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Systemic clonidine activates neurons of the dorsal horn, but not the locus ceruleus (A6) or the A7 area, after a formalin test: the importance of the dorsal horn in the antinociceptive effects of clonidine

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Abstract
Purpose. In order to clarify the principal site for the antinociceptive effects of clonidine, we investigated the nociceptive behavior and neural activity (c-fos staining) of the dorsal horn (DH), locus ceruleus (LC), and A7 area after a formalin test in normal saline- or clonidine-injected rats.
Methods. Thirty-six rats were divided into 6 groups as follows: formalin test + saline (FS); formalin test + clonidine (1 mg·kg^{-1}) (FC1); formalin test + clonidine (10 mg·kg^{-1}) (FC10); saline (S); clonidine (1 mg·kg^{-1}) (C1); and clonidine (10 mg·kg^{-1}) (C10). Normal saline or clonidine was injected intraperitoneally 30 min before the formalin test. In the FS, FC1, and FC10 groups, 10% formalin was injected into the left rear paw. All rats were killed 2.5 h after normal saline or clonidine injection. Sections of the lumbar spinal cord, LC, and A7 area were processed for c-fos immunohistochemistry using the avidin–biotin peroxidase complex method. To evaluate the sedative effects of clonidine, we investigated the loss of righting reflex (LORR) for 90 min in 6 other rats as follows: clonidine (1 mg·kg^{-1}) ($n = 3$) and clonidine (10 mg·kg^{-1}) ($n = 3$).
Results. The FC10 group showed fewer nociceptive behaviors and higher c-fos expression in the DH, but not in the A7 area, as well as lower c-fos expression in the LC than rats in the FS and FC1 groups ($P < 0.05$). The C10 group showed lower c-fos expression in the LC than that of rats in the S and C1 groups ($P < 0.05$). No rats exhibited LORR.
Conclusion. The antinociceptive effects of clonidine might be mediated primarily by neural activity in the DH.

Key words Clonidine · Formalin test · Dorsal horn · Locus ceruleus · A7

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Introduction

Clonidine, an α_2-adrenergic agonist, has been used in various clinical settings and has been shown to exert excellent analgesic effects, especially in cases involving epidural or intrathecal administration [1,2]. Eisenach et al. [3] reported that intrathecal, but not intravenous, clonidine reduced experimental thermal- or capsaicin-induced pain and hyperalgesia in normal volunteers. Guo et al. [4] reported that dexmedetomidine, another α_2-adrenergic agonist, produced an antinociceptive effect via direct effects on the locus ceruleus (LC) in rats. However, it has also been reported that LC lesions had no effect on the antinociception produced by clonidine in rats [5]. Here, we attempted to determine which area is the principle site for the antinociceptive effects of clonidine.

Only areas A5, LC (A6), and A7 have been shown to send projections to the spinal cord among all of the characterized noradrenergic cell groups (A1–A7). The majority of A5 neurons are thought to innervate the intermediolateral cell column of the spinal cord and mediate cardiovascular effects [6]; therefore we did not investigate A5 neurons. In this study, antinociceptive effects and the neural activity of the dorsal horn, LC, and A7 areas were investigated in normal saline- or clonidine-injected rats by using the formalin test and immunohistochemical staining for c-fos.

Materials and methods

All experimental methods were approved by our institutional animal care committee. Thirty-six adult male Sprague–Dawley rats, weighing 300–350 g, were divided into six experimental groups of six rats each: formalin test + normal saline (FS group), formalin test +

clonidine (1 mg·kg^{-1}) (FC1 group), formalin-test + clonidine (10 mg·kg^{-1}) (FC10 group), normal saline (S group), clonidine (1 mg·kg^{-1}) (C1 group), and clonidine (10 mg·kg^{-1}) (C10 group). Three milliliter of either normal saline or clonidine (Sigma Chemical Co., St. Louis, MO, USA) was injected intraperitoneally into the animal 30 min before the formalin test. Ten percent formalin (3.7% formaldehyde solution, 0.1 ml) was injected subcutaneously via a 26-gauge needle into the plantar surface of the left rear paw of the FS, FC1, and FC10 group rats. The formalin test observations were carried out immediately after the formalin injections had been administered. Pain behavior was observed for the first 5 min of each 10-min period between 0 min (immediately after formalin injection) and 55 min, i.e., 0–5 min, 10–15 min, and so on. Behavior was rated according to the criteria of Dubuisson and Dennis [7], and the pain score was calculated using the following formula: pain score = $(T1 + 2 \times T2 + 3 \times T3)/300$, where $T1$, $T2$, and $T3$ are the durations (s) spent in categories 1, 2, or 3, respectively, during each 300-s block. Categories: 0 = normal weight-bearing on the injected paw; 1 = limping during locomotion or resting the injected paw lightly on the floor; 2 = elevation of the injected paw; 3 = licking, biting, or shaking the injected paw. Because it is well known that the time-course of formalin-induced pain behavior is biphasic, we defined the early and late phases as 0–15 min and 20–55 min after formalin injection, respectively, and reported the sum of the pain scores during each phase.

