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Effects of Locus Coeruleus/Subcoeruleus Stimulation on the Tail Flick Reflex and Efflux of Noradrenaline into the Spinal Cord Superfusates

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ABSTRACT

Inhibition of the nociceptive Tail Flick Reflex (TFR) was observed with electrical stimulation of the locus coeruleus/subcoeruleus (LC/SC) in the male Sprague−Dawley rats under light anesthesia, and the involved neurotransmitter (s) were characterized. Electrical stimulation of LC/SC induced the analgesia with the stimulation threshold (intensity of the current, given for 100 usec and in 100 Hz frequency, which caused the TF latency longer than 6.5 sec) around 55 uA. Intrathecal administrations of α_2 antagonist, yohimbine (30 ug) or opioid antagonist, naloxone (20 ug) increased the stimulation threshold by 147% and 123% respectively (from 55 uA to 135 uA, and from 54 uA to 123 uA;$P<0.01$, $n=5$, each). The basal TF latency without stimulation (3.1 sec) was reduced by the antagonists (to 2.5 sec by yohimbine, $P<0.05$, $n=5$; to 2.6 sec by naloxone, $P<0.1$, $n=5$), vehicle only did not show any effect. Noradrenaline (NA) in the spinal cord superfusates measured with HPLC was increased by the LC/SC stimulation, from 4.18 ng/ml before to 7.74 ng/ml after stimulation ($P<0.05$, $n=10$).

The result suggest that analgesia induced by LC/SC stimulation is mediated, at least in part, by the noradrenergic system in which α_2 receptor is involved, as well as the opioid system.

Key Words: Locus coeruleus/subcoeruleus, Electrical stimulation, Tail Flick Reflex, Noradrenaline

INTRODUCTION

The origin of noradrenergic fibers in the spinal cord is known as dorsolateral pons (DLP), of which the locus coeruleus/subcoeruleus (LC/SC) is the major locus of the noradrenaline containing cell bodies. A direct coeruleospinal pathway was reported in rats to connect the LC/SC and the spinal cord, using fluorescence histochemistry (Nygren and Olson, 1977).

This coeruleospinal pathway has been suggested to be involved in the modulation of the nociceptive transmission, and the transmitter involved was suggested to be noradrenaline. Intrathecally administered noradrenaline and α-adrenoceptor agonists produced the antinociception (Reddy *et al.*, 1980; Howe *et al.*, 1983). Electrical stimulations given to the LC/SC inhibited the spinal nociception tested on the TFR (Jones *et al.*, 1984), and neuronal activity of spinal dorsal horn produced by peripheral noxious stimuli was decreased by LC/SC stimulation in cats (Hodge *et*

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al., 1981) and in the rats (Jones and Gebhart, 1986). The neurotransmitter involved in the LC/SC stimulation-induced analgesia was suggested to be noradrenaline based on the profile of the antagonist effects (Jones and Gebhart, 1986). This analgesia generated from the LC/SC was suggested to be independent of the opioid system because the LC lesion did not affect the antinociceptive potency of the morphine (Ossipov *et al.*, 1985).

Still the direct *in vivo* evidence is lacking for the release of noradrenaline during the analgesia induced by the LC/SC stimulation. Using a spinal superfusion system, the K^+ evoked release of serotonin and noradrenaline from rat and cat spinal cord was measured using HPLC with an electrochemical detector (Yaksh and Tyce, 1980). Hammond *et al.* (1985) also reported that efflux of 5-hydroxytryptamine and noradrenaline into spinal cord superfusates during stimulation of the rat medulla.

The purpose of this experiment is (1) to characterize descending inhibition of the nociceptive TFR induced by LC/SC stimulation and (2) to measure the concentration of noradrenaline in spinal superfusates before and after LC/SC stimulation.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing around 350 g were used in this experiment. Rats were initially deeply anesthetized for cannulation of the femoral vein, catheterization in the spinal cord and craniotomy with an intraperitoneal injection of pentobarbital sodium (40~50 mg/Kg). The rats were subsequently maintained in a light-anesthetized state (with corneal and flexion reflexes present) throughout the experiment with a continuous intravenous infusion of pentobarbital sodium (4~8 mg/Kg/h). Body temperature was maintained at 36~37℃ with an electric heating pad.

