INVESTIGATION OF CA1 NEURONAL ACTIVITY DURING CONTEXT FEAR CONDITIONING WITH MINIATURE FLUORESCENCE MICROSCOPES

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Abstract. Plastic changes in the neurons of the amygdala during learning in fear conditioning and their contribution to the modifications of behavior are well known, but the impact of hippocampal neurons in this behavioral task is not well studied to date. Recently a novel technique for simultaneous recording of calcium signal in multiple neurons in the brain of awake freely moving animals by miniature fluorescent microscope (miniscope) was developed. With the use of the miniscope, we have investigated neuronal activity in the CA1 area of hippocampus during memory formation and a recall in the task of contextual fear conditioning and correlated it with recorded mice behavior. Three epochs during learning were analyzed in mice behavior and brain activity: 120 s before, 2 s during, and 30 s after the electric shock. Memory retrieval was induced by placement of the animals for 180 s in the same context 24 h and 48 h after learning. The total amount of the neurons recorded in three mice was 507 during learning and 401 during memory retrieval. The patterns of neuronal activity were analyzed and discussed.

Keywords: long-term memory, fear conditioning, miniscope, calcium imaging, hippocampus, learning, neuron.

List of Abbreviations

CFC – Context Fear Conditioning

CMOS – Complementary metal–oxide–semiconductor

CNMF – Constrained Nonnegative Matrix Factorization

GRIN lens – Gradient-index

Introduction

Uncovering the mechanisms of formation and maintenance of long-term memory is one of the main goals of modern neurobiology. The changes in individual neurons and synapses during learning are now studied in detail on molecular and physiological levels (Kandel *et al.*, 2014). However, according to the modern views on memory formation, the long-term memory is not stored in the modifications of the activity of single neurons, but rather in the coordinated activity of the wide neuronal networks distributed among the different brain regions (Frankland & Bontempi, 2005). In the last few years, the growing body of research on the population coding of memory traces have emerged, in which the organization of the neural networks formed in learning and reactivated during memory recall was studied (Grewe *et al.*, 2017; Zhang & Li, 2018). Such studies became possible because of the rapid development of the new techniques for direct imaging of neuronal activity with the miniature fluorescent microscopes which allow to image calcium transients with neuronal resolution in the brain of awake and freely moving animals (Ziv *et al.*, 2013; Ziv & Gosh, 2015).

Miniscopes are a miniaturized version of epifluorescence microscope, where light-emitting diode is a light source and a miniature CMOS sensor is used for recording of the signal. The GRIN lens implanted to the certain region of the brain provides an optical pathway from the neural tissue to the sensor. With GRIN lens, the recording of fluorescent signal is possible even from the deep structures of the brain (Aharoni *et al.*, 2019).

In last 5 years the number of papers in various fields of neurobiology made with technique of miniature microscopes imaging is growing exponentially (Cai *et al.*, 2016; Liang *et al.*, 2018; Grewe *et al.*, 2017; Fustinana *et al.*, 2021).

Increasing popularity of the method is caused by the ability of miniscopes to simulta-neously record multiple neurons in awake animal, which can move freely and behave naturally.

Fear conditioning is a classical model of associative learning in small rodents. In this task animal forms an association between the neutral context or stimulus (e.g. sound) and the negative stimulus — weak electrical shock to the foot. As a result of such learning procedure, animals show fear reaction in the previously safe context or in response to the initially neutral stimulus. Amygdala is a brain region known as a main subject of plastic changes during learning in fear conditioning. Multiple studies have investigated the changes in amygdala neurons activity as a result of learning in auditory fear conditioning. Nevertheless, not only amygdala is required for learning in the context fear conditioning, but the hippocampus is also involved (Tovote *et al.*, 2015). Several questions remain unexplored. What is the specific mechanism of hippocampus participation in the context aversive memory formation? Do the hippocampal neurons encode properties of the unconditional stimulus – electric shock? Which way does their activity change after learning and during memory reactivation? The answers to all these questions are important, but still studied not enough.

