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Effect of 5-HT on pain modulation of substance P in spinal cord of rats

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AIM: To study the effect of serotonin (5-HT) on pain modulation of substance P (SP) in spinal cord of rats. **METHODS**: Using immunohisto-chemistry and measurement of pain threshold. **RESULTS**: The c-*fos* expression evoked by intrathecal injection (it) SP 10 μg and sc 5 % formaldehyde (For) 150 μL in the hindpaw was densely distributed in the laminae Ⅰ, Ⅱ, Ⅴ, and Ⅵ of spinal dorsal horn. The pain threshold in the SP group was decreased while the pain intensity rating measured by behavioral method in the For group was increased. The c-*fos* expression induced by it 5-HT 20 μg was mostly distributed in the spinal dorsal horn in laminae Ⅱ — Ⅳ and the pain threshold was increased. SP and For induced c-*fos* expressions in the spinal cord and the pain responses were reduced by 5-HT and increased by 5-HT depletor fenclonine 300 mg·kg^{-1}. **CONCLUSION**: SP mainly played an algogenesia in the spinal cord. 5-HT inhibited the c-*fos* expression in the spinal cord evoked by SP and participated in pain modulation of SP.

KEY WORDS serotonin; substance P; proto-oncogene proteins c-*fos*; formaldehyde; fenclonine; spinal cord; immunohisto-chemistry; pain threshold

Proto-oncogene c-*fos* belongs to a family of cellular immediate early genes. Its expression within some neurons of spinal cord can be induced by noxious stimulation, such as formalin[1-3], and is regarded as a marker for neuronal activity following noxious stimulation[1,2]. The c-*fos* protein has been viewed as "the 3rd messager" molecule in coupling short-term signals elicited by extracellular stimulation tolong-term alteration in cellular phenotypes by regulating expression of specific target genes[4]. Serotonin (5-HT), a neurotransmitter of CNS, participates in analgesia and inhibits noxious response[5,6]. While substance P (SP) is an excitatory neurotransmitter released from nociceptive primary afferent nerve terminals, and takes a double effect of analgesic and algogenic in pain modulation of the spinal cord[7]. This experiment is to compare the c-*fos* expressions evoked by SP, 5-HT, and formaldehyde (For) in spinal cord, to comprehend the relationship between the 3 stimulators, and to understand the effect of SP on pain modulation in spinal cord.

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MATERIALS AND METHODS

Experiments were performed on 62 Wistar rats, ♀ and ♂, weighing 210±*s* 10 g.

SP (Sigma) and 5-HT (Sigma) were both dissolved in artificial cerebrospinal fluid (pH 5.5). Fenclonine (Fen, Koch-Linht) was dissolved in 0.9 % NaCl containing NaOH and then the solution was neutralized with HCl. Formaldehyde (For) was dissolved in 0.9 % NaCl. Anti-c-*fos* serum was purchased from Cambridge Research Biochemicals and ABC kits from Vector.

Experimental procedures The rats were divided into control and experimental groups. Control group (8 rats): 2 rats receiving no stimulation; 2 rats were injected in the right hindpaw with isotonic saline (150 μL, 2 h); 2 rats were intrathecally injected (it) solution which dissolved SP and 5-HT, 1 h; 2 rats were it solution dissolved Fen, 3 d. The treatment groups: rats were it SP (7 rats) and 5-HT (6 rats); 6 rats were it Fen and 7 rats were injected in the right hind-

paw with For.

Intrathecal cannulation In rats under pentobarbital anesthesia, a cannula (PE-10 tubing) was inserted through the cisterna magna 7 cm to the L_4 segment in the spinal subarachnoid space[8]. A recovery period of 5—7 d was allowed, and only those rats showing no motor impairment were used for experiments. Drugs were injected in a volume of 15 μL and flushed in with 5 μL saline. Injections were delivered within 5 min. The solution of same volume was given to the control group without drugs.

Measurement of pain The method of the electrical stimulation of the tail[9] was applied and injection of For into hindpaw was carried out according to the behavior method. The rats were sc 5 % For 150 μL in the right hindpaw. The pain intensity rating (PIR) which is a standard guide for measuring pain response was recorded according to the four marks of pain response intensity of Dubuisson's[10]. The pain response is positively correlated to the PIR.

