·论著·

Nociceptive responses of anterior cingulate cortical ensembles in behaving rats

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KEY WORDS Emotion; Cingulate cortex; Neurons; Pain

SUMMARY **Objective:** To confirm the role of anterior cingulated cortices (ACC) in the coding of pain affect by exploring the neural ensemble coding pattern within the anterior cingulate cortex in behaving rats with a multichannel recording technique. **Methods:** In five adult male Sprague-Dawley rats, two arrays of eight stainless steel microwires were bilaterally implanted into ACC. Noxious radiant heat stimulation was applied to the tail, bilateral fore-paws and hind-paws of freely moving rats. Neuroelectric signals were obtained from the microwires and sent to a multichannel recording device via cables and connectors. The time stamps of neuronal activities were stored on a personal computer for off-line analysis. **Results:** Noxious heat stimuli evoked predominantly excitatory and sustained neural activity within ACC, reflecting the processing of pain unpleasantness; pain-related anticipatory responses could be seen near the stimulation start, indicating the behavioral preparation for escape; ACC neurons had broad receptive fields by showing quite similar pain-related responses to stimuli on either side of the hind-paw, suggesting that they are not eligible for the localization of a stimulus. **Conclusion:** ACC has played a major role in processing the affective-motivational aspect of pain.

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清醒大鼠前扣带皮层神经元群的伤害性反应

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[关键词] 情绪; 扣带皮层; 神经元; 疼痛

[摘 要] 目的: 利用多通道记录技术在清醒大鼠前扣带回(ACC)记录神经元群的痛反应模式, 以证实 ACC 在痛觉情绪编码中的作用。方法: 在 5 只成年雄性 SD 大鼠的双侧 ACC 脑区植入微电极阵列, 在大鼠术后清醒状态下给予尾部及四肢足底伤害性辐射热刺激, 神经元的放电信号经由电缆和连接器传送至多通道记录系统。结果: 伤害性辐射热刺激在大鼠 ACC 引起以兴奋为主的反应, 该反应持续时间较长, 可能与痛情绪有关; 刺激开始附近 ACC 神经元有与痛刺激相关的预期性反应, 可能与准备逃避的动机有关; 同侧和对侧肢体刺激引起的 ACC 神经元的反应差别不显著, 表明神经元感受野大, 不适合精确定位。结论: ACC 主要参与编码痛觉的情绪动机成分。

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Converging lines of evidence support the role of the anterior cingulated cortex (ACC) in encoding the motivational-affective aspect of pain[1-3]. It is well accepted that ACC receives nociceptive input mainly from the medial thalamic nuclei[4-5]. Previous neurosurgical evidence showed that cingulumotomy can be effective in relieving fear and anxiety of patients with intractable pain, in which marked emotional factors appeared to contribute to the intolerable situation[6]. Current functional imaging studies provide direct support for the hypothesis that ACC may be preferentially involved in the motivational-affective aspect of pain perception. Using hypnotic suggestion, Rainville and colleagues modulated pain unpleasantness without affecting pain intensity, revealing that pain unpleasantness was specifically associated with ACC but not the somatosensory cortex[7]. Data from electrophysiological recordings in rabbits, rats and humans are consistent with findings from brain imaging results[4,8,9]. Nociceptive neurons within ACC have large receptive fields and no somatotopic organization, suggesting that ACC is not suitable for processing the location information of noxious stimulus.

However, brain imaging study puts major emphasis on the spatial activation pattern across the cortical areas, and single-neuron recordings capture limited

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information on the electrophysiological properties of individual ACC neurons. In the present study, we used a many-neuron microwire array recording technique to explore the pain-related ensemble coding pattern within ACC, with an aim to further confirm the involvement of ACC in subserving the motivational-affective aspect of pain.

1 Materials and Methods

1.1 Animals and surgery

Five adult male Sprague-Dawley rats (300 - 350 g) were used in this study. Animals were housed under 12-h dark-light cycle for at least one week before surgery, with food and water available *ad libitum*. Animals were initially anesthetized with intraperitoneal injections of ketamine (100 mg/kg), and transferred to a Kopf stereotaxic apparatus. After removal of the skin and soft tissue, small craniotomies were made over the anterior cingulate cortex. Coordinates for the craniotomies were according to the atlas of Paxinos and Watson[10] as follows: 3.2 mm anterior to bregma, 0.8 mm lateral to midline, and 2.8 ventral to the skull surface. Two Arrays of eight stainless steel Teflon-insulated microwires (50-μm diameter, Biographics Inc. Winston Salem, NC) were bilaterally lowered into the target areas. The microelectrode arrays were secured onto the cranium with dental cement using skull screws as anchors. Animals were administered penicillin (60 000 U, i.m.) before surgery to prevent infection, and housed individually after surgery.

