AN ELECTROPHYSIOLOGICAL METHOD FOR DETECTING VISUAL TOXICITY IN UNRESTRAINED RATS

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Abstract—Methods for recording the electroretinogram (ERG) and the visually evoked potential (VEP) in unrestrained rats were developed, and used to test the long term toxicity of diaminodiphenoxybutane (DAPB). The electrodes for recording the ERG and VEP were implanted chronically in the sclera of the eye and on the surface of the visual cortex, respectively. Both the ERG and VEP were recorded from unrestrained rats using a slip ring that allows the rat free movement without twisting the cables. Fifty responses to repetitive photic stimulation at 10-sec intervals were averaged by means of a minicomputer. The waveforms of the ERG consisted of an initial negative wave (a-wave), followed by a positive wave (b-wave) with 3 to 4 oscillatory potentials on the rising slope. The VEP consisted of an initial positive wave (P1 wave), followed by a large negative wave (N1 wave) and late positive and negative deflections. The amplitudes of these components of the ERG and VEP varied considerably from animal to animal, whereas these components within the same animal were reproducible within a 20% variation in amplitudes and a 10% variation in latencies measured from ERGs and VEPs obtained repeatedly for 7 weeks in 6 control rats. Using this technique, the effects of DAPB, a retinotoxic compound, on ERG and VEP were studied. DAPB produced a marked dose-dependent decrease in the amplitudes and prolongation in the latencies of the ERG after a single intravenous injection at doses of 50 and 70 mg/kg. The VEP was also suppressed in parallel with the change of the ERG. These results indicate that this recording technique for ERG and VEP is useful for evaluating the visual toxicity of drugs in unrestrained rats.

It is well known that the visual system is a target of various kinds of drugs. In order to assess the visual toxicity of drugs in preclinical toxicity studies, the electroretinogram (ERG) and the visually evoked potential (VEP) have been recorded from the retina and visual cortex, respectively. The ERG is recognized to allow the early detection of retinal dysfunction (1), and the VEP furnishes useful information on the conduction pathways from the retina to the cortex (2). Therefore, the simultaneous recordings of both the ERG and VEP may predict the site for toxic action of drugs.

The ERG is typically recorded from an electrode placed in contact with the cornea using a contact-lens type or saline-soaked wick electrode in rats (3, 4). For this purpose, anesthetized or restrained animals

1) The main part of this paper was presented in the 8th International Congress of Pharmacology, Tokyo (July, 1981).
were used because of the necessity for giving a constant photic stimuli to the animal's eye and keeping an electrode in constant contact with the cornea. We also had recorded the ERG in a restrained rat without general anesthesia using a contact-lens type electrode (3) and realized that it is difficult to record a stable ERG in long term toxicity studies. Furthermore, many investigators have demonstrated that anesthetics modify waveforms of the ERG as well as the VEP (5-9). In addition, it has been reported that restraint produces severe stress in animals (10) and hence may enhance the toxicity of drugs. Thus, anesthetics or restraint may lead to errors in evaluating the visual toxicity—particularly long term toxicity—of a test compound.

Recently, a method for recording the ERG from noncorneal electrodes, chronically implanted in the supraorbital bone, employing a computer-signal averaging technique was demonstrated to be useful in monkeys (11). However, there is virtually no literature concerning the method for recording the ERG from noncorneal electrodes in rats.

Therefore, the present investigation was undertaken to establish a method for recording the ERG in unrestrained rats with chronic noncorneal electrodes. Furthermore, to assess the sensitivity and reliability of the method, we investigated the effects of diamino-diphenoxylbutane (DAPB), a retinotoxic compound, on the ERG and VEP.

MATERIALS AND METHODS

Animals: Adult, male Wistar rats weighing 450 to 550 g at the start of the experiment were used. Each rat was maintained individually in a stainless steel cage and had free access to tap water and laboratory rat diet (CLEA Japan Inc., CE-2). The animals were housed in a room having a constant 12/12 hr light-dark cycle with the light on (400-500 lux) from 8 a.m. to 8 p.m. The temperature was maintained at 23±3°C and the relative humidity at 55±10%.

