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# Dynamic neuronal responses in cortical and thalamic areas during different phases of formalin test in rats

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**Abstract**

Although formalin-induced activity in primary afferent fibers and spinal dorsal horn is well described, the forebrain neural basis underlying each phase of behavior in formalin test has not yet been clarified. The present study was designed to investigate the cortical and thalamic neuronal responses and interactions among forebrain areas during different phases after subcutaneous injection of formalin. Formalin-induced neuronal activities were simultaneously recorded from primary somatosensory cortex (SI), anterior cingulate cortex (ACC) and medial dorsal (MD) and ventral posterior (VP) thalamus during different phases (i.e., first phase, interphase, second phase and third recovery phase starting from 70 min after injection) of formalin test, using a multi-channel, single-unit recording technique. Our results showed that, (i) unlike the responses in primary afferent fibers and spinal dorsal horn, many forebrain neurons displayed monophasic excitatory responses in the first hour after formalin injection, except a small portion of neurons which exhibited biphasic responses; (ii) the response patterns of many cortical and thalamic neurons changed from excitatory to inhibitory at the end of the second phase; (iii) the direction of information flow also changed dramatically, i.e., from cortex to thalamus and from the medial to the lateral pathway in the first hour, but reversed in phase 3. These results indicate that the changes of activity pattern in forebrain networks may underlie the emerging and subsiding of central sensitization-induced pain behavior in the second phase of formalin test.


*Keywords:* Formalin; Primary somatosensory cortex; Anterior cingulate cortex; Thalamus; Nociception

## Introduction

The formalin test was introduced by Dubuisson and Dennis (1977) as a means of providing prolonged noxious stimuli. Biphasic nociceptive responses elicited by formalin injection have been observed intensively in rodent, i.e., rats and mice. In contrast, monophasic behavioral responses can be found in cats (Dubuisson and Dennis, 1977). An early brief phase (phase 1) starts immediately after the formalin injection and lasts for about 5 min followed by a period of little or no pain for 10–15 min (interphase). A later tonic phase (phase 2) is observed at 20–60 min. The nociceptive response decreases again about 60 min after s.c. formalin injection, called the third recovery phase (Porro et al., 2003).

Although the mechanisms underlying phase 1 and 2 have been widely investigated, changes leading toward the recovery phase have been largely overlooked. It has been hypothesized that the first phase is a response to the direct stimulation of nociceptors (Dubuisson and Dennis, 1977; McCall et al., 1996), while the second phase is either related to the sensitization of central nociceptive neurons induced by the initial afferent input during the first phase (Dickenson and Sullivan, 1987b; Vaccarino et al., 1993; Yashpal et al., 1996, 1998) or the development of inflammation (Abbadie et al., 1997; Fu et al., 2000; Hunskaar and Hole, 1987; Hunskaar et al., 1986; Pitcher and Henry, 2002; Shibata et al., 1989). Although the peak pain-related behaviors occur between 20 and 35 min post-injection (Dubuisson and Dennis, 1977; Sevostianova et al., 2003; Wheeler-Aceto et al., 1990), formalin-induced edema does not reach its peak until the 4th

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or the 5th hour (Brown et al., 1968; Wheeler-Aceto et al., 1990). Thus, the temporal discrepancy between formalin-induced behaviors and inflammation is intriguing. Porro et al. (2003) found that in the third recovery phase the formalin-induced activation declined in most forebrain structures but metabolic rates were increased in regions of endogenous antinociceptive systems. However, mechanisms underlying the rapid decreases of pain-related behavior at the end of phase 2 in formalin test remain unclear.

Nevertheless, neural activities from various levels of the nervous system differ in response to subcutaneous injection of formalin. Formalin injection produces a biphasic activation of Aδ- and C-fibers (McCall et al., 1996; Puig and Sorkin, 1996) as well as WDR neurons in the spinal cord (Chapman and Dickenson, 1995; Chapman et al., 1994; Diaz and Dickenson, 1997; Dickenson and Sullivan, 1987a,b; Haley et al., 1990; Henry et al., 1999; Pitcher and Henry, 2002) that parallels temporally the dual nature of the behavioral response. On the other hand, the spontaneous discharges of neurons in several brainstem structures increase only for the first few minutes after formalin injection and no late phase has been observed (Pertovaara and Tukeva, 1989; Shima et al., 1987). Trigeminal brain stem nociceptive neurons respond to formalin stimulation either with an early and short-lasting phase or with two phases separated by a short period of quiescence (Raboisson et al., 1995). These results suggest that nociceptive neurons in different regions of the nervous system have certain heterogeneity in response to formalin injection. However, changes in the neuronal discharges in the telencephalon and diencephalon have not been studied. Little is known about the detailed role of cortical–thalamic network in discriminating the time profile of behavior during the exposure to formalin injection.

The purpose of the current study is to investigate the ensemble neural activities within the forebrain network underlying formalin-evoked phasic nociceptive behaviors. Our working hypothesis is that the cortical–thalamic network might be an important part of the central mechanism that underlies the emerging and submerging of the phase 2 nociceptive hypersensitivity, which is essential to form the typical three-phase behavior observed in formalin test.

## Materials and methods

### *Animals*

Eight adult male Sprague–Dawley rats weighing 300–350 g were used in this experiment. Animals were housed individually and maintained under a reversed dark–light cycle (lights on at 7:30 pm) with a room temperature of 22 ± 1°C. Food and water were available ad libitum. Animals were handled daily for at least a week before electrode implantation surgery. The experiments were approved by the Institutional Animal Care and Use Committee of Peking University. All experiments were conducted in accordance with the guidelines for pain investigation in conscious animals (Zimmermann, 1983).

