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INVOLVEMENT OF THE PERIAQUEDUCTAL GREY MATTER AND SPINAL 5-HYDROXYTRYPTAMINERGIC PATHWAYS IN MORPHINE ANALGESIA: EFFECTS OF LESIONS AND 5-HYDROXYTRYPTAMINE DEPLETION

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1 Electrolytic lesions of the periaqueductal grey matter (PAG) were made in rats. The analgesia produced by intraperitoneal injection of morphine (10 and 20 mg/kg), tested by the tail flick and hot plate methods, was substantially reduced in the lesioned rats. Baseline pain thresholds were unaffected by the lesions.

2 The lesion effects were not due to damage to the dorsal raphé nucleus. The extent of histologically determined damage to the dorsal raphé and the resulting decrease in striatal 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations did not correlate with the reduction in morphine analgesia produced by the lesion. Furthermore, microinjections of 5, 6-dihydroxytryptamine (5,6-DHT) into the dorsal raphé nucleus produced a similar fall in 5-HIAA levels but had no effect on morphine analgesia.

3 Selective destruction of the periventricular catecholamine system produced by microinjection of 6-hydroxydopamine (6-OHDA) caused a slight decrease in morphine analgesia, thus raising the possibility that catecholamines may be involved in the action of morphine in the PAG.

4 5,7-Dihydroxytryptamine-induced lesions of the spinal cord 5-hydroxytryptaminergic pathways reduced cord 5-HT concentration by 70% and markedly attenuated morphine analgesia as determined by the tail flick test.

5 These experiments provide additional evidence that the PAG is a major site of action of opiates in producing analgesia. Furthermore, they have demonstrated the probable involvement of spinal 5-hydroxytryptaminergic pathways in the mediation of opiate analgesic effects.

Introduction

Investigations into central nervous system mechanisms of morphine analgesia have focused attention on two supraspinal systems, the periaqueductal grey matter (PAG) and the descending 5-hydroxytryptaminergic innervation of the spinal cord.

The possible involvement of the PAG in morphine analgesia is suggested by the finding that microinjections of morphine into the PAG or electrical stimulation of this area produces analgesia (Herz, Albus, Metys, Schubert & Teschemacher, 1970; and see Mayer & Price, 1976). More recently, high concentrations of opiate receptors (Kuhar, Pert & Snyder, 1973) and enkephalin (Simantov, Kuhar, Pasternak & Snyder, 1976) have been described in the PAG. Three studies (Bevan & Pert, 1974; Harvey, Schlosberg & Younger, 1974; Yeung, Yaksh & Rudy, 1975) have failed to demonstrate that the PAG is necessary for the analgesic actions of systemic morphine, since lesions of the PAG failed to reduce morphine analgesia. However, these brief reports presented no histological detail. We have re-examined this question by observing the effects of extensive, histologically verified, destruction of the PAG on morphine analgesia. A preliminary report of part of this study has been published (Dostrovsky & Deakin, 1977).

Two monoaminergic systems lie in the ventral part of the PAG, the periventricular system of catecholamine neurones and the 5-hydroxytryptaminergic cell bodies of the dorsal raphé nucleus. Both were damaged by the PAG lesions and this could contribute to any effect of lesions in this area. Therefore the effects of selective destruction of these neurones by intracerebral microinjections of 6-hydroxydopa-

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mine (6-OHDA) or 5,6-dihydroxytryptamine (5,6-DHT) on morphine analgesia were investigated. The remainder of the PAG was unaffected by these lesions.

The descending 5-hydroxytryptaminergic innervation of the spinal cord has also been implicated in morphine analgesia and lesion experiments have purported to show that this system is necessary for the production of analgesia following systemic morphine administration. However, in one study (Vogt, 1974) unusually low doses of morphine were used and in the study of Proudfit & Anderson (1975) the effects of the lesion in depleting spinal cord 5-HT concentrations were not confirmed. In the present study, the system was selectively destroyed by microinjections of 5,7-dihydroxytryptamine into the rostral spinal cord and the effects on morphine analgesia and on spinal cord 5-HT concentrations were examined.

Methods

Male Sprague-Dawley rats weighing between 280 and 300 g were used throughout the experiments. The rats were anaesthetized with tribromoethanol (2.5%; 1 ml/100 g i.p.) for operative procedures. Analgesia tests began one week post-operatively. Each experimental and control group consisted of 10 animals except for Group I PAG controls which consisted of 8 animals (see figure legends).

