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Original Article

Alteration of neuronal activity after digit amputation in rat anterior cingulate cortex

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Abstract: Phantom limb pain is experienced by nearly 50 - 80% of the patients following limb amputation. The anterior cingulate cortex (ACC) is a part of the limbic system that is an essential component in mediating the affective and emotional component of pain responses. To explore the role of ACC in the phantom limb pain, we recorded evoked excitatory postsynaptic potentials (EPSPs), cortical network activity and electrophysiological properties of pyramid neurons in adult rat ACC before and after a third hind paw digit amputation using *in vivo* intracellular or extracellular recording and staining techniques. The recorded neurons were morphologically identified as pyramidal neurons in the ACC region. The spontaneous activity of ACC neurons significantly reduced with a more percentage of down state after amputation, this is correlated with a decrease in spontaneous spikes in medial thalamus. However, the amplitude of the evoked EPSPs was increased significantly shortly after amputation and lasted for up to 7 days. This potentiation is associated with an increase of paired-pulse facilitation (PPF), suggesting the involvement of presynaptic component in this process. No significant difference in membrane properties was observed after amputation. On the other hand, administration of Complete Freund's Adjuvant (CFA) into the hind paw, a model of inflammatory pain, induced the potentiation of EPSPs in ACC neurons at 7 days after injection. These results demonstrate that digit amputation induced a long-lasting potentiation of synaptic transmission and decrease of cortical network activity in ACC in rats, which might contribute to the phantom limb pain.

Keywords: Phantom pain, cortex, pyramidal neurons, EPSPs, in vivo, intracellular recording

Introduction

Limb amputation is a result of traffic accidents, surgical amputation or cancer treatment. Nearly all amputees report non painful phantom sensation in the absent limb and about 50 - 80 % suffer from phantom limb pain [1, 2]. Amputation leads to reorganization within the somatosensory cortex and other cortex both in animal and humans [3, 4]. It has been reported that there is a positive relationship of cortical reorganization and phantom limb pain [5, 6]. However, molecular and cellular mechanisms contributing to plastic change in the neocortex after amputation remain to be investigated.

The Anterior Cingulate Cortex (ACC) is a part of limbic system and responds to nociceptive stimuli in animals [7, 8] and painful stimuli in humans [9-12]. Neurons within the ACC in the rabbit and rat have been characterized as nociceptive responsive. These neurons have large or whole body receptive fields and bilateral nociceptor innervations. Additionally, prolonged noxious stimulation induces expression of the *c-fos* protooncogenes bilaterally in the ACC [13]. Anatomical studies have shown that the ACC is innervated by neurons in the medial thalamus and amygdala [14-16]. The above studies suggest that ACC might influence other brain areas that are involved in emotional information processing and reactions to noxious stimulation. It has been reported that lesion of the ACC significantly reduced animal's sensitivity to noxious stimulation [17]. In human, the unpleasantness of pain is abolished in patients with neurosurgical lesions of the ACC (cingulotomy) [18, 19]. It is conceivable that ACC plays an important role in the processing of pain and encoding of negative affect.

The present study was designed to examine the contribution of ACC to phantom pain by using the digit amputation model. We studied the cortical activity such as neuron properties, cortical network activity and excitatory synaptic transmission in the ACC neurons using *in vivo* intracellular recording and staining techniques on adult rats.

Methods

Preparation of animals

All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Indiana University Institutional Animal Care and Use Committee. Male adult Wistar rats weighting 200 - 350g (Harlan, Indianapolis, IN. USA) were used for all experiments. The rats were anesthetized with 1 - 2% halothane in a gas mixture with 33% oxygen balanced with nitrogen. Body temperature was maintained at 37 °C using a thermostatically regulated heating blanket.