### *Surgery*

Following anesthesia with ketamine (100 mg/kg, i.p.), animals were transferred to a Kopf stereotaxic apparatus. A sufficient level of anesthesia was maintained throughout the surgery such that animals had no spontaneous movements and were not responsive to noxious stimuli, the blinking reflex was absent and breathing was slow and regular. Supplementary doses (one-third of the original) of ketamine were given when necessary. Four small craniotomies were made for microelectrode array implantation. According to the atlas of Paxinos and Waston (1998), stereotaxic coordinates were: (1) for primary somatosensory cortex (SI), 1.0 mm posterior to bregma (−1.0 A), 2.0 mm lateral to midline (L) and 2.0 mm ventral to the skull surface (V); (2) for anterior cingulate cortex (ACC), 3.2 A, 0.8 L and 2.8 V; (3) for medial dorsal thalamus (MD), −2.3 A, 0.8 L and 5.5 V; (4) for ventral posterior thalamus (VP), −3.0 A, 3.0 L and 6.0 V. Arrays of eight stainless steel Teflon-insulated microwires (50-μm diameter, Biographics, Winston-Salem, NC) were slowly lowered into the target areas. The microelectrode arrays were secured onto the cranium with stainless steel skull screws and dental cement. Animals were administered penicillin (16,000 U, i.m.) before surgery to prevent infection. Rats were allowed 1 week to recover from this surgical procedure.

### *Experimental procedures*

Testing sessions were carried out in a quiet room, with the room temperature kept at 25 ± 1°C. Each animal received control stimulus followed 140 min later by formalin injection to record continuously the responses of the same neurons to both kinds of stimuli. Rat was first placed in a plastic test chamber (44 × 44 × 44 $cm^3$) for at least 10 min to accommodate to the environment. Spontaneous neuronal firings were recorded for 20 min as the baseline data (see Fig. 1). A 27-gauge needle was used as sham stimulus to prick the plantar surface of the hind paw contralateral to the recording site. After that, rats were immediately placed back to the test chamber for a recording session of 140 min (with the last 20 min as baseline data for formalin session). Since Okuda et al. (2001) suggested that 10% formalin may elicit stronger endogenous antinociceptive responses of various supraspinal structures such as areas in cortex, thalamus and brainstem than 5% formalin, a subcutaneous injection of 10% formalin (50 μl, 1:10 dilution of 37% formaldehyde solution in 0.9% saline) was then delivered into the plantar surface of the same paw. Rats were again returned to the chamber immediately for another 120-min recording session of formalin-induced behaviors and neuronal responses. The whole procedure is shown in Fig. 1. Pain intensity was determined by measuring the time spent in licking the affected paw every 5 min after injection.

### *Data acquisition*

Neuronal activities were detected by the microwires and passed from the headset assemblies to a preamplifier via two

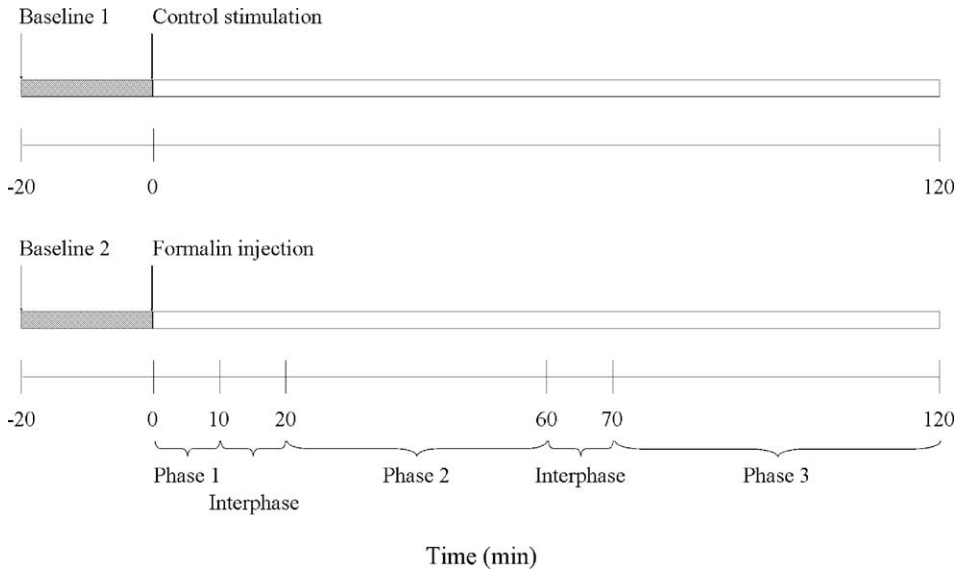


Fig. 1. Experimental procedures. Each animal was exposed to both the sham stimulus and the formalin injection in the same day. The formalin injection was delivered 140 min after sham stimulus. The narrow lines at −20 min show the time points when recording started, while the thick lines at 0 min indicate the time of control stimulation or formalin injection.

light-weight cables and a commutator. Neuroelectric signals were filtered (0.5 and 5 kHz, 6 dB cutoff) and sent to a multi-channel spike-sorting device. The recording was paused at the time of prick or injection. Neuronal spikes were monitored on a computer and picked up by setting proper high and low windows for amplitude and short and long windows for duration with a PC-based software *Magnet* (Biographics, Inc.). The time resolution for data collection was 50 kHz. The time stamps of the spike activities were saved into a database file for off-line analysis. Data were analyzed with commercially available PC-based programs *STRANGER* (Biographics, Inc. USA). The identity of clearly sorted single neurons was verified by graphical capture of waveforms. Inter-spike interval histograms were used to assure that only one neuron was recorded.

### *Data analysis*

The results of the formalin test were sectioned into 3 phases according to the behavioral response. Phase 1 of the formalin test corresponded to the first 10 min after formalin injection, phase 2 corresponded to 20–60 min after injection, and phase 3 was the last 50 min of the observation period. There were 10-min interphase periods between each two successive phases. Licking activity was recorded on a video tape. Frame by frame analysis of behavior was performed off-line. Discharges of each neuron were counted in 60-s bins using the analysis program NeuroExplorer (Plexon, Inc., Dallas, TX) to construct peri-stimulation time histograms (PSTHs), with a time range from 20 min before to 120 min after the stimulation (control or injection of formalin). Firing rates in each bin were transferred into $Z$ scores. In order to detect the neuronal response patterns to formalin injection or control stimuli over time, the whole period of observation was sectioned into 1-min time bins. An increase or decrease of firing rates over two-fold of the standard deviation of the baseline for at least five consecutive bins (i.e., 5 min) was considered as an excitatory or inhibitory response, respectively. A clustering analysis (K-means, SPSS) was performed to classify neuronal responses depending on the similarities in patterns of excitation or inhibition induced by injection of formalin. Neuronal firing rates were normalized to visualize activity patterns in neuronal populations. Numbers of neurons exhibiting excitatory or inhibitory response to the formalin or control stimulation at each time bin were counted.