Surgical procedure: Each rat was anesthetized with ketamine (100 mg/kg, i.m.) and electrodes for ERG and VEP recordings were implanted surgically.

For ERG recordings, 2 stainless steel wires (0.08 mm in diameter) were implanted in the sclera of the left eye. An active electrode was placed in the sclera 3 mm from the limbus and a reference electrode 1 mm from the limbus (Fig. 1). The wires were coated with silicone rubber except at the tip and led beneath the skin to a miniature socket (Honda Tsushin, MM-8F) fastened to the skull with dental cement (Yata Chemical Ind. Co. Ltd., POLISET). For VEP recordings, 2 stainless steel screws were threaded through the calvarium to make contact with the dura. An active electrode was placed 7 mm posterior to the bregma and 4 mm lateral to the midline and a reference electrode was placed 1 mm anterior to the bregma and 1 mm lateral to the midline. These electrodes were connected to the same miniature socket that was used for the ERG electrodes. In addition, a silicone rubber cannula for drug injection was implanted into

![Fig. 1. Electrode placements for ERG recordings. Very fine stainless steel wires (0.05 mm in diameter) were implanted in the sclera as an active and a reference electrode and were led to a miniature socket fastened to the skull.](image-url)
the right external jugular vein as described by Weeks (12). Animals were allowed at least 2 weeks to recover from surgery prior to recordings.

**Apparatus and procedure:** The experimental arrangement for recording the ERG and VEP in an unrestrained rat is shown schematically in Fig. 2. All recordings were made in a darkened and electrically shielded room. A rat with chronically implanted electrodes was placed in a cylindrical recording chamber (25 cm in diameter and 30 cm in height). The whole inner surface of the chamber was painted white in order to provide an even distribution of flashes. A slip ring (Airflyte Electronics Co., Electro-Cannular Slip Ring) was used to allow the rat relatively free movement without twisting the cables in the chamber. A photostimulator (Nihon Kohden, MSP-2R) was used to give a light flash stimulus of 100 μsec duration. A xenon lamp (diameter=20 cm) was placed on a sound-attenuating chamber (10 cm in height) consisting of 2 transparent resinous filters to eliminate the auditory noise delivered by the xenon lamp. The sound attenuating chamber was piled on the recording chamber. Both the ERG and VEP evoked by flashes were amplified and averaged with a signal processor (San-ei, 7TO7A; time constant =0.1 sec, sweep time=200 msec) and photographed with a camera (King, CRT). All the recordings were made between 9:00 a.m. and 5:00 p.m.

The amplitudes of the ERGs were measured from the baseline to the lowest point of the a-wave (a-wave amplitude) and from the lowest point of the a-wave to the highest point of the b-wave (b-wave amplitude). The P1-N1 amplitude of the VEP was measured from the peak of the first positive wave (P1 wave) to the peak of the first positive wave (P1 wave).

![Fig. 2. Experimental arrangement for recording ERG and VEP in unrestrained rats. A rat with chronically implanted electrodes for ERG and VEP was placed in recording chamber. ERG and VEP were evoked by flashes delivered by a xenon lamp, amplified with signal processor and photographed. A slip ring allows the rat free movement without twisting the cables.](image-url)
negative wave (N₁ wave). The latency was measured as the interval between the stimulus onset and the peak of the corresponding a- and b-waves of the ERG and N₁ wave of the VEP (Fig. 3).

**Drug administration:** Diaminodiphenoxyl-butane•2HCl synthesized in our Medicinal Research Laboratories was dissolved in physiological saline. The drug was administered intravenously through the cannula in doses of 50 and 70 mg/kg (3 animals/group). Six rats treated with physiological saline served as the control. Each animal was given a single dose.

ERG and VEP recordings were made at 24 hr before and 1, 3, 7, 14, 21, 28, 35, and 49 days after administration. The effects of DAPB were evaluated by a two-way analysis of variance (ANOVA) for repeated measures. If there was a significant difference, post-drug values were compared with the pre-drug value using the procedure of Dunnett for multiple comparison (13, 14).

