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ERG in *Drosophila*

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1. Introduction

The fruitfly *Drosophila melanogaster* has been a workhorse of genetics for over a hundred years, following the first report of a white-eyed mutant by T.H. Morgan (Morgan, 1910). Since then, systematic mutagenesis and subsequently, more sophisticated methods of genetic modifications have been implemented to isolate strains with specific defects in various proteins (Ashburner et al., 2005; Greenspan et al., 2004). Finally, the newly developed techniques enabled the researchers to mark, activate or silence specific cells or cell lines (Hampel et al., 2011; Gohl et al., 2011). Genetic modification and elimination of eye-specific proteins have led to fundamental discoveries in the visual pathway and the deciphering of the mechanisms of phototransduction in great detail (Pak, 2011). The prominent compound eyes of *Drosophila* have allowed for an easy selection of the visual mutants on the basis of the eye color, external eye morphology, presence of the deep pseudopupil, phototaxis, and the time course and the amplitude of ERG (Pak, 1995). *Drosophila* ERG is an excellent method for physiological evaluation of genetically induced protein modifications. It is a particularly robust and technically feasible technique which can yield high throughput and reproducible results. The limitations of ERG have become very obvious in the last 20 years due to the development of the whole-cell patch-clamp in isolated ommatidia (Ranganathan et al., 1991; Hardie, 1991) and the advanced application of sharp intracellular electrodes (Juusola and Hardie, 2001). However, ERG in *Drosophila* is an informative and well understood signal. It remains the method of choice in many studies, especially when the molecular genetic tools are extensively applied to studying cellular processes in the photoreceptor model, and when the phenotypes of numerous transgenic fruitfly strains need to be physiologically characterised in a reasonable time.

2. The visual system of *Drosophila*

The visual system of *Drosophila* consists of the three ocelli, the compound eyes and the visual ganglia – the lamina, the medulla, the lobula and the lobula plate. The retina of a compound eye contains ca. 800 ommatidia. Each ommatidium is a cluster of eight photoreceptor cells, supporting cells and pigment cells. The pigment cells contain the red screening pigment which optically isolates the ommatidia. The photoreceptor cells contain an additional, yellow screening pigment which translocates transversely in the photoreceptor soma thus conveying a pupillary response . The yellow and the red screening pigments are absent in commonly used white-eyed mutants.

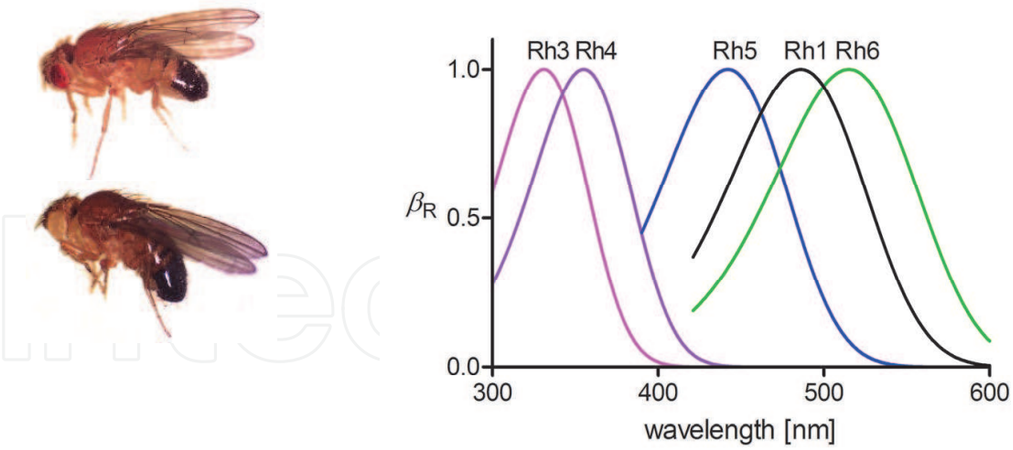

Fig. 1. Left, red and white eyed *Drosophila*. Right, relative absorbance of the five rhodopsins expressed in peripheral photoreceptors (R1-6: Rh1) and in the central photoreceptors (R7y: Rh4; R7p: Rh3; R8y: Rh6; R8p: Rh5). Shown are only the α-bands; Rh1, Rh5 and Rh6 have an additional β-band in the UV (peak height ca. 20% of the α-band peak).

Photoreceptor cells have three morphologically distinct regions: the light sensing *rhabdomere,* the *soma* with the nucleus, endoplasmic reticulum (ER), the mitochondria and other organelles, and the *axon*. The soma and the axon are sometimes referred to as the light-insensitive *basal membrane*, i.e. the membrane portion which is equipped with voltage gated (mostly K^+) channels, which dynamically shape the light response. This is not to be confused with the *basement membrane* (sometimes also the *basal lamina*), an extracellular structure separating the photoreceptor bodies from the higher order neurons in the *lamina*, an optical neuropil.

The light-sensitive part of the photoreceptor cell - the rhabdomere - is composed of ~30.000 microvilli, each 1-2 μm long and 60 nm wide. The microvillus harbours all the molecules that are necessary for the transduction of a successfully captured photon of light to a *quantum bump* – the unitary depolarization of the photoreceptor cell membrane. The microvillar membrane is packed with about 1000 visual pigment (rhodopsin) molecules, making the rhabdomere optically dense and behave as a light guide. The rhabdomeres of an ommatidium together form an open rhabdom. The rhabdomeres of the six outer photoreceptor cells R1-6 function as separate optical waveguides, which surround a central waveguide formed by the superimposed rhabdomeres of the central photoreceptors R7 (positioned distally in the ommatidium) and R8 (positioned proximally). The receptors R1-6 form an achromatic channel for detection of light contrasts and motion, while R7 and R8 receptors mediate colour vision. The R1-6 contain the Rh1 rhodopsin, which absorbs maximally in the blue, at 486 nm, and the R7&8 cells express the rhodopsins Rh3-6, maximally absorbing at 331, 355, 442 and 515 nm; the UV-sensitive rhodopsin Rh2 is expressed in the ocelli (Salcedo et al., 1999). The rhodopsin content in the eyes depends on the amount of carotenoid precursors in the food. Elimination of carotenoids from the feeding media can result in dramatic reduction of rhodopsin in the eyes (Goldsmith et al., 1964; Harris et al., 1977). On the other hand, a carotenoid enriched diet results in high sensitivity of R1-6 cells in the UV, due to the incorporation of an additional chromophore in the opsin molecule which acts as an antenna pigment molecule (Kirschfeld et al., 1983). Maximised rhodopsin content, as well as structural integrity of photoreceptors, can be easily

