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Neurochemical studies on the mesolimbic circuitry of antinociception

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Previous studies using the technique of microinjection into brain nuclei indicated that the periaqueductal gray (PAG), nucleus accumbens, habenula and amygdala play an essential role in pain modulation and that these nuclei possibly act through a 'mesolimbic neural loop' to exert an analgesic effect, in which Met-enkephalin (MEK) and β-endorphin (β-EP) have been implicated as the two major opioid peptides involved in antinociception. In the present study performed in rabbits, intracranial microinjection was supplemented with push–pull perfusion and radioimmunoassay to determine whether the release of enkephalins (ENK) and β-EP was increased in these nuclei when the putative neural circuit was activated by morphine administered into one of the nuclei. The results showed: (1) microinjection of morphine into the PAG increased the release of ENK and β-EP in the N. accumbens, and vice versa; (2) microinjection of morphine into the N. accumbens increased the release of ENK and β-EP in the amygdala, and vice versa; (3) morphine microinjected into the PAG caused an increase in the release of ENK and β-EP in the amygdala and vice versa, although the release of ENK in PAG was statistically not significant. These results indicate that PAG, N. accumbens and amygdala are connected in a network served by a positive feedback circuitry.

INTRODUCTION

Since the first demonstration by Reynolds that focal brain stimulation of the periaqueductal gray (PAG) could produce analgesia strong enough to perform surgery in rats in 1969[26], and the characterization of two enkephalins from porcine brain by J. Hughes et al.[17], multiple lines of evidence have established the existence of an endogenous antinociceptive system in the brain. The importance of certain brain nuclei in mediating opioid analgesia was evidenced by the findings that profound analgesia could be elicited by microinjection of morphine into PAG, nucleus accumbens or amygdala[7,16,27,33,42], and that the analgesic effect induced by systemic morphine and acupuncture stimulation was markedly attenuated by the opioid antagonist naloxone microinjected into one of these nuclei[7,8,41]. It has been postulated that these nuclei might form a mesolimbic loop of analgesia in which Met-enkephalin (MEK) and β-endorphin (β-EP) play important roles; if one nucleus in this loop is activated, other nuclei would be activated in succession and opioid peptides would be released in each nucleus[13,14]. This hypothesis has been supported by the findings that microinjection of naloxone or antisera against MEK and β-EP into one nucleus could block the action of morphine injected into another, which suggests that opioids, especially MEK and β-EP were released in the later nucleus[15,32,36]. Direct neurochemical evidence of ENK and β-EP release, however, remains to be provided. The present study was carried out to investigate if morphine acting on any one of the 3 nuclei, PAG, N. accumbens and amygdala would increase the release of ENK and β-EP in the other two nuclei. Push–pull perfusion and radioimmunoassay (RIA) techniques were used for this purpose.

MATERIALS AND METHODS

Animal preparation

Male rabbits weighing 2.0–3.0 kg, were anaesthetized with pentobarbital and implanted stereotaxically with 4 stainless steel cannulae directed to two of these 3 nuclei, PAG (P9.5, L1.0, H12.5–13.0), N. accumbens (A5.0–6.0, L1.2, H10.5–11.0) and amygdala (AP0–A1, L5.5–6.0, H15.0–15.5) in both sides according to Sawyer et al.[28]. The cannulae for injection were of 0.8 mm o.d. with the lower end located 2.0 mm dorsal to the site of injection. The cannulae for perfusion were of 0.9 mm o.d. with the lower end reaching the target nucleus. Altogether there were 6 groups of animals (PAG–N. accumbens, N. accumbens–PAG, N. accumbens–amygdala, amygdala–N. accumbens, amygdala–PAG, PAG–amygdala). Intracranial surgery was performed one week before the perfusion experiment.

The animals were conscious and restrained in hammocks during the experiment. Intracerebral injection was performed through an injection tube of 0.4 mm o.d. 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). Cerebral perfusion was performed by inserting an inner tube into the cannula, and protruding 0.5 mm beyond the cannula tip. Two peristaltic pumps were used for push–pull perfusion: one to push 37 °C artificial cerebrospinal fluid (CSF) into the brain at a rate of 100 μl/min, and another to pull the fluid synchronously[25]. The perfusate was col-

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lected in tubes containing 200 μl 1 N HCl. The outflow hose and the tube collecting perfusate were kept cold by ice water. At the beginning of the experiment, morphine (10 μg/μl or 20 μg/μl) or normal saline (NS) (1 μl) was injected into one nucleus. After a time lag of 20 min, perfusion in another nucleus was started and lasted for 30 min. The perfusates were lyophilized and kept in −20 °C.