Two hours and thirty minutes after normal saline or clonidine intraperitoneal injection (i.e., 2 h after formalin injection in the formalin-test groups), all rats were deeply anesthetized with pentobarbital (60 mg·kg^{-1} i.p.) and killed. The animals were perfused with 500 ml phosphate-buffered saline (PBS) (pH 7.4), followed by 500 ml 4% paraformaldehyde fixative. After perfusion, the lumbar spinal cord, medulla oblongata, and pons were removed and postfixed in the same fixative for 2 h. Twelve sections (40 μm) of the lumbar spinal cord and the entire LC (A6) and A7 area were sliced, and these sections were processed for c-fos immunohistochemistry using the avidin–biotin peroxidase complex (ABC) method described by Hsu et al. [8]. We used c-fos as a marker for all activated neurons [9]. The tissue sections were washed with 3% hydrogen peroxidase, and were then incubated for 1 h at room temperature in a blocking solution of 3% normal goat serum. The sections were incubated overnight at 4°C in PBS containing a polyclonal primary antibody to FOS (AB-2, 1:1000 dilution; Oncogene Research Products, San Diego, CA, USA). The samples were then processed according to the standard protocol employed for the ABC method (Vectastain kit; Vector Laboratories, Burlingame, CA, USA), using diaminobenzidine tetrahydrochloride as a chromogen. All the labeled cells in the surface (laminae I–II) and in the deep (laminae III–VI) laminae of the dorsal horn, as well as those in the A6 and A7 area were counted. Throughout the data-collection phase, the investigator did not know each animal's condition.

To assess the sedative effects of clonidine, six other rats were divided into two groups of three rats each as follows: 1 mg·kg^{-1} clonidine and 10 mg·kg^{-1} clonidine. Loss of the righting reflex (LORR) was defined as a lack of response to placement of the animal in a supine position. Rats were gently checked every minute to determine the LORR during the 90-min period following the intraperitoneal injection of clonidine.

The statistical analyses were performed using the Kruskal–Wallis test (Shirley–Williams) and 2-way analyses of variance (Bonferroni's post-hoc test) for assessing nociceptive behavior and the immunohistochemical study, respectively. A P value of less than 0.05 was considered to be statistically significant.

Results

There was no significant difference in the early phase of the formalin test among the FS, FC1, and FC10 groups. However, the FC10 group exhibited a lower score than the FS group in the late phase (20–55 min) of the formalin test ($P < 0.05$) (Fig. 1).

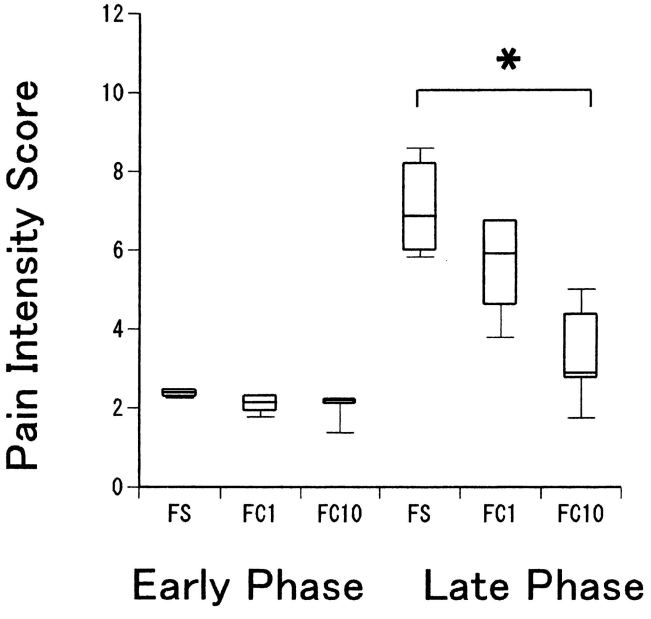

Fig. 1. Pain intensity scores in the early phase (0–5 min) and late phase (20–55 min). *FS*, formalin test + normal saline group; *FC1*, formalin test + clonidine 1 mg·kg^{-1} group; *FC10*, formalin test + clonidine 10 mg·kg^{-1} group. Median pain scores are indicated with *horizontal bars*. The *vertical bars* indicate the range, and the *horizontal boundaries of the boxes* represent the first and third quartiles. *$P < 0.05$ vs formalin test + saline group

