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ABSTRACT

The process of learning induces plastic changes in neuronal network of the brain. Our earlier studies on mice showed that classical conditioning in which monocular visual stimulation was paired with an electric shock to the tail enhanced GABA immunoreactivity within layer 4 of the monocular part of the primary visual cortex (V1), contralaterally to the stimulated eye. In the present experiment we investigated whether the same classical conditioning paradigm induces changes of neuronal excitability in this cortical area. Two experimental groups were used: mice that underwent 7-day visual classical conditioning and controls. Patch-clamp whole-cell recordings were performed from *ex vivo* slices of mouse V1. The slices were perfused with the modified artificial cerebrospinal fluid, the composition of which better mimics the brain interstitial fluid *in situ* and induces spontaneous activity. The neuronal excitability was characterized by measuring the frequency of spontaneous action potentials. We found that layer 4 star pyramidal cells located in the monocular representation of the "trained" eye in V1 had lower frequency of spontaneous activity in comparison with neurons from the same cortical region of control animals. Weaker spontaneous firing indicates decreased general excitability of star pyramidal neurons within layer 4 of the monocular representation of the "trained" eye in V1. Such effect could result from enhanced inhibitory processes accompanying learning in this cortical area.

Keywords: Mouse, classical conditioning, brain slices, visual cortex, neuronal activity

1. INTRODUCTION

Modification of sensory experience, including learning, induces plastic changes of neuronal networks in the respective cortical areas. These changes are caused by modification of physiological properties of neurons and interneuronal connections and can finally result in alterations of cortical representational maps of relevant sensory stimuli[1,2,3,4]. For example, visual deprivation in mice induces selective reconfiguration of layer 4 circuitry of the primary visual cortex (V1) manifested by pronounced changes of general neuronal excitability in response to alterations in net excitatory and/or inhibitory synaptic drive[5,6]. Short lasting classical conditioning involving pairing of sensory stimulation of one row of whiskers on mouse snout with a mild electric shock to the tail results in spatial enlargement of representation of the stimulated facial vibrissae in layer 4 of the somatosensory barrel cortex[7]. It has been further shown, using both neurochemical[8] as well as electrophysiological[9,10] methods, that this process is connected with up regulation of local GABAergic inhibitory systems within this cortical area. However, in this case, stronger inhibition can be counterbalanced by enhanced intrinsic membrane excitability of excitatory neurons[11].

The above examples show that plastic reorganizations of neuronal networks include complex interplay between modification of both excitatory and inhibitory connections as well as alteration of intrinsic properties of neuronal membranes. Our recent experiments on mouse visual system have shown that classical conditioning, similar to that used previously by Siucinska and Kossut[7] for vibrissae-barrel system, in which monocular visual stimulation is paired with an electric shock to the tail, enhances GABA immunoreactivity within layer 4 of the monocular part of the primary visual cortex receiving sensory information from the stimulated eye[12]. In the present study we investigate how electrophysiological features of the layer 4 excitatory neuronal network changes after this simple Pavlovian training. In mouse and rat the vast majority of layer 4 excitatory cells in V1 are star pyramidal neurons[13]. Therefore, as a first step we

Photonics Applications in Astronomy, Communications, Industry, and High Energy Physics Experiments 2017,
edited by Ryszard S. Romaniuk, Maciej Linczuk, Proc. of SPIE Vol. 10445, 104452I
 · doi: 10.1117/12.2281019

have decided to study general neuronal excitability of star pyramidal cells, which is a very sensitive marker[5,6,11] of physiological changes in the network.

2. MATERIALS AND METHODS

2.1. Animals

Experiments were performed on young (7-week-old) C57 mice. The animals were kept in a temperature-controlled room with a 12-hour light/dark cycle and had free access to food and water. All experimental procedures were carried out in accordance with regulations and standards of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Procedures were approved by the First Warsaw Local Ethics Committee for Animal Experimentation (permission no. 647/2014) and complied with the Polish Animal Protection Law.

