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Canine S- and M/L- cone electroretinograms

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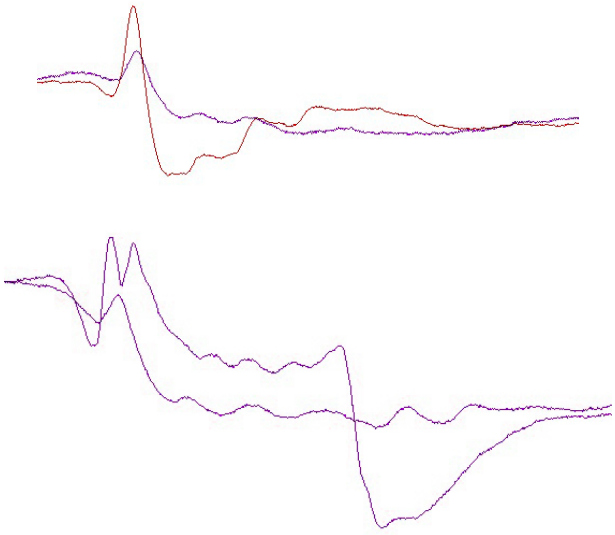

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Canine S- and M/L- cone electroretinograms
S- och M/L-tapp-drivna elektroretinogram hos hund

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SUMMARY

Full-field flash electroretinography is an electrodiagnostic method used to examine the function of retinal cells. Light stimulation of the eye elicits electrical potentials in the retina. By aid of a corneal electrode and a reference electrode close to the back of the eye, the electrical potentials can be recorded and presented as an electroretinogram (ERG). ERGs driven by mainly one type of cones can be used to examine the function of a single cone class. In human beings, studies have shown the cone class sensitive to light of short wavelengths, the S-cones, to be more vulnerable to acquired damage than the other cone classes (Daley *et al.*, 1987; Gouras *et al.*, 1993; Greenstein *et al.*, 1989).

Dogs have two cone classes, S-cones, and M/L-cones (most sensitive to medium to long wavelengths). Hitherto, no guidelines or protocols for separating S- and M/L-cone driven ERGs in the dog have been published. Hence we know little about how each of these two cone classes are affected in different canine retinal diseases and there may be diseases still unrevealed due to the lack of proper diagnostics. The aim of this study was to separate and examine S- and M/L- cone driven canine ERGs.

In this study, S- and M/L-cone driven ERGs were recorded using chromatic stimuli and selective chromatic adaptation in six healthy Beagle dogs. The method means that a light stimulus with a wavelength that maximally stimulates one cone class is presented on a bright chromatic background that saturates the rods and strongly supresses the responses from the cone type/types that are not of interest. We used a violet light stimulus (411 nm) on a steady, bright, red background light (627 nm) to elicit responses mainly driven by the canine S-cones. To examine the pure M/L-cone driven ERG, the violet light was instead used as background light whilst the red light was used as the stimulus. In addition, a second experiment was performed where we used the two stimuli on a steady, bright, blue background (470 nm) that would suppress the S- and M/L-cones almost equally, as well as bleach the rhodopsin completely.

Selective chromatic adaptation provided non-univariant responses despite the use of equal relative light intensities for the stimuli, which suggests that we were stimulating two different cone mechanisms. The S-cone driven ERG had longer b-wave implicit times and lower b-wave amplitudes, which seemed to saturate at lower stimulus intensities compared to the M/L-cone driven ERG, which had shorter b-wave implicit times and higher b-wave amplitudes that increased over a larger range of stimulus intensities. A prominent d-wave (response to the cessation of light) was seen on the M/L-cone driven ERG, whilst this was absent, or at least not obvious, on the S-cone driven ERG. Our results are in agreement with the results from a study of feline cone ERGs (Zrenner & Gouras, 1979).

SAMMANFATTNING

Elektroretinografi är en elektrodiagnostisk metod som används för att undersöka funktionen hos celler i retina. Metoden bygger på att ljusstimulering av ögat ger upphov till elektriska potentialer över retina. Registrering av dessa signaler sker via en corneaelektrod samt en referenselektrod nära ögats laterala kant och presenteras i form av ett elektroretinogram (ERG). Selektiva ERG huvudsakligen drivna av en viss typ av tappar kan användas för att undersöka funktionen hos en viss tapp-klass i taget. Studier på människa har visat att S-tapparna, tapp-klassen känsliga för ljus av korta våglängder, är mer sårbara än människans två andra tappar, både vid flertalet primära ögonsjukdomar och vid sekundär påverkan på näthinnan vid systemiska sjukdomar (Daley *et al.*, 1987; Gouras *et al*., 1993; Greenstein *et al*., 1989).

Hunden har två typer av tappar, S-tappar, samt M/L-tappar, de senare är mest känsliga för ljus av mellanlånga till långa våglängder. Hittills har inga riktlinjer eller protokoll som gör det möjligt att separera hundens S- och M/L-tapp-drivna ERG publicerats. Således är det idag okänt hur S- respektive M/L-tapparna hos hund påverkas vid olika patologiska processer i retina och det kan finnas sjukdomar som ännu inte upptäckts på grund av avsaknad av metoder för att kunna diagnosticera dem. Syftet med denna studie var att separera och undersöka S- och M/L-tappsdrivna ERG hos hund.

I studien registrerades S- och M/L-drivna ERG med hjälp av kromatiska stimuli och selektiv kromatisk adaptation hos sex friska beagle-hundar. Detta innebär att ett ljusstimulus av en våglängd som maximalt stimulerar en viss tapp-klass presenteras på ett starkt bakgrundsljus som både mättar stavar och optimalt trycker ner responsen från tapp-klasserna som inte är av intresse. Vi använde ett violett ljus (411 nm) på ett starkt, rött bakgrundsljus (627 nm) för att erhålla ett huvudsakligen S-tappsdrivet ERG. För att erhålla ett M/L-tappsdrivet ERG användes istället det röda ljuset som stimulus och det violetta ljuset som bakgrundsljus. Dessutom presenterades dessa två stimuli, samt ett blått (470 nm) stimulus, på en stark blå (470 nm) bakgrund som hämmade S- och M/L-tapparna ungefär lika mycket, samt mättade stavarna fullständigt.

Selektiv kromatisk adaptation gav upphov till ERG-kurvor med skilda utseenden, trots likvärdiga stimulusljusintensiteter, vilket indikerar att vi stimulerade två olika cellmekanismer. Den S-tapp-drivna kurvan hade en längre b-vågsimplicittider samt lägre b-vågsamplituder, vilka tycktes mättas vid lägre ljusintensiteter jämfört med den M/L-tappsdrivna kurvan, som följaktligen hade kortare b-vågsimplicittider och högre b-vågsamplituder. Medan en tydlig d-våg (en respons på då ljuset släckts) sågs när M/L-tapparna stimulerades, kunde en sådan inte urskiljas på S-tappsdrivna ERGn. Resultaten överensstämmer väl med en tidigare studie av S-tapps-drivet ERG på katt (Zrenner & Gouras, 1979).

CONTENT

ABBREVIATIONS

ERG	electroretinogram
S-cones	cone class sensitive to light of short wavelengths (absorption maximum at 429 nm in the dog)
M/L-cones	cone class sensitive to light of medium and long wavelengths (absorption maximum at 555 nm in the dog)
λ_m	medium wavelength in nanometers
LED	light emitting diode
OP	oscillary potentials

INTRODUCTION

Dogs play an important role in human society, not only as loved family members, but also as working and service animals. Many of these tasks require normal canine vision. Moreover, the use of dogs as animal models for human retinal diseases is increasing (Petersen-Jones *et al.*, 2006). Hence, not only good understanding of the physiology and pathophysiology of the canine retina is required, but also that methods for assessing normal and abnormal retinal function are well standardised in this species.

Studies in human beings have shown one cone class, the S-cones, to be more liable to acquired damage than the two other types of cones, the M- and L-cones. This has been seen in primary ophthalmic diseases, such as retinitis pigmentosa (Greenstein *et al.*, 1989), but also in ophthalmic disorders secondary to systemic diseases, for example type I diabetes and intoxications. (Daley *et al.*, 1987; Gouras *et al.*, 1993; Zrenner *et al.*, 1986)

Electroretinography is a method that has been used to objectively examine the functionality of the retinal cells in several species, including human and dog, for more than a century (Ekesten, 2013). The electroretinogram (ERG) displays electrical potentials arising in the retinal cells in response to light stimulation. Techniques for obtaining ERGs driven by mainly one type of cones, to enable examination of the function of the cone classes separately, have, for example, been described in human beings and the cat (Gouras & MacKay, 1990; Zrenner & Gouras, 1979), but not in the dog, to the author's knowledge. Hence, we know little about how the two canine cone classes, the S- and the M/L-cones, are selectively affected in retinal disease.