**RESULTS**

**Waveforms of ERG and VEP:** Typical waveforms of the ERG and VEP recorded from an unrestrained rat with chronically implanted electrodes are shown in Fig. 3. The ERG consisted of an initial negative wave (a-wave) followed by a positive wave (b-wave). On the rising slope of the b-wave, 3 to 4 oscillatory potentials were observed. The typical VEP consisted of an initial positive (P₁ wave) and negative (N₁ wave) waves, followed by slow positive and negative deflections. Animal-to-animal variations in these waves of the ERG and VEP were relatively large, whereas day-to-day variations were small in the same animal as will be described later.

**Effects of interstimulus interval variation:**
The effects of the interstimulus interval on the ERG were investigated after dark adaptation for 1 hr in 4 non-treated rats. The photic stimulation was given at interstimulus intervals of 3, 10, 30, and 60 sec with an intensity of 2 joule, and the responses were averaged in sets of 50. Typical waveform changes of the averaged ERG are shown in Fig. 4. The amplitude of each component increased in parallel with the prolongation of the interstimulus interval. Moreover, oscillatory potentials were relatively obscure at 3-sec intervals, but these became clear at intervals of 10 sec or more. On the basis of these results, the ERG was evoked at 10-sec intervals in the following study.

**Effects of photostimulus intensity variation:** The effects of photostimulus intensity on the ERG were investigated after dark adaptation for 1 hr in 4 non-treated rats. In this experiment, averaged ERGs were obtained by 50 light flashes at 10-sec intervals with intensities of 0.3, 2.0, and 20 joule. As shown in Fig. 5, the amplitudes of a- and b-waves peaked at 2 joule and the latency of the a-wave shortened in relation to the photostimulus intensity. On the other hand, the latency of the b-wave did not show any definite trend. On the basis of these results, the ERG was evoked with an intensity of 2 joule in the following study.

**Effects of dark adaptation:** After the
animal had remained for 5 min, 1 hr, or 14 hr in the dark, the ERGs were recorded 4 times under the same conditions at 2- to 4-day intervals. Although amplitudes of the ERG a- and b-waves evoked by the first light stimulus showed some tendency to increase in proportion to the prolongation of the dark-adaptation, both amplitude and latency of the averaged ERG remained relatively constant over the duration of dark-adaptation (Fig. 6).

Based on these results, the ERG was recorded after dark-adaptation for 5 min in the following study. In addition, although the waveforms of the ERG became more stable in parallel with the number of responses, 50 ERGs were averaged in routine visual toxicity studies. The VEPs were simultaneously recorded with the ERG.

**Effects of DAPB on ERG and VEP:** Changes in amplitudes and latencies of the ERG a- and b-waves and the $P_1-N_1$ amplitude and $N_1$ latency of the VEP of the control and DAPB treated rats are shown in Fig. 7 (ERG) and 8 (VEP), respectively.

In 6 control rats, the pre-drug values (mean±S.D.) of the ERG a- and b-wave amplitudes were $80±22$ and $186±38$ $\mu$V and the latencies were $6.2±0.3$ and $49.2±3.5$ msec, respectively. The values for each animal were reproducible within a 20% variation in amplitudes and a 10% variation in latencies for 7 weeks as compared with the pre-drug values. The pre-drug values of the VEP $N_1$ latency and $P_1-N_1$ amplitude were $27.0±1.2$ msec and $206±68$ $\mu$V, respectively. These values were reproducible within a 10% variation in both latencies and amplitudes when recorded simultaneously with the ERG.

In rats receiving 50 mg/kg of DAPB, the amplitudes of the ERG a- and b-waves were...
depressed 7 days after dosing, and the peak latencies of these waves and the N₁ wave of the VEP were slightly prolonged. Thereafter, these gradually recovered to the pre-drug values. In rats receiving 70 mg/kg, the amplitudes of both the ERG and VEP were depressed 7 days after dosing. At the same time, the peak latencies of the ERG a- and b-waves and the VEP N₁ wave were markedly prolonged. Thereafter, the latencies of these waves gradually recovered to the pre-drug values, whereas the amplitudes of these waves in both the ERG and VEP did not show any significant improvement even 49 days after dosing.