2.2. Visual classical conditioning

The whole training procedure was performed in the way similar to that described for earlier study[12]. For two weeks prior to conditioning, associative pairing of visual stimulus and electric shock, the mice were placed in a restraining apparatus for 1 to 10 minutes (time increased gradually), twice a day to become accustomed to physical restraint. Then the animals (n = 4) were subjected to left eye visual monocular training (MT), based on the classical conditioning protocol used in the previous study[12]. We will refer to this group as (MT [CS-UCS]). Before each training session, right eye was covered with a small occluder and the mouse was placed in the restraining apparatus, 25 cm in front of the flat 22″ CRT monitor (NEC, MultiSync FP2141SB, vertical refresh rate 160 Hz) used for presentation of visual stimuli. The monitor screen (occupying 80 degrees of the visual field horizontally and 58 degrees vertically) was centered at the mouse vertical midline, and extended up to 40 degrees temporally on either side of the midline and from -13.5 to 44.5 degrees vertically. The ViSaGe MKII Stimulus Generator (Cambridge Research Systems) was used for generation of the conditioned visual stimulus [CS] - a moving square wave gratings.

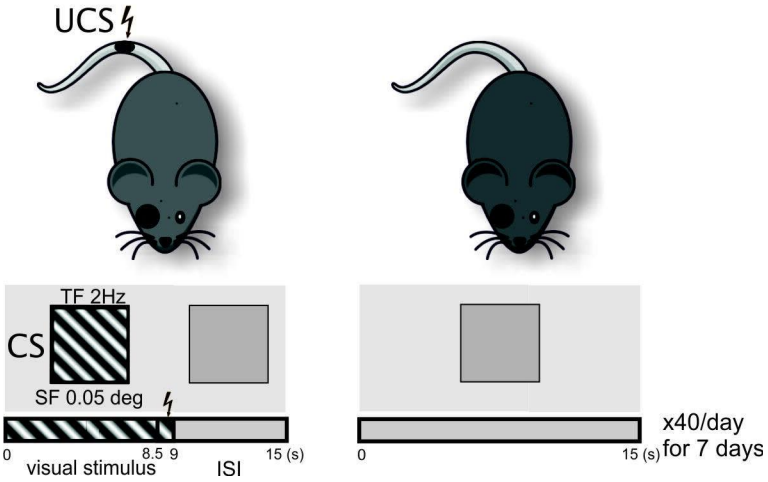

Figure 1. Visual classical conditioning. Left eye of mouse was covered with an opaque mask and visual stimuli, square-wave black and white gratings (CS), were presented to the animal in conjunction with electric shocks (UCS). A single trial consisted of visual stimulation, lasting for 9 seconds, terminated with an electric shock applied to the tail. The trial was repeated after a 6-second interval. The whole training consisted of 40 trials per day and lasted 7 days.

The luminance of the light strips was 72 cd/m2, whereas that of the dark strips was 8 cd/m2 (Michelson contrast 80%). The spatial frequency of the grating was 0.05 cycles/degrees and temporal frequency was 1 Hz. Visual stimuli with such spatio-temporal parameters were previously shown to be effective in activation of the mouse primary visual cortex[14,15],

and were effective in activation of group of GABA-ergic neurons in the mouse primary visual cortex in the similar visual classical conditioning procedure in our previous study[12]. The grating moved perpendicular to its orientation, in two directions, changing direction every 1 second. The stimulus was shown for 9 seconds every 15 seconds. In the intervals the screen was uniformly grey with a luminance of 32 cd/m2 – mean luminance of the visual stimulus. The conditioned stimulus was paired with a mild electric shock (unconditioned stimulus [UCS]) of 0.5 mA applied to the tail during the last second of each trial. The unconditioned stimulus lasted 0.5 seconds and terminated at the same time as visual stimulation. After a 6-second interval, the trial was repeated. The conditioning lasted 10 minutes each day (40 trials) and was continued for 7 consecutive days. This procedure will be referred to as "visual classical conditioning" (Fig. 1). The whole training procedure of visual classical conditioning was conducted under computer control (Spike2 software, Cambridge Electronic Design, Cambridge, UK).