Electrolytic lesions

Anodal lesions were placed in the PAG by passing 250–300 mA for 30 s through an electrode insulated to 1 mm above the tip. Two groups of experimental and burr hole control animals were prepared. In Group I the co-ordinates were AP + 0.5 and 1.0; V + 4.6; L 0.5 mm bilaterally, relative to the interaural plane with the incisor bar 1 mm above the interaural plane. In Group II a wider AP separation was used: AP + 0.5 and 1.3; V + 4.6; L 0.5 mm. Recovery was uneventful and no deaths occurred. Both groups received two doses (10 and 20 mg/kg) of morphine. A sequential design was used in Group I animals with two weeks elapsing between doses. In Group II animals, a crossover design was used with one week between injections.

5,6-Dihydroxytryptamine lesions of dorsal raphé nucleus

Microinjections of 5,6-DHT (4 μg) in 2 μl 1% ascorbic acid in saline (0.9% w/v NaCl solution) vehicle were made at 1 μl/min into the dorsal raphé nucleus. Coordinates were AP + 0.75; V + 3.8 mm in the mid line.

5,7-Dihydroxytryptamine lesions of spinal cord

Rats were pretreated with protriptyline 25 mg/kg 30 min pre-operatively to prevent non-specific damage to noradrenergic neurones (Hole, Fuxe & Jonsson, 1976). The rostral spinal cord was exposed between C1 and C2 and stabilized by traction applied to the hind limbs, the head being held in a stereotactic frame. Injections of 5,7-DHT (6 μg) in 2 μl 1% ascorbic acid saline vehicle were made bilaterally approximately 0.7 mm off the mid line and 1.7 mm below the surface. No motor impairment was produced in these animals. Control animals underwent the same procedure except for the microinjection.

6-Hydroxydopamine lesions of the periaqueductal grey matter

Rats were pre-treated with chlorimipramine (25 mg/kg i.p.) to prevent non-specific damage to 5-hydroxytryptaminergic neurones. Microinjections of 6-OHDA (4 μg) in 2 μl 1% ascorbic acid saline vehicle were made into the PAG between the two AP coordinates of the electrolytic lesions: AP + 0.7; V + 4.6; L 0.5 bilaterally.

Biochemical determinations

5-HT and 5-HIAA were assayed by the method of Curzon & Green (1970). 5-HT and 5-HIAA were assayed in the corpora striata of the PAG Group I and 5,6-DHT raphé lesioned animals. This area was chosen to reveal maximal effects of dorsal raphé nucleus damage since it is a major termination of the dorsal raphé projections (Jacobs, Wise & Taylor, 1974; Lorens & Guldberg, 1974).

Noradrenaline and dopamine were assayed in amygdala, cortex and hypothalamus by the method of Cuello, Hiley & Iverson (1973).

Brain areas were dissected out on a cold petri dish by cutting two 2 mm thick coronal sections anterior to the pituitary. The striatum was dissected from the anterior slice and the other areas from the posterior slice. Samples were rapidly frozen on dry ice and maintained at −70°C until assay.

Analgesia testing

Experimental and control animals were tested in random order and were indistinguishable. The identity of each animal was unknown to the experimenter at the time of testing.

Tail flick latencies were measured with the animals in loose fitting perspex restrainers, their tails protruding into a groove above which was focused 250 W projector lamp. A switch turned on the lamp and started an electronic timer. These were switched off

when the rat moved its tail. A 10 s cut-off point was used to prevent tissue damage.

The hot plate was maintained at 55°C ± 0.5°C. The most objective pain reaction was licking the hind paws and the latency to this reaction was measured with a hand-held stopwatch. However, the first reaction was usually a sudden lurch of the body and the latency to this sign was also recorded.

Hot plate and tail flick latencies were measured together before and 30 min after a single injection of morphine. The effects of morphine are presented as % maximum possible effect according to the formula:

$$\frac{\text{Post morphine latency} - \text{baseline latency}}{\text{Cutt off latency} - \text{baseline latency}} \times 100$$

Latencies in text are means ± standard deviation.

Technical failures prevented the testing of all groups of animals on the hot plate.

Histology

Brain stems were fixed in formol saline and alternate 30 µm coronal sections were cut on a freezing microtome. Sections were stained with cresyl violet and lesions were drawn with a camera lucida.

