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·Original Article·

Differential effects of long and short train theta burst stimulation on LTP induction in rat anterior cingulate cortex slices: Multi-electrode array recordings

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Abstract: Objective There is substantial evidence supporting the notion that the anterior cingulate cortex (ACC) is an important limbic structure involved in multiple brain functions such as sensory perception, motor conflict monitoring, memory, emotion and cognition. It has been shown that long term potentiation (LTP) is an important synaptic model of neural plasticity in the ACC, however, little is known about the spatiotemporal properties of ACC at network level. The present study was designed to see the LTP induction effects across different layers of the ACC by using different conditioning stimuli (CS) protocols. **Methods** A unique multi-electrode array recording technique was used in the acutely-dissociated ACC slices of rats. Long and short train theta burst stimulation (TBS) paradigms were applied in layer V-VI as the CS and the LTP induction effects were compared across different layers of the ACC. Briefly, both long and short train TBS are composed of bursts (4 pulses at 100 Hz) with a 200 ms interval, however, the former (TBS1) was with 10 trains and the latter (TBS2) was with 5 trains. After test stimulation at layer V-VI in the ACC, network field potentials (FPs) could be simultaneously recorded across all layers of the ACC. **Results** The waveforms of FPs were different across different layers. Namely, positive-going waveforms were recorded in layer I and negative-going waveforms were recorded in layers V-VI, in contrast, complex waveforms were localized mainly in layers II-III. Following application of two CS protocols, the induction rate of LTP was significantly different between TBS1 and TBS2 regardless of the spatial properties. TBS1 had more than 60% success, while TBS2 was less than 25% in induction of LTP. Moreover, both the 2 CS protocols could induce LTP in layers II-III and layers V-VI without layer-related difference. However, no LTP was inducible in layer I. **Conclusion** The present findings indicate that stimulation protocols may, at least in part, account for a large portion of variations among previous LTP studies, and hence highlight the importance of selecting the best LTP induction protocol when designing such experiments. Moreover, the present results demonstrate the prominent superiority of multi-electrode array recording in revealing the network properties of synaptic activities in the ACC, especially in comparing the spatiotemporal characteristics between different layers of this structure.

Key words: long term potentiation; anterior cingulate cortex; theta burst stimulation; multi-electrode array recordings; rat

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1 Introduction

As a part of the limbic system, the anterior cingulate cortex (ACC) is supposed to play important roles in various sensory, memory, emotion and cognitive functions[1,2], which is consistent with its widespread anatomical connections with

other brain structures[3-5]. Especially, several lines of evidence from animals to human studies have suggested that the ACC serves as an important limbic structure for the central process of pain[6,7]. Stimulation of ACC neurons by delivering electrical currents or local glutamate microinjection causes fear memory or triggers aversive behaviors[8,9], whereas lesions of the ACC could significantly reduce animals' sensitivity to noxious heat in the hot-plate test as well as persistent nociception and hypersensitivity induced by bee venom injection[10,11]. Disruption of the ACC could also prevent the formalin-induced aversive memory behaviors[12]. In addition, electrophysiological recordings and neuroimaging studies also show that neurons within the ACC respond to noxious stimuli[13-18]. Importantly, neuronal and synaptic plasticity occurring within the ACC after injury has been recently proposed to contribute substantially to the pathophysiogenesis of chronic pain[7,19,20].

