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Clinical Guideline for Assessing Flash Visual Evoked Potentials in Laboratory Dogs and Normal Data for Beagle Dogs

by D Torres & MC Tovar*

Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Murcia, Spain

**Correspondence: Desiree Torres, DVM, PhD*
C/ Félix Martí Alpera, nº 50, 7ºA. Cartagena 30205, Murcia, Spain.
Mob. +34 658019373
Fax +34 868884737
E-mail: denisets7@hotmail.com

Summary

Visual evoked potentials (VEPs) are useful to evaluate the visual pathway integrity from ganglion cells of the retina to the visual cortex. VEP could be applied to evaluate the effects on optic nerve function following ophthalmologic treatments and in toxicity studies. The aims of this research were to design a clinical protocol for testing flash-VEPs during sedation and dissociative anaesthesia in laboratory dogs, and to propose updated normal data for beagles.

The Flash-VEP technique was used in 12 beagle dogs after dark adaptation, pupil dilation and an anaesthetic protocol. Two tests were performed for each eye after monocular stimulation. Flash-VEP waveforms consisted of 2 positive (P1, P2) and 2 negative (N1, N2) peaks sequenced as P1, N1, P2, and N2, where P2 was the most prominent peak. Peak times (ms) and peak-to-peak amplitudes (μV) were measured to evaluate the time from light stimulation of retina to cortical response and their electrical intensity, respectively. No statistically significant difference was observed between right and left eye data from all dogs (Student's *t* test, $p \leq 0.05$), with the exception of P2 peak times.

The protocol described allows clinicians to accurately record Flash-VEP in dogs. These results are reliable and reproducible, and could be valuable for other laboratories and future studies as standard background data to compare with when testing VEP in beagle dogs.

Introduction

The most common electrodiagnostic technique used in veterinary ophthalmology, to evaluate retinal function, has been electroretinography. Nowadays, however, visual evoked potentials (VEPs) have been increasingly undertaken by specialised clinics to complete the visual system evaluation. In particular, this test records visual evoked responses as a result of light stimulation of retina and the conduction of the response along the visual pathway. These potentials are registered by scalp electrodes, filtered and averaged by an electrodiagnostic system and, finally represented as a waveform and data. Therefore, to obtain a typical VEP waveform it is necessary for there to be a normal conduction of visual information through all the visual pathway components (eyeball including retina, the optic nerve, optic chiasma, optic tract, lateral geniculate nucleus, optic radiations, primary visual cortex and visual association cortex). Consequently, this VEP procedure provides a complementary technique (together with ERG and clinical findings) for the diagnosis of central blindness due to a variety of pathological changes to the visual pathway.

Nevertheless, there are certain factors that limit the use of this test: the equipment cost, the need of a clinician with specific qualification, the complex calibration of the system, the difficulty of interpretation of the results, and the inability to correlate the

results with the signal sources. Lately, the availability of VEP equipment has extended their veterinary use. However, there is a need for standard protocols to perform this technique in laboratory dogs and also for specific normal data for each breed.

Several researchers have proposed VEP protocols for dogs (*Sato et al., 1982; Sims et al., 1989; Strain et al., 1990*), but some procedures seem to be invasive (intracranial screw electrodes by *Sato et al., 1982*), stressful (systems to restrain conscious dogs' movements by *Sato et al., 1982* and *Strain et al., 1990*), or too long to do within the working day (need to train dogs to adapt to a semi-restraining support, and also spend 60 min in dark adaptation by *Sato et al., 1982*). Hence our study was conducted in order to establish a short, non-invasive and simple clinical protocol to obtain objective and reliable values of visual pathway integrity.

Normal VEP data for beagle dogs have been reported about twenty-five years ago (*Strain et al., 1990*), but our study proposes updated data for this breed associated with changes to the protocol (dark adaptation time, pupil dilation, sedation-anaesthesia protocol, interocular difference evaluation). These data could be helpful because calibration of the electrodiagnostic equipment is a complex procedure. Nevertheless, it is advisable that each laboratory calibrates its own electrodiagnostic equipment because it can influence the results of the test (*Odom et al., 2010*).