In vivo recording

Intracellular recording and staining were performed as described in previous publications [20, 21]. Briefly, anesthetized rats were fixed in the stereotaxic apparatus. A craniotomy was performed in the region above the ACC or the medial thalamus, and the dura was cut and removed from the exposed area of cortex. Cerebrospinal fluid was drained via a cisternal puncture to reduce brain pulsation. The bipolar stimulus electrodes were made from stainless pins, insulated except within 1.0 mm of the tips. The electrodes were separated by 1.0 mm and placed into the ipsilateral ACC anterior to the recording site with an angle of 25° to the vertical, 2.2 mm from the dura surface. The electrodes stereotaxic coordinates relative to Bregma are as follows: intracellular electrode, AP 1.0 - 3.7 mm; ML, 0.5 -1.0 mm; DV, 2.0 - 3.2 mm; and extracellular electrode, AP -2.5 - -3.5 mm; ML, 0.2 -1.0 mm; DV, 5.0 – 6.0 mm.

The intracellular recording electrodes were pulled with a Kopf pipette puller (model 750, David Kopf Instruments, Tujunga, CA, USA) from glass capillaries. The tip impedance is 40 - 70 MΩ when filled with a solution of 4% neurobiotin (Vector, CA, USA) in 2 M potassium acetate. A stimulator (Master-8, A.M.P.I, Israel) was used to deliver stimulus pulse, the stimuli were constant current pulse of 0.1 ms duration ranging from 0.3 - 3 mA. The microelectrode was advanced slowly with a motorized micromotion controller (ESP300, Newport Corporation, CA, USA) into the ACC at 2 μm increments to impale the ACC pyramidal neurons after impalement, the neurons with a stable membrane potential of -60 mV or greater and action potential amplitudes of at least 60 mV were selected for further study. For extracellular recording in the medial thalamus, we use similar microelectrodes. The tip impedance is 30-40 MΩ when filled with 2 M NaCl solution. The electrode was lowered into the medial thalamus until robust activity was detected.

Signals were amplified using the bridge model of Axoclamp 2B amplifier (Axon Instruments, CA, USA) and stored on a Macintosh computer using Axodata program (Axon Instruments, CA, USA). Records were analyzed off-line using Axograph (Axon Instruments, CA, USA). The bridge balance and neuronal resistance was monitored from the oscilloscope and adjusted appropriately.

Histological identification of recording sites

After recording, neurobiotin was iontophoresed into the cell by applying depolarizing current pulse (2Hz, 450 ms, 1.0-1.5 nA. 10 min). Then the animal was deeply anaesthetized and perfused transcardically with 0.01 M PBS followed by 4% paraformaldehyde phosphate-buffered solution. Serial coronal section (80 μm) of the ACC were incubated in 0.01 M PBS containing 0.1% horseradish peroxidase-conjugated avidin-D (Victor, CA, USA) and stained with 3,3'-diaminobenzidine and hydrogen peroxide. Recording sites were identified based on the atlas of Paxinos and Watson.

Digit amputation and CFA injection

After deeply anaesthetized, the third digit of the right hind paw was amputation [22]. Any bleeding was stopped with ethanol gel. Inflammatory model of pain was induced with Complete Freund's Adjuvant (CFA) suspended in a oil/saline (1:1) emulsion and injected (100 μl) into the plantar surface of the right hind paw of anaesthetized rats.

