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Differential patterns of *c-fos* mRNA expression in the amygdaloid nuclei induced by chemical somatic and visceral noxious stimuli in rats

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Abstract

Pain includes a negative affective component, although the neural system is unclear. The amygdala including the lateral (La), basolateral (BL) and central (Ce) nuclei is thought to play a key role in emotional responses. In this study, we analyzed the *c-fos* mRNA expression, as a marker of neuronal activity, induced by two types of pain, chemical somatic and visceral noxious stimuli, in each amygdaloid nucleus in unanesthetized rats. We found that intraplantar injection of formalin as a chemical somatic noxious stimulus increased *c-fos* mRNA expression in the La and BL, but not Ce. On the other hand, intraperitoneal injection of acetic acid as a chemical visceral noxious stimulus induced it highly in the Ce, moderately in La and hardly in BL. These results suggest that distinct amygdaloid nuclei are activated by chemical somatic and visceral noxious stimuli, which might differentially contribute to emotional responses by them.

Keywords: Visceral pain; Somatic pain; *c-fos* mRNA; Central nucleus of amygdala; Basolateral nucleus of amygdala; Formalin; Acetic acid

Painful stimuli evoke pain sensation as well as its unpleasant emotional feelings. Although the neural systems of the sensory component of pain have been extensively studied, those of the negative affective component are less clear. The amygdala is a forebrain structure composed of several distinct nuclei including the lateral (La), basolateral (BL) and central (Ce) nuclei, and is thought to be a key neural substrate underlying emotional responses such as anxiety, fear and depression [7]. Electrophysiological studies indicated that painful stimuli activated the amygdaloid neurons in rats [3,14]. Lesions of the amygdala decreased emotional-related pain reactions such as post-stress analgesia and shock-induced hyperalgesia without affecting the basal nociceptive threshold [6]. These findings suggest that the amygdala could be involved in receiving, integrating and encoding pain information, all of which are considered to contribute to emotional responses.

Intraplantar (i.pl.) injection of formalin into the hind-paw evokes nociceptive behaviors such as lifting, licking, shaking or biting. These nociceptive responses are accompanied by inflammation, and caused by cutaneous as well as deep somatic stimuli. On the other hand, intraperitoneal (i.p.) injection of acetic acid produces inflammation of the wall of the abdominal cavity and evokes a sustained writhing behavior caused by visceral stimuli. Both of these methods have been extensively used as well established animal models for chemical somatic and visceral pain, respectively, in rodents. The induction of immediate early *c-fos* gene is frequently used as an indirect method of gauging neural activation, and its distribution in response to noxious stimulation is generally correlated with the nociceptive pathways mapped by electrophysiological and tract-tracing methods [8]. In this study, we investigated the distribution of the activated amygdaloid neurons by i.pl. injection of formalin as a chemical somatic noxious stimulus and i.p. injection of acetic acid as a chemical visceral noxious stimulus to unanesthetized rats by using in situ hybridization analysis of *c-fos* mRNA induction.

Male Sprague–Dawley rats weighing 180–220 g were used. They were kept at a constant ambient temperature of 24 ± 1 °C under a 12 h light/dark cycle with free access to food and water. For i.pl. injection of formalin, each rat was placed in a Plexiglass cylinder 30 cm in diameter and 50 cm in height to acclimatize it to the experimental environment

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for 30 min. Then, the unanesthetized rats were given an i.pl. injection of 2% formalin or saline at a volume of 100 μl into the right hind-paw, and immediately returned to the cylinder. I.pl. formalin evoked characteristic nociceptive behaviors such as lifting, licking, shaking or biting consisting of two temporally distinct phases. For i.p. injection of chemical acetic acid, each rat was placed in a Plexiglass box 50 cm in diameter and 30 cm in height and allowed to acclimatize for 30 min. Then, the unanesthetized rats were given an i.p. injection of 2% acetic acid or saline at a volume of 1 ml, and immediately returned to the box. After 1 h, the rats were sacrificed by decapitation, and the brains were rapidly removed and frozen in powdered dry-ice. Coronal sections (16 μm) including the amygdala (1.8–3.8 mm caudal to the bregma [15]) were prepared in a cryostat, thaw-mounted onto gelatin-coated slides and stored at −80 °C until use.