The group of control mice (CONTROL, n=4) underwent the procedure of habituation to physical restraint in the similar way as (MT [CS-UCS]) group and then mice were placed in a restraining apparatus in front of uniformly grey monitor with a luminance of 32 cd/m2 for 10 minutes a day for 7 days.

2.3. Slice preparation and electrophysiology

Twenty-four hours after the end of the training mice were decapitated under inhalation anaesthesia with 4% of isoflurane (Baxter, USA). Brains were quickly removed and immersed in cold (0.5 ºC), artificial cerebrospinal fluid (ACSF) of the following composition (in mM): KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 24, $MgSO_4$ 4, $CaCl_2$ 0.5, D-glucose 10, sucrose 219 (300 – 308 mOsm). This solution (as well as the ACSF used for incubation and recording) was saturated with 95% O_2/5% CO_2 to pH 7.3-7.4. Coronal slices (350 μm) containing primary visual cortex, were cut from the right hemisphere on the Leica VT1000S vibrating blade microtome. Slices were stored in the submersion-type incubation chamber filled with ACSF containing (in mM): NaCl 126, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 24, $MgSO_4$ 3, $CaCl_2$ 1, D-glucose 10. A single slice was transferred to submerged recording chamber mounted on the upright microscope (Olympus BX61WI, Japan) and perfused (2 - 2.5 ml/min) with warm ACSF (32 ± 0.5 ºC) of composition similar as in the incubation chamber but with equal 2 mM concentrations of $MgSO_4$ and $CaCl_2$ (further termed: standard ACSF).

Monocular part of the primary visual cortex was identified within the slice under the visual guidance with low magnification 4x objective in relation to mouse brain atlas[16]. Single neurons were visualized using long working distance water-immersion 20x objective, near-infrared (775 nm) differential interference contract optics (IR-DIC) and Hamamatsu C7500 video camera. Whole-cell recordings were performed from visually identified neurons within layer 4 of monocular part of V1. Recording pipettes (4-6 MOhm) were prepared from standard-wall (1.2 mm O.D) borosillicate glass capillaries, and were filled with (in mM) K-gluconate 120, KCl 4, NaCl 2, HEPES 10, EGTA 0.1, Mg-ATP 4, Na-GTP 0.5; osmolarity: 280-290 mOsm; pH: 7.2-7.3. For morphological verification of the dendritic tree, the pipette solution contained also fluorescent dye Lucifer Yellow (50 μM) and neurons were filled by diffusion during the 30-60 min recordings.

Signals were recorded using the Axopatch 200B amplifier (Molecular Devices, USA) working in the "fast current clamp" mode. Signals (low-pass filtered at 5 kHz) were digitized at 20 kHz using Digidata 1322A interface and analyzed in pCLAMP10 software (Molecular Devices, USA). Electrophysiological recordings were performed by an experimenter blind to the treatment groups. Response characteristics of the recorded neurons were evaluated with intracellular injections of 500 ms long rectangular current pulses. Before each current step the membrane potential was adjusted to -70 mV. Classification of the recorded neurons as star pyramidal was based on the presence of regular firing pattern in response to the above-described current pulses and on characteristic morphology of their dendritic tree. The presence of an apical dendrite extending out of the layer 4 into supragranular layers was used as a criterion of pyramidal cells[13,17,18]. Modified ACSF (3.5 mM KCl, 0.5 mM $MgCl_2$, 1.0 mM $CaCl_2$) of the composition close to the interstitial CSF *in situ*[19,20] was used to record spontaneous firing (according to[5,6,21]).

Throughout the text, the averaged data are presented as means ± SEM. The effect of training on the passive membrane properties and frequency of spontaneous action potentials was assessed by unpaired Student t-test.

