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PHOTODYNAMIC EFFECTS AND IRREVERSIBLE DAMAGE IN AUTOACTIVE NEURONS OF *HELIX POMATIA* EVOKED BY ROSE BENGAL AND METHYLENE BLUE EXPOSED TO VISIBLE LIGHT

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Photosensitization of different excitable tissues is a phenomenon being known since a long time and analyzed in details (LIPPAY, 1929; 1932; ROSENBLUM, 1960; LYUDKOVSKAYA and PEVSNER, 1964; LÁBOS, 1965; LÁBOS and TURCSÁNYI, 1966; LAKATOS and KOLLÁR-MORÓCZ, 1967; LAKATOS, 1969; SPIKES, 1967). Investigations at cellular level were started on squid giant axon (ARVANITAKI and CHALAZONITIS, 1953; CHALAZONITIS and CHAGNEUX, 1961). The natural photosensitivity of *Aplysia*-neurons (CHALAZONITIS, 1954; 1964; ARVANITAKI and CHALAZONITIS, 1960; 1961) due to endogenous neuronal pigments includes in a certain sense similar processes, but such phenomena in *Helix* neurons — because of their low pigment content — can be osberved only sporadically.

The cited authors generally emphasize that such a photoactivation is suitable tool when looking for electron processes be involved in the excitation. At the same time, it is also known that the photodynamic effect is noxious or even lethal (RAAB, 1902) and only the moderate effects are reversible.

Applying laser-pulses in the presence of methylene blue (ARVANITAKI et al. 1967) it succeeded to evoke discharges of *Helix* neurons, but long-lasting experiments were not carried out and effects of rose bengal on neurons — one of the most potent sensitizing dye — have not been reported yet. For these reasons in the present paper the photodynamic response evoked in the presence of methylene blue and rose bengal will be described up to its irreversible phase on molluscan neurons.

Methods

Spontaneous electrical activity of single or in some cases two neurons situated near the dorsal surface of the suboesophageal ganglion-complex was studied in the presence of 50—100 μg/ml rose bengal (Fluka, abbreviation: RB) and methylene blue (Merck, MC) in dark and in exposure to light. The composition of the physiological solution was the following: 3.0 g NaCl, 0.35 g KCl, 2.4 g $MgCl_2 \cdot 6\,H_2O$, 1.5 g $CaCl_2 \cdot 2\,H_2O$, 0.2 g $NaHCO_3$ pro lit. The recording was carried out with micropipettes of 4—10 MΩ filled with 2.5 M KCl, FET-input stages, and Tektronix 502 oscilloscope. The optical system had the following characteristics: tungsten-lamp of 500 watts; upper illumination from 50 cm; 15 mm heat-filter of BG 17 type; the surface of the focused spot was about 10 mm^2; effective illumination was near to 50 000 lx. The

exchange of solution was carried out at 100 lx. This latter illumination did not evoke any modification of neuronal activity even in the presence of sensitizing dyes.

The conditions of the leading off made possible experiments lasting for at least 5—6 hours without any essential decrease in the amplitudes of spike. In order to facilitate the input of stains to the cells, the thin layer of connective tissue covering the neurons was very carefully removed. To avoid mechanical excitations during the exchange of solution, it was carried out in a chamber of two compartments. The suction and exchange have taken place in the compartment where the ganglion was not present. The two parts of the chamber were communicating through a narrow gap.

50 neurons originating from about 40 ganglia were utilized in the experiments. In *Fig. 1* the localization of the neurons demonstrated in this paper is shown.

