Voltage-sensitive dye imaging of the visual cortices responding to electrical pulses at different intervals in mice *in vivo*

Yuki Hayashida, Kozo Takeuchi, Naohiro Ishikawa, Yuka Okazaki, Fehevari Tamas, Hiroki Tanaka, Tetsuya Yagi.

Abstract— **Properties of the neural responses to electrical stimulus pulses delivered at various inter-pulse intervals were examined in the visual cortices of mice in vivo, with utilizing the voltage-sensitive dye imaging technique. Our experimental results provided the relationships between the inter-pulse intervals and the stimulus-evoked transient depolarizations, which may offer insight into the design of effective and efficient stimulation for cortical visual prostheses.**

I. Introduction

It is known for several decades that a visual sensation of small spots of light, commonly called phosphenes, can be artificially induced in humans through electrical stimulation of the primary visual cortex (V1) [1][2]. Although many challenges have been posed in the clinical development of cortical visual prostheses, some of those should be overcome in the near future by the state-of-the-art technology, e.g. multielectrode array [3] or fully implantable stimulator device [4]. On the other hand, previous psychophysical or behavioral studies in primates, including humans, provided truly invaluable, but somehow limited information regarding the effect of stimulus parameters on the phosphene perception [5]. And the underlying neural responses of visual cortices to the electrical stimuli have remained much less understood on experimental animals [6][7]. Such a situation seems to be in contrast with the development of the cochlear implant [8][9].

Based on previous studies on the intracortical microstimulation in humans, one of the important stimulus parameters to modulate the characteristics of phosphenes is thought to be the temporal sequence of stimulus pulses applied. For instance, the perceived phosphenes faded in less than one second during the single continuous pulse train with the inter-pulse frequency of 75-200 Hz; whereas the phosphene lasted for ~2 sec when the intermittent interval of ~25 msec was inserted between the pulse trains of 125 msec duration each with the same inter-pulse frequency [10]. These observations could be explained by the dynamics of neuronal responses to the repetitive pulses, and/or of the subsequent synaptic transmissions.

A number of recent studies showed that basic response properties observed in the visual cortices of higher-order mammals are also present in the mouse cortex [11]. Besides, the visual areas located on the smooth-surfaced cortex can be advantageous for the functional imaging of neural activities over those areas simultaneously. Thus, in the present study, the neural response properties of visual cortices to the electrical pulses were examined in mice in vivo, with utilizing the voltage-sensitive dye (VSD) imaging technique [12]. We investigated the effect of inter-pulse intervals on the stimulus-evoked responses with using two types of temporal sequences of stimuli.

II. Methods

A. Animal surgery

All animal care and experimental procedures in the present experiments were approved by the committee for animal researches of Osaka University, and performed in accordance with the guidelines from The Physiological Society of Japan. Adult C57BL/6 mice (8 to 10 weeks, Clea Japan Inc.) were anesthetized via intraperitoneal (i.p.) injection of Urethane (125 wt.%, 5 μl/g b.w.) and administered with atropine (10 μg i.p.) and dexamethasone (20 μg i.p.). Animals were then secured with a stereotaxic apparatus. The rectal temperature was maintained at 37 ^{o}C with a blanket and heat pad, and electrocardiogram (ECG) was monitored throughout experiments. Craniotomy was made on the right posterior parietal bone to expose the cortical visual areas (1-5 mm posterior from the Bregma) with the dura mater intact. A thin (1 mm) silicon tube with an inner diameter of 6.5 mm was attached to the parietal bone to make a chamber that encircled the exposed dura. The chamber was filled with an artificial cerebrospinal fluid (aCSF) supplemented with the VSD, RH1691 (1 mg/ml with 0.125% dimethylsulfoxide), and heparin. After 90 min of the staining, the cortical surface was washed with aCSF for >30 min to remove the residual dye. aCSF was composed of (in mM) 125 NaCl, 2.5 KCl, 0.9 NaH_2PO_4, 5 Na_2HPO_4, 1.2 $CaCl_2$, 1.0 $MgCl_2$, 2.5 $_D$-glucose. During the optical imaging mentioned below, the chamber was filled with a constant volume of aCSF, which was replaced with a fresh aliquot every 1-2 hours. After the imaging experiment described below we verified by making the slice preparations that mostly the layer II/III was stained with RH1691 [13].