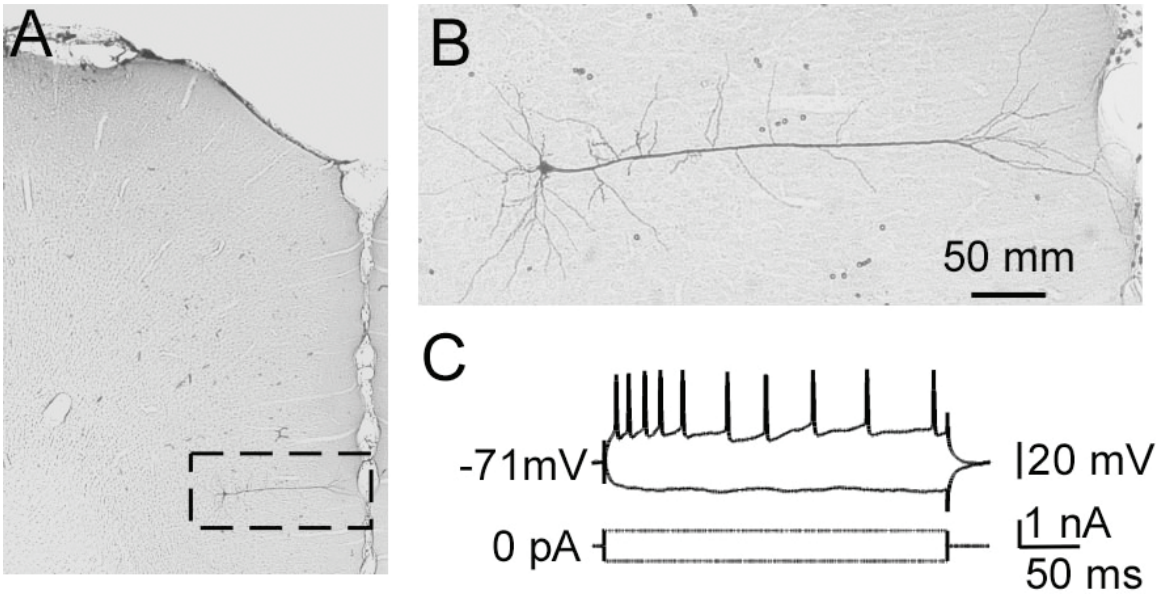

Figure 1. Identification of pyramid neurons in the anterior cingulate cortex (ACC). A. location of a pyramidal neuron intracellular stained with neurobiotin in a coronal section. B. Higher magnification of the neuron (in square) in A. C. Injection of depolarizing current pulse induces repetitive action potentials. Significant spike frequency adaptation observed in this cell suggests that this is a pyramidal neuron.

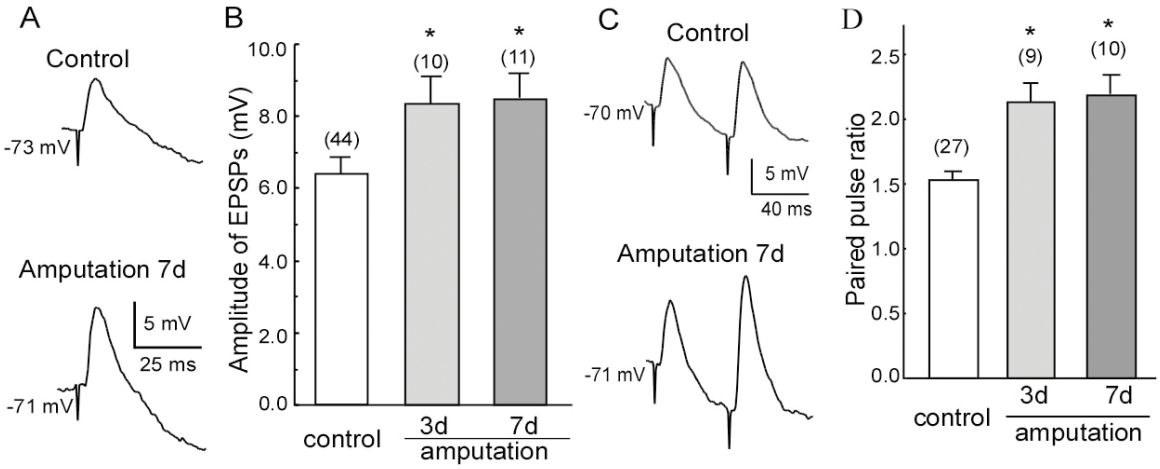

Figure 2. Amputation causes long-lasting potentiation of evoked postsynaptic potentials (EPSPs) in the ACC. A. Representative traces of EPSPs before and after amputation. The traces are the average of four consecutive sweeps. B. Group data showing the amplitude of EPSPs before and after amputation of the third digit of the contralateral hind paw. EPSPs were enhanced for up to 7 days. The stimulation intensity is 2 times of the threshold intensity to evoke EPSPs. C. Representative traces (average of four sweeps) of paired-pulse facilitation (PPF) before and after amputation. D. PPF in ACC was increased after 3d of digit amputation and lasted up to 7 days following the surgery. PPF was examined with a 50 ms inter pulse interval and measured as the ratio of the slope of the second EPSP to the slope of first EPSP. *P < 0.05 compared to the control group (Numbers of cells are denoted in parentheses).