checked by observing the clarity of the deep pseudopupil (optical superposition of rhabdomere images or shadows in the geometrical centre of the eye). Optically, the compound eyes of *Drosophila* are of the apposition type. However, different R1-6 cells in six adjacent ommatidia are arranged so that their visual axes are pointing to the same direction, and their axons project to a common neuron in the lamina. Such information integration, which improves the image quality in dim light, is typical of the higher dipteran insects and is named neural superposition (Kirschfeld and Franceschini, 1968). The central R7 and R8 receptor axons bypass the lamina and form chemical synapses in the medulla (Cajal and Sanchez, 1915; Fischbach and Dittrich, 1989).

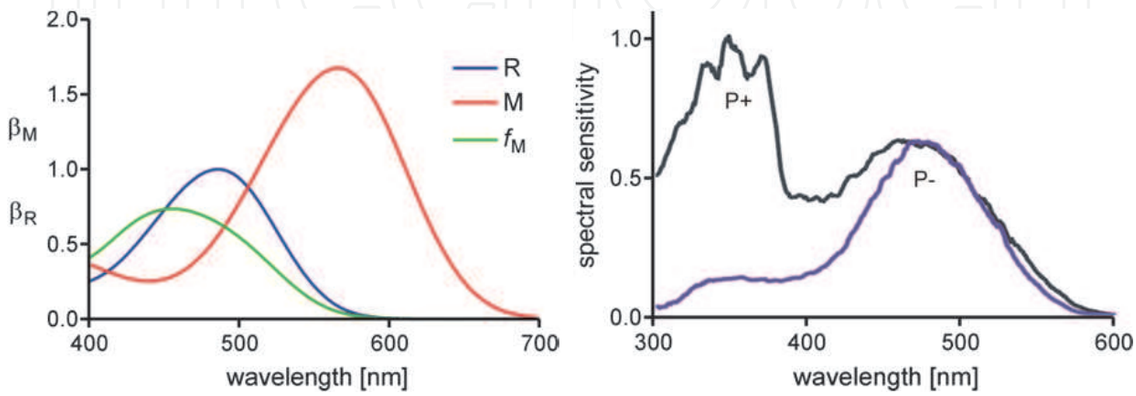

Fig. 2. Left, Spectral properties of the main visual pigment, Rh1, of *Drosophila*. The photosensitivities (β) of the two thermostable states, rhodopsin (R) and metarhodopsin (M), normalized to the rhodopsin peak, and the metarhodopsin fraction f_M in the photoequilibrium created by monochromatic stimuli with wavelenght λ. The spectra were calculated with the template functions of Govardovskii et al. (2000) using peak wavelength values 486 and 566 nm for R and M, respectively. Right, Spectral sensitivities measured via ERG of white-eyed *Drosophila* bred on a carotenoid-rich (P+) and carotenoid-deprived (P-) medium. The spectral sensitivity in P- exhibits the β-peak due to absorption by Rh1 in the UV, while the P+ has an much higher sensitivity in the UV with a fine vibronic structure (triple peaks) due to the incorporation of an additional chromophore into the Rh1 molecule.

Fruitflies are able to detect light photons at wavelengths between 300 nm and 650 nm. Upon photon absorption, their rhodopsins (R) are converted to a thermostabile metarhodopsin (M) isoform. In the case of Rh1, the absorbance peak of M is shifted to longer wavelengths (bathochromic shift) and the absorbance amplitude is higher by a factor of 1.6 (Ostroy et al., 1974; Salcedo et al. 1999). A M molecule can be converted back to R by another photon. The conversion of M to R is facilitated by the leaking of red stray light through the long-pass filtering red screening pigments (Stavenga et al., 1973; Stavenga, 2002). After a sufficient period of time, a photoequilibrium between M and R is created which depends on the spectral composition of the illumination. Short-wavelength monochromatic light can create large amounts of M. M, which triggers phototransduction (Hardie and Raghu, 2001), is quenched by the binding of arrestin. Rhodopsin to arrestin ratio has been estimated as 2.7:1 (Satoh et al., 2010). Thus, high fraction of M can out-titrate the available arrestin (Minke et al., 1975; Hamdorf, 1979; Dolph et al., 1993; Belušič et al., 2010; Satoh et al., 2010). In the white-eyed mutants illuminated with conventional light sources, the photoequilibrium with high metarhodopsin fraction (f_M) can be established within seconds. Thus, a state of

maximal receptor depolarization and absolute desensitisation is created, which can persist for many hours in the dark. This phenomenon is called the PDA, prolonged depolarizing afterpotential. A PDA can be abolished with long wavelength light. Usually, the PDA is created with blue light (460-490 nm) and interrupted with orange to red light (580-640 nm). In R7 photoreceptors, PDA can be created with UV and abolished with blue light (Stark, 1977). PDA has never been observed in R8 receptors.

3. The ERG signal

3.1 Recording preparation

The ERG is measured in immobilised living fruitflies. Fruitflies (and other flies) can tolerate extended periods of anoxia without any subsequent changes in the ERG (Agam et al., 2000). Therefore, living animals can be anaestethised by exposure to gaseous CO_2 or N_2 at room temperature or by chilling on ice. Subsequently, they are mounted to a holder, (e.g. a microscope slide or pipette tip) with wax, agarose or putty. Frequently, Krönig's mixture of bees wax and resin (colophony) 3:1 is used due to its excellent adherence to the chitin and its low melting temperature. The waxing is best performed with miniature soldering devices, such as a 12 V soldering needle (Stannol, Germany), driven at 3-4 V. It is important that the animal's abdomen with the stigmata is left untreated during the immobilization procedure in order to avoid suffocation. Suffocation is first indicated by the absence of the neural transient components of the ERG, but a somewhat slowed receptor component can persist for many minutes. The ERG can be recorded in virtually intact animals, and a stable measurement is possible for many hours or even days, if sufficiently high humidity is provided in the air surrounding the animal. The signal is usually recorded with glass micropipettes filled with physiological saline – 0.9% NaCl or insect salines of different compositions, yielding a resistance of a few kΩ to a few MΩ. An ERG can be recorded also with a chlorided silver wire or salinated cotton wicks. Direct illumination of the silver wire must be avoided since a photoelectric artifact of a few mV is easily introduced. The signal is amplified unipolarly in DC mode, with the recording electrode contacting the compound eye dipped into a droplet of EKG gel, or inserted just below the cornea. The grounded reference electrode is dipped into a gel droplet or inserted in a non-illuminated part of the body. Similarly, an ERG can be also obtained from the ocelli.