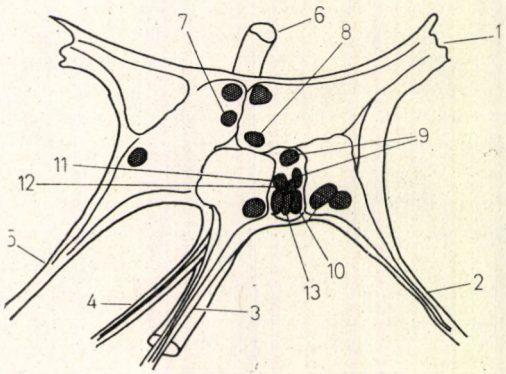

Fig. 1. A — Rough sketch of dorsal surface of suboesophageal ganglion. 1 — cerebropleural stub; 2 — n. intestinalis; 3 — n. analis; 4 — n. pallialis sin.; 5 — n. pallialis dext.; 6 — aorta; 7 — 170/8; 8 — 197/8 or 324/1 or 196/7; 9 — 190/2; 10 — 203/4; 11 — 195/1; 12 — 192/5; 13 — 196/6; 321/5; 198/1 (numbers of protocol).
Those neurons are demonstrated here whose reaction will be shown in the further figures (code-number see there)

Results

1. Effects of rose bengal in dark and light

In *Fig. 2* records obtained during an experiment carried out on a neuron discharging in constant intervals. During the control period of 25 min, the frequency was 22.6 $\pm$ 2 cpm. Exchanging the medium of incubation with rose bengal of 50 μg/ml, similarly for a period of 25 min, 21.4 $\pm$ 1.9 cpm frequency was observed. The resolution of these frequency-measurements was 20 sec. It is seen that these two values do not deviate significantly from each other. An illumination was applied after this period of 2×25 min. As an effect of the exposure to visible light, the frequency has doubled in 10—15 min and synchronously the peak to peak amplitudes have decreased. During a further 25—30 min the spikes get rarer and returning to the control a significant decrease in the amplitudes, the potential of resting membrane and

that of the overshoot was observed. In the given case, the decrease of frequency is fluctuating but that of the amplitude is monotonous.

In all experiments (25—30 neurons) we could notice a transient increase of frequency, a decrease of 25—30 mV in the membrane potential, an initially slow and after a rapid-decrease of amplitude and a diminuation of overshoot as well. In cases when the initial (control) amplitude of the action potential

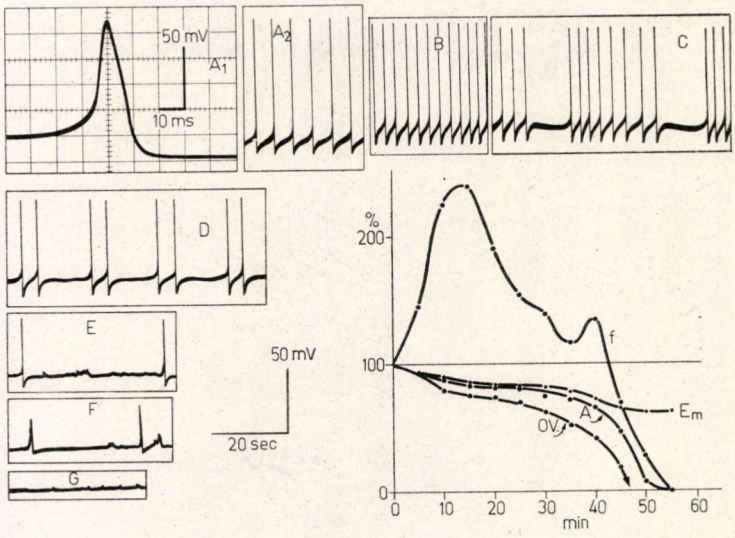

Fig. 2. Effect of 50 μg/ml RB in visible light. Neuron — 190/2. A_1 and A_2: control in RB and in dark. B—G: records in the 20, 30, 40, 42, 45 and 50th min of exposition. Time course of frequency (f), amplitude (A), membrane potential (EC) and overshoot (ov) changes in the per cent of control. The controls: 21 cpm, 107 mV, 54 mV or 33 mV (100 per cent)

was already smaller (peak to peak 60—80 mV) the above enumerated changes have taken place more quickly *(Fig. 3)*.

In *Fig. 3* the A_5—A_7 or B_7—B_8 squares demonstrate the terminal phases of the effect. These alterations are almost general at the end-period of the photo-dynamic effect. In such cases a polymorph oscillation of low amplitude appears. Some sufficently constant values of amplitude can be measured alternating bi- or multimodally. Synaptic noise, axon-spikes and abortive discharges of soma equally occur. In the final square of the *Fig. 3* a quasi-sinus oscillation is demonstrated yet. These diverse oscillations have amplitudes of about 1—20 mV.