The aim of this study was to separate and examine S- and M/L-cone driven responses of the canine ERG.

LITERATURE REVIEW

Structure of the canine retina

The retina can be subdivided in the neuroretina and the retinal pigment epithelium (Figure 1) (see Ofri, 2013 for review). The retinal pigment epithelium provides nutrition from the choriocapillaris to the outer layers of the neuroretina and phagocytises old photoreceptor segments. The neuroretina consists of several cell layers. The photoreceptors are located in the outermost layer of the neuroretina, adjacent to the pigment epithelium. On the opposite side of the neuroretina, closest to the vitreous body, is the ganglion cell layer. The axons of the ganglion cells form the optic nerve.

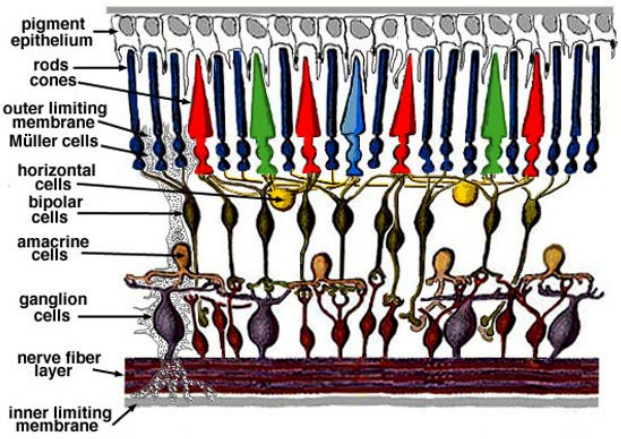

Figure 1. *Structure of the retina. Courtesy to http://webvision.med.utah.edu/*

In between the ganglion cell and the photoreceptor layers several cell types are interspersed (see Ofri, 2013 for review). The bipolar cells connect the photoreceptors and ganglion cells vertically. There are several types of bipolar cells. Anatomically they are divided into cone bipolar cells and rod bipolar cells. Physiologically, they can be classified as ON- and OFF-bipolar cells. The ON- and OFF-bipolar cells enable detection of dark objects on light backgrounds and vice versa. Greatly simplified, while ON-bipolar cells are activated (depolarised) by light stimulation of their central receptive fields, OFF-bipolar cells are inhibited by light stimulation in the central receptive fields and activated (depolarised) at cessation of the light stimulus.

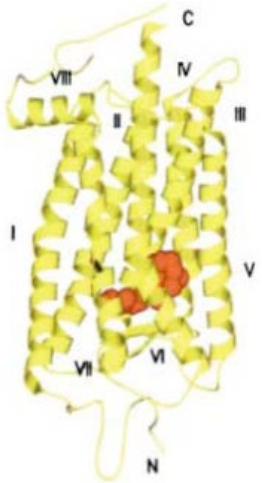

Figure 2. *The photopigment rhodopsin, composed of scotopsin (yellow) and 11-cis-retinal (red). Courtesy to http://webvision.med.utah.edu/*

While the bipolar cells link neuronal signals vertically, two cell types integrate and modulate the neuronal signals horizontally; the horizontal cells connect photoreceptors and the amacrine cells have a similar function in between ganglion cells. In addition, Müller cells provide support and maintenance.

Both excitatory and inhibitory synapses exist between the different cell types in the retina. When exposed to light, the photoreceptors produce a signal, modulated by other neuroretinal cells, transferred to ganglion cells and transmitted through the optic nerve to the brain, where the image is interpreted.

The photoreceptors

There are two types of photoreceptors, rods and cones (for reviews, see Ofri, 2013; Smith, 2006). The photoreceptors are specialized

neurons that contain photopigments, composed of an apo-protein, an opsin, and a light absorbing chromophore, 11-cis-retinal (Figure 2). The opsin determines which wavelength that the photopigment can absorb. The photopigment of rods is called rhodopsin and its opsin is named scotopsin. Scotopsin has a maximum absorption at about 500 nm in several species, while the types of opsin in cones vary amongst species.

The energy absorbed when light hits the retina initiates the phototransduction cascade starting with photoisomerization of 11-cis-retinal, which leads to a conversion of the chromophore, into all-trans-retinal. The new configuration is incompatible with the scotopsin, which leads to a split of the rhodopsin, and further conformational changes in the scotopsin. This chain reaction, often called the bleaching process, results in a hyperpolarisation of the cell.

While light stimulation causes bleaching of the rhodopsin, darkness enables regeneration of the photopigments. At reconstruction of the rhodopsin, all-trans-retinal is reconverted into 11-cis-retinal, which allows reconnection of scotopsin and 11-cis-retinal. The majority of the regeneration process occurs in the retinal pigment epithelium. Under normal conditions, the bleaching and regeneration processes in the retina occur simultaneously, which enables adaptation to the existing level of illumination in the environment. The phototransduction cascade and regeneration of cone photopigments resemble the rod phototransduction.

The rods constitute the majority of the photoreceptors in the canine retina (Mowat et al., 2008). Peripherally in the retina, Mowat et al. could measure a rod density of 305,000 cells/mm^2 and a cone density of 7,500 cells/mm^2, which correlates to a rod-to-cone-ratio of 41:1. In the area centralis, an area of high photoreceptor density located at a point 1,5 mm temporal and 0,6 mm superior to the optic disc, the rod-to-cone-ratio was however slightly lower. In this area the rod density was 501,000 cells/mm^2 with a cone density of 23,000 cells/mm^2, which provides a rod-to-cone-ratio of 22:1.

Rods and cones have properties that make them ideal for different light conditions (Ofri, 2013; Smith, 2006). Rods are extremely sensitive to light, which enables vision under dim light conditions, whereas cones are about 25-200 times less sensitive, which makes them suitable for vision under daylight conditions. Under background light intensities of less than 0.03 cd/m^2, vision is essentially rod driven (scotopic vision). Under background illumination of 0.03-3cd/m^2, both rods and cones function well (mesopic vision). Bright light conditions causes saturation of the rods, because all rhodopsin is bleached, which results in cone driven vision (photopic). In summary, cones provide high visual acuity and colour vision under daylight conditions. They respond rapidly to repeated stimuli; human cones have response kinetics that is 2-4 times faster than those of rods.

With increased light intensity of the background light, the cones will obtain a lower sensitivity, which reduces their ability to detect small changes in stimulus intensity (Ofri, 2013). On the other hand, light adaptation speeds up the cone responses to stimuli and increases the visual acuity (Tsang & Gouras, 1996).

Colour vision

Basis of colour vision

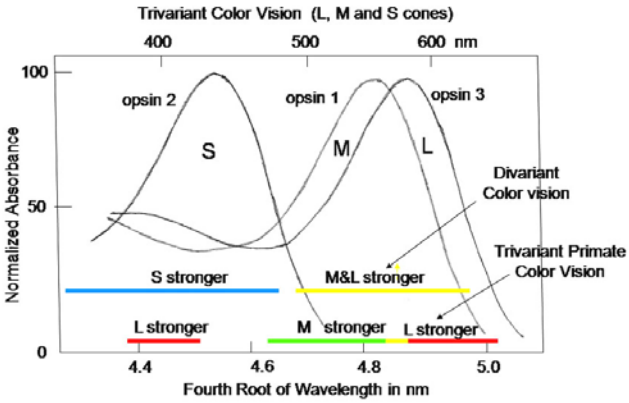

Figure 3. *Trichromatic color vision. Courtesy to http://webvision.med.utah.edu/*

The basis of colour vision lies in the number of cone classes in the retina and their spectral sensitivity to light of different wavelengths (Ofri, 2013; Gouras, http://webvision.med.utah.edu/, 2009). Each cone class has its own opsin, which decides the spectral sensitivity of the cone. Many reptilian, avian and fish species have four types of cones, allowing them tetrachromatic colour vision. The primates of the Old World, including human beings, have three cone classes, the S-, M- and L-cones and trichromatic vision (Figure 3). S-cone opsin has its peak of sensitivity at short wavelengths, around 430 nm, while the peak of M-cone opsin is around 520 nm and L-cone opsin has a sensitivity peak at about 560 nm. The M-and L-cones are also sensitive to shorter wavelengths, due to beta-band absorption of the photopigment.

The majority of the mammals, however, are dichromatic with two cone classes, S- and M/L-cones (Ofri, 2013).