To identify the interactions between ACC, SI, MD and VP following formalin injection, a statistical technique called partial directed coherence (PDC) was used. PDC is a frequency-domain approach involving the key concept of Granger-causality to uncover the direct influence on certain neuronal group by another (Granger, 1969). The process of PDC analysis has been described in detail elsewhere (Baccalá and Sameshima, 2001; Fanselow et al., 2001; Sameshima and Baccalá, 1999; Yang et al., 2005). To be brief, we computed the first principal component (PC1) of the activities in each brain area to calculate PDC (in 1–50 Hz range) around the stimulation. Results for PDC were normalized according to the 20-min pre-stimulus baseline data.

### *Histology*

After completion of the recording, subjects were overdosed with pentobarbital and 10–20 μA of anodal current was passed for 10–20 s through the recording electrodes to deposit iron ions. The animals were then sacrificed and perfused with 4% paraformaldehyde–5% potassium ferricyanide solution, and their brains were extracted. The brains were post-fixed with the same solution used for the perfusion. Coronal sections of 40 μm thick were cut through the SI, ACC and thalamus. Recording sites were determined under a light

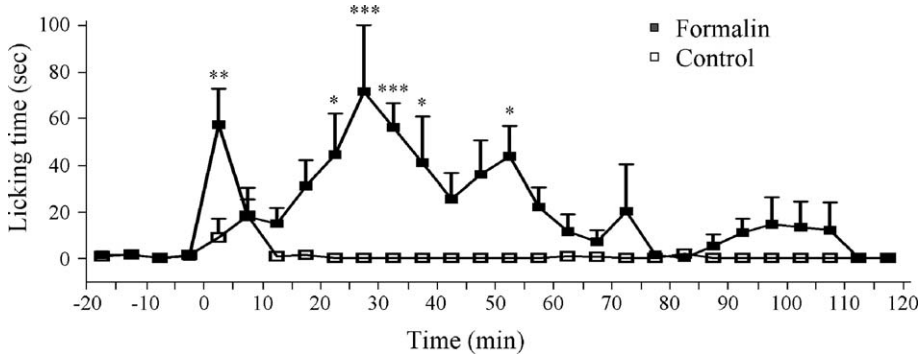


Fig. 2. Time courses of the licking activity observed after either s.c. injection of formalin (50 μl; filled square) into the plantar side of the hind paw or control stimulation (open square) at time zero ($n = 8$). Data are presented as the mean ± SEM per 5 min. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with control group.

microscope. The iron deposits were easily identified as blue dots.

## Results

### *Formalin-induced nociceptive behaviors*

Formalin-elicited licking activity was summarized in Fig. 2. This nociceptive behavior consisted of an early response of 5-min duration (phase 1), a late response of 40 min (phase 2) and absence of response between 5 and 20 min (interphase). Licking activity decreased during phase 3 and presented no significant differences from control.

### *Changes in the number of excitatory and inhibitory neurons over time*

A total of 183 single neurons were recorded in the ACC, MD, SI and VP from eight Sprague–Dawley rats (35 ACC, 45 MD, 57 SI, and 46 VP). In all recorded neurons, there were