The neurons modulated periodically (Br-like) are especially sensitive to this light-effect. In the case demonstrated in *Fig. 4* a short, transient increase of frequency, depolarization and finally a getting rare of frequency can be observed. The whole effect lasts for 10—15 min.

To verify that the observed destroying effects of rose bengal in light is not an accidental phenomenon caused by other influences, but it is in fact a photodynamic effect — we have recorded synchronously the alterations of

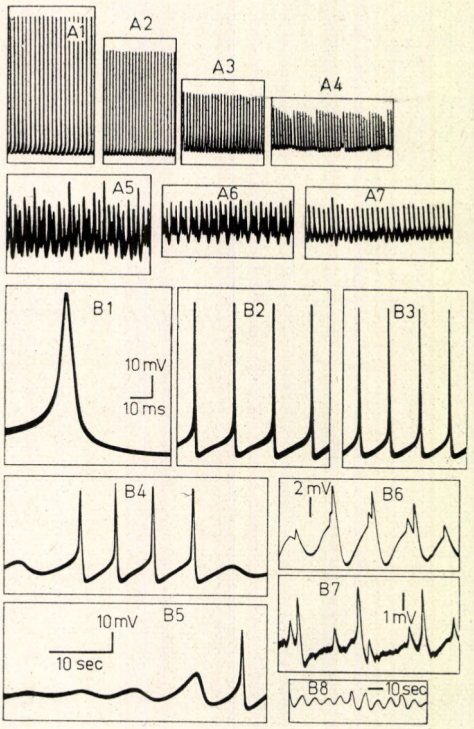

Fig. 3. Effect of 50 μg/ml rose bengal. A_1—A_7: neuron 192/5; B_1—B_8: neuron 170/8* A_1 — dark control in rose bengal; A_2—A_7 in the 10, 15, 17, 25, 30, 35 min of illumination. Calibration — A_1—A_4: 50 mV, 5 sec; A_5—A_7: 2 mV, 5 sec; B_1 — dark-control; B_2—B_5: 5, 10, 20, 32 min. of exposition; B_6—B_8: 40—50th min

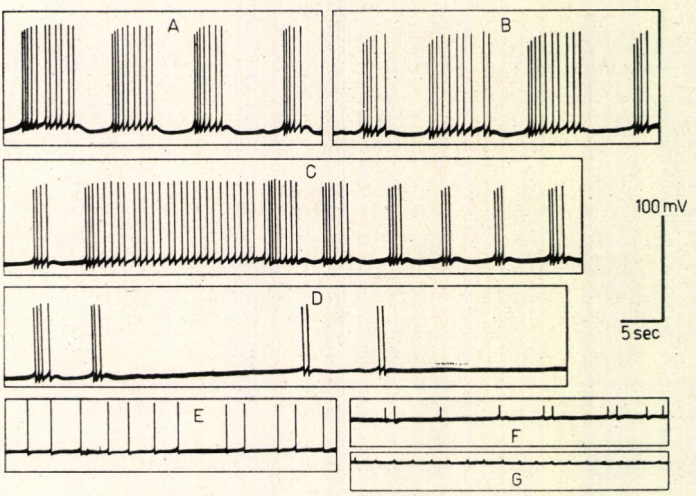

Fig. 4. Effect of 50 μg/ml RB on a cell of periodic activity (Br-like). Neuron 203/4. A — control; B — RB, in dark; C—G — RB in light: 1st, 2nd, 3rd, 10th and 12th min.

two different neurons *(Fig. 5)*. It is observable that in the cases of the two neurons (whose activities are, on the other hand, correlative) the decrease of amplitude, transient 2—5 fold increase of frequency and its terminal decrease are parallel events. In *Fig. 5D* and *5E* correlating periods are demonstrated. In *Fig. 5D* a seizure-like sequence of spike lasting for 2 sec is seen when in the other neuron a synaptic activation is notiecable. Such an activition of extremely high frequency in the given case between the 10th and 20th minutes of the exposition appeared 6 times and in all cases EPSP-s emerged in the record of the other neuron.

