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BRIEF COMMUNICATION

Neurochemical and Morphological Evidence of an Antinociceptive Neural Pathway From Nucleus Raphe Dorsalis to Nucleus Accumbens in the Rabbit

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MA, Q. P. AND J. S. HAN. *Neurochemical and morphological evidences of an antinociceptive neural pathway from nucleus raphe dorsalis to nucleus accumbens in the rabbit.* BRAIN RES BULL **28**(6) 931–936, 1992.—Previous studies using a pharmacological approach suggested a neural pathway emanating from the periaqueductal gray (PAG) to the nucleus accumbens relevant to antinociception. This was investigated with neurochemical and histochemical methods in the present study. Push–pull perfusion and radioimmunoassay were used to measure the release of immunoreactive-(ir) enkephalin (ir-ENK) and ir-β-endorphin (ir-β-EP) in the nucleus accumbens after microinjection of morphine into the PAG and the nucleus raphe dorsalis (NRD) of the rabbit. Morphine administration elicited an increase in ir-ENK and ir-β-EP in the nucleus accumbens. Horseradish peroxidase (HRP) retrograde tracing in combination with 5-hydroxytryptamine (5-HT) immunocytochemistry revealed a serotonergic projection from the NRD and ventral PAG to the nucleus accumbens in the rabbit. About 7% of the serotonin-positive cells in the NRD and ventral PAG send fibers directly to the nucleus accumbens, with an ipsilateral dominance. These results indicate the existence of a serotonergic pathway from the NRD to the N. accumbens involved in opioid analgesia.

Nucleus raphe dorsalis Nucleus accumbens Morphine β-Endorphin Enkephalins 5-Hydroxytryptamine Analgesic pathway

ELECTRICAL stimulation of (6,7,16,19), and microinjection of morphine into (2,11,27), the periaqueductal gray [PAG, including the nucleus raphe dorsalis (NRD)] increased pain threshold. Microinjection of morphine or serotonin [5-hydroxytryptamine (5-HT)] into the nucleus accumbens also increased pain threshold (4,5,30). Moreover, the serotonin antagonist cinanserin microinjected into the nucleus accumbens could attenuate the analgesic effects of morphine injected into the PAG of rabbits, and the opioid antagonist naloxone injected into the nucleus accumbens could block the analgesia elicited by microinjection of serotonin into the same site (9,26,28). These reports support the existence of an antinociceptive serotonergic pathway from the PAG to the nucleus accumbens that could induce the release of opioid in the nucleus accumbens. The aim of the present study was to examine whether morphine injected into the PAG can increase the release of opioid peptides in the nucleus accumbens with push–pull perfusion of the latter nucleus for radioimmunoassay of enkephalins (ENK) and β-endorphin (β-EP) and whether there is a serotonergic projection from the PAG to the nucleus accumbens with retrograde tracing in combination with immunocytochemistry. We found that microinjection of morphine into the PAG increased the release of immunoreactive-(ir) ENK and ir-β-EP in the nucleus accumbens and that there is a serotonergic projection from the ventral PAG and the NRD to the nucleus accumbens in the rabbit.

METHOD

Animal Preparation

The neurochemical study was carried out on 20 male rabbits weighing 2.0–3.0 kg. Animals were anesthetized with 3% pentobarbital (30 mg/kg, IV) and implanted stereotaxically with four stainless steel cannulae directed bilaterally to the PAG (P9.5,

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L1.0, H12.5–13.0) and the nucleus accumbens (A5.0–6.0, L1.2, H10.5–11.0) and one into the left and one into the right side of each nucleus, according to the atlas of Sawyer et al. (22). The cannulae directed to PAG were 0.8 mm outer diameter with the lower ends located 2.0 mm dorsal to the site of injection, which was used for microinjection. The cannulae directed to nucleus accumbens were 0.9 mm outer diameter with their lower ends reaching the nucleus, which were used for perfusion. The cannulae were fixed on the skull with dental acrylic. Intracranial surgery was performed 1 week before the perfusion experiment. Kanamycin sulfate (0.25 g, IM) was injected after surgery and following each experiment.