Quantification of the membrane potential fluctuation

Quantification of the membrane potential fluctuation was accomplished by sampling the spontaneous activity at 1 kHz for 20 s and counting number of samples at each membrane potential. The data were presented as all-point histograms. This gives the proportion of the time spent by the neurons at each membrane potential. The best fit of the sum of two Gaussian distributions was used to fit the data using Axogragh 4.9 (Axon Instruments Inc.) [23]:

Table 1. Membrane property of ACC neurons before and after amputation

groups	Spike height (mV)	Spike width (mS)	Spike threshold (mV)	Rheobase (nA)	Input resistance (MΩ)	Time constant (mS)
cont (31)	79.42±1.50	0.97±0.03	-52.00±0.70	0.38±0.03	21.69±1.03	6.65±0.30
3d (10)	79.00±2.92	0.97±0.05	-49.90±1.52	0.36±0.04	18.45±1.01	5.32±0.36
7d (9)	77.89±2.63	1.11±0.08	-53.22±0.64	0.38±0.02	18.58±1.16	5.48±0.50

Values are means±SEM, with number of neurons in parentheses.
Spike height is measured from the resting membrane potential. Spike width is measured at the half of the action potential. Input resistance is derived from the linear portion of the I-V curve (0~-0.5 nA). Time constant is derived from transients of hyperpolarizing pulse (-0.3 nA).

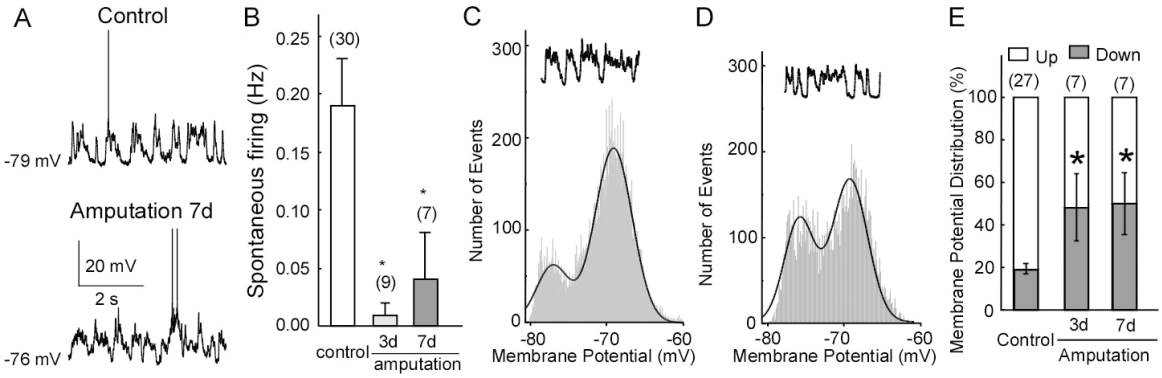

Figure 3. Spontaneous firing rate in the ACC was decreased after amputation. A. Representative traces of membrane potential in ACC pyramidal neurons before and after amputation. B. Group data shows that the spontaneous firing rate was significantly reduced after amputation. C & D. Histograms shows the number of events at various membrane potentials of representative pyramidal neurons before (C) and after amputation (D). Thick lines are the best fit of the sum of two Gaussian distribution to the histograms. The two peaks of the histogram represent the Down and Up states of the neuronal activity. The insets are membrane potential traces. E. Group data showing the proportional distribution of the Down states are increased while the Up states are decreased after amputation. *P < 0.05 compared to the control group.

$Pr(r) = a \exp [-(v-\mu_1)2/2\sigma_1^2] / \sqrt{2\pi}\ \sigma_1 + b \exp [-(v-\mu_2)2/2\sigma_2^2] / \sqrt{2\pi}\ \sigma_2$

Where a, b are the index of the time the neuron spent in Up and Down states, respectively, μ_1, μ_2 are average membrane potential and σ_1, σ_2 are refer as the variance of the two states.

Data and analysis

Data are presented as the mean ± SEM. ANOVA followed by post hoc test or unpaired t-test was used for statistical comparisons. P < 0.05 was considered significant.

Result

In vivo intracellular recording were performed on 65 rats, a total of 125 pyramidal neurons in ACC with stable resting potentials of -60 mV and more negative in this study. We identified pyramidal neurons by injecting depolarized currents into neurons to induce action potentials. The typical firing pattern of pyramidal neurons showed significant firing frequency adaptation [24, 25]. They were further confirmed as ACC pyramidal neurons based on the location and morphology of intracellular stained neurons (**Figure 1**).

