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Effect of midazolam, medetomidine, and butorphanol tartrate combination anesthetic on electroretinograms of mice

Yukihiro Miwa,[1,2] Kazuo Tsubota,[2] Toshihide Kurihara[1,2]

[1]Laboratory of Photobiology, Keio University School of Medicine, Tokyo, Japan; [2]Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan

Purpose: To evaluate electroretinogram (ERG) responses under anesthesia with midazolam, medetomidine, and butorphanol tartrate (MMB) combination compared with pentobarbital sodium and ketamine/xylazine (KX).
Methods: Six-week-old male C57BL/6J mice were divided into MMB-, pentobarbital sodium-, and KX-administered groups. Following overnight dark adaptation, an ERG was performed. The parameters sensitivity (*S*), log maximum amplitude (R_{max}), R_{max}, and time delay to the onset (T_d) of the ERG a-waves were computed based on the Lamb and Pugh model. The parameters light intensity at half maximum amplitude (*K*), R_{max}, and *n* of the ERG b-waves were computed based on the Naka-Rushton equation. The amplitude and the implicit time of oscillatory potentials (OPs) were quantified.
Results: The T_d of the dark-adapted a-waves was statistically significantly larger under anesthesia with the MMB combination and pentobarbital sodium compared to KX. The *K* of the dark-adapted b-waves was statistically significantly larger under anesthesia with pentobarbital sodium compared to the MMB combination. The amplitude of the dark-adapted OPs was statistically significantly larger under anesthesia with the MMB combination compared to pentobarbital sodium. The implicit time of the dark-adapted OPs was statistically significantly smaller under anesthesia with the KX combination compared to pentobarbital sodium.
Conclusions: The results suggested that ERG responses, especially in OPs, are greatly affected by the type of anesthetic. It is important to consider the sensitive responses influenced by the selection of anesthetics when ERG is performed.

Electroretinogram (ERG) is one of the most important methods for evaluating retinal function. To date, isoflurane, pentobarbital sodium (PS), and ketamine/xylazine (KX) are widely used for general anesthesia in animal experiments that include ERG [1-3]. To use isoflurane, a calibrated vaporizer is needed, because the anesthetic is supplied through the respiratory tract to animals. Furthermore, local exhaust ventilation is also needed to reduce the exposure of the examiner to the anesthetic gas. Pentobarbital sodium is an anesthetic which can be conveniently used by intraperitoneal administration. However, pentobarbital sodium is known to inhibit respiration and decrease blood pressure at the depth of anesthesia necessary for the ERG procedure [4]. The combination anesthetic of ketamine and xylazine is the most commonly used for mice. KX combination anesthetic can be used with intraperitoneal administration, and has a safe antagonist [5,6]. However, KX combination anesthetic has some problems, such as a high mortality rate depending on the strain and gender of the mouse [3,7]. Thus, it is desired to perform ERG on animals with a safe anesthetic without a special device requirement.

Correspondence to: Toshihide Kurihara, Laboratory of Photobiology, Department of Ophthalmology, Keio University School of Medicine; 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Phone: +81-3-5363-3204; FAX: +81-3- 5363-3274; email: kurihara@z8.keio.jp

Recently, midazolam, medetomidine, and butorphanol tartrate (MMB) combination anesthetic has been widely used with intraperitoneal administration in animal experiments [8,9]. An advantage of MMB combination anesthetic is the presence of an antagonist (atipamezole hydrochloride) that helps safer recovery from anesthesia, and there is no change in mortality due to the strain or gender of the mouse. However, there is little information on the effect of MMB combination anesthetic on ERG in animals, including mice. In this study, we compared ERG responses obtained from different anesthetic conditions to explore the effect of the MMB combination.

METHODS

Animals: All procedures were approved by the Ethics Committee on Animal Research of the Keio University School of Medicine and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the Institutional Guidelines on Animal Experimentation at Keio University, and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for the use of animals in research. C57BL/6J mice were obtained from CLEA Japan, Inc. (6 weeks old, male, Tokyo, Japan). The mice were divided randomly into the MMB-administered group (n=5), the pentobarbital sodium-administered group (n=6), and the KX-administered group (n=8).