Results

Electrolytic lesions of periaqueductal grey matter

Immediately following the electrolytic lesion, as the rats were recovering from the anaesthetic, the lesioned animals displayed a bizarre hyper-reactive behaviour consisting of frequent non-directed jumps. In between jumping the rats were still, but could usually be triggered into a jump by a loud noise or unexpected movement. This behaviour subsided after a few hours and subsequently it was not possible to distinguish lesioned animals from controls.

PAG lesions had no effect on baseline tail flick latencies (PAG lesioned = 3.3 ± 0.6 s; control = 3.7 ± 0.7 s) but PAG lesioned animals showed a reduced analgesic response to morphine 10 and 20 mg/kg (Figure 1).

When the animals were tested on the hot plate, before morphine administration, the PAG lesioned animals took significantly longer to lick their hind paws than controls. The time taken by normal animals to lick their hind paws when placed on the hot plate was 7.0 ± 1.6 s and PAG lesioned animals took 11.0 ± 4.0 s ($P < 0.001$, 2 tailed t test). However, lesioned animals clearly reacted vigorously to the hot plate and the mean latency to first pain reaction (body lurch or paw lifting) was not significantly different between the groups (PAG lesioned = 3.7 ± 1.0 s; control = 3.5 ± 0.6 s). In contrast to the results of the tail flick test, 10 mg/kg morphine had little analgesic effect on control animals or lesioned animals when they were placed on the hot plate and no significant difference was found between these groups. However, the lesioned animals showed a reduced response to morphine at 20 mg/kg compared to control animals (Figure 1).

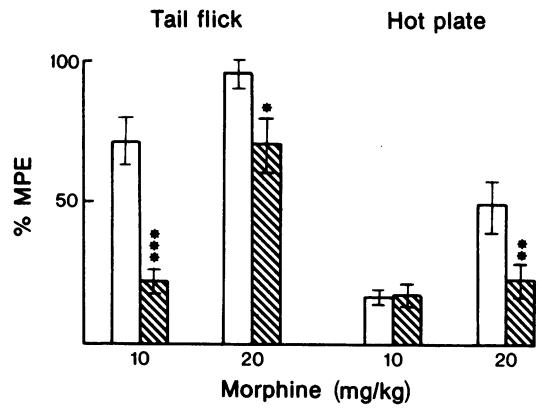

Figure 1 Effects of periaqueductal grey matter (PAG) lesions on morphine analgesia in tail flick and hot plate tests. Hatched columns are lesioned groups, open columns are control groups. Analgesia expressed as % M.P.E. = % maximum possible effect (see Methods). Data from Group I and II (see Methods) combined since no significant differences between these groups were obtained. 20 control animals, 18 PAG lesioned animals. $^{*}P < 0.025$; $^{**}P < 0.01$; $^{***}P < 0.0005$, Fisher's exact probabilities test.

Histological examination revealed extensive danage to the PAG (Figure 2). This was consistently achieved in the middle of the rostro-caudal extent of the PAG.

The role of periventricular catecholamine system damage in periaqueductal grey matter lesion effects

Injections of 6-OHDA into the PAG reduced the effects of 10 mg/kg morphine on the tail flick test (Figure 3). However, the effect was variable and the difference just failed to reach significance on a one tailed test ($P < 0.07$ one tailed Fisher's exact probabilities test). Hypothalamic and cortical noradrenaline and dopamine concentrations were unaffected by the 6-OHDA lesions; however, a significant reduction in amygdala noradrenaline concentrations (6-OHDA = 382 ± 88 ng/g; control = 471 ± 60 ng/g; $P < 0.05$ one tailed t test) but not of dopamine, was observed. None of the regional concentrations of catecholamine correlated with morphine analgesia.

