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BRIEF COMMUNICATION

Naloxone Blocks Opioid Peptide Release in N. Accumbens and Amygdala Elicited by Morphine Injected Into Periaqueductal Gray

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MA, Q.-P., Y.-S. SHI AND J.-S. HAN. *Naloxone blocks opioid peptide release in N. accumbens and amygdala elicited by morphine injected into periaqueductal gray*. BRAIN RES BULL **28**(2) 351–354, 1992.—It has been proposed that a serial, unidirectional circuit from the PAG to the N. accumbens and the amygdala are involved in antinociception and that enkephalins (ENK) and β-endorphin (β-EP) act as neurotansmitters in this circuitry. In the present study, we measured the release of ENK and β-EP by simultaneous push-pull perfusion of the N. accumbens and amygdala after microinjection of morphine into the PAG. Morphine administration elicited an increase in immunoreactive ENK and β-EP in both the N. accumbens and the amygdala, which was antagonized in each nucleus by perfusion with naloxone. These data suggest that the three nuclei were not serially connected and that they may take part in one and the same antinociceptive system with an "all or none" character.

Periaqueductal gray Nucleus accumbens Amygdala Enkephalins β-Endorphin

MICROINJECTION of morphine into the periaqueductal gray (PAG), nucleus accumbens and amygdala increased the pain threshold (7, 11, 20, 24, 29). The opiate receptor antagonist naloxone injected into these nuclei attenuated the analgesic action of morphine administered systematically (7,27). Several reports support the existence of a unidirectional loop through the three nuclei with enkephalins (ENK) and β-endorphin (β-EP) as important analgesic neurotransmitters involved in pain modulation (8, 9, 23, 25). Neurochemical studies recently conducted in this laboratory have also confirmed our previous suggestion that morphine injected into the PAG could induce the release of enkephalins and β-endorphin in the N. accumbens or the amygdala, and microinjection of morphine into the N. accumbens could increase the release of opioid peptides in the amygdala (14–16). The aim of the present study was to examine whether a unidirectional loop exists and the putative sequence of the three nuclei in the loop, with simultaneous perfusion of the N. accumbens and amygdala for radioimmunoassay of ENK and β-EP after microinjection of morphine into the PAG. We found that microinjection of morphine into the PAG increased the release of ENK and β-EP in the N. accumbens and the amygdala and naloxone administered into N. accumbens and amygdala antagonized the effects of intra-PAG injection of morphine. Our results did not support the existence of the proposed unidirectional circuitry.

METHOD

Animal Preparation

Sixteen male rabbits weighing 2.0–3.0 kg, were anaesthetized with pentobarbital (30 mg/kg) and implanted stereotaxically with 6 stainless steel cannulae directed bilaterally to the three nuclei, PAG (P9.5, L1.0, H12.5–13.0), N. accumbens (A5.0–6.0, L1.2, H10.5–11.0) and amygdala (APO-A1, L5.5–6.0, H15.0–15.5), one into the left and one into the right side of each nucleus, according to Sawyer et al. (21). The cannulae for injection were of 0.8 mm outer diameter with the lower end located 2.0 mm dorsal to the site of injection in the PAG. The cannulae for perfusion were of 0.9 mm outer diameter with their lower ends reaching the N. accumbens or the amygdala. The cannulae were fixed on the skull with dental acrylic. Intracranial surgery was performed one week before the perfusion experiment. Kanamycin sulfate (0.25 g, IM) was injected after surgery and following each experiment.

Intracerebral injections were performed through an injection tube of 0.4 mm outer diameter extending 2.0 mm beyond the tip of the cannula, the injection volume being 1 μl to be finished within 8 min via a slow injection apparatus (Palmer, England). Cerebral perfusion was performed by inserting a smaller tube of 0.4 mm outer diameter into the cannula protruding beyond the cannula tip for 0.5 mm. Two peristaltic pumps were used for push-pull perfusion: one to push 37°C artificial cerebrospinal fluid (CSF) into brain at a rate of 100 μl/min, and another to pull the fluid synchronously (19). The perfusate was collected in tubes containing 200 μl 1 N HCl. The outflow hose and the perfusate were kept in ice water. At the beginning of the experiment, morphine (10 μg/μl) or normal saline (NS) 1 μl was injected bilaterally into the PAG, while the N. accumbens

and amygdala were perfused bilaterally with artificial CSF or CSF containing 3 μM naloxone. After 20 min, perfusate was collected for 30 min, yielding approximately 3.0 ml of perfusate for each nucleus. Perfusates from each side were examined separately as a single sample. During the perfusion experiments, the animals were awake, and restrained in hammocks with eyes covered by blinders. The perfusates were lyophilized and stored at −20°C.

