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Research article

# An inhibitory pathway controlling the gating mechanism of the mouse lateral amygdala revealed by voltage-sensitive dye imaging

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## HIGHLIGHTS

- EC stimulation induces large and long-lasting hyperpolarizing signals in the La.
- This hyperpolarization is analyzed by VSD imaging spatially and temporally.
- We identify an inhibitory pathway toward the La via the m-ITC.

## ARTICLE INFO

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## ABSTRACT

The lateral amygdala nucleus (La) is known as a gateway for emotional learning that interfaces sensory inputs from the cortex and the thalamus. In the La, inhibitory GABAergic inputs control the strength of sensory inputs and interfere with the initial step of the acquisition of fear memory. In the present study, we investigated the spatial and temporal patterns of the inhibitory responses in mouse La using voltage-sensitive dye imaging. Stimulating the external capsule (EC) induced large and long-lasting hyperpolarizing signals in the La. We focused on these hyperpolarizing signals, revealing the origins of the inhibitory inputs by means of surgical cuts on the possible afferent pathways with four patterns. Isolating the medial branch of EC (ECmed), but not the lateral branch of EC (EClat), from the La strongly suppressed the induction of the hyperpolarization. Interestingly, isolating the ECmed from the caudate putamen did not suppress the hyperpolarization, while the surgical cut of the ECmed fiber tract moderately suppressed it. Glutamatergic antagonists completely suppressed the hyperpolarizing signals induced by the stimulation of EC. When directly stimulating the dorsal, middle or ventral part of the ECmed fiber tract in the presence of glutamatergic antagonists, only the stimulation in the middle part of the ECmed caused hyperpolarization. These data indicate that the GABAergic neurons in the medial intercalated cluster (m-ITC), which receive glutamatergic excitatory input from the ECmed fiber tract, send inhibitory afferents to the La. This pathway might have inhibitory effects on the acquisition of fear memory.

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## 1. Introduction

The amygdala is an important brain structure for emotional behavior and learning [13]. Fear conditioning is a widely-used experimental model to examine emotional and learning processing in animal brains. The lateral amygdala nucleus (La) is known as a gateway for emotional learning that interfaces sensory inputs from the cortex and the thalamus [14]. Inhibitory circuits are known to control the amygdala's functions, such as acquisition, expression, and extinction of conditioned fear [6,21,22]. Inhibitory inputs to the La control the strength of sensory inputs and interfere with the initial step of the acquisition of fear memory. Two groups of GABAergic neurons in the amygdala are known: local interneurons that are scattered within the local neuropil [17], and intercalated cells organized in clusters (intercalated clusters) surrounding the amygdala complex [15,16,18,20,23,24]. Although inhibitory inputs to the individual principal neurons in the amygdala have been analyzed electrophysiologically [4,26,31,37,38], how sensory inputs

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induce inhibitory responses in the La, and how inhibitory responses propagate in the La, are still unclear because of the technical limitations of patch clamp recording.

Optical imaging techniques overcome these limitations to investigating propagations in a wide range of neuronal interactions, and have been applied in the study of excitatory circuits of several brain regions [7,9,11,12,29,33,34]. In this study, using optical imaging techniques, we investigate neuronal activities in the La, focusing particularly on inhibitory responses. To identify the origins of the inhibitory inputs, we perform various patterns of knife-cut operations of the possible afferent pathways evoking hyperpolarization in the La. In addition, we investigate the effects of glutamatergic antagonists on the inhibitory responses in the La, and show an inhibitory pathway from the medial intercalated cluster (m-ITC) to the La.

## 2. Materials and methods

### 2.1. Slice preparation and staining procedure

The experimental protocol was reviewed and approved by the National Institute of Health Sciences (NIHS) in Japan, following the guidelines in the National Research Council's 'Guide for the Care and Use of Laboratory Animals'. All experiments were approved by the NIHS' ethics committee. Male mice (C57BL/6 J, 7–22 weeks old, Japan SLC, Inc., Japan) were deeply anesthetized with halothane and quickly decapitated. Coronal slices containing the amygdala complex (400 μm) were prepared using a vibrating microtome (Campden Instruments Ltd., Loughborough, UK) in ice-cold artificial cerebrospinal fluid (ACSF). The ACSF was composed of the following (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.0 $NaH_2PO_4$, 26.2 $NaHCO_3$, and 11.0 glucose; this was oxygenated with a mixture gas of 95% $O_2$ and 5% $CO_2$ (pH 7.4). The slices were immediately soaked in the oxygenated ACSF containing a voltage-sensitive dye (VSD), di-4-ANEPPS (50 μM, Invitrogen Molecular Probes Inc., Oregon, USA) for 10 s, and then transferred to a filter that absorbed the staining solution and subsequently to another filter that absorbed the normal ACSF for at least an hour before the experiment.