Colour vision in the dog

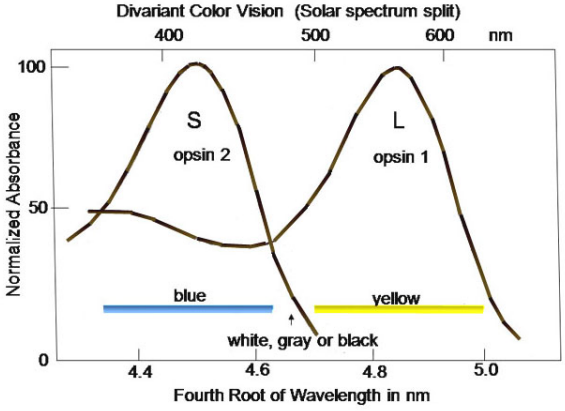

Figure 4. *Dichromatic color vision. Courtesy to http://webvision.med.utah.edu/*

Dogs have dichromatic colour vision (Figure 4) (Jacobs *et al*., 1993; Neitz *et al*., 1989). The S-cones and the M/L-cones have peak spectral sensitivities at about 429 nm and 555 nm, respectively. The majority, 88-91 %, of the canine cones are M/L-cones (Mowat *et al.*, 2008). Neitz *et al.* (1989) found that dogs have a difficulty to discriminate a 480 nm light from an achromatic light, because this is a wavelength where the absorption spectra of the two cones intersect and hence, both cone classes are equally stimulated. In addition, the dogs were unable to discriminate between wavelengths longer than about 520 nm, indicating a lack of S- cone influence at longer wavelengths (Neitz *et al*., 1989).

Basic electroretinography

In 1865, Holmgren was able to measure an electric potential over the eye as a response to illumination of the retina (Holmgren, 1865). Fifteen years later he was able to conclude that this potential was derived from the retina (Holmgren, 1880).

Today there are several types of electrodiagnostic examinations to evaluate the function of the visual system (see Ekesten, 2013 for review). In veterinary ophthalmology, the full-field flash electroretinogram (ERG) is the most widely used electrodiagnostic test.

Canine ERGs are often recorded on fully anesthetised animals (Ekesten *et al*., 2013; Ekesten, 2013). A corneal lens electrode is placed on the cornea (Figure 5) and a reference electrode is placed lateral to the temporal canthus of the eye. To reduce noise in the signals from the eye, a ground electrode is placed on the top of the skull.

It is of great importance that the retina is illuminated evenly. An lid speculum and stay sutures keep the eye open, the third eyelid out of the way and the direction of gaze relatively stable. Pupils are dilated prior to the recordings and a full-field stimulator, such as a ganzfeld stimulator, is preferably used to obtain a uniform distribution of the light stimulus and background light over the entire visual field and hence over the retina. Light stimulation of the eye results in electrical signals that are amplified, AD-converted and stored as waveforms, electroretinograms, in computerised data acquisition system. There is a recently updated guideline for clinical electroretinography in the dog, in which recommendations regarding light stimuli and background illumination are presented (Ekesten *et al*., 2013).

The ERG provides an objective, non-invasive measurement of retinal function and displays the activity of all types of retinal cells (Ekesten *et al*., 2013; Ekesten, 2013). Clinically, the canine ERG is useful in the early diagnosis of inherited or acquired retinal diseases. Moreover, the ERG enables examination of the retinal function when the fundus is obscured, for example prior to a cataract surgery, and it can be used to rule out a retinal disease as the cause to sudden loss of vision in a patient.

Anatomical and physiological variations causes large differences in the ERGs between species (Narfström, 2006). Even amongst the domestic dog, the appearance of the ERG may vary to such an extent that it is recommended to obtain baseline values from each breed and for at least three age groups.

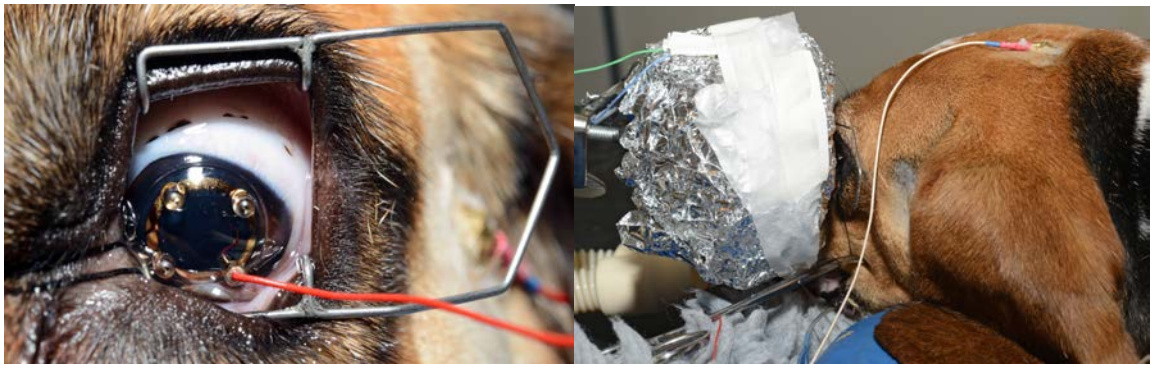

Figure 5. *a) Conjunctival stay sutures (black threads next to the nasal canthus), a lid speculum and a corneal contact lens electrode in place. b) A dog being prepared for an ERG recording. The shaved area aborally from the temporal canthus shows the intended position of the reference electrode. A monoocular mini-ganzfeld is used for light stimulation.*

The components of the canine electroretinogram

The initial negative component of the ERG is called the a-wave (Figure 6) and reflects the hyperpolarization of the photoreceptors as a response to light stimuli (see Ekesten, 2013 for review). The first positive component of the ERG is the b-wave (Figure 6), which is primarily related to the depolarisation of the bipolar cells (Frishman, 2006).

Superimposed, mainly on the ascending limb of the b-wave, are small wavelets named the oscillatory potentials (OPs) (Wachtmeister, 1998). The origin of each of the oscillations is still not well understood, but studies indicate that amacrine cells play an important role in their generation.

The d-wave is a response that can be seen in light-adapted (photopic) ERGs, as a response to cessation of the light stimulus (Figure 6) (Sieving *et al*., 1994). The OFF-bipolar cell, which depolarises at stimulus cessation, provides the largest contribution to this wave. When a light stimulus of short duration is used, the d-wave is superimposed over the b-wave. Using longer duration stimuli allows separation of the b- and d-waves. The d-wave has been observed in the light-adapted ERG in many species, including human beings and the cat (Sieving *et al*., 1994; Zrenner & Gouras, 1979), whereas any comprehensive reports on the d-wave of the canine ERG have not been published to the best of our knowledge.

The intensity, wavelength, frequency of presentation and duration of the stimulus as well as background illumination and the state of retinal adaptation all affect the waveform of the ERG (Gouras, 1970). A stronger light stimulus will cause a shorter implicit time and a larger amplitude on the ERG than a weak stimuli of the same wavelength, frequency and duration, at an equal background light (Gouras, 1970). An increased background light generates a reduction in implicit time and a reduction in amplitude (Gouras & MacKay, 1989).

There are several additional components in the canine ERG, some of which can be revealed only by using certain stimulation and recording paradigms. These will however not be described in this paper, for further information see for instance Frishman (2006) and Ekesten (2013).

Measuring amplitudes and implicit times of the ERG

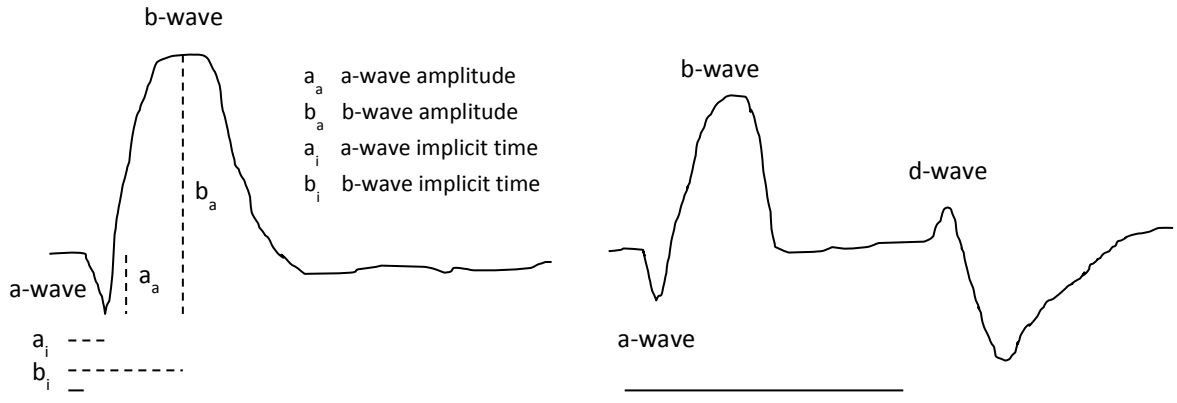

Figure 6. *Schematic drawings of ERG-responses to a 5ms (left) and a 100 ms (right) stimulus. The solid horizontal lines at the bottom of the figure show the durations of the stimuli.*

The amplitudes and implicit times of the a- and b-waves are the most essential parameters when interpreting an ERG (Figure 6) (Ekesten, 2013). The amplitude of the a-wave is measured from the prestimulus baseline to the trough of the a-wave and the amplitude of the b-wave is measured from the trough of the a-wave to the peak of the b-wave by convention. The implicit time of the a-wave is measured from the onset of the stimulus to the trough of the a-wave and the implicit time of the b-wave is measured from the onset of the stimulus to the peak of the b-wave. Measurements of the d-wave are beyond the scope of this report.