In situ hybridization for *c-fos* mRNA was conducted as previously described [13]. Briefly, ^{35}S-labeled antisense RNA probe to *c-fos* (Stratagene, La Jolla, CA) was synthesized in the presence of [α-^{35}S]UTP (Amersham, Buckinghamshire, UK) using standard transcription methods, and alkaline hydrolyzed to about 250 bases. Sections were fixed in 4% formalin, treated with 1 μg/ml proteinase K and then immersed in 0.25% acetic anhydrid. After prehybridization, sections were hybridized to the RNA probe at 55 °C for 18 h. They were washed, incubated with ribonuclease A (50 μg/ml) and then washed again. The slides were dipped in autoradiographic emulsion NTB-3 (Kodak, New York, NY). After 4 weeks of exposure, they were developed with D-19 (Kodak), fixed and lightly counterstained.

The cells positive for *c-fos* mRNA were counted in the La, BL and Ce. Twenty-four sections, that is, 12 sections from a noxious stimulus-given and corresponding saline-treated control rat, were processed in a single in situ hybridization experiment. The cells which bore 15 or more microautoradiographic silver grains on each cell body were considered to express the *c-fos* mRNA. The numbers of cells positive for the *c-fos* mRNA were counted mainly in lefthand (contralateral), as well as righthand (ipsilateral) (i.pl. formalin), or lefthand (i.p. acetic acid) La, BL and Ce of the three to four sections at a level of 2.8 mm caudal to the bregma from three animals. To define amygdaloid nuclear groups, the atlas of Paxinos and Watson [15] was used. The differences in the numbers of *c-fos* mRNA-positive cells were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test. Differences with $P < 0.05$ were considered significant.

In the amygdala of untreated control rats, no expression of *c-fos* mRNA was observed in any nuclei (Fig. 1B). In the contralateral amygdala of rats that received an i.pl. saline, *c-fos* mRNA was weakly expressed in the ventromedial part of the La and anterior part of the BL, while no expression of *c-fos* mRNA was observed in the Ce (Fig. 1C). On the other hand, i.pl. formalin markedly increased the expression of *c-fos* mRNA in the contralateral La and BL, but not Ce. The *c-fos* mRNA-positive cells were seen mainly in the ventromedial and dorsolateral, rather than ventrolateral, parts of the La, and anterior, rather than posterior, part of the BL (Fig. 1D). In the La and BL, one-way ANOVA showed a significant difference in the numbers of *c-fos* mRNA-positive cells ($F_{2,6} = 46.7$, $P < 0.001$ and $F_{2,6} = 16.9$, $P < 0.01$, respectively), while there was no difference in the Ce ($F_{2,6} = 1.24$, $P > 0.05$) (Fig. 2A). Post hoc comparison showed significant increases in the La and BL of the rats that received i.pl. formalin compared with untreated ($P < 0.001$ and $P < 0.01$, respectively) or saline-treated rats ($P < 0.001$ and $P < 0.01$, respectively). The induction of *c-fos* mRNA was not different between the contralateral and ipsilateral amygdaloid nuclei (data not shown), consistent with a previous report [17].