Spinal nociceptive tail flick reflex (TFR)

The TFR was evoked by focused radient heat applied to the underside of the tail every 1 minute at one of the 5 tail positions approximatly 1 cm apart. The position along the tail was systematically varied in order to minimize tissue damage to the tail. Inhibition of the TFR was defined as a TF latency more than 6.5 sec to minimize damage to the skin of the tail. TFR test was performed alternatively with and without the supraspinal stimulation with the interval of 1minute.

Brain stimulation

the electrode was introduced to unilateral LC/SC area using a pulse motor microdrive manipulator (Narishige, Japan) on the stereotaxic instrument. Ready-made insulated tungsten electrodes (125 μm diameter, 5M ohm, A-M systems) or stainless steel electrodes (250 μm, 5 M ohm, A-M systems) were used for brain stimulation. The coordinate of LC/SC was 3.0 mm posterior to lambda, 1.0~1.1 mm lateral, depth 6.0 mm to duramater with incisor bar -10 mm to horizpntal plan. Monopolar, cathodal constant current stimulation at a frequency of 100 Hz and pulse duration of 100 μsec was used in threshold determination. The stimulation intensity was increased stepwise until the TFR was inhibited, and the intensity of the stimulation did not exceed 300 μA or the intensity where it caused spastic movements in animal. At each experiment, TFR test was performed 10 sec after brain stimulation which continued until either the TFR was evoked or 6.5 sec had elapsed.

Intrathecal administration

Catheterization of the spinal subarachnoid space was done by the method of Yaksh and Rudy (1976). A PE 10 catheter (A-M systems, USA) was inserted through a transverse slit in the atlanto-occipital membrane and threaded caudally 7~8 cm to the lumbar enlargement for intrathecal drug administration. 30 μg yohimbine HCI (Sigma), 20 μg naloxone HCI (RBI) (both are dissolved in 10 μl artificial CSF) or the vehicle only were administrated in flow rate 0.8ul/min, by automatic micro injector.

Measurement of noradrenaline (NA) contents in the spinal cord

Desipramine HCI (RBI), an inhibitor of NA uptake, was administered by intra peritoneal injec-

tion (10 mg/Kg) 1 hour prior to superfusion. A PE 10 catheter was inserted through a transverse slit in the atlanto occipital membrane and threaded caudally 7~8 cm in the subarachnoid space. This catheter was connected with inflow syringe at 100 μl/min for infusion of artificial cerebrospinal fluid (CSF). After 30 minutes superfusion for washout,superfusates was then subsequently collected for 5 minutes at cisterna magna area using 20 gauge stainless-steel cannula by suction syringe on ice. Electrical stimulation of 100 usec duration, 100 Hz frequency and 100 uA intensity, which is nearly two times of basic threshold intensity, was given to LC/SC. Electrical stimulation continued for 1 or 5 minutes. The concentrations of NA were determined in each sample of superfusates as described previously (Park and Park, 1993) with a slight modification. Briefly, 5 μl of concentrated perchloric acid was added to 500 μl of collected superfusates, then it was centrifuged and the supernatent was collected. 3,4-dihydroxybenzamine (DHBA) 20 ng was added to 200 μl supernatent as internal standard, then filter the supernatent using ultrafilteration membrane (30,00 NMWL Filter unit, Millipore). For the HPLC analysis, a C-18 reverse phase column (Nova-pak) of 4 μm bid size and 0.5×12 cm dimension was used. The composition of the mobile phase was 0.1 M NaH_2PO_4, 0.1 mM EDTA, 1mM sodium octylsuphate, and 9% methanol (pH 3.6). The flow rate was 1 ml/minute. The electrochemical detector was used for the detection.

When using micro HPLC (BAS 200,USA) with the C18 microbore column (BAS, Sepstik, USA), same buffer was used in flow rate 30 μl/min by 2 μl injection volume including 100pg DHBA. The separation was performed at 45° C, with the detection temperature at 50° C. Quantitation of NA was done by calculating the peak area and multiplying it by the conversion factor obtained from the standard curves of NA and DHBA.