Immunohistochemistry Using c-*fos* antibody, we detected the c-*fos* protein expression in the spinal cord by immunohistochemistry. After above experiment, rats were deeply anesthetized with ip sodium pentobarbital 50 mg·kg^{-1}. The rats were perfused transcardially with 100 mL saline followed by 4 % paraformaldehyde PB solution 1 L. There moved lumbar enlargements of spinal cord were transferred to a 20 % sucrose solution at 4 ℃. Tissue sections (40 μm) were cut on a freezing microtome when the sample sank down to the bottom of the container and processed for immunohistochemistry using the ABC technique. Briefly, after being incubated in c-*fos* antibody (1:2000) for 48 h at 4 ℃, the sections were successively incubated in biotinylated anti-goat IgG (1:200) and avidin-biotin-HRP complex (1:100) for 3 h at room temperature (25 ℃), which were then visualized with the glucose oxidase-DAB-Ni protocol. In controls, c-*fos* antibody was replaced by normal goat serum or PBS (0.01 mol·L^{-1}) and processed as the experimental group.

Statistics Counts of immunoreactive cells were made over the L_3—L_4 in 4 sections taken from each rat, and the mean number of c-*fos*-like immunoreactivity (FLI) of each rat was recorded. The experiment data were analyzed using *t* test (PDA-2) by computer.

RESULTS

The laminas of rat spinal cord were divided according to the atlas of Molander[11]. To describe easily, we combined laminae I and II into superficial; laminae III and IV into nucleus proprius; laminae V and VI into neck; the rests were ventral, including laminae VII—X (Fig 1A).

Little c-*fos* protein immunoreactivity was found in control and Fen-treated rats (Fig 1A, 1G; Tab 1).

Effect of SP and For on pain threshold and c-*fos* of spinal cord SP (10 μg) it reduced the pain threshold in the rats. One h

Tab 1. Effect of 5-HT on pain threshold by electric stimulation of rat tail and c-*fos* protein-like immunoreactivity neurons in spinal cord of rats evoked by SP and formalin (For). $n = 6-8$, $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control; $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* SP; $^gP > 0.05$, $^hP < 0.01$ *vs* For.

	Number of c-*fos*-like immunoreactive neurons				Changes of pain threshold/%
	I — II	III — IV	V — VI	VII — X	
Control	3.6±1.2	0.6±0.7	0.1±0.4	0.1±0.4	100
SP	42.4±8.2^c	16.4±3.8^c	37.3±9.8^c	20.3±7.7^c	80.5±6.3^b
For	48.0±9.7^c	14.8±4.1^c	36.5±10.7^c	24.5±8.1^c	
5-HT	4.3±1.8^a	17.8±5.8^c	10.3±4.4^c	9.8±3.3^c	139.6±7.6^c
5-HT+SP	20.0±5.5^f	16.6±4.0^d	19.5±5.7^f	16.8±3.5^d	105.9±3.3^e
5-HT+For	26.8±9.0^f	12.3±4.5^g	21.1±5.9	21.0±6.4^g	
Fen	4.0±1.4^a	0.5±1.1^a	0.3±0.7^a	0.1±0.4^a	
Fen+SP	60.6±13.5^f	21.3±8.2^d	54.8±10.4^f	22.5±8.0^d	62.7±5.6^f
Fen+For	72.0±17.2^c	18.8±6.3^g	63.8±11.2^c	30.0±8.0^g	

