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Lack of evidence for increased descending inhibition on the dorsal horn of the rat following periaqueductal grey morphine microinjections

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1 Recordings were made from 18 neurones in the dorsal horn of the rat, anaesthetized with halothane. All cells received A- and C-fibre inputs and responded to innocuous and noxious stimuli applied to their excitatory receptive fields located on the extremity of the ipsilateral hindpaw. Transcutaneous application of suprathreshold (mean 3.2T) 2 ms square-wave pulses to the centre of the receptive fields resulted in responses to A- and C- fibre activation being observed; a mean 32.4 ± 6.0 C-fibre latency spikes were evoked per stimulus.

2 A high dose (20 μg) of morphine in 0.5 μl sterile saline, microinjected into the periaqueductal grey matter (PAG) had no effect on the C-fibre-evoked activity of thirteen cells (72%) and facilitated 5 neurones (28%). Microinjection sites covered most of the PAG particularly the caudal medioventral zone.

3 A relatively high dose (6 mg kg^{-1}, i.v.) of systemic morphine chloride, sufficient to elicit the direct spinal action of the opiate, inhibited all 5 cells tested.

4 We conclude that there is little evidence that the supraspinal action of morphine includes increased descending controls and depression of dorsal horn neurones in the rat.

Introduction

The relationships between the activity of dorsal horn nociceptive neurones and the analgesic effects of morphine have been extensively studied (see Duggan & North, 1984; Le Bars *et al.*, 1987). However, whereas the direct spinal actions of morphine and other opiates would seem to be due to a depression of these neuronal activities, the action of supraspinal morphine is as yet controversial. Since many of the sites in the midbrain and brainstem which support morphine analgesia also give rise to descending inhibition induced by electrical stimulation, it has been presumed that opiates simply mimic these inhibitions (Liebeskind *et al.*, 1976; Mayer & Price, 1976; Basbaum & Fields, 1978; 1980; Fields & Basbaum, 1978; 1984). Direct testing of this premise, gauging the effects of microinjections of morphine on dorsal horn neurones has led to conflicting results. Two groups have reported neuronal depression following morphine microinjected into the periaqueductal grey matter (PAG) (Bennett & Mayer, 1979; Gebhart *et al.*, 1984) whereas we have found contrary results (Dickenson & Le Bars, 1987) as have other groups using either brainstem microinjections (Llewellyn *et al.*, 1986), intracerebroventricular administration (Sinclair, 1984; Bouhassira *et al.*, 1986) or other experimental approaches such as reversible block of descending inhibition in the presence of morphine (Duggan *et al.*, 1980). In our previous studies we have used 5 μg of morphine in the microinjection, a dose effective in behavioural testing of analgesia in rats (Yaksh & Rudy, 1978; Dickenson & Le Bars, unpublished results). However, since the presence of anaesthesia could possibly reduce sensitivity to opiates, we describe here the interactions between dorsal horn nociceptive neurones and a much higher dose (20 μg) of microinjected morphine. We have also tested the effect of a dose of systemic morphine (6 mg kg^{-1}, i.v.), known to elicit the spinal inhibitory action of morphine (Le Bars *et al.*, 1980b) on the same cells.

We have chosen to monitor convergent neurone activity for two major reasons; (i) These cells are most probably involved in the transmission and integration of nociceptive information at the spinal level since they are clearly influenced by converging excitatory and

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inhibitory mechanisms in a fashion which relates to clinical observations (see references in Le Bars *et al.*, 1986). For example, viscero-somatic convergence can explain clinical referred pain and most of the manipulations which result in hypoalgesia or analgesia in humans also result in a reduction of the nociceptive responses of these convergent neurones in animals. These procedures include systemic and intrathecal morphine administration, dorsal column stimulation, transcutaneous electrical stimulation, stimulation of periaqueductal, periventricular and thalamic structures and heterotopic noxious stimulation. (ii) Secondly, these cells are strongly modulated by supraspinal structures; in particular electrical stimulation of the PAG induces marked inhibitory effects on dorsal horn convergent neurones (Guilbaud *et al.*, 1972; Liebeskind *et al.*, 1973; Oliveras *et al.*, 1974; Duggan & Griersmith, 1979; Hayes *et al.*, 1979; Carstens *et al.*, 1980; Yezierski *et al.*, 1982). Thus this type of neurone appears appropriate for an effect to be found following PAG morphine.