With regard to the plasticity in the ACC, synaptic and cellular mechanisms of long-term potentiation (LTP), a widely used synaptic model for learning and memory[21,22], have been widely studied using brain slice preparations. Here, it is worthwhile to note that different conditioning stimuli (CS) protocols have been used for inducing LTP of synaptic responses in the ACC[2]. LTP of excitatory synaptic responses could be recorded in the ACC using both field potential recording and whole-cell patch-clamp recording techniques[23,24]. For field potential recording from adult mouse ACC slices, glutamatergic synapses in the ACC can undergo LTP in response to theta burst stimulation (TBS, 5 trains of burst with 4 pulses at 100 Hz, 200 ms interval), a paradigm more closely mimicking the activity of ACC neurons under *in vivo* conditions[23,25]. Unlike that in the hippocampus, strong tetanic stimulation in the ACC does not cause reliable LTP induction[23,26]. On the other hand, whole-cell patch-clamp recordings allow a better investigation of synaptic mechanisms for LTP in the ACC[24,27]. In this case, LTP can be induced using 3 different CS protocols, including the pairing training protocol[24,27,28], the EPSPs-APs (excitatory postsynaptic potentials-action potentials) protocol[24,27,29], and the strong TBS 5 trains of burst with 4 pulses at 100 Hz, 200 ms interval, repeated 4 times at intervals of 10 s) protocol[24,27]. It is important to keep in mind that LTP recorded through field recording or whole-cell patch-clamp recording may employ different intracellular signaling pathways for induction and expression[2]. Furthermore, not every CS protocol is suitable for inducing sustained LTP in a certain brain region[30]. The magnitude of LTP induced by different protocols also varies a lot[31]. Thus, it seems quite necessary to compare the effects of different CS protocols on LTP induction and maintenance in the ACC area. Furthermore, previous anatomical and cytoarchitectural studies have suggested that different layers of the ACC contain distinct cell types and projecting fibers, probably resulting in layer-related difference in ACC function[6,32,33]. Unfortunately, fewer reports have been conducted to examine in detail this layer difference within the ACC slices.

Given the above statements, the aim of the present study was to evaluate potential differences in the induction rate and magnitude of LTP across the 3 major layers (layer I, layer II-III, layer V-VI) of ACC slices by different CS protocols, emphasizing the effect of length of TBS trains. Specifically, 2 types of LTP induction protocols were compared (see Fig. 1 for the schematic illustration): long train TBS (TBS1), 10 trains of burst with 4 pulses at 100 Hz (200 ms interval); short train TBS (TBS2), 5 trains of burst with 4 pulses at 100 Hz (200 ms interval). The field potential recording on ACC slices was accomplished by a state-of-the-art multi-electrode array (MEA) recording technique, namely the Panasonic's MED64 system[34-37], with the following advantages: (1) detect the activity of neuronal networks in both space and time[38-41]; (2) record and stimulate the electrophysiological activity of many sites simultaneously[39,40,42,43]; (3) stable recordings that are less sensitive to factors such as mechanical vibrations[39-41].

2 Materials and methods

2.1 Animals Experiments were carried out on male albino Sprague-Dawley rats provided by Laboratory Animal facilities of both Capital Medical University (CCMU) and the Fourth Military Medical University (FMMU). The rats were 3-4 weeks old and fed in standard laboratory condition (12 h light/12 h dark, temperature 22-26 °C, air humidity 55-60%) with *ad libitum* water and rat chow. The experimental proce-