Methods to separate rod- and cone driven ERGs

Taking advantage of the different properties of the photoreceptors, it is possible to obtain an ERG that is selectively rod- or cone-driven (Figure 7) (Ekesten, 2013). Stimulation with low light intensities of a dark-adapted retina will essentially provide a rod-driven response, as the cones are not activated under these conditions. In this paper however, the focus will lie on cone-driven ERGs, hence rod-driven ERGs will not be further discussed.

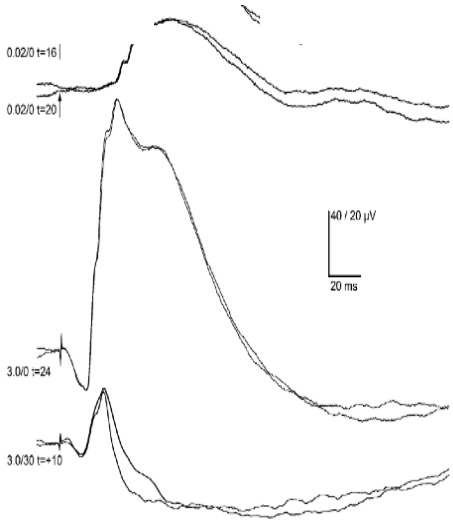

Figure 7. *Modified after Ekesten et. al, 2013. The top record shows a rod-driven ERG, the medium a mixed rod- and cone ERG and the bottom record a light adapted cone ERG.*

Bright background light of 10 millilamberts will saturate the rods and provide a cone-driven response (Gouras, 1970). In the guidelines for clinical electroretinography in the dog, a background light intensity of 30 cd/m^2 is recommended to obtain cone-driven responses (Ekesten et al., 2013). Furthermore, it is recommended the dog to be light-adapted for 10 minutes prior to recording. Another method to separate the cone response from the rod response is by taking into advantage of their different response kinetics. In human ERGs, flickering stimuli with frequencies above 30 Hz have been shown to provide cone-driven responses (Gouras, 1970).

Selective chromatic adaptation

Obtaining an ERG driven by solely one cone class entails a greater challenge than separating rod- and cone driven ERGs, as the properties of the cone classes show greater resemblance than those of rods and cones.

Selective chromatic adaptation is a technique that has been employed to obtain selective cone driven ERGs in several species, including cat (Schuurmans & Zrenner, 1981; Zrenner & Gouras, 1979), monkey (Schuurmans & Zrenner, 1981; Mehaffey & Berson, 1974) and human (Simonsen & Rosenberg, 1995). The method implies that a light stimulus of a wavelength that maximally stimulates the cone class of interest is presented on a chromatic background that saturates the rods and supresses the responses from the cone types that are not of interest by light-adapting them (Ekesten, 2013)

Zrenner and Gouras (1979) stimulated perfused cat eyes with 444 nm (dark orchid) and 583 nm (yellow), respectively, presented on a bright yellow background. The 583 nm stimulus provided a cone ERG with a short b-wave implicit time and a prominent d-wave. On the contrary, stimulation with 444 nm yielded a longer implicit time for the b-wave and no detectable d-wave. The b-wave amplitude in response to the 444 nm stimulus saturated at low light levels, whereas the b-wave amplitude of the 583 nm stimulus increased with increased energy levels and did not saturate even at the highest intensities used in the study. Hence, the two wavelengths provided non-univariant responses, indicating two separate cone mechanisms as origin of the responses.

Gouras and MacKay (1990) described a method to evaluate the human S-, M- and L-cones in the same ERG, opposed to selective cone driven ERGs. A bright achromatic (white to the human eye) background light was used and the light stimuli were varied in their spectral composition. The bright background intensity did not only saturated the rods, but also speeded up the response from the M- and L-cones, resulting in an early, united M- and L-cone b-wave and a later S-cone b-wave. This method was modified by Simonsen and Rosenberg (1995), who used selective chromatic adaptation to reduce the interference between the S-cones and the M- and L-cones. A stimulus of 440 nm presented on a yellow background light, led to a higher S-cone amplitude, whereas the M- and L-cone amplitude was unchanged or even reduced.

Two different light stimuli can produce an identical response from a cone, regardless of the wavelengths of the stimuli if the energy absorbed by the opsin is equivalent (Gouras, http://webvision.med.utah.edu/, 2009). The Silent Substitution method (Estevez & Spekreijse, 1982; Mortlock *et al.*, 2005; Sawusch *et al*., 1987) implies matching of two stimuli of different wavelengths and light intensities so that they stimulate one type of cones to the exactly the same extent. When pulses of the two stimuli are presented in counter phase, the excitation of the cone class to which the stimuli are equivalent is kept constant; hence an isolated ERG response from the other cone class can be obtained (Arden & Berninger, 2006).

Mortlock *et al*. (2005) used a steady background to light-adapt the L-cones. On this background, two stimuli with different wavelengths were matched to be equivalent for the M-cones, hence, an isolated S-cone response was obtained. The silent substitution method can also be used to produce an almost pure S-cone response in the human ERG with a minimum of M- and L-cone intrusion.

Cone degeneration in retinal diseases

A canine retinal disease that is known to primarily affect the cones is day blindness, i.e. hemeralopia, which has been described in the Alaskan Malamute by Aguirre and Rubin (1975). Hemeralopia is a recessively inherited disease (Rubin *et al*., 1967). Clinical signs arise at eight to ten weeks of age and include difficulties to see in daylight whilst in dim light, the vision seems to function well (Petersen-Jones & Narfström, 2013). No abnormal features are found on ophthalmoscopy. However, the ERG shows absence of cone responses. Histopathological examination has shown a progressive cone degeneration. At seven weeks of age, some cones are reported to be affected while others seem normal. By six months of age,

all cones are affected. In the end stage of the disease, at four years of age, there is a pure rod retina.

Cone dystrophy has been reported in the Miniature Poodle and the German Shorthaired Pointer, the latter in which the disease was shown to be caused by the same mutation as in the Alaskan Malamute. Single cases of cone degeneration have also been reported in the Australian Cattle dog, the Chihuahua and the Rhodesian Ridgeback.

As mentioned in the introduction, studies in human beings have shown the S-cones to be more liable to acquired damage than the M- and L-cones (Greenstein *et al.*, 1989; Daley *et al*., 1987; Gouras *et al*., 1993; Zrenner *et al*., 1986). If the canine S- and M/L-cones are affected differently in various diseases affecting the retina is not known today, as there are no protocols or guidelines for examining selectively S- and M-/L-cone-driven canine ERGs.

MATERIAL AND METHODS

Dogs

The ERGs were recorded from six Beagle dogs from the Clinical Science Department at the Swedish University of Agricultural Science (Table 1). All experiments adhered to the guidelines from the Association for Research in Vision and Ophthalmology (ARVO) for use of research animals and ethical approval was obtained from the Uppsala Regional Ethical Review Board before the experiments started.

Prior to the experiments all dogs underwent a general physical examination, including assessment of general condition, posture and gait, hydration status, auscultation of the circulatory and respiratory system, palpation of lymph nodes and abdomen. In addition, an ophthalmologic examination including testing of menace responses, palpebral and corneal reflexes, pupillary light reflexes, dazzle reflexes, cotton ball test and doll's eye reflex, Schirmer's tear test, tonometry (Tono-Pen XL, Medtronic Solan, Jacksonville, USA), slit-lamp biomicroscopy (Kowa SL-15, Kowa Optimed Deutschland GmbHand, Düssseldorf, Germany) and direct and indirect ophthalmoscopy (Heine Beta 200, HEINE Optotechnik, Herrsching, Germany and Topcon ID-10, Topcon Corporation, Tokyo, Japan) after dilation of the pupils was performed. The results of the examinations are shown in Table 1.

Table 1. *Clinical and ophthalmological examinations of the six Beagle dogs included in the study. n.a.d. = no abnormalities detected*

	Sex	Age	Clinical findings	Ophthalmologic findings
Dog 1	intact female	3	n.a.d.	n.a.d.
Dog 2	intact female	3	n.a.d.	n.a.d.
Dog 3	intact female	3	mild tonsillitis	mild distichiasis
Dog 4	intact female	3	n.a.d.	n.a.d.
Dog 5	intact female	3	n.a.d.	n.a.d.
Dog 6	intact female	3	n.a.d.	n.a.d.