Histology

At the end of experiment, anodal electrolytic lesions (5uA, continuous current for 5 minites) were made by lesion generator (Stoelting Co, USA) to confirm the sites of stimulation. The brain was removed and immediately frozen and cut in 30 um coronal sections and stained with cresyl violet for histological veification of the site of stimulation.

Statistical analysis

All data are presented as means ± the standard errors of the means. Statistical comparisons were made using student's paired t-test.

RESULT

Throughout the whole experiment, the intact TFR (latency 3.1 ± 0.3 sec) was verified after the insertion of electrodes. Brain stimulation continued until either the TFR was evoked or 6.5 sec had elapsed, since TF latency immediately after LC/SC stimulation for 10 sec was 5.7 ± 0.1 sec (n =3). To determine LC/SC stimulation threshold intensity, 3 consecutive inhibitions of the TFR (each followed by a control TFR by 1 minute interval) was established before drugs were administered intrathecally.

Descending inhibition of nociceptive TFR by LC/SC stimulation

Figure 1A shows the effects of antagonists on the LC/SC threshold intensity. In the yohimine group, the control threshold intensity which increase the TF latency (>6.5 sce) was 55.1±7.5 μA before the drug was introduced. This threshold intensity increased to 135.9 ± 40.4 uA ($P<0.01$, n= 5) 10 minutes after the intrathecal administration of yohimbine (30 μg). Naloxone (20 μg) also increased the threshold intensity from 54.0±6.1 uA before to 120.7±37.3 uA ($P<0.01$, n=5) 10 minutes after the administration. In control group, the threshold intensity of 50.0±4.8 uA was hardly changd to 55.8±7.2 uA after vehicle administration ($P>0.1$, n=4). Real surface temperature was measured by thermocouple from TF latency value (Fig.1B). TF latency of 2 sec corresponds to 39.3℃ ±0.3, TF latency of 3 sec corresponds to 48.2℃± 0.4 and TF latency of 6 sec corresponds to 55.7℃ ±0.4. The nociceptive thermal threshold greatly increased from 45℃ (TFR=3.1) at resting state to 57° C (TFR=6.5) during LC/SC stimulation (n= 6).

Tonic descending inhibition of the TFR

The intrathecal administration of yohimbine

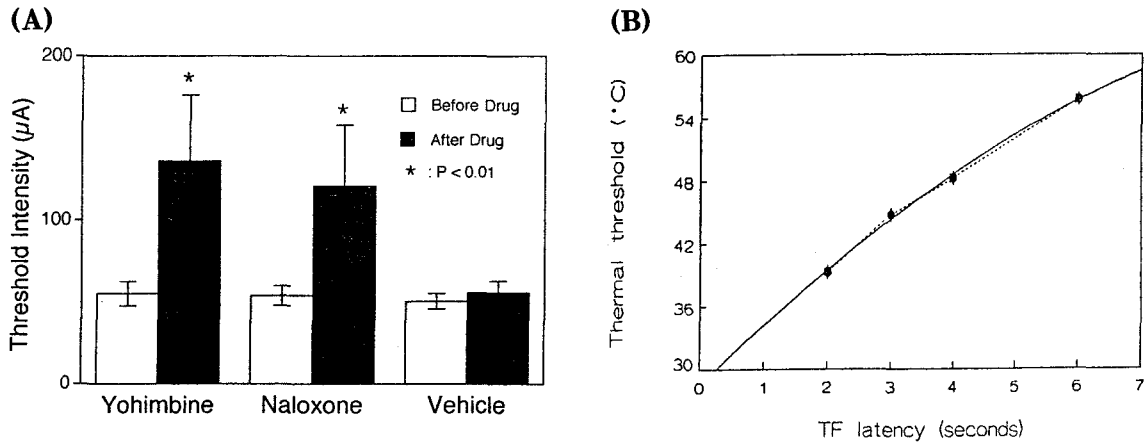

Fig. 1. (A) The effects of 30 μg yohimbine, 20 μg naloxone and vehicle on the LC/SC threshold intensity which increase TF latency (beyond 6.5 sec). (B) The nociceptive thermal threshold corresponds to TF latency.

