Spatiotemporal characteristics of pain-associated neuronal activities in primary somatosensory cortex induced by peripheral persistent nociception

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Abstract

The primary somatosensory cortex (S1 area) is one of the key brain structures for central processing of somatic noxious information to produce pain perception. However, so far, the spatiotemporal characteristics of neuronal activities associated with peripheral persistent nociception have rarely been studied. In the present report, we used c-Fos as a neuronal marker to analyze spatial and temporal patterns of pain-related neuronal activities within the S1 area of rats subjected to subcutaneous (s.c.) injection of bee venom (BV) solution, a well-established animal model of persistent pain. In naive and saline-treated rats, c-Fos-labeled neurons were diffusely and sparsely distributed in the hindlimb region of S1 area. Following s.c. BV injection, c-Fos-labeled neurons became densely increased in superficial layers (II–III) and less increased in deep layers (IV–VI). The mean number of c-Fos positive neurons in the layers II–III began to increase at 1 h and reached a peak at 2 h after BV treatment that was followed by a gradual decrease afterward. The time course of c-Fos expression in the layers IV–VI was in parallel with that of the superficial layers, but with a much lower density and magnitude. The present results demonstrated that BV-induced peripheral persistent nociception could evoke increased neuronal activities in the S1 area with predominant localization in layers II–III.

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Overwhelming evidence has been derived from human and animal studies to support active involvement of primary somatosensory cortex (S1 area) in subserving somatosensory-discriminative aspects of pain experience, such as location, duration, quality and intensity coding of pain [3,17]. Anatomical studies have revealed that the S1 area receives projections from thalamic nociceptive neurons [5,27]. Several electrophysiological experiments have demonstrated that part of neurons across the S1 area of anesthetized and awake monkeys respond to noxious stimuli, the activity of which correlates well with duration and intensity of the stimulus [5,17]. Albeit with these extensive research efforts, it still remains unclear of the spatial and temporal patterns of population neuronal activities associated with peripheral nociception within this region.

In the past 20 years, c-Fos expression has been widely employed as a functional marker to monitor neural activity in the spinal cord in response to various types of noxious stimulation [6,13,15,19,20,22]. To our best knowledge, however, the existing evidence for noxious stimulation-evoked activation of c-Fos in the S1 area comes principally from only three literatures [1,7,26]. Nevertheless, all of these three studies have not explored c-Fos expression in the S1 area of the rat following peripheral persistent pain stimulation. Therefore, the current study was designed to clarify the spatial and temporal characteristics of pain-associated neuronal activities in the contralateral S1 area by immunohistochemical localization of the c-Fos protein at different time points after subcutaneous (s.c.) injection of bee venom (BV) solution into one hindpaw of conscious rats, a well-established animal model of persistent inflammatory pain [4,18].

Adult male Sprague–Dawley albino rats (180–220 g, Animal Center of Fourth Military Medical University, Xi’an, PR China) were used in the present experiment. The animals were housed with food and water available ad libitum, and kept under controlled conditions of temperature (23–25 °C) and light/dark cycle (12 h/12 h). The whole experimental procedures were approved by the Institutional Animal Care and Use Committee of Fourth Military Medical Uni-
sity and were consistent with the guidelines of the International Association for the Study of Pain for pain research in conscious animals [28]. As described elsewhere [4], a volume of 50 μl BV solution dissolved in 0.9% sterile saline (4 μg/μl, Sigma, St. Louis, MO) was administered into the posterior plantar surface of the right hindpaw of rats to produce a kind of persistent pain stimulation (n = 5 rats/each time point). Another parallel group of rats received an equal volume of 0.9% normal saline injection (vehicle control, n = 4 rats/each time point). Animals without any treatment were assigned as the naive group (n = 4 rats).

For tissue collection, at indicated time points after s.c. saline or BV injection (1, 2, 3 and 4 h), the rats were sacrificed by an overdose injection of sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 200 ml normal saline followed by 500 ml 4% paraformaldehyde in 0.01 M phosphate buffer (PB). After perfusion, the brain was removed immediately and postfixed in the same fixative overnight at 4 °C, and then cryoprotected in 0.1 M PB containing 30% sucrose until the tissue block sank onto the bottom of the container. Finally, the brain tissue including S1 area of the hindpaw was cut into coronal sections with 45 μm thickness on a freezing microtome (CM1900, Leica, Germany) and collected in 0.01 M phosphate buffer saline (PBS). During the cutting process, it was ensured that the microtome sections collected for further analysis corresponded to stereotaxic coordinates of S1 hindlimb area by means of identifying anatomical landmarks according to the atlas of Paxinos and Watson [24].