3.2 Stimulus

The stimulus for obtaining an ERG is typically a simple light pulse, lasting between 10 ms and 10 s. The light source can be a DC operated halogen lamp, a xenon arc lamp, or LED. AC driven lamps and conventional fluorescent bulbs are inadequate since they contain flickering components at 50 Hz or 100 Hz which a fly eye actually perceives as flicker, resulting in oscillations of the signal. The light from the lamps is usually filtered with long-pass (OG515, OG580, Schott, Germany) or short-pass light filters (BG family of filters, Schott, Germany). Monochromators inserted between the light and the preparation can be operated between 300 and 650 nm to produce stimuli for the measurements of spectral sensitivity or for selective light adaptation of different photoreceptor classes. Superluminescent LED sources can deliver light from 300 nm on, capable of saturating any fly photoreceptor. The intensity of the stimuli is adjusted with neutral density filters or metal wedges. In order to cover the entire dynamical working range of the *Drosophila* ERG, the intensity of the light stimuli needs to be adjusted over a range of 6 orders of magnitude, in steps no larger than

0.5 log unit. LED sources can be driven with voltage operated current sources, which allow for fast changes of intensity modulation and thus provide e.g. sinusoidally modulated stimulus or white noise, spectrally flat up to 500 Hz (fast enough even for the fastest insect photoreceptors).

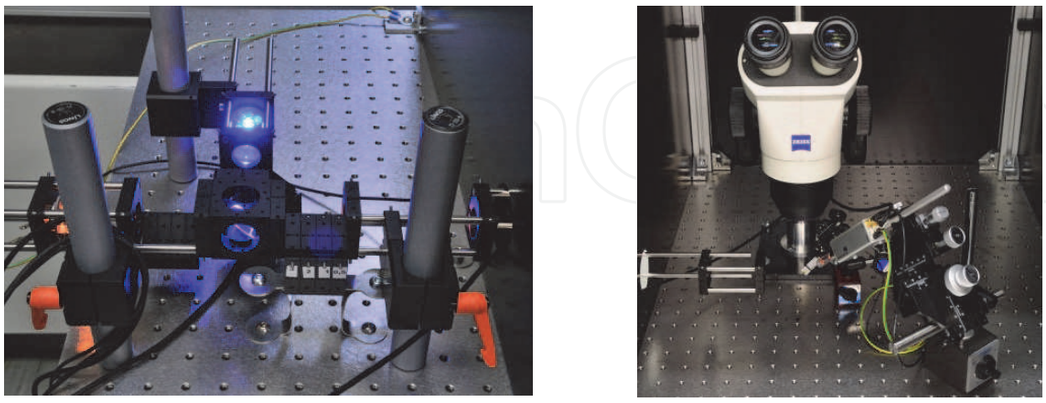

Fig. 3. A simple setup for *Drosophila* ERG, constructed by the author and dr. Olaf Voolstra. Left, Stimulator made of two superluminescent LED sources (480 nm and 580 nm, Roithner Lasertechnik, Austria), three sets of latchable neutral density filters and a 50% neutral density filter (45° inclined) as a beam mixer. Optics and mechanics by Qioptiq, Germany. Right, Faraday cage with an optical breadboard (Thorlabs, Germany), binocular (Zeiss, Germany), a micromanipulator (Märzhauser, Germany) with the recording headstage (NPI, Germany), an improvised *Drosophila* yoke made from a micropipette holder (WPI, USA), with a 50 µm diameter Ag/AgCl wire (Goodfellow, UK) attached as a reference electrode. The objective lens of the stimulus setup protrudes from the left wall of the cage. Photo: O. Voolstra, University of Hohenheim, Stuttgart, Germany.

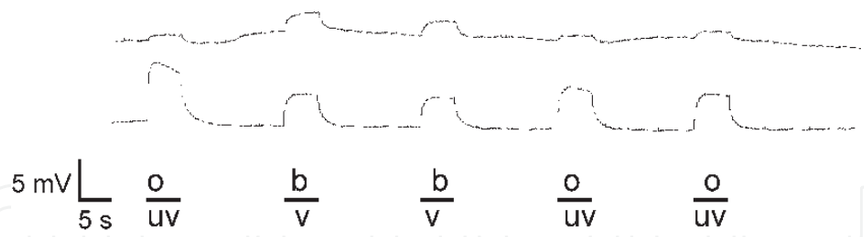

Fig. 4. The photoelectric effect. Signal in a dead animal, recorded with the Ag/AgCl wire directly illuminated with the stimulating light (stimuli corresponding to the upper trace: o - 580 nm; b - 480 nm; stimuli corresponding to the lower trace: uv – 360 nm; v – 420 nm).

3.3 Red-eyed and white-eyed fruitflies

The ERG is usually recorded in a few varieties of white-eyed *Drosophila*. White-eyed fruitflies are preferably used in biochemical experiments since the red pigment from the wild-type eyes colours the homogenates and the gels. In physiological experiments, white-eyed mutants are often preferred because they lack the pupillary response, in wild type mediated by the movement of yellow pigment granules within the photoreceptors. Moreover, uniform »ganzfeld« illumination of the entire compound eye is easily achieved in the translucent retina of white-eyed mutants. The angular sensitivity of the photoreceptors

in the white-eyed mutants is extended more than tenfold (Streck, 1972), so that image formation is heavily impaired. The photoreceptor cells of the white-eyed mutants are otherwise physiologically unaffected. However, the red screening pigment in the wild-type flies has an important role as a long-pass, red filter so that illuminations with broadband light allow long wavelength photons reaching the photoreceptors. These photons convert metarhodopsin to rhodopsin so that the metarhodopsin levels in the photoreceptors are kept sufficiently low to be manageable by the limited amount of arrestin molecules. In the white-eyed fruitflies, this is not the case. Under white light, the metarhodopsin levels in white eyes can become so high that the photoreceptors remain depolarized for long periods of time. Furthermore, in physiological experiments, short wavelength light is frequently used as a stimulus. White eyed flies then must be regularly exposed to orange or red light in order to interrupt any possible prolonged depolarizations.