Intracerebral injection was performed through an injection tube of 0.4-mm diameter extending 2.0 mm beyond the tip of the outer cannula, the injection volume being 1 μl to be completed in 8 min via a slow-injection apparatus (Palmer, England). Upon perfusion of the nucleus, a smaller tube of 0.4-mm outer diameter was inserted into the cannula and extended 0.5 mm beyond the cannula tip (18). Two peristaltic pumps were used for perfusion: one for infusing 37°C artificial cerebrospinal fluid (CSF) (Na^+ 160.4 mM, K^+ 3.0 mM, Mg^{2+} 0.5 mM, Ca^{2+} mM, Cl^{-1} 140 mM, HCO_3^- 20 mM, HPO_4^{2-} 0.4 mM, $(NH_2)_2CO$ 0.013%, bovine serum albumin 120 μg/ml, bacitracin 30 μg/ml) into brain tissue through the inner tube at a rate of 100 μl/min and the other for pumping out the perfusate synchronously via the space between the inner tube and the guide cannula. The perfusate was collected in tubes containing 200 μl 1 N HCl. The outflow hose and 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 nucleus accumbens was perfused bilaterally with artificial CSF. After 20 min, perfusate was collected for 30 min, yielding approximately 3.0 ml perfusate. Perfusate from each side was examined separately as a single sample. During the perfusion experiments, animals were awake and restrained in hammocks with their eyes covered by blinders. The perfusates were lyophilized and stored at −20°C.

Each animal was used for two experiments at an interval of 4 days. Animals were randomly injected with morphine or saline. 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 identification of sites for microinjection and perfusion. Data were utilized only from rabbits with both injection and perfusion sites located within the target nuclei. Data were presented as mean ± SEM. The contents of opioid peptides in the perfusates of the morphine and NS groups were compared using Student's t-test (two-tailed). A p value less than 0.05 was considered statistically significant.

The histochemical study was performed on seven male rabbits weighing 2.0–3.0 kg. Animals were also anesthetized with intravenous injection of pentobarbital. A glass micropipette of 50-μm outer diameter was introduced stereotaxically to the nucleus accumbens to deliver a single injection of 0.05–0.2 μl 30% horseradish peroxidase (HRP) (Sigm VI) dissolved in 0.9% saline. The injection was completed within a period of 30 min. After a survival time of 48 h, animals were deeply anesthetized and perfused through the ascending aorta with 500 ml warm (about 30–37°C) normal saline, followed by 2,000 ml 4% paraformaldehyde and 1,000 ml 10% sucrose, both in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed and sectioned immediately, or put into 30% sucrose in 0.1 M PB at 4°C and sectioned the next day, into 30-μm frontal cryostat sections.

Radioimmunoassay

Antileu-enkephalin (LEK) serum, which possesses a 100% crossreactivity with met-enkephalin (MEK) yet does not crossreact with β-EP, dynorphin A, and dynorphin B in measurable amounts was prepared jointly by the Navy General Hospital and our laboratory. Anti-β-EP serum was prepared by our laboratory (the crossreactivities with LEK, MEK, dynorphin A, and dynorphin B were all less than 0.1%). LEK and β-EP were labeled with [^{125}I] according to chloramine T method. Standard curves and samples were all measured in duplicate. Each tube contained 3,000 cpm (LEK) or 10,000 cpm (β-EP) [^{125}I]-labeled peptide, 100 μl 1:3,200 diluted antiserum, and 100 μl redissolved perfusate (original volume 500 μl). The reaction volume was 300 μl. After incubation for 24 h at 4°C, the bound and free peptides were separated by polyethylene glycol 6000 (for LEK) or active charcoal (for β-EP). Radioactivity was measured with a τ-counter. Under the conditions used, the sensitivity of the assay for LEK (corresponding to a representative value that can be distinguished from maximum binding with 95% confidence) was 10 fmol with an IC_{50} of 100 fmol LEK/tube, and the sensitivity for β-EP was 1 fmol with an IC_{50} of 10 fmol/tube.

HRP Histochemistry and Immunocytochemistry

The sections were treated with tetramethylbenzidine (TMB) and the reaction was intensified by 1% cobalt chloride and diaminobenzidine (DAB) (20). Immunocytochemical staining was carried out according to Sternberger's peroxidase–antiperoxidase (PAP) method (24). The procedure follows the consecutive steps: incubation (A) in rabbit anti-5-HT serum (1:8,000) for 48 h at 4°C, (B) in sheep antirabbit immunoglobulin (Ig) G serum (1:30) for 30 min, and finally (C) in PAP complex (1:10) for 30 min; the sections were washed in 0.01 M phosphate-buffered saline (PBS, pH 7.4) after each step. Hereafter, the sections were treated with DAB to visualize the precipitation product.