Preparations

Pupil dilation was obtained by topical administration of cyclopentolate (Cyclogyl, 1%, eye drops, Alcon Laboratories, Inc. Fort Worth, Texas, USA) and tropicamide (Mydriacyl, 0.5%, eye drops, Alcon Laboratories, Inc. Fort Worth, Texas, USA). Two drops of each drug were administered 5 minutes apart and was then repeated after 15 minutes. The last administration was approximately 30 minutes prior to the ERG. Pupil dilation was evaluated before and after each experiment.

Artificial tears (Comfort Shield, 0.15%, Vétoquinol Scandinavia, Åstorp, Sweden) were used as coupling agent between the contact lens electrode and the cornea and to keep the eye moist. The eyelids were kept open using a lid speculum and stay sutures (silk 4-0) kept the third eye out of the way.

The dogs were premedicated with 0,04-0,05 mg/kg acepromazine (Plegicil vet, 10mg/ml, Pharmaxim, Helsingborg, Sweden) intramuscularly. Anaesthesia was induced with 0,6-0,8 mg/kg propofol (Lipuro 10 mg/ml, B. Braun Melsungen AG, Melsungen, Germany) intravenously and maintained by inhalation of isoflurane (Attane vet, Isofluran, 1000mg/g, Piramal Health Care UK Limited, Northumberland, Great Britain). Hydration was maintained using Ringer's acetate (Baxter, healthcare Corporation Chigao, Illinois, USA) at a dose corresponding to 40 ml/kg and day. The dogs were positioned in ventral recumbency. Oxygen saturation and pulse rate was monitored using a battery-operated pulse-oximeter (TuffSat, GE Healthcare, Louisville, CO, USA) throughout the experiments.

Equipment for stimulation and registration

A corneal contact lens electrode (JET-lens electrode, Universo, Switzerland) was placed on the cornea. A reference electrode (Gold Disc Electrodes, F-E5GH, Natus Neurology Incorporated, West Warwick, RI, USA) was put approximately 3 cm from the lateral canthus and a ground electrode (same model as for the reference electrode) was placed at the top of the skull. The skin electrodes were attached using a conductive paste (Ten20 Conductive, D.O Weaver & Co, Aurora, CO, USA). The impedance was measured with an impedance meter (F-EZM5, GRASS, Astro-Med inc., West Warwick, RI, USA) and kept below 5 kOhms.

The signals were amplified by an isolated biological amplifier (Iso-Dam, World Precision Instruments Inc., Sarasota, FL, USA) and by a combined amplifier and AD-converter (PowerLab/8SP, AD Instruments Ltd, Castle Hill, Australia). A special software (Scope 4, AD Instruments Ltd, Castle Hill, Australia) was used to display, store and analyse the data.

A monocular, customized, mini-ganzfeld was used for presenting both background light and light stimuli. The mini-ganzfeld had built-in light emitting diods (LEDs) (Table 2). The LEDs were driven by a signal generator (Siglent; SDG-5082, Ferner elektronik AB, Järfälla, Sweden). The background light was driven and kept constant by the signal generator whilst the light stimulus was presented at rate of 0.5 Hz.

Table 2. *Specifications of the light emitting diodes (LEDs) used for light stimulation of the eye*

Type	Colour	Wavelength range	λ_m	Manufacturer
Luxeon K2 red	red	620.5-645 nm	627 nm	Philips Lumileds, San Jose, California
YSF-B319EY	blue	460-480 nm	470 nm	Yoldal CO Zhonghe City, Taipei Country
OCU-400 411 OS	violet	407-415 nm	411 nm	OSA Opto Light GmbH Berlin, Germany

Calculation of stimulus intensity

The relative number of photons (n) in each light stimulus was calculated as the quotient of the total relative energy divided in the energy of a photon. The energy of a photon (E) is

$$E = hc \div \lambda$$

where c is the speed of light (299,792,458 m/s), h Planck's Constant ($6.626\ 069\ 5729 \times 10^{-34}$ J•s), and λ the wavelenght in nm. However, the wavelengths emitted by the LEDs constituted a rather symmetrical distribution on each side of the peak wavelength, hence the peak wavelength was used when calculating the number of photons.

Light intensities of stimuli and background light were measured prior to the experiments, with a photometer (IL 1700, International Light Ltd, Newburyport, MA, USA). The light intensities were measured without any filter and as the photometer was not calibrated for this, the values obtained were in "relative irradiance", which differ from the true irradiance by a constant factor. Hence, the number of photons calculated from the relative energy is a relative and not an absolute number. Background light intensities were however also measured in human scotopic cd/m^2, using a filter calibrated for human scotopic vision.

Protocols

Two main protocols for light stimulation were used (Tables 3 and 4). In summary, the first protocol was used to examine ERG responses to violet, blue and red stimuli on a blue background and the second protocol for selective chromatic adaptation; violet light presented on a bright red background and vice verse.

The dogs were adapted for ten minutes to each different background light intensity. A recovery period of 2 minutes was used between different stimulus intensities. The relative intensity of each stimulus was increased for each background intensity and stimulus wavelength (Table 5). A response to both a 5 ms and a 100 ms stimulus was recorded for each intensity. Data for a second study (C. Kristensson, Evaluation of the retinal ON- and OFF responses in the dog ERG, ISSN 1652-8697) were collected from each experiment, however only the data relevant for this paper is presented here.

Table 3. *Summary of protocols for chromatic stimuli presented on blue background (470 nm) of three different intensities. The intensity of each background is presented in log relative photons/m^2/s and below each background intensity, the wavelengths of the light stimuli are presented in nm. Both short (5 ms) and long (100 ms) stimuli were recorded for each stimulus and background. The x marks that an ERG was obtained using these particular settings.* λ_m *= peak wavelength (presented in nm)*

	Background illumination of 470 nm λ_m								
	11.50 log rel. photons/m^2 & s			11.80 log rel. photons/m^2 & s			11.97 log rel. photons/m^2 & s		
	Stimulus λ_m			Stimulus λ_m			Stimulus λ_m		
Subject	411	470	627	411	470	627	411	470	627
1		x			x			x	
2	x	x	x						
3	x	x	x				x	x	x
4	x	x	x	x	x	x			
6				x					

Table 4. *Protocol for selective chromatic adaptation, subjects 1 and 6*

Stimulus wavelength	Background illumination	
	411 nm (violet) 13.7 log relative photons	627 nm (red) 14.38 log relative photons
411 nm (violet)		x
627 nm (red)	x	

Table 5. *The range of relative intensities (in log relative photons/m^2) for each wavelength of the light stimulus*

	log relative photons/m^2	
Wavelength of stimulus	5 ms stimulus	100 ms stimulus
411 nm	10.40-11.39	11.70-12.70
470 nm	9.64-11.61	10.94-12.91
627 nm	10.92-12.08	12.22-13.38

Dog 5 had a miotic pupil at the end of the ERG session and was therefore excluded from the study.

RESULTS

Altered chromatic stimuli presented on a blue background

Prominent a- and b-waves were seen in ERGs in response to violet, blue and red stimuli presented on a rod-saturating blue background (Figure 8). The b-wave amplitudes were slightly lower to violet stimuli than to blue and red stimuli, despite that the number of photons were almost equal for all three lights.

B-wave amplitudes grew higher following increased stimulus intensities for all three wavelenghts. Blue stimulus, which stimulates both S- and M/L-cones almost equally, produced a larger a-wave than violet and red stimuli. The b-wave was uni- or bi-phasic in response to the lower stimulus intensity and bi- or tri-phasic at the higher intensity. No obvious differences in b-wave implicit times could were observed.