Long-lasting potentiation of EPSPs in the ACC caused by amputation

Peripheral tissue/nerve injury causes central sensitization. Previous studies found that amputation of a third digit of the hind paw caused different immediate-early genes activated. In the present study we found a long-

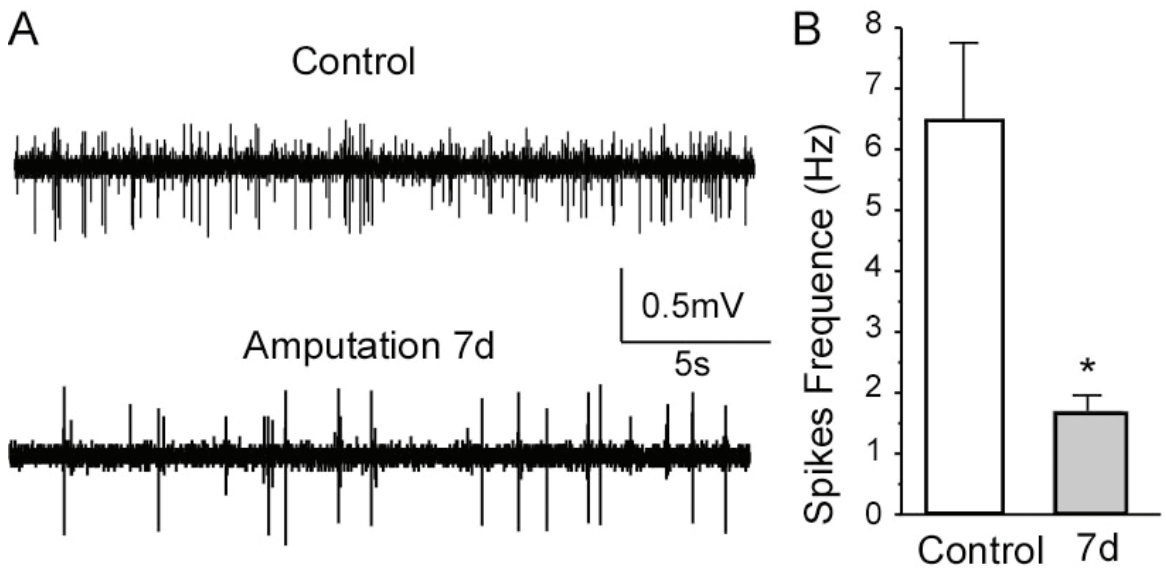

Figure 4. Spontaneous activity in medial thalamus is decreased after amputation. A. Representative traces of extracellular unit recording before and 7 days after amputation. B. Histograph demonstrating the spike frequency is decreased after amputation of the third digit of the contralateral hind paw. *P < 0.05 compared to the control group.

lasting (at least 7 days) potentiation of EPSPs in ACC after amputation (**Figure 2B**). The amplitude of EPSPs was significantly increased from 6.06 ± 0.32 mV of control leves (n=44) to 8.35 ± 1.09 mV (n=10) and 8.16 ± 0.53 mV (n=11), after 3d or 7d amputation, respectively (P < 0.05). No significant differences in membrane properties of pyramidal neurons in ACC before and after amputation (**Table 1**).

To determine whether the potentiation of EPSPs after amputation is mediated through presynaptic or postsynaptic mechanism, paired-pulse facilitation (PPF) ratio test was considered to reveal the change of PPF of these neurons following amputation. As show in **Figure 2D**, the PPF ratio increased from 1.55 ± 0.07 of control (n=27) to 2.15 ± 0.15 (n=9) and 2.21 ± 0.15 (n=10) after 3d or 7d of amputation respectively, (P < 0.05). This suggests the involvement of the presynaptic mechanism.

