Electroretinography: A biopotential to assess the function / dysfunction of the retina.

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Abstract. The Electroretinography (ERG) is a noninvasive technique that allows the assessment of functional integrity of the retina. The ERG recordings are biopotencials acquired in the corneal surface as a response of retinal tissue against controlled light stimuli. In clinical ophthalmology ERG is not commonly used but nowadays, because of the high incidence of degenerative diseases of the retina (RD), its use should be increased. Like other biopotentials as electrocardiography (ECG), electroencephalogram (EEG) and electromyography (EMG), ERG is a low amplitude signal, in this case a few hundred of microvolts (μV), which must be fitted and processed. The ERG signals are affected in morphology in the presence of pathologies that affects the integrity of the different retinal cell groups, for example due to some RD. In advanced cases of RD recordings can be abolished in the time domain; and yet in them it is believed that there is relevant clinical information making the ERG a great potential diagnostic tool.

1. Introduction

Biopotentials are electrical signals that can be measured between two points as electrical potential differences at the cellular level, tissue or an organism. Living tissue, when is adequately stimulated, responds with variations in its ionic composition causing local changes in current that varies with time. In some cases these local changes generate a depolarization that exceeds a threshold and can spread generating what is known as action potential. These variations, local and propagated can be registered with appropriate instrumentation and get analogical signals commonly called biomedical signals. In the field of Biomedical Engineering the importance of these kind of signals for diagnosis or research of different diseases is well known. ECG, EEG, EMG, among others are the most known and used biomedical signals, and their names depend on the cell group or tissue whose electrical activity is recorded.

Along human history there have been attempts to record all kinds of biopotentials and often those intentions were thwarted by the limitations of the electronics and instrumentation of the time. Gotch Francis (1903) and then Einthoven and Jolly (1908) were the first to determine the morphology of the electrical signal from the retina in response to flash light stimulus. It was then, in 1933, that Ragnar Granit identified different components in the cat's ERG, which later earned him the 1967 Nobel Prize

in Physiology and Medicine. While some conceptions and descriptions were changed over the years, its foundations persist.

Even today some limitations due to electronics are remained. But if the minimum requirements that should be requested to the instrumentation are well known, reliable and reproducible recordings can be achieved. These limitations may be the reason why in clinical practice ERG technique is rarely used, even with the great need to find a diagnosis tool to know the existence of a degenerative disease of the retina (RD) and a clinical monitoring to know its evolution. In pathological conditions, the ERG recordings are diminished in amplitude and its latency time is increased, thus in advanced stages of the disease abolished recordings in the time domain can be found [1]. Our hypothesis is that the ERG could be a useful tool for confirming the diagnosis of RD, as it is known they are altered from very early stages of the disorder.

We believe that using basic techniques of signal processing such as Fourier Transform, the frequency spectra and hidden information in them can be analyzed.

1.1. The retina

The retina is a photosensitive tissue that belongs to the Central Nervous System (CNS) that lines the back of the eye. It consists of the neural retina and the retinal pigment epithelium (see Table 1). The thickness of the neural retina is between 100 and 200μm depending on the species. In the outermost layer photoreceptor cells (rods and cones) can be found. These types of cells perform the process of phototransduction, where light (photons) is transformed into a nervous signal that is sent to the brain through the optic nerve for integration and interpretation. The embryonic origin of the retina is the neural tube, the entire retina stems from its outgrowth. It consists of two layers that are called optical cup. The outer layer of the optical cup originates the pigment epithelium, while the inner layer gives rise to the rest of the retina [2].

Table 1. Cells and layers of the neural retina

Layer	**Cells**
Outer	Photoreceptor: rods and cones
Medial	Amacrine, bipolar and horizontal
Inner	Ganglion

