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Distinct contributions of reactive oxygen species in amygdala to bee venom-induced spontaneous pain-related behaviors

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Abstract

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, play essential roles in physiological plasticity and are also involved in the pathogenesis of persistent pain. Roles of peripheral and spinal ROS in pain have been well established, but much less is known about ROS in the amygdala, a brain region that plays an important role in pain modulation. The present study explored the contribution of ROS in the amygdala to bee venom (BV)-induced pain behaviors. Our data show that the amygdala is activated following subcutaneous BV injection into the left hindpaw, which is reflected in the increased number of c-Fos positive cells in the central and basolateral amygdala nuclei in the right hemisphere. Stereotaxic administration of a ROS scavenger (tempol, 10 mM), NADPH oxidase inhibitor (baicalein, 5 mM) or lipoxygenase inhibitor (apocynin, 10 mM) into the right amygdala attenuated the BV-induced spontaneous licking and lifting behaviors, but had no effect on BV-induced paw flinch reflexes. Our study provides further evidence for the involvement of the amygdala in nociceptive processing and pain behaviors, and that ROS in amygdala may be a potential target for treatment strategies to inhibit pain.

Keywords

Reactive oxygen species; bee venom; pain; amygdala

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1. Introduction

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, play essential roles in physiological plasticity [10,17], whereas under pathological conditions, the formation of ROS has been shown to be critical in apoptosis, stroke pathology, spinal cord injury and neurodegenerative disorders [6,22]. Moreover, ROS are also implicated in pathological pain states, including neuropathic pain, inflammatory pain and visceral pain [9,11,13,16,19,32,33]. Whereas previous studies mainly focused on the roles of peripheral and spinal ROS in the pathogenesis of inflammatory and neuropathic pain [7,16,33,37], the pain-related function of supraspinal ROS is still an understudied area [11,13,19,21].

As a part of the limbic system, the amygdala has traditionally been associated with negative emotions such as fear [14] and plays an important role in emotional responses to pain and in pain modulation [24–26]. The amygdala is composed of lateral (LA), basolateral (BLA) and central (CeA) nuclei [31], which serve distinct functions in the processing of pain-related information [24]. LA-BLA receive and integrate polymodal sensory, including nociceptive, information, which is transmitted to the amygdala output region (CeA) that also receives a nociceptive teaching signal from the brainstem parabrachial area [24]. These subnuclei of amygdala have been shown to be differentially activated in the formalin test and the acetic acid-induced visceral pain model based on different patterns of c-fos mRNA expression [23]. Our previous work showed that ROS in amygdala are involved in experimental models of visceral and arthritis pain [11,13,19]. However, the role of ROS in the amygdala in other experimental pain models remains to be determined.

The bee venom (BV) test is a well-established experimental pain model. It is produced by subcutaneous injection of a BV solution, which can induce long-lasting spontaneous pain-related behaviors such as the spinally mediated paw flinch reflex and the supraspinally mediated paw licking and lifting behaviors [4,5,18,30,38]. The present study first mapped c-Fos changes in different subnuclei of amygdala following BV injection and then tested pharmacological agents to investigate the roles of ROS in the amygdala in BV-induced spontaneous pain-related behaviors.

2. Materials and Methods

2.1. Animals

All experiments were conducted on male albino Sprague-Dawley rats (weighing 180–220g, 8–9 weeks old) purchased from the Laboratory Animal Center of Fourth Military Medical University (FMMU). The animals were housed in groups of 4–6 and maintained under standard conditions (12 h dark/light circle, temperature 22–26°C, air humidity 40–60%) with food and water available *ad libitum*. The experimental protocols were approved by the Institutional Animal Care and Use Committee of FMMU and animals were maintained and cared in line with EC Directive 86/609/EEC and the guidelines set forth by the International Association for the Study of Pain [39]. Every effort was made to minimize the number and suffering of the animals.