3.4 ERG waveform

The ERG recorded from the retina in the described configuration (DC, unipolar, measuring electrode in the retina, grounded reference in the body) is a rather complex signal, composed of several fast transient components, and several rising and decaying sustained components (Stark and Wasserman, 1972). The photoreceptors contribute a corneal-negative, slow, sustained plateau component at medium light intensities. The second-order neurons L1 and L2 in the lamina contribute fast transient »on« and »off« components, at the beginning and at the end of the stimulus (Coombe, 1986). At light intensities which elicit maximal responses, the ERG is more complex due to fast adaptation processes of the photoreceptor cells. The receptor response is further transformed at the synapse and in the lamina, resulting in additional transients in the ERG (Järvilehto and Zettler, 1971; Laughlin et al., 1987).

In order to understand the nature and origin of the ERG time course in detail, it is necessary to delineate the underlying responses of the photoreceptor cells and second order neurons. All photoreceptor cells (R1-8) respond to a simple light pulse with a sustained depolarization, occurring with a delay as short as 3 ms. This so-called receptor potential lasts as long as the stimulus. Its amplitude is proportional to the logarithm of the stimulus intensity. At high light intensities, the large depolarization of the photoreceptor at the onset of the pulse is reduced after 50-100 ms by fast adaptation mechanisms to a lower level. This results in a sharp spike at the beginning of the receptor potential. In light adapted flies, the fast adaptation is frequently followed by a damped oscillation of the receptor potential plateau. The receptor potential is detected by the ERG electrode in the extracellular space as a corneal-negative signal, with a somewhat smaller amplitude (e.g. -20 mV between the retina and the body, instead of the 40 mV of the transmembrane depolarization), and its time course resembles an inverted receptor potential. The photoreceptors R1-6 convey the receptor potential to second order neurons in the lamina, the L1 and L2. These neurons transform the receptor potential to a sequence of decaying transient components and thus seem to work analogously to the on- and off-bipolar cells in the vertebrate retina (Joesch et al., 2010). The laminar neurons act as high-pass filters with strong amplification at high frequencies, thus greatly amplifying small changes in the receptor potential (Autrum et al., 1970; Uusitalo and Weckström, 2000). The combined signal from L1 and L2 is very well detected by the ERG electrode and contributes a corneal-positive »on« transient at the beginning of the light pulse, and a corneal-negative »off« transient at the end of the pulse.

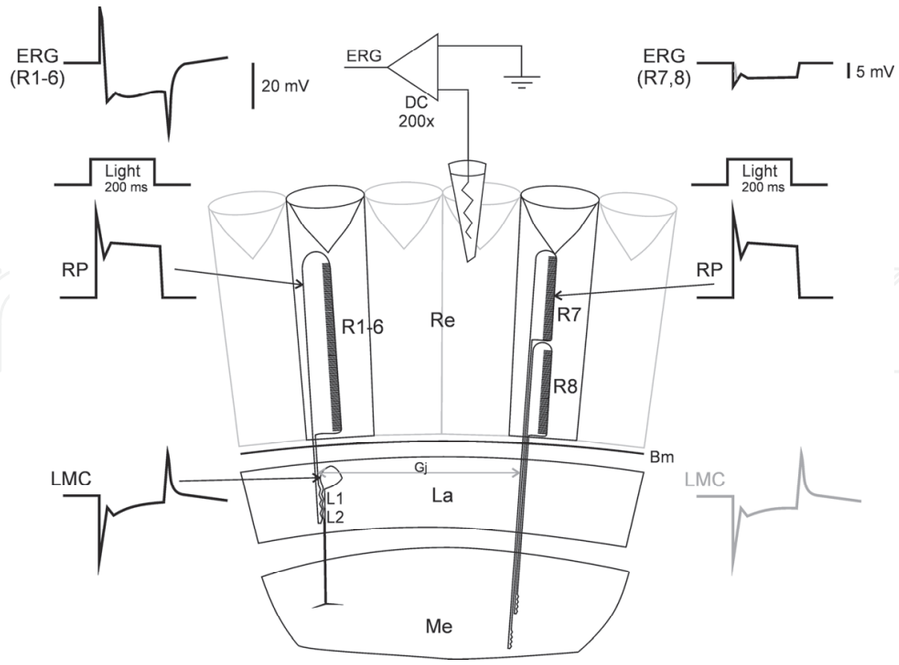

Fig. 5. Origin of the ERG waveform in the retina (Re), recorded in DC mode, unipolarly across the basement membrane (Bm). Left, the ERG from the photoreceptors R1-6, which is an extracellular summation of the inverted receptor potential (RP) from R1-6, and the inverted signal from the large monopolar cells (LMC) L1 and L2 in the lamina (La). Right, the ERG from the photoreceptors R7 and R8, which is a scaled-down, inverted receptor potential from R7 and R8. The large LMC transients are absent because the R7 and R8 form synapses in the medulla (Me). Sometimes, the ERG from R7 and R8 is accompanied with an on-transient (ERG R7,8, grey trace), since R7 and R8 form gap junctions (Gj) with the R1-6 axons in the lamina.

The photoreceptors R7 and R8 form chemical synapses in the medulla and the synapsing higher order interneurons do not contribute any signal to the ERG. Yet, light responses of the R7 and R8 cells are sometimes accompanied by transients, which are presumably due to electrical synapses between the R7 and R8 and the axons of R1-6 and accompanying R1-6 signals to L1 and L2 (Shaw, 1984; Juusola, personal communication).