The goal of the present work was to study the activity of the hippocampal CA1 neurons during learning in the context fear conditioning task and during retrieval of memory about dangerous context.

Materials and Methods

Animals. The work was carried out in male C57Bl/6 mice. Experiments were wade in intact animals of age 8–9 weeks. Mice were kept in cages in groups of five, with ad libitum access to food and water. At the day of experiment, the home cages with mice were moved to experimental room 30 min before the experiment starts. Experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and the Ethical Committee of the Institute of Higher Nervous Activity and Neurophysiology of Russian Academy of Sciences approved the protocol.

Context Fear Conditioning (CFC). Mice were placed into the experimental cage for 120 s, then electrical shocks of different magnitude were applied, and then mice had 30 s more to behave. Mice were divided in four groups varying in applied current of electric shock: 0 mA (control), 0.3 mA, 0.45 mA, 0.6 mA (n = 5 in every group). 24 and 48 hours after learning we have made two consequent tests of mouse's long-term memory. Mice were placed to the learning context (experimental cage) for 180 s.

Mouse behavior during learning and testing was recorded with a conventional web-camera (Logitech HD Pro Webcam c920). Video recordings were analyzed with ezTrack software (Pennington *et al.*, 2019), scoring duration of freezing (specific type of behavior, reflecting fear in rodents).

Calcium Imaging. To study the activity of hippocampal neurons, we have made calcium imaging with miniature fluorescent microscopes (UCLA miniscope, http://miniscope.org). At least 8 weeks prior to imaging mice were injected with the AAV2-based viral vector encoding protein calcium sensor GCamp6s sequence under control of CAG promoter (AAV-CAG-GCamp6s) into CA1 area of hippocampus. Next, after recovery we have implanted a 1.8 mm diameter GRIN lens over the CA1 hippocampus area to lead the optical way from the neurons to the miniscope. Last, the mounting plate for the miniscope was installed on the skull. All surgical manipulations were made under general isoflurane anesthesia. At least 4 weeks after last surgery mice were trained and tested according to the CFC protocol (above) with the electric shock magnitude 0.6 mA. During learning and testing we recorded the fluorescent signal of genetically encoded calcium sensor by miniscope.

Video recordings of calcium signal were analyzed with Minian software (Dong *et al.*, 2021, preprint biorxiv), based on CNMF-e algorithm (Zhou *et al.*, 2018). Calcium events from processed data were selected by threshold excess of median + 4 median absolute deviations (MAD).

Statistical analysis was made in GraphPad Prizm 7.0 software. The data are presented as mean ± SD.

Results

At the day of learning, all mice have actively explored the experimental cage showing no signs of fear. The overall duration of freezing before electric shock did not exceed 2% (Fig. 1A). After electric shock all groups, except the control one, demonstrated short episodes of freezing, by average less than 10% of time. 24 hours after shock, at first testing, mice from experimental groups that have received 0.45 mA and 0.6 mA electric shocks displayed significantly more freezing duration in comparison with the control group (p = 0.02 and p = 0.04 respectively, one way ANOVA, post-hoc Tukey). 24 hours after first testing we have made second testing in the same conditions. Mice from experimental group 0.6 mA showed significantly more freezing (Fig. 1A) in comparison with mice of groups 0.3 mA and 0.45 mA (p = 0.04 and p = 0.009 respectively, one way ANOVA, post-hoc Tukey).

In experiments with mice with implanted miniscopes we used current magnitude 0.6 mA for electric shock. During testing of the memory about dangerous context mice showed more freezing than before the electric shock (baseline 11.7% ± 4.5% vs. Test 1 28.9% ± 13.8%, p > > 0.05, Wilcoxon signed-rank test), Fig. 1B.

Using the miniscopes we have recorded activity of neurons in the CA1 area of hippocampus from 3 mice during FC learning and retrieval of memory about dangerous context. During learning we have totally recorded 507 neurons, in average 169 ± 144.8 neurons per animal. During retrieval of memory, we have recorded 401 neurons, in average 133.6 ± 53.3 neurons per animal. It's worth to note that 32% of all neurons recorded during learning were active both at the time of learning, and during memory retrieval (total 164 neurons, in average 54.7 ± 35.9 per mouse).