1.2 Experimental procedure

Experiments started 7 days after surgery. Animals were placed in a plastic chamber (44 cm ×44 cm ×44 cm) and allowed to move freely during the entire recording session. Lightweight cables connected the detachable headset to a rotating commutator on the ceiling of the chamber to allow for the animal's free movements. Noxious radiant heat from a 12.5-W projector bulb was used as painful stimulation, which was applied to the rats' tails, bilateral fore-paws and hind-paws through a glass floor (1 mm thick). The stimulation was stopped by manually turned off the radiant heat when escape responses happened. A time stamp series (resolution, 1 ms) marking stimulus starts (and ends) was recorded and synchronized with the neural spike recordings. The interstimulus intervals were no less than 20 s. Sham stimulations were randomly inserted among real painful stimulations (i.e., illuminated the lamp without focusing on the body parts). The neural responses around the sham stimulations were recorded as controls.

1.3 Electrophysiological recording

Neuroelectric signals were obtained from the stainless steel microwires, and passed from the headset assemblies to a preamplifier via two light-weight cables and a commutator. The signals were then filtered (0.5 and 5 kHz, 6 dB cutoff) before being sent to a multichannel spike-sorting device. Spike activities were monitored on a computer with a time resolution of 20 microseconds, picked up by setting proper parameters for amplitude and duration, and recorded into a database file with a PC-based software Magnet (Biographics, Inc., Winston-Salem, NC). Data were then analyzed with a commercially available PC-based program (STRANGER, Biographics, Inc., Winston-Salem, NC). The identity of clearly sorted single neurons was verified by graphical capture of waveform. The time stamps of these waveforms were then stored on a personal computer for off-line analysis.

1.4 Data analysis

Bin counts for each trial (0.1 s bin size) were calculated using a commercially available analysis program NeuroExplorer (Plexon, Inc., Dallas, TX) and the results were exported to Matlab in a spreadsheet form. Neural responses to painful stimulation were evaluated using a sliding-window averaging technique[11], in which a 1-s time window was slid through the entire period of a trial (10 s before to 10 s after stimulation events) at 0.1-s step. The bin counts of each trial within each window were compared with those of a preset 3-s control window (baseline firing level) 10 s before the stimulation event by Student's t-test. The differences were considered significant when it reached a P level no larger than 0.005 in three consecutive steps, thus to achieve a global significance of $P < 0.05$. We used '*Surprise*' (negative natural logarithm of the probability), an information theory concept, to illustrate the averaged significance of changes in single neuronal responses over time[12] relative to a control period. The 'P values' produced by the aforementioned sliding-window method were converted into surprise to highlight the significance of the responses distributed over a time period. In addition, a clustering technique (K-means, SPSS, Inc.) was utilized to sort single neurons depending on the similarities in response patterns of excitation or inhibition around stimulation events. A principal components analysis (PCA), an effective tool for classifying neural ensemble responses, was used to map the nociceptive information distributed across a group of neurons within ACC[13].