1.2. Electroretinography

It is known that sensory receptor cells generate membrane potentials when they receive an excitatory stimulation. The extracellular potentials can be recorded noninvasively. This is the result of local conductance changes in the membrane of the activated cells originating input and output currents. These currents also flow through the extracellular space and create an extracellular potential. Extracellular current flowing through the conductive fluid surrounding a cell, whose activation has caused a local stream, is mainly directed towards the less activated cell parts; thus, when neurons are arranged in that way all of them are activated synchronously due to extracellular currents flowing in the same direction. The change of the extracellular potential resulting, called potential field, can be large enough so that it can be registered in a distal surface, for example the cornea [2]. Therefore the ERG is an electric potential generated by the retinal response to a flash of light and it is a measure of the average of biopotentials that reflects the medium and outer retinal layers functionality [1, 3-5], in addition it can be recorded in a distant tissue. The most studied parameters of this biopotential are the amplitude (microvolts μV) and the latency time (ms) [1, 3] of the main waves (a- and b-wave). Under dark-adapted conditions, in response to a stimulus such a bright flash, the a- and b-waves are generated. They are due to the activity of photoreceptor cells [6] and depolarization of the bipolar cells respectively [7]. It is considered that the ERG recordings are a powerful diagnostic tool to determine the progresive loss of

retinal cells function in the clinical practice and in the investigation field, caused by different types of RD.

It is important to know the contribution of each type of retinal cells in the ERG recordings in order to determine if the ERG is an efficient tool for the assessment of the normal and pathological retinal activity. Radially oriented neurons (photoreceptors and bipolar cells) contribute more to the ERG than the cells that are oriented irregularly or laterally on the retina (horizontal and amacrine cells) [2].

The signal recording consists of different stages: a) acquisition, b) fitting and amplification and c) the signal processing. The acquisition should be performed following the standards of the International Society for Clinical Electrophysiology of Vision (ISCEV) for the clinical full field Electroretinography [8].

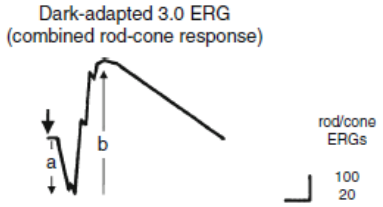

Figure 1: Response in dark-adapted conditions. ISCEV Standard (2008) [5]

1.3. Retinal degeneration.

RD is defined as the pathological process that causes death of retinal cells inducing the loss of tissue structure and possible blindness. Some of the diseases that are related to light stimullation:

1.3.1. Retinitis pigmentosa (RP): In most cases, the rods are most affected causing visual impairment ranging from mild retinal dysfunction (night blindness, reduced visual field side) to a possible total blindness [9-12].

1.3.2. Age related macular degeneration (AMD): It is one of the most common causes of irreversible blindness that affects about 50 million people worldwide. In humans is defined as an ocular disorder that destroys the macula, the part of the retina with the highest concentration of cones, responsible for visual acuity and color vision [10].

*1.3.3. Light pollution: A*rtificial lighting has drastic consequences for human such as retinal damage and disrupt of biological rhythms [13, 14]. Light pollution by day could accelerate the processes of death in RP and increases the incidence of AMD.

In early stages of the RD the main changes are the reduction of the amplitude of the main scotopic response waves the a and b-wave (upon dark adaptation), with preservation of the photopic response (upon adaptation to light). In more advanced stages, both responses are affected in different ways, showing an important decrease in the a-wave. In stages near blindness, minimum ERG responses are registered barely differentiable from noise (≈5μV), which can be considered abolished recordings characteristic of the disease [15, 16]. This abolished registers could be hiding relevant information in time domain that could be useful for an early diagnosis. There are different types of ERG recordings that allow this analysis [17], however there is no clear definition of the changes they suffered due to the evolution of the RD, since no studies have been conducted on which and in what exact time of the disease the ERG recordings are altered.

1.3.4. RD Models: They are widely used to study the mechanisms of cell death in RP and AMD. Different methods of exposure (white light, monochrome, high or low intensity) in different animals (fish, amphibians, mammals (rat, mouse, etc.)) are used as models for different types of studies. In our laboratory we have demonstrated RD in an animal model causing photoreceptor cells (rods) death by

constant exposure to 200 lux LED light. This could be due to constant activation of the phototransduction cascade by exposure to low intensity light without interruption.

1.4. Diagnosis of RD.

Nowadays there is not a technique for early detection of RD. When symptoms of diseases associated with RD appear may be a temporary affection of the visual system with the possibility of conducting a treatment to stop the progression or the permanent loss. Because of this in the clinical and experimental research, different early diagnostic techniques are being sought. That would allow the ophthalmologist to perform an accurate diagnosis in early stages of the disease.

