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Research Paper

Effect of stimulation and lesion of the thalamic nucleus submedius on formalin-evoked nociceptive behavior in rats

LI Yang[1,2], YUAN Bin[1], TANG Jing-Shi[1,*]

[1]Department of Physiology and Pathophysiology, Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education, Xi'an Jiaotong University School of Medicine, Xi'an 710061, China; [2]Department of Medicine, Tibet Nationalities Institute, Xianyang 712082, China

Abstract: The aim of the present study was to examine whether the thalamic nucleus submedius (Sm) was involved in the modulation of persistent nociception. Using an automated movement detection system to measure nociceptive behavior (agitation) induced by subcutaneous injection of formalin into the hind paw pad, the effects of electrical stimulation or electrolytic lesion of the Sm on the agitation response were examined in conscious rats. Unilateral stimulation (100 μA, 5 min) of the Sm ipsilateral or contralateral to the formalin-injected paw produced a significant inhibition of agitation response in the second phase, while stimulation of thalamic structures more than 0.5 mm away from the Sm had no effect on agitation response. Bilaterally electrolytic lesion of the Sm did not significantly influence the number of agitation events induced by formalin injection in the first phase or the second phase. These results suggest that the Sm is not only involved in the modulation of phase nociception, as reported previously, but also of persistent nociception. The present study provides novel evidence for the participation of the Sm in descending modulation of pain.

Key words: thalamic nucleus submedius; persistent nociception; formalin test; rat

电刺激和损毁丘脑中央下核对大鼠福尔马林诱发伤害性行为的影响

李 杨[1,2]，袁 斌[1]，唐敬师[1,*]

[1]西安交通大学医学院生理学与病理生理学系，环境与疾病相关基因教育部重点实验室，西安 710061；[2]西藏民族学院医学系，咸阳 712082

摘 要：本文旨在研究丘脑中央下核(thalamic nucleus submedius, Sm)是否参与持续伤害感受性调制。以自动运动检测系统记录大鼠一侧后爪皮下注射福尔马林诱发的伤害性行为(烦乱反应)为指标，观察电刺激和电解损毁 Sm 对烦乱反应的效应。结果显示， 电刺激(100 μA, 5 min)同侧或对侧 Sm 明显抑制福尔马林诱发的第二时相的烦乱反应，而刺激 Sm 外邻近结构(超过 0.5 mm)对烦乱反应无明显效应。电解损毁双侧 Sm 对第一或第二时相的烦乱反应均无影响。结果提示，Sm 不仅参与急性时相性伤害感受性调制，也参与持续性伤害感受性调制。本研究为 Sm 参与下行痛调制提供了新的证据。

关键词：丘脑中央下核；持续性痛；福尔马林试验；大鼠
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Anatomic studies in rats and cats have established that the thalamic nucleus submedius (Sm) receives a major projection from the medulla and spinal dorsal horn lamina I and projects to the ventrolateral orbital cortex (VLO)[1-5]. The VLO contains neurons that project to the periaqueductal gray (PAG)[6,7], a region involved in descending the modulation of nociception[8]. Previous studies have demonstrated that bilaterally electrolytic lesion of the Sm facilitates the rat tail flick reflex and attenuates analgesia produced by peripherally electrical stimulation[9,10]. Furthermore, electrically or chemically evoked activation of the Sm depresses the tail flick reflex, jaw-opening reflex and nociceptive response of spinal dorsal horn neurons; these effects can be eliminated by electrolytic lesion or by

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*Corresponding author. Tel: +86-29-82655172; Fax: +86-29-82656364; E-mail: jstang@mail.xjtu.edu.cn

microinjection of inhibitory transmitter γ-aminobutyric acid (GABA) into the VLO or PAG[11-14]. These findings suggest that the Sm-VLO-PAG pathway is involved in the modulation of nociception and that the activation of this pathway may lead to activation of the descending inhibitory system in the brainstem and depression of the nociceptive input at the spinal level. However, it is unclear whether the Sm is also involved in the modulation of persistent nociception. To clarify this issue, the effects of electrical stimulation and electrolytic lesion of the Sm on nociceptive behavior (agitation) induced by the injection of formalin into the hind paw pad of conscious rats were examined in the present study.

