Photostimulation of Single Cones

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INTRODUCTION

As far as we know, no experimental study exists concerning the functional activity of single cones of the vertebrate retina. It has not been directly proved that the cones are photosensitive, although such an assumption has been generally accepted mainly because of morphological reasons.

On the basis of psychophysical studies on vision, of light absorption measurements of the retina or extracts of it, and of electrophysiological studies on the activity of the second and third order of retinal neurons, a number of more or less likely conclusions have been drawn concerning the functional properties of the cones. The spectral absorption measurements made by Rushton (1-3), offer evidence for the presence of two photopigments in the human foveal cones. Hanaoka and Fujimoto (4), made measurements of the spectral absorption of the outer segments of isolated single cones (outer segment plus ellipsoid), separated from the fish retina. These authors claim the existence of five different photopigments which would indicate the presence of a corresponding number of different types of cones. The spectral response curves recorded from the second order of retinal neurons of fish retina (Motokawa (5), Svaetichin (6), Motokawa, Ókawa, and Tasaki (7), Tomita et al. (8), Mitarai et al. (9), MacNichol and Svaetichin (10), Watanabe and Tosaka (11)), indicate the existence of at least three or possibly five different types of cones.

For any theory of vision, a knowledge of the functional characteristics of the cones is of fundamental importance. All relevant theories of color vision are based on the assumption of the existence of cones having different spectral sensitivities, although this has not yet been directly proved.

All visual functions in photopic conditions are proportional to the logarithm of the illumination intensity. This is a basic law for vision, being observed in measurements relating for instance “brightness discrimination,” “visual acuity,” and “critical fusion frequency” to the intensity of illumination. Furthermore, the frequency of the spikes and the amplitude of the slow potentials recorded from the retina are also proportional to the logarithm of the...
intensity of the light stimulus. However, it has not been possible to decide whether this relationship is a characteristic that has to be assigned to the photoreceptors themselves or to the retinal neuronal networks (see the review by Jahn and Wulff (12). It has neither been clearly demonstrated whether the cones of one type of retina all have the same thresholds, and if the cones react in an "all-or-none" or a "graded" fashion.

The aim of this work has been to develop a method for microillumination which would make it possible to study some of the functional characteristics of the cone which are important to determine in order to understand the visual mechanisms. So far only white light has been used for microillumination in these experiments; however, we intend to work with different wavelengths of light. Some preliminary measurements of the spectral absorption of single cones in the surviving isolated retina have been done. A previous report on the present studies was given at the 21st International Congress of Physiological Sciences which was held in Buenos Aires, 1959 (Svaetichin and Krattenmacher (13)).

METHODS

The technical procedure of working with the isolated fish retina has been described in detail in previous papers (Svaetichin (14), Svaetichin and Jonasson (15), MacNichol and Svaetichin (10)). In the present experiments we used shallow water fishes of the Centropomidae and Gerridae species.

The general optical system, the electronic equipment, and the microelectrode technique used in these investigations have also been described in detail in the above mentioned publications. The additional optical arrangement used for the microstimulation is schematically shown in Fig. 1. The image of an illuminated small sooted platinum diaphragm (diameter 60 μ) was projected by means of an apochromatic microscope objective, and a spot of light, 4 μ in diameter, was formed within the ellipsoid of a single cone. A set of interchangeable diaphragms of progressively larger diameters was also mounted, allowing the projection of progressively bigger light spots, illuminating in this way different numbers of cones.

The main photostimulator, which was described in the papers referred to above, and which is equipped with a well corrected optical system (symmetrical aplanatic lenses), was used for the illumination of the small diaphragm. The use of a prism beam splitter enabled the simultaneous illumination of the retina from the second light source.

The small diaphragm and the objective lens used for the microillumination were mounted on Leitz micromanipulators. This arrangement enabled an accurate focusing and centering of the system and made it possible to move the light spot properly from one cone to another. The focusing of the spot into the
right level of the retina (into the cone ellipsoid), proved to be of importance for the illumination of one cone exclusively. When the optical system was properly adjusted, it was possible to illuminate one member of a double cone at a time.