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Y. Hayashida is with Graduate school of Engineering, Osaka University, Suita, Osaka 565-0871, Japan (correspondence to; phone: +81(6)6879-7786; fax: +81(6)6879-7784; e-mail: hayashida@eei.eng.osaka-u.ac.jp).

T. Takeuchi, N. Ishikawa, F. Tamas were with the doctoral course in Graduate school of Engineering, Osaka University.

H. Tanaka and Y. Okazaki were with Center for Advanced Medical Engineering and Informatics, Osaka University, Osaka 565-0871, Suita, Japan. H. T. is now with Graduate school of Frontier Informatics, Kyoto Sangyo University, Kyoto, Kyoto 603-8555, Japan.

T. Yagi is with Graduate school of Engineering, and with Center for Advanced Medical Engineering and Informatics, Osaka University.

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C. Optical imaging

Under a epi-fluorescence microscope with a 1.6x objective lens (PlanApo M-series, Leica), the exposed cortex was illuminated with the excitation light (640±14 nm) and the emitted fluorescence was recorded through a dichroic mirror (660 nm) and a barrier filter (675 nm) by a CMOS imaging system (MiCAM-Ultima, BrainVision) at 1000 frames/sec with 100x100 pixels. The observation field was 6.25x6.25 mm on the cortex. The timings of the image acquisition, electrical stimulation, and excitation light exposure were controlled by a trigger signal generated from the R-wave of ECG.

With respect to each pixel in the acquired image, the relative change of the membrane potential of the population of cells was quantified as $\{F(t) - F_0\}/F_0$, where F_0 is the basal fluorescence intensity, and $F(t)$ is the fluorescence intensity at time t (This is referred to as $\Delta F/F$ in the following text). The $\Delta F/F$ was measured with and without the electrical stimulation, and averaged for 8-16 trials. The averaged $\Delta F/F$ obtained with stimulation was subtracted by that without stimulation. High frequency noise in the $\Delta F/F$ signal was reduced off line by using a temporal Bessel filter (3rd-order, 100-Hz cutoff) and a spatial Gaussian filter (5x5 pixel, σ~1).

B. Intracortical microstimulation

Glass microelectrodes with tip diameters of 3-6 μm were used for intracortical microstimulation. The electrode shank was filled with aCSF in contact with a Ag/AgCl wire and the tip impedance was 1-2 MΩ at 1 kHz when measured in aCSF. The tip of the electrode attached to a micromanipulator (NMN-21 Narishige) was advanced into the layer 2/3 around the center of the primary visual cortex (V1). A stimulating reference electrode (Ag/AgCl pellet) was inserted in the mouth, or placed in the head chamber. Cathodic-first biphasic current pulses (200 μs/phase in pulse width) were delivered through the glass microelectrode by using an isolated current generator (STG4008 or STG4002, Multi-channel systems). Amplitude of the current pulse was set at 2-3 times above the threshold for evoking a depolarizing response around the stimulation site; which corresponded to 20-60 μA/phase. We used also two types of pulse-train stimuli: fixed-interval pulse trains and random-interval pulse trains. In the former, the current pulses were delivered at fixed frequencies, ranging from 10 to 80 Hz, for a duration of 500 msec. In the latter, the current pulses were delivered at intervals randomly selected from a preset list: 10, 25, 50, 100, 125, 250 and 500 msec. Either one of these intervals was selected three times in one stimulus sequence of 4-sec duration. Hence the total of 22 pulses was included in one sequence, and the mean pulse frequency was about 5 Hz. In each experiment, five to eleven different random sequences of pulse trains were used. The data sets obtained those multiple sequences were averaged and used for analyses (e.g. Fig. 5), in order to reduce a possible effect of the context, if any, and rather examine the effect of inter-pulse intervals, on the responses. The electrical stimulation of either single pulse, fixed-interval pulse train, or random-interval pulse train was delivered more than 10-25 sec after the precedent stimulation.

