Footshock-induced activation of the claustrum-entorhinal cortical pathway in freely moving mice

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Short title: Footshock-induced activation of the CLA-MEC pathway

Summary

Footshock is frequently used as an unconditioned stimulus in fear conditioning behavior studies. The medial entorhinal cortex (MEC) contributes to fear learning and receives neuronal inputs from the claustrum. However, whether footshocks can induce a neuronal response in claustrum-MEC (CLA-MEC) projection remains unknown. Here, we combined fiber-based Ca^{2+} recordings with a retrograde AAV labeling method to investigate neuronal responses of MEC-projecting claustral neurons to footshock stimulation in freely moving mice. We achieved successful Ca^{2+} recordings in both anesthetized and freely exploring mice. We found that footshock stimulation reliably induced neuronal responses to MEC-projecting claustral neurons. Therefore, the footshock-induced response detected in the CLA-MEC projection suggests its potential role in fear processing.

Keywords

Claustrum • Fear • Footshock • Fiber photometry • GCaMP

Introduction

Fear, which can elicit defensive behaviors to avoid or reduce harm, is an important emotion for survival [1, 2]. Fear is regulated by multiple brain regions and complex neural circuits. The amygdala nuclei are essential for the acquisition and expression of fear [3, 4]. Fear responses are involved in dispersed brain networks, such as the sensory cortex, the medial prefrontal cortex, the hippocampus and the entorhinal cortex [1, 5]. Footshock-induced pain is frequently used as the unconditioned stimulus in fear conditioning experiment paradigms [4, 6]. The claustrum, a slender region underneath the cortical area, has a neuroanatomical basis that allows it to participate in high-order functions [7, 8]. The medial entorhinal cortex (MEC), which has been reported to participate in fear learning, receives neuronal projections from the claustrum [9-11]. Thus, the neuronal projection from the claustrum to the MEC (CLA-MEC) may be involved in fear processing [9]. However, whether footshock can induce any response in the CLA-MEC projection remains unknown.

Real-time monitoring approaches are needed to answer this question. Traditional electrophysiological recording provides the highest temporal resolution in neural activity recording. Combined with optogenetics, optrodes provide cell-type or projection information for recorded

neurons [11-14]. However, the application of this technique is limited by a low recording efficiency. Two-photon microscopy provides a high recording efficiency but is restricted to cortical areas in anesthetized or head-fixed animals [15, 16]. Fiber photometry combined with genetically encoded Ca^{2+} indicators provides a simple but efficient method for projection-specific recording in freely moving animals [17, 18]. Recent studies have developed recording methods in the claustrum in freely moving mice [19, 20]. However, the real-time recording of a specific CLA-MEC projection under specific behavioral tasks is still lacking.

Here, we utilized fiber-based Ca^{2+} recording to investigate the possible role of the CLA-MEC projection in footshock-induced neural responses. We observed a stable labeling of MEC projection neurons in the claustrum by local injection of retrograde AAV carrying the Ca^{2+} indicator GCaMP6m gene into the MEC. The method was then validated by Ca^{2+} recording in anesthetized and freely moving mice. Moreover, we performed Ca^{2+} recording of MEC projection neurons in the claustrum with the application of footshock stimuli in freely moving mice. Robust neuronal responses of these neurons to footshock stimuli were observed, suggesting a possible role of CLA-MEC projection in fear-related information processing.

Methods

Animals

Eleven adult male C57BL/6J mice (aged between 8-12 weeks) were used for all described studies. Mice were housed in groups under 12-hour light/dark cycle conditions, with *ad libitum* access to food and water. All experiments were performed according to institutional animal welfare guidelines and were approved by the Third Military Medical University Animal Care and Use Committee.

Optical setup

A custom-built fiber photometry system was used to record Ca^{2+} activity. Excitation light with a wavelength of 488 nm (Coherent, OBIS 488 LX-50 mW) was delivered to tissue by an optical fiber with a diameter of 200 μm (Doric Lenses, MFP_200/230/900-0.48). The optical fiber was glued into a short metal cannula (ID. 0.51 mm, OD. 0.82 mm, length 18 cm) with a fiber tip extending ~3.5 mm out of the cannula. Emission fluorescence was detected with an avalanche photodiode

(Hamamatsu Photonic, Si APD, S2382). Data acquisition was controlled by user-customized software on the LabVIEW platform (LabVIEW 2014, National Instruments).