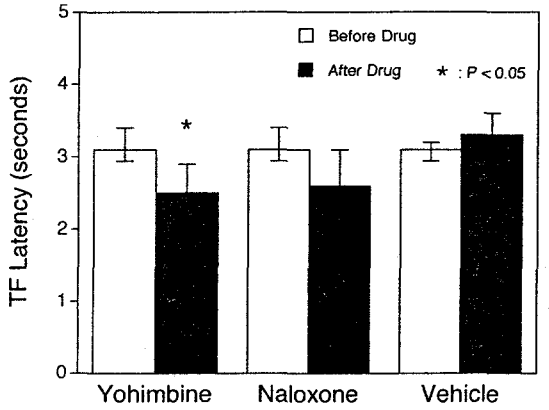

Fig. 2. The effects of 30 μg yohimbine, 20 μg naloxone and vehicle on the control TF latency.

also showed the presence of the tonic descending inhibition of the TFR by a NA component (Fig. 2). The mean pre drug control TF latency in animals receiving yohimbine and nloxone was both 3.1±0.3 sec. It was reduced to 2.5±0.4 sec ($P<0.05$, n=5) and to 2.6±0.5 sec ($P<0.1$, n=5) after the intrathecal administration of yohimbine and naloxone respectively. In control group, reducing of TF latency was not observed; TF latency was 3.1±0.1 sec and 3.3±0.3 sec ($P>0.1$, n=4) before and after vehicle administration respectively.

Histological findings

At the end of the experiments, anodal electrolytic lesions were made to mark the site of electrical stimulation. Intact normal (A) and lesion (B) of LC/SC could be differentiated in the same animal (Fig.3). TF latency could be increased by electrical stimulation from wide areas of the pons (Fig.4): anteropostriorly, from 9.16 mm to 10.3 mm posterior to the bregma, and vertically from 6.4 mm to 8.6mm based on horizontal plane passing through bregma and lambda on the surface of the skull according to atlas of the Paxinos and Watson (Academic Press, New York, 1982). Threshold intensities were variable in DLP. 3sites (marked with▲) which had more than 300 uA of intensity were outside LC/SC, but the others which had low intensity (between 35 and 80 uA) located within LC/SC except two sites, lateral parabrachial and pontine reticular area.

Changes of noradrenaline concentration by LC/SC stimulation

Desipramine (10 mg/Kg), NA uptake blocker was injected by intra peritoneal injection 1 hour before superfusion. After washout period for 30 minutes, sampling of spinal superfusates began subsequently for 5 minutes before and after LC/

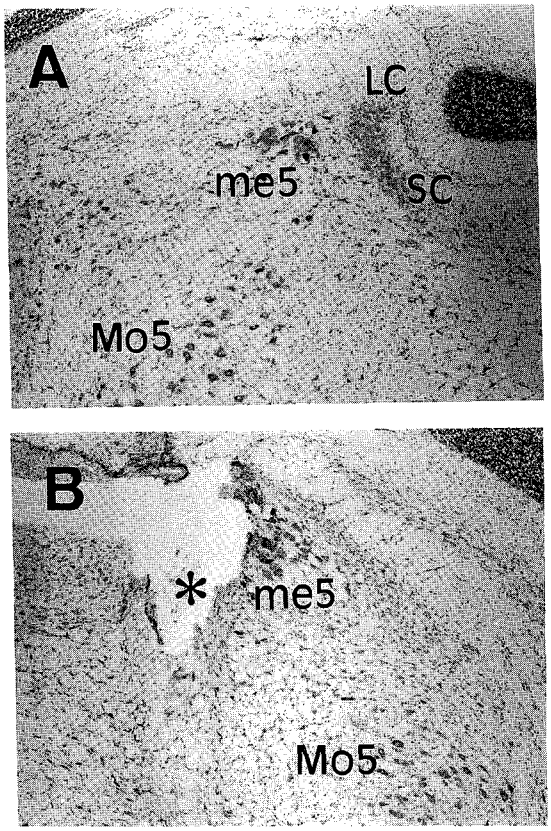

Fig. 3. Representative photomicrograph of a coronal section through the LC/SC. Normal LC/SC (in figure A) and lesion of LC/SC (marked with *, in figure B) could be seen at the same slide. Mesencephalic trige-mimal tract (me5) and parts of motor trigeminal nucleus (Mo5) could be seen.