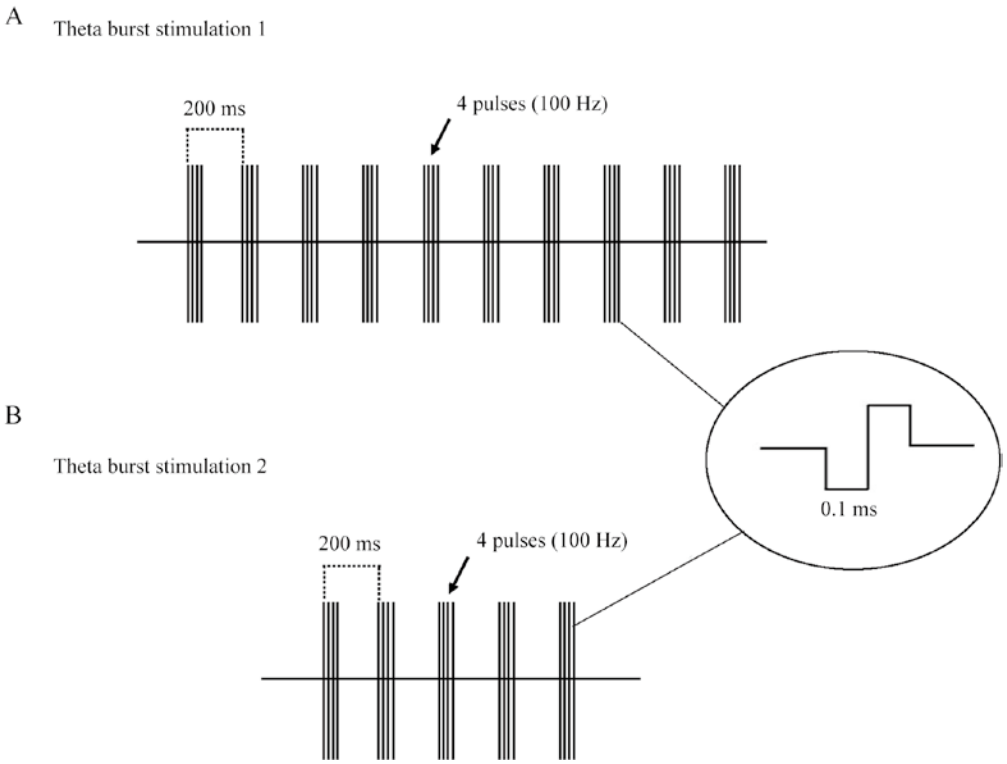

Fig. 1 The 2 TBS protocols for induction of LTP in the ACC. A: Protocol 1 (TBS1) is composed of long train of burst stimulation (4 pulses, 100 Hz) repeated 10 times with an interval of 200 ms. B: Protocol 2 (TBS2) consisted of short train stimulation that contains 4-pulses burst (100 Hz) repeated 5 times with an interval of 200 ms. Each single pulse is a pair of 0.1 ms negative-positive square waveforms.

dures were approved by the Institutional Animal Care and Use Committee at both CCMU and FMMU. All animals were maintained and cared for according to the guidelines set forth by the International Association for the Study of Pain[44]. The number of animals used and their suffering were greatly minimized.

2.2 Slice preparation The general procedures for preparing acute ACC slices were similar to those described previously for hippocampal slices[34-36]. Rats were decapitated after anesthesia with 4% sodium pentobarbital (0.1 mL/100 g, i.p.). Subsequently, the whole brain was quickly removed and placed into a cold bath of oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mmol/L): NaCl 124, KCl 3.3, KH_2PO_4 1.2, $MgSO_4$ 2.4, $CaCl_2$ 2.5, $NaHCO_3$ 26 and glucose 10 (pH 7.35-7.45). After cooling for about 1-2 min, appropriate portions of the brain were then trimmed and the remaining brain block was glued onto the stage of a vibrating tissue slicer (Dosaka, DTK-1000), where coronal ACC slices (350 μm) were obtained and transferred to a chamber continuously perfused with oxygenated ACSF at room temperature. Slices were allowed to recover for at least 2 h before electrophysiological recording was attempted.

2.3 Preparation of the multi-electrode array A commercial 64-channel multi-electrode dish system (MED64, Panasonic, Japan) was used for extracellular field recordings in this study. Procedures for the preparation of the Multi-Electrode Dish (Panasonic, MED probe) were almost the same as described previously[34]. The size of each electrode in the array was 50 μm × 50 μm, and all the 64 electrodes were arranged in an 8 × 8 square pattern with an inter-electrode distance of 300 μm to cover a total area of 4.4 mm^2. For detailed composistion and manufacture of the MED64 probe, please refer to Oka *et al*.[34]. Before use, the surface of the MED64 probe was treated with 0.1% polyethyleneimine (Sigma, St. Louis, MO; P-3143) in 25 mmol/L borate buffer (pH 8.4) overnight at room temperature. This coating helped establish sufficient adhesion of the slice

to the probe surface, resulting in enough perfusion by the recording buffer (2-3 mL/min) to keep the slice healthy for more than 6 h of fEPSP recording. The probe surface was rinsed 3-5 times with sterile distilled water before immediate use in each experiment. In general, the MED64 probes could be re-used for approximately 30-40 recording sessions with a mean duration of 4-6 h. Electrode properties could be maintained constant by carefully cleaning the probe with deionized water following each recording session.