Virus injection

The mice were anesthetized with 1.5% isoflurane in pure oxygen for 3-5 min. mice were then moved onto a stereotactic apparatus with a heating pad that maintained a temperature of approximately 37 °C during the entire surgery, and anesthesia was maintained by continuous delivery of isoflurane. The hair at the top of the head was shaved, and an 8-10-mm-long incision was made along the midline. Then, one small craniotomy (0.5 × 0.5 mm) above the MEC (AP: -4.9 mm, ML: 3.5 mm) was made. A glass micropipette with a tip diameter of ~10 μm was inserted down to a depth of 2.5 mm (from the dura) to infuse the virus. Approximately 300 nL of retroAAV-GCaMP6m viral solution was gradually injected while the micropipette was slowly lifted to a depth of 1.5 mm. Each injection took 5-10 min. The micropipette was kept in place for 5 min before being slowly withdrawn. After virus injection, the scalp wound was closed with surgical sutures, and each mouse was kept in a warm plate. Mice were then returned to their home cage for recovery. Meloxicam oral suspension (Metacam) was provided in drinking water for three days after surgery.

Fiber recording in anesthetized mice

Recording experiments were conducted approximately one month after virus injection. The preprocessed fiber probe was slowly inserted through another craniotomy above the claustrum (AP: 1.1 mm, ML: 2.5 mm) to a depth of ~2.7 mm. After the fiber probe was secured to the skull by UV-curing hardening dental cement (Tetric EvoCeram, 595953WW), the concentration of isoflurane was increased to 1.8%. Following an adaptation period of 10 min, Ca^{2+} recordings were performed for 10 min at each anesthesia level, and the initial 3 min of each level was excluded for data analysis. The concentrations of isoflurane were 1.8%, 1.5%, 1.2%, and 0.8%.

Fiber recording in freely moving mice

The cannula was fixed to the mouse skull with dental cement after recording data from the mice in an anesthetized state, and then the mice were returned to their home cages for recovery. Meloxicam oral suspension (Metacam) was provided in drinking water immediately after surgery

for three days. Freely moving mice were placed into an opaque plastic rectangular box (30 cm × 17 cm) for 30 min. Ten sound stimulation trials (8944 Hz pure tone, 1 s, 70 dB sound pressure level, 3 min inter-sound interval) were played for mice. Subsequently, mice were moved to an electric shock box (50 cm × 50 cm) for 10 footshock trials (1 s, 0.6 mA, 3 min inter-shock interval). In the meantime, a camera was set above the recording box to monitor the behaviors of the mice. Ca^{2+} signals and mouse behaviors were recorded simultaneously. Event markers were used to synchronize the Ca^{2+} signals and behavior videos.

Histology and imaging

All experimental mice were perfused after recording to confirm the virus expression areas and fiber positions. Mice were perfused with phosphate-buffered saline (PBS) for ~ 5 min and then with 4% paraformaldehyde for 15-20 min to ensure complete tissue fixation. Brain samples were collected and placed in 4% paraformaldehyde overnight at 4 °C. Brain tissue was sectioned into 50-μm-thick slices with a freezing microtome and then stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were collected with a fluorescent microscope using a 2.5× or 4× objective.

Data analysis and statistics

The Ca^{2+} data were acquired at a sampling rate of 2000 Hz and analyzed as previously described [18, 21, 22]. A Savitzky–Golay finite-impulse smoothing filter (50 side points and 3 polynomial orders) was first applied to the data. Then, the relative fluorescence change was calculated by $\Delta f/f = (f - f_{baseline})/f_{baseline}$, where $f_{baseline}$ was the baseline fluorescent intensity. A transient was identified as a Ca^{2+} event if the amplitude was three times larger than the standard deviation of the baseline segment.

Nonparametric and 1-way ANOVA with *post hoc* Tukey's multiple comparisons tests in MATLAB (MATLAB 2016b, MathWorks) were used for comparison. All summarized data were from individual mice and plotted as the mean ± SEM.