1 METERIALS AND METHODS

1.1 *Animal preparation*

The experiments were performed on 31 male adult Sprague-Dawley rats weighing 220-350 g provided by the Medical Experimental Animal Center of Shaanxi Province, China. The animals were anesthetized with sodium pentobarbital (50 mg/kg body weight) and the animal's head was positioned in a stereotaxic frame. A small craniotomy was performed over the thalamus and a bipolar electrode (diameter 180 μm, tip cross-section exposed) was stereotaxically placed in the Sm (2.3-3.0 mm caudal to Bregma, 0.6-0.9 mm lateral to Bregma, 6.5 mm below the cortical surface)[15] and fixed on the skull with 4 microscrews and dental cement. In the lesion experiment, a pair of insulated steel wire electrodes (base diameter150 μm, tip diameter 70 μm exposed 200 μm) was placed onto the bilateral Sm. The electrolytic lesion was induced by passing 0.2 mA anodal direct current through each electrode for 15 s with a reference electrode placed in the wound margin. Then, the lesion electrodes were removed and the wound was closed. After the animal recovered from anesthesia, sodium penicillin (200000 U/d, for 3 d, intraperitoneally) was administered to prevent wound and intracerebral infection. The experiments were performed according to the Guidelines of the International Association for the Study of Pain[16].

1.2 *Formalin test*

A simple automated movement detection system for the formalin test in conscious rats has been described in great detail previously[17,18]. Briefly, 7 d after surgery, the animals were moved to the laboratory and placed in a polycarbonate box (H, 16 cm; L, 16 cm; W, 10 cm) for about 30 min to adapt to the experimental environment. Then, formalin (5%, 50 μL) was administered subcutaneously (s.c.) into the rat hind paw pad and immediately replaced in the box, which was placed on a spring balance (weight range 0 to 2 kg). Nociceptive behavior (agitation) elicited by the formalin injection, such as licking, flinching, shaking, elevating, dysphoria, clutching and favoring the affected paw, induced movement of the spring balance that was transformed into electrical signals via an electromagnetic transducer. The electrical signals were amplified, filtered (1.0 kHz), displayed on an oscilloscope and fed into a window discriminator and a computer system that allowed quantitative recording of the number of agitation events and construction of a time histogram over a 60-minute observation. An interval of at least one week was allowed before the same animal was tested again, to ensure that the swelling or small blisters had disappeared in the formalin-injected paw. If not, the animal was excluded from the study. In some experiments, the response to formalin injection was monitored by measuring the total duration (s) of licking/lifting of the injected hind paw every 5 min during a 60-minute observation after formalin injection, as reported previously[18,19].

1.3 *Intracerebral stimulation*

Electrical stimulation, consisting of 200 ms trains of 0.3 ms pluses at 40 Hz, twice/s for 5 min, 100 μA, was delivered to the Sm contralateral or ipsilateral to the affected paw during 25-30 min or 35-40 min after formalin injection to observe the effect on formalin-evoked nociceptive behavior.

1.4 *Histology*

At the end of the experiment the stimulation site was marked by passing a 0.1 mA anodal direct current through the stimulation electrode for 10 s. Under deep anesthesia the animal was perfused transcardially with 0.9% normal saline followed by 10% formalin containing 2% potassium ferrocyanide. The brain was then removed and fixed in fresh formalin for 3-7 d. Sections of 80 μm were cut with a freezing microtome, mounted, and stained with cresyl violet. Examples showing the stimulation site and the bilateral lesion sites in the Sm were shown in Fig.1.

1.5 *Data analysis*

All data were expressed as means±SEM. Time-course curves of the effects of stimulation or lesion of the Sm on agitation response elicited by formalin were plotted by calculating the mean agitation event rate (Hz) per 5 min during 60-minute observation and analyzed statistically by repeated measures of analysis of variance (RM ANOVA) with a *post hoc* multiple comparison (Bonferroni *t*-test or Tukey test) for analysis of the differences over the entire