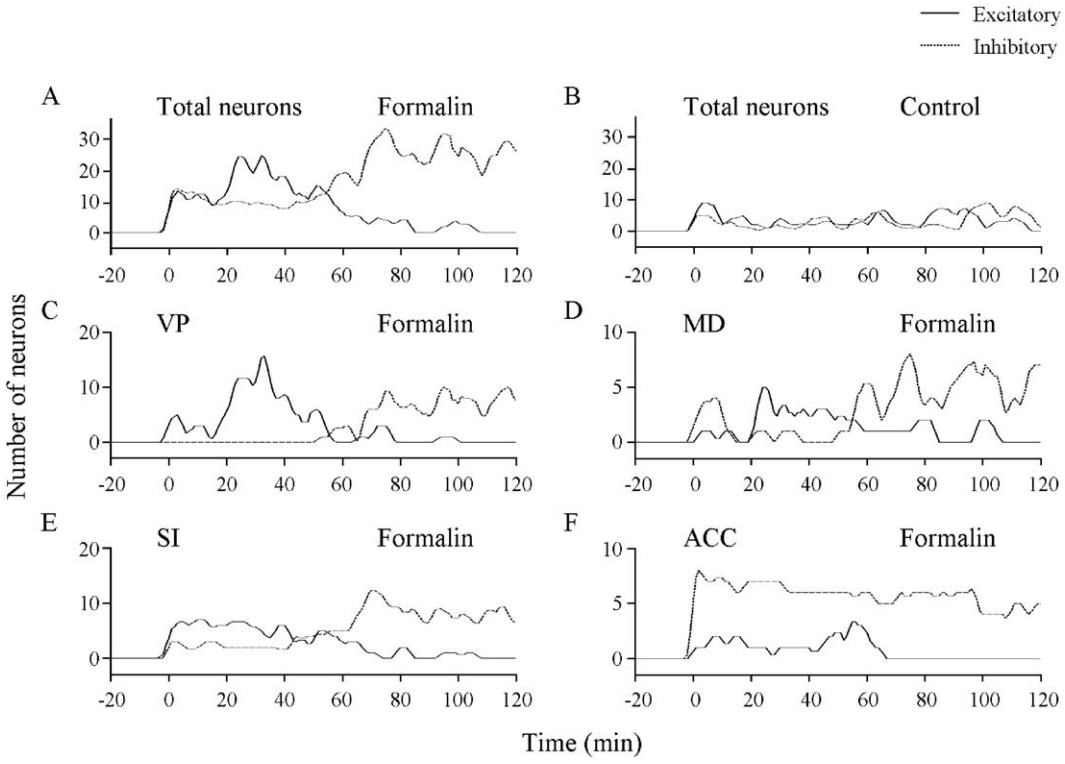


Fig. 3. Numbers of neurons exhibiting excitatory or inhibitory responses to formalin injection over time. (A) Total neurons. After an initial peak within 5 min, the total numbers of excitatory neurons increased further during 20–60 min and then declined. More neurons were inhibited during the last 60 min. (B) There were only a few neurons showing responses to control stimuli. (C) The neurons in VP exhibited exclusively excitatory responses in the first hour and inhibitory responses in the second hour. (D) There were more excitatory neurons in MD over 20–60 min than inhibitory ones. In the last 60 min, the responsive neurons were predominantly inhibitory. (E) There were more excitatory neurons in SI within the first hour than inhibitory and less within the second hour. (F) The excitatory neurons in ACC presented only during the initial hour while the inhibitory neurons presented over the entire period of observation. Solid lines denote the number of excitatory neurons, and dashed lines indicate inhibitory neurons.

more excitatory responses during the initial hour after formalin injection but more inhibitory ones in the second hour, as shown in Fig. 3A. The amount of excitatory neurons in VP and MD further increased 20 min after formalin injection (Figs. 3C, D). In SI and ACC, the excitatory responses appeared only during the first hour, but inhibitory ones existed throughout the period of observation (Figs. 3E, F). The number of inhibitory neurons in SI further increased 60 min later. In contrast, the inhibitory

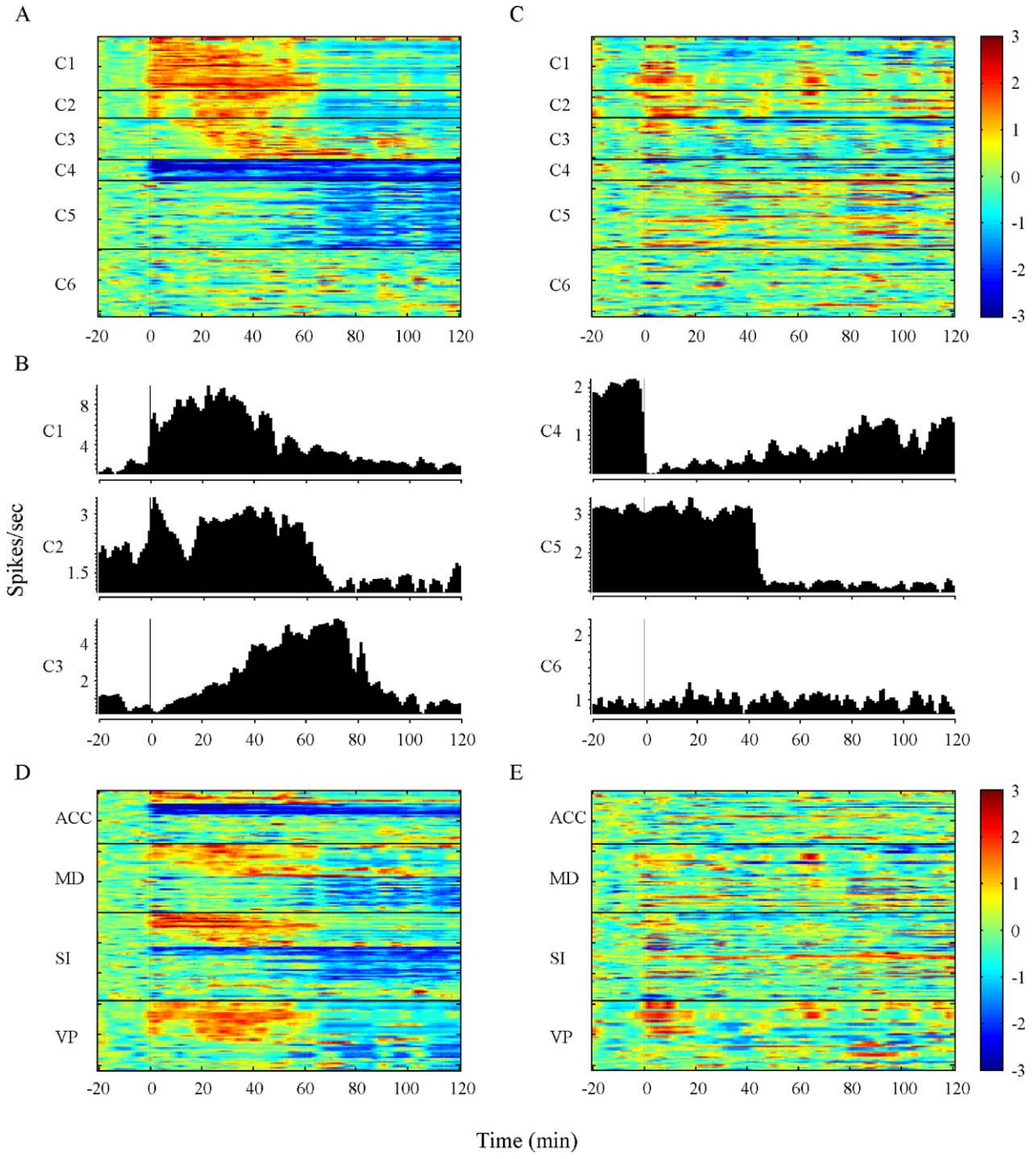


Fig. 4. Temporal distribution patterns of neural activity to formalin injection. Cluster plots depicted the neural response patterns to formalin over 2 h. Each horizontal line represents change of normalized firing rates of one neuron. Cold color (blue) represents decrease, while warm color (red) represents increase in firing rates. (A) Six types of firing patterns. Neurons in cluster 1 exhibited tonic, long-lasting excitatory response which lasted 20 to 60 min. Neurons in cluster 2 displayed biphasic response during the first 60 min similar to the nociceptive behavior in terms of time course. Cluster 3 neurons were characterized by delayed excitatory responses appearing 10 to 30 min after injection. Cluster 4 consists of neurons inhibited dramatically and persistently throughout the entire period of observation. The firing rates of neurons in cluster 5 started to decrease around 50 min after injection and continued for the remaining period of observation. Cluster 6 neurons failed to show any evident responses. (B) Examples of rate histograms for each cluster. (C) Response patterns of each cluster induced by control stimulation. Neurons were arranged according to the order in A. (D) Temporal coding patterns arranged by brain regions. Predominant neuronal response patterns in ACC, MD, SI and VP were excitatory, characteristic of monophasic in the first hour and inhibitory in the second hour. Neurons with delayed inhibitory response situated mainly in SI and MD. Neurons showing biphasic response were mainly located in VP and MD. (E) Temporal response patterns of different regions to control stimulation.