Materials and Methods

Animals

Twelve beagle dogs were selected as a representative sample, 11 male and 1 female, aged from 2- to 5-years old, with body weights between 10-21 kg. All animals belonged to the Laboratory Animals Service of Murcia University (Support Service to the Research (SAI); no. REGA IS300305440012), which have been maintained and used in accordance with the EU guidelines concerning the protection of animals used in experimentation (86/609/EEC).

All dogs were clinically healthy, and a complete ophthalmologic examination was also performed 24 hours before VEP testing. This examination comprised: menace response, obstacle course, cotton-ball test, pupillary light reflexes, indirect ophthalmoscopy to examine the back of the eye, direct ophthalmoscopy with a slit lamp, and electroretinography to evaluate retina cells.

A 40-min clinical protocol was designed for VEP testing. It was composed of the following stages:

Preparation of the room

The room selected for electrodiagnostic recordings was in complete darkness, and the environmental temperature was controlled to avoid hypothermia of the anaesthetised dog.

Preparation of the dog

A 12-hour fasting period was necessary, prior to sedation-anaesthesia, to avoid emesis during the testing process. Then a proper sedation-anaesthesia protocol was selected to guarantee dog's relaxation, and to avoid electrical muscle activity that creates signal artefacts in the recordings (*Bichsel et al., 1988*).

The drugs selected for sedation were a combination of medetomidine hydrochloride (Domtor®, Orion Pharma, Espoo, Finland) (0.01 mg/kg) and butorphanol tartrate (Torbugesic®, Fort Dodge Animal Health, Iowa, USA) (0.3 mg/kg) intramuscularly applied. This pre-anaesthetic medication reduces the dose of the anaesthetic agent required to keep the dog relaxed, and also avoids the seizures induced by dissociative drugs (*Flaherty, 2009*). The anaesthetic protocol was completed with ketamine hydrochloride (Imalgene 500®, Merial Laboratory S.A., Barcelona, Spain) intravenously administered at a single dose of 5 mg/kg of body weight by catheterisation of the cephalic vein. This dissociative drug was selected in order to keep the dog unconscious during the entire test, and to relocate the eye in central position, which is very important due to the ventromedial position of the eye induced by medetomidine (*Clark, 2009*). In addition, oxygenation was provided during the experimental procedure, the dog was kept warm by using heat pads to avoid hypothermia, and ventilation was also controlled by monitoring the dog.

While the dog was relaxed, administration of a mydriatic drug was necessary to guarantee full dilation of the pupil; when a greater area of the retina is stimulated, more intense responses are obtained in a shorter time (*Itoh et al., 2010*). Then, 1% tropicamide eyedrops (Colircusí tropicamida®, Alcon Cusi, S.A., Barcelona, Spain) were instilled every 5 min for 15 min. Afterwards, the dog was gently placed in sternal recumbence on the examination table and the base of the head was kept horizontal.

During the preparation of the dog, a minimum of 20 min of dark adaptation was performed prior to light stimulation. Furthermore, all the recording procedure was performed in darkness to guarantee

the activity of rods. This type of photoreceptor predominates in dogs' retinas and consequently produces a stronger VEP response than by only stimulating cones.

Electrode placement

Before fixing the electrodes, skin was shaved, cleaned and disinfected with alcohol to provide good electrical connection between the electrodes and the skin. Both eyelids were also spread with Barraquer eyelid speculums to avoid blinking, due to the brightness of the flash which is not sufficient to penetrate the eyelids.

Three gold disc scalp electrodes were selected (*Odom et al., 2010*) because they are of good quality, non invasive (avoids possible infections caused by needle electrodes) and provide excellent recordings (*Perez-Salvador, 1999*). These were located over the scalp midline according to a modification of the International 10/20 System for humans, and fixed with a proper amount of conductive paste (TEN 20 conductive*, Weaver and company, Colorado, USA). Then the active electrode (O_z) was located over the occipital protuberance midline to register cortical responses; the reference electrode (F_z) was fixed over the forehead midline just between the eyes; and finally a ground electrode (C_z) was placed over the vertex midline between both ears to complete the electrical circuit.