Chemicals

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, U.S.A.

Radioimmunoassay

Anti-LEK serum was prepared jointly by the Navy General Hospital and this laboratory, which possesses a 100% cross-reactivity with Met-enkephalin, yet does not cross-react with β-EP, dynorphin A and dynorphin B at measurable amount. Anti-β-endorphin serum was prepared by this laboratory, the 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 the chloramine T method at this laboratory. Standard curve and samples were all measured in duplicate. Each tube contained ^{125}I-labeled peptide 3000 cpm (LEK) or 10,000 cpm (β-EP), 100 μl 1:3200 diluted antisera and 100 μl redissolved perfusate (original volume 500 μl). The reaction volume was 300 μl. After incubation of 24 h under 4 °C, the bound and free peptides 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.

Data analysis

Each animal was used for two experiments at an interval of 4 days The experiments using morphine or saline were arranged randomly. After completion of the experiment, the animal was killed by an overdose of pentobarbital and its head was immersed in 10% formalin for 4 weeks. The brain was then taken out and cut serially into frontal sections at 0.5 mm on a freezing microtome for the identification of the sites for microinjection and perfusion. Only in rabbits with both injection and perfusion sites located within the target nuclei, will the data be used for statistical analysis. Since PAG, N. accumbens and amygdala are relatively large nuclei and easy to be located correctly, the animals with incorrect placement of the injection or perfusion sites were too few to form a group (one or two in each group) for statistical analysis. Therefore, we intentionally implanted the injection cannulae outside the target nucleus in order to investigate the site-specificity of morphine action. The 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.

RESULTS

The results are summarized in Fig. 1, and the sites of injection and the sites of perfusion are shown in Fig. 2. The following are more detailed descriptions.

The effects of morphine injected into PAG on the contents of ir-ENK and ir-β-EP in the perfusates of N. accumbens

Twenty rabbits were divided into two groups, one group (n = 9) was given an intra-PAG injection of 1 μl of NS as control, the other group (n = 11) was given an intra-PAG injection of 10 μg/1 μl of morphine. The results are shown in Fig. 1A. In the two groups receiving intra-PAG injection of NS or morphine, the contents of ir-ENK in the perfusates ot N. accumbens were 0.63 ± 0.63 fmol/0.5 ml and 103.0 ± 37.1 fmol/0.5 ml, respectively. The content in the morphine group is significantly higher than that in the NS group ($P < 0.05$). Radioimmunoassay of β-EP revealed that the content of ir-β-EP in the morphine group (10.9 ± 3.89 fmol/0.5 ml) is also significantly higher than that in the NS group (1.79 ± 0.68 fmol/0.5 ml) ($P < 0.05$). It is obvious from these results that intra-PAG injection of morphine could increase the release of ir-ENK and ir-β-EP in N. accumbens. Fig. 2A shows the sites of injection in PAG and sites of perfusion in N. accumbens.

In 6 rabbits morphine was injected into the cerebral cortex or superficial part of the colliculi, the content of ir-ENK in the perfusate was 5.85 ± 2.38 fmol/0.5 ml, which is significantly lower than that of intra-PAG injection of morphine ($P < 0.05$). The content of ir-β-EP was 2.05 ± 0.36 fmol/0.5 ml, which is also significantly lower than that of intra-PAG injection of morphine ($P < 0.05$). These results indicate the site-specificity of the action of morphine.