3. RESULTS

The results are based on recordings from 13 star pyramidal neurons located in layer 4, among which 6 belonged to the monocular part of the primary visual cortex (V1), contralateral to the stimulated eye of MT [CS+UCS] mice and 7 to the same cortical area of control animals (see Materials and Methods). Behavioural index of learning was obtained from the analysis of heart rate. It revealed 8 % reduction of heart rate from the first to the last training session ($P < 0.05$, paired Wilcoxon test), indicating the formation of association between CS and UCS (see also[12]).

The basic parameters characterizing passive membrane properties of the recorded neurons: the average resting membrane potential and input resistance, did not differ significantly between the two experimental groups. The mean values obtained respectively for trained and control group were -74.3 ± 1.6 versus -74.5 ± 1.3 mV for membrane potential and 239 ± 55 versus 254 ± 20 MΩ for membrane resistance ($P > 0.8$ for either comparison, t-test).

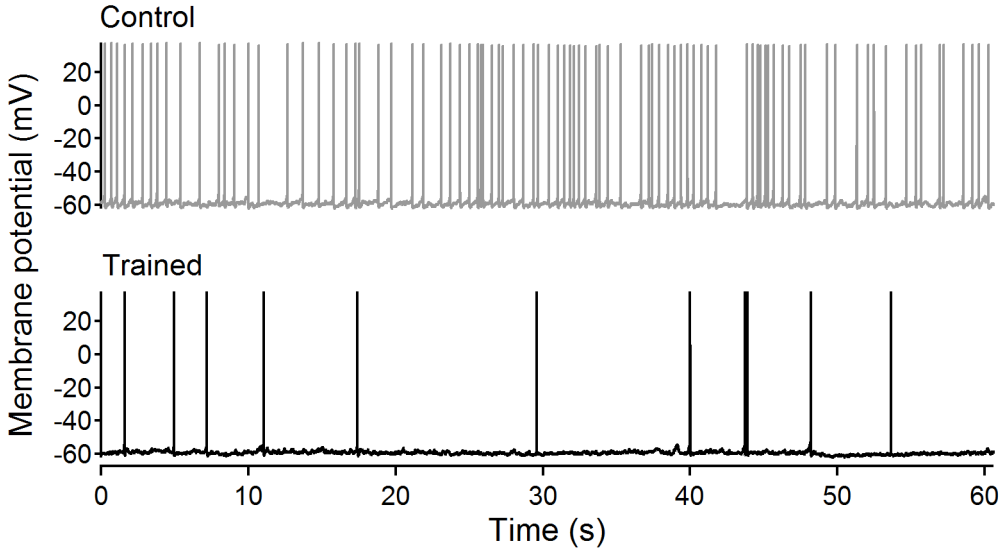

Figure 2. Decreased firing rate of star pyramidal neurons in MT [CS+UCS] mice. Typical examples of spontaneous action potential activity in control (upper trace) and trained (lower trace) animal.

To obtain a general measure of neuronal excitability we evaluated the frequency of spontaneous action potentials. In line with previous observations in the visual cortex[5,6], in standard ACSF the recorded neurons generated no spontaneous spikes at rest, and even with steady depolarization up to -40 mV they fired none or only few action potentials. However, when standard ACSF was replaced with the modified one *(see Materials and Methods)* whose composition better mimics the brain interstitial fluid *in situ,* after 8-10 minutes (necessary for saturation of the recording chamber and the slice with the modified ACSF) the investigated cells started to fire spontaneously at membrane potential close to -60 mV[5,6]. To better compare results obtained for different cells (according to[5,6]), the measurements of spontaneous firing frequency were done in each neuron at the same membrane potential of -60 mV (this was achieved by manual injection of small, usually depolarizing, DC current through the recording electrode) and at this baseline level neurons were allowed to respond freely to spontaneous fluctuations of the incoming synaptic input. Measurements of firing frequency were taken during 60 s time periods in order to obtain reliable results for cells with very low firing rate.

