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Non-Competitive NMDA-Receptor Antagonists and Anoxic Degeneration of the ERG B-Wave *In Vitro*

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Summary

Studies have been undertaken to see if the non-competitive NMDA antagonists, ketamine, MK-801 and dextromethorphan would preserve the b-wave of the electroretinogram (ERG) *in vitro*. The drugs had no effect on the ERG b-wave, nor prolonged its survival postmortem. The present results support previous evidence suggesting that NMDA-receptors are not involved directly in synaptic transmission between photoreceptors and ON-bipolar cells. Further, loss of the b-wave in postmortem anoxia does not appear to be mediated via NMDA-receptors.

During studies on survival of phototransduction in postmortem rat and human retinae, we have found that, following regeneration of visual pigment, PIII responses of the ERG could be recovered to a large extent in previously-bleached, freshly isolated rat retinae, and partially recovered in human retinae up to 58 hours postmortem.[1–3] We also noted that the b-wave was apparent not only in the rat retina but also in some human retinae up to 43 hours postmortem.[2,3] Its survival, however, was poor compared with PIII. The b-wave is an indicator of synaptic transmission between photoreceptors and second order retinal neurones, principally, if not totally, ON-bipolar cells.[4,5] Excitatory amino acids mediate the transmission and, in man as well as lower order vertebrates, L-glutamate is likely to play a major role.[6–8] Recently, it has been reported that dextromethorphan, a non-competitive antagonist of excitatory amino acid receptors of the NMDA (N-methyl-D-aspartate) type, can protect the rabbit retina against ischaemic damage and preserve the b-wave *in vivo*.[9] Furthermore, NMDA is known to cause spreading depression[10] and to have neurotoxic actions[11] in the retina *in vitro,* and both of these actions can be blocked by non-competitive NMDA-receptor antagonists such as ketamine or MK-801 [(+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine]. Therefore, we considered it possible that loss of the b-wave from the retina postmortem might also be due to anoxic damage involving NMDA receptors. To investigate this, we have attempted to protect rat retinae against postmortem damage by pretreating rats with systemic injections of ketamine or dextromethorphan, and we have also studied the effects of these drugs and MK-801 on the ERG b-wave *in vitro*.

Materials and Methods

Medium

Earle's medium (composition in mM−NaCl 116, KCl 5.4, $NaHCO_3$ 26.2, NaH_2PO_4 1.0, $MgSO_4$ 0.8, $CaCl_2$ 1.8 and glucose 5.5) was pregassed with a moistened mixture of 95% O_2/5% CO_2 and had a final pH of 7.2 at room temperature. Fetal calf serum was added to a concentration of 2% for perfusion studies.

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The electroretinogram

Isolated retinae were placed, ganglion cell layer downwards, onto Whatman No. 1 filter paper and both surfaces of the tissue/filter paper preparation were perfused at 7ml/min with 400 ml of recirculating perfusion medium. The medium was gassed continuously with a moistened mixture of 95% O_2/5% CO_2 and maintained at 35°C. Photoresponses were recorded with cotton wick and Ag/AgCl electrodes, connected to a high input impedance preamplifier with a band pass of 0–200 Hz. A 1.5 mm diameter L.E.D. stimulus, wavelength 550 nm, was used. Duration was 0.2 sec. and maximum intensity (log I = 0) 2.0×10^4 quanta/um^2.

Application of ketamine, MK-801 and dextromethorphan

(1) Pretreatment of rats with ketamine and dextromethorphan:

Female albino Wistar rats, weighing 200 to 300 grams, were maintained in total darkness overnight before use. Some animals were injected intraperitoneally with 100 mg/kg ketamine[12] under dim red light, and after approximately five minutes they had lost their righting and other reflexes. Other animals were injected with 40 mg/kg dextromethorphan[13] and after 10 minutes the animals, together with the control ones, were anaesthetised with ether. All three groups were then killed by cervical dislocation, and the enucleated eyes stored at room temperature for the times indicated in the text. The Guiding Principles in the Care and Use of Animals (DHEW publication, NIH 80–23) were followed throughout.