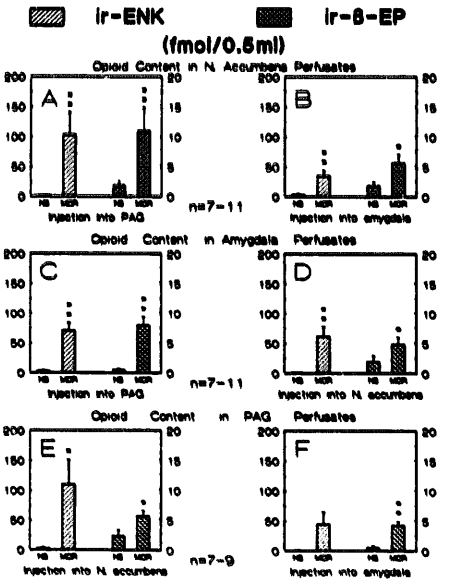

Fig. 1. A: the effects of morphine microinjected into PAG on the contents of ir-ENK and ir-β-EP in the perfusates of N. accumbens. B: the effects of microinjection of morphine into amygdala on the contents of ir-ENK and ir-β-EP in the perfusates of N. accumbens. C: the effects of morphine injected into PAG on the contents of ir-ENK and ir-β-EP in the perfusates of amygdala. D: the effects of microinjection of morphine into N. accumbens on the contents of ir-ENK and ir-β-EP in the perfusates of amygdala. E: the effects of morphine injected in N. accumbens on the contents of ir-ENK and ir-β-EP in the perfusates of PAG. F: the effects of morphine microinjected into amygdala on the contents of ir-ENK and ir-β-EP in the perfusates of PAG. *, $P < 0.05$; **, $P < 0.01$.