Experimental Design

Each animal was used for two experiments at an interval of four days. The animals were randomly perfused with CSF or CSF containing naloxone, and injected with morphine or saline. There were 4 groups (each contained 8 experiments, or 16 samples): the first received an intra-PAG injection of 1 μl of NS and their N. accumbens and amygdala were perfused with artificial CSF (NS-CSF group), the second received an intra-PAG injection of 1 μl of NS and their N. accumbens and amygdala were perfused with artificial CSF containing 3 μM naloxone (NS-NX group), the third received an intra-PAG injection of 1 μl of morphine and their N. accumbens and amygdala were perfused with artificial CSF (MOR-CSF group), the fourth received an intra-PAG injection of 1 μl of morphine and their N. accumbens and amygdala were perfused with artificial CSF containing 3 μM naloxone (MOR-NX group).

After completion of the experiment, each animal was sacrificed with an overdose of pentobarbital and its head was immersed in 10% formalin for 4 weeks. The brain was removed and cut serially into frontal sections at 0.5 mm on a freezing microtome for the identification of the sites for microinjection and perfusion. Data were utilized only from rabbits with both injection and perfusion sites located within the target nuclei. (Six rabbits were rejected for incorrect placement of injection or perfusion cannula.) Data were expressed as mean ± S.E.M. The contents of opioid peptide in the perfusates of morphine group and NS group were compared using Student's *t*-test (two-tailed). A p value less than 0.05 was considered statistically significant.

Chemicals Used in the Experiment

Morphine chloride was produced by Qinghai Drug Company, China. Leu-enkephalin (LEK) and bacitracin were products of Sigma Chemical Company. β-Endorphin was purchased from Peninsula Laboratories, USA.

Radioimmunoassay

Anti-LEK serum was prepared jointly by the Navy General Hospital and our laboratory, which possesses 100% cross-reactivity with Met-enkephalin, yet does not cross-react with β-EP, dynorphin A and dynorphin B in measurable amounts. Anti-β-endorphin serum was prepared by our laboratory (cross-reactivities with LEK, MEK, dynorphin A and dynorphin B were all less than 0.1%). ^{125}I labelling of LEK and β-EP was performed with chloramine T method in our laboratory. Standard curves and samples were all measured in duplicate. Each tube contained ^{125}I-labelled peptide 3000 cpm (LEK) or 10000 cpm (β-EP), 1:3200 diluted antisera 100 μl and redissolved perfusate 100 μl (original volume 500 μl). The reaction volume was 300 μl. After incubation of 24 h under 4°C, the bound and free peptide were separated by polyethylene glycol 6000 (for LEK) or active charcoal (for β-EP). The radioactivity was measured by a γ-counter. Under the conditions used, the sensitivity of the assay for LEK (corresponding to a representative value which can be distinguished from maximum binding with 95% confidence) was 10 fmol with an IC_{50} of 100 fmol LEK/tube, and that for β-EP was 1 fmol with an IC_{50} of 10 fmol β-EP/tube.

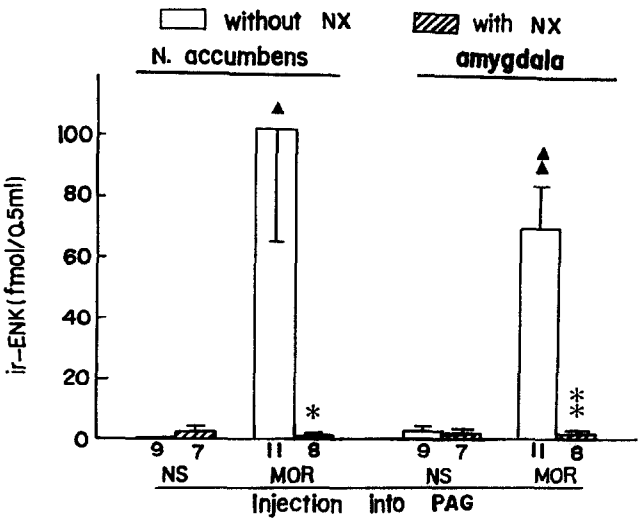

FIG. 1. The effects of morphine microinjected into the PAG on the contents of ir-ENK in the perfusates of the N. accumbens and the amygdala. ▲$p<0.05$, ▲▲$p<0.01$, compared with NS-CSF group. *$p<0.05$, **$p<0.01$, compared with MOR-CSF group. MOR: morphine. NS: normal saline. NX: naloxone.

RESULTS

The results of ir-ENK content in perfusate of the N. accumbens and the amygdala are shown in Fig. 1. The content of ir-ENK in the perfusates of the N. accumbens in MOR-CSF group was significantly greater than that of NS-CSF group ($p<0.05$), whereas the ir-ENK content in MOR-NX group was significantly lower than that of MOR-CSF group ($p<0.05$). There was no significant difference between NS-CSF and NS-NX groups ($p>0.05$). The ir-ENK content in the perfusate of the amygdala in MOR-CSF group was significantly greater than that of NS-CSF group ($p<0.01$) and the ir-ENK content in MOR-NX group was significantly lower than that of MOR-CSF group ($p<0.01$). These results indicate that microinjection of morphine into the PAG could increase the release of ir-ENK in the N. accumbens and the amygdala and that naloxone injected into the two nuclei could block the effects of morphine.