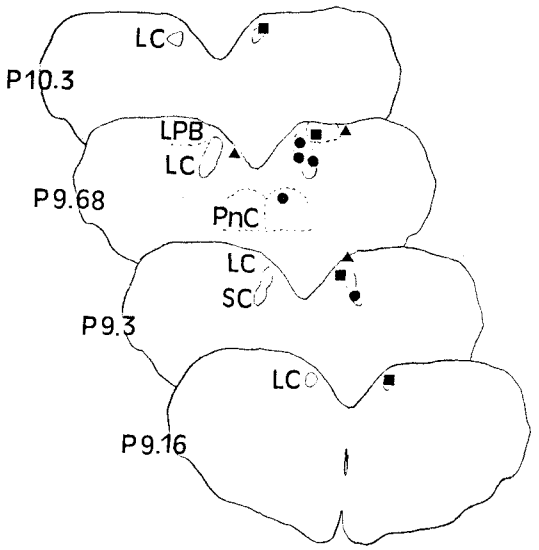

Fig. 4. The stimulation sites are portrayed on the serial coronal brain sections in the dorsolateral pons (DLP). The sites which showed low intensity (lower than 80 uA) were mostly within LC/SC area except parabrachial nuclei and pontine reticular area (in coronal section, P9. 68) Numbers to the left of each section indicate distance in mm poterior to the bregma according to the Atlas of Paxinos and Watson. Three kinds of marks indicate stimulation sites; lower than 60 μA(●), between 60 and 80 μA(■) and over than 300 μA(▲) of threshold intensity.

Abbreviations:

LC/SC; locus coeruleus/subcoeruleus
LPB; lateral parabrachial nuleus
PnC; pontine reticular nucleus

SC stimulation at 100 μl/min flow rate of artificial CSF. Figure 5 shows that representative peaks of authentic standard (A), of spinal superfusates before (B) and after LC/SC stimulation (C) using micro HPLC. Significant increases of NA concentration in superfusates were observed at the 7 experiments from totoal 10 experiments. Average concentration of NA increased from 4.18 ± 1.03 ng /ml to 7.74 ± 1.84 ng/ml ($P<0.05$, $n=10$) by LC/SC stimulation. Peak of dopamine was also detected, there was no significant change in the dopamine concentration by LC/SC stimulation (data are not shown).

DISCUSSION

Descending inhibition of nociceptive TFR by LC/SC stimulation and neurotransmitter (s) mediating that inhibition was characterized in this experiment. The connection between LC/SC and spinal cord was well confirmed by anatomical studies. Björklund and Skagerberg (1982) reported that LC/SC is responsible for 30~40% of the total spinal noradrenaline innervation in rat. In immunocytochemical study, Westlund *et al.* (1983)