Equipment organisation

All electrodes were connected to a single recording channel differential amplifier and this to the central unit. A mini-ganzfeld was also connected to the central unit. This was a handle photostimulator lamp to provide monocular stimulations which are used to detect functional asymmetries between both eyes.

The equipment employed in this laboratory was RETIsystem* (Roland Consult, Germany) with RETIport 32 as testing software. Equipment for humans was used because this was the one available for our study; nowadays there are many VEP equipments adapted to animals which are very similar in calibration, and this guideline might be applicable to both.

Calibration of the system

To prepare the testing system it was necessary to select Flash-VEP technique (other VEP techniques such as pattern or onset/offset are available) for light stimulation. This is the recommended technique for recording VEP in anaesthetised dogs, because other techniques, for instance pattern screens, need the active attention of the dog. Furthermore, Flash stimulations also provide good recordings in humans with media opacities (*Odom et al., 2010*), which means that it could be of value in dogs with these problems.

Calibration of many stimulation and recording parameters was also necessary. In this study, the recommendations from the International Society for Clinical Electrophysiology of Vision (ISCEV) for humans (*Odom et al., 2010*) were followed as summarised in Table 1.

Light stimulation

Flashes of white light were the stimulus emitted from a mini-ganzfeld held about 5 cm in front of the eye. At closer distances the eyelids were touched which caused digital artefacts, and farther distances resulted in less intense responses. Then monocular stimulations were performed by covering the contralateral eye with a dark patch. Two measures were taken from each eye in the same testing session to assess repeatability (*Odom et al., 2010*). We always checked

Table 1. Stimulation and recording parameters for Flash-VEP calibration[a]

Parameters	
Stimulus type	White Flash
Environmental luminosity	Darkness
Stimulation	Monocular
Eye-Miniganzfeld distance	5 cm
Flash strength	3 cd·s·m^{-2}
Background luminance	30 cd·m^{-2}
Stimulation frequency	1 flash/s (1Hz)
Electrode impedance	5-7 KΩ
Filters (-3 dB)	1 Hz (low freq) – 100 Hz (high freq)
Nº sweeps averaged	64
Sweep time	250 ms

[a] Adapted from the ISCEV recommendations (*Odom et al., 2010)*

the right eye first (two measurements) and then the left eye (two measurements).

Recording and averaging system

The active electrode recorded the signals evoked by the visual cortex in response to the visual stimulus. High pass and low pass filters allowed extraction of visual-origin signals from background cerebral activity unrelated to the visual stimulation; visual signals were then amplified by the differential amplifier (*Odom et al., 2010*).

The last stage was to send the filtered responses to the central unit in a PC, where the signals were averaged and represented as a waveform.

Analysis and interpretation

All waveforms obtained were analysed in a specific way for each dog. First, each waveform was analysed to study the general morphology, the number of peaks and their polarity, the sequence of appearance, and their size. Then, all waveforms were compared between both eyes in a given dog and between dogs to find abnormalities and asymmetries.

Peak time and amplitude values were measured for each wave as suggested by ISCEV (*Odom et al., 2010*). Peak times, measured in ms, represented the time from light stimulation of retina to visual pathway cells' response. This measure was taken from the beginning of the flash stimulation to each peak. Amplitudes, measured in μV, showed the voltage or electrical intensity of cellular responses. P1N1 and P2N2 represent the difference in voltage between peaks P1-N1 and P2-N2, respectively.

Finally, descriptive statistical analysis of data was performed by using SPSS Statistics 16® software (SPSS Inc. Illinois, USA) to establish range of normality, mean, standard error of mean (SE), and standard deviation (SD) for a sample of 48 measures (n=48); 2 measures from each eye in 12 dogs. Mean value was taken as representative when SD ≤ R/3. In addition, mean interocular difference value and Student's *t* test for paired samples ($p<0.05$) were used to evaluate the difference between right and left eye measures in each dog.