The destroying effect of rose bengal proved to be irreversible.

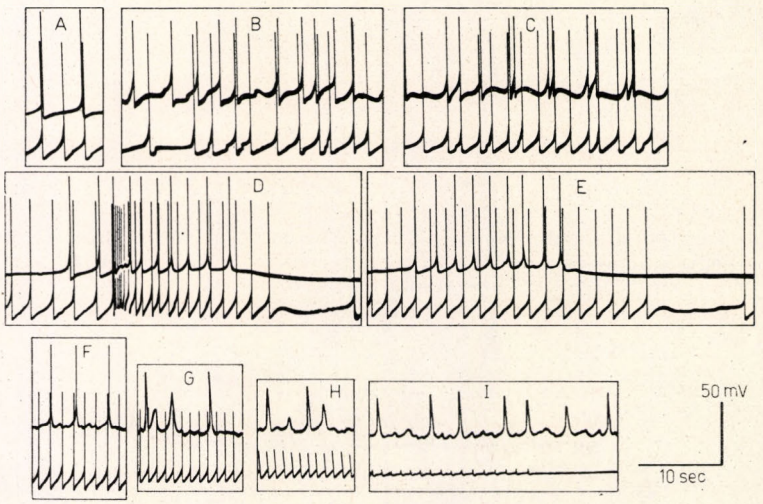

Fig. 5. 50 μg/ml RB. Two neurons of correlating activity. A — control in RB; B — I in light (5, 7, 10, 12, 20, 30, 40, 50th min of exposition). Neurons: 196/6 and 196/7

2. Effects of methylene blue in dark and light

Effects of 50 μg/ml methylene blue at the applied illumination are detectable already after an exposition of 5—10 min. It is observable furthermore that the dyestuff has effects even in dark, which effects become more explicit in light.

In the case illustrated in *Fig. 6* during the dye-free, control period and in the presence of the stain in dark or under the illumination, the amplitude of the action potential, its duration at the half-height or the frequency of discharge were measured. The values obtained are the following.

	98	92	80 mV (10—10 measurements)
18.6 ± 0.9	23.4 ± 1.4	28.6 ± 1.2	msec (11—11 measurements)
6.3 ± 0.45	6.48 ± 0.70	7.19 ± 2.27	spikes/20 sec (20—27—58 samples)

Changes can be observed in the shape of action potential already in dark. Both the decrease of amplitude and the prolongation are statistically significant (t-test, $P < 0.05$). It is noticeable, furthermore, that the relative dispersions

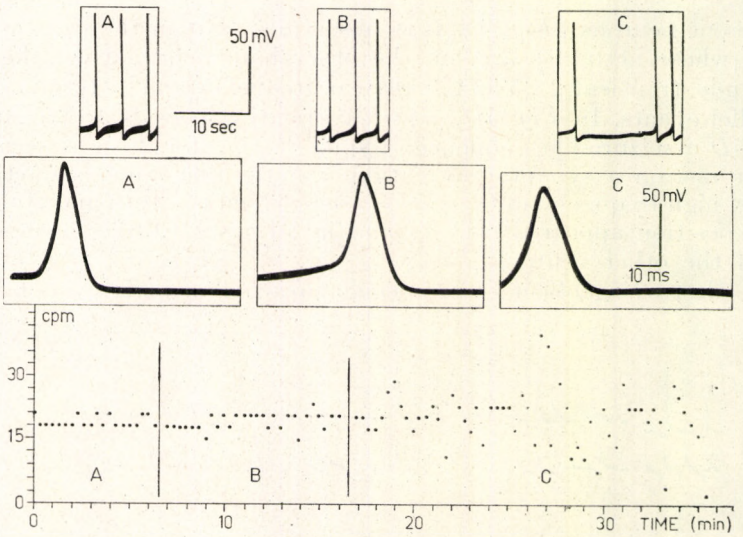

Fig. 6. Effect of 100 μg/ml MC. Neuron: 321/5. A: control; B: MC in dark; C: MC in light. The lower diagram demonstrates the frequency change in time for A, B and C periods. Time resolution is 20 sec