Chemicals

Morphine chloride was produced by Qinghai Drug Company (Qinghai, China). LEK and bacitracin were products of Sigma Chemical Company (St. Louis, MO). β-EP was purchased from Peninsula Laboratories, Belmont, CA. Rabbit anti-5-HT serum was purchased from Immunonuclear Corporation, Stillwater,

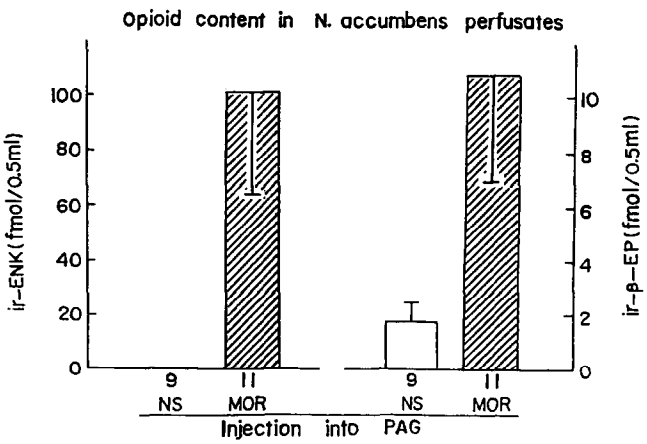

FIG. 1. Effects of microinjection of morphine into PAG on the contents of opioid peptides in the perfusates of nucleus accumbens. NS, normal saline; MOR, morphine.

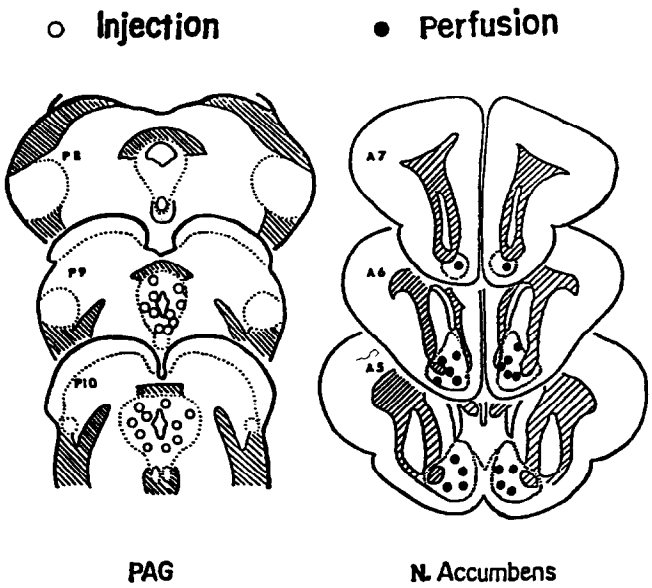

FIG. 2. Sites of injection in the PAG and sites of perfusion in the nucleus accumbens.

MN. Sheep antirabbit Ig G serum and PAP complex were products of Beijing Institute of Biological Products (Beijing, China).

RESULTS

Effects of Morphine Microinjected into the PAG on the Content of ir-ENK and ir-β-EP in the Perfusates of the Nucleus Accumbens

Twenty rabbits were divided into two groups: One group received an intra-PAG injection of 1 μl NS as control (NS group) and the other received an intra-PAG injection of 10 μg morphine. The results are shown in Fig. 1. The content of ir-ENK in the perfusates of the nucleus accumbens in the NS group was actually undetectable with the present ENK radioimmunoassay (RIA) (less than 10 fmol/0.5 ml), whereas that in the morphine group was 103.0 ± 37.1 fmol/0.5 ml. The ir-ENK content in the morphine group was significantly greater than that of the NS group ($p < 0.05$). The content of ir-β-EP in the NS and morphine groups were 1.79 ± 0.68 fmol/0.5 ml and 10.9 ± 3.89 fmol/0.5 ml, respectively. The latter was also significantly greater than the former ($p < 0.05$). Figure 2 shows the sites of injection in the PAG and the sites of perfusion in the nucleus accumbens.