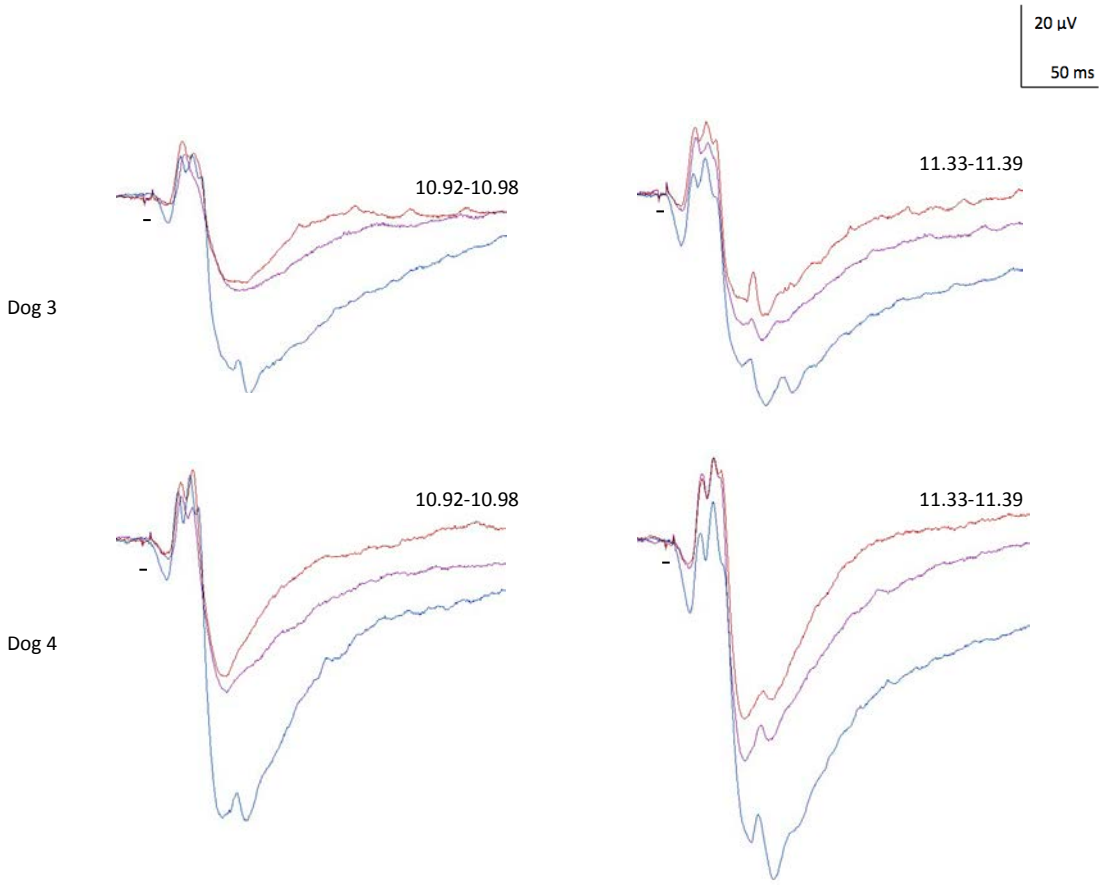

Figure 8a. *ERGs in response to violet (λ_m 411 nm), blue (λ_m 470 nm) and red (λ_m 627 nm) stimuli, presented on a steady blue (λ_m 470 nm) background light with an intensity of 11.50 log relative photons/m^2 and second. ERG responses produced by the three different chromatic stimuli of approximately equal light intensities are superimposed on each other to show the similarity. The solid horizontal lines show the duration of the stimuli (5 ms). Stimulus intensities, presented in log relative photons/m^2, are shown to the right of each record.*

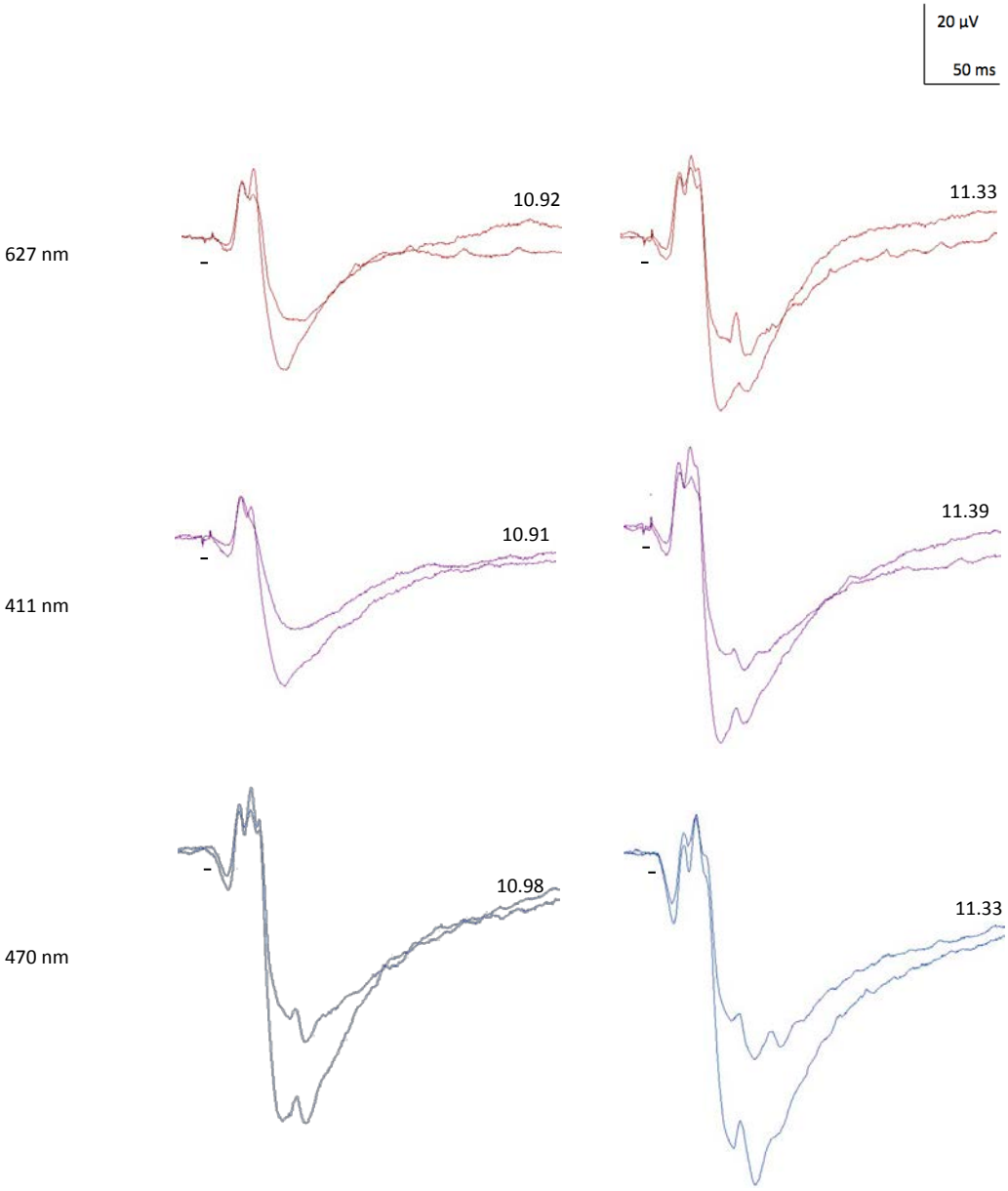

Figure 8b. *ERGs in response to red (λ_m 627 nm), violet (λ_m 411 nm) and blue (λ_m 470 nm) stimuli presented on a steady blue (λ_m 470 nm) background with an intensity of 11.50 log relative photons/m^2/s. ERG records from two dogs are superimposed on each other to show the similarity. The solid horizontal lines show the duration of the stimuli (5 ms). The stimulus intensity, in log relative photons/m^2, is shown to the right of each record.*

Figure 9 shows b-wave amplitudes and implicit times in response to violet and red stimuli presented on a blue background. The amplitudes for both stimuli increased with increased light intensities of the stimuli, red stimulus producing somewhat higher amplitudes. The implicit times appear to increase with increasing stimulus intensity (at least at higher intensities), but our sparse data is rather scattered.