III. Results

A. Response of the cortical visual areas to the single pulse stimulus

Fig. 1A shows a typical spatio-temporal pattern of VSD signal in response to the single pulse stimulation in V1. With a relatively large current amplitude (60 μA/phase), the VSD signals corresponding to membrane depolarizations were induced not only in V1, but also in extra-striatal areas, which decayed toward the resting level in several tens of msec (Fig. 1B). The secondary activated area is clearly discernible (at 10 msec post-stimulus, indicated by arrow 'b') and presumed Latero-Medial (LM) area [12, 14], indicating the signal transmission from V1 to the secondary visual area. Fig. 1B shows the effect of current intensity on the responses in V1 (left panel) and the presumed LM (right panel). The peak amplitude of the VSD signals became larger as the current amplitude was increased, and saturated around 60 μA/phase in this case. The response delay between these two areas was 5-10 msec, consistent with the one observed with electrical recordings from single cells [15].

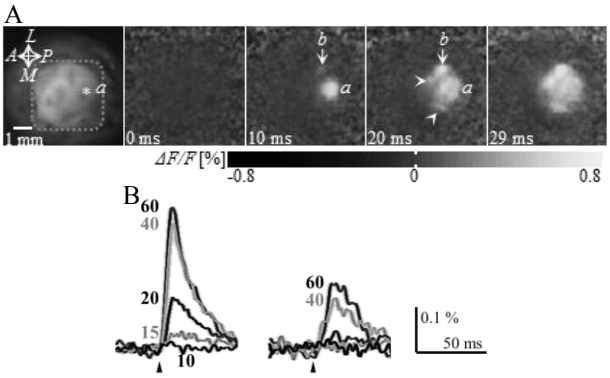

Figure 1. VSD imaging of the response of the cortical visual areas to a single current pulse in the mouse in vivo. A, The upper left-most panel is the fluorescence image emitted from the exposed cortex (encircled with gray dotted line). An asterisk indicates the simulation site in V1. *L*, lateral; *M*, medial; *A*, anterior; *P*, posterior. The other four panels show the image of $\Delta F/F$ recorded at the time frame indicated at the bottom of each panel. An arrow and arrowheads indicate distinct regions exhibiting significant $\Delta F/F$ changes; *b* corresponds to a presumed secondary visual area, LM. B, Time courses of the $\Delta F/F$ changes induced with different current amplitudes (10, 15, 20, 40, 60 μA/phase) in V1 (left) and the presumed LM (right). Triangles indicate the onset of the stimulus pulse.

B. Time course of the responses to fixed-interval pulse trains

Previous clinical experiments on volunteer human patients revealed that the stimulus-evoked phosphenes changed theirs appearance depending on the frequency of repetitive pulse stimulation [16]. Fig. 2 shows an example of the responses of V1 (a, black traces) and the presumed LM area (b, gray traces) to the fixed-interval pulse trains. For comparison with the response to the single pulse, the same current amplitude was used in this recording (i.e. 60 μA/phase).

The responses to the 10-Hz and 20-Hz trains (upper two panels) were periodically modulated in amplitude; namely, depolarization arose and peaked at approx.10 msec after the

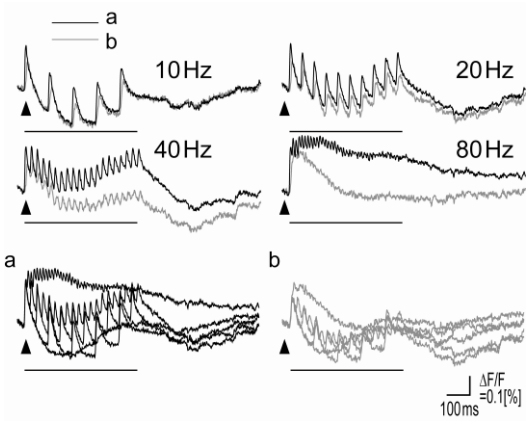

Figure 2. Time courses of the responses of V1 and LM areas to fixed-interval pulse trains. Each of the upper four panels superimposes two traces recorded in V1 (a, black) and LM (b, gray). The onset and duration of stimulation are indicated by an arrowhead and a horizontal line, respectively, in each panel. Each of the lower two panels superimposes the four traces from above panels and the one recorded with with the single pulse stimulus, for V1 (a, the left panel) and LM (b, the right panel).