In six rabbits, morphine was injected into the cerebral cortex or superficial part of the colliculi. The content of ir-ENK in the perfusates of the nucleus accumbens was 5.85 ± 2.3 fmol/0.5 ml, which was significantly lower than that of intra-PAG injection of morphine ($p < 0.05$). The ir-β-EP content was also significantly lower than that of intra-PAG injection (2.05 ± 0.36 vs. 10.9 ± 3.89, $p < 0.05$), suggesting a site specificity of morphine action. These results indicate that morphine injected into the PAG can increase the release of opioid peptides in the nucleus accumbens.

Serotonergic Projection from the NRD and Ventral PAG to the Nucleus Accumbens

The center of peroxidase activity in the HRP injection site was located within the nucleus accumbens and the diffusion range did not go obviously beyond the boundary of the nucleus accumbens. HRP reaction products were shown as black punctate granules in the cytoplasm, whereas the cytoplasm of 5-HT-positive neurons was homogeneously stained in light brown. Hence, double-labeled perikarya could be easily identified. In the sections that were not immunocytochemically processed after HRP visualization, HRP-labeled cells were scattered along the entire rostrocaudal extent of the NRD, with an ipsilateral dominance. HRP-labeled neurons were also observed in the ventral part of the PAG at its middle and caudal levels. 5-HT-positive neurons were distributed in the entire extent of the NRD. Many 5-HT-positive neurons were located in the ventral part of the PAG beyond the boundary of the NRD. HRP-labeled 5-HT-positive neurons were observed in the NRD and nearby ventral PAG, mostly in the ipsilateral side (Fig. 3). Double-labeled cells were fusiform, triangular, or multiple-polar with diameters ranging from 15–35 μm. In some large double-labeled neurons, distinct HRP granules were present in their processes (Fig. 4). To get an idea on the ratio of HRP-single-labeled, 5-HT-single-positive, and double-labeled cells, five sections of the mesencephalic part were taken randomly, one from each animal. The results are shown in Table 1. The rank order of the three kinds of cells is 5-HT-single-positive (91%), double-labeled (7.4%), and HRP-single-labeled (1.6%). In other words, most (82.6%) the NRD neurons innervating nucleus accumbens are serotonergic. The ratio of double-labeled neurons located at ipsilateral vs. contralateral wings was about 2.5:1 (9.8 vs. 4.1%, χ^2 test, $p < 0.05$). The percentage of double-labeled neurons contained in the ventromedial part of the NRD was 8.9%, markedly higher than that of contralateral wing (χ^2 test, $p < 0.05$) and not sig-

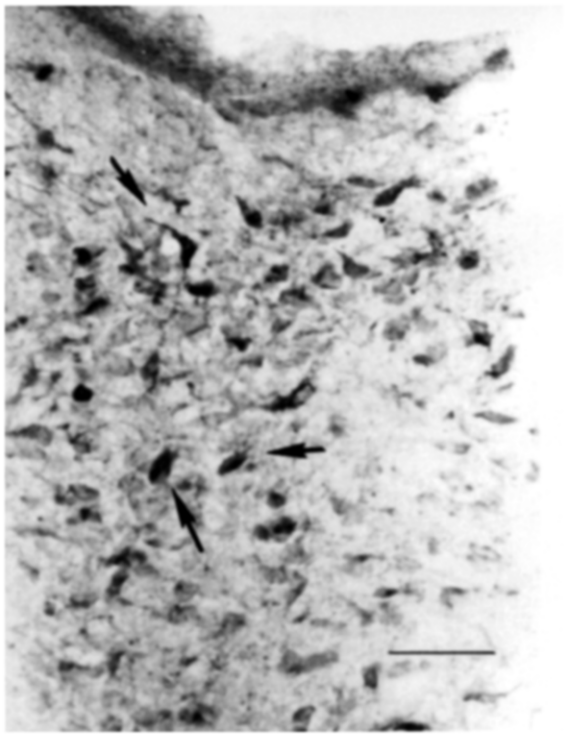

FIG. 3. 5-HT- and HRP-double-labeled neuron in NRD after microinjection of HRP into the nucleus accumbens. Dense punctual black granules of HRP products could be observed in the homogenous brownish cytoplasm (shown by arrows). Bar, 100 μm.