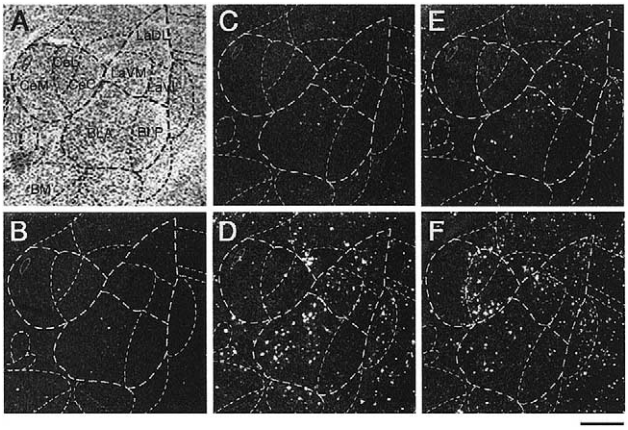

Fig. 1. Effects of chemical somatic or visceral noxious stimuli on the expression of *c-fos* mRNA in the amygdala. (A) Photomicrograph of cresyl violet-stained coronal section through the amygdala of non-treated animals. (B–F) Representative photomicrographs showing *c-fos* mRNA expression in the lefthand amygdala of non-treated control animals (B); chemical somatic stimulated animals 1 h after i.pl. injection of saline (C) or formalin (D); chemical visceral stimulated animals 1 h after i.p. injection of saline (E) or acetic acid (F). CeM, medial division of Ce; CeL, lateral division of Ce; CeC, capsular part of Ce; LaDL, dorsolateral part of La; LaVM, ventromedial part of La; LaVL, ventrolateral part of La; BLA, anterior part of BL; BLP, posterior part of BL; BM, basomedial nuclei of amygdala, Scale bar: 500 μm.

In the amygdala of rats that received i.p. saline, *c-fos* mRNA was weakly induced in the ventromedial and dorsolateral parts of the La, and anterior part of the BL, while no expression of *c-fos* mRNA was observed in the Ce (Fig. 1E). On the other hand, i.p. acetic acid induced *c-fos* mRNA highly in the Ce, moderately in La and hardly in BL. The *c-fos* mRNA-positive cells were seen mainly in the lateral division and capsular part, rather than the medial division, of the Ce (Fig. 1F), consistent with previous electrophysiological findings that the majority of the neurons in the lateral division and capsular part of the Ce responded exclusively or preferentially to painful stimuli [3,14]. In the La and Ce, one-way ANOVA showed a significant difference ($F_{2,6} = 8.1$, $P < 0.05$ and

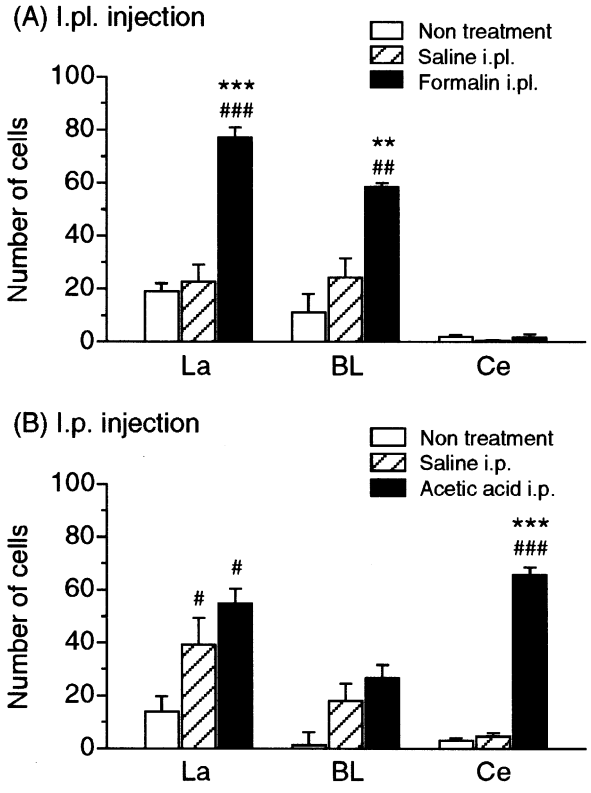

Fig. 2. The mean number of cells positive for the *c-fos* mRNA in the lefthand La, BL and Ce 1 h after i.pl. (A) or i.p. injection (B). Each column represents means ± SEM in the sections from three animals. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the non-treated control animals; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the saline-treated animals by the Student–Newman–Keuls post hoc test.