Results

Waveform and data analysis

A total of 48 waveforms were obtained and compared. Waveform morphologies resembled an 'M' form (Figure 1) and consisted of four peaks sequenced as: P1, N1, P2, and N2. This nomenclature was according to the polarity (P= positive, N= negative) and the appearance order of the peaks. P1 and N1 were constant peaks in all dogs. P2 was the most prominent peak in all dogs and the most varying. However, P3 was not present in all dogs and that is the reason why P3 was not included in our results.

Waveforms were similar between two measures taken in each eye and even between eyes in a given dog (Figure 1). In addition, all waveforms were compared between dogs and no remarkable difference was apparent (Figure 2). Moreover, no peak or wave was absent or significantly delayed, nor were exaggerated responses obtained.

Descriptive statistical values as range, mean, standard mean error (S.E), and standard deviation (S.D) for peak times and amplitudes are presented in Table 2 and 3, respectively.

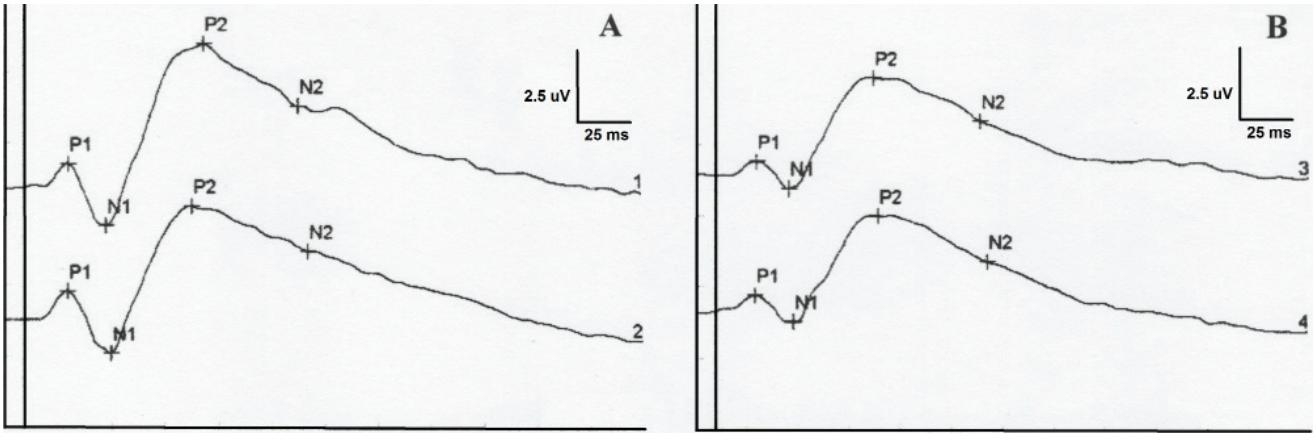

Figure 1. Flash-VEP waveforms from a beagle dog (A: right eye, B: left eye) showing 'M' shape and composed by 4 peaks: P1, N1, P2, and N2. It can be noticed that the earliest peak has positive polarity. Two measures have been obtained from each eye. It can be seen the similarity of the waveforms from the same eye and between eyes in a given dog.

