Supraspinal Contribution to Development of Both Tonic Nociception and Referred Mirror Hyperalgesia

A Comparative Study between Formalin Test and Bee Venom Test in the Rat

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Background: The roles of descending facilitatory pathway from the rostral medial medulla (RMM) in development of persistent spontaneous nociception and hyperalgesia were evaluated in the bee venom (BV) test and the formalin test.

Methods: Bilateral lesions of the RMM with ibotenic acid, a soma-selective neurotoxin, were performed to study their effects on the spontaneous pain-related behaviors and hyperalgesia, which were determined by counting the number of flinching reflex per 5 min (1 h) and by measuring paw withdrawal thermal latency (PWT) and mechanical threshold (PWM) to radiant heat and von-Frey filaments to both hind paws in conscious rats, respectively.

Results: 1) Bilateral lesions of the RMM produced a similarly significant inhibition of persistent spontaneous flinching reflexes in the BV test and the formalin test; however, the inhibitory effect occurred in the late 50 min (11–60 min), but not the first 10 min (0–10 min) following intraplantar injection of either BV or formalin. 2) Bilateral lesions of the RMM prevented the development of the BV-induced referred mirror heat hyperalgesia occurred in the noninjected paw, but had no effect on the primary heat and mechanical hyperalgesia occurred in the injected paw.

Conclusions: The present results provide a new line of behavioral evidence that tonic activation of descending facilitatory pathway contributes to the establishment of 1) the BV and formalin-induced persistent spontaneous nociception; and 2) the BV-induced referred mirror heat hyperalgesia and the central sensitization, but not the primary heat and mechanical hyperalgesia.

SUBCUTANEOUS injection of bee venom (BV) into one hind paw of a rat produces not only a prolonged, persistent monosynaptic spontaneous nociceptive behavior, but also a subsequent primary heat and mechanical hyperalgesia in the injected paw and contralateral heat hyperalgesia (also referred to as referred mirror hyperalgesia) in the noninjected paw.1–3 It has been found that the BV-induced persistent spontaneous nociception (PSN), primary heat or mechanical hyperalgesia, and the referred mirror heat hyperalgesia may share different neural mechanisms at both peripheral and spinal levels.2,4–6 Moreover, the mirror heat hyperalgesia has also been proved to be induced by temporal spinal summation of ongoing inputs of primary afferents from the injection site and maintained by the central sensitization.7,8

It has been demonstrated that descending facilitatory pathways from the rostral medial medulla (RMM), including the gigantocellular reticular nucleus (Gi) and dorsal paragigantocellular nucleus (DPGi), play a key role in the transmission and processing of nociception induced by tissue and nerve injuries.9–11 For example, the RMM lesion prevented chemical injury-induced secondary, but not primary, hyperalgesia.9,12 However, to our best knowledge, the roles of the descending facilitatory pathway from the RMM in tissue injury-induced PSN and referred mirror hyperalgesia have not been investigated yet in these previous studies. Thus, the present study is designed to determine whether tonic activation of descending facilitatory pathway from the RMM contributes to the formalin and the BV-induced PSN and mirror hyperalgesia.

Materials and Methods

Animals

The experiments were performed on Sprague-Dawley albino male rats weighing from 250 to 300 g. The animals were provided by Laboratory Animal Center of the Fourth Military Medical University (FMMU) and use of the animals was reviewed and approved by the FMMU Animal Care and Use Committee. The International Association for the Study of Pain guidelines for pain research in conscious animals were followed.13 The animals were housed in plastic boxes in groups of 3 at 22–26°C with food and water available ad libitum in the colony room. A 12:12 h light dark cycle with lights on at 8:00AM was maintained and testing was done between 9:00AM and 6:30PM. The animals were acclimatized to the laboratory and habituated to the test boxes for at least 30 min each day for 5 days before testing. The rats were randomly divided into two groups: the lesion group contained rats with bilateral microinjections of ibotenic acid into the RMM (n = 13); the control group contained rats with bilateral microinjections of phosphate buffer saline (PBS) into the RMM (n = 13). Furthermore, each group was subdivided into two groups: the BV injection group (n = 7) and the formalin injection group (n = 6).
**Destruction of Bilateral RMM**