We have also estimated the diffusion of the drug during the microinjection by autoradiographic means (Dickenson & Le Bars, 1987) and so have attempted to map the periaqueductal grey matter for effects on nociceptive processing in the dorsal horn. A preliminary account of this work has appeared (Dickenson & Le Bars, 1983).

Methods

Electrophysiology

The methods have previously been described in detail (Dickenson & Le Bars, 1987) and only a brief account will be given here. Male Sprague Dawley rats weighing 230–270 g were anaesthetized with halothane (3% in 66% N_2O, 33% O_2), tracheal and jugular cannulae inserted and the lumbar dorsal horn exposed by laminectomy. The rats were placed in a head-holder and a craniotomy made over the PAG. The animals were then paralysed with gallamine triethiodide and the halothane level reduced to 0.5%. Heart rate was continuously monitored and core temperature maintained at 37 ± 0.5°C by means of a homeothermic blanket system. Single unit extracellular recordings were made in the lumbar spinal cord from dorsal horn neurones receiving A- and C-fibre inputs from ipsilateral hindpaw receptive fields. Neurones were responsive to both innocuous and noxious stimuli, typical convergent (or multireceptive) cells, and were located in both the superficial and deep laminae of the dorsal horn. Activity was evoked by transcutaneous electrical stimulation of the receptive field (2 ms at 0.66 Hz) and the C-fibre evoked activity separated on latency measurements and quantified in blocks of 50 trials. Following a stable control sequence the morphine was microinjected into the midbrain. A cannula (230 μm external, 170 μm internal diameter) previously inserted stereotaxically into the PAG was used for the injections by connecting to a motor driven Hamilton syringe. An injection of 20 μg of morphine in 0.5 μl sterile saline was made over a 6.5 min period. The neuronal activity was then monitored at 5 min intervals for up to 1 h. At this stage, the effects of systemic morphine (6 mg kg^{-1}, i.v.) was tested on 5 neurones, together with the effects of naloxone (0.6 mg kg^{-1}, i.v. 20 min after the morphine). Following cessation of the experiment the rat was deeply anaesthetized and perfused transcardially by saline followed by 10% formalin, 30 μm thick frozen sections were cut through the midbrain, stained with cresyl violet, and the microinjection sites plotted onto four representative sections of the PAG according to the atlas of Paxinos & Watson (1982).

Autoradiography

In order to provide information as to the diffusion of morphine at the tip of the cannulae, an additional 3 rats were prepared in conditions identical to those used for electrophysiological experiments. [^{3}H]-morphine with specific activity of 24 Ci $mmol^{-1}$ (Amersham) was added to cold morphine to achieve the concentration used in the present study, and microinjected within the PAG (20 μg, 0.5 μl) over a period of 6.5 min; 30 min later, the level of halothane was increased to 3% for 5 min then the spinal cord and the brain were quickly removed and frozen in isopentane at − 70°C. Serial 16 μm thick sections were cut on a cryostat and every fourth section was thawmounted on a slide at 50°C. For autoradiography, we used the technique described by Young & Kuhar (1979) for localization of receptors, and the techniques used were identical to those described previously (Dickenson & Le Bars, 1987).

Results

Autoradiography

The spread of the injection was gauged by autoradiographic means following microinjection of [^{3}H]-morphine (20 μg; 0.5 μl) and killing the animal at 30–40 min. No labelling was observed over the spinal cord sections. The general impression given by the brain autoradiograms was a large diffusion of [^{3}H]-morphine within a zone about 1.25–1.5 mm around the tip of the cannula. Interestingly labelling was not found along the track of the cannula and was spherical or oblong.