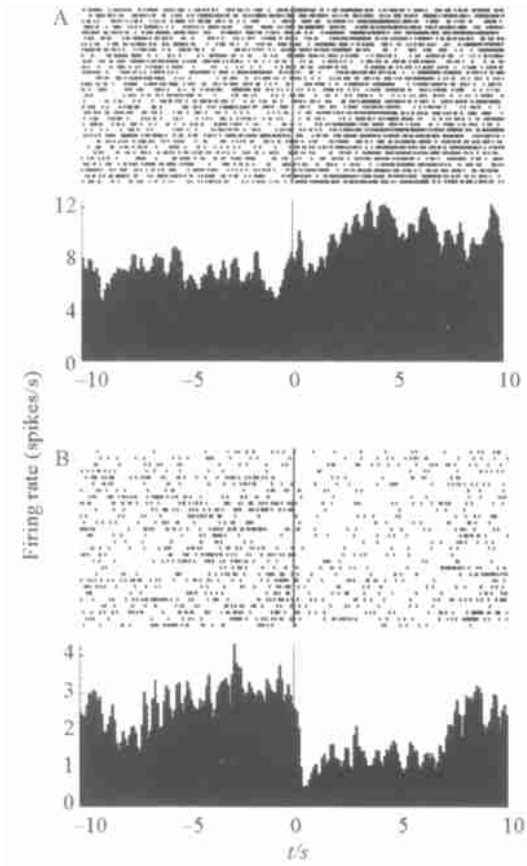
1.5 Histology

At the end of the experiment, rats received an overdose of ketamine. Recording sites were marked by iron electrophoretic deposit (10 - 20 μA DC current, 10 - 20 s duration, anode at the electrode) at the tip of selected wires. Animals were then perfused with 4% (mass fraction) paraformaldehyde. The brains were post-fixed in a solution of 5% (mass fraction) potassium ferricyanide/4% paraformaldehyde for several days. Coronal sections (40 μm) were cut through the cingulate cortex. Recording sites were determined under a light microscope. The iron deposits were easily identified as blue dots.

2 Results

A total of 73 neurons were isolated in rat ACC during pain stimulation. Of these neurons, 70%, 32%, and 18% produced significant spike activities with the application of painful stimuli to hind-paws,

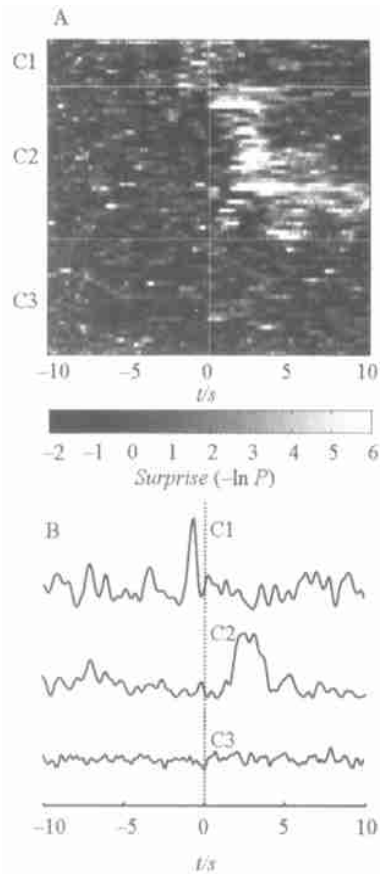
fore-paws, and tails, respectively. Units that increased their activities after painful stimuli were termed as excitatory responses; those that decreased their activities were considered inhibitory. It should be noted that excitatory nociceptive responses were predominant and inhibitory responses were seldom encountered. Fig. 1 depicted the typical excitatory and inhibitory responses of ACC neurons. Sham stimulations never induced any significant changes except for some anticipatory responses around the start of stimulation.



Time = 0, corresponds to the time of stimulus onset.

Figure 1 Typical excitatory (A) and inhibitory (B) response of ACC neurons evoked by noxious radiant heat

Our result indicated that ACC neurons had distinct temporal coding pattern of noxious stimuli. It can be seen in Fig. 2A that the cluster plot classified the ACC neurons into three categories according to their responses to noxious heat: (1) those with early or anticipatory responses (C1), i.e., generated responses within the first 1 - 2 s after or even prior to painful stimulus application, accounted for about one sixth of total neurons, (2) those with late responses (C2), i.e., produced stronger activities around 2 - 4 s following stimulus application and lasted for at least 2 s, accounted for nearly a half, (3) those with no significant responses (C3), accounted for the remaining one third. The above results were supported by principal components analysis (PCA) in Fig. 2B, where it provided a linear mapping of neural ensemble activities. This method searched for significant trends occurring across the entire neuronal population. As shown in Fig. 2B, three major components were most common in the pain-evoked responses, with one showing early responses (top line), and the other two demonstrated later ones (middle and bottom lines).