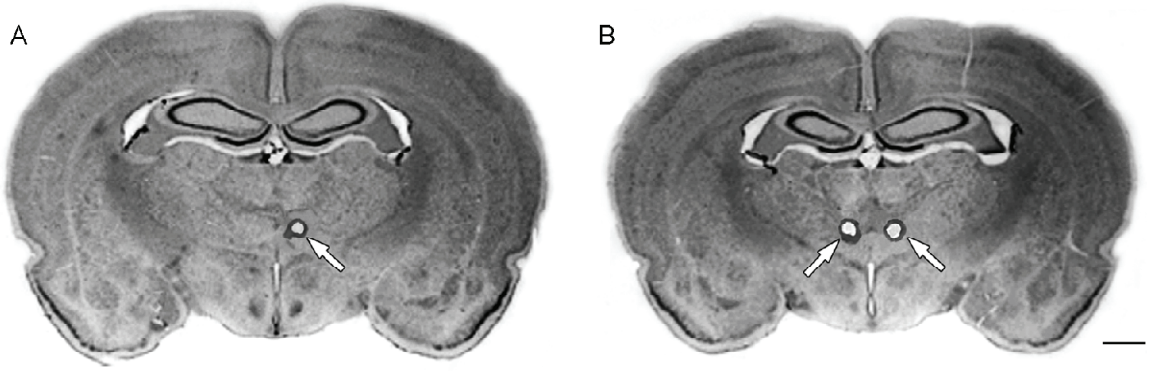

Fig. 1. Photomicrographs showing the locations of the stimulation site (*A*) and the bilateral lesion sites (*B*) in the thalamic nucleus submedius (Sm). The arrows indicate the stimulation or lesion sites. Scale bar, 1 mm.

observation or at each time point among different groups. $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Formalin-evoked agitation response

Subcutaneous injection of formalin into the rat hind paw pad produced a typical two-phase nociceptive behavior (agitation) clearly quantified by the automated movement detection system. The first phase (early phase) began immediately after formalin injection and lasted approximately 5-8 min and was followed by a low active or silent period of 6-10 min. The second phase (late phase) began 15 min after formalin injection and lasted more than 50 min. The formalin-induced nociceptive behavior was consistent with those reported previously, either with the automated detection system or manual detection methods[19-21], as shown in Fig.2.

2.2 Inhibitory effect of electrical stimulation of the Sm on nociceptive behavior induced by formalin

2.2.1 Stimulation of the contralateral Sm

During the late phase (25-30 min and 35-40 min) of the agitation response after formalin injection, a unilateral electrical stimulation delivered to the Sm contralateral to the formalin-injected paw, significantly depressed the agitation response. An example was shown in Fig.3*A*. The mean agitation event rates during the 25-30-minute and 35-40-minute stimulation were (12.3±1.9) Hz and (10.3±1.4) Hz (n=10), respectively, which were significantly lower ($P<0.01$, $P<0.001$) than that of pre-stimulation [(17.1±1.1) Hz and (19.7±1.6) Hz], as shown in Fig.3*B*. As a control experiment, the effect of electrical stimulation of the Sm on duration of paw licking/lifting induced by formalin was also examined using the manual recording method. A significant inhibition was observed during the same stimulation period. The duration of paw licking/lifting during the

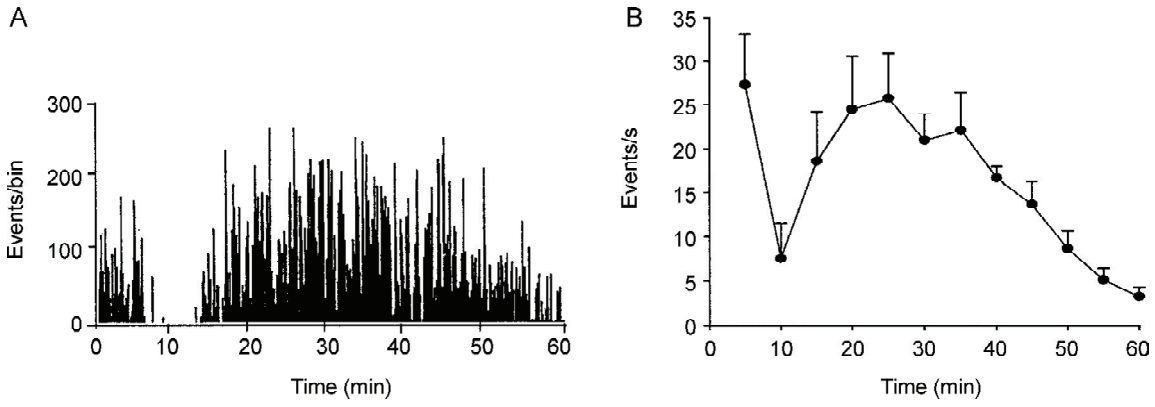

Fig. 2. Typical biphasic nociceptive response (agitation) induced by formalin (5%, 50 μL) subcutaneously injected into the unilateral hind paw pad measured by an automated movement detection system. *A*: A representative time histogram of agitation events recorded by this system. Bin width=3 s. *B*: Time course curve of the mean event rates (Hz) of the agitation response after formalin injection per 5 min during 60-minute observation in 14 rats.