![Diagram showing the general optical assembly for photostimulation of single cones. The image of a small diaphragm (down to 60 μ in diameter) was projected into the retina by means of an objective lens. The second light source was used for background illumination.](image)

**RESULTS**

*Illumination of One Cone*

The fish retina is very convenient for experimental work on single cones since the cones are unusually large and they can easily be exposed for microscopical inspection in the isolated retina. For this purpose, the retina, previously separated from the eye, was carefully deprived of its superficial layer of pigment epithelium together with most of the outer segments of the rods by touching its external surface with a strip of dry filter paper. The exposed cone layer was then inspected under the microscope by using transmitted light which entered the retina from the vitreous side. Once the pigment and the rods were successfully removed down to the level of the distal tips of the cones, these were observed to stand straight and to have their longitudinal axes in the same direc-
tion as the optical axis of the microscope, forming a very regular pattern. In order to preserve the regular pattern and this normal position of the cones, the pigment had to be removed very carefully so as to avoid losing the natural support which the prolongations of the pigment cells give to the cones.

Since the light, which entered the cone through its myoid and ellipsoid, was totally reflected in the interior of the receptor, the narrow (2μ) distal tip of the outer segment of each cone showed up as a bright spot. In the micrograph of Fig. 2 A, the retina is shown when illuminated with a circular spot of light which had a diameter of about 150 μ. Single and double cones appeared as bright spots forming a regular pattern. This pattern can be seen more clearly in the electron micrograph reproduced in Fig. 2 B. This electron micrograph shows a cross-section at the level of the ellipsoids. We wish to thank Dr. Gloria Villegas for the permission to print this picture from her original work.

Response Evoked by Stimulation of One Cone

After developing an optical method to illuminate one cone only, the question arose as to whether it would be possible to record any electrical activity from the retina when such a restricted light stimulus was used. The slow potential
recorded from the second order of retinal neurons of the fish retina was named the “graded photopic response” or g.p.r. by Svaetichin and MacNichol (16), and an alternative term, the “S potential” has been proposed by Motokawa (see Tomita, Murakami, Sato, and Hashimoto (17)). It actually proved to be possible to record this type of response from the second order of retinal neurons by photostimulation of one cone only. For such an experiment, fish retinas were selected which gave the L type of response, having an amplitude of at least 30 mv.

The experimental procedure was as follows. A large area of the retina was illuminated with an intensity of light enough to produce a large response, the amplitude however, being below the level of saturation (i.e., being smaller than the maximal response seen in Fig. 3). The diameter of the illuminated area of the retina was then stepwise reduced by placing a series of different diaphragms in the light path. Once the light spot was reduced to a diameter of about 4 μ, illuminating one cone only, the response showed an amplitude of a few hundred microvolts, and reached close to one millivolt in some cases. In order to reduce the effect of light scattering from the beam of microillumination, a constant weak background illumination was applied on the retina with the aid of the second light source.

Light Absorption in Single Cones

It is worth mentioning that when inspecting the retina under the microscope, no difference was observed in the color of the light which passed through the individual cones. During this visual examination, the retina was kept in a moist chamber with oxygen passing through it, and judging from the electrical activity which could be recorded from the second and third order of retinal neurons, it was seen that the retina was still in a good functional state. The retinas used in these experiments showed the luminosity (L) and the chromatic (R-G) types of spectral response curves.

Assuming that color vision is based on the presence of cones which show differences in their spectral absorptions, one would expect to see differently colored cones. The concentration of the photopigments in these receptors appears to be very low. However, it might still be possible to detect differences in the absorption when using a photomultiplier unit, and such experiments are being carried out so as to measure the spectral absorption of functioning single cones in the intact isolated fish retina.

Thresholds of Cones. Area of Convergence

When any single cone was illuminated within a radius of about 50 μ (this distance differed somewhat depending on the species of fish used) from the tip of the recording microelectrode, the evoked L responses all showed equal amplitudes, and the cones had the same thresholds. When the light spot was
moved further away, the amplitude of the recorded response showed a lower amplitude and became zero at a radius of about 150 to 400 μ; the radius being smaller in the retina of the Centropomidae sp. than in the retina of the Gerridae sp.