2.2. Immunohistochemistry

Two hours after receiving subcutaneous BV (0.2 mg/50 μ l, Sigma, USA) or physiological saline injection into the left hindpaw, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal) and perfused transcardially with physiological saline followed by 4% paraformaldehyde. The whole brain was removed, post-fixed in the same fixate for 4 h and then transferred to a 30% sucrose solution in 0.01M phosphate buffer for cryoprotection. Coronal brain sections (40 μ m thick) containing the amygdala were cut on a cryostat (CM1900, Leica, Germany). Every fifth slice was collected, yielding about 10–15 sections per rat. Brain slices were washed in 0.01 M phosphate-buffered saline (PBS) and incubated in 3% hydrogenperoxide for 10 min, and then incubated for 1 h in 1% bovine serum and 0.2% Triton X-100 in 0.01 M PBS. Sections were incubated overnight with rabbit anti-c-Fos polyclonal antibody (1:200, Cell Signaling). Sections were then washed and subjected to incubation with biotinylated goat anti-rabbit secondary antibody (1:200, ZSGB-Bio) for 3 h. Sections were washed again and incubated in avidin-biotin complex (1:200, Sigma) for 3 h. After several rinses in PBS, the immunostaining reactions were developed with an ABC kit (ZSGB-Bio, P.R. China). Reactions were stopped by repeated washes in PBS. Brain sections were mounted on slides and coverslipped. The number of c-Fos positive cells in CeA and BLA was counted with Image-Pro Plus digitizing software (Olympus, Japan).

2.3. Intra-amygdala drug application

The rat was anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. A craniotomy over the right amygdala was performed and a guide cannula (outer diameter: 0.64 mm; inner diameter: 0.45 mm) was implanted on the dorsal margin of the CeA, using the following coordinates (in mm): 2.5 caudal to bregma; 4.0 lateral to midline; depth, 7.5. The cannula was fixed to the skull with dental acrylic. The behavioral tests were carried out 5 days after the surgery. For drug application, a microinjection tubing probe was inserted through the guide cannula so that the probe protruded by 1 mm. The probe was connected to a PE-10 polyethylene tube filled with dissolved drug solution. The drug solution was pushed into the brain tissue by injecting 1 μ l air into the polyethylene tube with a microsyringe. Then the polyethylene tube was heat sealed and the probe was left in place for 10 min before the behavioral tests. The following drugs were used: Tempol, baicalein, apocynin (Tocris). Drugs were dissolved in DMSO (30% in deionized water) and diluted in ACSF (containing [in mM] 117 NaCl, 4.7 KCl, 1.2 NaH_2PO_4 , 2.5 CaCl_2 , 1.2 MgCl_2 , 25 NaHCO_3 and 11 glucose) at 1:100 to their final concentration for injection into the amygdala.

2.4. Histology

At the end of behavioral tests, all rats that received intracerebral cannulation were perfused as previously described above (see “Immunohistochemistry”). Brain sections containing the amygdala were sectioned at 20 μ m and mounted on gel-coated slides. Brain sections were stained with 0.1% cresyl violet for identification of the tip of the cannula.

2.5. Quantitative measurement of pain-related behaviors

A 30 × 30 × 30 cm transparent plastic box with transparent glass floor was placed on a supporting frame 30 cm high above the experimental table. The animals were habituated to the environment before the initiation of the experiment. Following s.c. BV injection into the left hindpaw, the number of spontaneous paw flinches, a spinally mediated reflex [4,18,38], and the lifting and licking time, which are supraspinally mediated [30], were counted for each 5-minute time block for 60 minutes.

2.6. Statistical analysis

All data were expressed as mean ± SEM. One way ANOVA (post-hoc Fisher's least significant difference) and two-way ANOVA with Bonferroni post-hoc tests were used where appropriate. $P < 0.05$ was chosen as the criterion for statistical significance.