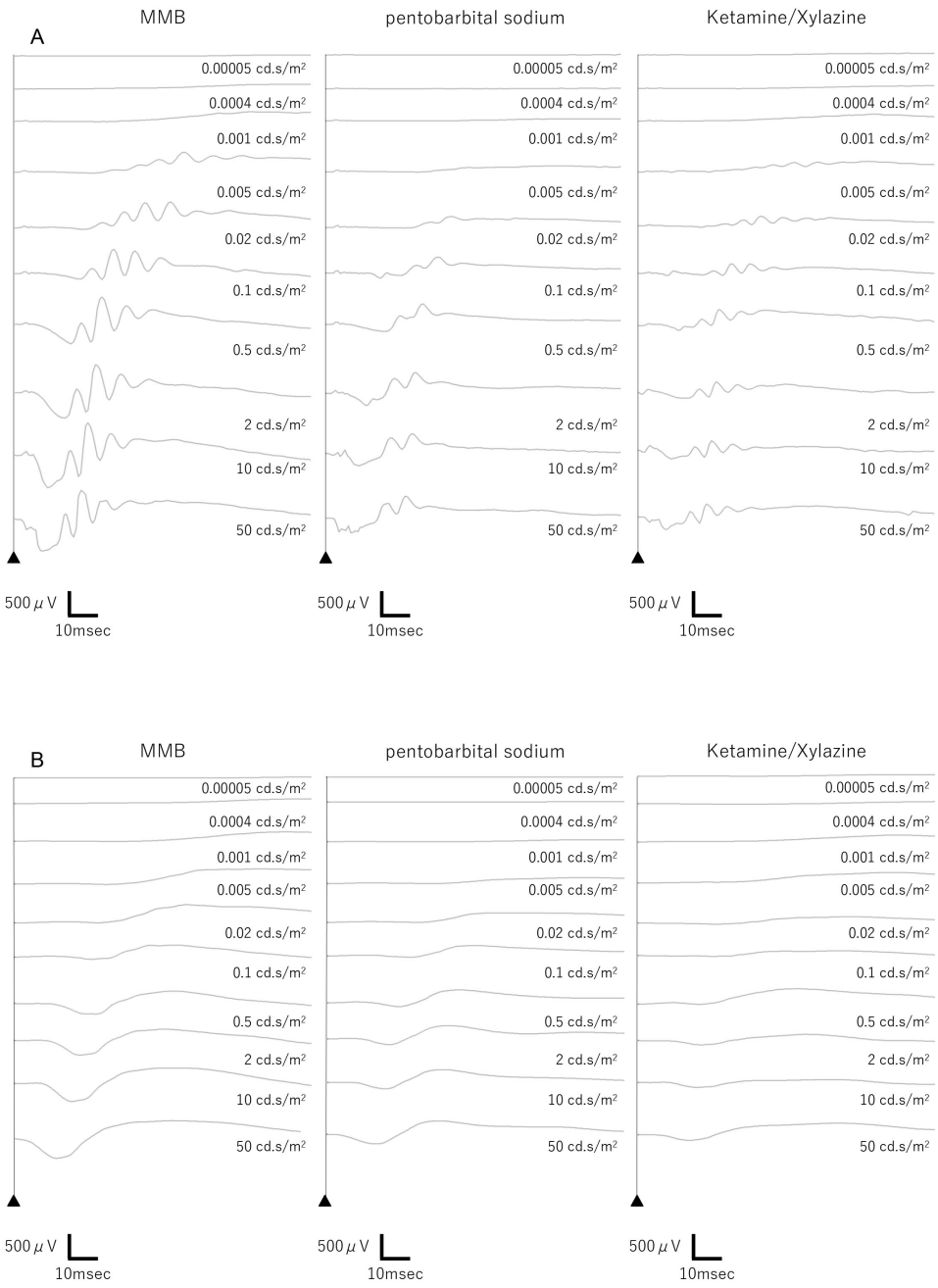

Figure 1. Dark-adapted ERGs. **A**: Representative electroretinogram (ERG) recordings performed with midazolam, medetomidine, and butorphanol tartrate (MMB; left), pentobarbital sodium (center), and ketamine/xylazine (KX; right). **B**: The waveforms processed with a 30 Hz low-pass filter for b-wave fitting.

Drugs: The MMB combination anesthetic contained midazolam (4 mg/kg, SANDOZ, Yamagata, Japan), medetomidine (0.75 mg/kg, Nippon Zenyaku Kogyo Co., Ltd, Fukushima, Japan), and butorphanol tartrate (5 mg/kg, Meiji Seika Pharma, Tokyo, Japan). Each drug was contained at a ratio of 1.00:1.07:1.33 in the MMB combination anesthetic. Pentobarbital sodium (64.8 mg/kg, Kyouritu Co., Ltd., Tokyo, Japan) and the combination of ketamine (ketamine hydrochloride, 80 mg/kg, Daiichi Sankyo Pharmaceutical Ltd., Tokyo, Japan) and xylazine (ROMPUN, 10 mg/kg, Bayer) were used for the comparison.