The results of ir-β-EP content in the perfusate of the N. accumbens and the amygdala are shown in Fig. 2. The content of ir-β-EP in the perfusates of the N. accumbens in MOR-CSF group was significantly greater than that of NS-CSF group ($p<0.05$), and the ir-β-EP content in MOR-NX group was significantly lower than that of MOR-CSF group ($p<0.05$). The ir-β-EP content in the perfusates of the amygdala of MOR-CSF group was significantly greater than that of NS-CSF group ($p<0.01$), while the ir-β-EP content in MOR-NX group was significantly lower than that of MOR-CSF group ($p<0.01$). These results suggest that morphine microinjected into the PAG increases the release of ir-β-EP in the N. accumbens and the amygdala and naloxone blocks the effects of morphine. Figure 3 shows the sites of injection in the PAG and the sites of perfusion in the N. accumbens and the amygdala.

DISCUSSION

The PAG is known to be a strategic site for morphine-induced analgesia (3, 4, 17). The N. accumbens (7,27) and

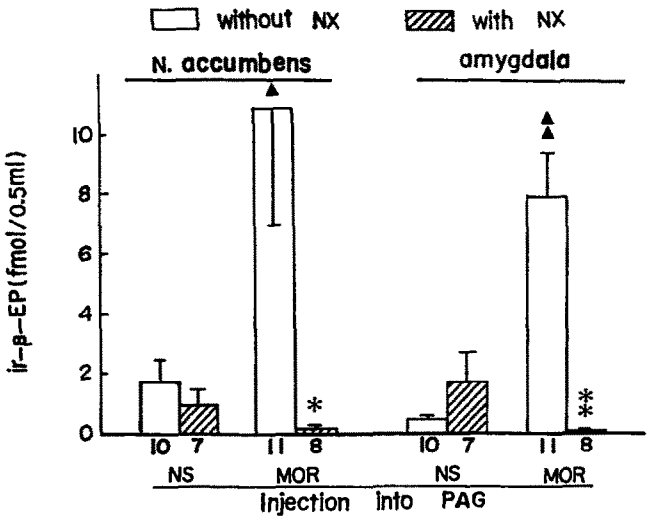

FIG. 2. The effects of morphine microinjected into the PAG on the contents of ir-β-EP in the perfusates of the N. accumbens and the amygdala. $^{\blacktriangle}p<0.05$, $^{\blacktriangle\blacktriangle}p<0.01$, compared with NS-CSF group. $^{*}p<0.05$, $^{**}p<0.01$, compared with MOR-CSF group. MOR: morphine. NS: normal saline. NX: naloxone.

amygdala (20, 22, 27, 29) have also been implicated by pharmacological data as neural structures involved in the modulation of nociception. Previous studies from our laboratory suggested the existence of an serotonergic pathway from the PAG to the N. accumbens and a pathway from the N. accumbens to the amygdala to be involved in mediating morphine analgesia. The involvement of ENK and β-EP in the two pathways has been demonstrated by evidence that (a) the analgesia elicited by microinjection of morphine into the PAG was blocked by ENK or β-EP antibody injected into the N. accumbens (8,23), and (b) the analgesia elicited by microinjection of morphine into the N. accumbens could be blocked by ENK or β-EP antibody injected into the amygdala (25). Morphological data revealed that the N. accumbens was innervated by nerve fibers originating from the ventral PAG and nucleus raphe dorsalis (NRD) (2, 5, 6), and the amygdala was innervated by nerve fibers emanating from the

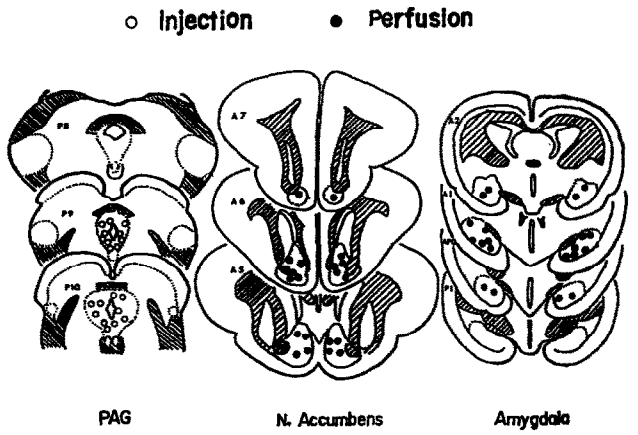

FIG. 3. The sites of injection (tip of injection tube) in the PAG and sites of perfusion (tip of perfusion tube) in the N. accumbens and the amygdala.