Under sodium pentobarbital-induced anesthesia (50 mg/kg, intraperitoneal), rats were placed in a stereotaxic instrument. After a midline incision, bilateral openings were made in the skull with a dental drill to make bilateral microinjections of a soma-selective neurotoxin ibotenic acid (0.3 μg/0.3 μl, Sigma Chemical, St. Louis, MO) or vehicle (PBS, 0.3 μl) into the RMM by lowering a 1-μl microinjection needle into the targets. According to stereotaxic coordinates suggested by Urban et al., the final coordinates for the injection site were calculated relative to the interaural line: −2.0 mm (rostral-caudal), 0.5 mm (medial-lateral), and −9.0 mm (dorsal-ventral). The solutions were manually delivered over 30 s, and the injection needle remained at the target site for additional 5 min. Five days after the RMM treatment, the behavioral testing was performed. After completion of the study, all rats were given an overdose of pentobarbital (100 mg/kg, intraperitoneal) and perfused intracardially with 150 ml physiologic saline followed by 500 ml 4% paraformaldehyde and the injection sites and lesion area in each animal were identified on coronal sections of the brain stem (40-μm thick) stained with 1% cresyl violet (Nissl stain).

**Intraplantar Administration of BV or Formalin**

The BV was lyophylized whole venom of Apis mellifera (Sigma Chemical) dissolved in 0.9% sterile saline. A volume of 50 μl saline containing 0.2 mg BV was used for the whole study, because it was shown that 0.2 mg in 50 μl was the optimal dose to produce a prolonged nociceptive behavioral response. For subcutaneous injection of formalin, 50 μl 2.5% formalin (0.925% paraformaldehyde diluted in 0.9% sterile saline) was used in the present study, based on our previous study.

**Measurement of PSN**

A transparent acrylic test box (30 × 30 × 30 cm) with a transparent glass floor was placed on a supporting frame of 50 cm high above the experimental table to allow the experimenters to observe the paws of the animals without obstruction. The rat was placed in the test box for at least 30 min before administration of BV or formalin. The spontaneous nociceptive behavioral response of rats was determined by counting the number of paw flinches during each 5-min interval during a 1-h time course following injection of BV or formalin.

**Examination of Hyperalgesia**

Paw withdrawal thermal latency (PWTL) and paw withdrawal mechanical threshold (PWMT) of both hind paws were examined prior to the RMM lesions and prior to or 4 h after BV injection, respectively. For examination of PWMT, mechanical stimuli were applied by 11 individual monofilaments with bending forces at 58.5, 78.4, 98.0, 147.0, 196.0, 294.0, 343.0, 392.0, 441.0, 490.0, and 588.0 mN. The rat was placed on a metal mesh floor covered with a plexiglas chamber (20 × 20 × 25 cm) and von Frey filaments were applied in an upgrade intensity order from underneath the metal mesh floor to the testing sites of the bilateral hind paws. A single von Frey filament was applied 10 times (once every several seconds) to each testing site. A bending force sufficient to evoke 50% of the paw withdrawal occurrence was expressed as PWMT. Following measurement of PWMT, rats were moved to the surface of a 2-mm thick glass plate covered with the same acrylic chamber. The latency of paw withdrawal reflex to heat stimuli (PWTL) was measured with an RTY-3 radiant heat stimulator (Xi’an Fenglan Instrumental Factory, China), as described previously. The radiant heat source was a high-intensity projector halogen lamp bulb (100 W, 4 V, 4th ventricle; DPGi, dorsal paragigantocellular nucleus; Gi, gigantocellular reticular nucleus; g7, genu of the facial nerve; Sp5, spinal nucleus of trigeminal nerve. Scale bar in A = 750 μm.
10.5 V) positioned under the glass floor directly beneath the targeting area on the hind paw. The distance between the projector lamp bulb and the lower surface of the glass floor was adjusted to produce a light spot on the floor surface with 5 mm diameter. Five stimuli were repeated to the same site and the mean PWTL was obtained from the latter 3 or 4 stimuli based on the consistency of the values. The interstimulus interval for each heat test was more than 10 min at the same region and 5 min at the different paw. To avoid excessive tissue injury, manual cutoff of the heat stimulus was performed if heat stimulation with 30 s failed to evoke a paw withdrawal reflex.