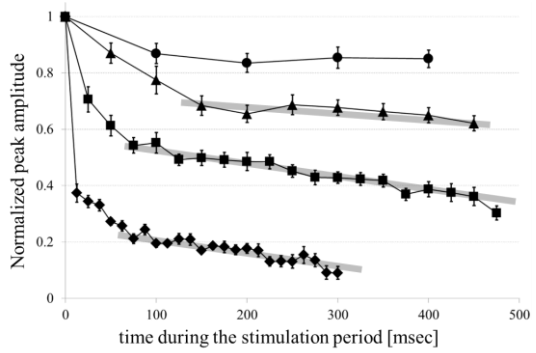

Figure 3. Decline of the peak amplitude of the transient depolarizations in V1 during the train stimulation. The mean ± s.e.m. is plotted for 11 mice. Different marks represent the different stimulus frequencies: circle, 10 Hz; triangle, 20 Hz; square, 40 Hz; rhombus, 80 Hz. The peak amplitude was below the detectable range after 300 msec with the 80-Hz train.

onset of each pulses, and then decayed until the next pulse. In the response to the 40-Hz train (left panel in middle row), the depolarization accumulated due to the short inter-pulse interval and the transient depolarization gradually became smaller in amplitude during the stimulation period. With the 80-Hz train (right panel in middle row, Fig. 2), the depolarizing response was least temporally modulated and mostly sustained, the level of which gradually decayed with time even during the stimulation period. The responses in the presumed LM area (gray traces) and those in V1 (black traces), were similar in time course with the low frequency stimuli (10 and 20 Hz), but deviated from each other with the high frequencies (40 and 80 Hz). Particularly in the response to the 80-Hz train, the depolarizing response of the LM rapidly decayed to the resting level in a few hundred msec. The overall time courses of the responses in V1 and the LM are compared as in the bottom two panels in Fig. 2. The transient and sustained components of depolarization were induced in V1 dependently on the stimulus frequency (a), while the sustained component was largely attenuated when transmitted from V1 to the LM area.

Fig. 3 plots the normalized peak amplitude of the transient depolarizations in V1 with respect to the time during the stimulation period. The rate of the decline in amplitude was faster with the higher frequency of the stimulus train. With the 10-Hz train (circles), the peak amplitude reached a plateau after the second pulse. On the other hand, with the higher frequencies, the amplitude became significantly smaller after a few pulses, and then continued to decline during the stimulation period with a relatively slow rate (gray lines). These results showed that the development of unresponsiveness of V1to the repetitive pulses may occur with, at least, two different phases.

C. Time course of the responses to random-interval pulse trains

Under natural visual environment, electrical stimulation applied to the visual cortex would not be a long-lasting, fixed-frequency pulse trains. Rather, the inter-pulse intervals in the stimulus are supposed to be changed time to time depending on images that the patient receives. Fig. 4 shows an example of the responses of V1 (middle, black trace) and the presumed LM area (bottom, gray trace) to a random-interval pulse train (upper trace). In this recording, the current amplitude was selected to be relatively moderate (20 μA/phase) so that the responses were evoked even near the end of the 4-sec stimulation period.

As similar to the responses to the fixed-interval pulse trains (Fig. 2), each of the stimulus pulses induced a transient depolarization in V1. When compared with these V1 responses, transient depolarizations of the LM were significantly smaller in amplitude for the most stimulus pulses, but could be larger for the pulses applied with the short pre-stimulus intervals (e.g. asterisks in upper trace).

In Fig. 5A, the relationships between the peak amplitude of the transient depolarizations and the pre-stimulus intervals were plotted for V1 (filled circle, a) and the LM (open circle, b). In these plots, the peak amplitude of each depolarizing response was normalized by that of the initial depolarization induced by the first pulse in a given stimulus period. As shown

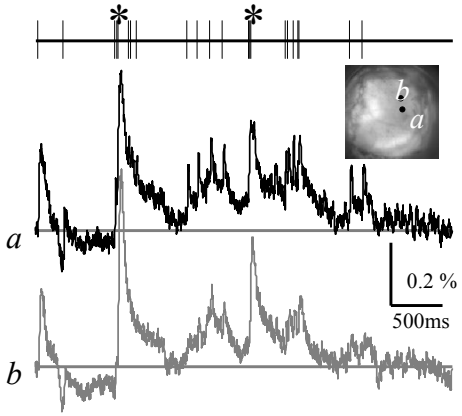

Figure 4. Time courses of the responses of V1 and LM areas to a random-interval pulse train. Upper, middle and bottom traces were the timing of the stimulus pulses, the response in V1, and that in the presumed LM area, respectively. Inset, the raw fluorescence image of the exposed cortex of the mouse used in this recording.