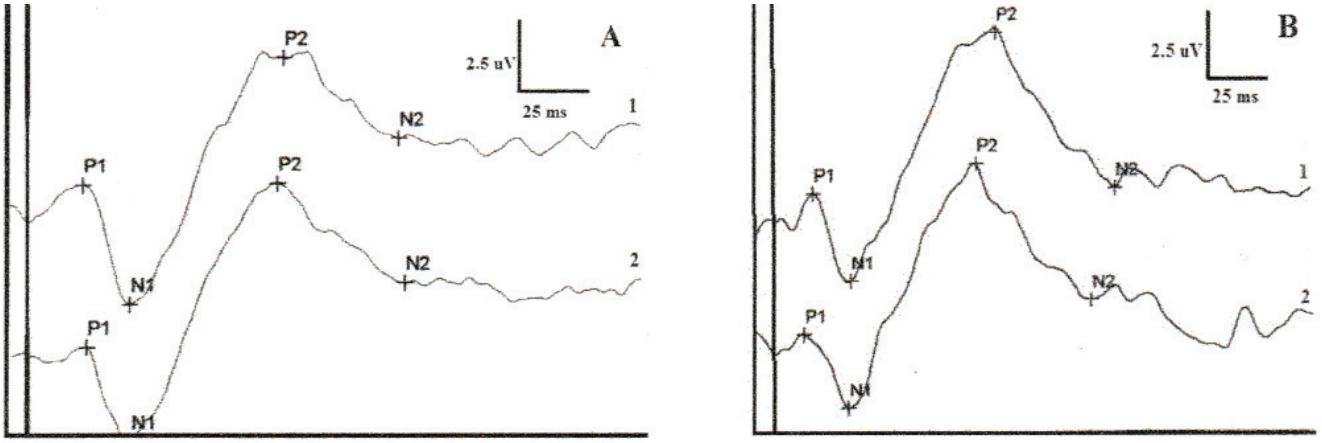

Figure 2. Flash-VEP waveforms from two different dogs (beagle A and beagle B). It has been represented an averaged waveform from 2 measures taken in right eye (1) and left eye (2). As can be appreciated, there is a clear similarity between waveforms from different dogs.

Table 2. Flash-VEP peak time descriptive statistical data and *p* paired t-test values

	P1 peak time (ms)		N1 peak time (ms)		P2 peak time (ms)		N2 peak time (ms)	
	RE	LE	RE	LE	RE	LE	RE	LE
N	12	12	12	12	12	12	12	12
Mean	16.41	17.41	35.25	37.33	93.08	90.33	141.75	143.00
S.E.	0.92	0.64	1.02	1.36	2.17	2.30	4.11	3.11
S.D.	3.20	2.23	3.54	4.73	7.53	7.99	14.25	10.77
Range	(8-21)	(13-22)	(30-40)	(32-49)	(79-103)	(77-100)	(120-161)	(128-158)
Mean interocular difference	1.00		2.08		2.75		1.25	
p	0.305		0.116		0.025		0.554	

RE= right eye; LE= left eye; S.E. = standard error of mean; S.D. = standard deviation

Table 3. Flash-VEP amplitude descriptive statistical data and *p* paired t-test values

	P1N1 amplitude (μV)		P2N2 amplitude (μV)	
	RE	LE	RE	LE
N	12	12	12	12
Mean	2.17	2.39	2.31	2.76
S.E.	0.28	0.31	0.38	0.39
S.D.	0.99	1.08	1.32	1.36
Range	(0.74-4.16)	(0.89-3.75)	(0.40-6.03)	(1.46-5.12)
Mean interocular difference	0.21		0.45	
p	0.405		0.187	

RE= right eye; LE= left eye; S.E. = standard error of mean; S.D. = standard deviation

Regarding peak times, mean data were representative (SD ≤ R/3), but later peaks such as P2 and N2 showed greater S.D and S.E than the previous ones. However, mean interocular differences were insignificant (from 1 to 2.75 ms) as shown in Table 2. Furthermore, paired t-test analysis between both eyes only showed a slight significant difference for P2 peak times.

Amplitudes, demonstrated less S.D (less than 1.4 μV) and S.E (less than 0.4 μV) than peak times. In addition, amplitude data presented great stability intra-individually, since mean interocular differences were less than 0.5 μV and paired t-tests showed no significance (see Table 3). Analysing these results, very low values (< 0.2 μV) for peak amplitude were suggested to be abnormal as were exaggerated responses.

Discussion

VEP protocol quality and influential factors

Adult dogs were selected for this study because many authors (*Kimotsuki et al., 2006*) have demonstrated the influence of age on VEP morphology and data (young dogs and senior dogs showed changes in data due to poor development of the visual system and the reduction of myelinisation of the central nervous system respectively). However, a future study including normal data from different age groups could be of great value.