Results

Specific labeling of claustral neurons that project to the MEC by GCaMP6m

We used the fiber photometry system [18, 21, 22] to monitor the population Ca^{2+} activity of claustral neurons that project to the MEC (CLA^{MEC}-projecting neurons, Fig. 1a). We expressed the

genetically encoded Ca^{2+} sensor GCaMP6m [23] specifically in CLA^{MEC}-projecting neurons by injecting retroAAV-syn-GCaMP6m [24] into the MEC (Fig. 1b,c). Then, an optical fiber was implanted above the claustrum to record the Ca^{2+} activity from CLA^{MEC}-projecting neurons (Fig. 1b, d). The expression of GCaMP6m was verified and was restricted in the MEC by *post hoc* histology (Fig. 1e). Robust expression of GCaMP6m in the claustrum was also confirmed (Fig. 1f).

Population Ca^{2+} recordings of MEC-projecting claustral neurons in anesthetized mice

Next, we determined whether the Ca^{2+} signals in the claustrum could be recorded by optical fibers with our labeling method. The Ca^{2+} signals of CLA^{MEC}-projecting neurons were recorded under different anesthesia levels by isoflurane. Figure 1g shows examples of Ca^{2+} signals from CLA^{MEC}-projecting neurons at decreasing anesthesia levels (1.8%, 1.5%, 1.2%, and 0.8%). We observed slow oscillation-associated population Ca^{2+} events, similar to the observations that have been previously described in the cortex [21, 25-27], in CLA^{MEC}-projecting neurons. The amplitude and frequency of the Ca^{2+} events changed with different anesthesia levels. The amplitude (Δf/f) decreased from 0.13 ± 0.05 to 0.05 ± 0.01 when the isoflurane concentration decreased from 1.8% to 0.8% (Fig. 1h, repeated measures 1-way ANOVA with Tukey's multiple comparisons test, F = 16.37, 1.8% vs. 0.8%, $P = 0.006$; 1.5% vs. 0.8%, $P = 0.0002$; 1.2% vs. 0.8%, $P = 0.007$). Meanwhile, the frequency increased from 0.36 ± 0.05 Hz to 3.29 ± 0.35 Hz under these conditions (Fig. 1i, repeated measures 1-way ANOVA with Tukey's multiple comparisons test, F = 37.27, 1.8% vs. 1.5%, $P = 0.03$; 1.8% vs. 1.2%, $P = 0.00004$; 1.8% vs. 0.8%, $P = 0.00003$, 1.5% vs. 1.2%, $P = 0.004$; 1.5% vs. 0.8%, $P = 0.002$). Thus, the retrograde labeling method can be combined with fiber photometry for real-time neural activity recording of CLA^{MEC}-projecting neurons.

Population Ca^{2+} response in MEC-projecting claustral neurons induced by footshock in freely behaving mice

To investigate the response induced by footshock in freely moving mice, we conducted optical fiber recordings of CLA^{MEC}-projecting neurons together with behavioral video surveillance. We performed the recordings at least 5 days after fiber implantation. Mice were placed in a white rectangular box for free exploration, with an infrared camera placed above them to monitor their behavior (Fig. 2a). Figure 2b shows a 200-s recording of Ca^{2+} signals (black trace) synchronized with body movements (gray trace). Mouse locations during the corresponding recording period were

177 automatically tracked and are plotted in Figure 2c. According to both the example in Figure 2b and
178 statistical analysis in Figure 2d, we found significantly higher Ca^{2+} signals during locomotion than
179 during grooming behavior (Fig. 2d, two-sided Wilcoxon rank-sum test, z = 6.3, p = 2.88e-10).