An approximation can be made of the mean concen-

tration of morphine in the brain. We injected 20 μg of morphine base in 0.5 μl (140 mM) which appeared to diffuse in a 2.5–3 mm diameter sphere (a volume of 8–14 μl). The mean concentration in such a sphere was high, in the 25–50 mM range.

About one half of the labelling was concentrated in this theoretical volume. In the analysis of data, and for clarity of presentation we have arbitrarily chosen the theoretical volume of 0.5 μl (492 μm radius) for the drawings of the diffusion of morphine (see Figures 1, 3 and 5); it must be recognized that this is an underestimate of the real diffusion of the drug.

Electrophysiology

A total of 18 neurones, all receiving A- and C-fibre inputs were recorded and the effects of injection of morphine at various midbrain sites gauged on the electrically evoked activity of these cells. Their excitatory fields were found on the extremity of the ipsilateral hindpaw and could be activated by both noxious (pinch, radiant heat) and innocuous (hair movement, stroking, light pressure) stimuli. By application of transcutaneous electrical square-wave stimuli of 2 ms duration to the centre of their

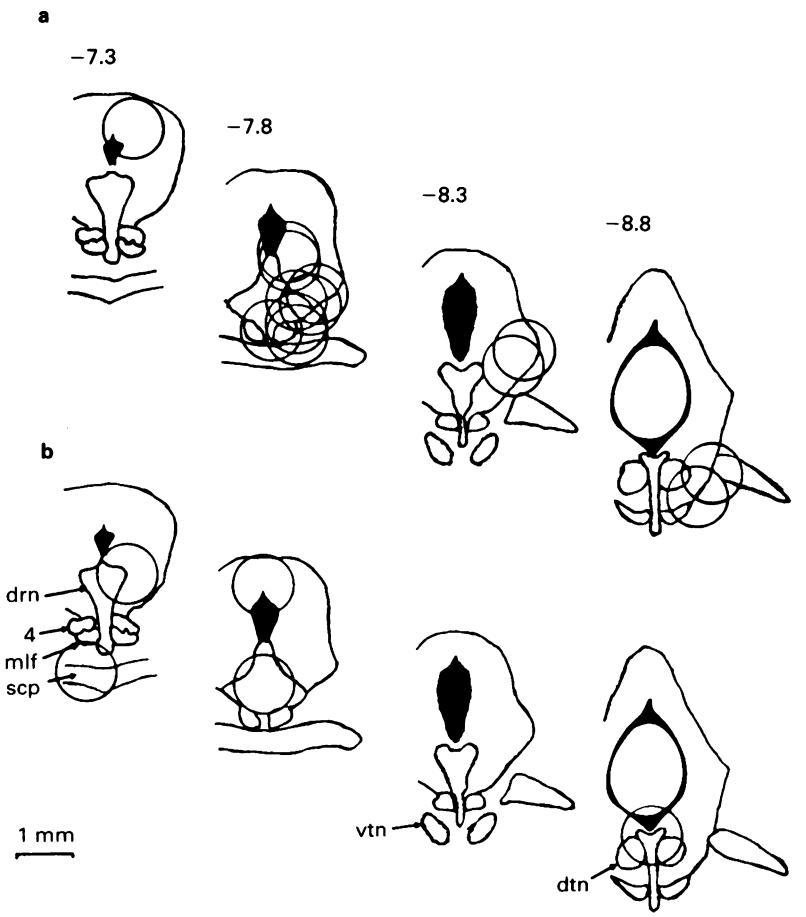

Figure 1 Location of microinjection sites with reference to the two types of effects of periaqueductal grey (PAG) morphine observed on C-fibre-evoked responses. The corresponding mean curves are respectively shown in Figure 6a and b. (a) Unaffected responses (13/18); (b) facilitated responses (5/18). Drawing from the atlas of Paxinos & Watson (1982); antero-posterior coordinates are indicated on the upper part of the figures with reference to bregma. Abbreviations: drn: dorsal raphé nucleus, 4: trochlear nucleus; mlf: medial longitudinal fasciculus; scp: superior cerebellar peduncle; vtn: ventral tegmental nucleus; dtn: dorsal tegmental nucleus.