3. Results

3.1. BV-induced c-Fos expression in the amygdala

Two hours after s.c. saline injection into the left hindpaw, there were scatter c-Fos positive cells in the amygdala (CeA and BLA) (Fig. 1), with no difference of the number of c-Fos positive cells between the right and left amygdala. Importantly, two hours after s.c. BV injection into the left hindpaw, the number of c-Fos positive cells increased significantly in the right BLA ($F_{1,44} = 26.008$, $P < 0.001$) and CeA ($F_{1,44} = 21.477$, $P < 0.001$) compared to that received s.c. saline injection, and there were more c-Fos positive cells in the right amygdala (BLA, $F_{1,44} = 31.941$, $P < 0.001$; CeA, $F_{1,44} = 27.685$, $P < 0.001$) than that in the left. Increased c-Fos positive cells in CeA were mainly localized in the laterocapsular part (Fig. 1), which has been termed the “nociceptive amygdala” [26]. No difference of the number of c-Fos positive cells was detected between the right and left amygdala.

3.2. Effects of a ROS scavenger, NADPH oxidase inhibitor and lipoxygenase inhibitor on BV-induced pain-related behaviors

Consistent with our previous findings [5], s.c. BV injection resulted in paw flinch reflexes, which lasted about 60 minutes, and paw licking or lifting behaviors, which lasted about 40 minutes. Stereotaxic administration of a ROS scavenger (tempol, 10 mM/1μl), which is a superoxide dismutase mimetic, or a 5- and 12-lipoxygenase inhibitor (baicalein, 5 mM/1μl), or a NADPH oxidase inhibitor (apocynin, 10 mM/1μl) into the right amygdala 10 min before s.c. BV injection had no effect on the BV-induced paw flinch reflex ($F_{4,34} = 0.177$, $P = 0.949$; Fig. 3A and B). However, the paw lifting and licking behaviors were inhibited significantly ($F_{4,34} = 10.803$, $P < 0.001$; Fig. 3C and D).

3.3. Histological analysis

Histological analysis showed that the tips of the cannulae were mainly located within the region of the CeA, and with several tips located in BLA, which were on the lateral margin of CeA (Fig 2).

4. Discussion

In the present study, we found that following subcutaneous injection of BV solution into the left hindpaw, the number of c-Fos positive cells increased significantly in the right CeA and BLA, indicating neuronal activity increases in the amygdala in this pain model. Stereotaxic administration of a ROS scavenger, NADPH oxidase inhibitor or lipoxygenase inhibitor into the right amygdala attenuated BV-induced supraspinally mediated paw lifting and licking behaviors, but had no effect on BV-induced spinal paw flinch reflexes. Our results show that ROS in amygdala play differential roles in BV-induced pain-related behaviors.

The amygdala is composed by several subnuclei that serve distinct functions in the processing of pain-related information. The CeA receives nociceptive information directly from the spinal cord and the parabrachial area [8], whereas the LA receives polymodal sensory information from thalamus and cortex [28,29,31]. The LA-BLA network is believed to attach emotional significance to sensory information [28,29,31], and this highly processed information is then transmitted to the CeA, which modulates pain behaviors through projections to descending pain control center [26,27,35]. The involvement of amygdala in the processing of pain-related information has been previously demonstrated using electrophysiology [24–26], biochemistry [2], and neuronal activity markers such as c-Fos [36]. c-fos mRNA in the amygdala increased remarkably following noxious visceral or somatic stimulation [23] but suggested that the activation pattern in subnuclei of the amygdala can be different, depending on the type of the noxious stimulus. Intraplantar injection of formalin induced c-fos mRNA expression in the LA and BLA, but not CeA, although it should be noted that synaptic plasticity was measured in the CeA in the formalin pain model [1]. Intraperitoneal injection of acetic acid resulted in increased c-fos mRNA expression in the CeA rather than BLA [23]. In the present study, we found that BV injection increased the number of c-Fos positive cells in the BLA and CeA, particularly the laterocapsular part (CeLC), which further suggests that the activation of subnuclei of amygdala may be stimulus specific. Therefore, it is important to study roles of different subnuclei of amygdala in different experimental pain models.