(2) Application of ketamine, MK-801 and dextromethorphan *in vitro:*

Ketamine (final concentration 150 to 300 μM),[14] MK-801 (100 to 200 μM)[15] or dextromethorphan (100 to 800 μM)[16] was added to the perfusion medium during measurement of the ERG. In these experiments control responses to light stimuli were recorded within 5 minutes of the death of the animal.

Application of APB in vitro

For comparison, the effect of DL-APB (2-amino-4-phosphonobutyric acid) on the ERG was also studied, as this compound has been shown to act selectively on ON-bipolar cells and thus eliminates the b-wave.[17,18]

Drugs

Ketamine and MK-801 were generous gifts from Parke-Davis Veterinary (Pontypool) and Merck, Sharpe and Dohme (Harlow), respectively. Dextromethorphan and APB were purchased from Sigma Chemicals.

Results

All experiments were individually performed with at least three animals. Typical representative observations are shown throughout, as a given treatment protocol resulted in very similar observations in all preparations in a group.

Figure 1 shows that the threshold and amplitude of both the a- and b-waves of retinae from rats which were not pretreated with ketamine were almost unaffected by the presence of up to 300 μM ketamine in the perfusion medium (n = 5). Similarly, MK-801 (up to 200 μM) was without effect (n = 3). Figure 2 shows that, at concentrations of 100 and 200 μM,[16] dextromethorphan had no effect on the ERG, including the b-wave (n = 4). Only when the concentration of dextromethorphan was increased to 400 or 800 μM, did the drug exert a non-specific,

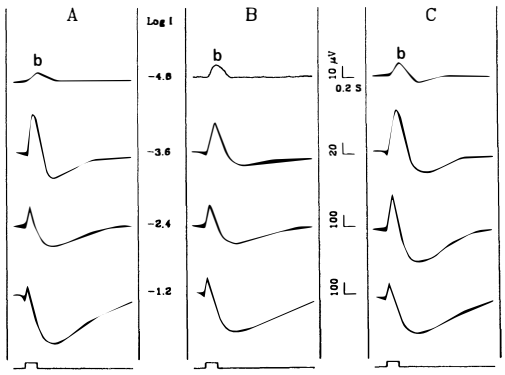

Fig. 1. *Effect of ketamine on the ERG* in vitro. *The ERG was recorded in a dark-adapted, isolated rat retina. Ketamine was added to the perfusion medium to a final concentration of 150 μM. A) Before, B) 4 minutes after, and C) 16 minutes after the application of ketamine. Note: increasing the concentration to 300 μM did not change the responses (not shown). The sweep duration was 2 s and stimulus duration 0.2 s, as indicated by the markers beneath the records. Responses obtained at different stimulus intensities (Log I = −4.8 to −1.2) are shown. b = b-wave.*

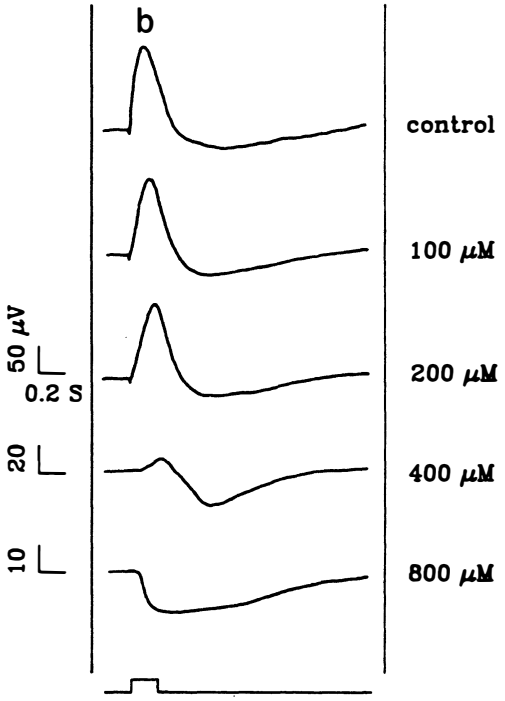

Fig. 2. *Effect of dextromethorphan on the ERG* in vitro. *The ERG responses were recorded in a dark-adapted, isolated rat retina before and 10 minutes after applications of dextromethorphan with increasing concentrations. Note: after the responses were suppressed, no recovery was observed throughout 2 h of reperfusion with non-dextromethorphan medium. The sweep duration was 2 s and stimulus duration 0.2 s. Stimulus intensity: log I = −2.4. b = b-wave.*