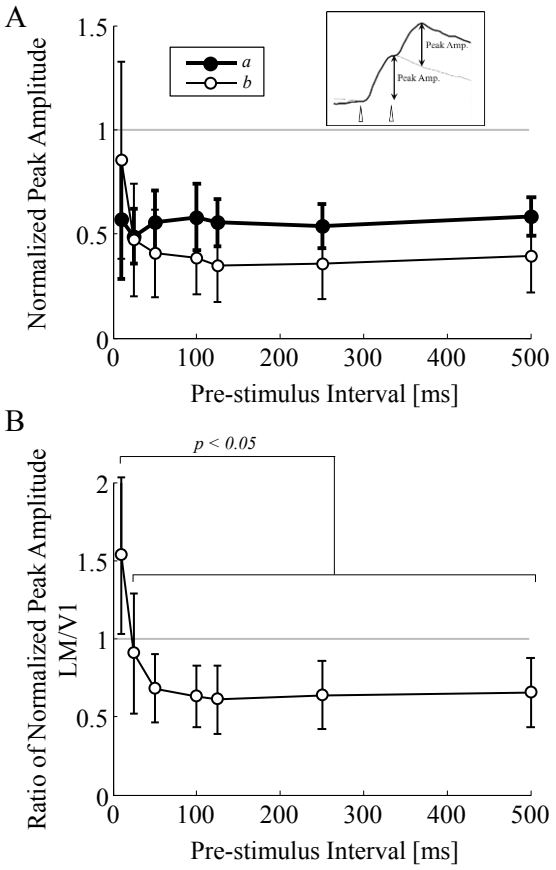

Figure 5. Effect of the pre-stimulus interval on the response amplitude. A, The normalized peak amplitude of the responses in V1 (a, filled circles) and the presumed LM (b, open circles). The mean ± s.d. is plotted for the data sets obtained with 11 different stimulus sequences. Inset, an example of the response to the two pulses (indicated by triangles) with the shortest inter-pulse interval. The peak amplitude of the second depolarization was measured by subtracting the template response (thin line). B, Ratio of the normalized peak amplitude of the LM response to that of the V1 response, mean ± s.d.

in the Fig., the normalized amplitude in the V1 responses was less than one (~0.6) and almost constant regardless of the pre-stimulus interval. On the other hand, the normalized amplitude of the LM response was larger as the pre-stimulus interval was shorter, and was close to one at the shortest interval (10 msec). The ratio of the LM response amplitude to the V1 response amplitude was calculated as the transfer ratio from V1 to the LM, as shown in Fig. 5B. The transfer ratio was larger at the shorter pre-stimulus interval, and greater than one at 10 msec. The difference between the ratios at 10 msec and at the others were statistically significant (P <0.05, n=9, one-way ANOVA followed by Bonferroni test). Such a relatively high transfer ratio at the short pre-stimulus is possibly due to the short-term enhancement of the synapses from V1 to the LM, in which a certain class of neurons exhibits the paired-pulse response compatible with the present data [17].

IV. Conclusion

In this study, we addressed a systematic analysis on the effect of an important parameter of electrical pulse stimuli, i.e. the inter-pulse interval, on the stimulus-induced neural activities in the visual cortices in mice in vivo. The present results showed nonlinear effects (Figs. 3 and 5B) of the repetitive pulses on the responsiveness of V1 and V2 in mice. We have not known how these response nonlinearities can relate visual perception of mice. And it certainly remains to be further studied if the results obtained from mice are somehow applicable to primates. Nonetheless, a possible nonlinear interaction between temporally consecutive responses should be taken into account for the future development of cortical visual prostheses in humans.

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