Changes in spontaneous activities in the ACC after amputation

The membrane potential of pyramidal neuron spontaneously shifted between two relatively constant subthreshod levels, a hyperpolarized Down state and a depolarized Up state. Action potentials were intermittently triggered from the noisy membrane potential fluctuation in the Up state. After 3d and 7d amputation, spontaneous firing rate decreased significantly from 0.18 ± 0.04 Hz of control (n=30) to 0.01 ± 0.01 Hz (n=9) and 0.04 ± 0.04 Hz (n=7) respectively (p< 0.05, **Figure 3B**). The proportion of the Up state decreased significantly from 0.81 ± 0.02 of control (n=27) to 0.52 ±0.16 (n=7) and 0.50 ±0.15 (n=7) at 3d or 7d following amputation. (p< 0.05, **Figure 3C-E**).

Changes in spontaneous activities in the medial thalamus induced by amputation

Cortical neurons *in vivo* are constantly bombarded by synaptic inputs including inputs from thalamus, so we recording the spontaneous spikes from the medial thalamus for 2 min by using the extracellular *in vivo* recording techniques. The spike frequency is decreased from 6.45 ± 1.32 Hz of control (n=13) to 1.69 ± 0.26Hz after amputation of the third digit of the contralateral hind paw (n=12, P< 0.05, **Figure 4**).

Changed synaptic transmission in the ACC from CFA injection

To test if other forms of chronic pain influence the synaptic transmission in the ACC, we selected CFA-induced inflammatory pain as an example. In this model, after injection of CFA into the hind paw, hyperalgesia and edema are present for appropriately 1 to 2 weeks. In the present

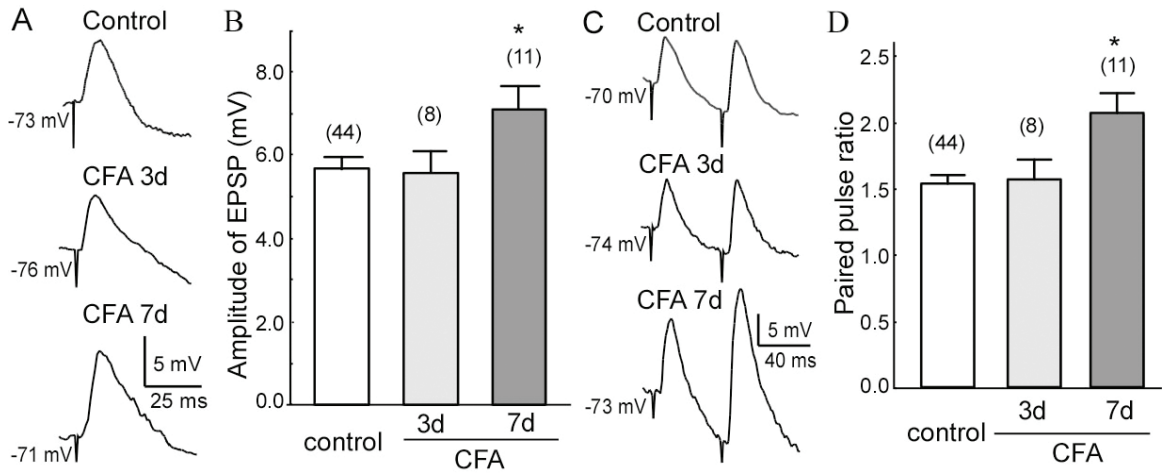

Figure 5. Complete Freund's adjuvant (CFA) injection induced a potentiation of EPSPs in the ACC. A. Representative traces of EPSPs recording at different time points after CFA injection into the plantar surface of the contralateral hind paw. B. Group data show that the amplitude of EPSPs of ACC neurons is significantly increase at 7 days after CFA injection. C. Representative traces of paired-pulse facilitation (PPF) at different times after CFA injection. D. Group data showing the increase of PPF in ACC neurons at 7 days after CFA injection. *P < 0.05 compared to the control group.

study, no significant changes in EPSP was observed in ACC neurons 3d after CFA injection. However, the amplitude of EPSPs was significantly increased 7d after CFA injection (**Figure 5B**) and the PPF ratio also increased at the same time point (n= 11, p < 0.05, **Figure 5D**). These results suggest that the CFA induced inflammatory pain might have different mechanisms in ACC as compared with the pain induced by amputation.