excitatory receptive fields, responses due to peripheral activation of A- and C-fibre could be observed. The mean C-fibre threshold for these cells was 3.9 ± 0.6 mA and at a stimulation strength of a mean 3.2 times threshold, 12.4 ± 2.4 A-fibre latency and 32.4 ± 6.0 C-fibre latency spikes were evoked per stimulus. All these neurones were studied fully for at least 55 min following the microinjection of morphine. One experiment was carried out with each animal. The neurones tested fell into two groups, so that 13 cells were not influenced and 5 were facilitated. The injection sites corresponding to these units are shown on Figure 1.

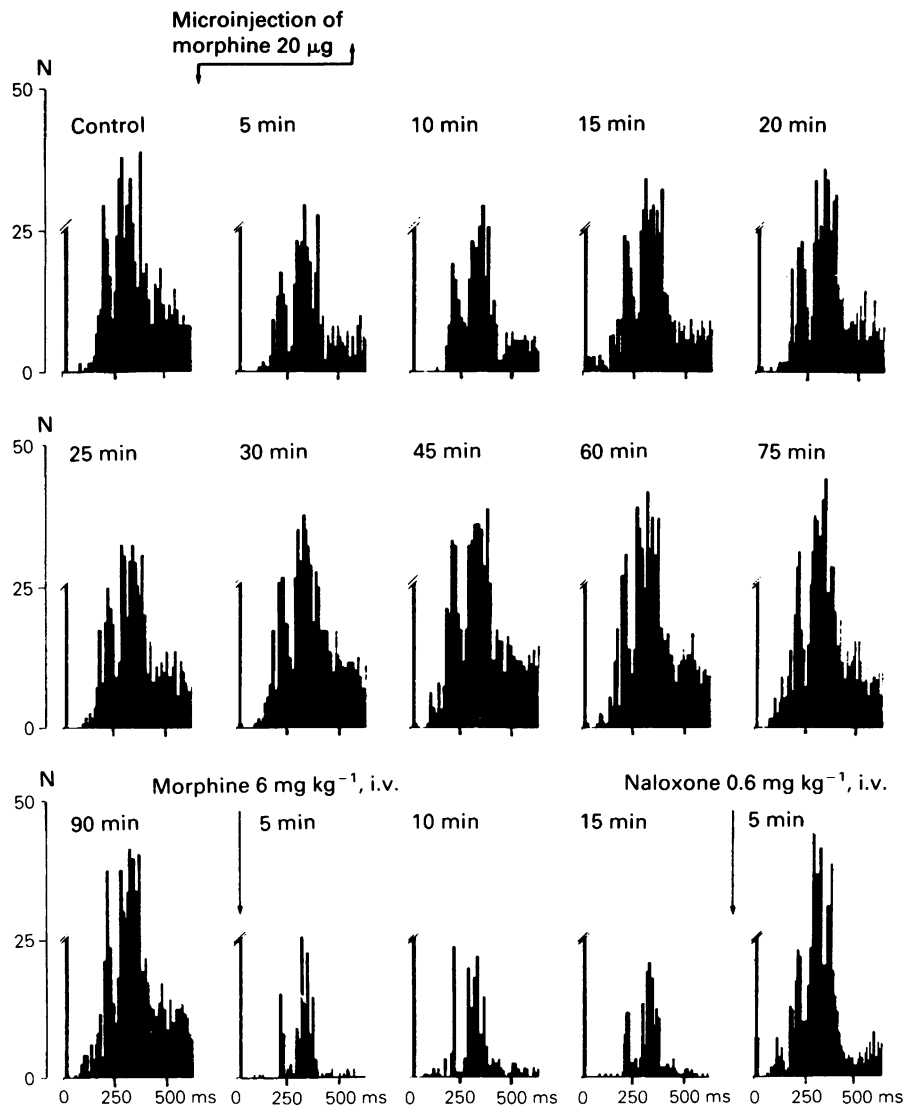

Figure 2 Individual example of the lack of effects of periaqueductal grey (PAG) morphine upon the C-fibre-evoked response of a convergent neurone. Post-stimulus histograms (ordinate scale: number of spikes obtained with 50 trials and a bin width of 5 ms). The earlier Aα responses are truncated for clarity of presentation. PAG morphine resulted in only an early (15–10 min) slight depression of the responses which then remained almost constant during the 90 min recording (see the corresponding curve in Figure 3c). The systemic administration of morphine strongly depressed the neuronal responses in a naloxone-reversible fashion.