Dark adaptation prior to light stimulation was essential because the retina of dogs is mostly composed of rods, about 95% (*Curtis & Lightfoot, 1993*), which respond to luminance changes (this is in contrast to the human retina which is dominated by cones). In previous examinations (data not published), we found that larger amplitudes and shorter peak times were recorded with longer dark adaptation times. However, more studies are necessary to investigate this finding. At least 20 min of dark adaptation was necessary to obtain waveforms with typical morphology and reproducible results. Shorter adaptation times registered weak responses and nearly flat waveforms. We think that a 1h-dark adaptation time (as used by *Kimotsuki et al., 2005* and *2006*) is too long and less practical for a clinical protocol. Strain *et al.* (*1990*) reported a study with no dark-adaptation but, in our laboratory, recordings in similar conditions showed low intensity signals (data not published).

Regarding the use of mydriatic drugs, the ISCEV (*Odom et al., 2010*) suggests not using these drugs in Flash-VEP techniques for humans but, in our studies with dogs, faster waves with higher intensity were obtained due to the stimulation of a greater area of retina and consequently the transmission to a larger area of the visual pathway. This difference between protocols might be due to the greater number of cones in the central retina of humans that makes possible the recording of VEP without mydriatics. Furthermore, a recent study (*Itoh et al.,* 2010) demonstrates the great influence of mydriasis on Flash-VEP recordings in dogs. Another advantage of using mydriatics was to avoid miosis caused by flashes (*Sims et al., 1989*).

According to several authors (*Sato et al., 1982; Sims et al., 1989; Margalit et al., 2003; Kimotsuki et al., 2005; Itoh et al., 2010*) an anaesthetic protocol was essential to obtain relaxation of the dog and avoid electrical artefacts from muscle activity, blinking, or ocular movements. Nevertheless, it is important to take into account that all sedative or anaesthetic drugs can cause depressive effects in the CNS, as shown in many reports (*Sato et al., 1982; Strain et al., 1990 and 1991*). Despite this fact, the dissociative protocol produces lower cortical depression than inhalatory anaesthesia as demonstrated by Margalit *et al.* (*2003*). Moreover, as reported by Clark (*2009*), the ventromedial rotation of the eye caused by medetomidine was compensated with ketamine which relocated the globe in central position. Local anaesthesia was also rejected since Ropo *et al.* (*1992*) suggested that these drugs could alter the electrical activity of the optic nerve.

In most of the reports referenced (*Strain et al., 1990 and 1991; Kimotsuki et al., 2005 and 2006*) needle electrodes or subdural electrodes (*Margalit et al., 2003*) were employed, but scalp electrodes are non-invasive and provided good quality recordings.

Studies using 3 electrodes were reported several years ago (*Strain et al., 1990, 1991; Kimotsuki et al., 2005, 2006; Itoh et al., 2010*) and showed good results like ours. Regarding electrode placement, a modification of the International 10/20 System for electrode placement in humans over the scalp's midline was useful to record VEP in dogs. The occipital protuberance midline was also the best place to fix the active electrode because it correlates with the part of the visual system that receives the majority of visual information (*Ofri et al., 1994*; *Kimotsuki et al., 2005*), and also where clear signals were recorded with no impedance problems. The negative electrode was located between the eyes since, in our opinion, this place does not correlate with the visual cortex and has less influence from the light stimulus due to the distance from the positive one. Finally, the ground electrode was placed between both ears. Although it could be placed in another position by the ears (*Kimotsuki et al., 2005*) or forehead (*Strain et al., 1990 and 1991*), this position provided a good electric circuit.

Concerning light stimulation, the eye to mini-ganzfeld distance affected the eye stimulation, because distances greater than 10 cm did not allow proper rod stimulation and, consequently responses were slower and smaller. Moreover, covering the non stimulated eye was necessary to assess monocular recordings. In this way, previous tests (data not shown) without covering the contralateral eye with a dark patch showed larger responses due to the combined responses of both eyes. Furthermore, low responses when the uncovered eye was stimulated may have been due to rods previously stimulated by

the environmental light coming from the other eye stimulation, and so the rods were saturated.