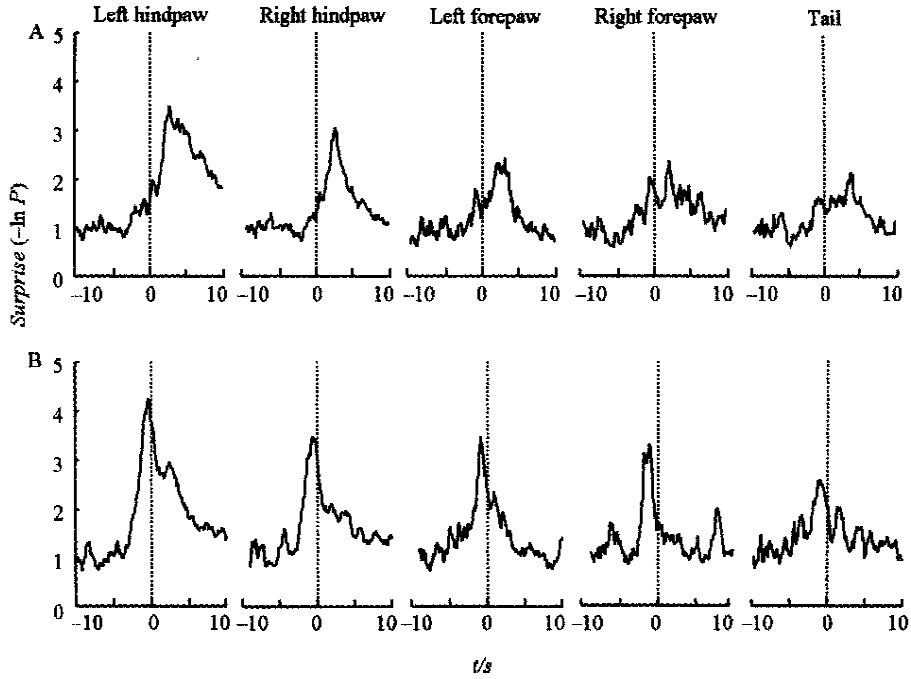
Each neuron is displayed as a row in the gray scale image (white for the highly significant and black for non-significant). C1 - C3 indicate the three clusters of recorded ACC neurons; B, Principal components analysis (PCA) drawn from neural ensemble activities.

Figure 2 Neuronal activities rearranged in clusters

The important features of ACC are also depicted in Fig. 3. With the application of painful stimuli to different extremities, we can see clear overall pain-related anticipatory responses around or even prior to the stimulation onset (Fig. 3A), which may reflect the preparative motivational response to pain stimuli. For all the stimulated parts, ACC neurons produced the strongest responses 0.5 - 1.0 s before avoidance movement (Fig. 3B). However, spontaneous paw movements never elicited any significant response in our recorded ACC neurons (data not shown). This indicated that the strongest response might represent the processing of the nociceptive information related to pain rather than motor response.

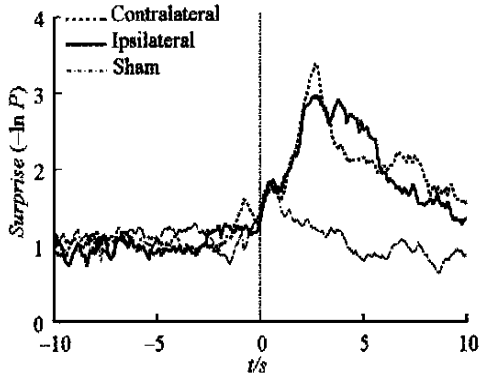
In addition, we examined the cutaneous receptive fields of ACC neurons. The noxious heat stimuli were delivered to five body parts, i.e., bilateral fore- and hind-paws as well as tails. Of the units that produced pain-related responses, more than 90% re-

sponded to nociceptive stimuli applied to more than three parts. As an illustration, Fig. 4 displays the average responses of unilateral ACC neurons to painful stimuli applied to ipsi- or contralateral hind-paws. As we can see, ACC neurons exhibited quite similar pain-related responses to stimuli on either side of the hind-paws without obvious contralateral bias.



A, Painful stimuli induced universal anticipatory responses near the stimulation start (time = 0) with the noxious stimulation on all of the parts; B, The strongest neuronal activity occurred 0.5 - 1.0 s before the avoidance behavior (time = 0).

Figure 3 Surprise plots characterizing the pain responses of ACC neurons with the application of pain stimuli to left hind-paw, right hind-paw, left fore-paw, right fore-paw and tail, respectively



Neurons on either side of ACC show bilateral pain-related responses without contralateral bias.