2.4 Electrophysiological recordings After incubation, one slice was positioned on the MED64 probe in such a way that the right ACC was in the center of the recording dish mounted on the stage of an inverted microscope (IX71, Olympus, Japan). The anatomic locations of those recorded slices were equivalent to AP: +2.28 to -1.56 mm, ML: 0.0 to 0.9 mm, and DV: 1 to 4 mm, according to the rat atlas of Paxinos and Watson[45]. Once the slice was settled, a fine mesh anchor (SHD-22L, Harvard, USA) was carefully disposed to ensure stabilization of the slice during recording. A microphotograph of one ACC slice positioned on the MED64 probe was shown in Fig. 2A.

For the electrophysiological recordings, the probe with immobilized slices was connected to the stimulation/recording component of MED64. The slice was continuously perfused with oxygenated, fresh ACSF at the rate of 2-3 mL/min with the aid of a peristaltic pump (PERI-STAR™, WPI, USA). After a 20-min recovery of the slice, one of the 64 available planar microelectrodes was selected from the 64-switch box

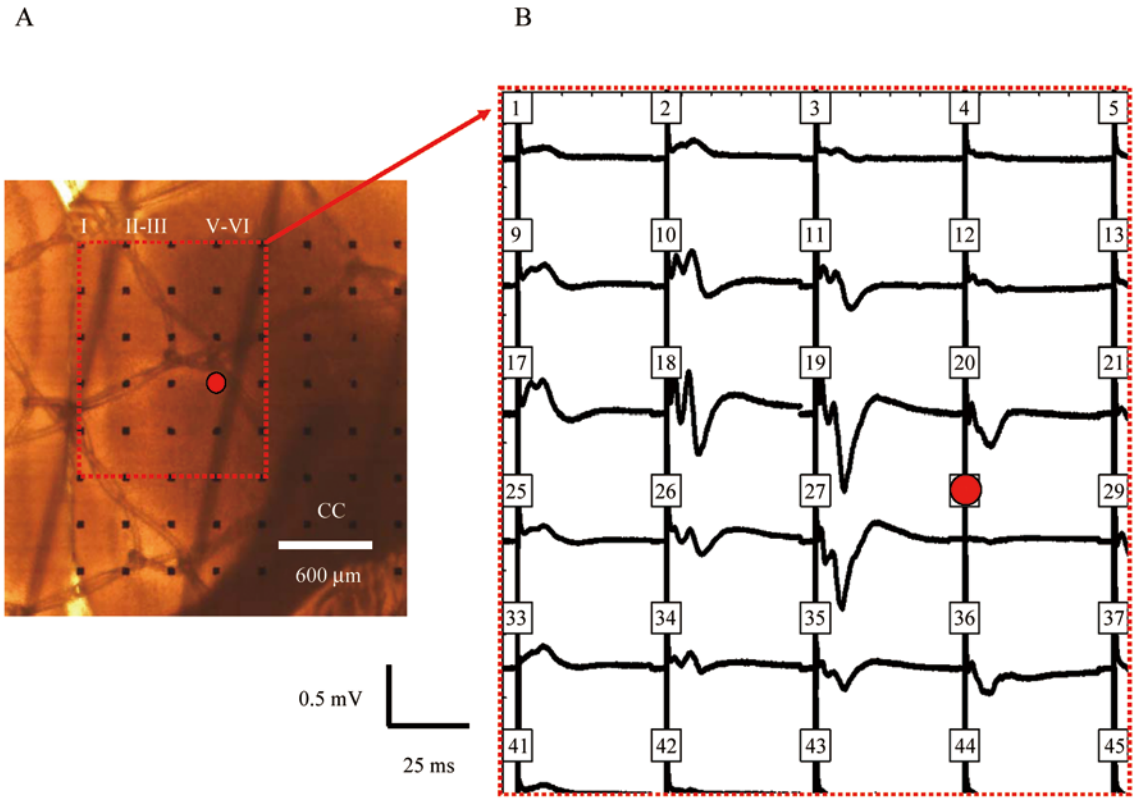

Fig. 2 Network responses in the ACC recorded with a MED-64 probe. A: A photomicrograph shows the relative position of one ACC slice on the MED-64 electrode (inter-electrode distance, 300 μm). cc, corpus callosum. Scale bar: 600 μm. B: A typical 2 dimensional map of evoked field potentials and multiunit activities in the ACC slice shown in A. Generally, 3 types of field potentials could be recorded across all layers of ACC slices by the MED64 probe. Scale bar represents 25 ms, 0.5 mV. For both A and B, the red circle indicates the stimulus site and the red square represents the recording area on the ACC slice.

for stimulation following visual observation, through a charge-coupled device camera connected to the inverted microscope. When one of the 64 planar microelectrodes was selected as the stimulating electrode, the remaining 63 sites were used for recording the extracellular field potentials. If not specified, monopolar, biphasic constant current pulses (70 μA, 0.1 ms) generated by the data acquisition software were applied to the layer V-VI of the ACC at 0.1 Hz. The layer V-VI was chosen to be stimulated for the reason that neurons within this layer receive sensory inputs from the thalamus and also send fibers to the superficial layers[3-5,32,46]. The evoked potentials were amplified by a 64-channel amplifier and then digitized at a 20 kHz sampling rate. The digitized data were displayed on the monitor screen and stored on the hard disk of a micro-computer. To improve the signal-to-noise ratio, 5 successive responses were averaged automatically in real time by the recording system.