The *Drosophila* ERG is probably shaped also by the pigment cells which respond to the changes in the ionic composition of the extracellular medium next to the photoreceptors by actively pumping ions (Hamdorf et al., 1978). The receptor potential plateau during long stimuli thus slowly decays, which is reflected in a not fully sustained ERG. The ERG at high light intensities is often accompanied by corneal-positive afterpotentials, in the dark after the stimulus, which is probably due to the hyperpolarizing action of the activated Na^+/K^+ pump in the receptor membrane (Jansonius, 1990).

4. Quantitative analysis of ERG

4.1 ERG amplitude and stimulus-response relationship

The ERG recorded with an electrode in the distal retina and the reference elsewhere in the body is actually measured as a change in the voltage drop across the basement membrane.

The lateral resistance between the ommatidia is low, so that the ERG is not a local phenomenon. The basement membrane separating the retina and the lamina functions as a blood-brain barrier and has a much larger resistance; even in the dark, a voltage drop across the eye of 30-50 mV exists (Heisenberg, 1971). Illumination of only a few ommatidia of a red-eyed fruitfly creates an ERG amplitude of only a few mV, even at high light intensities. In a white-eyed fly, the ERG in a brightly illuminated eye can measure more than 40 mV peak-to-peak, with a sustained (DC) component of as much as 30 mV. The extraordinary large amplitude of the ERG in the uniformly illuminated eye is a result of the concerted activity of a large number of photoreceptors, which produce large currents running across the high resistance of the basement membrane.

Other measuring configurations allow for isolation of specific ERG components. First, if the measuring electrode is advanced into the retina, the basement membrane is penetrated at ca. 120-150 μm from the cornea. This is marked by a sudden voltage drop of ca. 15 mV and a change in the ERG shape. At a depth of between 120-200 μm below the cornea, the receptor component becomes negligible, so that the neuronal transients dominate the ERG response . Further advancement of the electrode leads to a complete loss of the ERG signal. If the reference electrode is placed deep into the receptor layer, just above the basement membrane, the ERG attains the shape of inversed receptor potentials and no neural transients are detectable in the signal. The effects of different recording configurations are described in great detail by Heisenberg (1971).

Both the photoreceptor and the neuronal ERG components are graded with respect to the stimulus intensity. The ERG amplitude is graded over a very wide range of stimulus intensities, spanning more than 6 log units. The relationship between the logarithm of light intensity and the sustained ERG component is very well described by the Hill equation

$$V/V_{max}=I^n/(I^n+I_{50}{}^n) \quad (1)$$

where V/V_{max} is the normalized ERG amplitude, I is the logarithm of the relative light intensity ($\log(I/I_{max})$; at $\log I=0$, $V=V_{\max}$,, n is the Hill slope (slope of the sigmoid curve at the half maximum) and I_{50} is the light intensity producing 50% of the maximal ERG amplitude. The Hill coefficient or Hill slope n is used to describe the dynamic working range of a photoreceptor cell. If measured intracellularly, n in *Drosophila* photoreceptors is typically around 0.6 (Wu and Pak, 1978). The Hill slope as estimated from the ERG in white eyed fruitflies in the dark adapted state is as low as 0.49 (Belušič et al., 2010). This means that the estimated photoreceptor dynamic working range via ERG is 5-6 log units or orders of magnitude, and 3-4 log units via intracellular measurements. The dynamic working range via ERG is obviously exaggerated by 2 log units or 100-fold. The difference between the two methods is probably due to the different illumination conditions.

In intracellular measurements, a single cell is illuminated on-axis, and the whole dynamic range of a photoreceptor, from single quantum bumps to saturation is easily studied. In the ERG, oblique illumination with a beam of a limited numerical aperture is most often used, so that a population of the photoreceptors is not illuminated directly, but rather with the scattered light penetrating the retina. The photoreceptors in the white eyed mutant still retain a fraction of directional sensitivity (Streck, 1972) and are progressively recruited as the oblique beam gets stronger. Thus, the saturation state can be reached only at very high light intensities, often higher than available. The problem is even more pronounced in the red eyed flies, but can be overcome by using a hemispherical diffuser around the compound eye, providing real »ganzfeld« illumination. Anyhow, the measurement of the entire

dynamic working range is feasible with proper illumination, allowing for very precise estimates of light sensitivity in different populations of the fruitfly.

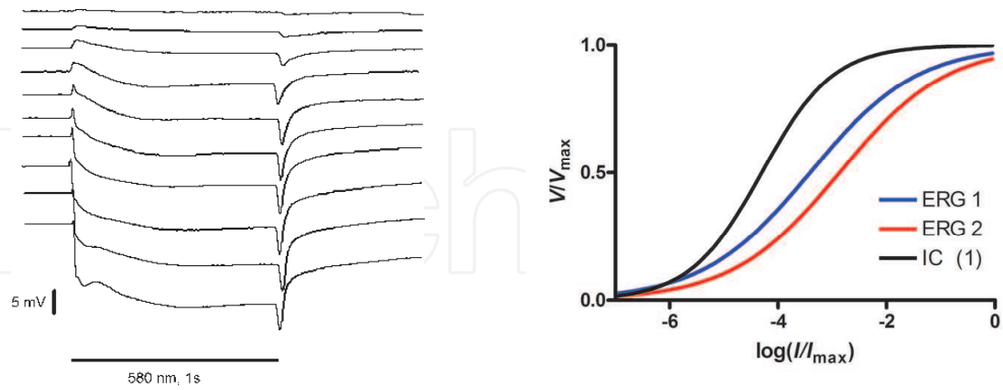

Fig. 6. The stimulus-response relationship. Left, Sequence of ERG recordings from a wild-type, white-eyed fruitfly; stimulus intensity attenuated from -4.5 log ($10^{-4.5}$) to 0 log (full light) in 0.5 log steps. The response is not saturated. Right, The »V-log *I* curve« as obtained with ERG and with intracellular recordings. Curves are plotted with the Hill equation (1) with the Hill slope n = 0.49 (ERG 1,2) and 0.66 (IC (1)), respectively, and I_{50} = -3.4 (ERG 1), -2.9 (ERG 2) and -4.3 (IC). The intracellular curve (IC (1)) corresponds to a hypothetical photoreceptor in the most illuminated part of the eye in ERG 1. The ERG curves are shallower than the IC curve due to non-ideal illumination of the retina and the progressive recruitment of the shaded photoreceptors at high light intensities. The working dynamic range of the photoreceptors (3-4 orders of magnitude, IC) as measured intracellularly is exagerrated in the ERG (5-6 orders of magnitude, ERG). The light sensitivity in the flies of ERG 1 is higher than in the flies of ERG 2 by 0.5 log units, i.e. 3.16 fold. Such differences between fly strains can be reliably measured with appropriate instrumentation and skills.