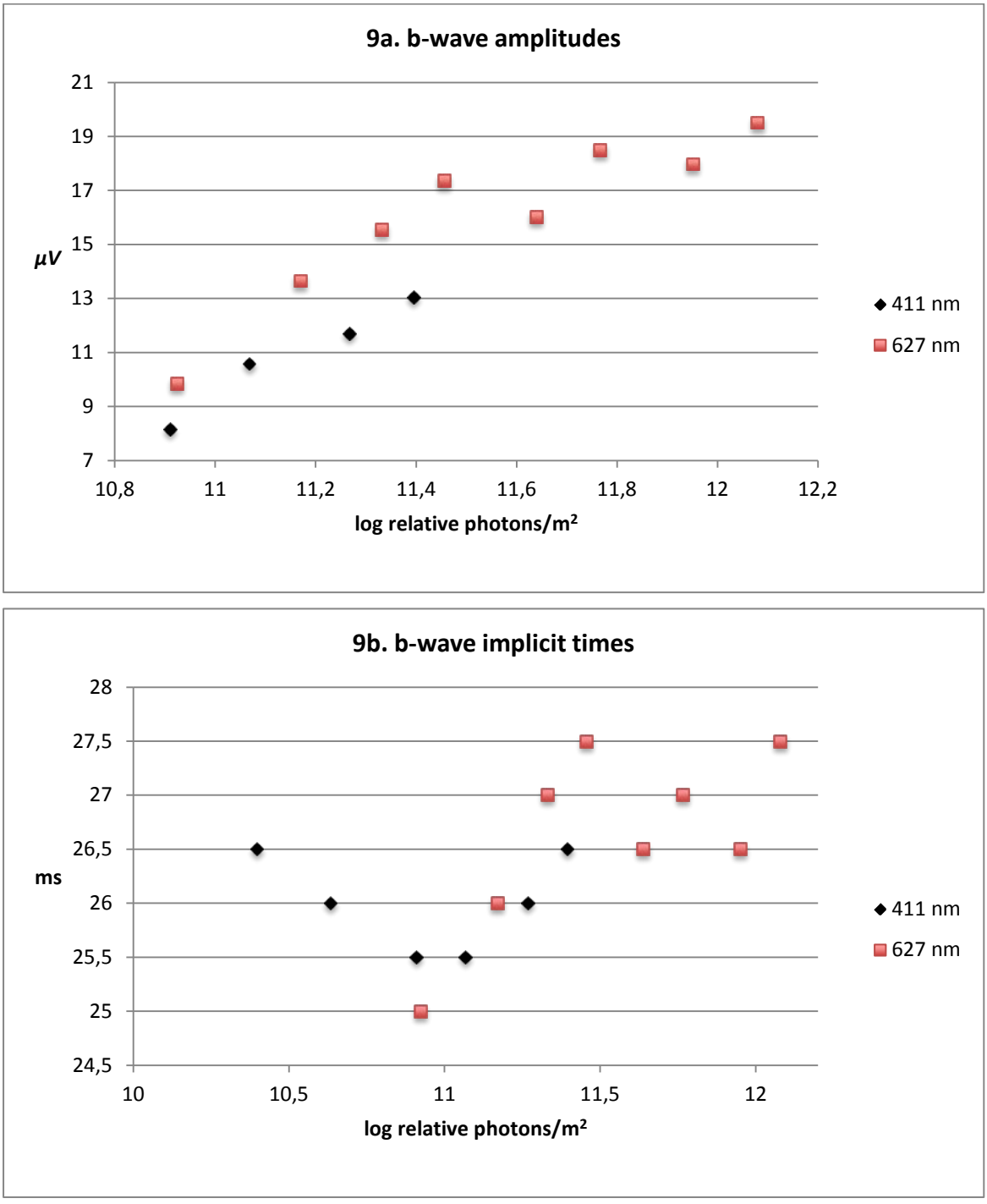

Figure 9. *B-wave amplitudes (a) and implicit times (b) produced by violet (λ_m 411 nm) and red (λ_m 627 nm) stimuli as a function of stimulus intensity. The stimuli were presented on a blue (λ_m 470 nm) background with an intensity of 11.97 log relative photons/m^2/s.*

Selective chromatic adaptation

The violet stimulus presented on the rod-saturating and M-/L-cone suppressing red background produced smaller b-wave amplitudes compared to responses to red stimuli on a rod-saturating and S-cone suppressing violet background when approximately equal stimulus intensities were employed (Figure 10). While the b-wave amplitude to red stimuli increased sigmoidally with increasing stimulus intensities, the amplitudes of the b-wave in response to the violet stimulus were similar throughout the range of intensities (Figure 11).

The b-wave implicit times were longer to violet stimuli than to red stimuli for all the light intensities in the study (Figure 11b).

Figure 10. *ERGs in response to violet (λ_m 411 nm) and red (λ_m 627 nm) stimuli presented on chromatic adaptation fields. The red stimulus was presented on a steady, violet background with the intensity of 13.7 log relative photons/m^2 and second. The violet stimulus was presented on a steady, red background light with the intensity of 14.38 log relative photons/m^2 and second. Records produced by red and violet stimuli of approximately equal light intensities are superimposed on each other. The stimuli intensities are shown to the right of each record, in log relative photons/m^2. The solid horizontal lines show the duration of the stimuli (5 ms).*

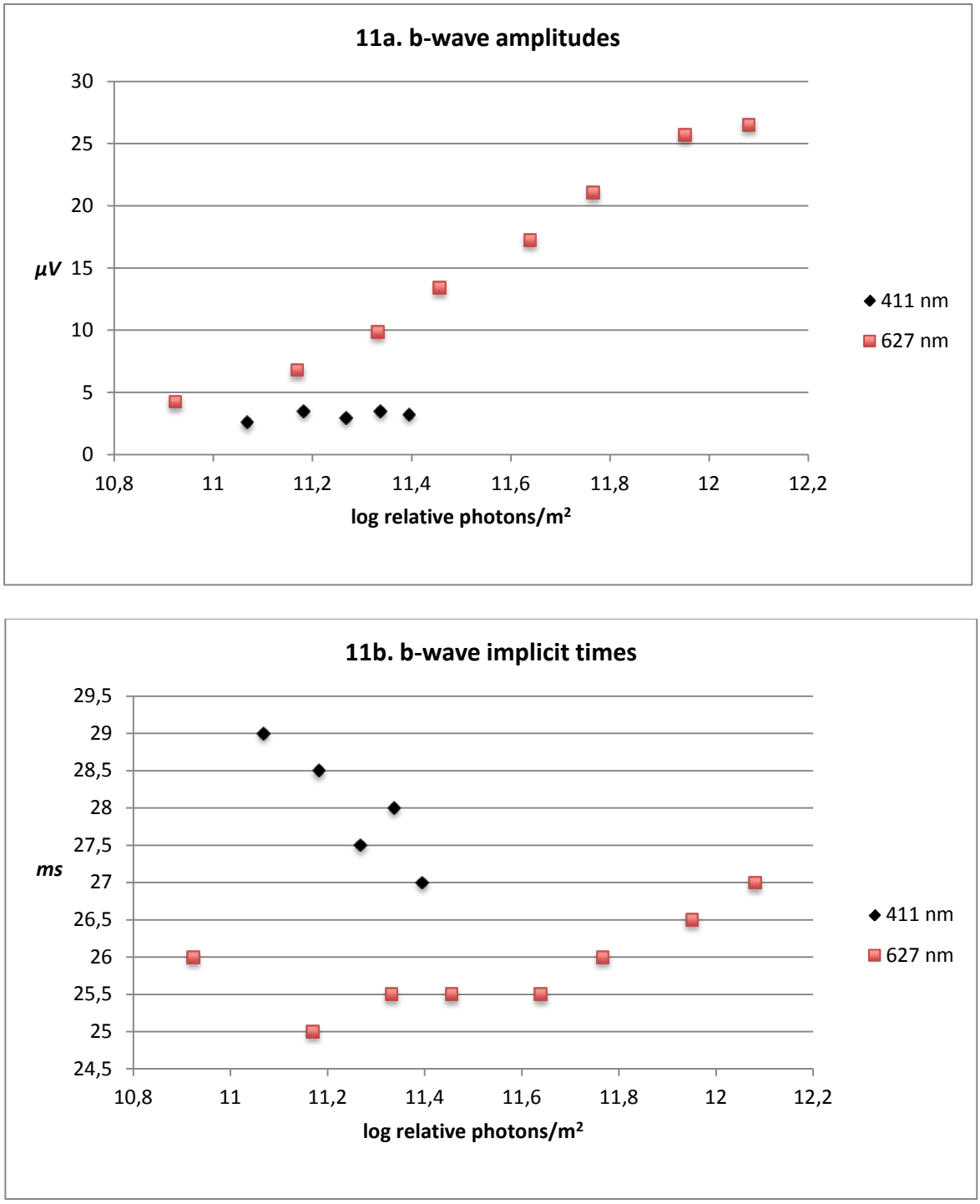

Figure 11. *B-wave amplitudes (a) and implicit times (b) in response to violet (λ_m 411 nm) and red (λ_m 627 nm) light stimuli of different intensities. The violet stimulus was presented on a red background light with 14.38 log relative photons/m^2/s., whereas 13.7 log relative photons/m^2/s violet background was used for the red stimulus.*

Responses to increased stimulus duration

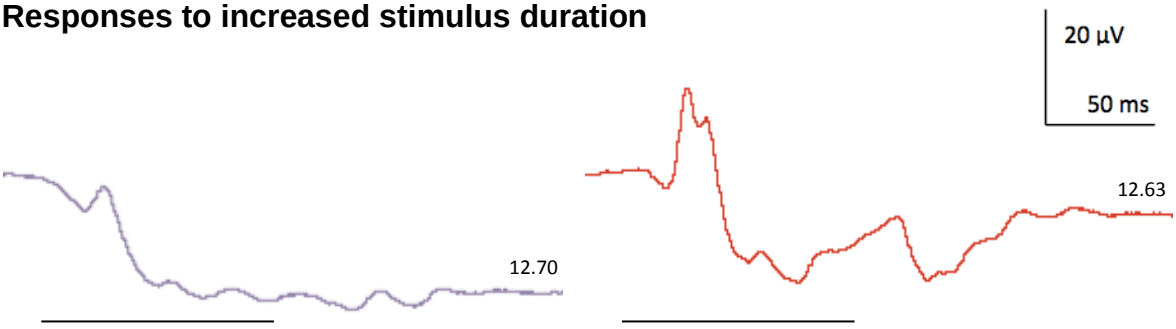

Figure 12. *ERG recordings in response to violet (λ_m 411 nm) and red (λ_m 627 nm) stimuli of approximately equal light intensities. The violet stimulus was presented on a steady, red background light with the intensity of 14.38 log relative photons/m^2/s. The red stimulus was presented on a steady, violet background with the intensity of 13.7 log relative photons/m^2/s. The stimuli intensities are shown to the right of each record, in log relative photons/m^2. The solid horizontal line below each record shows the 100 ms stimulus.*

The b-wave amplitude in response to a 100 ms red stimulus (Figure 12) was higher than that to the red stimulus of 5 ms duration. In contrast, a 100 ms violet stimulus caused no increase in b-wave amplitude compared to the 5 ms violet stimulus.