Many authors (*Uzuka et al., 1989; Perez-Cobo et al., 1994; Odom et al., 2010*), in their studies with other species, prefer binocular stimulation to study the global function of the visual system, but we prefer to use monocular stimulations in order to detect asymmetries between eyes.

VEP waveform and data

All the results showed a typical waveform that resembles an 'M' letter, as demonstrated in other works (*Ropo et al., 1992; Perez-Cobo et al., 1994; Margalit et al., 2003; Odom et al., 2010; Itoh et al., 2010*). The ISCEV identified four peaks in humans sequenced as: N1, P1, N2, and P2, but many studies in dogs (*Strain et al., 1990 and 1991; Margalit et al., 2003; Kimotsuki et al., 2005 and 2006*) showed a sequence like ours: P1, N1, P2, and N2. Strain *et al.* (1990, 1991) and Kimotsuki *et al.* (2005, 2006) registered a waveform with 5 peaks (P1, N1, P2, N2, and P3), but P3 was very difficult to standardise in our study. Maybe the cause was the difference in equipment; the particular calibration of each type of equipment could modify the results (*Odom et al., 2010*). All referenced studies in dogs showed the prominence of P2 and N2.

The global analysis of peak times presented great similarity with other works in dogs (*Strain et al., 1990 and 1991; Kimotsuki et al., 2005 and 2006*), where P1 and N1 were the most stable values (smaller SD), and P2 and N2 showed more SD, although without being significant, perhaps due to an artefactual influence in the later peaks. Thus, from our knowledge, measures of all peaks have to be taken into consideration, not only N2 and P2 as used by Itoh *et al.* (2010). Our peak time values for P1 and N1 were greater than reported by Strain *et al.* (1990) and Kimotsuki *et al.* (2005), probably due to an anaesthesia effect. However, there was no significant difference between two measures taken in each eye nor for the interocular difference for a given dog (Figure 1 & 2), except P2 that showed a slightly interocular difference ($p \leq 0.05$, but not too significant $p \geq 0.01$) possibly due to the special sensitiveness of this peak to light stimulation factors. One of the most important findings was the difference between humans (*Odom et al., 2010*) and our dogs' peak times, since the earliest peak appears at 30 ms and 16 ms, respectively. The reason could be, as reported by Strain *et al.* (1990), that there are many interspecies peak time differences, and presumably for amplitudes as well.

Regarding wave amplitudes, several investigators reported (*Sato et al., 1982; Uzuka et al., 1989; Perez-Salvador, 1999*) P1-N1 and P2-N2 as the most interesting, but amplitudes presented more variability than peak times. Our results differ from other studies (*Strain et al., 1990; Kimotsuki et al., 2005*) as our mean amplitudes were less (possibly due to central nervous system depressing effect of anaesthesia) and our ranges of normality and SD were less. Additionally, by checking twice both eyes, we demonstrated no significant interocular difference for a given dog nor a significant difference between two measures in the same eye ($p \geq 0.05$) that verify data reproducibility.

Conclusion

It can be concluded that all recorded VEP responses were very similar in waveform morphology, all peaks were constant in appearance, and data showed no significant interocular difference. None of the reports referenced have provided interocular data and t-test normal values for all Flash-VEP peaks in beagle dogs. We consider it is very important to estimate these measures for detecting possible asymmetries between visual pathways derived from both eyes. We encourage all laboratories to study morphology of the waveform (number of peaks and polarity), peak time values (to estimate the time of response to light), and amplitude values (to analyse the intensity of the response) in Flash-VEP studies to assess visual pathway function in order to complement electroretinography (ERG) and neurologic techniques. VEP should be considered normal when the waveform is characteristic, peak times are constant and within a normal range, and amplitudes are not exaggerated. Finally, we propose our clinical protocol to be used in future studies to obtain reliable VEPs for beagles; our results for clinically normal beagles may provide useful background reference data for future dog studies.

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