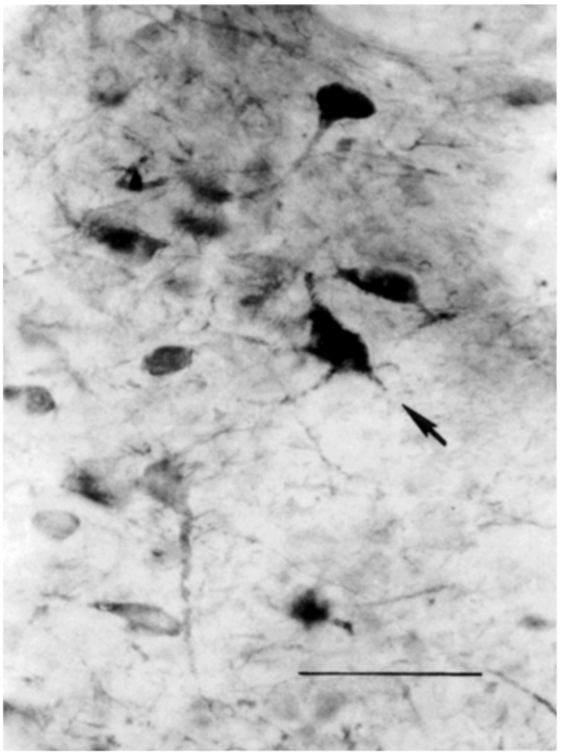

FIG. 4. Large 5-HT- and HRP-double-labeled cells in the NRD after injection of HRP into the nucleus accumbens (shown by an arrow). 5-HT-single-labeled cells can also be seen. Bar, 100 μm.

nificantly different from that of ipsilateral wing (χ^2 test, $p > 0.05$). The coronal section of the NRD where the three types of cells were counted is shown schematically in Fig. 5.

DISCUSSION

Marked analgesia could be elicited by morphine microinjected into the PAG or nucleus accumbens in rats (4,11,27) and rabbits (9,26,29,30), and naloxone microinjected into the PAG or nucleus accumbens could attenuate the analgesic effect induced by systemic morphine and acupuncture stimulation in rats (4,5,27) and rabbits (28). A serotonergic antinociceptive pathway from the PAG to the nucleus accumbens has been suggested by the findings that microinjection of cinanserin into the nucleus accumbens blocked the analgesic action of morphine injected into the PAG in the rabbit (9,10). Moreover, microinjection of naloxone into the nucleus accumbens could block the analgesic action of serotonin injected into the same site, whereas microinjection of cinanserin into the nucleus accumbens has no effect on the analgesia elicited by morphine injected into the same site (26). These previous findings indicate that microinjection of morphine into the PAG induces the release of serotonin in the nucleus accumbens and the released serotonin elicits analgesia through releasing opioid peptides (9,10,26).

Although many pharmacological data support the existence of an antinociceptive pathway from the PAG to the nucleus accumbens, there are still some questions remaining: One is whether intra-PAG injection of morphine really increases the release of opioid peptides in the nucleus accumbens. The results of the present study confirmed our previous suggestion that microinjection of morphine into the PAG could increase the release

TABLE 1

RATIO OF THE THREE TYPES OF CELLS IN THE NRD AFTER INJECTION OF HRP INTO THE NUCLEUS ACCUMBENS AND 5-HT IMMUNOCYTOCHEMICAL STAINING

	HRP-L	5-HT-L	Double-L	Total
IPSI wing	11 (2.1%)	452 (88.1%)	50 (9.8%)	513 (100%)
CONT wing	6 (1.2%)	482 (94.7%)	21 (4.1%)	509 (100%)
VENT part	4 (1.2%)	293 (73.2%)	29 (8.9%)	326 (100%)
Total	21 (1.6%)	1,227 (91.0%)	100 (7.4%)	1,348 (100%)

HRP-L, HRP-single-labeled cells; 5-HT-L, 5-HT-single-labeled cells; double-L, HRP- and 5-HT-double-labeled cells; IPSI wing, wing of the NRD ipsilateral to the injection side; CONT wing, wing of the NRD contralateral to the injection side; VENT part, ventromedial part of the NRD. The numbers represent the number of the cells in the five sections counted. The rostrocaudal level of these sections corresponds to the level shown in Fig. 5.

of opioid peptides in the nucleus accumbens. Since neither the PAG nor the nucleus accumbens possesses β-endorphinergic perikarya, other pathways besides the direct projections from the PAG to the nucleus accumbens must exist to elicit the increase of β-EP. It is possible that serotonergic fibers emanating from the NRD can activate the β-endorphinergic neurons in the hypothalamic arcuate nucleus to release β-EP in the nucleus accumbens. This postulation was supported by the findings that β-EP antiserum injected into nucleus accumbens blocked the analgesia elicited by intra-PAG injection of morphine and that the action of β-EP antiserum disappeared in animals whose arcuate nuclei were destroyed (10).