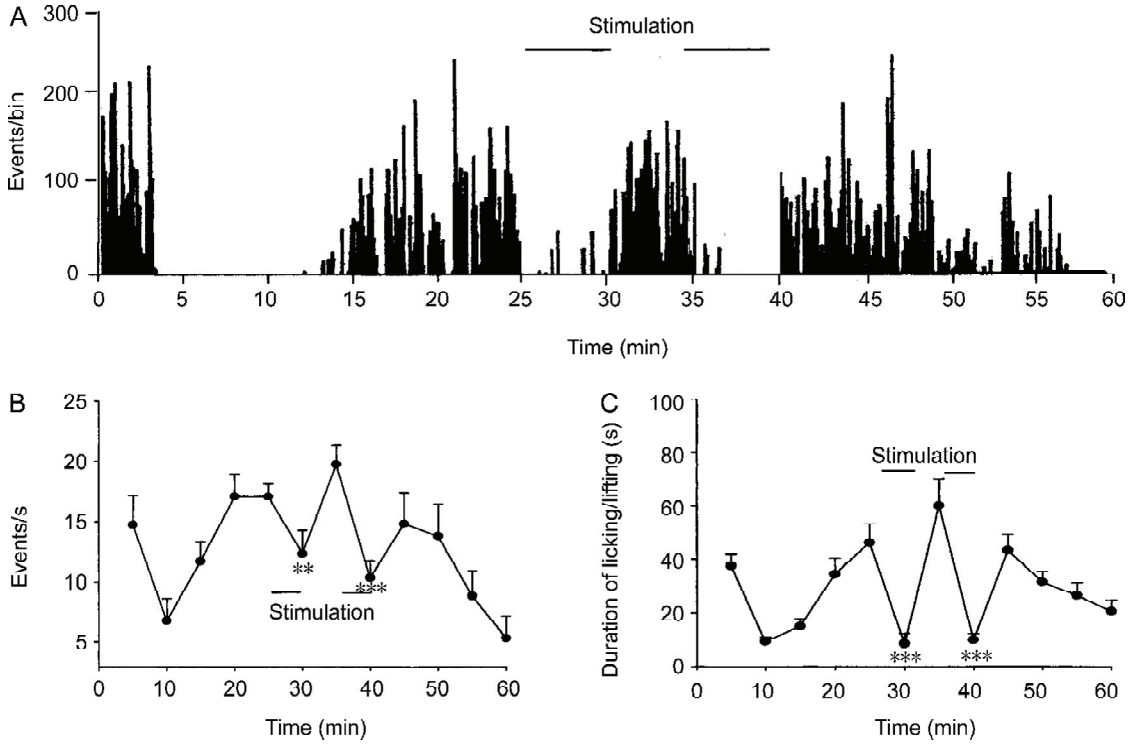

Fig. 3. Inhibitory effect of electrical stimulation of the Sm contralateral to the affected paw on the nociceptive response induced by formalin during 25-30-minute and 35-40-minute periods. *A*: Representative time histogram of the agitation events recorded by the automated movement detection system. Bin width=3 s. *B*: Time course curve of the mean event rates (Hz) of the agitation response after formalin injection per 5 min during 60 minute recorded by the automated movement detection system. $^{**}P<0.01$, $^{***}P<0.001$ compared with pre-stimulation (n=10). *C*: Time course curve of the mean duration (s) of licking/lifting the affected paw per 5 min during 60 minute recorded manually. $^{***}P<0.001$ compared with pre-stimulation (n=10).

25-30-minute and 35-40-minute stimulation were (8.3±3.9) s and (9.7±2.4) s (n=10), respectively, which was significantly lower ($P<0.001$) than that of pre-stimulation [(46.1±7.1) s and (60.1±10.0) s], as shown in Fig.3*C*.