Discussion

Peripheral deafferentation or amputation could cause massive plastic changes within cortical and subcortical structures [3]. In the present study, we found that amputation of a single digit of one hind paw caused long-lasting changes in sensory responses in the ACC, a region critical for processing pain information in the CNS, may serve as an important synaptic mechanism for enhanced nociceptive transmission after amputation, which might contribute to the phantom pain. We also provide evidence that administration of CFA into the hind paw, a model of inflammatory pain, induced the potentiation of EPSPs in ACC neurons at 7 days after injection. These results suggest that the induction and the time course of amputation-induced long-lasting potentiation are different from those of inflammatory pain.

Among many physiological functions of the ACC, its role in pain and pain-related cognitive functions has been intensely investigated [26]. The ACC has been suggested to contribute to the perception of pain, the learning process associated with the prediction/avoidance of noxious sensory stimuli as well as pathological phantom pain. Electrophysiological recordings from both animals and humans demonstrate that neurons within the ACC respond to noxious stimuli [8, 10]. In the present study, we demonstrated that both amputation and inflammatory pain models could enhance the synaptic response in the ACC.

Glutamate is a major excitatory transmitter within the ACC [22, 27]. The long-term plasticity of glutamatergic synapses, such as long-term potentiation and long-term depression, is well documented in the hippocampus [28-30]. However, only a few studies have been performed in the ACC slices *in vitro*. Sah & Nicoll [27] reported that delivery a brief tetanic stimulation to the afferent fibers (callosal inputs) led to a synaptic potentiation lasting less than 1 hour. In another study, evoked field EPSPs remained enhanced for 2 hours after the amputation [31]. In the present study, the enhanced EPSPs could last for at least 7days after the amputation. This indicates the reorganization of the cortex. The changes of spontaneous firing rate after amputation indicate that the functional connection between ACC and other cortical region has been changed after amputation.

The idea underlying investigations of the relations between LTP and PPF is that PPF should be changed following LTP induction if presynaptic mechanisms contribute to LTP express. Schulz et al, suggested the transmitter release is proportional to the product of the number of neurotransmitter release sites and the probability of neurotransmitter release at those sites. In our study, the PPF ratio change significantly 3 days after amputation, would be due to the increase in the number of effective release sites [32, 33]. This means the generation of new synapses, and supported by other studies [34, 35].

In the intact brain, neurons are under constant bombardment by a complex barrage of excitatory and inhibitory synaptic inputs. A large part of this connectivity originates from the cortex itself, but inputs are also received inputs from subcortical structures, such as the thalamus [36, 37]. As a result, neurons *in vivo* experience fluctuating membrane potentials. The Down state defines a quiescent period during which little activity occurs, whereas the Up state corresponds to an active cortical state with depolarized membrane potentials and sometimes action potential firing [38]. In this study, the membrane potential of the pyramidal neuron also showed the spontaneous fluctuation. This is consisted with previously studies [24, 39]. Amplitude of the fluctuation did not change after amputation. Instead, amputation primarily affected the dynamic of the fluctuation, increasing the proportion of the membrane potential spent in the Down state. This change in proportion accounted for the decreased spontaneous firing rate.

The origin of this phenomenon is not precisely known. Anatomical studies have shown that medial thalamus afferent terminals form asymmetric synapses with dendritic spines arising from the apical dendrites of pyramidal neurons whose somata reside in layers III and V of the prefrontal cortex [37]. Another study revealed a high density of terminal of medial thalamus in ACC, and demonstrated that the medial thalamus is the major thalamic relay that transmits nociceptive information to the ACC [40]. In neocortical slices, spontaneous activity can arise within the intracortical connection [41, 42], and this cortical network activity is generated through a dynamic balance of excitation and inhibition [38]. After amputation, change in dynamic of the fluctuation demonstrated the change of excitatory and inhibitory connection between the ACC and other cortical or subcortical structure. In consistent with this, we found that the spikes frequency in the medial thalamus is decreased. This indicated that the input from medial thalamus to the ACC is weakened.

CFA-induced inflammatory pain is a kind of chronic pain model that can persist for approximately 1 to 2 weeks [43]. In this model the hind paw of the animal is intact. In the present study, CFA injection needs a longer time to induce potentiation of EPSPs in ACC neurons, which suggests that the CFA-induced pain might have a different mechanism from the phantom pain.

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