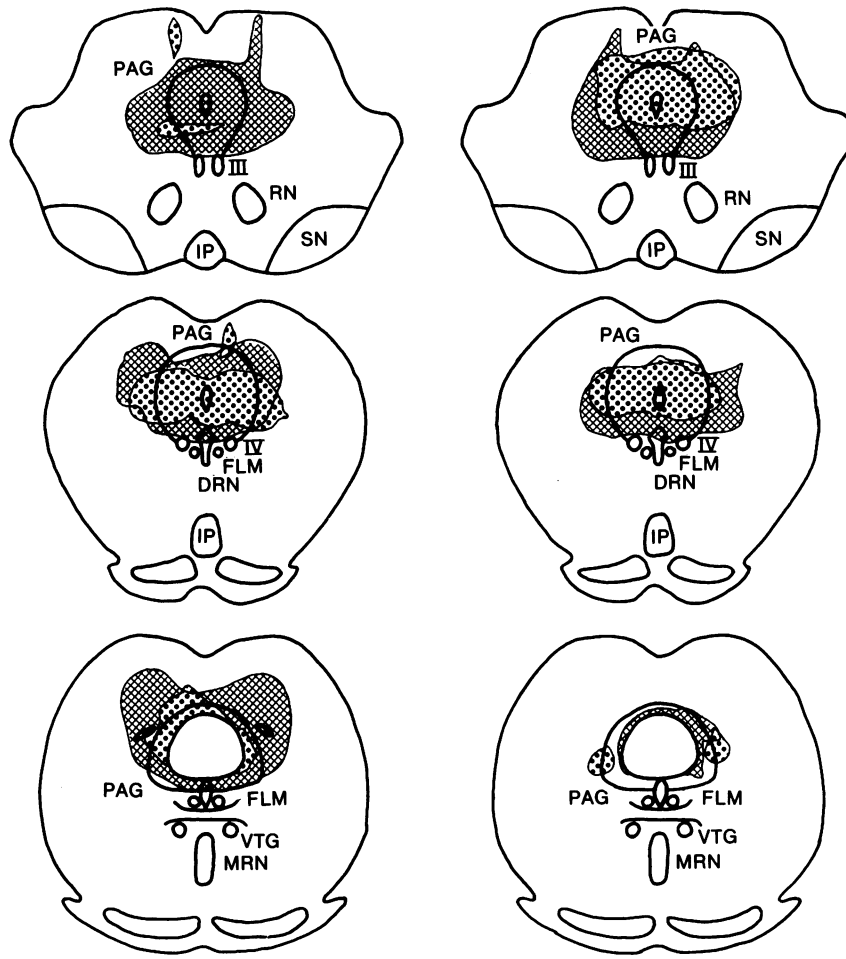

Figure 2 Extent of periaqueductal grey matter (PAG) lesions. Cross hatching represents the largest lesion and stippling the smallest lesion at anterior, middle and posterior levels of the PAG. Group I on left hand side and Group II on right. Consistent lesioning of the middle part of the rostro-caudal extent of PAG was achieved. In one Group I animal the lesion did not reach the most rostral level shown. In 3 Group I and 5 Group II animals the lesion did not reach the most caudal level shown. Abbreviations: III—nucleus of III cranial nerve; IV—nucleus of IV cranial nerve; DRN—dorsal raphé nucleus; FLM—fasciculus longitudinalis medialis; IP—interpeduncular nucleus; MRN—median raphé nucleus; PAG—periaqueductal grey; RN—red nucleus; SN—substantia nigra; VTG—ventral tegmental nucleus of Gudden.

The role of dorsal raphé nucleus damage in periaqueductal grey matter lesion effects

Three indices of damage to the dorsal raphé nucleus were measured in 10 of the PAG lesioned animals: striatal 5-HT, striatal 5-HIAA, and rankings of histological encroachment of lesion onto the dorsal raphé nucleus. Table 1 demonstrates that electrolytic lesions of the PAG reduced striatal 5-HT and 5-HIAA levels by 27% and 34% respectively.

Correlation coefficients were calculated between striatal 5-HT and 5-HIAA and histological intactness of the nucleus; all three measures were significantly intercorrelated. Correlation coefficients of these measures of dorsal raphé nucleus damage with the analgesic effects of morphine at both doses and both