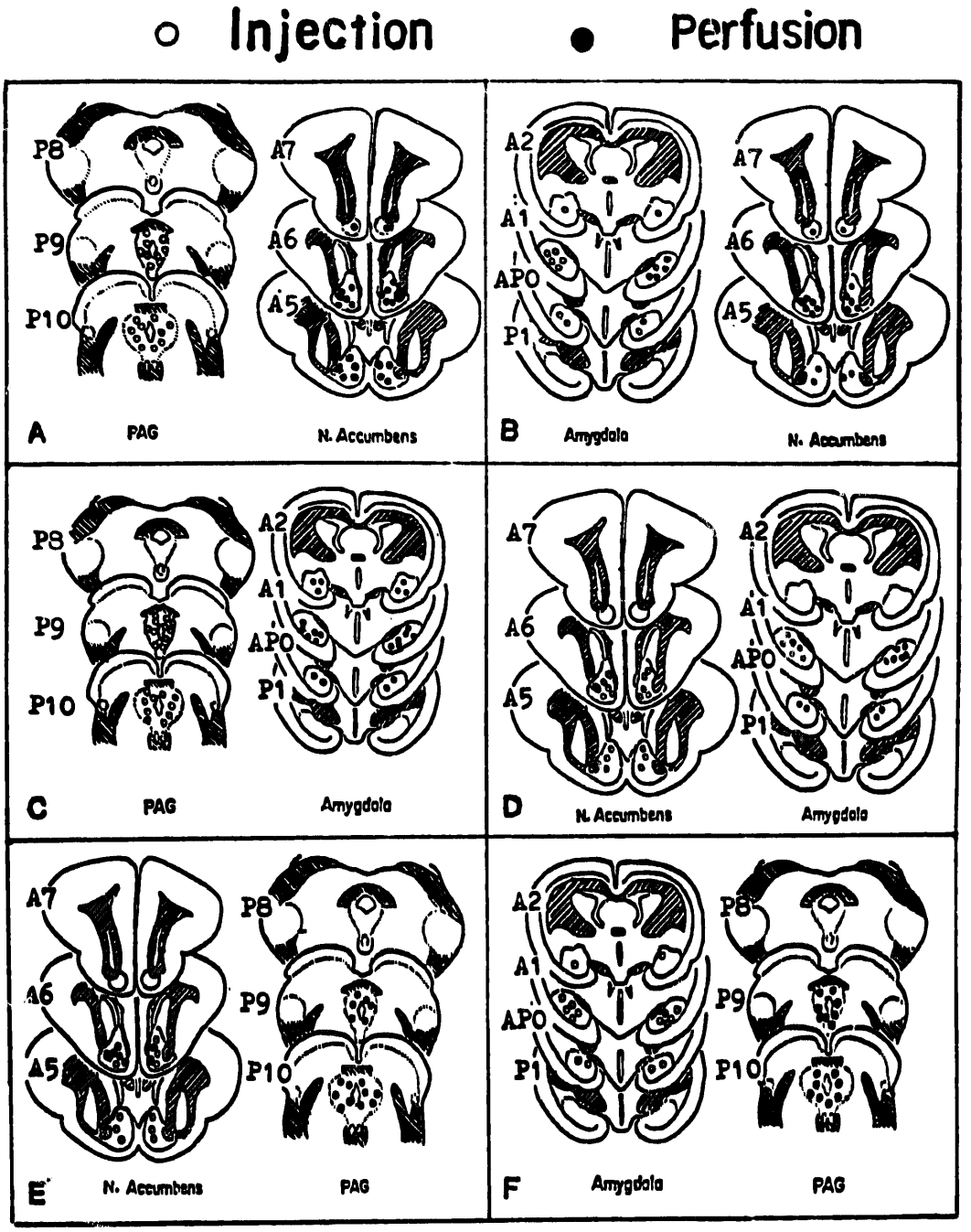

Fig. 2. A: the sites of injection (tip of injection tube) in PAG and sites of perfusion (tip of perfusion tube) in N. accumbens. B: the sites of injection in amygdala and sites of perfusion in N. accumbens. C: the sites of injection in PAG and sites of perfusion in amygdala. D: the sites of injection in N. accumbens and sites of perfusion in amygdala. E: the sites of injection in N. accumbens and sites of perfusion in PAG. F: the sites of injection in amygdala and sites of perfusion in PAG.

The effects of morphine injected into N. accumbens on the contents of ir-ENK and ir-β-EP in the perfusates of PAG

Sixteen rabbits were divided into two groups. One group was given 1 μl of NS as control, the other received an injection of 20 μg/1 μl of morphine into N. accumbens. Fig. 1E shows the results of the two groups given NS or morphine. The contents of ir-ENK were 1.87 ± 1.58 fmol/0.5 ml and 109.7 ± 40.8 fmol/0.5 ml, respectively. The ir-ENK content in the morphine group was significantly higher than that in the NS group ($P < 0.05$). The ir-β-EP contents were 2.29 ± 1.00 fmol/0.5 ml and 5.63 ± 0.89 fmol/0.5 ml, respectively. The content in the morphine group is also significantly higher than that in the NS group ($P < 0.05$). It is clear from these results that morphine microinjected into N. accumbens could accelerate the release of ir-ENK and ir-β-EP in PAG. Fig. 2E shows the sites of injection in N. accumbens and

the sites of perfusion in PAG.

In 6 rabbits morphine was injected into the area of the caudate nucleus and the corpus callosum (about 5 mm dorsal to N. accumbens), the content of ir-ENK in the PAG perfusate was 41.9 ± 19.2 fmol/0.5 ml, which was lower than that in the group with morphine injected into N. accumbens, but the difference was statistically not significant ($P > 0.05$). The content of ir-β-EP was 2.44 ± 0.37 fmol/0.5 ml, which is significantly lower than that in the group with morphine injected into N. accumbens ($P < 0.05$). These results also indicate the site-specificity of the action of morphine.

The effect of morphine injected into N. accumbens on the contents of ir-ENK and ir-β-EP in the perfusates of amygdala

Sixteen rabbits were divided into two groups, one group ($n = 7$) was given an injection of 1 μl of NS as control, the other ($n = 9$) received an injection of morphine 20 μg/1 μl into N. accumbens. Fig. 1D shows the results. The content of ir-ENK in the perfusates of amygdala in the NS group was 0.43 ± 0.43 fmol/0.5 ml as compared to 61.6 ± 16.3 fmol/0.5 ml in the morphine group, which is significantly higher than that in the NS group ($P < 0.01$). Ir-β-EP contents were 1.88 ± 0.98 and 4.80 ± 1.12 fmol/0.5 ml, respectively. The content in the morphine group is also significantly higher than that in the NS group ($P < 0.05$). It is obvious from these results that morphine injected into N. accumbens did increase the release of ir-ENK and ir-β-EP in amygdala. Fig. 2D shows the sites of injection in N. accumbens and the sites of perfusion in amygdala.