Electroretinogram: ERG was performed as previously described [10]. Full-field flash ERG responses were recorded using a Ganzfeld dome, an acquisition system, and light-emitting diode (LED) stimulators (PuREC, MAYO Corporation, Inazawa, Japan). Following overnight dark adaptation, the mice were anesthetized with MMB combination, pentobarbital sodium, or KX combination anesthetic under dim red light. A mixed solution of 0.5% tropicamide and 0.5%

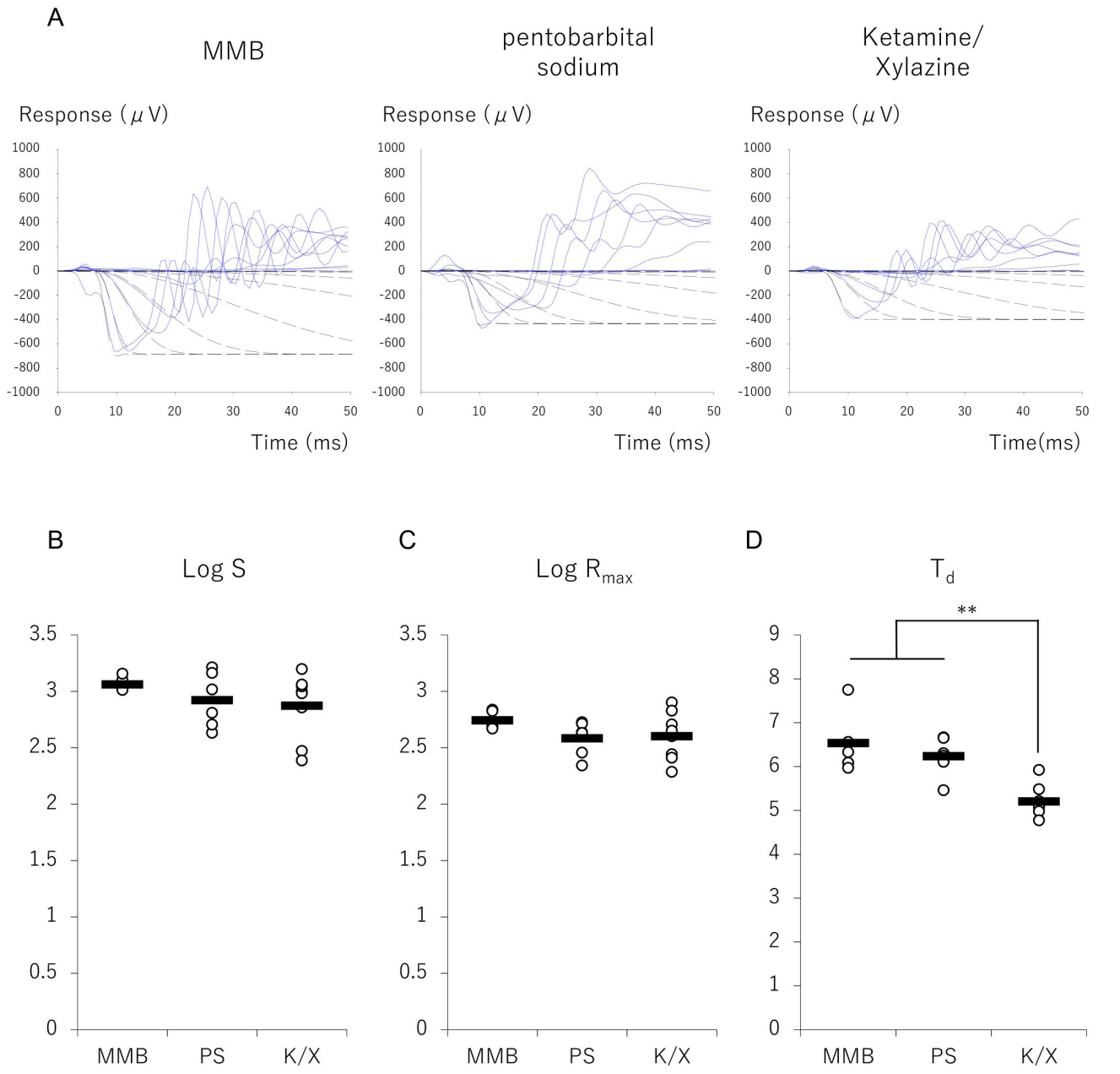

Figure 2. Fitting of dark-adapted ERG a-waves. **A**: Representative a-wave fitting waveforms of midazolam, medetomidine, and butorphanol tartrate (MMB), pentobarbital sodium, and ketamine/xylazine (KX). The log sensitivity (*S*), log maximum amplitude (R_{max}), and delay before the onset of the a-wave (T_d) parameters of each group are shown in **B**, **C**, and **D**, respectively. **D**: Note that the T_d of the mice administered MMB and pentobarbital sodium was larger than that of the mice administered KX. Statistically significant differences are indicated by ** for $p<0.01$ using one-way factorial ANOVA (ANOVA) followed by Tukey's test.