180 We next performed fiber recordings of CLA^{MEC}-projecting neurons during the application of
181 sound or footshock in freely moving mice (Fig. 2e). We found that sound stimulation could not
182 induce any Ca^{2+} signal (four example trials from one mouse in Figure 2f, left; summary of 19 trials
183 from 3 mice in Fig. 2g), while footshock stimulation induced clear and stable responses from
184 CLA^{MEC}-projecting neurons (four example trials from one mouse in Fig. 2f, right; summary of 21
185 trials from 3 mice in Fig. 2h). The onset latency of these footshock-induced Ca^{2+} responses was 48.3
186 ± 4.8 ms (21 trials from 3 mice). The peak amplitude (Δf/f) of footshock-induced Ca^{2+} responses
187 was significantly larger than that of sound stimulation (Fig. 2i, two-sided Wilcoxon rank-sum test, z
188 = 5.3, p = 1.28e-7).
189
190 **Discussion**
191 In this study, we utilized fiber photometry to investigate the neural responses in CLA-MEC
192 projection to footshock stimulation. We used a retrograde AAV carrying the genetically encoded
193 Ca^{2+} indicator GCaMP6m to specifically label MEC-projecting neurons in the claustrum. To test the
194 effectiveness of the retrograde labeling, we first performed Ca^{2+} recording in the anesthetized state
195 by implantation of an optical fiber probe in the claustrum of retroAAV-GCaMP6m-injected mice.
196 We found that the amplitude of the population Ca^{2+} events decreased with decreasing isoflurane
197 concentration. In contrast, the frequency increased in this process. Then, we demonstrated Ca^{2+}
198 recordings of CLA^{MEC}-projecting neurons in freely moving mice. Stable neuronal responses induced
199 after footshock but not sound stimulation were detected in the CLA^{MEC}-projecting neurons. These
200 Ca^{2+} responses after footshock could result from locomotion, pain sensation or startle reflex caused
201 by footshock [28]. Future work is needed to differentiate these possibilities.

202 The claustrum has been hypothesized to be involved in higher-order processes, depending on
203 its dense connection to and from the associative cortex [9, 10, 29, 30]. Moreover, it has been
204 suggested to participate in multiple brain functions, such as sensory information integration,
205 attention and consciousness [29-32]. The projection from the claustrum to MEC has been less
206 studied. Kitanishi et al. reported that CLA-MEC projection was activated by novel context and

modulated contextual memory [9]. However, the real-time activity pattern of CLA-MEC projection has not been clearly investigated. Here, we showed that a fiber-based recording method combined with retrograde AAV labeling can achieve real-time recording of specific projections in freely behaving mice, which will aid our understanding of the function of specific neural projections.

Overall, we demonstrated that fiber-based Ca^{2+} recording combined with retrograde AAV labeling is ideal for real-time monitoring of CLA-MEC projection in freely moving mice. With this approach, we found a stable and reliable response of CLA^{MEC}-projecting neurons induced by footshock stimulation. These findings may lead to a clearer understanding of neural circuits in fear learning and pain.

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Conflicts of interest

There are no conflicts of interest.