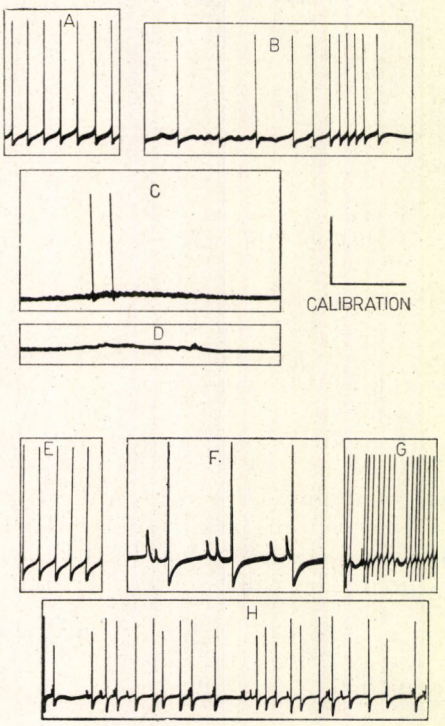

Fig. 7. Effect of 100 μg/ml MC in visible light. A—D — neuron 197/8; E—G: neuron 195/1. A — MC, dark, 5th min. B—D — MC, light 2nd, 5th and 8th min. E — control in MC; F—H: between the 40th and 50th min of exposition. Calibration — A—E: 50 mV, 10 sec; F: 25 mV, 4 sec; G: 25 mV, 40 sec; H: 60 mV, 10 sec

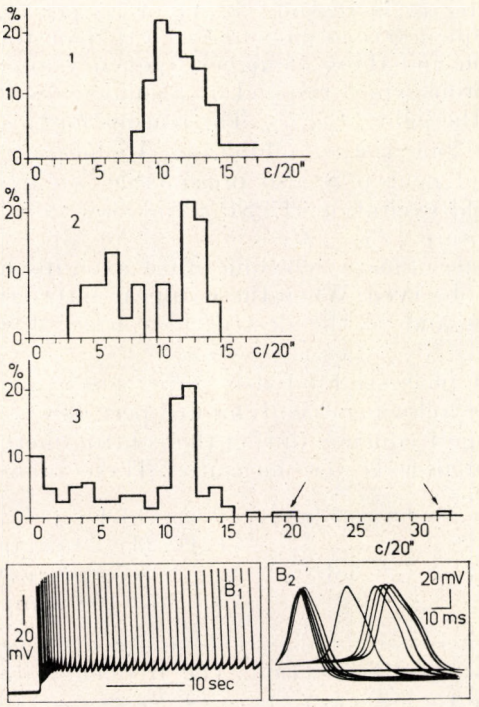

Fig. 8. Effect of 50 μg/ml MC. Neuron: 198/1. A — frequency-distribution; resolution is 20 sec; 1, 2, 3 — stain-free control, dark control in the presence of stain, in light. B_1 and B_2 — seizure-like burst in light; B_2 — shows the changes of shape, during the sequence

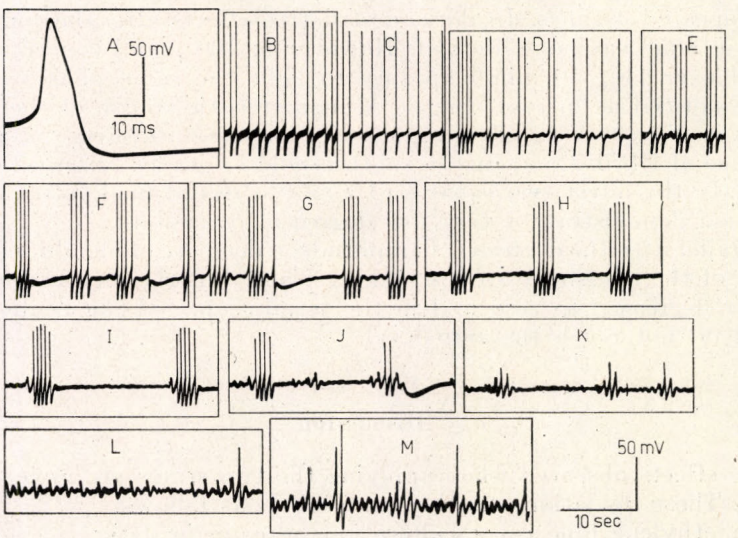

Fig. 9. Effect of 100 μg/ml MC in light. Neuron 324/1. A, B: dyestuff-free control; C — MC in dark 25th min; D—K: MC in light (1, 5, 12, 15, 25, 30, 35 and 40th min; L—M — records newly after some hours of total darkness