The ERG in *Drosophila* is a very sensitive method. In the white eyed, wild-type flies, the half maximal intensity (I_{50}) corresponds to the photon catch rate of 2.9×10^4 photons s^{-1} per receptor cell (Belušič et al., 2010). Since the signal is detectable already at the light intensities that are three log units lower, i.e. $10^{-3} \times I_{50}$, it follows that at the threshold of the ERG, the rate of photon absorption per receptor is as low as 10 s^{-1}, when the individual quantum bumps are still discernible in the intracellular recordings. The weak supratreshold ERG signal is however characterized only by the neural transients. Namely, the *Drosophila* eye belongs to the neural superposition type, typical of the higher dipteran insects. This means that the axons of the six R1-6 photoreceptors in the adjacent ommatidia which are directed into the same point in the space project to the common neuron in the lamina. Thus, a laminar neuron receives the input from a larger area, covered by six photoreceptors, without much loss in the angular resolving power, and the limited sensitivity of the eye due to the apposition optical design is greatly enhanced. In addition, laminar neurons strongly amplify the small changes in the receptor potential: even the single quantum bumps are faithfully transmitted to the brain (Dubs et al., 1981). Due to the nature of the neural summation of photoreceptor responses, the amplification and the filtering properties of the laminar neurons, i.e. the neuronal component of the ERG signal is detectable even at very dim light, where the photoreceptor response is dominated by single photon absorptions.

4.2 The dynamic properties of ERG

The dynamic properties of the ERG should be treated with great caution. Firstly, *Drosophila* is a poikilothermic animal and the rates of its physiological processes depend strongly on the temperature. The eyes of the flies (*Calliphora vicina*, *Drosophila melanogaster*) can operate at temperatures of up to 45°C, and the ERG can be detected even at -10°C. The ERG parameters exhibit temperature quotients Q_{10} between 2 and 4 (Hamdorf and Keller, 1962; Stušek et al., 2000). Therefore, it is very important to control the temperature of the preparation and avoid possible heating of the eyes with broadband light sources, which contain infrared light. Secondly, the latencies and rates of depolarization and repolarization depend strongly on the state of the light adaptation and on the effective illumination intensity and tend to become shorter and quicker with the increasing light adaptation. Neural transients are strongly high-pass filtered and amplify the fast components of the receptor response. The amount of the neural ERG transients depends on the quality of the preparation, the ability of the animal to ventilate and on the depth of the electrode within the retina. The visual system of a fruitfly can follow the stimulus flicker of up to 80 Hz at the room temperature, but the signal at the high frequencies originates exclusively in the lamina neurons. If the contribution of the neurons is diminished for any of the aforementioned reasons, the ERG waveform will become slower and the flicker response will cease (flatten) at lower stimulus frequencies.

The speed of repolarization as measured in the ERG seems to reflect the amount of arrestin available in the microvilli (Lee et al., 2003; Satoh et al., 2010) or the ability of arrestin to bind to metarhodopsin (Elsaesser et al., 2010). In the dark adapted state, microvilli contain only 25% of the total arrestin available. With light adaptation, the remaining arrestin is progressively translocated from the soma to the rhabdomere, and the repolarization is accelerated (Satoh et al., 2010).

4.3 The PDA paradigm

Extended or very bright illumination of a fruitfly eye can create any desired fraction of metarhodopsin (f_M) between 0 and 0.7. The easiest way to establish a defined photoequilibrium is to use bright monochromatic light, since the absorbance characteristics of R and M are well known and allow for the precise, wavelength dependent setting of f_M. f_M >0.37 will create a PDA, i.e. maximally depolarize the photoreceptor cell for many hours, even in the dark. PDA can be created with light at wavelengths below 520 nm, and is quickly abolished with orange or red light above 580 nm, which brings the f_M nearly to 0 and leads to repolarization of the photoreceptors. During the blue-light induced PDA, receptors R1-6 are inactivated, but the responses of R7 and R8 can still be elicited with blue, violet or UV pulses (Minke et al., 1975). In the UV-sensitive R7y and R7p receptors, PDA can be created with UV light and abolished with blue light (Stark, 1977). PDA in R8 receptors has never been observed. During the PDA in R1-6, it is not only possible to isolate the ERG of R7 and R8, but also of R8y exclusively in the mutant sevenless (*sev*, lacking R7y, R7p and R8p). PDA represents the maximal metabolic load to photoreceptors and reflects their ability to sustain maximal depolarization for an extended period of time.

The most frequently applied sequence of light pulses presented during a PDA assay is orange – blue – blue – orange – orange (OBBOO), each pulse 5-10 s long, with 10-20 s dark intervals. The first O pulse converts all M to R; the first B creates high f_M and PDA; the second B is to make sure the f_M is maximal or to further increase f_M if the first B was too weak or short; the second O reconverts M back to R and and starts the repolarization; the

last O pulse is applied to check if the preceding O was sufficient to bring f_M next to 0. PDA has been traditionally used to characterise the phenotypes of visual mutants. This protocol also allows for separation of the photoreceptor activation from inactivation and deactivation.