$F_{2,6} = 331.0$, $P < 0.0001$, respectively), while there was no difference in the BL ($F_{2,6} = 1.47$, $P > 0.05$) (Fig. 2B). Post hoc comparison showed significant increases in the La, but not BL and Ce, of the saline-treated rats compared with untreated rats ($P < 0.05$). I.p. acetic acid significantly increased the number of *c-fos* mRNA-positive cells in the La compared with untreated rats ($P < 0.05$), while it was not significant compared with the saline-treated rats. On the other hand, the number of *c-fos* mRNA-positive cells in the Ce was significantly increased compared with untreated or saline-treated rats ($P < 0.001$).

In this study, we found that i.pl. formalin to unanesthetized rats as a chemical somatic noxious stimulus induced *c-fos* mRNA in the La and BL, but not Ce, while i.p. acetic acid as a chemical visceral noxious stimulus induced highly in the Ce, moderately in La and hardly in BL, indicating that these two types of pain produced different patterns of *c-fos* mRNA expression in the respective amygdaloid nuclei. It has been reported that chemical somatic and visceral noxious stimuli induced different patterns of *c-fos* in the spinal cord [12] and supraspinal regions such as the periaqueductal gray, parabrachial, hypothalamic and thalamic nuclei [1,5,17]. Previous anatomical and electrophysiological studies revealed that multiple neural pathways are implicated as the principal routes providing sensory information to the amygdala. Somatic sensory, including noxious, information from the spinal dorsal horn is transmitted through the lateral thalamus to the cortical areas such as the primary and secondary somatosensory cortex and insular cortex, and then reaches the amygdala, particularly the La and BL [7,18,19]. On the other hand, the Ce is directly linked to nociceptive centers in the spinal cord and brain stem through the spino-(trigemino)-parabrachio-amygdaloid nociceptive pathway [2–4,14], which is reported to be involved in visceral pain [4]. Visceral sensory information also reaches the nucleus of the solitary tract, which contains neurons projecting to the Ce [20]. Taken together, it is proposed that chemical somatic noxious information by i.pl. formalin might be transmitted through the spino-thalamo-cortico-amygdaloid pathway to activate the La and BL neurons, while chemical visceral noxious information by i.p. acetic acid might be through the spino-(trigemino)-parabrachio-amygdaloid and/or the nucleus of the solitary tract-amygdaloid pathway to activate the Ce neurons. However, i.p. acetic acid weakly induced *c-fos* mRNA in the La and BL, raising the possibility that chemical visceral noxious information might also be mediated by other pathways [5].

A body of evidence has shown that respective amygdaloid nuclei play distinctive and dissociable roles in several emotional behaviors [9,10]. It has been suggested that the La and BL integrate different sensory inputs to execute different emotional responses [10]. The sequential processed information, in turn, is given to other amygdaloid nuclei such as the Ce [16]. On the other hand, the Ce is the major output nucleus in the amygdala, that is, the Ce efferents diffusely project to a number of forebrain and brainstem structures, and generate emotionally related behavioral responses [10,16]. It is known that cutaneous somatic pain, which is escapable and possible to control, characteristically evokes active emotional copying responses such as agitation, hyperactivity, fight-flight and hypertension, whereas visceral pain, which is inescapable and impossible to control by subjects themselves, evokes passive emotional copying responses such as behavioral quiescence, decreased reactivity to the environment, hypotension and nausea [1,11]. These differential emotional responses might be explained by the differential activation of the amygdaloid nuclei. Additional investigations are needed to elucidate the roles of the respective amygdaloid nuclei and their mechanisms in the negative affective component of pain.

Acknowledgements

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