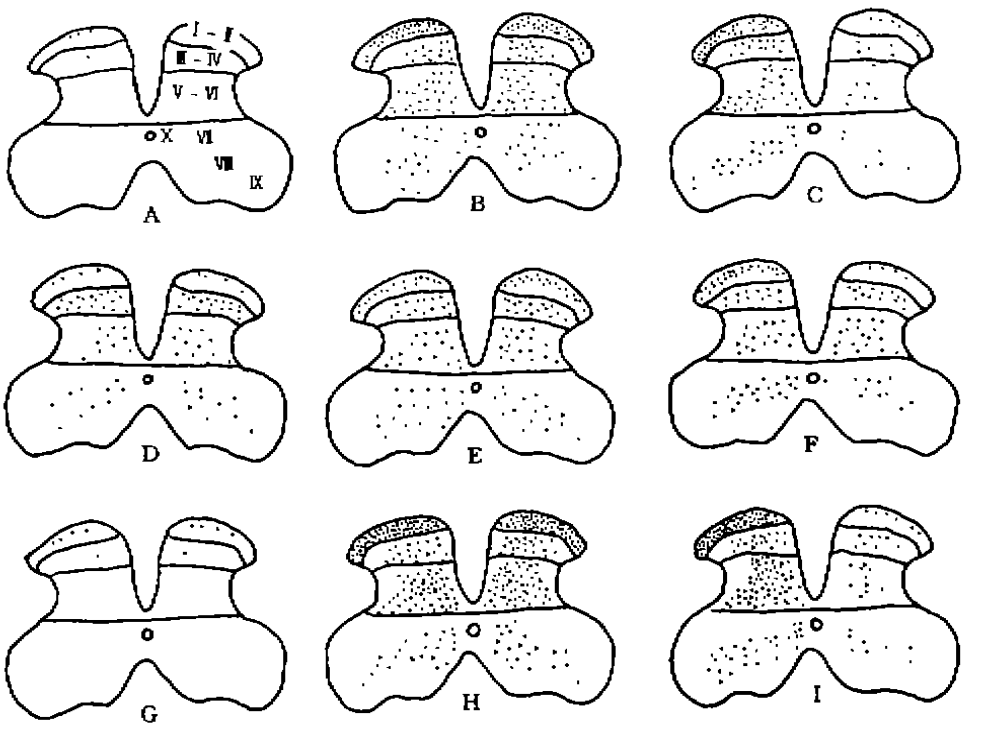

Fig 1. Distribution of FLI neurons in rat spinal cord after control (A), it SP (B), sc formalin (For) into one hindpaw (C), it 5-HT (D), it 5-HT+it SP (E), it 5-HT+sc For in hindpaw (F), ip Fen (G), ip Fen+it SP (H), it Fen+sc For in hindpaw (I).

after it SP, the FLI neurons were localized in laminae I, II, V, VI on both sides of the spinal cord (Fig 1B; Tab 1). After sc 5 % For 150 μL in the right hindpaw of the rats, the PIR was markedly increased (Fig 2). Two h after For, the FLI neurons were densely distributed in the superficial, moderately in nucleus proprius and neck, slightly in ventral of the lumbar spinal cord (Fig 1C; Tab 1).

Effect of 5-HT on pain threshold and c-*fos* of spinal cord 5-HT it 20 μg increased the pain threshold. One h after it 5-HT, the FLI neurons were mostly distributed in laminae II — IV of both sides of the spinal cord (Fig 1D, Tab 1).

Effect of 5-HT on pain response and c-*fos* of spinal cord evoked by SP and For After it 5-HT, it SP (1 h) or sc For (2 h) increased the pain threshold, reduced the PIR and c-*fos* expression in the spinal cord ($P<0.05$ or $P<0.01$), as compared with the SP

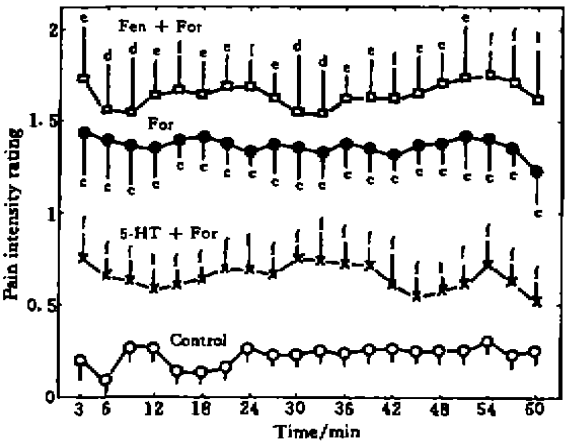

Fig 2. Effect of 5-HT (20 μg) on pain response produced by sc 5 % formalin (For) 150 μL in hindpaw. Control (○); For (●); 5-HT+For (×); Fen+For (□). $n=7-8$ rats. $\bar{x}\pm s$. $^{*}P<0.01$ *vs* Control; $^{\Delta}P>0.05$, $^{*}P<0.05$, $^{\dagger}P<0.01$ *vs* For.

or For group (Fig 1E, 1F; Fig 2; Tab 1).