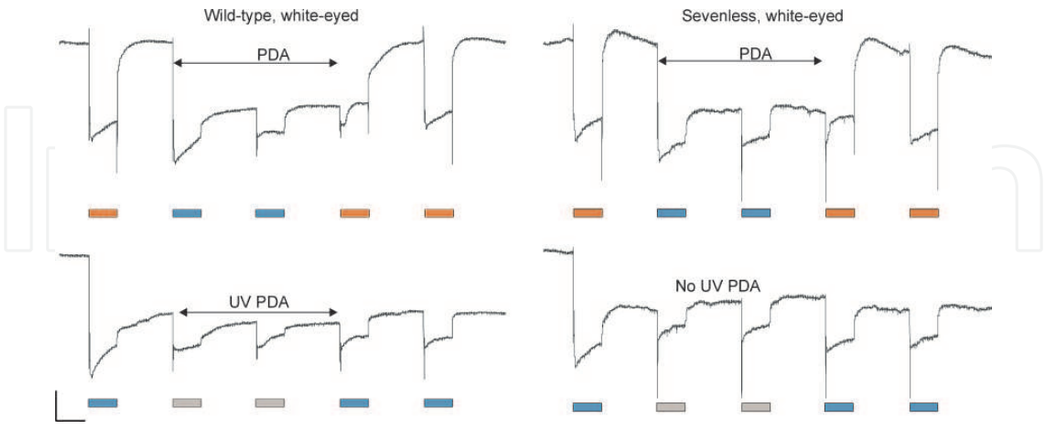

Fig. 7. The PDA paradigm. Left, PDA in the wild-type. Right, PDA in the *sevenless* mutant. Upper row, PDA elicited with blue light (473 nm, 5 s) and abolished with orange light (580 nm, 5 s). Lower row, PDA elicited with blue light (473 nm), additional PDA in R7 cells elicited with UV light (360 nm, 5 s; grey rectangles). In the wild type, PDA isolates the response of R7 and R8; additional UV isolates the ERG of R8y and R8p (lower row, left, second UV pulse). In *sevenless*, R7 and R8p cells are absent; »blue PDA« isolates the response of R8y (upper row, second blue pulse; lower row, UV pulses), which do not enter PDA under any condition. Calibration bars in the lower left corner, 5 s and 5 mV.

Sometimes the blue light pulse cannot provoke a PDA, photoreceptors repolarize vigorously, and the ERG returns back to the baseline. The inability to provoke a PDA can indicate low rhodopsin content due to the lack of carotenoids in the food. Rhodopsin levels are low also in the mutants of the Rh1 gene *nina*E. The *nina*E mutant has been named after its inability to enter a PDA, the property named »neither inactivation, nor activation«, abbreviated into *nina*. PDA is absent also in certain retinal degeneration mutants during early phases of degeneration of photoreceptors R1-6 (such as in the mutant *rdg*C, retinal degeneration C).

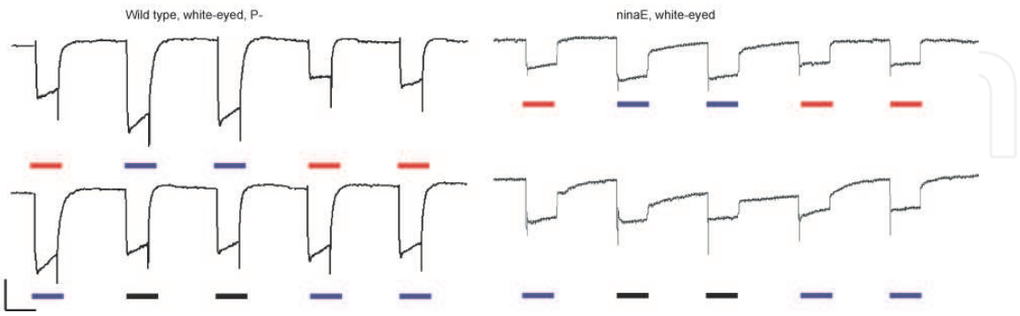

Fig. 8. Left column, absence of PDA in the white-eyed, wild-type fruitfly, reared without the carotenoids in the food. Note the transients indicating that the R1-6 receptors are functional. Right column, same paradigm applied in a *ninaE* fly. The absence of large transients indicates that only receptors R7 and R8 are functional. R7 enter PDA in the UV. Rectangles, 5 s stimuli (orange, 580 nm; blue, 480 nm; black, 360 nm). Calibration bars on the lower left indicate 5 s and 5 mV.

Fruitfly strains can be compared with respect to the amount of blue light, required to enter PDA. The amount can be set by varying pulse intensity or duration. A more precise method is to set a defined f_M in order to provoke PDA with a series of long pulses at different wavelengths. PDA creation depends on the ratio between rhodopsin and arrestin or the ability of arrestin to inactivate metarhodopsin. Thus, the quantity of arrestin can be calculated from the f_M required to provoke a half-maximal PDA (Belušič et al., 2010). PDA is created at low f_M in the mutants of the visual system specific arrestin, *arr*2.

In certain fruitfly mutants, the receptor potential cannot be sustained during the light pulse. The ERG returns to baseline, but a subsequent light pulse does not elicit a response: the photoreceptors are inactivated. Obviously, PDA cannot be created. Such ERG waveform has been named the *transient receptor potential* and was described in the first discovered visual mutant of the fruitfly, *trp* (Cosens and Manning, 1969). The mutant lacks the canonical TRP channel which mediates the major portion of the light-induced current in the photoreceptors. Similar phenotype can be observed in the mutants of the *ina*D class which have mutated or lack the scaffolding protein INAD. This protein anchors TRP and, as a consequence of the mutation, TRP is lost from the microvillar membrane. The abbreviation *ina* denotes the phenotype »inactivation – no afterpotential«, describing the absence of PDA and photoreceptor inactivation.

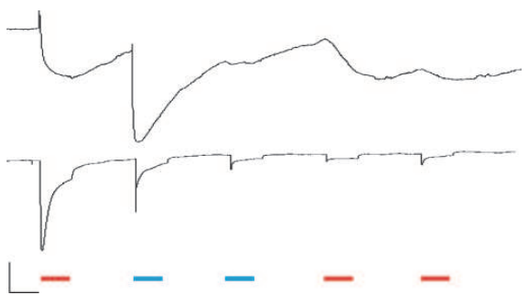

Fig. 9. Transient ERG caused by the transient receptor potential in a *inaD*^{1} mutant (upper trace) and in a *trp*^{P343} mutant (lower trace). Calibration bars, 5s, 3 mV.