phenylephrine (Santen Pharmaceutical Co., Osaka, Japan) was used to dilate the pupils. The active electrodes were recorded with contact lens electrodes, and the reference electrode was placed subcutaneously between the eyes. An electrode clipped to the tail served as a ground. ERG responses were obtained from the right eye of each animal. Scotopic responses were recorded under dark adaptation with various stimulus (Figure 1A). Photopic responses were recorded with various stimuli

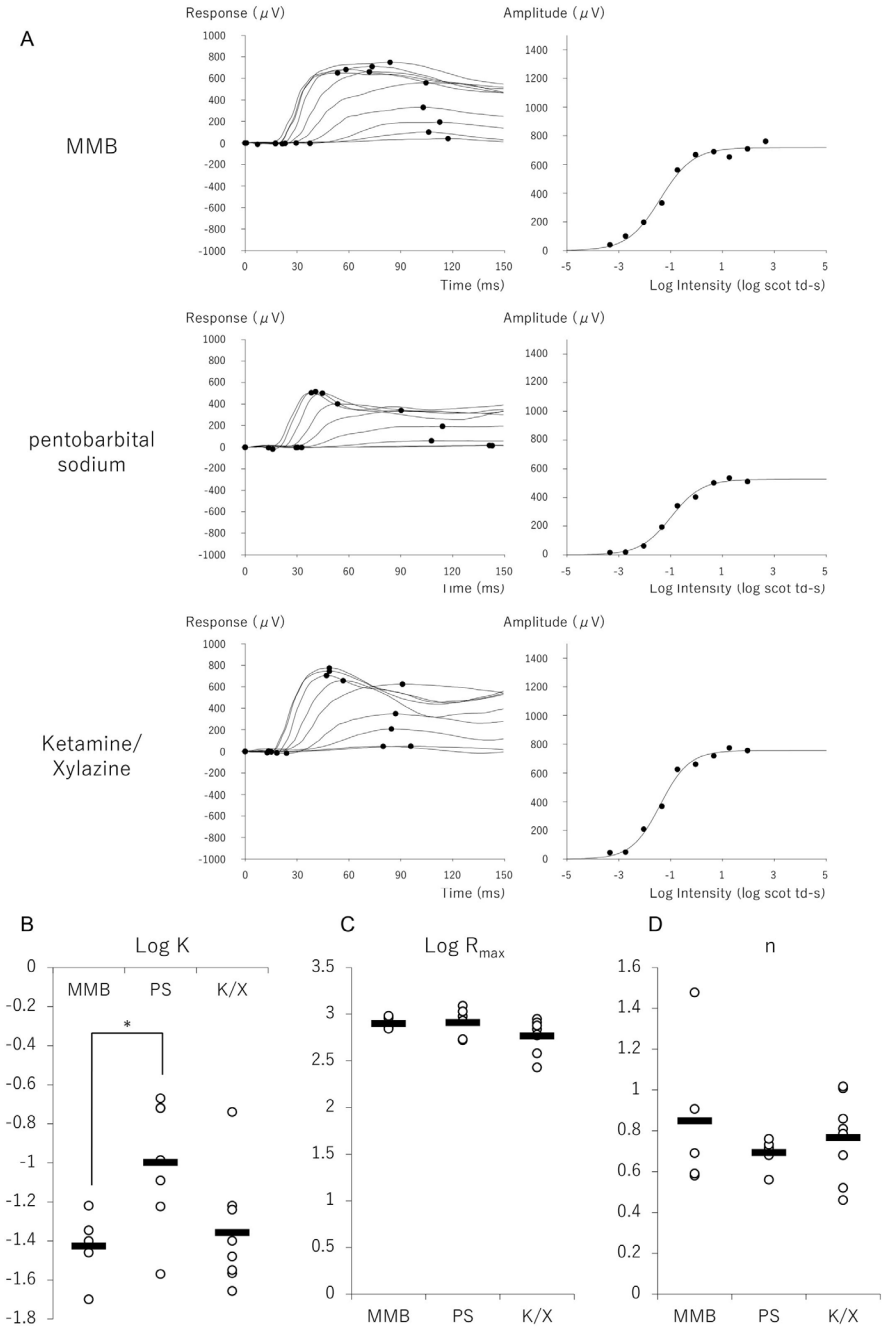

Figure 3. Fitting of dark-adapted ERG b-waves. **A**: Representative b-wave fitting waveforms of midazolam, medetomidine, and butorphanol tartrate (MMB), pentobarbital sodium, and ketamine/xylazine (KX). The log light intensity at half maximum amplitude (*K*), maximum amplitude (R_{max}), log R_{max}, and *n* parameters of each group are shown in **B**, **C**, and **D**, respectively. **B**: Note that the *K* of the mice administered pentobarbital sodium was larger than that of the mice administered MMB. Statistically significant differences are indicated by * for p<0.05 using one-way factorial ANOVA (ANOVA) followed by Tukey's test.

against a white background (30 cd/m^2). All mice were kept warm during the procedure using heat pads.