In another experiment in 3 rats, electrical stimulation of the adjacent structures, central medial thalamic nucleus (n=2) and ventrolateral thalamic nucleus (n=1), more than 0.5 mm away from the Sm, had no effect on formalin-induced nociceptive behavior (data not shown).

2.2.2 Stimulation of the ipsilateral Sm

In a group of 13 rats, electrical stimulation of the Sm ipsilateral to the injected paw also significantly attenuated agitation response induced by formalin during the 25-30-minute and 35-40-minute stimulation. The mean agitation event rates [(10.0±1.3) Hz and (9.3±1.6) Hz, n=13] were significantly lower ($P<0.001$) than that of pre-stimulation [(17.9±1.9) Hz and (20.8±3.3) Hz], but were not different from that of stimulation of the contralateral Sm [$F_{(1,231)}$= 1.021, P=0.324], as shown in Fig.4 and Fig.3*B*.

2.2.3 Repeatability of Sm stimulation-evoked inhibition

In 10 rats that received repeated formalin injections, with an interval of at least one week, followed by electrical stimulation (100 μA) of the Sm during 25-30-minute and 35-40-minute periods, the agitation response was depressed in a similar manner, and no significant difference was found

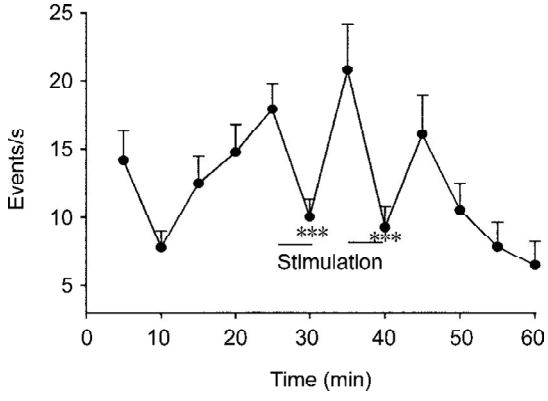

Fig. 4. Inhibitory effect of electrical stimulation of the Sm ipsilateral to the affected paw on the nociceptive response induced by formalin during 25-30-minute and 35-40-minute periods. $^{***}P<0.001$ compared with pre-stimulation. n=13.

in agitation response [$F_{(1,198)}$=0.567, P=0.461, n=10], suggesting that this effect was reproducible in the same animals, as shown in Fig.5.

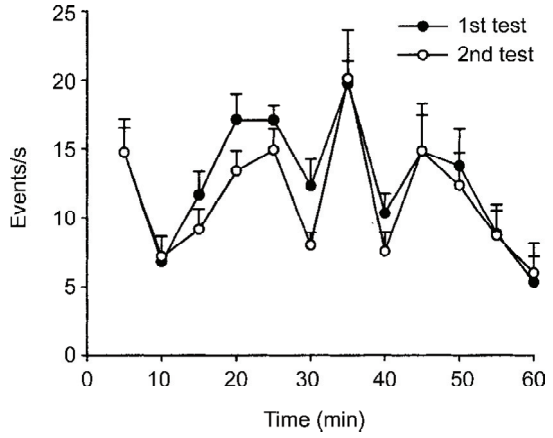

Fig. 5. Inhibitory effect of stimulation of the Sm on the event rates of agitation response induced by formalin injection was reproduced at an interval of one week in the same animal. No significant difference was found in the nociceptive response. n=10,

2.3 Effect of electrolytic lesion of the bilateral Sm on nociceptive behavior

One week after bilaterally electrolytic lesion of the Sm, the mean agitation event rate [(21.9±2.8) Hz] induced by formalin injection during a 60-minute observation was not significantly different [$F_{(1,77)}$=0.0002, P=0.99, n=8] from that of pre-lesion [(27.3±5.8) Hz]. At any time point there was no significant difference between pre- and post-lesion, as shown in Fig.6.