response in VP and MD occurred exclusively during the second hour (Figs. 3C, D). Only a few neurons showed responses to control stimulation (Fig. 3B).

*Temporal coding patterns of single neurons*

A cluster analysis revealed the neural response patterns of the cortex and thalamus to formalin injection. As shown in Fig. 4A, about 44% of neurons showed excitatory responses, while only around 7.7% exhibited inhibitory responses in the initial hour. However, the response patterns changed at the end of the second phase, where the predominant responses became inhibitory. Unlike biphasic activities recorded in spinal cord neurons, only about 10% neurons displayed biphasic response (in cluster 2) in the first hour after injection while 19% exhibited monophasic excitatory responses which lasted for 20–60 min (in cluster 1). Neurons in cluster 3 showed delayed excitatory responses starting 10 to 30 min after injection, part of which outlasted the cessation of the nociceptive behavior. Twenty-five percent of neurons started to display inhibitory responses at the beginning of phase 3 (in cluster 5). Examples of time histograms for each cluster are illustrated in Fig. 4B. However, after the control stimulation, only 25% of neurons showed transient activation (in clusters 1, 2 and 3, Fig. 4C).

Sorting neurons in each brain area according to the clusters revealed that most excitatory responses ceased in phase 3. Neurons with delayed excitatory responses were mainly situated in VP and MD. On the other hand, cluster 5 neurons which showed inhibitory responses only in the second hour were located in SI and MD. In addition, 17% of neurons in VP and 9% in MD showed biphasic responses corresponding to behaviors during the initial 60 min. Typical biphasic neuronal activities in VP are illustrated in Fig. 4B (C2). Neurons with persistent and strong inhibition were mainly seen in SI and ACC (43% of them were in SI and 50% in ACC). For control stimulation, sparsely distributed responding neurons were mainly found in VP (Fig. 4E).

*Change of information flow after formalin injection*

The changes of information flow among four recorded regions were determined by PDC analysis. Fig. 5A shows an example of normalized PDC between SI and VP observed after formalin injection. The mean values of coherence across 1–50 Hz were calculated at each time point (Fig. 5B). The amount of information flow from SI to VP increased in the first hour then returned to baseline during phase 3, whereas that from VP to SI did not show any evident changes until the beginning of phase 3 where it increased significantly.

To elucidate the general information flow between cortex and thalamus (i.e., ascending or descending) as well as between the lateral and the medial pain pathway (i.e., from lateral to medial or from medial to lateral), we merged the data that belong to the same category (e.g., we pooled data of VP → SI, VP → ACC, MD → ACC and MD → SI together as ascending information). The time courses of average PDC changes in each direction were illustrated in Fig. 6. The areas under the average PDC curves during each phase were compared between opposite directions. The direction of information flow reversed at the end of phase 2 either between cortex and thalamus or between the lateral and medial pathway. Within phase 2, the amount of descending information was significantly larger than ascending direction; however, this direction predominance reversed in phase 3 (Fig. 6A). Similarly, the amount of PDC from the medial to the lateral pathway was significantly larger than that in the opposite direction in the first hour, but the direction of information flow also reversed during phase 3 (Fig. 6B). No significant differences were found between any opposite directions after control stimuli except during the first 20 min where there was larger amount of descending PDC (Figs. 6C, D).

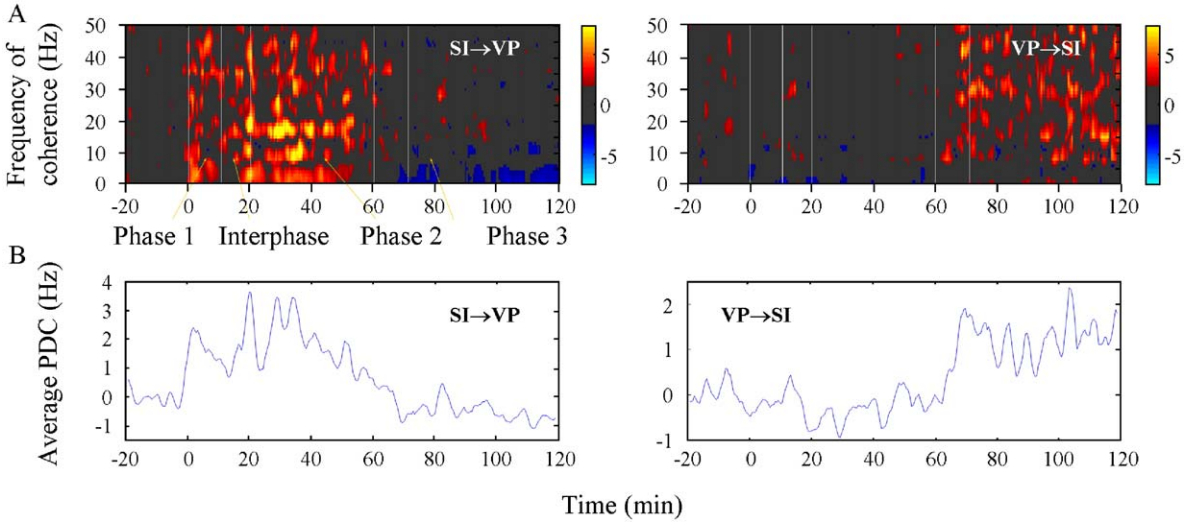


Fig. 5. An example of the amount of partial directed coherence observed between SI and VP after formalin. (A) Normalized PDC from SI to VP (left) and from VP to SI (right) during different phases of formalin test. (B) Mean coherence across 1–50 Hz at each time point. The amount of PDC from SI to VP increased during the first hour then dropped to baseline during phase 3, whereas that from VP to SI failed to show any evident changes during the first hour and increased in phase 3.