Figure 4 Average responses of unilateral ACC neurons to application of real painful stimuli to ipsi- or contralateral hind-paw or sham stimuli

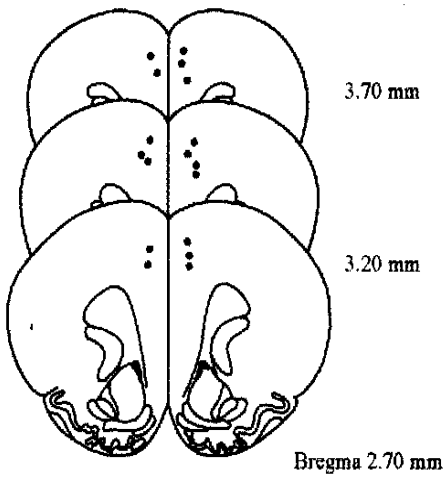
The histological results in Fig. 5 indicated the location of microwires In the cingulate cortex, most of the iron deposits were found in the anterior areas.

3 Discussion

In the present study, by using a multichannel recording technique, we simultaneously recorded multiple single neurons within ACC in behaving rats, and revealed the specific temporal activation of ACC neuron ensembles. Our results demonstrated that: (1) nearly half of ACC neurons produced pain-evoked responses characteristic of long duration, (2) about one sixth presented anticipatory pain responses, and (3) the nociceptive neurons within ACC had large and bilateral receptive fields. These results strongly suggested the participation of ACC in encoding the motivational-affective aspects of pain.

Previous study has demonstrated ACC's involvement in pain processing in the following aspects: (1) coding the affective dimension of noxious stimuli, (2) motor response selection for noxious stimuli, and (3) learning associated with the prediction and avoidance of noxious stimuli[14]. Data about ACC's involvement in the motivational-affective dimension of pain come mostly from brain imaging and neurosurgical studies[7, 15, 16]. Few studies provided direct evidence regarding the affective component of pain in rodents until Johansen *et al*. proposed the use of a formalin-induced conditioned place avoidance pain model[17]. Our findings showed that ACC neurons displayed sustained pain responses of both excitatory and inhibitory kinds. These responses peaked at 0.5 - 1.0 s before avoidance movement and maintained several seconds after the end of nociceptive stimulation. In contrast, results from Ploner *et al*[18] and our own study (to be published elsewhere) showed that the primary somatosensory cortex (SI) neurons had intensive responses with short duration, a property corresponding to the encoding of sensory-discriminative dimension of pain. Compared with the results of SI, the long-lasting response of ACC may reflect the animal's persistent aversive sensation when undergoing noxious stimuli. Another possible explanation of the observed ACC responses is that they may represent motor pro-

cessing. But analysis of spontaneous paw movement did not reveal any significant changes in neural activities. Thus, these later ACC neural activities (C2 in Fig. 2A) should primarily reflect the aversive responses, though they may also implicate the preparation of pain-evoked escape behavior.



The black dots labeled the position of iron deposits at the tips of selected microwires.

Figure 5 A schematic drawing indicating the location of recording sites in ACC

A close association between ACC and the prediction of pain has been indicated by a number of neuroimaging and electrophysiological studies[19,20]. Our results revealed the anticipatory response of ACC during the expectation of pain. As shown in Fig. 2A, there are quite a few neurons that increased their activity at the start of thermal stimulation or even preceded the stimulation (C1). Logically, these early responses were anticipatory but not nociceptive, because a certain time period was needed before the radiant heat became noxious and the nociceptive signal was transmitted to the sensory thalamus and cortical areas. One reason why rats could predict random thermal stimulation is that the radiant heat stimulator we used shed some light into the behavior box when it was turned on. The other is that the manipulation of the stimulator arm (i. e., to move the arm toward where the rat located) may be detected by the animal. These may serve as cues of noxious stimuli after repeated application. The anticipatory nature of these early responses was supported by the fact that sham stimulation also evoked similar early responses (Fig. 4).

Evidence for the involvement of ACC in pain affect was provided by the large receptive fields of its neurons. Single neuron recordings in the ACC of rats and rabbits revealed nociceptive neurons that were not organized somatotopically and had large receptive fields[4,9]. According to our results, more than 90 % of responding neurons had broad receptive fields that included no less than three of the five stimulated body parts. On the other hand, a bilateral activation without contralateral bias was observed in response to unilateral painful stimuli on either side of the limbs. These results indicated that ACC neurons were not primarily involved in processing stimulus location. In conclusion, the present study showed that ACC neurons can be activated by noxious heat stimuli by using a multichannel recording technique. The pain-evoked responses were distributed over a wide time range, reflecting the signaling of pain unpleasantness. With additional findings of the anticipatory response and broad receptive fields of its neurons, we provided further evidence demonstrating the ACC's involvement in the motivational-affective dimension of pain.

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