toxic effect on the responses—irreversibly suppressing both the b-wave and PIII component (Figure 2). Such high concentrations of dextromethorphan are known to exert non-specific effects.[16] In addition to the above observations, intraperitoneal injection of 100 mg/kg ketamine prior to tissue isolation had no direct effect on the a- or b-waves of retinae which had been isolated immediately (n = 3).

In contrast to the effects seen with non-competitive NMDA antagonists, addition of 10 to 100 μM APB to the perfusion medium reversibly blocked the b-wave (Figure 3) (n = 3), thus showing that the system was responsive to a drug that acts selectively on ON-bipolar cells.[17,18]

To see if ketamine or dextromethorphan could prolong survival of the b-wave during postmortem anoxia, dark-adapted eyes, taken from rats previously injected with the drug, were left in the dark at room temperature for 30 and 60 minutes, respectively, and then the retinae were isolated. For comparison, eyes from rats without pretreatment with the drug were kept for the same periods of time. Following 30 minutes storage and subsequent perfusion with oxygenated medium, a b-wave gradually became manifest, taking about 10 minutes to reach its peak of 10 uV: it then declined (Figure 4) (n = 3). No b-wave was seen in eyes which had been stored *ex vivo* for 60 minutes (n = 3). Prior intraperitoneal administration of ketamine or dextromethorphan made no difference to the above observations (Figure 4).

Discussion

In this study we have used the b-wave of the erg as an index of synaptic transmission from photoreceptors to second-order retinal cells. Although it is possible that the b-wave arises remotely from these second-order neurones, it is generally agreed that generation of the b-wave involves second-order neurones and that blockade of neurotransmission between photoreceptors and ON-bipolar cells abolishes the b-wave.[4,5,17,18] The observation of partial recovery of the b-wave in the rat retina after 30 minutes anoxia (Figure 4) is in agreement with Winkler[19] who reported that a b-wave could be regained by re-perfusion

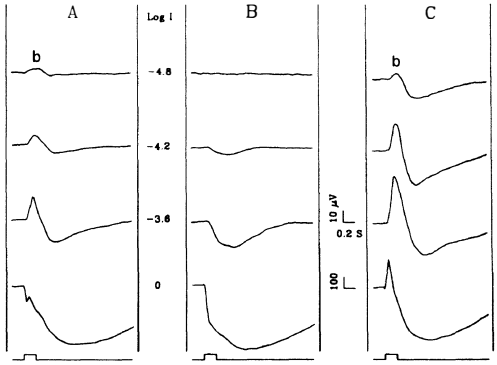

Fig. 3. *Effect of APB on the b-wave* in vitro. *The ERG was recorded in a dark-adapted, isolated rat retina. APB (final concentration 100 μM) was added to the perfusion medium, as described in the text. A) Before and B) 2 minutes after the application of APB. C) 2 minutes after reperfusion with non-APB medium. Note: reducing the concentration of APB to 10 μM produced similar effects on the b-wave. The sweep duration was 2 s and stimulus duration 0.2 s. b = b-wave.*