For the LTP induction, stable baseline responses were first recorded for at least 30 min before delivering the conditioning stimulus to ACC area. The test stimulation intensity (70 μA) used in the present study had been proved in our pilot experiments to be able to evoke about 50% of the maximum amplitude of field potentials. As for the LTP induction protocol, it is widely accepted that the TBS paradigm resembles the *in vivo* conditions and has been suggested as a method to establish a link between artificial and natural synaptic activities[30]. In addition, LTP induced by TBS appears to be more robust and stable than that induced by other means[47,48]. In our experiments, 2 TBS were adopted protocols to induce the LTP in ACC area. The long train protocol (TBS1) consisted of 10 trains of burst with 4 pulses at 100 Hz, 200 ms interval (Fig. 1A), while the short train protocol (TBS2) had the same settings of the train, but the number of trains was decreased to 5 (Fig. 1B). To standardize tetanization strength in different experiments, the TBS intensity was identical to that of the test stimulation. After TBS, the test stimulus was repeatedly delivered once every 10 min for more than 2 h to allow for the observation of any changes in LTP magnitude and duration.

2.5 Data analysis Data were analyzed off line by the MED64 Conductor. For quantification of the LTP induction, the amplitude of field potentials was normalized and expressed as a percentage of the averaged baseline value. Data sets included results from only one slice per rat (n = number of slices) and were presented as the mean±SEM. When necessary, the statistical significance was determined by one-way ANOVA, followed by the appropriate *post hoc* tests using Statistical software. Paired or two-sample Student's t test was used when comparisons were restricted to two means. $P < 0.05$ was considered as statistically significant.

3 Results

Generally, there were 3 types of field potentials recorded in different layers of the ACC area by the MED-64 probe following electrical test stimulation of layer V-VI: positive-going in layer I, dual-going (or complex) in layer II-III and negative-going in layer V-VI, respectively (Fig. 2B).