Statistical Analysis

All results were expressed as mean ± SD. The data between experimental and control groups were compared by using ANOVA followed by post hoc analysis (Scheffé test). P < 0.05 was considered to be statistically significant.

Results

In line with some previous reports, bilateral microinjections of ibotenic acid into the RMM produced an obvious lesion of neurons as determined by histologic observation. Figure 1A shows an example of the result following bilateral lesions of the RMM, the injection sites and lesion area were confined to an area in the giganto-cellular reticular nucleus (Gi) and the dorsal paragigantocellular nucleus (DPGi) (fig. 1A). The summary of 13 pairs of lesions within the RMM is shown in figure 1B. The hatched area in both sides of the scheme represents the size of the area where the lesions could be clearly identified according to histologic location in 13 animals receiving ibotenic acid (fig. 1B).

Effect of Bilateral RMM Lesions on BV or Formalin-induced PSN

As described in our previous reports,1–3 subcutaneous injection of BV into the plantar surface of one hind paw of control group rats produced a persistent, prolonged spontaneous flinching reflex in a monophasic manner (39.76 ± 2.04 times/5 min, n = 6) (fig. 2A). Compared with the control group, bilateral RMM lesions produced a significant inhibition of the BV-induced total flinching reflex (20.69 ± 2.63 times/5 min, n = 7) (fig. 2B). However, the time course observation showed that the RMM lesion-produced inhibitory effect was only statistically significant in the late 50 min, but without significant effect on that in the initial 0–10 min induced by either BV or formalin. **P < 0.01. Vertical bars: ±SD.

**Fig. 2. Effects of bilateral rostral medial medulla (RMM) lesions on the bee venom (BV)-induced (A, B) or the formalin-induced (C, D) persistent spontaneous flinching reflexes (PSFR).** Graphs A and C show the time courses of the BV- and the formalin-induced PSFR in control (open squares) and the RMM-lesioned (filled squares) groups, respectively. Graphs B and D show the mean numbers of the BV- and the formalin-induced PSFR per 5 min of a 1-h total time course (left column), the initial 10-min period (middle column) and the last 50 min period (right column), respectively, in control and the RMM-lesioned groups. ANOVA post hoc Scheffé test analysis showed that bilateral lesions of the RMM produced significant inhibition of the total PSFR and that in the late 50 min, but without significant effect on that in the initial 0–10 min induced by either BV or formalin. **P < 0.01. Vertical bars: ±SD.
that bilateral RMM lesions produced a similar significant inhibition of the formalin-induced flinching reflex in phase 2; however, the RMM lesions were not able to prevent occurrence of phase 1 response of the formalin test (fig. 2C, D).

**Effect of Bilateral RMM Lesions on BV-induced Hyperalgesia**

Compared with the values before surgery (baseline), bilateral RMM treatment with ibotenic acid or PBS did not alter baseline PWMT or PWTL of both hind paws (fig. 3A–C). The results are consistent with previous reports, suggesting that the RMM does not exert descending influence on the baseline nociceptive threshold under the physiologic conditions. Moreover, in good agreement with our previous results, subcutaneous BV injection in control rats produced a significant decrease in PWMT and PWTL of the injected paw (fig. 3A and B, table 1) and a significant decrease in PWTL of the noninjected paw (fig. 3C, table 1). However, bilateral RMM lesions significantly prevented the development of the BV-induced referred mirror heat hyperalgesia in the noninjected paw (fig. 3C, table 1), but had no effect on the BV-induced primary heat and mechanical hyperalgesia in the injected paw (fig. 3A and B, table 1).