In 5 rabbits morphine was injected into the caudate nucleus and the corpus callosum (about 5 mm dorsal to N. accumbens), the content of ir-ENK in amygdala perfusate was 38.0 ± 14.7 fmol/0.5 ml which is a little lower than that with morphine injection into N. accumbens, but the difference is statistically not significant ($P > 0.05$). Ir-β-EP content was 2.25 ± 0.27 fmol/0.5 ml, which is significantly lower than that with morphine injected into N. accumbens ($P < 0.05$).

The effect of intra-amygdala injection of morphine on the contents of ir-ENK and ir-β-EP in the perfusates of N. accumbens

Sixteen rabbits were divided into two groups, one group ($n = 7$) were given an intra-amygdala injection of 1 μl of NS, the other ($n = 9$) received an intra-amygdala injection of 20 μg/1 μl of morphine. In the NS and morphine groups the contents of ir-ENK in the perfusate of N. accumbens were 2.41 ± 1.41 and 34.6 ± 8.4 fmol/0.5 ml, respectively. The latter is significantly higher than the former ($P < 0.01$). The contents of ir-β-EP were 1.79 ± 0.64 and 5.58 ± 1.39 fmol/0.5 ml, respectively. The content in the morphine group is also significantly higher than that in the NS group ($P < 0.05$). The results as shown in Fig. 1B strongly suggest that intra-amygdala injection of morphine is capable of increasing the release of ir-ENK and ir-β-EP. Fig. 2B shows the sites of injection in amygdala and sites of perfusion in N. accumbens.

In 5 rabbits morphine was injected into the area of the globus pallidus and the internal capsule (about 5 mm dorsal to amygdala), the content of ir-ENK in the perfusates of N. accumbens was 4.53 ± 1.14 fmol/0.5 ml which is significantly lower than that of the intra-amygdala injection group ($P < 0.01$). The content of ir-β-EP was 1.32 ± 0.15 fmol/0.5 ml which is also significantly lower than that of the intra-amygdala injection group ($P < 0.01$). These results indicate the site-specificity of the action of morphine.

The effect of morphine microinjected into PAG on the content of ir-ENK and ir-β-EP in the perfusate of amygdala

Twenty-one rabbits were divided into two groups: one group ($n = 10$) was given an intra-PAG injection of 1 μl of normal saline as control (NS), another group ($n = 11$) received an intra-PAG injection of 10 μg/1 μl of morphine. The results were shown in Fig. 1C. The ir-ENK content in perfusates of amygdala of the group receiving NS was 2.16 ± 1.68 fmol/0.5 ml as compared to 70.3 ± 13.6 fmol/0.5 ml in the group of animals receiving morphine injection, the latter is significantly higher than the former ($P < 0.01$). Ir-β-EP contents were 0.45 ± 0.15 fmol/0.5 ml and 7.90 ± 1.44 fmol/0.5 ml, respectively. The content in the morphine group is also significantly higher than that in the NS group ($P < 0.01$). It is obvious from these results that intra-PAG injection of morphine could increase the release of ir-ENK and ir-β-EP in amygdala, Fig. 2C shows the sites of injection and perfusion.

When morphine was injected into the cerebral cortex or the superficial part of the colliculi ($n = 5$), the content of ir-ENK in the amygdala perfusate was only 14.2 ± 4.46 fmol/0.5 ml, which is significantly lower than that in the case of intra-PAG injection of morphine ($P < 0.01$). The content of ir-β-EP was 0.33 ± 0.07 fmol/0.5 ml, which is also significantly lower than that of intra-PAG injection of morphine ($P < 0.01$). These results strongly indicate the site-specificity of the action of morphine.

The effect of morphine injected into amygdala on the contents of ir-ENK and ir-β-EP in the perfusate of PAG

Sixteen rabbits were divided into two groups. One

group (n = 7) was given an intra-amygdala injection of 1 μl of NS as control, the other group (n = 9) received an intra-amygdala injection of morphine 20 μg/1 μl. Fig. 1F shows the results of the two groups given NS or morphine. The contents of ir-ENK were 0.32 ± 0.32 fmol/0.5 ml and 44.0±20.2 fmol/0.5 ml, respectively. The ir-ENK content in the morphine group was higher than that in the NS group, but the difference was statistically not significant ($P > 0.05$). The ir-β-EP contents were 0.45 ± 0.30 fmol/0.5 ml and 4.20 ± 0.62 fmol/0.5 ml, respectively. The content in the morphine group is significantly higher than that in the NS group ($P < 0.01$). It is clear from these results that morphine microinjected into amygdala could promote the release of β-EP, and probably also ENK in PAG. Fig. 2F shows the sites of injection in amygdala and sites of perfusion in PAG.