To analyze the constitutive layers of somatosensory cortex, the sections from naïve rats were immunostained for NeuN by using a well-developed avidin–biotin–peroxidase complex (ABC) method [12]. Briefly, the sections were first treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidases and then rinsed three times in 0.01 M PBS (10 min for each time). The sections were further incubated with a mouse anti-NeuN monoclonal antibody (1:500, Chemicon, MAB377) in 0.01 M PBS (solution containing 2.5% Triton X-100) with 3% goat serum for 24 h at 4 °C. The primary antibody was detected with a biotinylated rabbit anti-mouse secondary antibody (Vector Laboratories, CA) at a 1:200 dilution followed by incubation with the ABC complex (1:200, Vector Laboratories, PK 6101). The reaction product was visualized with 0.01% hydrogen peroxide and 0.05% diaminobenzidine (DAB, Beijing Zhongshan Goldenbridge Company, PR China) in 0.05 M Tris–HCl buffer (pH 7.6). Between incubations the sections were rinsed three times in 0.01 M PBS, each for 10 min. The sections were then mounted on slides, air dried, dehydrated in ascending alcohols, cleared in xylene and coverslipped. Finally, immunolabeling of NeuN in the S1 region was examined with a microscope (BX51, Olympus, Japan) via a charge-coupled device camera (DP71, Olympus, Japan).

For double immunofluorescence staining, the sections were incubated with rabbit anti-Fos polyclonal antiserum (1:1000, Santa Cruz, sc-52) as the primary antibody and cy3-conjugated goat anti-rabbit IgG (1:200, Sigma) as the secondary antibody for labeling c-Fos. Double staining was then performed using mouse anti-NeuN monoclonal antibody (1:1000, Chemicon, MAB377) as the first antibody and FITC-conjugated rabbit anti-mouse IgG (1:200, Sigma) as the secondary antibody for labeling NeuN. When PBS or normal serum was used instead of c-Fos or NeuN primary antibody, no positive staining was discerned. Photomicrographic images were obtained under a laser scan confocal fluorescent microscope (Olympus FV1000, Japan).

Observers blinded to the treatment performed all steps of the image capture and cell counting. A total of about 40 sections from each animal were evaluated and averaged from 4 to 5 animals per time point in each group. In general, 25 consecutive planes (per 1.8 μm) were captured per section via confocal microscope scanning. For each section, 5 images every 5 μm interval (which was believed to be large enough to avoid the overlap of the stained nucleus of a single cell body) were selected for counting. To further avoid the possibility of overlapping of c-Fos-labeled neurons, we adopted the averaging values of the cell counts for each section. Image-Pro Plus digitizing software (IPP 6.0, Olympus, Japan) was used to set a threshold (based on optical density and size of the object) such that only those cells that were clearly positive under the microscope could be recognized by the software. The numbers of c-Fos-labeled nuclei in layer I–III (superficial layers) and layer IV–VI (deep layers) were counted respectively in the hindlimb representative region of S1 area based on the borderline localized by double-labeling with NeuN and c-Fos. All results were expressed as mean ± S.E.M. The statistical significance of the data was tested with one-way ANOVA followed by individual post hoc multiple comparisons (Fisher’s PLSD test). A P-value less than 0.05 was set as the significant level.

The hindlimb representative area in the somatosensory cortex was identified as a region located 2.0–3.2 mm lateral to the midline in the coronal section, extending from +0.36 to −2.28 mm relative to bregma [24]. Fig. 1(A) illustrates a microphotograph of NeuN labeling of the rat brain at the level of −2.16 mm to bregma, focusing on the hindlimb area of contralateral S1 (S1HL). Immunostaining with NeuN clearly showed the layer-related structural organization of the S1 region, which could be generally divided into six layers (I–VI) (Fig. 1A, a). Confocal microscopy of NeuN immunofluorescence further confirmed this finding (Fig. 1B, left panel).