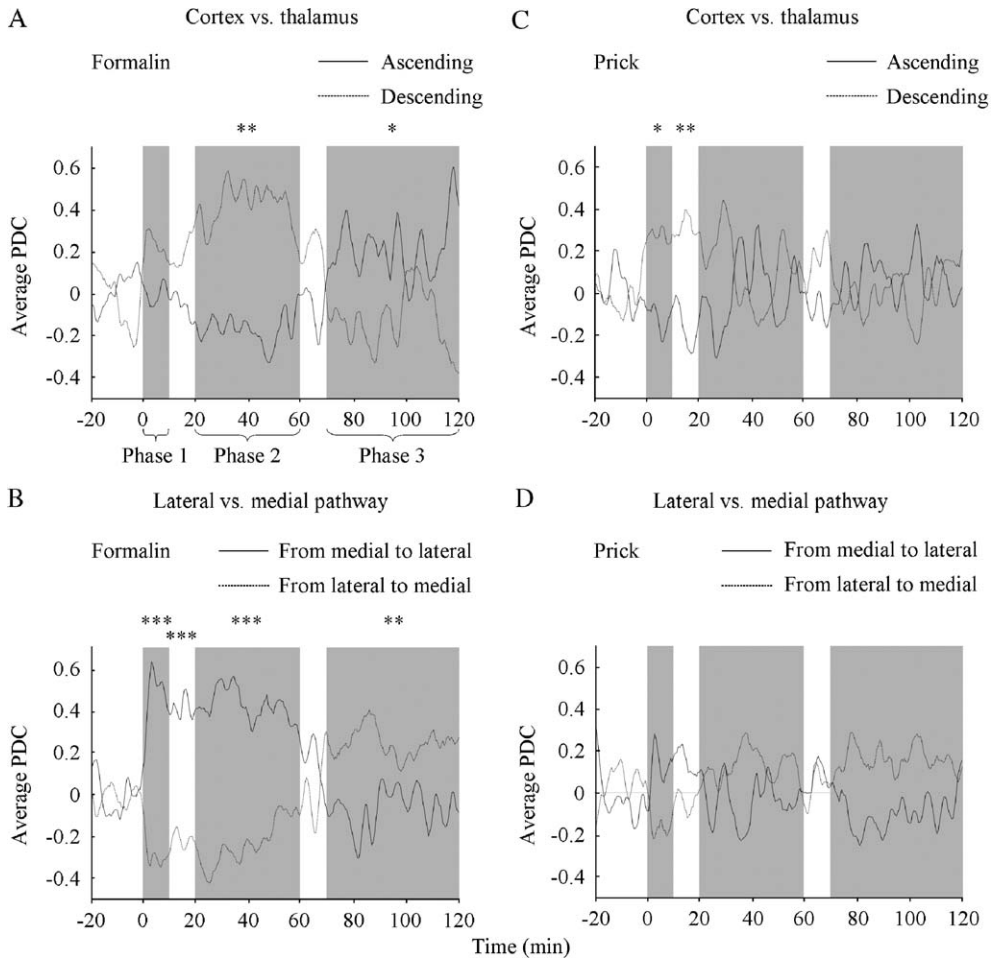


Fig. 6. Time courses of average PDC changes for all rats in the four directions after formalin injection and control stimuli. The direction of information flow reversed at the end of phase 2 either between cortex and thalamus or between the lateral and the medial pathway after formalin injection. (A) The amount of PDC from cortex to thalamus (descending) was significantly larger than that from thalamus to cortex (ascending) during phase 2, but significantly smaller during phase 3. (B) The amount of PDC from the medial to the lateral pathway was significantly larger than that from the lateral to the medial pathway during the first hour after formalin injection, but significantly less during phase 3. (C, D) There was no statistically significant difference in the amount of PDC between the two pairs of directions after control stimulation except that a significantly larger amount of PDC directed from cortex to thalamus during phase 1 and interphase.

*Histological localization of recording sites*

Potassium ferricyanide staining revealed recording sites as blue dots in the ACC, SI, MD and VP. The locations of recording sites included in this report were depicted in Fig. 7.

## Discussion

This study for the first time investigated the cortical and thalamic responses of many simultaneously recorded neurons to formalin injection using a multi-channel, single-unit recording technique (Nicolelis et al., 1995, 1998) in awake freely moving rats. Our major finding is that, unlike the responses of primary afferent fibers and spinal dorsal horn neurons, only 10% forebrain neurons showed biphasic activity. About 19% of cortical and thalamic neurons exhibited monophasic excitatory responses in at least part of the first hour after injection. In about 54% of neurons, either the excitatory responses were inhibited or new inhibitory responses emerged during the recovery phase. In addition, PDC analysis revealed that the direction of information flow between cortical and thalamic areas as well as between the medial and lateral pathways also reversed at the beginning of the third recovery phase. These results indicated that an active central process might occur in the cortical–thalamic network which brought the formalin rats into the third phase of the test.