The learning procedure is meaningfully divided into three epochs: exploration of unknown safe context (first 120 s), electric shock (next 2 s), and exploration of dangerous context after shock (last 30 s). Total number of active neurons in the first epoch was 1.7 times more than in the third epoch, after electric shock (before: total 477, in average 159 ± 145.4; after shock: total 278, in average 92.7 ± 67.9). It is important to note that the large fraction of neurons was active only before shock (45.2% of all neurons active during learning), few neurons were active only after shock (5.9%), and the rest of neurons were active all the time of recording (48.9%) (Fig. 2). Mean frequency of calcium events in permanently active neurons increased from 0.047 ± 0.038 in the first epoch to 0.097 ± ± 0.077 in the third epoch (p < 0.0001, Student's T test for paired samples, Fig. 2).

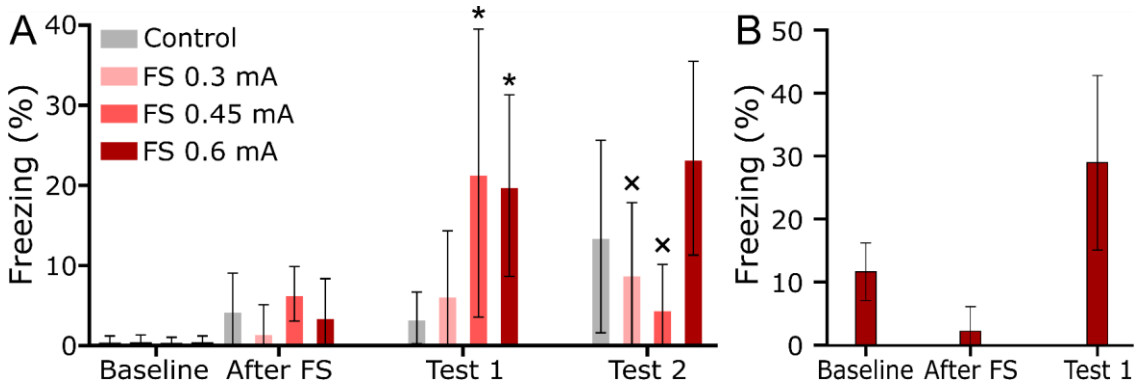

Fig. 1. Mice with and without implanted miniscopes had comparable expression of fear responses.
A. Intact mice displayed freezing behavior during re-exposure to the context (Test 1) on the following day if they received 0.45 or 0.6 mA, but not 0.3 mA footshock. During the second test only mice from FS 0.6 mA group demonstrated high freezing level. B. Mice with implanted miniscopes displayed strong freezing response to the learning context during test 24 hour after learning. * – p < 0.05, comparing to Control; × – p < 0.05, comparing to FS 0.6 mA (One-way ANOVA, post-hoc Tukey); data are mean ± SD