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Figures

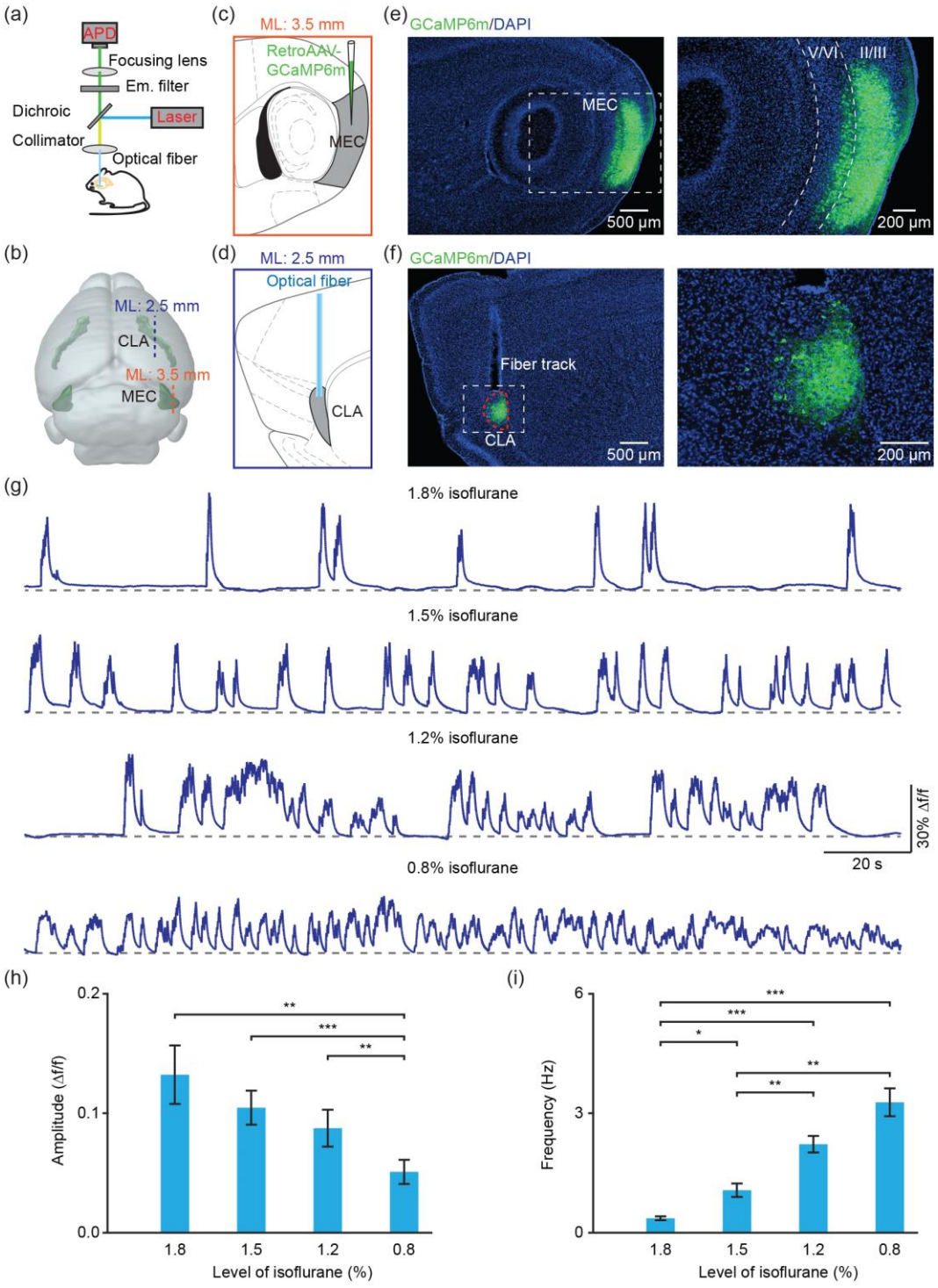

Figure 1 Population Ca^{2+} recordings of CLA^{MEC}-projecting neurons in anesthetized mice with GCaMP6m. (a) Schematic of the fiber photometry setup. (b-d) Experimental design of retroAAV-GCaMP6m injection in the MEC (c) and fiber implantation in the claustrum (d); ML: mediolateral, CLA: claustrum; MEC: medial entorhinal cortex. (e) *Post hoc* histological images showing the expression of GCaMP6m in the MEC. (f) *Post hoc* histological confirmation of GCaMP6m expression and optical fiber position in the claustrum. (g) Examples showing the population Ca^{2+} signals of CLA^{MEC}-projecting neurons at different anesthesia levels. (h) Amplitude of Ca^{2+} events of CLA^{MEC}-

projecting neurons at different anesthesia levels (n = 11 mice; repeated measures 1-way ANOVA with Tukey's multiple comparisons test, $^{**}P < 0.01$, $^{***}P < 0.001$). (i) Frequency of Ca^{2+} events of CLA^{MEC}-projecting neurons at different anesthesia levels (n = 11 mice; repeated measures 1-way ANOVA with Tukey's multiple comparisons test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