ERG a-waves were fitted to the following equation after being processed with a 300 Hz low pass filter. We computed the response (R) as a function of time (T) and flash intensity (I) based on the Lamb and Pugh model [11]. R_{max} is the maximum amplitude, S is the sensitivity, and T_d is the delay before the onset of the a-wave. The S and R_{max} data were transformed into log values for data analysis:

$$R(I,T) = \{1\text{-}\exp[-I \times S \times (T\text{–}Td)^2] \times R_{max}.$$

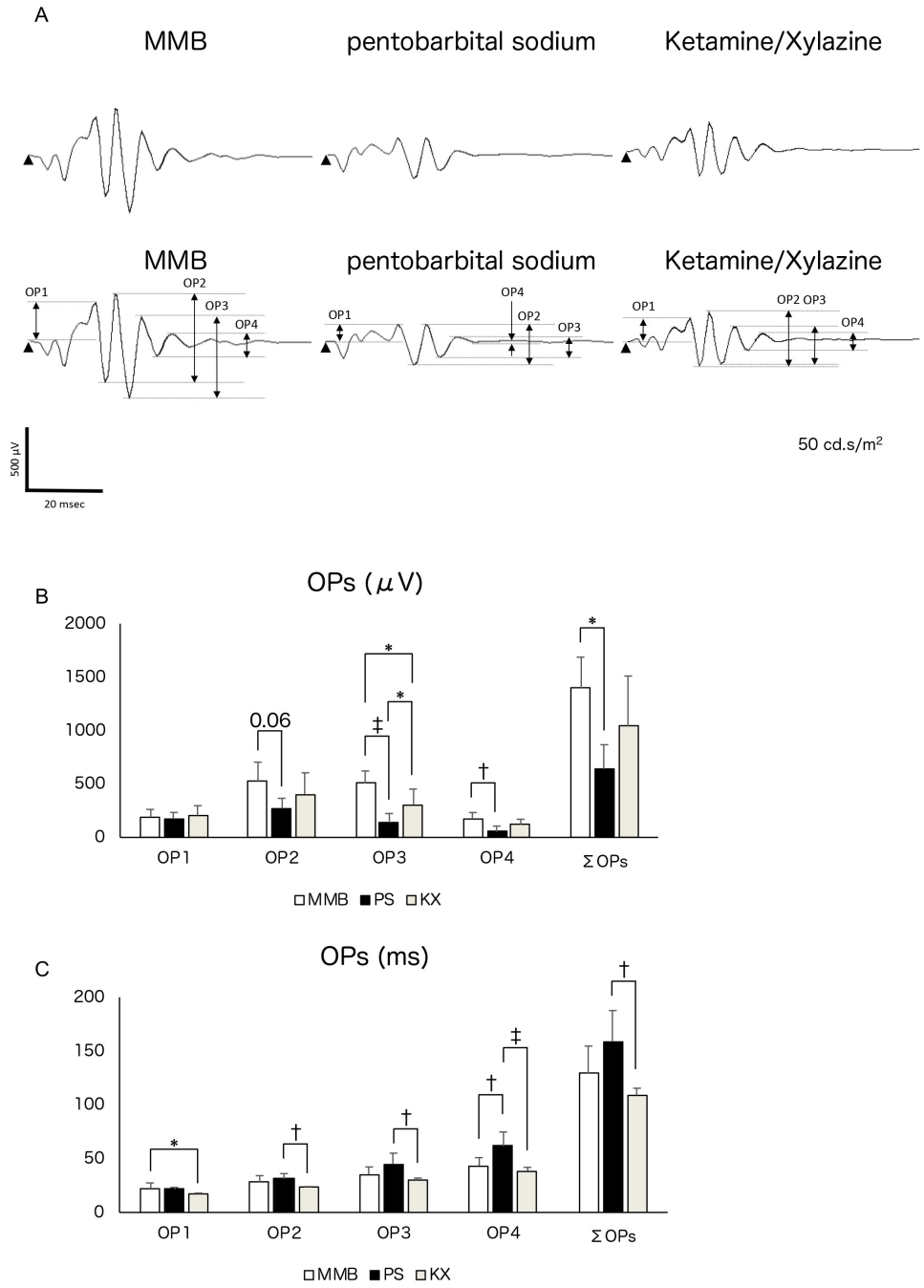

Figure 4. Dark-adapted ERGs representing scotopic OPs. Oscillatory potentials (OPs) were recorded under dark adaptation in response to a flash at intensity of 50 cd.s/m^2. **A**: Representative waveforms of electroretinogram (ERG) performed with midazolam, medetomidine, and butorphanol tartrate (MMB (left), pentobarbital sodium (PS; center), and ketamine/xylazine (KX; right). The waveforms were processed with a 65 Hz high-pass filter and a 300 Hz low-pass filter to quantify the OPs. **B**: The amplitude of the OPs. Note that the amplitude of the OPs in the mice administered MMB was statistically significantly larger than that in the mice administered pentobarbital sodium except OP1 and OP2. The amplitude of the OPs in the mice administered MMB was statistically significantly larger than that in the mice administered KX for OP3. The amplitude of the OPs in the mice administered KX tended to be larger than the amplitude of the mice administered PS. **C**: Note that the implicit time of the OPs in the mice administered PS was statistically significantly