In the case of LTP induction, the long train protocol (TBS1) elicited a clear LTP after its application onto the layer V-VI in ACC slices (Fig. 3A, upper panel). The success rate of LTP induction averaged 61.54 % (8/13) in either layer II-III or layer V-VI (Table 1). However, the TBS2 protocol could only induce LTP in 1-2 of the total 8 slices, with an induction rate being 25 % and 12.50 % in layer II-III and layer V-VI, respectively (TBS2 *vs* TBS1, $P < 0.01$; Fig. 3A, lower panel, Table 1). But both of these 2 CS protocols failed to produce LTP in layer I (Fig. 3, Table 1). Moreover, the induction probability of LTP, by either protocol, did not differ between layer II-III and layer V-VI ($P > 0.05$, Table 1).

During maintenance of LTP in layer II-III, the mean amplitudes of field potentials normalized to the baseline measured at 30 min and 120 min post-TBS1were (125.38 ± 5.42) % and (146.89 ± 7.64) %, respectively (n = 8, Fig. 3B). The values in layer V-VI were (123.36 ± 3.44)% and (151.34 ± 5.64) % (n = 8, Fig. 3B). Furthermore, the LTP induced by TBS1 occurred from post-TBS1 30 min and lasted up to 120 min in these layers. There was no significant difference in the time course or magnitude of LTP between layer II-III and layer V-VI ($P > 0.05$, Fig. 3B). Notably, due to the small induction rate of LTP with the short train protocol (TBS2), quantification of the LTP data by TBS2 could not be obtained.