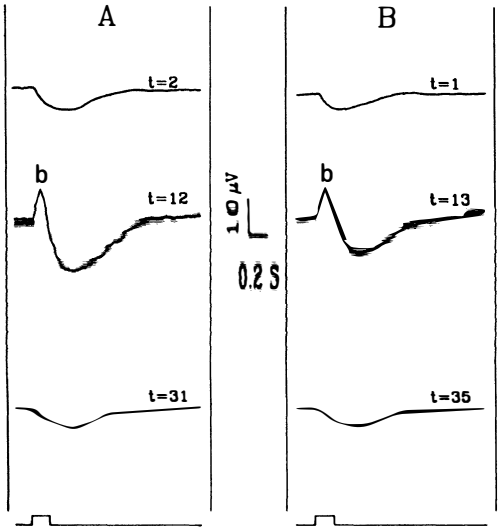

Fig. 4. In vivo *pretreatment with ketamine. Dark-adapted eyes were kept in the dark at room temperature for 30 minutes before measurement of the ERG: A) with and B) without prior intraperitoneal injection of ketamine (see the text for details.). The sweep duration was 2 s and stimulus duration 0.2 s. Stimulus intensity: log I = −2.7. t = time in minutes after the start of retinal perfusion. b = b-wave.*

with oxygenated medium within 60 minutes anoxia, with no recovery if re-perfusion was commenced after this 60 minute period.

The present results indicate that ketamine, MK-801 and dextromethorphan have no direct effect on the b-wave in the rat retina (Figure 1, Figure 2). It is unlikely that the lack of effect on the b-wave is due to an inability of these drugs to penetrate the tissue, as APB was effective in our preparation. A more likely explanation is that the b-wave is not mediated via NMDA receptors. This is in agreement with Bloomfield and Dowling[20] who found that NMDLA (N-methyl-DL-aspartate) had no clear effect on rabbit ON-bipolar cells, and with Coleman and Miller[21] who showed that D-2-amino-5-phosphonovaleric and D-2-amino-7-phosphonoheptanoic acids (competitive NMDA antagonists) have no effect on the ERG in the mudpuppy. Thus the current observation confirms that there is little or no synaptically activated NMDA-receptor component to ON-bipolar cell responses to light in the vertebrate retina.

Since neither the b-wave nor PIII was better preserved postmortem by pre-treatment of rats with ketamine or dextromethorphan (Figure 4), and both responses were not affected by *in vitro* application of NMDA-receptor antagonists as discussed above, it is unlikely that their postmortem decline involves NMDA-receptor activation. This is, at first sight, somewhat surprising in view of the previous positive finding relating to the protection of the b-wave by dextromethorphan upon transient ischaemia *in vivo*.[9] However, it is possible that different mechanisms underly *in vivo* ischaemia and postmortem anoxic damage or that dextromethorphan has other pharmacological effects on the rabbit retina than the proposed NMDA-receptor antagonism. According to Yoon and Marmor's observations in the rabbit,[9] dextromethorphan prevents the photoreceptor cells, particularly the outer segments, from degenerative changes caused by 60 minutes' ischaemia. This phenomenon is interesting, but the mechanisms of neuro-protection may not be via a blockade of NMDA receptors of photoreceptor cells, because NMDA has been shown to be ineffective in inducing membrane potential changes in photoreceptor cells.[20,22]

It has been shown in animals with a dual retinal circulation (such as the rat,[23] gerbil,[24] cat[25] and monkey[26]) that the initial degenerative changes, caused by ischaemia of the eye, occur in the inner retina. By contrast, in the rabbit[27,28] much of whose retina in effect only has a choroidal blood supply, interruption of the circulation causes ultrastructural abnormalities in photoreceptor outer segments within 30 minutes and in the inner segments and nuclei within two hours. Thus it seems that hypoxic damage to the retina, in terms of the sensitivity of geographic location, relates to its *in vivo* circulation conditions. It has also been suggested that dextromethorphan may induce an increase in cerebral blood flow or a decrease in cerebral metabolic requirements.[29] Therefore, it is possible that the protection of rabbit photoreceptor cells by dextromethorphan against ischaemia is related to a reduction of tissue metabolic rate rather than a blockade of NMDA receptors. This requires further investigation by comparing various species and pharmacological agents.

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Key words: b-wave, dextromethorphan, electroretinogram, ketamine, MK-801, NMDA antagonist, rat retina, excitatory amino acids, neurotoxicity.

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