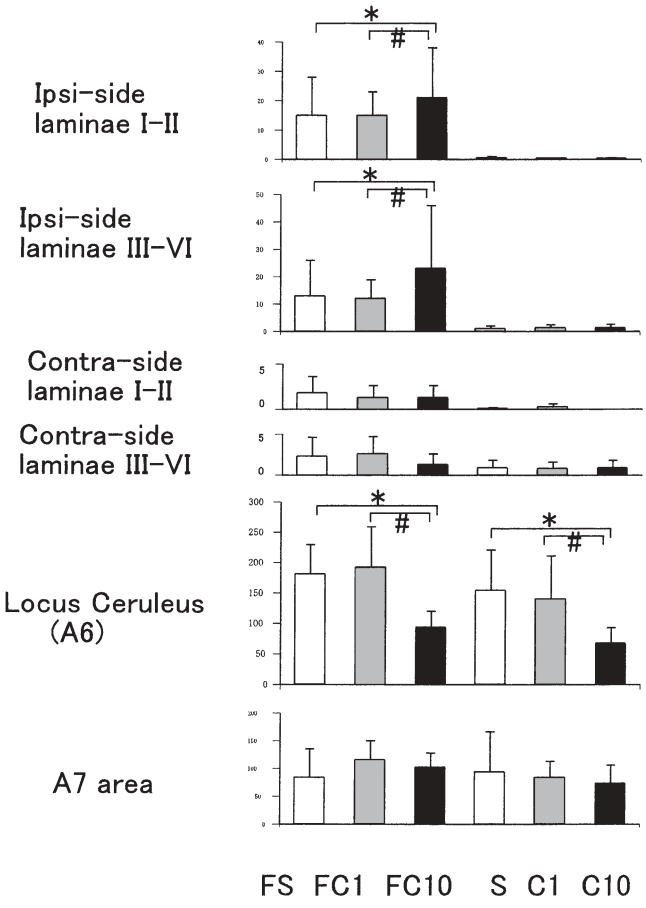

Fig. 2. Total c-fos-positive cell numbers in the dorsal horn, locus ceruleus (A6), and A7 area. *FS*, formalin test + normal saline group; *FC1*, formalin test + clonidine 1 mg·kg^{-1} group; *FC10*, formalin test + clonidine 10 mg·kg^{-1} group; *S*, normal saline group; *C1*, clonidine 1 mg·kg^{-1} group; *C10*, clonidine 10 mg·kg^{-1} group. *$P < 0.05$ vs formalin test + saline or saline group. $^{\#}P < 0.05$ vs formalin test + clonidine 1 mg·kg^{-1} or clonidine 1 mg·kg^{-1} group

Figure 2 shows the number of c-fos-positive cells in six animal groups. The FC10 group showed higher c-fos expression in the ipsilateral dorsal horn than did the FS and FC1 groups ($P < 0.05$). The S, C1, and C10 groups showed very few c-fos-positive cells in the dorsal horn. These results indicate that the c-fos expression of the ipsilateral dorsal horn was induced by the formalin injection and affected by the clonidine. The FC10 group showed a lower c-fos expression in the LC than the FS and FC1 groups ($P < 0.05$). The C10 group also exhibited a lower c-fos expression in the LC than the S and C1 groups ($P < 0.05$). However, there was no significant difference between formalin-injected groups (FC10, FC1, and FS) and control groups (C10, C1, and S) in the c-fos expression in the LC. These results suggest that the c-fos expression in the LC was affected by clonidine, but not the formalin injection. There was no significant difference among the six groups in terms of c-fos expression in the A7 area.

No LORR was observed in either of the clonidine-dosage groups. All rats showed immobility 5–10 min after clonidine injection; however, the animals maintained a prone position.

Discussion

Systemically administered clonidine inhibited the nociceptive behavior of rats, as determined by a formalin test, and increased c-fos expression in the ipsilateral dorsal horn, but not that of the A7 area. The neural activity (c-fos expression) of the LC was decreased by the systemic clonidine, irrespective of the formalin test.