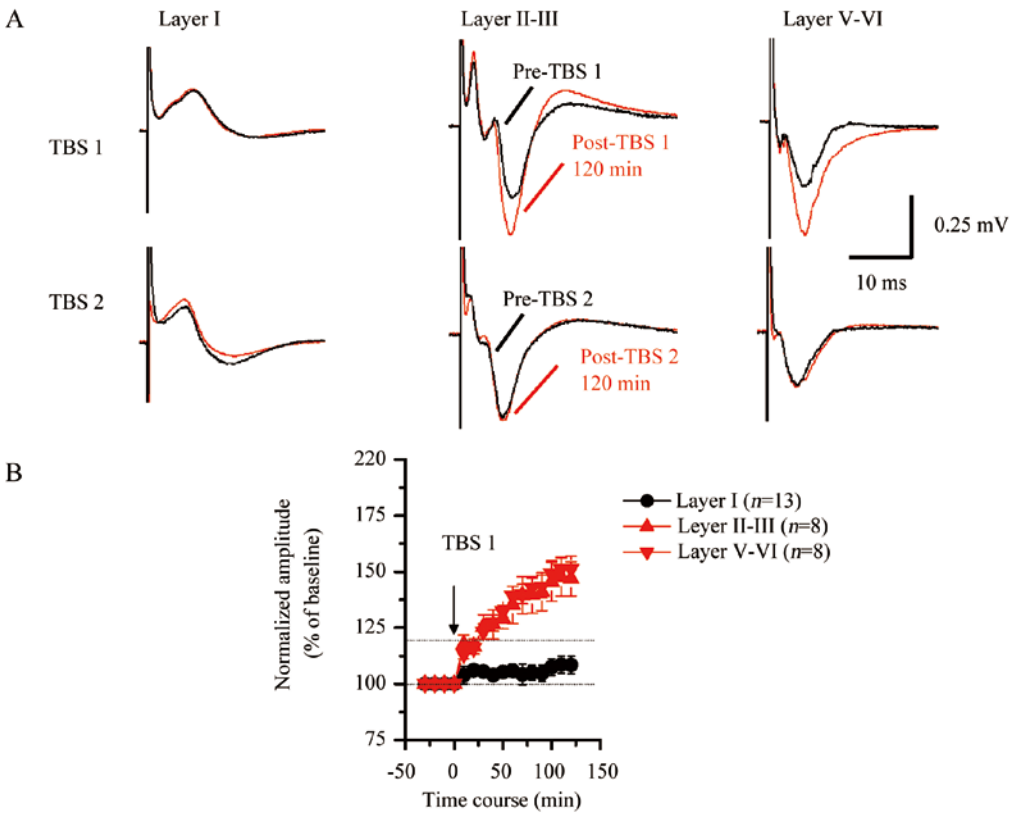

Fig. 3 Effects of 2 theta burst stimulation (TBS1 and TBS2) protocols on LTP induction in the ACC. A: Upper panels show trace examples of field potential responses within layer I (left), II-III (middle) and V-VI (right) of the ACC prior to (Pre-TBS1) or 120 min after TBS1 (Post-TBS1 120 min), respectively. Lower panels show trace examples of field potential responses within the 3 layers prior to (Pre-TBS2) or 120 min after TBS2 (Post-TBS2 120 min), respectively. It is clear that TBS1 induced a marked LTP at 120 min while TBS2 failed to produce LTP. B: Time course of cingulate LTP induced by TBS1 in the 3 layers of the ACC slices. There was no apparent difference in the magnitude and duration of LTP induced between layer II-III and layer V-VI, while no significant LTP was induced in layer I.

Table 1. Comparative analysis of LTP induction rate in layer I, layer II-III and layer V-VI of rat ACC slices between short and long train TBS

	Layer I	Layer II-III	Layer V-VI
TBS 1	0% (0/13)	61.54% (8/13)	61.54% (8/13)
TBS 2	0% (0/8)	25.00% (2/8)	12.50% (1/8)

Notes: ACC, anterior cingulate cortex; TBS1, theta burst stimulation 1; TBS2, theta burst stimulation 2; LTP, long-term potentiation. Number in parenthesis indicates successful induction of LTP from the number of slices used in the experiments.

4 Discussion

The planar multi-electrode array (pMEA) is a unique and well-established tool for investigating at a macroscopic level the electrophysiological properties of living brain slices containing intact networks of neurons, providing a bridge between single cell testing and behavioral studies[40,42]. Compared to traditional electrophysiology, the advantages of pMEA include: (1) gathering large amounts of otherwise undetectable spatial information on the internal dynamics of networks with multisite recordings[39,41]; (2) long-term analysis of the spatiotemporal distribution of network level electrical activity[38,40,42]; (3) stimulating and recording the electrophysiological activity of many sites within a slice[39,40]; (4) steady recordings that are less sensitive to factors such as mechanical vibrations[39,41]. Altogether, the pMEA technology represents a valuable tool for stably recording electro-

physiological data from multiple sites over extended periods of time from a variety of biological preparations[35,36,42]. Therefore, in the present study, we chose this pMEA recording technique to evaluate changes in LTP induction rate and magnitude in the ACC slices by 2 different stimulation protocols.

The MED64 produced by Panasonic has 64 electrodes in total, with the covered area of the array being about 4.4 mm^2, which is wide enough to cover the whole ACC region from layer I to layer V-VI (Fig. 2). One of the most remarkable characteristics of using the MED64 to record electrical activity in ACC slices is that the neuronal activity of every layer of ACC cells could be simultaneously obtained when one layer is stimulated. As delineated in Fig. 2, three types of field potentials could be instantaneously recorded across different layers of the ACC slices when stimulating the layer V-VI. Previous anatomical studies have revealed that the ACC area has different projecting fibers and cell types within different layers[3-6]. Additionally, the inter-layer connections have also been well investigated[32,33]. The defined relationship between these layer distinction and linkage in anatomy and 3 types of field potentials shown in the present study remains to be further elucidated. Interestingly, after TBS application onto the ACC slices, there was no dramatic difference in the LTP induction rate and magnitude of LTP between layer II-III and layer V-VI (Table 1, Fig. 3), implicating that the 2 layers of cells may receive the same source of inputs and thus function synchronously during synaptic plasticity. This unique advantage of MED64 recording in revealing fruitful spatial information is also embodied in other brain areas, such as the somatosensory cortex and the hippocampal formation (unpublished data).