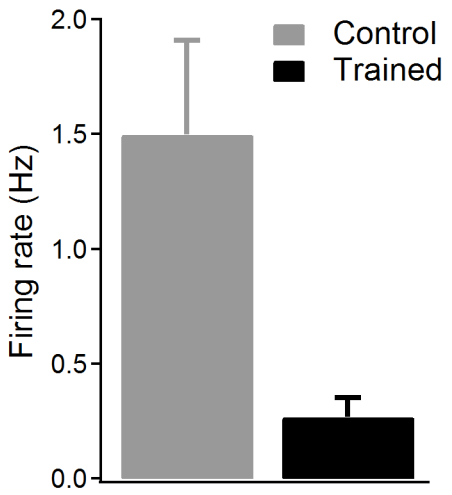

Figure 3. Mean values of firing frequency for two groups of mice used in this study. Mean firing rate of star pyramidal neurons calculated from trained mice (black bar) was several times smaller than that obtained for control animals (grey bar). P = 0.02, t-test.

The spontaneous firing frequency appeared robustly different between cells from the trained (MT [CS+UCS]) and control (habituated) mice. The typical single-cell examples of spontaneous firing are presented in Fig. 2. The mean values of firing frequency calculated for the whole groups of cells are shown in Fig. 3. The firing rate measured in layer 4 star pyramidal cells located within the monocular part of V1 in mice that underwent our simple associative learning was much lower than in similar cells from control animals. On average star pyramidal neurons which receive the sensory information from the stimulated eye in MT [CS+UCS] mice (n=6) generated action potentials at the frequency of 0.27 ± 0.08 Hz – several times less often than similar cells from the control (habituated) group of animals (1.5 ± 0.41 Hz, n=7). These two mean firing rates were significantly different (P = 0.02; tested with unpaired t-test).

4. DISCUSSION

The main result of this study shows that V1 layer 4 excitatory star piramidal cells located within the monocular representation of the stimulated eye of conditioned MT [CS+UCS] mice have lower frequency of spontaneous action potentials than neurons from the same cortical region of control (habituated) animals. Weaker spontaneous firing indicates on decreased general neuronal excitability characterizing excitatory cell network in layer 4 of the monocular representation of the "trained" eye in V1.

The general network excitability depends on several different factors. When neurons generate action potentials in response to spontaneous fluctuations of membrane potential, the firing rate results from the balance between the net excitatory and inhibitory synaptic drive[5,6] and from the intrinsic neuronal membrane excitability[11]. Our recent study has shown that the same visual training method upregulates a portion of the GABA inhibitory system within layer 4 of the monocular part of the V1 receiving sensory information from the stimulated eye[12]. This effect suggests that the main factor responsible for lower firing rate of layer 4 excitatory cells in the conditioned mice of current experiment could result from enhanced inhibitory processes in the investigated cortical area. However, in order to get a more complete picture of reorganization within layer 4 cell network after classical visual conditioning we need to explore in more detail the changes of electrophysiological properties of both inhibitory and excitatory inputs to layer 4 excitatory cells as well as differences in intrinsic membrane excitability of these neurons.

These studies will help us in the future to better understand the general mechanisms of plastic network reorganization in visual cortex not only after simple Pavlovian learning but also after changes in sensory visual experience or damages in the visual system. Additionally, these experiments will give us more information on the extent to which such behavioural learning paradigm induces similar results in two different cortical sensory areas: barrel cortex (see for instance[9,10,11]) and visual cortex. As these two cortical areas differ to some extent anatomically, the mechanisms of network reorganization could also not be the same.

ACKNOWLEDGMENTS

Supported by the Polish National Science Center grant Symfonia 1 (2013/08/W/NZ4/00691) and the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement no 665735 (Bio4Med) and by the funding from Polish Ministry of Science and Higher Education within 2016-2020 funds for the implementation of international projects (agreement no 3548/H2020/COFUND/2016/2).

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