The Association of affected people with RP from Euskadi ("AARPE"), established as a clinical examination protocol to confirm the diagnosis or monitor the evolution of the disease, the following list of tests:

1.4.1. Ophtalmological examination: It consists of inspection methods and evaluation of the integrity of the anatomical structures of the eye. Visual acuity, visual field, intraocular pressure (IOP), dark adaptation, color vision are measured.

1.4.2. Electrophysiological examination: It consists of an integrated assessment of the visual system functionality by measuring biopotentials in different anatomical structures that conducts the visual information. The techniques used are the ERG and visual evoked potentials (VEP).

The AARPE differentiate these techniques with those used for early diagnosis, and presumably the ERG "would allow early detection of new cases and the possibility of studying families or even isolated individuals from the initial stages when night blindness is the only symptom or even before symptoms appearance. The ERG can detect anomalies in phototransduction (electrochemical) before making changes in visual function (night blindness)". Furthermore it suggests that the ERG would be a useful tool to evaluate effects of treatments before detecting photoreceptors dysfunction. The intention is to capitalize the time earned with this tool to prevent or delay the arrival of early symptoms and further histological retinal damage [19].

2. Materials and Methods

2.1. Animals and induced retinal degeneration:

3 months aged male albino Wistar rats were bred in a bioterio under controlled temperature, humidity and illumination (80 lux) conditions. The animals were exposed to a 12:12 hs light-dark cycle (LD) with food and water *ad libitum*. Under these conditions LD control recordings were taken. Dark Control (DD): Animals were kept in total darkness for 4 days (DD4).

Experimental groups: Animals exposed to constant low intensity white-LED light (200lux) during 1 to 8 days: LL1, LL2, LL3, LL4, LL6, LL8 respectively.

2.2. ERG recording

ERG signals were recorded from control animals (n=40). The animals were anesthetized with ketamine hydrochloride and xylazine hydrochloride administrated intraperitoneally (xylazine, 200µl/100g) ¾ ketamine, ¼ xylazine (one hour of total anesthesia is achieved manteining pupilar reflex and flicker). Pupils were fully dilated with a drop of tropicamide (Alcon Laboratories 0.5%), 10 minutes before the placement of the electrodes and topical anesthetic proparacaine (Alcon Laboratories 0.5%) was applied to ensure good electrode-corneal interface and avoid flicker. The acquisition was made in both eyes simultaneously.

Corneal (monopolar) electrodes were adapted to the size of the rats' eyes. The electrodes were hand made with malleable silver wires loop-shaped placed and held in the bulbar conjunctiva to avoid motion artifacts. The reference and ground electrodes used were needle monopolar electrodes placed

subcutaneously in the ear and tail respectively. After positioning the electrodes to animals left 10 minutes in dark adaptation. The electrode placement was performed under red dim illumination. This maneuver does not significantly affects the dark adaptation. Scotopic ERGs were recorded from both eyes simultaneously and 10 responses to flashes of unattenuated white light (5 ms, 0.1 Hz) from a full-field photostimulator LED (light-emitting diodes) set at maximum brightness (3 cd s/m2 without filter) were amplified and filtered (1.5Hz low-pass filter, 300Hz high-pass filter, notch filter activated) and averaged (Akonic BIOPC, Buenos Aires, Argentina). All the acquisitions were always performed at the same time of the day, so there is no interference with the circadian rhythm of the rat.

2.3. Temporal Analysis

The a-wave amplitude was measured from the pre-stimulus baseline, down to the negative peak signal, while the b-wave amplitude was measured from the baseline pre-stimulus to the peak positive deflection due to depolarization of bipolar cells followed by the a-wave. Latency times were measured from the moment of stimulation to the point where the maximum amplitude values of the a and b-waves are taken, respectively called "a-wave latency" (TLA) and "b-wave latency "(TLB). Each record has length N = 510.