**Discussion**

The results present an interesting finding that tonic activation of descending pathway from the RMM of brain stem to the spinal dorsal horn is responsible for the development of both BV-induced and formalin-induced PSN and for the establishment of the BV-induced referred mirror heat hyperalgesia (central sensitization), but not the primary heat and mechanical hyperalgesia identified in the injection site. This adds further evidence for the involvement of the RMM-spinal dorsal horn (SDH) descending facilitatory system in the BV-induced referred mirror hyperalgesia as well as the BV-induced and formalin-induced persistent spontaneous nociception.

Although many previous studies have investigated the role of the RMM-SDH system in tissue or nerve injury-induced hyperalgesia, its role in some tissue injury-induced PSN has not been well determined. In the present study, we observed that bilateral RMM lesions produced a significant inhibition of the persistent spontaneous flinching reflexes induced by either subcutaneous BV or formalin, suggesting an involvement of the descending facilitation from the RMM in the developing processes of some chemical tissue injury-induced PSN. More interestingly, the time course observation showed that bilateral RMM lesions produced a similar significant blocking effect on the flinching reflexes occurring in the late 50 min period but without significant influence on

![Fig. 3. Effects of bilateral rostral medial medulla (RMM) lesions on the bee venom (BV)-induced primary mechanical (A) and heat (B) hyperalgesia in the injection site and the referred mirror heat hyperalgesia (C) in the noninjected contralateral hind paw of rats. Graphs A, B, and C show that the baseline values of paw withdrawal thermal latency (PWTL, s) and paw withdrawal mechanical threshold (PWMT, mN) of both hind paws could not be influenced by injections with either ibotenic acid (right panels for Lesion) or phosphate buffered saline (left panel for Control) in bilateral RMMs. As compared with the control groups (left panels in A and B), bilateral lesions of the RMM did not produce any influences on the established primary mechanical and heat hyperalgesia in the injection site (right panels in A and B). However, compared with the control group (left panel in C), bilateral RMM lesions resulted in a complete blockade of the referred mirror heat hyperalgesia in the contralateral hind paw (right panel in C). The results suggest that activation of the RMM-spinal dorsal horn descending pathways is involved in development of the mirror heat hyperalgesia, but not in primary heat and mechanical hyperalgesia. Baseline, PWMT and PWTL values prior to any treatment; After surgery, PWMT and PWTL values measured 5 days after the RMM injections of ibotenic acid or PBS; Surgery+BV, PWMT and PWTL values measured 2–4 h after subcutaneous BV injection in both PBS and ibotenic acid treatment. *P < 0.05; **P < 0.01; n.s., no significant. Vertical bars: ±SD.

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the immediate 0–10 min period of the BV response or phase 1 of the formalin response. This suggests that tonic activation of the RMM-SDH descending facilitatory system might be dependent upon the temporal summation of primary afferent inputs from the injured site to the spinal cord, and then to the RMM. Since we have found that the establishment of the BV-induced referred mirror heat hyperalgesia is a consequence of central sensitization, which requires a given time window for temporal summation of ongoing primary afferent barrage originating from the injured sites,7,8 we therefore infer that a time window is also required for establishment of functional synaptic connection between the RMM and the SDH, which in turn maintains the sensitized state of dorsal horn nociceptive neurons following the BV injection. This presumption is supported by the results of the present study, showing that the BV-induced referred mirror heat hyperalgesia is also maintained by an intact circuit from the RMM to the spinal dorsal horn. Taken together, we conclude that the RMM-SDH descending system can be activated by the ongoing primary afferent that is required for establishment of central sensitization in the spinal dorsal horn, which in turn leads to behavioral expression of referred mirror heat hyperalgesia and the remaining late period of PSN.

It has been proven that an intact RMM-SDH descending system is required for establishment of secondary hyperalgesia but not for primary hyperalgesia in several animal pain models.9 For example, the RMM lesions prevented the development of secondary hyperalgesia induced by intraarticular injection of carrageenan or kaokin into the knee and topical application of mustard oil to the hind leg, but not primary hyperalgesia induced by intraplantar injection of carrageenan into one hind paw.12 In the present study, we found that the bilateral RMM lesions could cause elimination of referred mirror heat hyperalgesia, but without such effect on the BV-induced primary heat and mechanical hyperalgesia. This suggests that the RMM-SDH descending system contributes to the secondary as well as the referred mirror hyperalgesia, but not to the primary hyperalgesia.9,12 Since the referred mirror heat or mechanical hyperalgesia can be observed in other animal models of pain,16–19 the involvement of RMM-SDH descending system in their developing processes needs to be further examined. It has been proposed that the referred mirror hyperalgesia is likely to be established by a functional synaptic connection between bilateral spinal dorsal horns through dorsal commissural fibers.7,8,20 It is interesting to find that the ascending spinoreticular and descending RMM-SDH pathways are also likely to be an alternative pathway responsible for development of referred mirror heat hyperalgesia identified in the BV test.