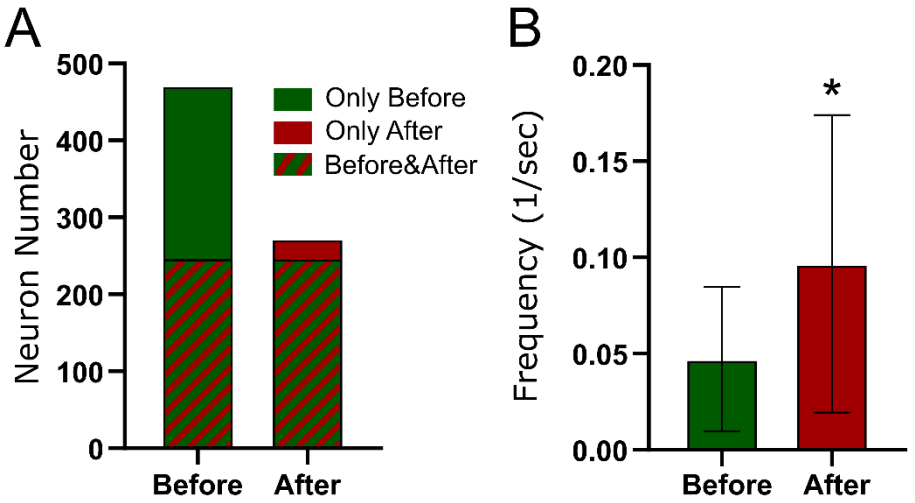

Fig. 2. Changes in CA1 neuronal activity during fear conditioning learning session.
A. Total number of active neurons decreases after footshock in learning session. B. Mean frequency of calcium events in CA1 neurons increases after footshock delivery during learning session. * – $p < 0.05$, comparing to the Before period (Student's T test for paired samples); data are mean ± SD

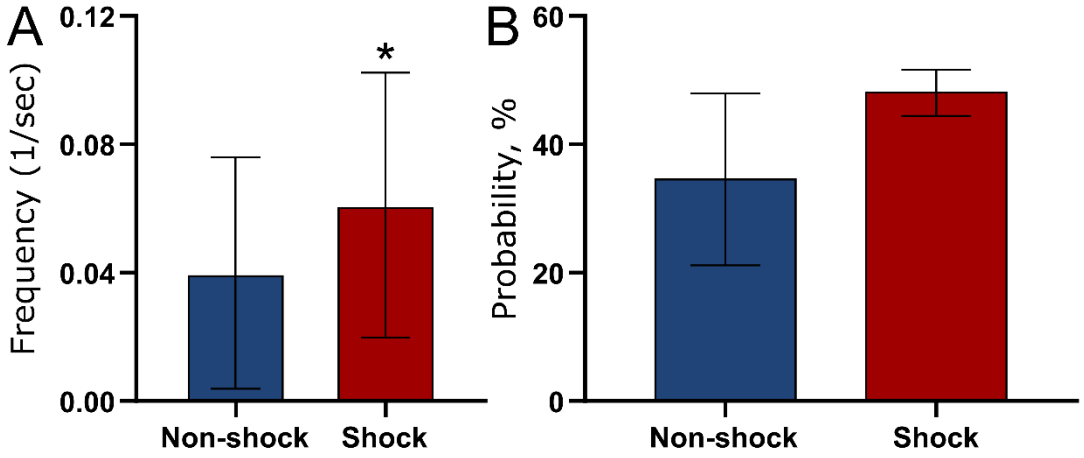

Fig. 3. CA1 neurons active during footshock presentation are more active during both learning and test sessions.
A. Mean frequency of calcium events in CA1 neurons active during footshock is higher than non-active during footshock neurons. B. Probability of reactivation during Test session is higher in neurons activated during footshock than non-active during footshock. * – $p < 0.05$, comparing to the Before period (Welch's T test); data are mean ± SD

Among all neurons active during learning, 13.2% were active during the electric shock (second epoch). Mean frequency (during last 30 s) of calcium events in these neurons was significantly higher than in the neurons that were silent during second epoch (0.039 ± 0.036 inactive vs. 0.061 ± 0.041 active; $p < 0.0001$, Welch's T test, Fig. 3A). One more metric to discuss is the probability of repeated activation of the neurons during memory testing. The probability of activation of the neuron during both learning and testing session was higher in neurons, which were active during electric shock, in comparison with neurons not being active during second epoch, Fig. 3B.

Mean frequency of calcium events was significantly higher during memory retrieval as compared with learning (0.046 ± 0.034 learning vs. 0.059 ± 0.039 testing; $p < 0.0001$, Welch's T test).