2.4. Frequency Analysis

Registers in time domain were saved with the .txt file extension. Then with the software MATLAB 2012b (Math-works, Natick, MA, USA) were imported and read. Fast Fourier Transform (FFT) was applied to the signals individually, as follows:

$$X(k) = \sum x(t) e^{-i(2\pi/N)tk}, \text{ k=0,1,…,N-1} \quad (1)$$

X(k) represents the FFT coefficients, x(t) is the ERG signal in the time domain, N is the number of samples. Each FFT coefficient conteins the weight of the frecuency component of the signal. Then the absolute value of X (k) can be plotted to show the frequency spectrum. N = 512 are taken with the addition of two equal endpoints at the position 510. The frequency analysis may be a useful tool to indicate events that in the time domain are not easily noticeable. Some main frequency components were identify and associated to the maximum power components [20-23], labeled 1, 2 and 3, and compared control signals against experimental groups.

2.5. Statistical Analysis

Mean values and standard deviations for the two main components of the ERG in the time and frequency domains were computed. To make comparisons a one way-ANOVA was performed and then a mean comparison tests were reported in each of the results. The statistical tool INFOSTAT (2013 version InfoStat Group, FCA, National University of Cordoba, Argentina) was used.

3. Results

The analysis in time and frequency domain are shown below. Also we show a comparison of the control recordings in the time domain against the RD ones.

3.1. Analysis in the time domain

As shown in Table 2 the analysis in the time domain allows the identification of the major components of the ERG recordings (a- and b-waves) by their amplitudes and latencies in normal registers, and in Table 3 the same analysis with the RD data is shown. In control recordings, the a-wave is at 42.18 ± 7.22 ms (mean ± standard deviation (SD) with n = 40) with an amplitude of -22.2 ± 6,7μV and b-wave 141.63 ± 27 , 61ms with an amplitude of 85.4 ± 29,82μV. Comparison with the recordings of the experimental groups were performed. Figure 2 shows the ERG registers of the control and experimental groups, where the progression of the RD is shown. Throughout the days of constant light exposure, the amplitudes tend to decrease, and the latency time increase, reaching abolished records (in time domain),

so that the main components are not easily identifiable; this is the reason why no values for the experimental groups LL8 and LL6 are presented. The results of statistical analyzes are presented in Figures 3 and 4. In Figure 3 the quantitative data obtained as mean ± SD of "n" values taken in each experimental group are expressed. A one-way ANOVA was conducted to determine homogeneity of variance of the data and then applied Tukey post-hoc test as a test for means comparison between groups with p-value <0.05. Apparently, the RD would be altering mainly the latency of the two principal waves as shown in Figure 4. As expected, in the a-wave of LL4 a decrease in its amplitude is observed, simultaneously with an increase in the latency time, both statistically significant versus the control group, LL1, LL2 and even LL3. This would indicate a critical event of photoreceptor cells dysfunction.

3.1. Analysis in the frequency domain.

The power spectra of control ERG signals were compared against the experimental model ones. In all cases three principal low-frequency power components are found (from now on called Components 1, 2 and 3). The results of the statistical analysis are shown in Figure 5. Quantitative data are expressed as mean ± SD obtained from the "n" values taken from each experimental group. A one-way ANOVA was conducted to determine homogeneity of variance of the data and then applied Tukey post-hoc test for comparison of means between groups with p-value <0.05. No significant differences in the component 1 from all groups were reported. Therefore just the analysis of the components 2 and 3 are reported. In both frequency components it is shown that in LL3 there would be a significant difference in comparison with the other experimental groups and finally also in LL8. The coefficient of variation (CV) from the analysis performed on the component 2 shows that the results would be more reliable than the ones from the component 3.