Corresponding experiments were done with the chromatic response; however, the diameter of the spot of light used in this case was 35 μ, covering about 10 cones. A smaller spot could not be used since the intensity of the monochromatic stimulus was not strong enough. Further, due to some seasonal malfunctioning of the fish, only chromatic responses smaller than 10 mv. could be obtained, and this only when a large area of the retina was illuminated. However, it could be shown that the area within which the chromatic response could be evoked was of the same order of magnitude as the one of the L type of response. Watanabe and Tosaka (11), have shown that the luminosity and the chromatic responses can be elicited when the stimulating light spot (diameter 1.5 mm.) was located at 0.7 mm. and even further away from the tip of the recording electrode. It would have been of great interest, in connection with the chromatic type of response, to be able to evoke a response by stimulating one cone only, since one might expect to get a polarity change of the response when moving the spot of light from one cone to another. The answer to this is still dependent on future experiments.

Non-Linear Stimulus-Response Relationship

It has been shown previously (15, 14) that the amplitude of the response recorded from the second order of retinal neurons is proportional to the logarithm of the intensity of the light stimulus when a large constant area of the retina was illuminated. A curve which shows this relation between the response amplitude and the logarithm of the light intensity when the number of illuminated cones was kept constant is shown in Fig. 3 (reproduced from Svaetichin (18)). In the present experiments it was observed that the response amplitude was in the same way proportional to the logarithm of the light stimulus when only a single cone was stimulated. Thus, the electrical response of the second order of retinal neurons was a graded one when a single cone was stimulated by light.

Response Amplitude Related to the Number of Illuminated Cones

The relation between the number of cones illuminated at constant light intensity and the amplitude of the L type of response has been studied in a series of experiments. To the left of each oscillographic recording in Fig. 4, a micrograph showing the area of the retina illuminated concentrically around the electrode tip is shown. The number in the right upper corner of each micrograph gives the number of illuminated cones. In this experiment a retina of the Centropomidae sp. was used. The diameters in micra of the illuminated
retinal areas were about 7, 18, 35, 80, 150, and 400. The corresponding response amplitudes in mv. were 0.7, 1.3, 2.5, 7.4, and 17.8. The vertical lines to the right of each oscillographic recording indicate the calibration in 10 mv., while the horizontal lines at the bottom of the same figure mark the light stimulus which was of a duration of 300 msec. The data obtained from Fig. 4 are presented in the upper graph of Fig. 5. The maximum number of cones illuminated in this experiment was about 900, and the corresponding amplitude of 17.8 mv. was taken as the 100 per cent amplitude. The graph at the bottom of Fig. 5 demonstrates similar experiments performed on the retina of the Gerridae sp. The black dots represent values obtained from ten series of experiments, carried out with responses of the L type, recorded from different cells in the same retina. In this case the maximum response taken as the 100 per cent amplitude corresponded to a stimulation of about 5900 cones.

The graph illustrating the experiment on the Gerridae sp. shows that when the concentric illuminated area around the tip of the microelectrode was enlarged (the light intensity per unit illuminated area being kept constant), the amplitude of the response increased linearly in proportion to the number of illuminated cones, until about 170 cones were illuminated (spot diameter, 160 μ). From there on this 1:1 ratio of the “response amplitude” to the “number of cones” changed gradually when the concentric area of illumination was further increased. When about 250 cones were illuminated (spot diameter,
Figure 4. Oscillographic recording of the L type of response when illuminating different numbers of cones. The duration of the light stimulus and the calibration of the amplifier are indicated. To the left of each recording a micrograph shows the illuminated area of the retina. The numbers indicate the cones illuminated.
400 μ) this ratio was about 1:4. Consequently, in the Gerridae sp. the effects of the cone synapses in the near concentric area around the tip of the micro-electrode were additive in respect to the electrical response produced in the second order of retinal neuron.  