*Cortex and thalamus mediate the decrease of nociceptive behavior during the recovery phase*

The licking activity declined at the end of phase 2 in the present study. Similar decrease of nociceptive behavior after formalin injection was observed by Porro et al. (2003). In parallel, the cortical and thalamic response patterns altered notably at the end of phase 2. Most excitatory neuronal responses disappeared; some neurons showed novel inhibitory reactions. These changes coincide with EEG shift from a vigilant pattern to a non-vigilant pattern approximately 50 min after formalin injection (Ichinose et al., 1999). These functional changes of the forebrain may explain the behavioral transition

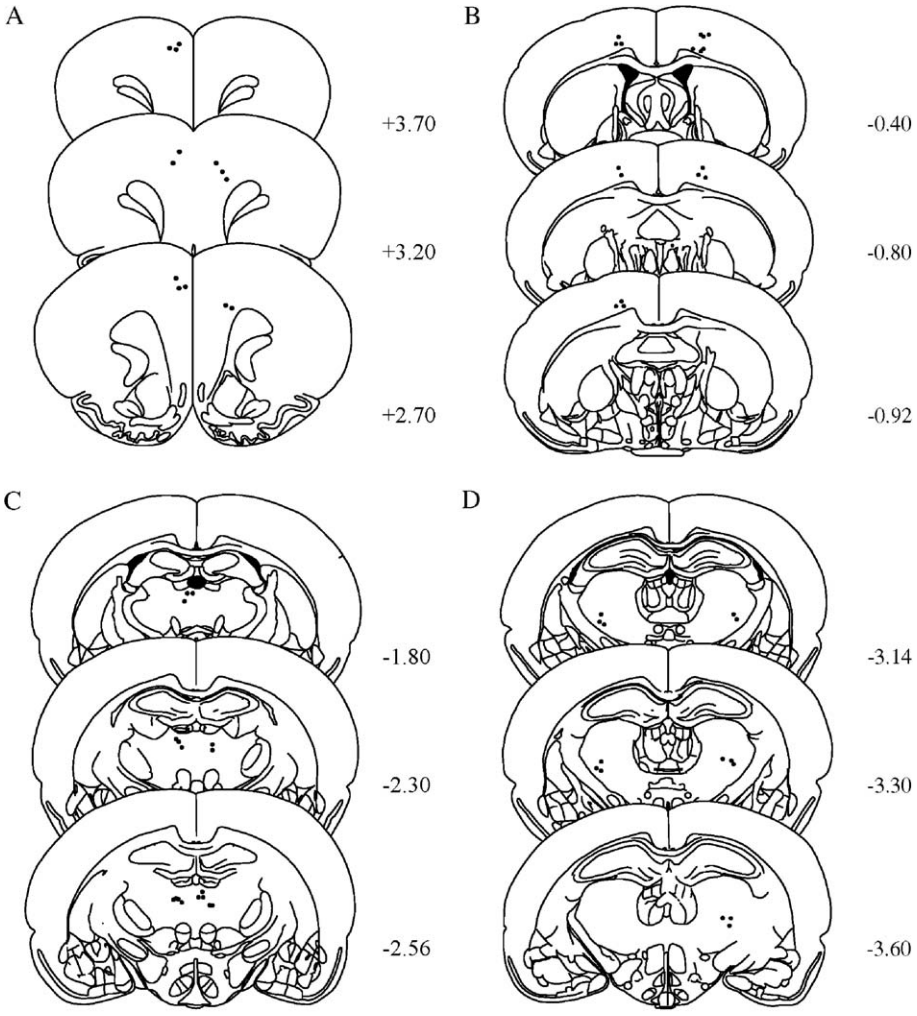


Fig. 7. A schematic drawing indicating the location of recording sites in ACC (A), SI (B), MD (C) and VP (D). The black dots labeled the position of iron deposits at the tips of selected microwires.

from active to inactive. Porro et al. (2003) described a decreased metabolic rate in extensive cortical and subcortical regions including contralateral SI, bilateral cingulate cortex and several thalamic nuclei during phase 3. Meanwhile, some brain regions in endogenous antinociceptive systems such as PAG, the arcuate hypothalamic nucleus, zona incerta (Porro et al., 2003), dorsal raphe nucleus (DRN) (Liu et al., 1998; Porro et al., 1991) and locus coeruleus (LC) (Lei et al., 2004; Liu et al., 1995; Porro et al., 1999; Wei et al., 2001) are activated. The inhibition of excitatory response in cortex and thalamus observed in the current study may be due to activation of the endogenous antinociceptive systems which can inhibit neurons in telencephalon and diencephalon directly through ascending antinociceptive projections (Andersen, 1986; Gura et al., 1991; Jiao et al., 1995; Reichling and Basbaum, 1991; Westlund et al., 1991) or indirectly by mobilizing descending inhibitory pathways to reduce nociceptive inputs from spinal cord.

In our study, we observed that some neurons in SI and MD commenced showing inhibitory responses at the end of phase 2. SI sends corticospinal projections to the superficial and deeper laminae of the dorsal horn in rats (Casale et al., 1988), which appears to exert bidirectional control on the activity of dorsal horn nociceptive neurons. Some neurons in SI can produce tonic descending cortical facilitation on activity of dorsal horn cells. Inhibition of the spontaneous firings of these cortical neurons would reduce the responsiveness of the dorsal horn neurons to noxious stimulus. Rampin et al. verified that, in animals with brachial plexus lesions, abnormal activity of the dorsal horn neurons decreases after cortical spreading depression (Rampin and Morain, 1987). Inhibition of somatosensory cortex can suppress spontaneous discharges in spinal dorsal horn for 30 min (Gorji et al., 2004). Thus, active inhibition of SI may be related to the decrease of nociceptive behavior during the recovery phase through directly inhibiting the spinal nociceptive neurons. Given the complex roles of MD in pain regulation, role of active inhibition of MD in the suppression of formalin-induced spontaneous pain has to be investigated further.

### *Response of cortical and thalamic single neurons*

Although subcutaneous formalin evokes a biphasic activation of Aδ and C fibers (McCall et al., 1996; Puig and Sorkin, 1996) as well as dorsal horn convergent neurons (Dickenson

and Sullivan, 1987a,b; Haley et al., 1990) which mirrors the behavioral response, it was not always the case in our forebrain recording. As described in the result section, only 10% cortical and thalamic neurons exhibited biphasic responses; many of them showed monophasic excitatory responses in certain period after formalin injection. The monophasic instead of biphasic response in the first hour has also been observed in VPM (Jung et al., 2004). Ichinose et al. (1999) reported that the cortical EEG exhibited fast and low amplitude waves which lasted for 35–70 min without interruption during the transient absence of nociceptive behavior. These results suggested that the response pattern of supraspinal nociceptive circuits may, to some extent, be different from that of spinal networks.