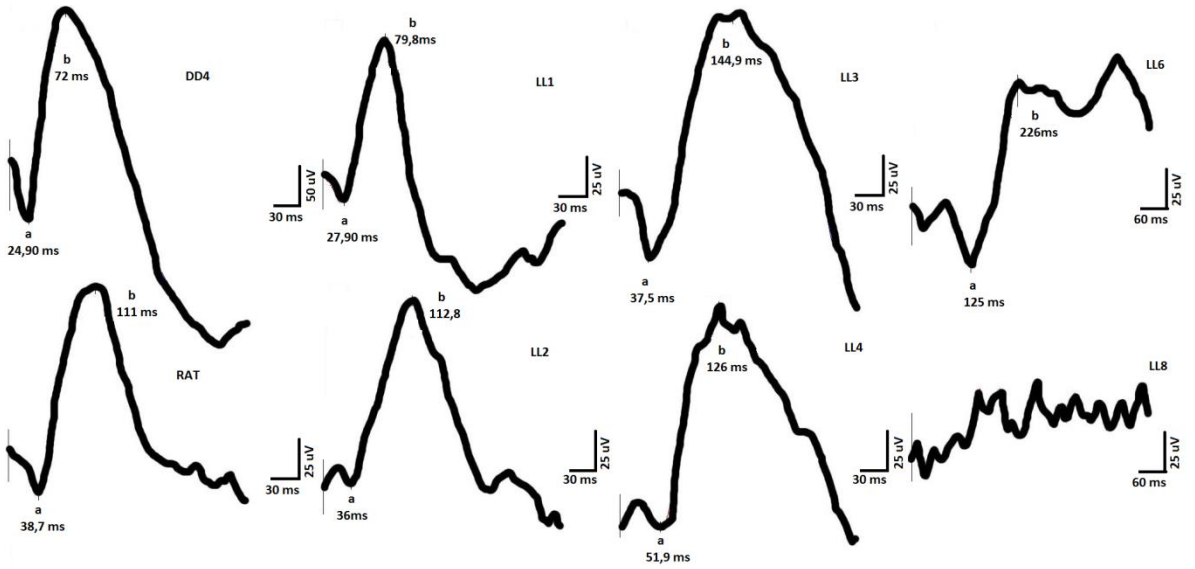

Figure 2: Control ERG vs RD.

Table 2: Analysis in time domain from the control group

Component	Latency time (ms)	Frequency (Hz)	Amplitude (μV)
a-wave	42,18±7,22	n/a	-24,1±9,27
b-wave	144,26±27,24	n/a	85,55 ± 29,82

Table 3: Results of the analysis in time domain from the experimental groups

	Component	Latency time (ms)	Amplitude (µV)
DD4	a-wave	56±14.18	-27.2±8.1
n=7	b-wave	135.43±20.16	83.77 ± 29.6
LL1	a-wave	36	-19.65
n=1	b-wave	133	93.1
LL2	a-wave	60	-20
n=3	b-wave	190	45
LL3	a-wave	60±0.94	-26±0.2
n=7	b-wave	214.67±2.31	59±0.9
LL4	a-wave	88.2±1.1	-10.77±2.62
n=7	b-wave	247.33±9.8	62.39±6.63

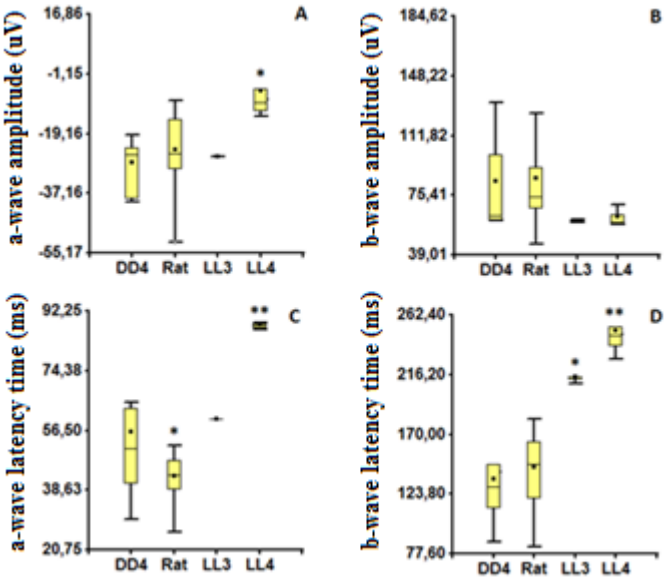

Figure 3: ERG characteristics comparison by experimental group. One-way ANOVA conducted and post-hoc test TUKEY *, **: p-value<0.05. (**A)** a-wave amplitude, (**B**) b-wave amplitude without significant differences, (**C**) a-wave latency time VC=14.9, (**D**) b-wave latency time VC=14.5