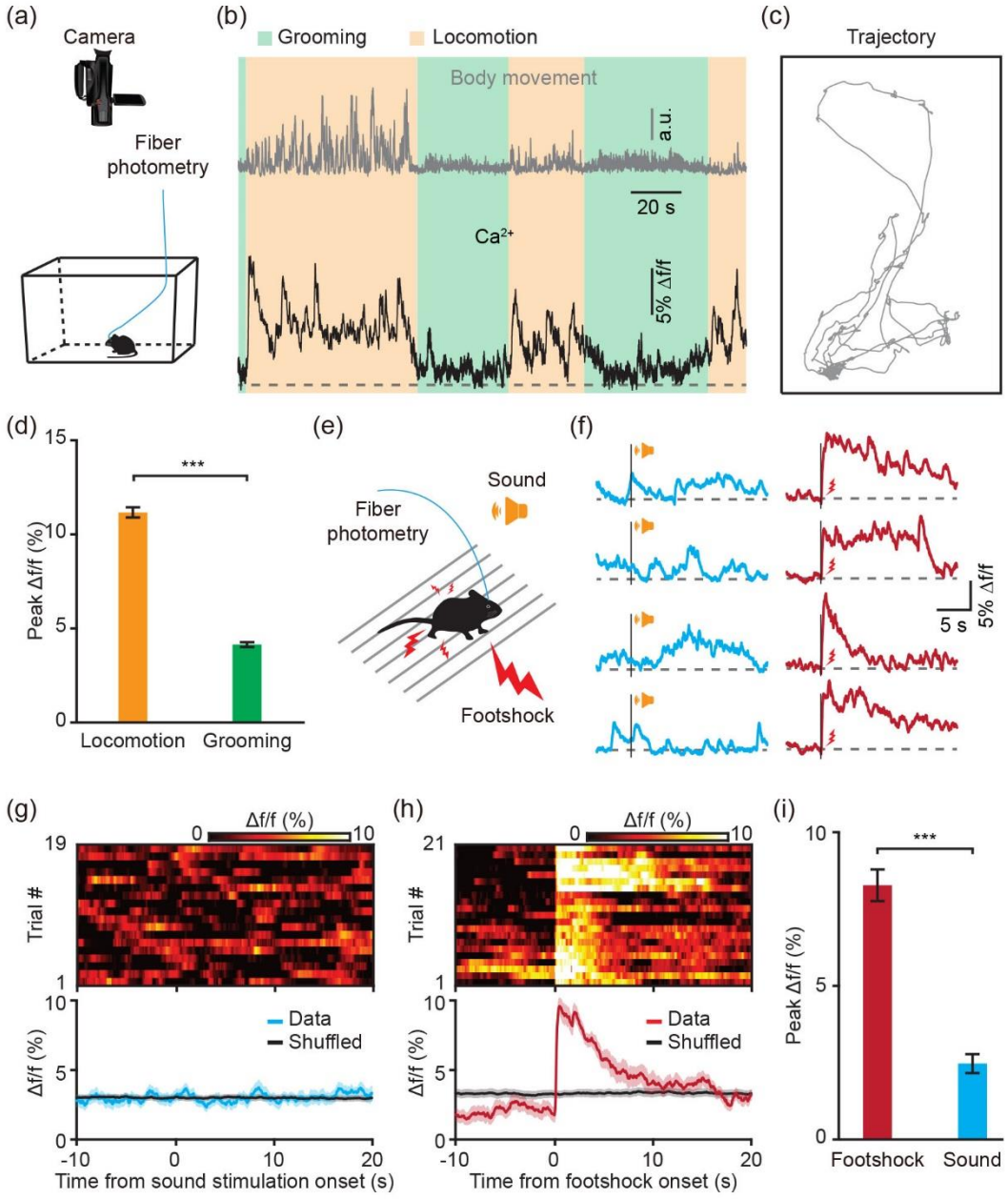

Figure 2 Population Ca^{2+} transients of CLA^{MEC}-projecting neurons induced by footshock stimulation in freely moving mice. (a) Diagram of the recording setup in freely moving mice. (b) Example showing the relative body movements (gray trace) and the Ca^{2+} transients recorded in CLA^{MEC}-projecting neurons (black trace) in a freely moving mouse; green shaded areas: grooming; yellow shaded areas: locomotion. (c) The corresponding locomotion trajectory of the recording period in b. (d) Summary of peak Δf/f during locomotion or grooming (locomotion: n = 31 trials from 3 mice; grooming: n = 24 trials from 3 mice, two-sided Wilcoxon rank-sum test, $^{***}p < 0.001$). (e) Schematic diagram of Ca^{2+} recording with footshock or sound stimulation. (f) Representative Ca^{2+} signals

induced after sound (blue) or footshock (red) in CLA^{MEC}-projecting neurons from one mouse. (g, h) Color-coded intensities (top) and average trace (bottom) of Ca^{2+} signals aligned to sound (g, 19 trials from 3 mice) or footshock (h, 21 trials from 3 mice) stimulation. The black trace is the shuffled data, and shaded areas represent s.e.m. (i) Summary of peak Δf/f during sound and footshock stimulation (sound: n = 19 trials from 3 mice, footshock: n = 21 trials from 3 mice, two-sided Wilcoxon rank-sum test, z = 5.1, $^{***}p < 0.001$).