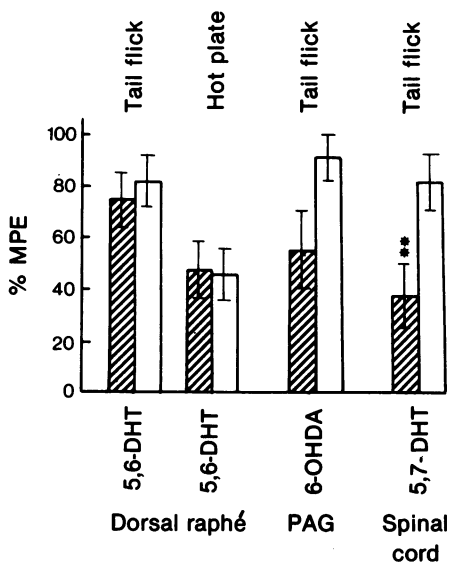

Figure 3. Effects of 5,6-dihydroxytryptamine (5,6-DHT) lesions of the dorsal raphé nucleus, 6-hydroxydopamine (6-OHDA) lesions of the periaqueductal grey matter (PAG) and 5,7-dihydroxytryptamine (5,7-DHT) lesions of the spinal cord on the analgesia produced by 10 mg/kg morphine sulphate. Hatched columns are lesioned groups, open columns are control groups. Standard errors represented by vertical bars. $^{**}P < 0.01$, Fisher's exact probabilities test. 10 animals per group.

analgesia tests were then calculated and found to be insignificant and non-systematic. Hence, there was no tendency for those animals with most damage to the dorsal raphé nucleus to show least analgesia following morphine.

The possibility that the effects of PAG lesions on morphine analgesia were due to damage to 5-HT containing cell bodies in the nucleus raphé dorsalis was further assessed by selective lesioning of these cell bodies with 5,6-DHT leaving the PAG intact. This produced a 25% reduction in striatal 5-HIAA concentrations (Table 1) but had no effect on morphine analgesia on the tail flick or hot plate (Figure 3) tests. Baseline hindpaw lick latencies were unaffected by 5,6-DHT lesions (5,6-DHT lesioned = 12.6 ± 3.7 s; control = 10.2 ± 1.7 s) and the increased paw lick latencies, in Group I PAG lesioned animals did not correlate with the indices of dorsal raphé damage.

The role of the descending 5-hydroxytryptaminergic system in morphine analgesia

5,7-DHT lesions of spinal cord 5-hydroxytryptaminergic fibres reduced cord 5-HT concentrations by 70% from 0.52 ± 0.09 μg/g to 0.16 ± 0.03 μg/g. The effect of 10 mg/kg morphine on the tail flick test was markedly reduced in the lesioned animals (Figure 3). Baseline latencies were unchanged and the experimental animals were indistinguishable from controls behaviourally.

Discussion

These experiments have demonstrated that PAG lesions attenuate morphine analgesia on two different tests of pain sensitivity, the tail flick and hot plate tests. However, the effects of morphine were not abolished in the lesioned animals and the response to 20 mg/kg was greater than the response to 10 mg/kg morphine. This could indicate that undamaged areas of the PAG mediated the residual response to morphine, or that other mechanisms of action, for example, a direct spinal action (Duggan, Hall &

Table 1 Effects of periaqueductal grey matter (PAG) lesions and 5,6-dihydroxytryptamine (5,6-DHT) lesions of the dorsal raphé nucleus on striatal 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations

	PAG Group I		*5,6-DHT dorsal raphé*	
	Lesioned	*Control*	*Lesioned*	*Control*
Striatal 5-HT (μg/g) ± s.e. mean	0.60** ±0.05	0.82 ±0.07	—†	—†
Striatal 5-HIAA (μg/g) ± s.e. mean	0.68** ±0.09	1.03 ±0.06	0.71* ±0.09	0.94 ±0.09

$^{*}P < 0.05$; $^{**}P < 0.01$.
†technical breakdown during 5-HT assay

Headley, 1976; Yaksh & Rudy, 1976) could be involved. The finding that PAG lesion reduced morphine analgesia is at variance with other studies (Bevan & Pert, 1974; Harvey *et al.*, 1974; Yeung *et al.*, 1975). Possible reasons for the discrepancy are difficult to assess because of the lack of histological and other detail in previous reports. One possibility is that the lesions in previous studies destroyed a smaller proportion of the PAG than in our study. Figure 2 shows that destruction of the PAG was extensive leaving only a variable amount of anterior PAG and the grey matter around the fourth ventrical intact.

PAG lesions did not affect pre-morphine sensitivity to pain but the behavioral reaction to the hot plate was altered in that the hind paw licking component was delayed in lesioned animals. However, other components of the pain response, paw flicking, paw lifting and escape attempts, were equally manifest and occurred at similar latencies to controls. This alteration in the reaction to the hot plate may be related to the finding of Harvey *et al.* (1974) of an analgesic effect of PAG lesions on hot plate reactivity as measured by paw lick latency. Other studies of PAG lesions on other pain sensitivity tests report no analgesic effects (Kelly & Glusman, 1968; Liebman, Mayer & Liebeskind, 1970; Bevan & Pert, 1974; Yeung *et al.*, 1975).