All the key players in *Drosophila* phototransduction have been so far studied via mutations which rendered them non-functional. Elimination of certain proteins resulted in very pronounced, easily discernible ERG phenotypes. These were the rhodopsin Rh1 (gene and mutant *ninaE*), the light operated channel TRP (*trp*), the scaffolding protein INAD (*InaD*), and the arrestin 2 (*arr2*). Mutation in the gene *norpA* which eliminated the enzyme phospholypase PLCβ resulted in the absence of receptor potential (no receptor potential; *norp*). However, a number of different mutations have yielded weak ERG phenotypes, converging phenotypes or phenotypes hard to explain. Such were the mutations which eliminated the TRPL channel (resulting e.g. in oscillations of the ERG), yielding an ERG phenotype similar to the one in the flies with arrestin 1 eliminated (Belušič, 2003). Elimination of the protein kinase C (eye specific PKC) gene *inaC* resulted in an inactivation phenotype, similar as in *inaD*.

A number of mutations that include protein eliminations, point mutations or ectopic protein expression result in a general degeneration phenotype. This phenotype is manifested by delayed repolarizations, responses that are diminished over time, and the gradual disappearance of the R1-6 response.

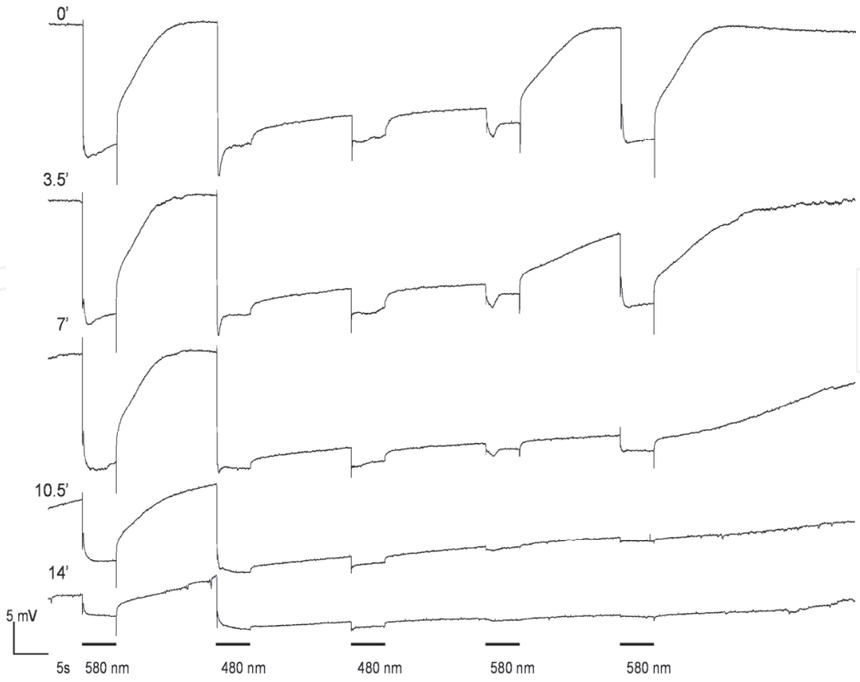

Fig. 10. ERG phenotype in a retinal degeneration mutant *rdgC*. The fly has been dark-raised for a week. Exposure to bright light in the ERG setup elicited rapid decay of the photoreceptors within minutes (numbers at the left of the traces), characterized by progressively slowed repolarization and decreasing amplitude.

5. Future applications of ERG

5.1 ERG in combination with other techniques

ERG in *Drosophila* is a feasible and robust technique that allows for simultaneous measurement of other physiological signals from the eyes. These are the measurement of ionic concentrations with the ion-selective electrodes (Agam et al., 2000), monitoring of pO_2 with carbon fibre electrodes (Meglič et al., 2009), spectrophotometrical measurement of redox states of mitochondria in the photoreceptors (Zupančič, 2003; Meglič and Zupančič, 2011), photometrical estimation of metarhodopsin to rhodopsin ratio (Belušič et al. 2010), or monitoring of GFP marked proteins in the eyes (Meyer et al,. 2006). Exclusive ectopic expression of UV rhodopsin in all functional photoreceptor classes allows for separate photoreceptor stimulation at short wavelengths and optical measurements at long wavelenghts (Liu et al., 2008).

5.2 *Drosophila* as a model for human disease

Drosophila is an animal model for numerous human diseases (Pandey and Nichols, 2011). Nearly 75% of human disease-causing genes are believed to have a functional homolog in the fruitfly. The human diseases available for research in *Drosophila* include Parkinson's disease, Alzheimer's disease, several cardiac diseases, amyotrophic lateral sclerosis, fragile X syndrome, hereditary spastic paraplegia, Huntington disease, hypoparathyroidism-retardation-dysmorphism syndrome, Machado-Joseph Disease, metabolic diseases, diabetes, obesity, microcephaly, neuronal ceroid lipofuscinosis, Niemann-Pick disease, polyglutamine

diseases, seizure disorders, epilepsy, spinal muscular atrophy, spinocerebellar ataxia 2, Werner syndrome, and X-linked mental retardation. Several mutations which are lethal if expressed in all somatic cells can be conveniently studied if expressed only in the visual system. This is easily achieved by the driver-reporter genetical systems (Greenspan et al., 2004), and the effects of expression can be elegantly followed by ERG.

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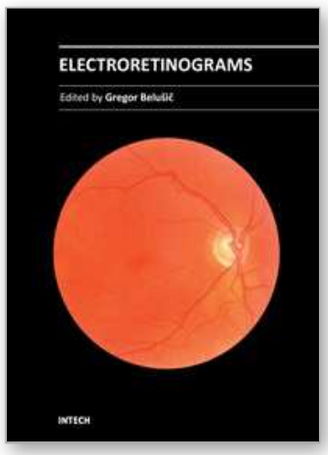

Electroretinograms

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Electroretinography (ERG) is a non-invasive electrophysiological method which provides objective information about the function of the retina. Advanced ERG allows to assay the different types of retinal receptors and neurons in human and animal models. This book presents contributions on the recent state of the ERG. The book is divided into three parts. The first, methodological part, reviews standard methods and normatives of human ERG, reports about the advanced spatial, temporal and spectral methods of stimulation in human ERG, and deals with the analysis of the multifocal ERG signal. The second part deals with the ERG in different diseases of the human visual system and in diabetes. The third part presents the ERG in the standard animal models of human retinal disease: mouse, rat, macaque and fruitfly.

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