delayed compared to the mice administered KX except OP1. The implicit time of the OPs in the mice administered PS showed a tendency for delay compared to the mice administered MMB. Statistically significant differences are indicated as *p<0.05, †p<0.01, ‡p<0.001 using one-way factorial ANOVA (ANOVA) followed by Tukey's test. Error bars indicate mean plus standard deviation (SD).

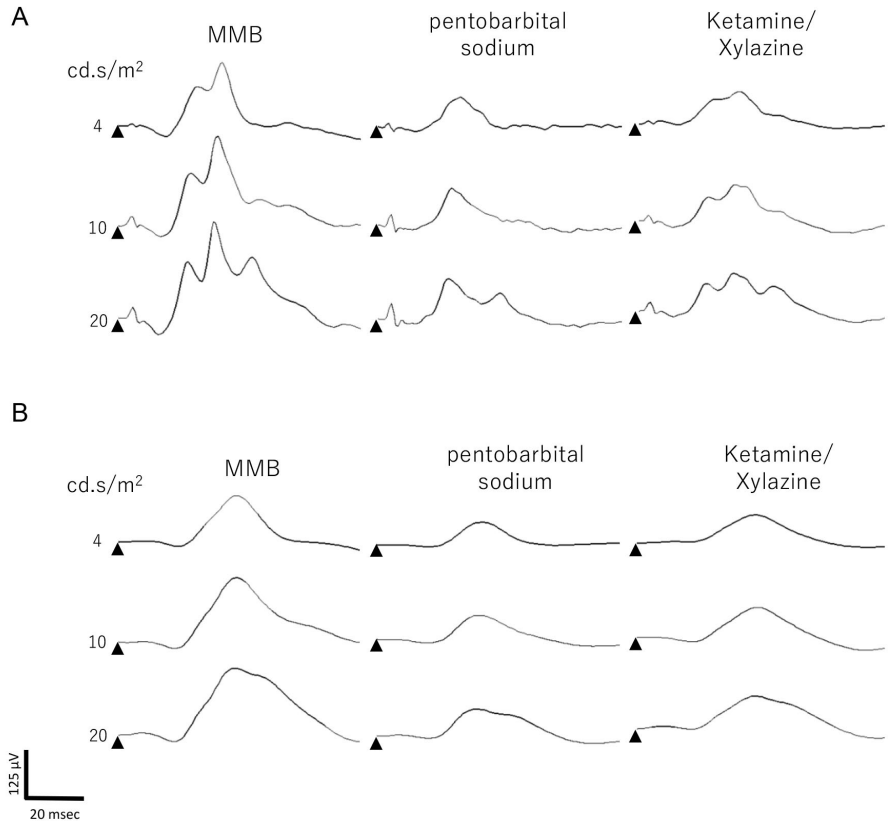

Figure 5. Light-adapted ERGs. **A**: Representative light-adapted electroretinogram (ERG) recordings performed with midazolam, medetomidine, and butorphanol tartrate (MMB), pentobarbital sodium, and ketamine/xylazine (KX) against a white background. **B**: The waveforms processed with a 30 Hz low-pass filter for b-wave fitting.

ERG b-waves were fitted to the Naka-Rushton equation after being processed with a 30 Hz low-pass filter (Figure 1B) [12]. R is the amplitude, R_{max} is the maximum amplitude, I is the flash intensity, K is the light intensity at half maximum amplitude, and n is a dimensionless compressive constant:

$$R = I^n / I^n + K^n \times R_{max}.$$

For the quantification of OPs, the responses were processed with a 65 Hz high-pass filter and a 300 Hz low-pass filter. The amplitude of OP1 was measured from the baseline to the peak of OP1. The amplitudes of OP2, OP3, and OP4 were measured from the lowest point of the immediately preceding negative wave to the peak of each waveform. Total OPs were expressed as ΣOP, which is the sum of OP1 to OP4. The implicit times of OPs were measured from the moment of the stimulation to the top of each OP.