N. accumbens (13). Recent studies from our laboratory provide direct neurochemical evidence that microinjection of morphine into the PAG did increase the release of ENK and β-EP in the N. accumbens, and microinjection of morphine into the N. accumbens increased the release of ENK and β-EP (14–16). It must be pointed out, however, that the data from these studies were not sufficient to prove a unidirectional loop.

In order to determine whether the three nuclei are really connected serially in a unidirectional loop, we have employed two approaches: (a) perfusing simultaneously N. accumbens and amygdala after microinjection of morphine, and (b) adding naloxone in perfusion fluid. It can be reasoned that if three nuclei are connected serially and morphine can activate them one after another, naloxone added into perfusion fluid would only block the release of opioid peptides in one nucleus (e.g., the last one among the three), N. accumbens or amygdala, induced by microinjection of morphine. The results of the present study showed that the release of opioid peptides in both the N. accumbens and the amygdala were reduced by naloxone, which does not support the postulation of a unidirectional loop. This is in line with certain previous findings. For example, the increase of β-EP in the perfusate of the N. accumbens and the amygdala could not be explained by these nuclei connected in a serial manner, since neither the PAG nor the N. accumbens and amygdala contain β-endorphinergic neuronal perikarya (12). The increase of β-EP could only be explained by the activation of other nerve structures connected in parallel manner, most probably the hypothalamic arcuate nucleus. These results indicate that the three nuclei might take part in one and the same complex network for antinociception, which possesses characteristics mimicking the "all or none" principle.

The existence of a complex network for antinociception is also suggested by the complexity of the system. For example, an ascending pathway from PAG to amygdala participating in the modulation of morphine analgesia has also been suggested by the previous findings that the antinociceptive effect elicited by morphine injected into the PAG could be attenuated by microinjection of naloxone, MEK antiserum or β-EP antiserum into the amygdala (26). Neuroanatomical studies have shown that the amygdala is innervated by fibers originating from the NRD (1, 5, 18). We have evidence, using HRP retrograde tracing and immunocytochemical techniques, that the NRD sends serotonergic fibers to amygdala which may be the neuroanatomical substrate for the ascending pathway (data to be published). This is in line with the finding that microinjection of cinanserin into amygdala could block the analgesic effect of systematically administered morphine (22). Recent studies from our laboratory also provide direct neurochemical evidence that morphine injected into the PAG increases the release of ENK and β-EP in the amygdala (16).

Both neuroanatomical and neurochemical data have revealed the existence of direct projections from the ventral PAG to the N. accumbens and the amygdala which may elicit opioid peptide release. It is not clear whether the two direct projections from the PAG to the N. accumbens and the amygdala elicit opioid peptide release separately. It seems unlikely for both pharmacological and neurochemical studies to indicate an interaction between the N. accumbens and the amygdala in eliciting opioid peptides release (9, 10, 14, 25). Because it is the NRD and ventral PAG that send direct projections to N. accumbens and amygdala, one might easily envisage that morphine injected into the PAG would diffuse to the ventral PAG and NRD to have effects on serotonergic neurons. This is in line with the finding that administration of morphine to the ventral half of the PAG produced significantly greater analgesia compared to the dorsal half of the PAG (28). Since most our previous pharmacological

studies did not differentiate the effects between dorsal vs. ventral locations and the number of samples was relatively small in the present study, we have not examined the differences between dorsal and ventral locations. It is possible that differences in opioid peptide release exist between dorsal and ventral locations.

Our results present neurochemical evidence that the three nuclei under investigation are not serially connected in a unidirectional loop. It is, as yet, unknown how such a complex system possesses "all or none"-like characteristics. Although we can not give a clear answer presently, we suggest that certain kinds of positive feedback mechanism may provide a basis for concerted antinociceptive activities. From this one could speculate that (a) microinjection of opioids into one nucleus would produce a cascade of opioid effects to mimic the result elicited by systemic injection of opioid agonist or (b) microinjection of naloxone into one nucleus would block the positive feedback mechanism and quench the cascade reaction. These are indeed what have been reported so far. For example, microinjection of 10 μg of morphine into ventral PAG of the rabbit was sufficient to produce an increase in escape response latency (ERL) over 100%, equal to the effect produced by 10 mg morphine in a 2-kg rabbit (28). On the other hand, analgesia induced by IV injection of 5 mg/kg of morphine would be almost totally blocked by naloxone (1–2 μg) injected into one of the 4 nuclei: PAG, N. accumbens, amygdala or habenula (27). The working hypothesis of a single unidirectional loop is oversimplified. Further studies are needed to elucidate the details of such a complex network.

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