In naïve rats, c-Fos immunoreactive (IR) neurons were distributed diffusely and sparsely in the cortical area from layer II to layer VI (Fig. 1B, middle panel). There was no detectable c-Fos response in the layer I. The c-Fos IR labeling was localized exclusively in the nucleus of neural cells and no labeling was detected in the cytosol of cells. In the merged picture, only a few Fos/NeuN IR double labeled neurons were discerned (6.2% of c-Fos-labeled neurons with NeuN labeling), indicating that the activated c-Fos IR neurons were minimal in the S1 area under the naive state (Fig. 1B, right panel). However, among these limited c-Fos positive neurons, a majority of them was localized in neurons (82.6% of NeuN-labeled neurons with c-Fos immunostaining). No appreciable changes were found in cortical c-Fos expression at 2 h after s.c. saline injection (Fig. 2A and C). However, at 2 h after BV-evoked persistent pain stimulation, a significant increase in the number of c-Fos IR cells was observed, the response being much stronger than that from naïve or saline-treated group (Fig. 2B and C). This increasing effect was most prominent in superficial layers (layer I–III, 354.69 ± 22.83, 35.9 ± 11.32, and 22.26 ± 6.01 for BV, saline and naive group, respectively, P < 0.01 vs. saline), with less influence in deep layers (layer IV–VI, 60.19 ± 12.17, 40.17 ± 11.14, and 20.54 ± 5.93 for BV, saline and naive group, respectively, P > 0.05 vs. saline). To note, in the layer I of the S1 area, we failed to find any change in the number of c-Fos IR neurons after s.c. BV injection.

Examination of the time course of c-Fos expression revealed no obvious difference at each time point after saline treatment (Fig. 2C). However, compared with the other two groups, c-Fos IR labeling in the contralateral S1 area began to increase at 1 h after s.c. BV injection. The number of c-Fos IR neurons in superficial layers reached a peak at 2 h, then declined and attained an even lower level at 4 h. In deep layers of S1 area, the number of neurons with positive c-Fos expression followed a similar time course to the superficial layers, but with a much lower density and magnitude (Fig. 2C). Over the last decades, c-Fos expression has become an important research tool for the study of neural activities of nociception [2,6,11]. However, so far, few studies have been conducted to evaluate possible changes in cortical c-Fos expression in the S1 area induced by peripheral noxious stimulus [1,7,26]. In one previous
In the present study, we also examined the time course of c-Fos expression in the contralateral S1 area in response to s.c. BV injection. We found that the number of c-Fos IR neurons began to increase at 1 h, and then reached a peak at 2 h followed by a decrease at 3–4 h after persistent noxious stimulation. This temporal profile of c-Fos expression was partially in line with our earlier immuno-histochemical experiments performed in the spinal cord [22]. In that study, persistent pain-induced spinal c-Fos induction also peaked at 2 h after s.c. BV injection and a layer-dependent difference of c-Fos expression was similarly detected primarily in terms of response duration (but not magnitude), with the deep (but not superficial) layers of spinal dorsal horn exhibiting a much longer response [22]. Unlike that previous study, the current results also revealed a certain amount of c-Fos expression in the S1 area from naïve and saline-treated rats. The exact reasons for this discrepancy are not fully understood. Speculatively, it might be attributed to a convergence from both nociceptive and non-nociceptive inputs at the cortical level [25]. Indeed, there is one previous study reporting that tactile experience in behaving rats could produce a profound increase of c-Fos expression in the barrel cortex [8]. The precise mechanisms underlying saline-induced c-Fos activation in the S1 area remain less characterized. However, in our previous immunoblotting studies, we also found such saline-elicited prolonged activation of ERK and JNK in the S1 area [10,21]. Accordingly, it may be the case that the saline injection process resulted in a form of short-term physiological pain, which maybe partially responsible for some extent of c-Fos induction in the cortex.

The nature of the extracellular signaling molecules that were recruited upon subjecting animals to painful stimulation and hence responsible for cortical c-Fos expression was not clear. Based on the above descriptions that peripherally induced persistent pain stimulation initiated an intense and long-lasting (at least 48 h) activation of both ERK and JNK in the somatosensory cortex in BV-inflamed rats [10,21], it is reasonable to suppose that these activated mitogen-activated protein kinases may serve as candidate upstream molecules responsible for the subsequent induction of c-fos gene expression [16]. Of course, this presumption still needs additional verification in future studies. On the other hand, it remains unknown what is the actual role of elevated c-Fos expres-
sion in the S1 area after s.c. BV-evoked peripheral persistent pain. In this context, there seem to exist two opposing views regarding the roles of spinal c-Fos in nociceptive processes: one is that Fos may contribute to sensitization of spinal nociceptive neurons accounting for behavioral hyperalgesia or allodynia [13,23]; the other is that Fos is probably involved in the inhibition of heightened or prolonged nociception [14]. Given this controversy, future experiments are certainly warranted to determine specialized roles of c-Fos protein in the S1 area during BV-evoked inflammatory pain processing. However, according to the present findings, we can at least conclude that BV-induced persistent pain is expected to induce a distinct spatial and temporal pattern of c-Fos expression in the S1 area, reflecting enhanced activation of pain-associated neuronal activities in the higher level of the neuraxis.

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