Statistical analyses: Significant differences were determined using one-way factorial ANOVA (ANOVA). Probability values of less than 0.05 were considered statistically significant. All results in this paper were expressed as the mean ± standard deviation (SD).

RESULTS

The results of the dark-adapted ERG a-waves fitting are shown in Figure 2. In the a-wave analysis, the T_d [F(2,16)=13.531, p<0.01] of the dark-adapted a-waves was statistically significantly larger under anesthesia with the MMB combination and pentobarbital sodium compared to KX (Figure 2D). In the results for S [F(2,16)=0.982, p>0.05] and R_{max} [F(2,16)=1.444, p>0.05], there were no statistically significant differences between any groups (Figure 2B,C).

The results of the dark-adapted b-waves fitting are shown in Figure 3. In the b-wave analysis, the K [F(2,17)=4.270, p<0.05] of the dark-adapted b-waves was statistically significantly larger under anesthesia with pentobarbital sodium compared to the MMB combination (Figure 3B). In the results for R_{max} [F(2,16)=2.39, p>0.05] and n [F(2,16)=0.601, p>0.05], there were no statistically significant differences between any groups (Figure 3C,D).

The results of the dark-adapted OPs stimulated with 50 cd.s/m^2 are shown in Figure 4. The amplitude of the OPs of the MMB-administered group was statistically significantly