In 5 rabbits morphine was injected into the area of globus pallidus and internal capsule (about 5 mm dorsal to amygdala), the content of ir-ENK in the PAG perfusate was only 0.15 ± 0.02 fmol/0.5 ml, which is lower than that of intra-amygdala injection, although statistically not significant ($P > 0.05$). The content of ir-β-EP was 2.44 ± 0.37 fmol/0.5 ml, which is significantly lower than that of intra-amygdala injection ($P < 0.01$). These results also indicate the relative site-specificity of the action of morphine.

DISCUSSION

The analgesic effects of the opioid peptides released

It has been shown that marked analgesia can be produced by intraventricular injection of either ENK[29] or β-EP[22]. Since the dose needed for intraventricular injection of exogenous ENK to produce an antinociceptive effect is large (over 100 nmol[29]), whereas in the present study the content of ir-ENK in the perfusate of N. accumbens after microinjection of morphine into PAG was only a fraction of a picomole, one would question if the quantity of the released ENK (and β-EP) is sufficient to produce any behavioral changes? We have estimated the amount of opioid peptide released from a nucleus during electroacupuncture stimulation by titrating the amount of antibody injected into the nucleus, which is sufficient to block the effects of electroacupuncture analgesia. Thus the C-terminal extended Met-enkephalin (Met-enkephalin-arg^6-Phe^7, MEAP) released in the PAG during 30 min of electroacupuncture stimulation was estimated to be 0.6–6 pmol[11], which is well within the range obtained in the present study if we take into account the efficacy of the antibody diffusing into synaptic cleft and binding to the peptide released. The large dose needed for exogenous ENK to produce analgesia has been mainly attributed to the rapid enzymatic degradation of the peptide en route to the target synapses. In fact, the amount of ENK needed to produce an analgesic effect can be greatly reduced when the ENK is co-injected with enkephalinase inhibitor thiophan or the aminopeptidase inhibitor bestatin[40].

The interactions between PAG and N. accumbens

PAG has long been shown to be a strategic site for morphine analgesia. A well-established mechanism for morphine analgesia is the activation of a descending serotonergic system from PAG via the brainstem raphe nuclei to the dorsal horn neurons to suppress nociceptive transmission[3,4,23]. Previous studies in our laboratory suggested the existence of an ascending serotonergic pathway from PAG to N. accumbens to be involved in mediating morphine analgesia. The involvement of enkephalins in this pathway was evidenced by the fact that the analgesia elicited by microinjection of morphine into PAG could be blocked by enkephalin antibody injected into N. accumbens[12,32]. A similar technique of antibody microinjection was also used to demonstrate the involvement of β-EP in this putative ascending pathway[35]. In the present study, we have shown direct neurochemical evidence to confirm that microinjection of morphine into PAG did increase the release of MEK and β-EP in N. accumbens. Pharmacological data have implicated the N. accumbens as one of the neural structures involved in modulation of nociception[7,8,41]. The participation of MEK and β-EP in a descending pathway from N. accumbens to PAG was suggested by the findings that the antinociceptive effect elicited by microinjection of morphine into the N. accumbens was markedly attenuated by the microinjection of naloxone, MEK antiserum or β-EP antiserum into PAG[15]. The results of the present study provided direct evidence that intra-accumbens injection of morphine can increase the release of ENK and β-EP in PAG. From these results one can infer that opioid peptides released in PAG will possibly promote the release of opioid peptides within N. accumbens, and vice versa, thus forming a positive feedback mechanism. It is understandable from this inference why the analgesic effect of morphine could be markedly attenuated by naloxone injected into N. accumbens, or PAG.