Another question of interest is whether there is a serotonergic projection from the NRD and the ventral PAG to the nucleus accumbens. The nucleus accumbens has been reported to contain enkephalinergic perikarya and nerve terminals (12) and serotonergic nerve terminals (21) in rats. It is well established that the nucleus accumbens, which lacks serotonergic perikarya, re-

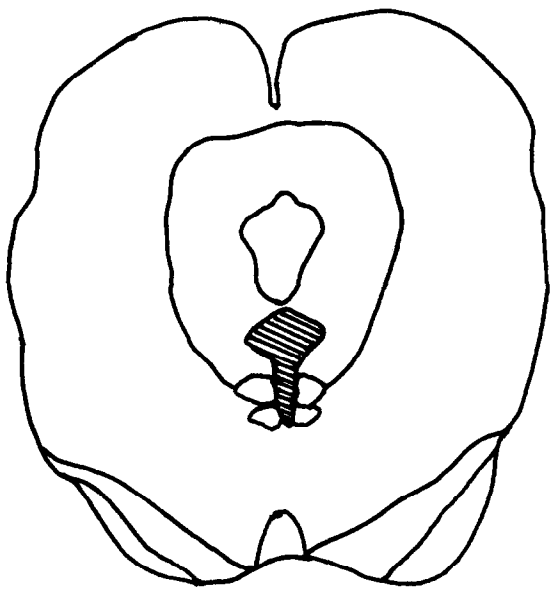

FIG. 5. Schematic drawing showing the mesencephalic level of the NRD (the hatched area) where the three types of cells were counted.

ceived direct projections from the NRD, which is the major origin of the ascending serotonergic fibers in rats (1,3,17,23). Therefore, we assume that the serotonergic terminals in the nucleus accumbens arrive largely from the NRD and that the action of cinanserin injected into the nucleus accumbens is possibly accomplished by blocking a serotonergic afferent from the NRD. However, since the NRD contains both serotonergic and non-serotonergic neurons the existence of serotonergic projections from the NRD to the nucleus accumbens needs direct demonstration. It has been reported that serotonergic neurons in the PAG send axons to the nucleus accumbens in the rat (13). This was confirmed by a similar study from our laboratory (data to be published). Since the hypothesis of an antinociceptive serotonergic pathway is mainly based on pharmacological experiments performed on rabbits, morphological evidences in the rabbit are needed. The results of the present study revealed the existence of a serotonergic projection from the NRD and the ventral PAG to the nucleus accumbens, which provides morphological evidence for the before-mentioned hypothesis. We found over 80% of the neurons sending axons to the nucleus accumbens were serotonergic, which constituted about 7% of the total population of 5-HT-positive neurons in the NRD and the ventral PAG. Our results are in keeping with the finding that more than 70% of NRD cell are serotonergic (25). The NRD of the rabbit occupies a large area ventral to the aqueduct (8). Because it is the NRD and the ventral PAG that send direct projections to the nucleus accumbens, one might easily envisage that morphine injected into the PAG may diffuse to the NRD and ventral PAG to have effects on the serotonergic neurons. This is in line with the finding that administration of morphine into the ventral half of the PAG produced significantly greater analgesia compared to the dorsal half of the PAG (29). 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. The putative serotonergic innervation of enkephalinergic neurons was supported by the finding that 5-HT-immunoreactive terminals may form excitatory synapses with ENK-positive dendrites in the nucleus accumbens of rats (14).

Concerning the possible mechanisms of morphine activation of serotonergic neurons in the NRD, we have done some initial study using immunocytochemistry in combination with in vitro receptor autoradiography (15). The preliminary results revealed numerous opiate receptors ([^{3}H]-etorphine binding sites) on some raphe serotonergic perikarya. If these receptors were really located on the cell surface, morphine will activate the NRD cells directly. If these receptors were located on the inhibitory axonal terminals innervating the serotonergic cells, morphine will activate the NRD cells through disinhibition.

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