Cells not influenced by morphine

This group of cells formed the largest population of neurones (72%) tested with 20 μg of PAG morphine. The injection sites corresponding to these cells are given in Figure 1a and with the exception of one site in the rostral dorsal PAG, all were clustered in the medioventral and lateroventral parts of the caudal PAG with many including the nucleus raphé dorsalis. A representative example is given in Figure 2 in the form of PSTH's: following the microinjection of morphine into the PAG a short lasting depression (5–10 min) was followed by a recovery which remained more or less constant over a 90 min period (see the corresponding curve in Figure 3c). Ninety minutes after the microinjection, we tested the effects of morphine chloride (6 mg kg^{-1} i.v.), on the neurones, a dose close to the ED_{50} for C-fibre inhibition following systemic morphine (Le Bars *et al.*, 1980b). The systemic opiate produced a clear and rapid depression of the cell fibre-evoked activity which was promptly reversed by systemic naloxone (0.6 mg kg^{-1}, i.v.). Hence this neurone and also the four others tested in this way clearly were responsive to the inhibitory effects of systemic morphine.

Examples of the responses of these cells to the microinjection are shown in Figure 3 in the form of inhibitory curves. It should be noted that these examples are of cells where the microinjections were in the dorsal, lateral and ventral PAG respectively. The cells in Figure 3a and b exhibited no change in C-fibre-evoked activity following the microinjection whilst, as already shown in Figure 2, the cell in Figure 3c was transiently depressed for 10 min following the injection but the response rapidly recovered. Such a transient depression was only observed with this unit and in none of the cells in this population was there any maintained inhibition. The overall results for these 13 neurones are given in Figure 6a and it is clear that the mean response was not altered in any way by the microinjection.

Cells facilitated by morphine

Five of the 18 cells (28%) were facilitated by the opiate with the C-fibre responses being clearly elevated above control levels. An individual example is shown in Figure 4 in the form of a PSTH series: note the transient decrease observed over the first 15 min which is followed by an increased activity which was particularly clear during the 30–40 min period (see the corresponding curve in Figure 5a). Examples of the responses of these cells to the morphine microinjection are shown in Figure 5; the clear elevations of activity shown in Figure 5 resulted from two microinjections including the nucleus raphé dorsalis. It should be noted that the elevated activity lasted generally about 1 h and then declined to control levels (Figure 5b). As already shown in Figure 4, the cell in Figure 5a was transiently inhibited for 15 min following the injection but such an effect was only observed with this unit. The overall results for these 5 neurones (Figure 6b) demonstrate these facilitatory effects. The microinjection sites for the total population of these cells are given in Figure 1b.