Similar experiments performed on the Centropomidae sp. which are illustrated in the upper graph of Fig. 5, show that the relationship between the response amplitude and the number of illuminated cones was a linear one only in the very central area, and that there was a rapid deviation from this linearity, which started when the number of illuminated cones was less than ten. In this respect, therefore, the functional organization of the retina shows a considerable species difference.

**Focusing-Defocusing Experiments**

An interesting phenomenon was noticed when the microillumination experiments were carried out on the retina of the Centropomidae sp. A light spot, having a diameter of 4 μ, was focused into one cone which was seen as a bright spot under the microscope. The focusing is schematically shown at the
level A to the right of Fig. 6. The recording shown in Fig. 6 A was obtained when the light beam was focused into the single cone. The focus of the beam of microillumination was then lowered towards the vitreous side of the retina as indicated at B, to the right side of Fig. 6. Owing to this defocusing, the area of illumination at the level of the cones was increased to a diameter of approximately 30 μ, which corresponded to the illumination of about ten cones. The recorded response which was evoked by this illumination of about ten cones

**FIGURE 6.** A. Oscillographic recording of the L type of response obtained when one single cone was illuminated. B. The response when the beam of microillumination was defocused from the level of the cones. To the right side, a scheme shows the focusing of the light beam into one cone exclusively (focus A), and the defocusing of it (focus B), in which case about ten cones were illuminated. E.L.M., external limiting membrane. S, synaptic endings of the cones.

is shown in Fig. 6 B. An additional defocusing, which moderately enlarged the area of illumination, did not produce any further increase of the response amplitude, while an extensive defocusing caused a reduction of the same. It is interesting to notice that the amplitude of the response B was somewhat more than three times larger than the one of the response A, although the light stimuli used for eliciting the responses evidently contained the same amount of radiant energy. It has to be pointed out that the intensity of the light stimulus used was well below the level of saturation of the response (i.e., corresponding to a response smaller than the maximal one seen in Fig. 3).
DISCUSSION

It will be discussed whether, on the basis of the experiments described in this paper, it can be concluded that the receptor process (of the cone) is non-linear, while the synaptic process is linear. In the experiments in which the relationship between the number of stimulated cones and the amplitude of the response was studied, it was shown that the spatial synaptic effects are additive in the near central area of illumination. The phenomenon described in connection with the focusing-defocusing experiments is easily explained if we assume that the synaptic mechanism, which causes the response of the second order of retinal neurons, is linear, while the receptor process in the cone is a non-linear one; the non-linearity is illustrated by the curve shown in Fig. 3. When the focus of the beam of microillumination was moved towards the vitreous side of the retina, so that ten cones were illuminated instead of one, the energy content of the light entering each of the ten cones was less than one-tenth of the energy which originally was focused into one cone only. If we assume that the non-linearity of the receptor process of the cone is reflected in the curve shown in Fig. 3, then it can be calculated from this curve that a reduction of the light intensity to one-tenth will cause a decrease of the response amplitude to approximately one-half of its original value. The summation of the spatial synaptic effects from the synaptic endings of ten cones would be expected to create a response of the second order of retinal neurons having an amplitude of five times the amplitude evoked when the total light energy was focused into one cone only. (If the response of the second order of retinal neurons is $A$ when the beam is focused into one cone, then the response is expected to be about one-half when one cone is stimulated with one-tenth of the light intensity; cf. Fig. 3. If we assume the summation of spatial synaptic effects from ten cone synapses, then the response amplitude would be expected to be $10 \times \frac{1}{2} A = 5A$). In the focusing-defocusing experiment illustrated in Fig. 6 the response amplitude, after defocusing (10 cones illuminated), was about three times higher than the one obtained when the total light beam entered one cone only. Hence, the expected response amplitude is higher than the one actually recorded. This difference can be caused by the deviation from the linearity of the summation of the spatial synaptic effects, such as was observed to occur in the Centropomidae $sp.$ retina already when less than ten cones were involved (see Figs. 4 and 5, upper graph). A minor part of the discrepancy may depend on the fact that one-third of the defocused beam of light due to morphological reasons was entering the space between the cones (see Fig. 2).