Discussion

To study neuronal activity during formation and retrieval of associative memory we have assembled custom-made rig for contextual fear conditioning, based on the previously published design (Amaral-Júnior et al., 2019). We have tested the rig, adjusted the parameters of learning in intact mice and found two current magnitudes – 0.45 mA and 0.6 mA, which as a reinforcement effectively triggered creation of a long-term memory about dangerous context. The current magnitude 0.6 mA that we have chosen for the following experiments is common and widely used value (Curzon *et al.*, 2009). One-time shock with current 0.45 mA is rarely used and one may propose weak memory as a result of such low intensity shock – just what we have found in second testing. It was shown previously that, depending on the duration of the exposition to the dangerous context, two different processes may develop: either reconsolidation or extinction of memory (Merlo *et al.*, 2014). According to this literature data, the duration of the first trial is critical for triggering either reconsolidation or habituation. In the second test in our experiments, mice in group 0.6 mA do not show a decrease in total duration of freezing that suggests formation of strong and long-term memory. In contrast, mice from group 0.45 mA demonstrated five times less freezing in the second test in comparison with the first one. Since the decrease in total duration of freezing occurs, one may suppose that the memory in that group of mice is weak and even short exposition to dangerous context without reinforcement by footshock leads to extinction of memory, which we observe as a decrease of the fear in the previously dangerous context. Extinction of memory can be also interpreted as appearance of a new extinction memory.

After preliminary experiments with CFC on control mice, we used resulting protocol for training mice with miniscopes, implanted into CA1 area of hippocampus. Neuronal activity was indicated by the changes in fluorescent calcium signal from genetically encoded calcium sensor GCaMP6s. Totally, we have recorded 507 neurons during learning and 401 neurons during first test of memory, with 32% of neurons being both active during learning and then reactivated during testing. These results are in compliance with data of ex vivo studies of Wiltgen's lab (Tayler *et al.*, 2013; Tanaka *et al.*, 2014). In these studies neurons being active during learning and retrieval were labeled by expression of immediately early gene c-fos. Although gene expression is rather different marker of neuronal activity, as compared to calcium imaging, they have also shown that 20–40% of neurons were reactivated by exposition of mice to the dangerous context.

Miniscope in vivo imaging not only allows researchers to count the number of active cells, but also to estimate the patterns of individual neuronal activity which is impossible with the approach of Wiltgen's lab. We have found that the mean activity of CA1 neurons during reactivation of memory is increased – the frequency of calcium events is significantly higher in testing vs. learning sessions.

Fear conditioning is a classical model for study of associative learning in mice and rats. However, most of studies are focused on plasticity in amygdala during auditory fear conditioning. In our work we have acquired new data about changes in activity of hippocampal neurons after electrical footshock in contextual fear conditioning. Detailed analysis of calcium events frequency in different epochs of learning procedure allowed us to identify the following patterns. Relatively high fraction of neurons, being active during exploration of new context, is silent after delivery of footshock. The neurons that still remain active after footshock, show higher frequency of calcium events. Similar results were demonstrated on the hippocampal organotypic slices (McKay *et al.*, 2009). The authors have analyzed intrinsic excitability of pyramidal neurons in hippocampus 24 hours after learning in context fear conditioning. In brain slices made from learned rats, neurons were significantly more excitable in comparison with slices from rats that just explored new context without electric shock (McKay *et al.*, 2009). The advantage of our study is that we have shown how neuronal activity changes by learning in vivo, not in artificial conditions of acute brain slices.

One more point of in vivo activity recording is a possibility to find neurons the activity of which is correlated to certain time periods during the learning process. We have found that among all neurons recorded during learning, about 13% were active during electric shock. It is important to note that calcium events frequency in these neurons before shock was significantly higher in comparison with neurons inactive during shock. On the one hand, one may assume that some neurons were initially more active and their activity just randomly coincided with the foot-shock delivery time interval. On the other hand, the so-called hypothesis of primed neurons exists, suggesting preferential inclusion of most active neurons into the memory trace (Yiu *et al.*, 2014). Our data may be interpreted as indirect evidence for this hypothesis. Moreover, we have shown that the probability of reactivation during memory retrieval is higher exactly in the neurons which were active during electric shock in comparison with neurons inactive during the foot-shock delivery.

To conclude, we have obtained new data about neuronal activity in the CA1 hippocampal neurons during negative memory formation and retrieval. The data acquired open up new opportunities for further analysis of population coding of memory trace – engram – and exploration of its stability and reactivation patterns.

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