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Research paper

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

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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.

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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.

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

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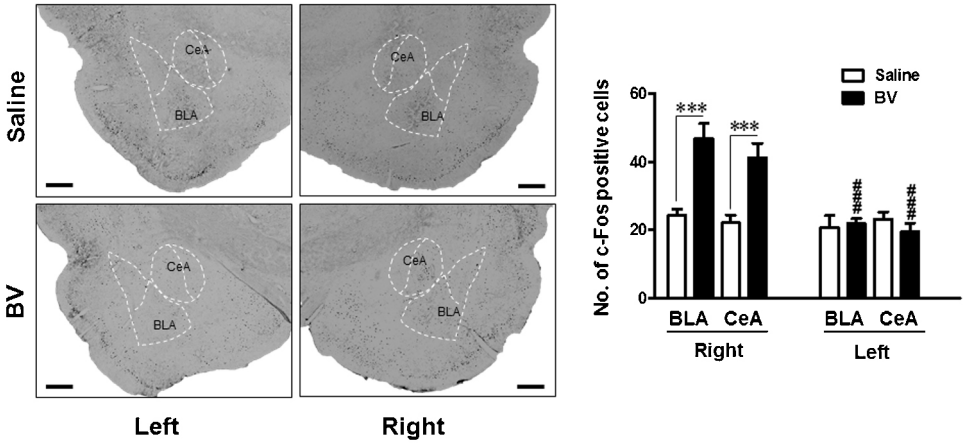

Fig. 1. c-Fos expression in amygdala following s.c. BV or saline injection. Panels on the left show representative images of c-Fos immunoreactivity in the amygdala following s.c. BV or saline injection. Scale bars: 500 μm. Bar histograms on the right show quantitative analysis of the number of c-Fos 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 ± SEM.

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 180–220 g, 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 μl, 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 0.01 M phosphate buffer for cryoprotection. Coronal brain sections (40 μm 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 μl 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