### 2.2. Experimental apparatus for VSD imaging

An epi-illumination macro zoom fluorescence microscopy (MVX-10 MacroView, Olympus, Japan), a LED light source with a 530 nm center wavelength (LEX2-Green, Brainvision Inc., Tokyo, Japan), a dichroic mirror (560 nm), an emission filter (BP 575–625 nm), and a CMOS imaging device (MiCAM ULTIMA-L, Brainvision Inc., Tokyo) were used for VSD imaging.

The decrease and increase in the fluorescent intensity from the preparation corresponded to the membrane depolarization and hyperpolarization, respectively. Each data acquisition consisted of 1024 images of consecutive frames (1.0 ms/frame). A coaxial needle electrode (TF203-047, Unique Medical Co. Ltd., Tokyo, Japan) was placed on the external capsule (EC). Electrical stimuli with 200-μs duration at various intensities from 15 to 90 μA were delivered at the 100th frame of each acquisition. To analyze the effects of deafferentation on the induction of the hyperpolarization, the stimulus intensity was adjusted to make the peak value of depolarization equal before and after the surgical cut. Methods to calculate optical signals and present images were described in previous papers [11,29,32].

### 2.3. Surgical cuts of afferent connections to the La

After recording the optical signals at various stimulation intensities, we performed knife-cut operations on the pathways assumed to be involved in the induction of the hyperpolarization in the La. Four afferent pathways to the La were cut under the macro scope observation, as follows: the La was isolated by the longitudinal cut from: (i) the lateral branch of the EC, (ii) the medial branch of the EC and (iii) the CPu, and by (iv) the transverse cut of ECmed at the dorsal part. For the sham operations, the slices remained intact but the same procedure was carried out. After the surgical cuts, the slices were stored in the recovery chamber at room temperature (at least 1 h).

### 2.4. Excitation and inhibition values

Images from the 251st–300th frames were stacked and averaged to determine regions of interest (ROIs), which were circles of 8 pixels in diameter. Two ROIs were defined for each experiment. One of the ROIs had the maximal hyperpolarization value in the center spot. The other was adjacent to the first, which had an adequately large hyperpolarization value within the region. For the after-operation analysis, the ROIs were centered on the spots that had the same distances from the position of the stimulating electrode and the EC as the before-operation analysis. The averaged optical signals of the two ROIs were used as representative data.

The excitation ($E$) value was determined as the largest value among all the values from the first to the 15th frame after electrical stimulation. The inhibition ($I$) value was determined by averaging 50 frames, from the 251st frame to the 300th frame after the electrical stimulation.

### 2.5. Statistical analysis

We defined the operation index (OI) as follows: $OI = [I/E]_a/[I/E]_b$, where a: after the operation, b: before the operation. The data were presented as mean ± standard error of the mean (SEM). Normality of the data was tested with the Shapiro–Wilk test, and subsequently one-way ANOVA followed by Dunnett's post-hoc test was carried out. In the pharmacological experiments, inhibition value was statistically analyzed using a paired $t$-test.

## 3. Results

### 3.1. Optical signal propagation after the EC stimulation

The anatomical nomenclatures related to our experiments are shown in Fig. 1A. Each white fiber bundle in the amygdala slice preparation was observed through the macro zoom microscope; thus, the ramifying point of the EC was identified. The La, BLA, and CeA of the amygdala complex were identified in the fluorescent image recorded by the system (Fig. 1B). In Fig. 1C, a typical example of the optical signal propagation is shown in pseudo-color representation. The depolarization started at the stimulation point and spread over the La within 3 ms after the stimulation (0–3 ms). It became stronger in the La and spread over the other regions, the BLA, CPu, and CeA (4–7 ms). Then the depolarization at the dorsal area of the La faded, while growing stronger in the other regions (8–11 ms). Following depolarization, a weak hyperpolarization was first observed in the La; the depolarization remained in the other regions, although it was weakened (12–15 ms). The hyperpolarization grew stronger, and the depolarization in the other regions gradually disappeared (16–19 ms). The hyperpolarization in the La lasted about 600–650 ms (the middle wave in Fig. 1B). The maximal value of the hyperpolarization was observed around 255 ms after the stimulation.

After the hyperpolarization started in the La at the area along the dorsal part of the ECmed (16–19 ms), it spread out in the La during the next 100 ms (20–119 ms), then it spread over the BLA and a narrow part of the CPu along the dorsal part of the ECmed

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