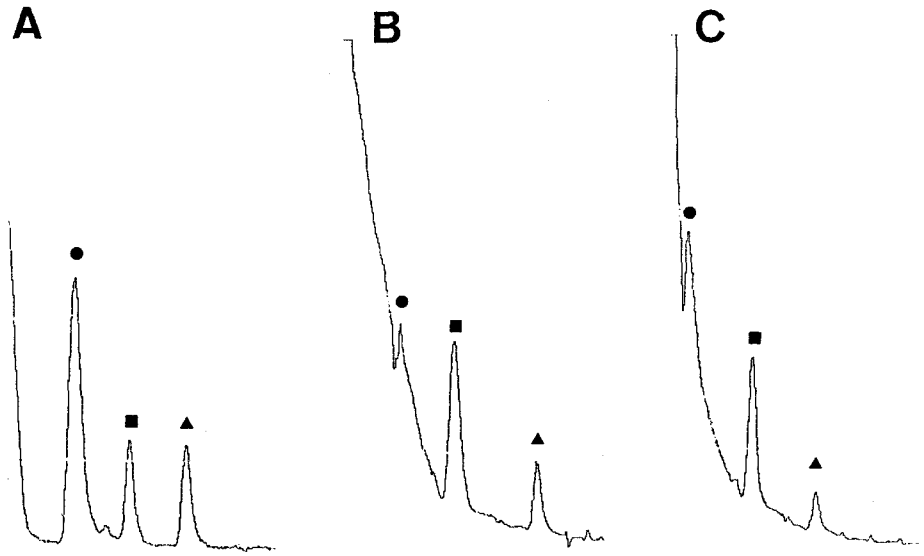

Fig. 5. Representative peaks of authentic standard (A), of spinal superfusates before (B) and after LC/SC stimulation (C) using micro HPLC (BAS 200, USA). The peaks are marked which chromatograph with NA (●), DHBA (■) and dopamine (▲). Figure A shows one of the authentic standard runs. From the left, the peak of NA (150pg), DHBA for internal standard (60pg) and dopamine (60pg) could be seen. Before stimulation, NA concentration was 8.4 ng/ml with 100pg DHBA. After stimulation, concentration of NA increased to 16.2 mg/ml. The concentration of dopamine was 14.9 ng/ml at resting release. On contrary to NA, concentration of dopamine did not increased after LC/SC stimulation. (Flow rate: 30 μl/min, injection volume: 2 μl, Colume size: 100×1 mm 3 μ C18 microbore column (BAS, Sepstik, USA) in oven temp 45℃)

reported that LC/SC in rat contains the major portion of neurons retrogradely labeled with dopamine-beta-hydroxylase antibody from the spinal cord, divided equallly between the LC and SC.

In our experiment, brain stimulation initiated 10 sec prior to the application of heat to the tail, since the optimal intervals between the onset of brain stimulation and heating of the tail was 10 sec (Sandkuhler and Gebhart, 1984). The effect of descending inhibition produced by focal electrical stimulation was maintained only for the period of stimulation. So, brain stimulation continued until either the TFR was evoked or 6.5 sec had elapsed. TFR was checked after the placement of electrode in LC/SC each time to rule out any mechanical damage by the electrode insertion. Most of sites which showed low threshold intensity were within LC/SC, but stimulation of part of parabrachial nuclei and of pontine reticular area also increased the TF latency (Fig.4). Both α_2 adrenoceptor antagonist (yohimbine) and opiod antagonist (naloxone) significantly increased the stimulation intensity required to inhibit the TFR. Both antagonists also tended to decrease the basal TF latency although it was not statistically significant for naloxone. This suggests that a descending noradrenergic system contributed to the tonic inhibitory influence on the TFR in resting state.

The current result with yohimbine is consistent with the previous report by Jones and Gebhart (1986) but that of naloxone was different. The cause of the discrepancy is not clear at present time. But, it is quite possible that a slight change in the stimulation method or the dose of drug result in the different state of analgesia, considering the complicated parallel pathways involved in the stimulation produced analgesia (SPA) or stress induced analgesia (SIA). Recently Watkin *et al.* (1992) reported both nonselective opioid antagonist naltrexone and combined blokade of all 3

classes (μ,δ, and κ) of opioid receptors antagonized all of the "non opioid" analgesias including tail shock anesthesia, hind paw foot-shock and cold water swim induced anlgesia. They suggested that all endogenous analgesia system might in fact be opioid at the level of spinal cord. Stress induced analgesia (SIA) is partly dependent on noradrenergic system. Luis *et al.* (1990) recorded LC neuronal activity changes before and after immobilization stress in rat and concluded that adaptive changes occurred in the noradrdnergic system following acute or chronic stress. Although SPA induced by LC/SC stimulation in our experiment is not a normal physiological situation, descending inhibition via centrifugal pathway is analogous to the SIA. It's probable that parallel activation of multiple spinal opioid processes occurred by NA released from noradrenergic terminal. Our data also support the work by Sawynok and Reid (1987) suggesting that central NA pathways were critical to the expression of morphine analgesia. Analgesic effect of morphine was reduced following spinal NA depletion. The increase of TF latency by LC/SC stimulation in our experiment is not due to the inhibition of motor component in the spinal dorsal horn. In previous works, either LC stimulation (Strahlendorf *et al.*, 1980) or iontophoretically administered noradrenaline on the spinal ventral horn facilitated their excitability (White and Newman, 1980) rather than inhibition of the motorneuron activities.