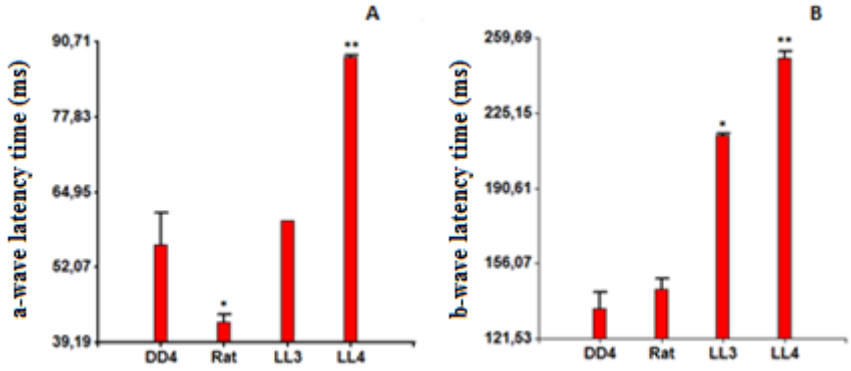

Figure 4: a- (A) and b-wave (B) latency time mean comparison.

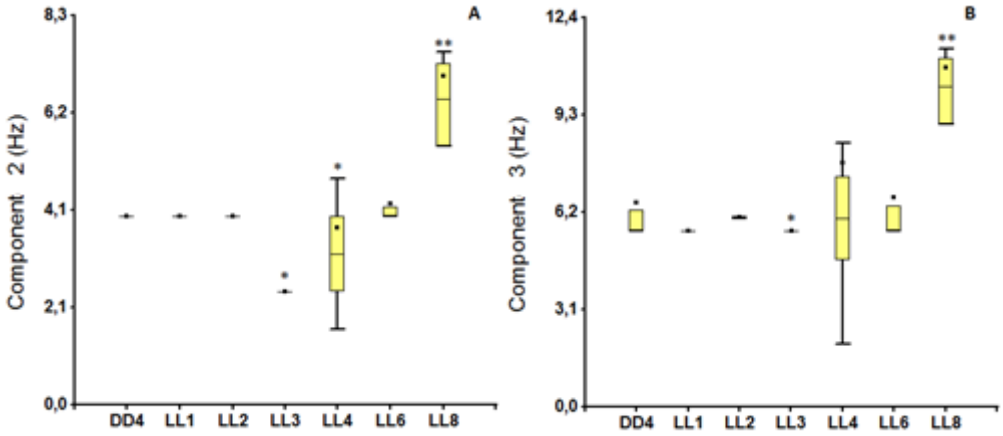

Figure 5: Comparison of frequency behavior. One-way ANOVA conducted and post-hoc test TUKEY: (A) Component 2 (*, **: p-value <0.05) VC=15.58 (B) Component 3 (*, **: p-value <0.05) VC= 25.07.

4. Conclusions

The RD is a major source of visual impairment caused by a lack of anatomical or functional integrity of the retina. The retina as a part of the CNS and is a sensible structure with little feasibility of regeneration in case of damage. The causes can be inherited or acquired, either by environmental factors or age. According to the World Health Organization (WHO) retinal diseases are the leading cause of visual disability in countries with medium-high and high socioeconomic development. The WHO suggests "make regular eye exams that allow early diagnosis of the disease and early treatment to prevent or delay the loss of visual function."

In the search of methods and noninvasive techniques for prevention and early RD detection in this paper we show that the ERG could be an appropriate tool to measure the function / dysfunction of the retina in its different cell layers.

From the results shown above we can conclude that both, in temporal and frequency analysis, the alterations caused by the RD become noticeable. According to Contin, et. al. (2013), there is a death of photoreceptors statistically significant by the 5th day (LL5) of constant light exposure. According to the results presented here it shows that LL4 could be a point of inflexion, where the cells of the retina, mainly photoreceptors, show a statistically significant dysfunction in both, time domain and frequency. Finding that point of inflection in the experimental model, the same analysis could be performed in

patients with a diagnosed RD and may apply some existing treatments. Finally we conclude that there is relevant information in the ERG signals that could be useful and as in the time domain is not evident, with the using of more powerful signal processing tools, a prolonged monitoring of the RD would be possible.

5. References

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