Concerning the morphological basis of such an interaction, both PAG and N. accumbens have been reported to contain enkephalinergic perikarya and terminals, as well as β-endorphinergic terminals[19,20]. N. accumbens has been demonstrated to be innervated by nerve fibers originating from nucleus raphe dorsalis (NRD)[2,5,6]. Fluorescent retrograde tracing and immunofluorohistochemical study have revealed that certain projections from NRD to N. accumbens are serotonergic[39]. This was confirmed by our recent study using the HRP retrograde

tracing in combination with immunocytochemical techniques (data to be published). It can be reasoned that intra-PAG injection of morphine may diffuse to the NRD to activate serotonergic neurons. This is in line with the findings that profound analgesia could be elicited by electrical stimulation of NRD[8,9], or injection of morphine into the ventral half of PAG produced significantly stronger analgesia than that into the dorsal half of PAG[42]. A direct nerve fiber tract projecting from N. accumbens to PAG has so far not been demonstrated. Electrophysiological data suggest that an indirect connection between N. accumbens and PAG is involved in pain modulation via the habenula[18,30]. Since neither PAG nor N. accumbens contain β-endorphinergic neuronal perikarya[19,20], the increase of β-EP in the perfusate could only be explained by activation of other nerve structures, most probably hypothalamic arcuate nucleus.

The interaction between N. accumbens and amygdala

The amygdala has been known to be related to emotion. The participation of amygdala in pain modulation was suggested by the findings that electrical stimulation of, or morphine injection into, amygdala increased pain threshold[27,38,43], whereas naloxone administration or destruction of amygdala resulted in attenuation of morphine or acupuncture analgesia[8,38,41]. A neural pathway from N. accumbens to amygdala mediating analgesia via release of enkephalins and β-endorphin was also indicated by the findings that the antinociceptive effect elicited by microinjection of morphine into N. accumbens was markedly attenuated by intra-amygdala injection of naloxone, MEK-antiserum or β-EP-antiserum[37]. Morphological observations have revealed the existence of enkephalinergic perikarya and terminals as well as β-endorphinergic terminals in amygdala[19,20]. Neurons have been found within nucleus accumbens which send fibers to amygdala[34]. The results of the present study demonstrate that microinjection of morphine into N. accumbens increases the release of ENK and β-EP in amygdala, which provides neurochemical evidence for such a connection.

Neural tract tracing study has revealed that N. accumbens receives fibers projected from amygdala[21]. The present study shows that morphine microinjected into amygdala can also increase the release of MEK and β-EP in N. accumbens. Taken together it implies that N. accumbens and amygdala might form a mechanism of positive feedback to potentiate the analgesic effects induced by morphine. The reciprocal innervation between N. accumbens and amygdala[21,34] might provide the neuroanatomical basis for such a feedback circuitry, but the possibility of a complicated multisynaptic relay through other nuclei cannot be excluded. The release of β-EP is most likely through the activation of β-endorphinergic neurons in the hypothalamic arcuate nucleus.

The interaction between amygdala and PAG

The existence of an ascending pathway from PAG to amygdala and a descending pathway from amygdala to PAG participating in the modulation of morphine analgesia through releasing MEK and β-EP has been suggested by the previous findings that the antinociceptive effect elicited by morphine injected into PAG could be attenuated by microinjection of naloxone, MEK antiserum or β-EP antiserum into amygdala, and vise versa[13,14]. Neuroanatomical studies have shown that the amygdala is innervated by fibers originating from NRD[1,5,24], but direct projections from amygdala to PAG or NRD have not been reported. Our study with HRP retrograde tracing and immunocytochemical techniques revealed that NRD sent 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[31]. The results of the present study provide direct neurochemical evidence that morphine injected into either of the two nuclei could increase the release of ENK and β-EP in the other nucleus.

The interaction between PAG, N. accumbens and amygdala

The results shown above presented unequivocal evidence that the 3 nuclei under investigation are reciprocally connected. Microinjection of opioid agonist into one nucleus triggered the release of opioid peptides (β-EP, in most cases also ENK) in the other two nuclei. 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, (b) microinjection of naloxone into one nucleus would block the positive feedback mechanism and quench the whole cascade. 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 of morphine in a rabbit of 2 kg[42]. On the other hand, analgesia induced by i.v. 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[12,15,31,32,41]. The working hypothesis put forward previously that the 4 nuclei are connected in a single unidirectional loop[12–15,41] seemed to be oversimplified since the con-

nection between any two of the nuclei being investigated in the study were found to be bidirectional. Further studies are needed to elucidate the details of such a complex network.

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