In the present study it is shown that the response amplitude of the second order of retinal neurons is proportional to the logarithm of the intensity of light
focussed into one cone. Solely on the basis of this experiment it would not be possible to determine clearly whether the non-linearity depends on the synaptic mechanism or on the receptor process. However, the focusing-defocusing experiment definitely shows that the receptor process is non-linear, whereas the spatial synaptic effects are linear within a limited area. Simple reasoning shows that the phenomenon observed in the focusing-defocusing experiments cannot be explained if we assume that the receptor process is a linear one, and further, that the situation would even be more absurd if we assume that the synaptic effect is non-linear. On the basis of the experiment in which the effect of the light intensity was studied when only one cone was illuminated and one synapse involved, it can be said that the local synaptic process within one synapse is graded and linear. The focusing-defocusing experiments conclusively show that the cones are photosensitive.

As indicated in this study, the proportionality of visual functions to the logarithm of the intensity of illumination is apparently determined by the characteristics of the cone itself. These fundamental characteristics probably depend on photochemical events. Vision theories, which are based on the assumption of a statistical distribution of different sensibilities of the cones are not supported by the present work since the thresholds of the cones proved to be all approximately the same. For the literature concerning these problems see the review by Jahn and Wulff (12).

In a theoretical study, Grundfest (19), made very interesting attempts to fit the findings on the fish retina into the general neurophysiological concepts. He stressed the similarity between the retinal response and the activity of the gland cells. Since the process in the cone synapse is a linear one, this might mean that the amplitude of the electrical response of the second order of retinal neuron is proportional to the amount of secreted transmitter substance or to the number of released microvesicles.

It is interesting to notice the species differences in respect to the slope and the shape of the curves relating the amplitude of the response recorded from a second order of retinal neuron to the number of cones stimulated (Fig. 5). In the curve obtained from the experiment on the Centropomidae sp. retina, the critical change of the initial linear slope to a non-linear one occurs with less than ten illuminated cones, whereas, in the curve obtained from the Gerridae sp. the critical change of the slope occurs at about 170 cones. Correspondingly, the area of convergence was also larger in the Gerridae sp. retina.

1 Note Added in Proof. On the basis of the above presented results, it is not possible to decide at which level of the receptor-bipolar system the semi-logarithmic relationship originates. However, we now realize that from the the experiments described by Svaetichin and MacNichol (16, Fig. 6, pp. 392-394) it can be concluded that the postsynaptic membrane responds in a non-linear manner, whereas the receptor process is linear. Further experiments done in this laboratory by R. Fatechand, G. Mitarai, E. Vallecalle, and J. Villegas and the present authors give additional support to this conclusion.
These differences between the two species of fish is probably connected to the degree of visual acuity which they have. The Centropomidae sp. is a very fast moving predatory shallow water fish, while the Gerridae sp. is a slow moving fish living close to the mud. One would expect from the behavior of these fishes that the Centropomidae sp. would have a higher visual acuity than that of the Gerridae sp. In this connection it might be worth mentioning that the four double cones surrounding the single one, anatomically form a unit consisting of nine elements (Fig. 2 B) and that this might functionally also be the unit where the visual acuity is high. Thus it seems reasonable to suggest that the visual acuity is determined by the deviation from the linearity of the spatial synaptic summation of synapses converging on a second order of retinal neuron. The earlier the deviation occurs, the smaller is the convergence, and the higher is the visual acuity.

SUMMARY

A method of illuminating individual cones in the isolated functioning fish retina is described. The electrical response (L type) of the second order of retinal neurons has been recorded when one cone is being illuminated. In evoking a response in the second order of retinal neurons, the cones in the center of the area of convergence proved to have equal thresholds. The amplitude of the response evoked by stimulation of one cone was graded and proportional to the logarithm of the light intensity. The relationship between the amplitude of the response and the number of cones illuminated was also studied. The spatial summation of the synaptic effects of the cones was linear for a certain limited area, which was different for different species of fish. At the bipolar level no inhibitory interactions between the receptive fields were observed.

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REFERENCES