In this study, no rat exhibited a LORR; however, all rats exhibited reduced locomotion. Since clonidine has sedative effects, the number of licking behaviors, as determined by the formalin test, might have decreased due to both sedative and analgesic effects. However, we believe that the effect of sedation is relatively slight, because the pain scores of the early phase were not affected by clonidine. Because the animals were similarly sedated during the early and late phases of formalin-induced pain, it appears reasonable to assume that the reduction of pain score by clonidine which we observed during the late phase represents the true analgesic effects of clonidine, but not its sedative effects.

Systemically administered clonidine was found to increase c-fos expression in the superficial and deep laminae of the dorsal horn. Thus, the principle site for the antinociceptive effects of clonidine could be either spinal or supraspinal. Spaulding et al. [10] rejected the notion that the antinociceptive effects of clonidine were associated with supraspinal structures in T6–8 spinally transected mice. Murata et al. [11] demonstrated that clonidine suppressed the noxiously-evoked activity of spinal-wide dynamic range neurons in T11–12 spinal cord-transected cats. In addition, Sawynok and Reid [5] examined the antinociceptive effects of clonidine by lesioning the LC; they reported that the mechanisms associated with the effects of clonidine did not involve the endogenous norepinephrine pathway, and spinal sites were found to be important for producing antinociception. Previous results, when taken together with our findings, suggest that the nociceptive effects of systemic clonidine might be mediated primarily by the dorsal horn.

In this study, an injection of clonidine (10 mg·kg^{-1}) decreased the pain score and increased c-fos expression in the DH. C-fos expression is usually derived by noxious stimulation. Here, it was unclear why c-fos expres-

sion in the DH did not decrease in the FC10 group. Neurons positive for c-fos immunoreactivity are known to be activated neurons [9]. We speculated that the c-fos-positive neurons included local inhibitory neurons. Along these lines, Hashimoto et al. [12] have shown that nitrous oxide administration increases the number of c-fos-positive cells in the spinal cord. The c-fos-positive cells induced by nitrous oxide were almost entirely co-localized with glutamic acid decarboxylase-positive cells. They concluded that the activation of GABAergic neurons in the spinal cord was involved in the antinociceptive effects of nitrous oxide. It is likely that the upregulation of c-fos expression not only indicates the magnitude of noxious substance-induced expression but also reveals the neuronal activities of antinociceptive systems.

Clonidine suppressed the late, but not the early, phase of the formalin-induced pain behaviors. It is well known that the late phase is dependent on the activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, while the early phase is not. Therefore, our result is consistent with the fact that the α-2-adrenoreceptor agonists decrease the NMDA-evoked responses of the neurons in the medulla and the spinal cord [13,14].

The major function of the LC is to control sleep, attention, memory, and vigilance [15]. The bilateral suppression of LC discharge activity led to a reduction in forebrain electroencephalographic activity [16]. The LC has been reported to be a major site for the hypnotic effects of α-2-agonists [17–19]. In this study, systemic clonidine produced hypnotic responses in rats and decreased the neural activity of the LC. Aghajanian and VanderMaelen [20] also reported that systemic clonidine suppressed the neuronal activity of the LC in an intracellular recording study. Our findings agree with those of many previous studies. The LC nucleus contains descending projections to the spinal cord, as well as a number of ascending projections [15]. The descending-projection neurons might be related to the antinociceptive effects of clonidine; however, there are only a few of these neurons. Changes in the activity of the descending-projection neurons might not have been detectable in our immunohistochemical study.

In light of the anatomical evidence reported thus far, A7 neurons might have the highest potential for the modulation of nociception, because the spinally projecting noradrenergic neurons of the LC and A7 cell groups innervate the ventral horn (VII–IX) and dorsal horn (I–IV), respectively [6]. Yeomans et al. [21] have reported that electrical stimulation of the A7 area induced antinociception in rats. However, clonidine did not show any significant changes in c-fos expression in the A7 area in this study. Since the S (normal saline and no formalin) group exhibited similar c-fos expression to that of the other groups, the basic neural activities of the A7 area might be high, and the clonidine-induced changes might be unremarkable.

Clonidine was not found to activate the descending noradrenaline antinociceptive pathway via the LC or the A7 area. The results of this study suggest that the antinociceptive effects of systemically administered clonidine might be mediated primarily by neural activity in the dorsal horn.

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