We have investigated the possible role of the periventricular catecholamine system in morphine analgesia because the cell bodies and fibres of this system travel in the PAG and were undoubtedly at least partly destroyed by electrolytic lesions of the PAG. We observed that 6-OHDA lesions of this system produced a slight antagonism of morphine analgesia of borderline statistical significance. Therefore, the possibility that the effects of electrolytic lesions of the PAG may have been due in part to destruction of the periventricular catecholamine system cannot be excluded. However, it should be noted that there was no relation between regional catecholamine concentrations and the effects of morphine. The effects of 6-OHDA lesions of the PAG on regional catecholamine concentrations are of interest and suggest that this system may have a noradrenergic projection to the amygdala. The reduction in amygdala noradrenaline concentrations is not due to damage to the nearby dorsal noradrenergic bundle since cortical noradrenaline concentrations were unchanged.

It is unlikely that the effects of PAG lesions could be due to damage to the adjacent dorsal raphé nucleus. No systematic relationship was found between the extent of damage to the nucleus or reduction in forebrain 5-HT and 5-HIAA levels and the reduction in morphine analgesia. Furthermore, our control pharmacological 5,6-DHT lesions of the dorsal raphé nucleus did not reduce morphine analgesia despite comparable reductions in striatal 5-HIAA concentrations. Other groups have also failed to obtain a reduction in morphine analgesia following electrolytic dorsal raphé lesions (Lorens & Yunger, 1974; Adler, Kostowski, Recchia & Samanin, 1975) with the exception of the study of Sasa, Munekiyo, Osumi & Takaori (1977).

A number of studies have provided evidence for the involvement of descending 5-hydroxytryptaminergic pathways, activated by morphine, which inhibit transmission of nociceptive input at the spinal level. Vogt (1974) has found that lowering brain 5-HT concentrations by injection of 5,6-DHT or intraperitoneal *p*-chlorophenylalanine reduces morphine analgesia. This effect was attributed to an action on descending 5-hydroxytryptaminergic pathways to spinal cord since spinal 5-HT levels were the most affected by drug treatments. It has been reported that iontophoretically applied 5-HT inhibits responses of lamina I spinal neurones to noxious stimuli (Randić & Yu, 1976). Deakin, Dickenson & Dostrovsky (1977) and Anderson, Basbaum & Fields (1977) have shown that systemically administered morphine leads to increased firing of some raphé magnus neurones which are believed to be primarily 5-hydroxytryptaminergic and which project to the spinal cord. Electrical stimulation of these neurones inhibits behavioural and electrophysiological responses to pain (Proudfit & Anderson, 1975; Beall, Martin, Applebaum & Willis, 1976). Finally, Ruda (1975) has demonstrated a projection from the PAG to the nucleus raphé magnus suggesting a possible link between PAG opiate receptors and the 5-HT innervation of the spinal cord. Our results demonstrate that selective lesions of the spinal 5-hydroxytryptaminergic pathways result in large decreases in morphine analgesia and provide additional support for the involvement of this pathway in morphine analgesia.

This study has provided further evidence for an action of opiates in the PAG and in activating descending 5-hydroxytryptaminergic pathways which act at the spinal level to block transmission of nociceptive stimuli. However, it remains to be demonstrated that the spinal 5-hydroxytryptaminergic system is the efferent pathway for opiate actions in the PAG. It is unlikely that these systems are the only ones involved in opiate-induced analgesia. Yaksh & Rudy (1976) have demonstrated a direct spinal action of morphine and iontophoretic application in the spinal cord has produced blockade of some electrophysiological responses to painful stimuli (Calvillo, Henry & Newman, 1974; Dostrovsky & Pomeranz, 1976; Duggan *et al.*, 1976). These results are borne out by the demonstration of high concentrations of opiate receptors and endorphins in the substantia gelatinosa layer of the dorsal horn (Kuhar *et al.*, 1973; Simantov *et al.*, 1976). Nevertheless, the dramatic de-

crease in the analgesic efficacy of morphine following lesions of the PAG and descending 5-HT system points to a major role for these supraspinal systems in morphine analgesia.

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