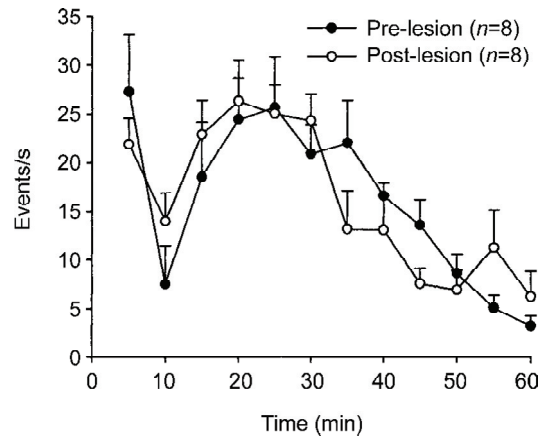

Fig. 6. Effect of bilaterally electrolytic lesion of the Sm on the agitation response induced by formalin injection one week after lesion. There was no significant difference during the entire observation between pre- and post-lesion.

3 DISCUSSION

The formalin test, as a model of tonic pain, has been commonly used in studies of nociception and its modulation in rats[19-23]. The present study has demonstrated that electrical stimulation delivered to the Sm depresses nociceptive behavior in the second phase using an automated movement detection system that qualitatively records the agitation response, and a manual recording method that records the duration of paw licking/lifting. This result is consistent with previous studies showing that electrically or chemically evoked activation of the Sm depressed the tail flick reflex, jaw opening reflex and nociceptive responses of neurons in the spinal dorsal horn[11-14]. These results suggest that the Sm is involved in the modulation of nociception, not only in the acute phase nociception but also in persistent nociception. Since the response in the first phase elicited in the formalin test is believed to be a result of direct activation of peripheral nociceptors, whereas the response in the second phase is mediated by a combination of low ongoing activity in the primary afferents and increased sensitivity of the spinal cord neurons (central sensitization)[22,23], which is similar to clinical symptoms with persistent and inflammatory pain. Thus, results of this study, that activation of the Sm depresses formalin-induced nociceptive behavior, may be of significant importance for elucidating the underlying mechanisms of the inflammatory and persistent pain.

Anatomical studies have established that the Sm receives not only contralateral but also ipsilateral projections from the spinal dorsal horn neurons[1-5]. Electrophysiological studies have demonstrated that Sm neurons responded to peripheral noxious stimulation and receptive fields of the neurons are very large and bilateral throughout the entire body[24]. Therefore, it is not surprising that electrical stimulation of the Sm, either contralateral or ipsilateral to the affected paw, depresses formalin-induced nociceptive behavior. Because electrolytic lesion or chemical depression of the VLO or the PAG eliminates the Sm-mediated antinociceptive effect[11-14], it is reasonable to presume that this effect may be produced by activation of the Sm-VLO-PAG pathway, leading to activation of the brainstem descending inhibitory system and depression of the nociceptive input at the spinal cord level.

Early evidence showed that intracerebrally electrical stimulation with 200 μA produced an effective current spread of about 0.5 mm from the electrode tip in rats[25,26]. Thus, the inhibitory effect of Sm activation on formalin-induced nociceptive behavior may be not a result of the

stimulation current spreading to other structures adjacent to the Sm, as reported previously by Zhang *et al*.[11] in the tail flick test in rats.

Recent studies have indicated that microinjection of morphine or the selective μ-opioid receptor agonist endomorphin-1 into the Sm depressed formalin-induced nociceptive behavior and the nociceptive response of neurons in the spinal dorsal horn. These effects were blocked by the non-selective opioid receptor antagonist naloxone or the selective μ-opioid receptor antagonist β-funaltrexamine hydrochloride (β-FNA)[27,28]. Also, microinjection of naloxone into the Sm blocked the analgesia induced by electroacupuncture stimulation of the acupoint[29]. These results suggest that the Sm is involved in the opioid-receptor-mediated antinociceptive effect or acupuncture analgesia, however, further studies are required to clarify the neurochemical mechanisms of the Sm-induced antinociceptive effect.

In addition, this study indicates that bilaterally electrolytic lesion of the Sm does not significantly influence nociceptive behavior in the first or second phase induced by formalin, suggesting that the Sm may lack a tonic inhibitory action on the nociceptive response under tonic pain conditions. It is inconsistent with that under phasic pain conditions, as reported by Zhang *et al*.[10] that electrolytic lesion of the bilateral Sm facilitates the rat tail flick reflex.

In summary, the present study suggests that the Sm is involved not only in the modulation of acute and phasic nociception, as reported previously, but also in the modulation of persistent nociception. It provides novel evidence for the participation of the Sm in descending modulation of pain.

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