in the three periods are increasing: 7—11 or 32 per cent. It can be easily followed on the frequency-time plots in *Fig. 6* that on the effect of light both periods of inhibition and those of higher frequency appear.

There are neurons which respond by a sudden cessation of their activity after switching on the light *(Fig. 7)*. The reasons for this may be the appearance of EPSP-s or long lasting inhibition. An increase of threshold in the pacemaker region of neuron is also presumable, as in some cases only the signs of subthreshold excitation (EPSP-s and slow depolarizing waves) have survived light exposure *(Fig. 7D)*.

In the late-phase of methylene blue effect axon-discharge-like depolarizations can often be observed. When these appear interferring with spikes, the frequency decreases and in the destructive phase of effect the amplitude decreases as well.

The fact, that phases inhibited by other mechanisms as classic IPSP-s demonstrated by the following case. Inhibited periods whose duration exceeded 2 sec and the average frequency during the control, methylene blue/dark and methylene blue/light periods were measured. These values are:

4.2 ± 1.2	10.6 ± 3.9	19.6 ± 14.4 sec (124—23—29 samples)
11.4 ± 1.4	8.7 ± 3.3	8.7 ± 5.5 spikes/20 sec (50—37—108 samples)

A prolongation of inhibited periods and a decrease of frequency were observed already in the dark period. The frequency distributions of the 3 periods demonstrate these facts *(Fig. 8A)*. Cases marked by arrows refer to the presence of bursts with high frequency. Such a burst is demonstrated in *Fig. 8B*. These bursts are similar to those paroxismal discharges observed in rose bengal and in visible light *(Fig. 5D)*.

In the demonstrated case of sensitization with methylene blue such bursts appeared 4 times. In dark, destructive effects after the application of methylene blue was not observed. However in 100 μg/ml concentration and after a longer (30—50 min) exposure to light the same phases of damage could be noticed as in rose bengal. A characteristic course of the alteration is shown in *Fig. 9*. A significant change of rhythm, a decrease of amplitude, appearance of EPSP-s and finally subthreshold or graded, grouped oscillation are seen. In the latest two squares of *Fig. 9* a record made after 3 hours of dark period demonstrate a very low degree of reversibility.

Parallel with the decrease of amplitude, a decrease of membrane potential and that of the overshoot are also taking place. But the membrane potential has never decreased to zero and in the terminal period the peaks of action potential do not reach the zero level.

Discussion

The effects observed when applying the two stains are in certain sense different. These deviations can be summarized as follows:

1. methylene blue causes alterations already in dark;
2. in the effect of methylene blue perhaps the inhibitory phenomena are dominating;

3. in the case of methylene blue to evoke destroying effects a little higher concentration or longer exposition is needed

(50 μg/ml MC$\sim$156 μM MC; 50 μg/ml RB$\sim$50 μM RB)

Effects of the two stains are similar in the destructive phase of depolarization character and in the appearance of seizure-like bursts from time to time.

The higher sensitivity of certain neurons (for example Br-cells) perhaps is concerned with their higher endogenous pigment content. However, in stain-free medium no destructive reaction was observed. Because of such differences in the sensitivity of the individual cells, quantitative examinations, for example measurements of dose-effect curves can be carried out only when a great number of the same identified neuron is studied.

The destructive effect on the soma membrane is irreversible and taking place in discrete steps. Presumably in such cases different patches of the soma-membrane discharge without coordination which manifests in a more or less disordered fluctuation of amplitude. A probable mechanism of this that both inward and outward cation-transport are damaged as both the overshoot and membrane-potential decrease. According to this later fact in some cases a decrease in the resistance of membrane was observed (unpublished).