After the first report of Crawley *et al.*(1979) about noradrenergic metabolite levels by LC stimulation, experiments of Yaksh and Tyce (1980) showed the possibility of collecting and measuring monoamines which overflow from spinal terminals. Increased concentration of monoamine during SPA also reported by Hammond *et al.* (1986). They reported that, in rats, pre treated with desipramine, the efflux of NA was increased during stimulation of the nucleus raphe magnus and the nuleus reticularis paragigantocellularis. In our experiment, increase of NA in the spianl cord superfusates also could be observed *in vivo* using HPLC by LC/SC stimulation and it's confirmed that NA is one of the putative neurotransmitters mediating SPA, and it was suggested that α_2 receptor is involved because yohimbine reduced the analgesic effect by LC/SC stimulation.

The opiate anlgesias are initiated supraspinally and different descending pathway activate different spinal opiate process. It was reported that the descending serotonin pathway interacted with a spinal κ system (VonVoighlander *et al.*, 1984), while a noradrenergic pathway interacted with μ site (Ossipov *et al.*, 1989;Ossipov *et al.*, 1990; Spanos *et al.*, 1989). Probably LC/SC stimulation induced analgesia in our experiment is due to noradrenergic activation as well as parallel activation of multiple spinal opioid process. Using specific opioid receptor antagonists, the study of sereial or parallel interaction mechanism between specific opioid receptors and nonopioid analgesia is necessary to elucidate pain modulatory system and to settle the concept of opioid and nonopioid analgesia in spinal level in future.

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=국문초록=

청반핵 자극으로 인한 노르아드레날린의 유리가 동통의 조절에 미치는 영향

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배외측 뇌교의 전기자극은 척수 수준에서 동통의 역치를 증가시키고, 또한 말초로 부터 오는 유해성 자극에 의해 야기되는 척수후각세포의 흥분성을 억제하여 진통효과를 나타낸다. 이러한 진통효과는 noradrdnergic 원심신경을 매개로 한다고 하였지만, 이직까지 이를 직접적으로 뒷받침하는 신경화학적인 증거는 보고되지 않았다. 따라서 본 실험은 뇌교에 위치한 청반핵(Locus coeruleus)의 전기자극시, 척수 수준에서 일어나는 동통조절효과를 쥐꼬리 회피 반사(Tail Flick Reflex)를 이용해 관찰하고, 이러한 동통조절효과는 어떤 신경전달물질을 매개로해서 일어나며, 또한 생체내에서 직접 유리 되는지 살펴보기 위해 Push Pull technique을 이용해 척수액내 noradrenaline의 농도 변화를 HPLC로 측정하였다.

청반핵자극시의 동통조절기전을 알아보기위해 yohimbine, naloxone 그리고 vehicle의 3 group으로 나누어 각 길항제의 효과를 실험하였다. 청반핵 자극시 TF latency의 증가(>6.5 sec)를 보이는 역치 자극강도가 yohimbine 30 μg을 척수내로 투여한 후에는 135 μA로서 안정시의 55 μA에 비해 147% 증가되어 유의한 변화를 보여주었다($P<0.01$, $n=5$). Naloxone 20 μg을 투여한 실험군에서도 초기 역치자극강도 54 μA에서 120 μA로서 123% 증가되어 역시 유의한 변화를 보여주었다($P<0.01$, $n=5$). 그러나 vehicle group에서는 투여 전,후 역치자극강도의 변화가 없었다. 청반핵 자극이 없는 안정상태에서의 TF latency 값은 모두 3.1 sec였고 yohimbine과 naloxone투여후에는 각각 2.5 sec, 2.6 sec로 감소되어, 긴장성 억제가 차단되는 것으로 나타났다(각각 $P<0.05$, $P<0.1$, $n=5$). 청반핵의 전기자극(100 μA)은 척수액내 noradrenaline의 농도를 증가시켰으나(평균 4.18 mg/ml에서 7.74 ng/ml로, $P<0.05$, $n=10$),dopamine의 농도는 증가되지 않았다. 이상의 결과는 청반핵 자극에 의한 척수내 동통조절효과는 opioid 계외에 부분적으로 noradrdnaline을 매개로해서 이루어지며, 이에 관여하는 수용체는 α_2 임을 보여주었다.