Interestingly, no differences of the expression of c-fos mRNA were detected between the left and right amygdala following intraplantar injection of formalin into the right hindpaw [23], whereas our study found more c-Fos positive cells in the CeA and BLA of the right than left amygdala. It is possible that the more pronounced increase in c-Fos expression in the right amygdala may be due to the side of BV injection, i.e., into the contralateral paw. Another possibility is that hemispheric lateralization of pain-related information processing in the amygdala, which has been suggested based on biochemical and behavioral [3] and electrophysiological [12] studies. Our pharmacological and behavioral experiments also confirm the right amygdala as an important site for antinociceptive drug effects.

Although ROS play essential roles in physiological plasticity, ROS have also been implicated in pain mechanisms. Peripheral or spinal administration of exogenous ROS can exert pronociceptive effects, whereas the administration of ROS scavengers can have antinociceptive effects in pain states [15,34]. Moreover, intracerebral administration of ROS scavengers into the CeA inhibited visceral and somatosensory nociceptive responses of

amygdala neurons induced by activation of metabotropic glutamate receptors [13,19] and attenuated visceral pain-related emotional responses [11]. Our present study further showed that intra-amygdala administration of a ROS scavenger, NADPH oxidase inhibitor or lipoxygenase inhibitor attenuated supraspinally mediated BV-induced lifting and licking behaviors. Consistent with our previous report that bilateral lesion of the amygdala exerts no effects on spinally mediated BV-induced paw flinch reflexes [20], the present study also found no evidence for the involvement of amygdala ROS in BV-induced spinal paw flinch reflexes. Taken together, ROS can contribute to the processing of pain-related information at different levels and inhibition of the synthesis of ROS or elimination of ROS with scavengers at different levels can inhibit pain-related behaviors that are mediated at different levels. It is possible that ROS needs to be targeted at different levels (systemically) to achieve maximum pain inhibition.

In conclusion, our study provides further evidence for the involvement of the amygdala in pain processing and pain modulation and suggests that ROS in amygdala may be a useful target for developing treatments for pain.

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Highlights

- The right amygdala can be activated by s.c. injection of BV into left hindpaw
- This activation is represented by increased c-Fos positive cells in right amygdala
- Intra-amygdala administration of ROS scavenger attenuated BV-induced flinch reflex
- NADPH oxidase and lipoxygenase inhibitor also attenuated BV-induced flinch reflex

Figure 1. c-Fos expression in amygdala following s.c. BV or saline injection

Panels on the left show representative images of c-Fos immunoreactivity in the amygdala following s.c. BV or saline injection. Scale bars: 500 μm. Bar histograms on the right show quantitative analysis of the number of c-Fos positive cells in the CeA and BLA. *** $P < 0.001$, BV compared to saline; ### $P < 0.05$, 0.001 left compared to right. Data are expressed as mean $\pm$ SEM.

Figure 2. Drug application sites in the amygdala

(A) Representative histological image of the drug application site. (B–E) Localizations of drug application sites for different compounds. Scale bars: 2 mm.

Figure 3. Effects of ROS scavenger, NADPH oxidase inhibitor and lipoxygenase inhibitor on BV-induced pain-related behaviors

(**A, C**) Time course of the effects of intra-amygdalar administration of a ROS scavenger (tempol), NADPH oxidase inhibitor (baicalein) or lipoxygenase inhibitor (apocynin) on BV-induced paw flinch reflex and licking and lifting behaviors. (**B, D**) Total number of paw flinches (**B**) and total duration of licking and lifting behaviors (**D**) based on data shown in **A** and **C**, respectively. *** $P < 0.001$ compared to DMSO (vehicle control). Data are expressed as mean $\pm$ SEM. Bar histograms show averaged data and individual data points (open circles).