In spite of huge numbers of previous studies on the LTP phenomenon in various brain structures, such as the hippocampus, ACC, and amygdala, one critical but largely overlooked point is that different LTP induction protocols might exert significant influences not only on the induction probability of LTP, but also on the duration and magnitude of established LTP[2,24,27,30,31,47]. In fact, an induction protocol-dependent difference has been reported in the involvement of NR2B-containing N-methyl-D-aspartic acid receptors in the hippocampal CA1 LTP[49]. When it comes to the LTP in the ACC, Zhuo and his colleagues have used the CS protocol of TBS2 in this study to successfully induce LTP in the field potential recordings on mouse ACC slices[23,25,50,51]. However, the data presented here showed that TBS1 elicited cingulate LTP more frequently and robustly than the TBS2 in layer II-III or layer V-VI except in layer I in rat ACC slices (Table 1, Fig. 3). A possible reason for this apparent discrepancy is the experimental animal species (mouse *vs* rat). Alternatively, these comparison data indicate that the number of bursts or the duration of train in TBS may indeed account greatly for the resultant LTP, in terms of either induction rate or the magnitude and duration. Therefore, it seems likely that long train of TBS (10 bursts) may be more appropriate to facilitate the LTP induction in the ACC area.

In summary, these observations strongly reinforce the assertion that different stimulation protocols may have profound effects on the LTP induction rate and magnitude, thus affecting final interpretation of the electrophysiological data regarding the LTP phenomenon in brain slices. Since LTP is believed to be the mostly accepted synaptic plasticity underlying information processing and storage, and even pathological pain hypersensitivity[52,53], these data emphasize the necessity of considering protocol-related variations when designing such experiments in future.

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长串与短串θ节律刺激在大鼠前扣带回皮质脑片诱导长时程增强效应的不同作用：平面微电极阵列记录技术

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摘要: 目的 大量研究显示前扣带回(anterior cingulate cortex, ACC)是一个多功能的边缘系统结构，参与诸如知觉，运动，情绪和认知等多种高级脑功能。有证据显示长时程增强现象(long-term potentiation, LTP)是研究ACC内发生神经元突触可塑性变化的重要模型。然而，迄今为止，关于ACC神经网络的时空特性仍少为人知。本研究旨在应用平面微电极阵列记录技术观察不同长度θ节律串刺激对诱发大鼠ACC区域不同层结构的LTP的影响。**方法** 采用平面微电极阵列记录技术，在急性分离的大鼠ACC脑片上进行记录。通过实验电刺激V-VI层，记录ACC各层场电位。然后应用两种θ节律串刺激(theta burst stimulation, TBS)作为条件刺激诱发ACC脑区LTP，并比较不同层的LTP诱出效果。两种刺激模式参数为：TBS1，100 Hz，4个双向方波脉冲为一串，重复10次，间隔200 ms；TBS 2，100 Hz，4个双向方波脉冲为一串，重复5次，间隔200 ms。实验刺激前扣带回深层可以分别在I层，II-III层及V-VI层同时诱出三种波形不同的场电位：I层为正向波，V-VI层为负向波，II-III层以复杂的波形为主。**结果** 在实验刺激位点给予两种不同的条件刺激后，长串TBS1条件刺激的LTP诱出率显著地高于短串TBS2条件刺激，即TBS1的LTP诱出率超过60%，而TBS2的LTP诱出率少于25%。此外，层间分析结果显示LTP主要发生在II-III层及V-VI层，I层不能诱出LTP。进一步分析表明，无论是LTP诱出率还是幅度在II-III层及V-VI层之间均无显著差别。**结论** 本实验结果提示同一刺激模式但重复刺激时间长短不同也可能导致LTP诱出率不同，这可能是为什么以往报导关于前扣带回LTP诱出率结果不同的一个关键原因之一，因此在设计此类实验时选择最佳刺激参数更为重要。另外，本实验证明平面微电极阵列记录技术在研究前扣带回皮质突触可塑性的网络特征，尤其是比较不同层结构中的时空特性具有单电极无法比拟的优势。

关键词：长时程增强；前扣带回皮质；θ节律刺激；平面微电极阵列记录技术；大鼠