After the cessation of the 100 ms red stimulus, a d-wave appeared followed by a large negative wavelet. No obvious d-wave could be seen in response to the 100 ms violet stimulus. However, when the same violet stimulus was presented on the blue background, a distinct d-wave was seen (Figure 13).

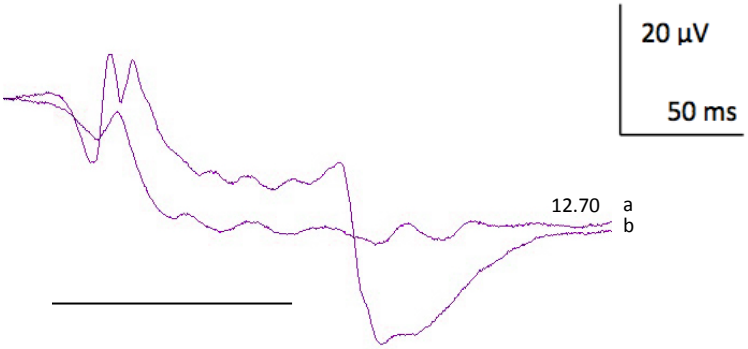

Figure 13. *ERG recordings in response to a bright 411 nm stimulus of 12.70 log relative photons/m^2 on a red (λ_m 627 nm – curve a) and blue (λ_m 470 nm - curve b) background. The intensities of the background lights were 14.38 log relative photons/m^2/s for the red light and 11.80 log relative photons/m^2/s for the blue light. The solid horizontal line below the records shows the 100 ms stimulus.*

DISCUSSION

We used a long-wavelength, red stimulus (627 nm) to obtain selective, canine M/L-driven ERGs, as we know that the canine S-cones cannot respond to wavelengths longer than 520 nm (Neitz *et al.*, 1989). A bright violet (411 nm) background was used to saturate the rods. This wavelength will also desensitise the M/L-cones because of their beta-band absorption, but we consider that the effect must have been small.

Our violet (411 nm) flash will not selectively stimulate the S-cones, due to the beta-band absorption of the M/-L-cones, but it should be a considerably more effective stimulus for the S- than the M/L-cones. However, as the M/L-cones constitute the majority of the cones in the canine retina (Mowat *et al*., 2008), their contribution to the ERG is larger than the S-cones. It is therefore of utmost importance to desensitise the M/L-cones as much as possible to minimise their ability to respond to the violet stimulus in order to obtain an ERG mainly driven by the S-cones. This was done using a bright chromatic background of 627 nm.

Selective chromatic adaptation provided ERG responses with different waveforms in response to violet and red stimuli of approximately the same number of photons. The two different chromatic stimuli employed will however not be equally well absorbed by the two photopigments. Based on the S- and M/L-cone spectral sensitivity functions presented in Jacobs *et al.*, 1993, we estimate the red stimulus to be less effectively absorbed by the M/L-cones (around 30 % of their maximum absorption capacity) than the violet stimulus by the S-cones (around 80% of their maximum absorption capacity). Hence, speaking in terms of the number of photons effectively absorbed by the two cone types, the 627 nm curve in Figure 11 would rather be displaced to the left in relation to the 411 nm curve. The fact that M/L-cone absorption at 627 nm wavelength is as low as 30% of maximal absorption, but the red stimulus still causes markedly larger amplitudes and a faster increase in amplitudes than the violet stimulus, makes it less likely that the response to the violet stimulus is produced by weakly stimulated M/L-cones. Furthermore, neither the implicit time-intensity functions to 411 and 627 nm stimuli appear univariant. Finally, the OFF-responses to 100 ms violet and red stimuli appear different throughout the entire range of stimulus intensities, suggesting that two different cone mechanisms were stimulated. Hence, we believe that we have obtained a mainly S-cone driven ERG in response to 411 nm stimuli.

The 470 nm background light was chosen to enable comparison of the ERG responses produced to the 411 nm and 627 nm stimuli presented on a background suppressing the S- and M/L-cones almost equally (Neitz *et al*. 1989) and, of course, saturating the rods. Both the waveforms and the b-wave amplitude as a function of stimulus intensity were similar for both stimulus wavelengths on the blue background. Therefore, we have no solid evidence that we were able to selectively stimulate the S-cones in that experiment. Despite the rather low sensitivity to the violet stimulus in the beta-band of the M/L-cones, the much larger number of these cones compared to the S-cones (Mowat *et al*., 2008), may have made their contribution to the violet response considerable. Hence, we believe that the red stimulus selectively drove the M/L-cones and the violet stimulus produced a mixed S- and M/L-cone response on the blue background.

Properties of the S- and M/L-cone electroretinogram

The results indicate that the canine S-cone driven ERG has a longer b-wave implicit time and a smaller b-wave amplitude that appears to saturate at lower stimulus intensities, compared to the M/L-cone driven ERG, which has a shorter b-wave implicit time and a higher b-wave amplitude that increases with increased stimulus intensities. The M/L-cone driven ERG has a prominent d-wave after the cessation of the light stimulus, while the d-wave was absent, or at least not obvious, in the S-cone driven ERG. Our results from the dog are in agreement with the results from the cat (Zrenner & Gouras, 1979). However, our study is marred by the low number of dogs and a larger population should be studied to confirm these results.

To further investigate the S-cone ERG a larger range of light intensities are however needed. In addition to this, to obtain a maximal response from the S-cones, a stimulus wavelength at around 429 nm would be optimal, as this is the absorption maximum for the S-cones (Neitz *et al.*, 1989).

Variable number of peaks of the b-wave

We observed b-waves with one, two or three peaks in our experiments. One possibility is these constitute an early M/L-cone b-wave and a later S-cone, as the M/L-cone response speeds up faster than do the S-cone response at high background light intensities (Gouras & MacKay, 1990). This is however less likely because b-waves with this appearance are seen in response to the 627 nm stimulus, which exclusively drives the M/L-cones. A rod component can also be excluded as the background illumination was bright enough to fully saturate the rods, according to the Guidelines for Clinical Electroretinography in the dog (Ekesten *et al.*, 2013). Another prospect was that a multiphasic appearance was caused by a delay of the bipolar OFF-responses even for the brief light stimulus of 5 ms. This was however rejected as the bi- and tri-phasic b-waves were seen on ERGs in response to stimuli with a duration of 100 ms, whereas distinct d-waves (OFF-responses) emerged after cessation of the stimuli. By using a high-cut (low-pass) filter on the ERG records, the peaks were filtered out suggesting that they were large OPs (see literature review for details).

Technical aspects

No filter for canine photopic vision was available for the photometer when we measured the light intensities. The CIE photopic filter would not have been appropriate, because its transmission spectrum is adjusted to human trichromatic vision. Hence, the intensities were measured without any filter, and the values obtained were in “relative irradiance” (based on the energy per area unit, W/m^2, detected by the photometer, but off from the true irradiance by a constant factor). Our calculated number of photons per area unit is therefore not the absolute number of photons, but a relative value that was used when we compared different intensities and different wavelengths. Hence, the relative number of photons in our study cannot be compared to absolute number of photons in other studies. The background light intensities were however also measured in scotopic cd/m^2 using a filter calibrated for human scotopic vision. We find this pertinent, because the absorption spectra of human and canine rhodopsin is quite similar and an important purpose of the background light was to saturate the rods.

CONCLUSIONS

The results indicate that almost selective S- and exclusive M/L-cone driven ERGs can be obtained using chromatic stimuli and selective chromatic adaptation. The S-cone driven ERG was slower and had lower amplitudes that appeared to saturate at lower light intensities than the M/L-cone driven. While the M/L-cone driven ERG had a prominent d-wave after the cessation of a 100 ms stimulus, the d-wave was absent, or at least not obvious, on the S-cone driven ERG. Although this was just a pilot study, our results suggest that our protocol can be used to study conditions where one class of cones in the canine retina is exclusively affected or more affected than the other. It would, for instance, make it possible to diagnose colour-blind dogs.

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