Based on the anatomic relationship between the spinal cord and the RMM,21,22 and based on our present and previous studies,1,7,8 we highly favor a positive feedback loop between the RMM and the spinal cord, which contributes to the development and maintenance of the BV-induced PSN and central sensitization. In this case, the ongoing primary afferent inputs from the BV injection site are conveyed to the spinal cord and then probably to the RMM through the ascending spinoreticular projection.22 It thus produces tonic activation of the descending facilitatory system, which is further maintained by temporal summation of the ongoing primary afferent in the dorsal horn of the spinal cord. Through bilateral descending projections, the activated RMM neurons might either facilitate the release of excitatory amino acids and substance P from the central terminals of primary afferent or enhance the hyperexcitable process of postsynaptic nociceptive neurons in the ipsilateral SDH eventually resulting in PSN. However, they might also cause hypersensitivity of the contralateral SDH neurons resulting in referred mirror heat hyperalgesia. It is also possible that the facilitation of ongoing primary afferent inputs to the spinal cord by the RMM activation may contribute to hypersensitivity of contralateral spinal dorsal horn neurons through an intercommunication mediated by dorsal commissural fibers within the spinal cord.20 As we have demonstrated in our previous and unpublished data, both PSN and mirror heat hyperalgesia induced by subcutaneous BV is involving persistent coactivation of various spinal receptors, ionic channels, and intracellular cascades, such as gluta-

### Table 1. Comparisons of PWTL and PWMT Values between Control and RMM-lesioned Groups

<table>
<thead>
<tr>
<th>PWTL, s</th>
<th>Control</th>
<th>RMM Lesion</th>
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<tbody>
<tr>
<td>Injection site</td>
<td>Baseline</td>
<td>After Surgery</td>
</tr>
<tr>
<td>PWTL, mN</td>
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<tr>
<td>Injection site</td>
<td>14.23 ± 1.35</td>
<td>13.85 ± 1.34</td>
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<tr>
<td>Contralateral hindpaw</td>
<td>13.59 ± 2.10</td>
<td>13.00 ± 1.44</td>
</tr>
<tr>
<td>Injection site</td>
<td>487.60 ± 44.12</td>
<td>522 ± 52.08</td>
</tr>
<tr>
<td>Animals, n</td>
<td>7</td>
<td>7</td>
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Values are from control and RMM-lesioned groups before and 5 d after surgery and 4 h after injection with bee venom. BV = bee venom; PWMT = paw withdrawal mechanical threshold; PWTL = paw withdrawal thermal latency; RMM = rostral medial medulla.

*P < 0.05; †P < 0.01 vs. values after surgery.
mate N-methyl-d-aspartate (NMDA)/non-NMDA and neurokinin1/2 receptors, N- and P/Q-subtypes of voltage-sensitive calcium channel, and intracellular messengers, such as protein kinase C, protein kinase A, cyclooxygenase 2, and nitric oxide synthesis. Therefore, it is reasonable to conclude that activations of the above interneuronal or intraneuronal chemical signal transduction pathways are likely to be involved in activation of bilateral RMMs, which in turn results in maintenance of both PSN and referred mirror heat hyperalgesia.

In conclusion, the present study provided a new line of behavioral evidence that tonic activation of the RMM-SDH descending facilitatory pathway contributes to the establishment of the following: 1) the BV and formalin-induced persistent spontaneous nociception, and 2) the BV-induced referred mirror heat hyperalgesia and the central sensitization, but not the primary heat and mechanical hyperalgesia.

References

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