On the base of above outlined facts it can be stated that the photodynamic experiment in its early phase only, or after slight influences may be adequate to investigate the electron processes which are perhaps included in nerve excitation.

Summary

Spikes of 50 autactive giant neurons situated superficially on the dorsal surface of suboesophageal ganglion of *Helix pomatia* were studied in the presence of 50—100 μg/ml rose bengal and methylene blue in dark and visible light applying heat filter.

In the presence of rose bengal, the photodynamic effect is manifesting in a long-lasting increase of frequency, slow depolarization, decrease of amplitude, membrane potential and overshoot. In the terminal phase the frequency is newly decreasing and polymorph subthreshold oscillations originating from different sources having multimodal amplitude and frequency distribution, appear or remain. The effects are irreversible.

Methylene blue already in dark causes appreciable alterations of shape and frequency. In visible light the effect is more explicit. In the early phase of the influence the inhibitory phenomena may be more than before. In visible light a similar destruction takes place as in the case of rose bengal. Its effect is also irreversible.

Seizure-like burst were observed in the presence of both stains.

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AUTOAKTIV *HELIX POMATIA* NEURONOK FOTODINÁMIÁS BEFOLYÁSOLÁSA ÉS IRREVERZIBILIS KÁROSITÁSA BENGÁLVÖRÖSSEL ÉS METILÉNKÉKKEL

Lábos Elemér

Összefoglalás

Helix pomatia suboesophagealis ganglionjának 50, dorzális felszíni, autoaktív, óriás neuronjának kisüléseit vizsgáltuk 50—100 μg/ml bengálvörös, ill. metilénkék jelenlétében, sötétben, ill. látható fényben, hőszűrés mellett.

A fotodinámiás hatás bengálvörös jelenlétében a frekvencia tartós növekedésében, lassú depolarizációban, az amplitúdó, a membránpotenciál és az overshoot esésében nyilvánul meg. A hatás késői fázisában a frekvencia újra esik, és több forrásból eredő polimorf, multimodális amplitúdójú és frekvenciájú küszöb alatti oszcillációk jelennek meg. A hatás irreverzibilis.

Metilénkék már sötétben is észrevehető alak- és frekvenciaváltozást okozhat. Fényben a hatás kifejezettebb. A hatás kezdeti fázisában a gátlási jelenségek fokozódhatnak. Fényben a bengálvörös hatásához hasonló destrukció zajlik le. A hatás irreverzibilis.

Mindkét festék jelenlétében megfigyeltünk paroxizmális jellegű kisüléssorokat.

ФОТОДИНАМИЧЕСКОЕ ВЛИЯНИЕ НА НЕЙРОНЫ HELIX POMATIA СО СПОНТАННОЙ АКТИВНОСТЬЮ И ИХ НЕОБРАТИМОЕ ПОВРЕЖДЕНИЕ БЕНГАЛЬСКИМ КРАСНЫМ И МЕТИЛЕНОВЫМ СИНИМ

Э. Лабош

Была исследована спонтанная активность 50 поверхностных гигантских нейронов в подглоточном ганглии *Helix pomatia* в присутствии 50—100 μг/мл бенгальского красного или метиленового синего в темноте или при дневном свете при исключении тепловых волн.

Фотодинамическое действие в присутствии бенгальского красного проявляется в длительном увеличении частоты, в медленной деполяризации, и в падении мембранного потенциала, амплитуды потенциала действия и overshoot-та.

В последующих фазах действия красителья частота снова падает и проявляются полиморфные, с мултимодальной амплитудой и частотой подпороговые осцилляции, возникающие из разных источников. Дествие красителья необратимо.

Метиленовый синий способен вызвать заметное изменение формы и частоты потенциалов уже и в темноте. При дневном свете действие более выраженное. В начальной фазе тормозные процессы могут усиливаться. При дневном свете смеют места превращения, подобные эффекту бенгальского красного.

В присутствии обоих красителей наблюдались разряды, подобные к пароксимальным.