After a 3-d depleting of 5-HT with ip Fen (300 mg·kg^{-1}), the pain threshold was reduced, and the PIR and FLI of spinal cord were increased following it SP (1 h) or sc For (2 h) (Fig 1H, 1I; Fig 2; Tab 1), as compared with SP or For group ($P < 0.05$ or $P < 0.01$).

DISCUSSION

In the present study, continuous tonic noxious stimulation induced by sc For in the hindpaw evoked the expression of c-*fos* protein in spinal cord, which was in agreement with the literature[1,2]. SP it also induced c-*fos* expression and reduced the pain threshod. FLI induced by SP, as that by For, were localized in all laminae of the spinal cord, but densely distributed in laminae Ⅰ, Ⅱ, Ⅴ, Ⅵ, which contain nociceptive cell. SP showed to be an excitatory neurotransmitter released from the primary afferent nerve terminals of the spinal cord and played an important role in pain transmission[6,7]. Microiontophoretic application of SP activated nociceptive neurons and induced a strong, although slow, excitatory action in the most of the units tested in the dorsal horn of cat spinal cord[12]. SP it to mice results in a pain behavioral response[6]. Therefore, c-*fos* expression induced by it SP in our experiment may be due to the activation of nociceptive neurons in the spinal dorsal horn. These findings indicate that SP in the spinal cord takes part in algogenic effect.

5-HT it in the rats increased the pain threshold, meanwhile the c-*fos* expression was enhanced. The FLI neurons were mostly distributed in laminae Ⅱ — Ⅳ of both sides of the spinal cord, differing from that induced by For. FLI evoked by electroacupuncture was also distributed in laminae Ⅱ — Ⅳ of the spinal cord[13]. 5-HT is a neurotransmitter participating in analgesia. 5-HT it could increase the pain threshold, and the analgesic effect could be alleviated by its blockers or antagonists[5]. The c-*fos* expression induced by 5-HT, therefore may be related with analgesia.

In our experiment, 5-HT inhibited pain response and c-*fos* expression in laminae Ⅰ, Ⅰ, Ⅴ, Ⅵ but not other part of laminae of spinal cord induced by SP or For. On the other hand, after depleting 5-HT by ip Fen, the c-*fos* expression evoked by SP or For was increased and the pain response was strengthened. 5-HT and SP coexisted in the same neurones of spinal cord[14], and 5-HT reduced SP responses on dorsal horn interneurones[15]. 5-HT it in mice can inhibit biting and scratching behavior evoked by SP[6]. It is indicated that c-*fos* expression reduced by 5-HT may be due to inhibit nociceptive neurons activated by SP in the spinal drosal horn. In our previous study, injection of For in the hindpaw could release SP by activating peripheral sensory nerve fibers[7]. So the decrement of the c-*fos* expression evoked by For in the spinal cord by 5-HT may be the result of inhibiting nociceptive neurons activated by SP.

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5-羟色胺对大鼠脊髓 P 物质痛觉调制的影响

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目的: 比较 SP、5-HT 与 For 诱发的脊髓内 c-*fos* 表达的异同, 以及它们之间的相互关系, 从而进一步了解 SP 在脊髓痛觉调制中的主要作用. **方法**: 用免疫组织化学法和痛阈测定法. **结果**: 发现大鼠 it P 物质(SP) 10 μg 和 sc 5 % 甲醛 (For) 150 μL 诱发的脊髓 c-*fos* 表达主要在背角 Ⅰ, Ⅱ, Ⅴ 及 Ⅵ 层, 同时 SP 使痛阈降低, For 使痛级均数(PIR)升高. 5-HT it 20 μg 引起的 c-*fos* 表达较多地分布于背角 Ⅱ—Ⅳ 层, 并可使痛阈升高. 5-HT 和 Fen 可分别减弱和增加 SP 及 For 诱发的脊髓 c-*fos* 表达及痛反应. **结论**: SP 在脊髓内可能主要起致痛作用, 5-HT 可抑制 SP 引起的脊髓 c-*fos* 表达, 从而参与 SP 的痛觉调制作用.

关键词 5-羟色胺; P 物质; 原癌基因蛋白 c-*fos*; 甲醛; 芬克洛宁; 脊髓; 免疫组织化学; 痛阈