All the rats receiving 50 or 70 mg/kg of DAPB showed no signs of visual dysfunction such as loss of the blink or pupillary reflexes or dilatation of the pupils.

Finally, a slight proliferation of connective tissue at the site of the ERG electrodes was revealed by autopsy about 10 weeks after the electrode implantation in all the control and DAPB treated rats. No other abnormalities were observed in the eyeball.

**DISCUSSION**

In many cases, the ERG has been recorded by a contact-lens type or saline-soaked wick electrode placed at the cornea in anesthetized or restrained rats (3, 4). However, as described in the introduction, anesthesia apparently exerts influences on the ERG and VEP (5-9), and restraint produces severe stress in animals (10). Therefore, it is very important to use unrestrained animals for studying the effects of unknown compounds on the ERG and VEP. In the present study, we could record stable ERG from relatively unrestrained rats for a long period.

The waveforms of the ERG are subject to distortions caused by the direction of gaze, blinking, electrode placements, and ocular rotation (15). These problems were overcome by the following procedures: 1) 50 responses to repetitive photic stimulation were averaged with the use of a computer-signal averaging technique to extract the low-amplitude responses from background “noise” and reduce the variability of each response, 2) the whole inner surface of the recording chamber was painted white in order to provide roughly equal distribution of flashes, and 3) the ERG electrodes were chronically implanted in the sclera of the eye throughout the recording period. As a result, typical waveforms of the averaged ERG consisting of an a-wave, a b-wave, and oscillatory potentials were reproducible within a 20% variation in amplitudes and a 10% variation in latencies for a long period. Such variability is so slight that few problems arise in assessing the effects of test compounds on the ERG. The variation of the averaged ERG measured repeatedly from the same animal was very small, whereas the variation among the animals was relatively large. This might be due to the variation of the ERG.
electrode placements.

The amplitudes of the ERG increase during the course of dark-adaptation and reach a maximum about 30 min after the start of the adaptation in rats (4). On the other hand, it is well known that light-adaptation is shaped more rapidly than dark-adaptation (16). In the present study, both amplitude and latency of the averaged ERG were not modified by the duration of the dark-adaptation, though the recordings were begun after the animal had remained in the dark for a duration ranging from 5 min to 14 hr. This might be because the amplitude of the ERG rapidly decreased to a certain level in the presence of repeated flash lights, and its level was maintained relatively constant during each averaged ERG recording. This explanation was supported by the finding that the single ERG response to the first light flash showed some tendency to increase in proportion to the prolongation of the dark-adaptation.

Aminophenoxyalkanes including DAPS have been known to produce visual dysfunction in several species (17–19). We demonstrated in a previous report that DAPS reduced visual function in rats and revealed that the primary site of action was the retina (3) as reported in other species such as monkeys, dogs, cats, and rabbits (17–19). In the present study, the ERG and VEP were depressed dose-dependently in rats receiving a single intravenous dose of 50 or 70 mg/kg of DAPS. The depressed responses recovered about 3 weeks after dosing in rats treated with 50 mg/kg, whereas the rats receiving 70 mg/kg did not show any significant improvement even by 7 weeks after dosing. These findings are coincident with the results in our previous study (3) in which we recorded single ERG to a light flash from restrained rats using a contact-lens type electrode after adaptation to darkness for 60 min. Therefore, the sensitivity and reliability of the recording method established in the present study might be comparable with those in the previous study.

No significant gross behavioral changes such as loss of blink or pupillary reflexes occurred even when marked depression of the ERG and VEP was observed in rats receiving DAPB. This finding was also in agreement with the results in our previous study and indicated again that it was almost impossible to detect visual toxicity in rats on the basis of gross behavioral signs.

From the facts described above, it is concluded that a stable ERG can be recorded repeatedly by electrodes chronically implanted in the sclera of the eye of relatively unrestrained rats for a long period. Furthermore, this recording method is useful for evaluating the visual toxicity of drugs, particularly in long term toxicity studies.

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