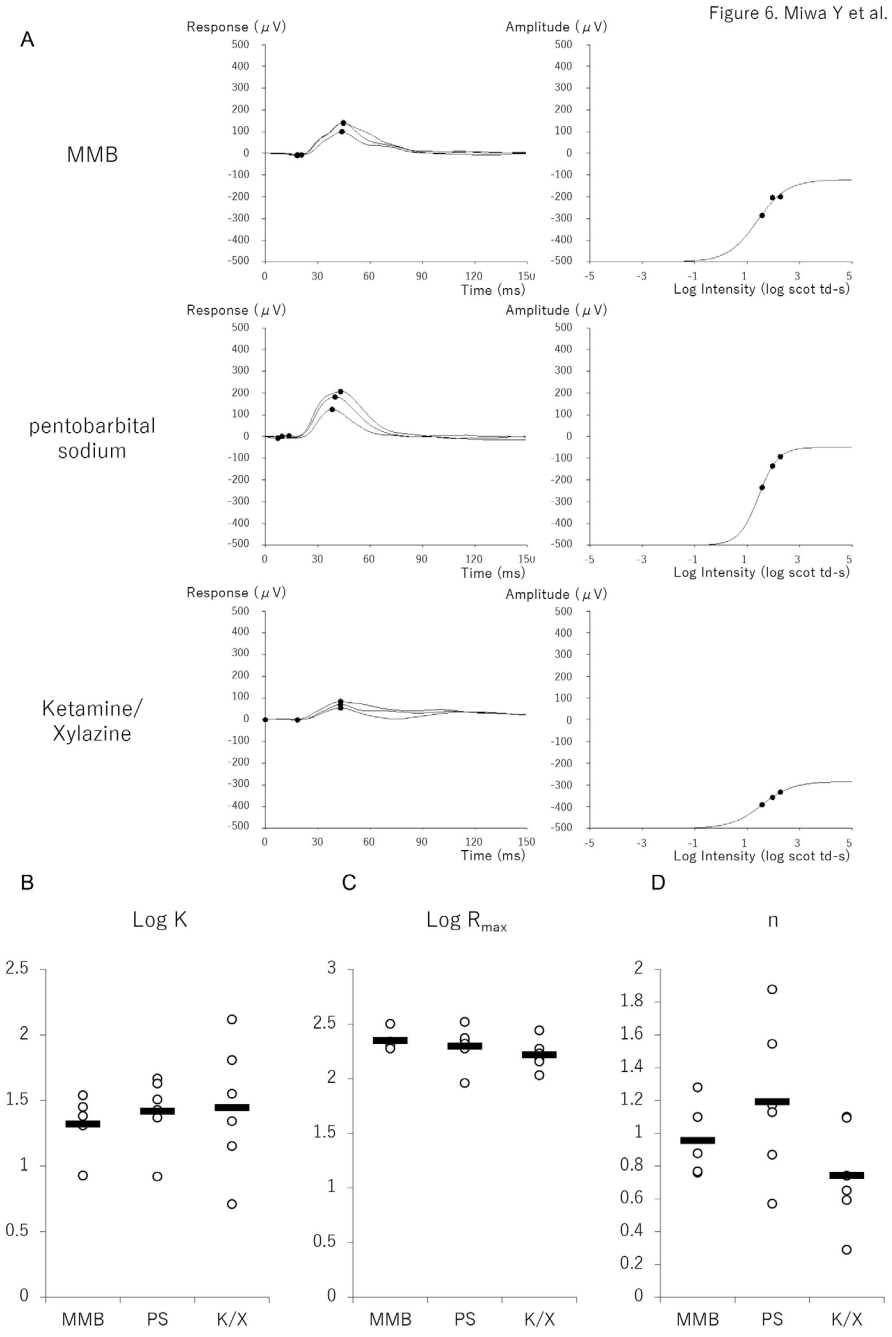

Figure 6. Fitting of light-adapted ERG b-waves. **A**: Representative light-adapted b-wave fitting waveforms of midazolam, medetomidine, and butorphanol tartrate (MMB), pentobarbital sodium, and ketamine/xylazine (KX). Log light intensity at half maximum amplitude (*K*), log maximum amplitude (R_{max}), and *n* parameters of each group are shown in **B**, **C**, and **D**, respectively. Note that there were no statistically significant differences between the three groups.

larger compared to that of the PS-administered group (Figure 4B). In addition, although there was no statistically significant difference except OP3, the amplitude of the OPs in the KX-administered group tended to be larger than that in the PS-administered group (Figure 4B). Furthermore, the implicit time of the OPs in the PS-administered group was statistically significantly extended compared to that of the KX-administered group, and the extension tendency was also observed compared to the MMB-administered group (Figure 4C).

Photopic responses were recorded with various stimuli against a white background, and processed with a 30 Hz low-pass filter (Figure 5). The results of the light-adapted b-waves fitting are shown in Figure 6. There were no statistically significant differences between any groups (Figure 6B–D), for *K* [F(2,14=1.77, p>0.05], *Rmax* [F(2,14=1.15, p>0.05], and *n* [F(2,14=2.38, p>0.05].

DISCUSSION

Several studies have described the influence of ERG waveforms caused by anesthetics [3,13-16]. There are several reports on the change in ERG waveforms when KX or pentobarbital sodium is administered [17]. However, there is no study which examined how ERG waveforms change when MMB is used. Sugimoto et al. reported that the ERG waveforms of mice administered pentobarbital sodium showed obscured OPs but did not have a statistically significant effect on a-wave and b-wave compared to those of mice receiving KX [18]. In this study, we examined the ERG of the mice receiving the MMB combination compared to mice receiving pentobarbital sodium and KX as general anesthesia. Consistent with the previous report, the OPs of the mice administered pentobarbital sodium showed a statistically significantly smaller amplitude than those of the mice administered MMB or KX (Figure 4B). The implicit time was also statistically significantly prolonged with administration of pentobarbital sodium (Figure 4C).

OPs are small wave groups that are superimposed on the ascending limb of the b-wave [19]. Studies have been conducted to explore the origins of OPs. For example, intravitreal administration of glycine damaging amacrine cells has been reported to show an attenuated amplitude of OPs. The result suggested that OPs could be derived from amacrine cells [20,21]. Midazolam contained in MMB belongs to the benzodiazepine class of drugs. It is known that benzodiazepine exerts a sedative effect by enhancing the action of gamma-aminobutyric acid (GABA) at the $GABA_A$ receptors [22]. Amacrine cells include dopaminergic and GABAergic cells [23]. The activation of $GABA_A$ receptors may be one of the mechanisms of the larger amplitude of the OPs. However, pentobarbital sodium was reported to suppress the amplitude of OPs by affecting bipolar cells and Müller cells in isolated rat retinas [24]. Thus, it is possible that the results of the present study may be emphasized by applying pentobarbital sodium as the comparison.

In this research, we also compared several key ERG parameters under scotopic and photopic conditions. As a result of the dark-adapted a-wave analysis, T_d indicating the response time to the light stimulus in the mice administered MMB and pentobarbital sodium was statistically significantly larger compared with that of the mice administered KX (Figure 2C,D). In the results of the dark-adapted b-wave analysis, the *K* was statistically significantly larger under anesthesia with pentobarbital sodium compared to the MMB combination (Figure 3B).

In conclusion, the present data suggest that OPs are greatly affected by anesthetics. Therefore, it is necessary to unify the anesthetics or to pay careful attention to the difference in ERG waveforms by the type of anesthetic. OPs are attenuated in retinal circulation disorders, such as diabetic retinopathy (DR) and retinal vein occlusion (RVO) [19,25,26]. Selecting the MMB combination for anesthesia may be useful for detecting small differences in OPs finely, especially to evaluate ERG in animal models of DR or RVO.

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