Lacking of a clearly defined interphase in many forebrain units might be due to the complex interactions between cortex and thalamus. Thalamic neurons relay noxious information to cortex (Gingold et al., 1991; Sikes and Vogt, 1992; Vogt et al., 1987), and can be in turn directly excited by corticothalamic projections (Deschenes et al., 1998; Liu et al., 1995). This excitatory feedback might trigger oscillations within corticothalamic network (Contreras et al., 1996; Steriade et al., 1972) and lead to a persistent activation during interphase (Coderre et al., 1994; Henry et al., 1999). In fact, the acutely spinally transected rats can preserve normal interphase. Based on the evidence above, the subsiding of pain behavior during the interphase might reflect an adaptation of the peripheral nociceptive stimulation of formalin, thus does not involve much active intervention of the thalamo-cortical network.

*Forebrain mechanisms of the second phase*

In the present study, delayed responses starting 10 to 30 min after injection were detected in some thalamic neurons (MD and VP), which have rarely been reported. One explanation for this phenomenon is expansion of the receptive field (RF) of some neurons into the injection site as a result of central sensitization. Another possibility is that the responsiveness of silent neurons to ascending noxious inputs, which have no responses to noxious stimuli in intact subjects, increases due to central sensitization. Indeed, thalamic neurons do have changes in their excitability reflected by reduced thresholds to mechanical and thermal stimuli, higher spontaneous activity, increased reactivity to afferent inputs and expansion of the receptive field after intra-plantar injection of other chemical irritants such as carrageenin (Guilbaud et al., 1987). These results indicate that central sensitization may occur at forebrain levels during phase 2.

The discharge frequency of neurons in peripheral nervous system and spinal cord during phase 2 is usually 1/5–1/3 of that in phase 1 (McCall et al., 1996; Puig and Sorkin, 1996). This ratio becomes larger in WDR neurons in spinal dorsal horn and reaches 1/2–2/3. Our results showed that the mean response of biphasic neurons in forebrain during phase 2 was as strong as that during phase 1. If the second phase depended on inflammation alone, the relative magnitude of phase 2 to phase 1 would not increase progressively with levels of the hierarchy. Thus, it is possible that the nociceptive signals in phase 2 are amplified during transmission due to central sensitization. However, the present results cannot unambiguously determine whether inflammation is involved in phase 2. Further work will be required to resolve the details of this phase.

Based on the result that more signals directed from the cortex to the thalamus than the opposite direction during phase 2, we postulate that, in addition to central sensitization, descending influence of cortex on thalamus also contributes to the thalamic activities during phase 2. It is known that the descending projection from cortex to thalamus is seven to ten times greater than the ascending projection from thalamus to cortex (Bourassa et al., 1995; Liu et al., 1995). The initial ascending nociceptive input would trigger top–down modulation on the thalamic activity to optimize the detection of incoming sensory input. In line with our hypothesis, Jung et al. found that formalin did not evoke any significant response in VPM between 15 and 40 min when SI was inactivated (Jung et al., 2004). Inactivation of the SI cortex also resulted in reduced thalamic responses to electrical stimuli (Yuan et al., 1986). Neurons in the medial thalamus are activated antidromically by neurons in ACC which project to medial thalamus (Hsu and Shyu, 1997). Taken together, the thalamic activity during phase 2 may be at least partially modulated by descending projections.

*Information flow among forebrain sites*

In the current experiment, there were larger amounts of information flowing from the medial to the lateral pathway during the first hour. The lateral pathway has been classically considered to encode the sensory dimensions of pain, while the medial pathway is thought to process the affective aspects of pain (Albe-Fessard et al., 1985; Hudson, 2000; Melzack and Casey, 1968; Schnitzler and Ploner, 2000; Treede et al., 1999). Most previous studies which demonstrated little influence of pain unpleasantness on pain intensity mainly focused on acute pain which elicits relative weak emotional response (Hofbauer et al., 2001; Rainville et al., 1997; Rainville et al., 1999). Nevertheless, formalin can evoke strong and long-lasting negative emotional response. Clinical studies have proved that sustained negative emotion, such as anxiety, fear and depression, could enhance pain intensity (Fernandez and Milburn, 1994; Fernandez and Turk, 1995; Huyser and Parker, 1999; Keefe et al., 2001). Pain sensation of rheumatoid arthritis patients increases with depression (Affleck et al., 1991). Meta-analyses demonstrated that antidepressant treatment is effective in relieving chronic pain (McQuay et al., 1996; Onghena and Van Houdenhove, 1992). These findings and our current result strongly suggested that negative emotion can enhance pain sensation; treatment aimed at negative emotion may contribute to relief of chronic pain.

Beyond our expectation, the amount of information flow from thalamus to cortex did not increase during the first hour compared to baseline. The cortex is continuously bombarded by incoming input from thalamus. Nociceptive information is conveyed from thalamus to cortex simultaneously along with other irrelevant information. Based on the theory of ‘egocentric selection’ proposed by Suga et al. (2000), the corticothalamic

projection could amplify the 'correct' input while inhibit irrelevant information at the same time to improve the detection of noxious stimuli (Rauschecker, 1998; Singer, 1977). Therefore, the total ascending information would be reduced due to the suppression of irrelevant information. When ascending noxious information decreases in phase 3, the suppressed inputs are unmasked, leading to an increase of total ascending information.

In summary, our results showed that, (i) following formalin injection, thalamo-cortical neurons exhibited monophasic, biphasic or delayed excitatory responses, as well as persistent or delayed inhibitory responses; (ii) a clear inhibitory transition happened at the beginning of the third phase, together with a reverse of information flow direction. We conclude that these changes of activity pattern in forebrain networks, together with related changes in the spinal cord and peripheral sensory fibers, may underlie the emerging and submerging of central sensitization-induced pain behavior in the second phase of formalin test, thus in charge of the formation of the typical three-phase nociceptive behavior in formalin test.

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