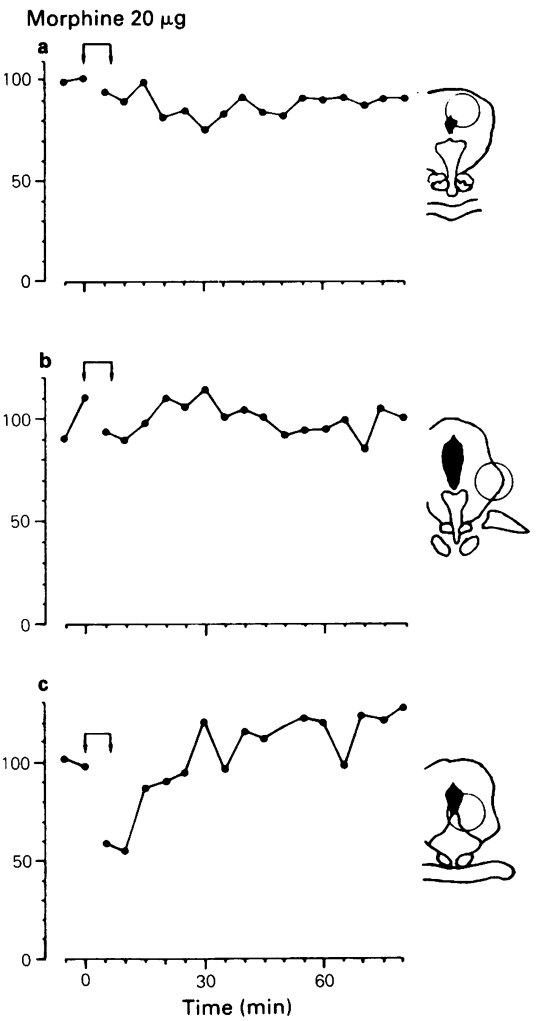

Figure 3 Individual examples of the lack of effect of periaqueductal grey (PAG) morphine upon the C-fibre-evoked responses. Curves are expressed in terms of percentage of the mean control pre-morphine values. In each case the microinjection site is drawn on the right of the corresponding curve on a representative midbrain section (see Figure 1).

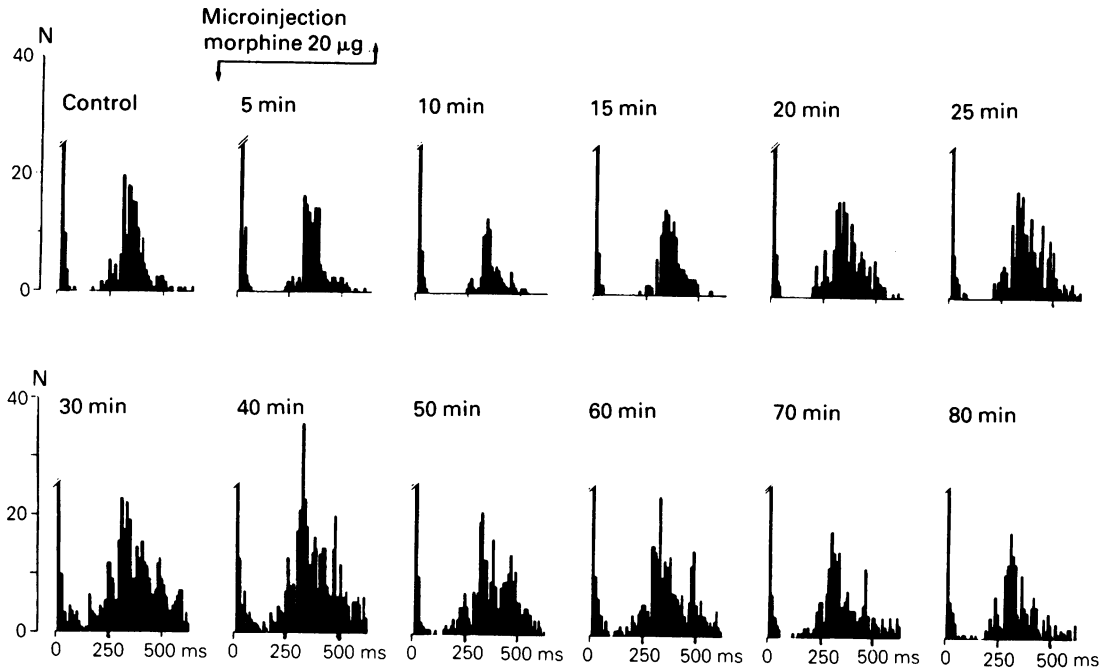

Figure 4 Individual example of the facilitatory effect of periaqueductal grey (PAG) morphine upon the C-fibre-evoked activity of a convergent neurone (presentation as in Figure 2). PAG morphine resulted in an early (5–15 min) weak depression of the responses but the major effect was a facilitation which peaked at 30–40 min (See the corresponding curve in Figure 5a).

Discussion

These results clearly show that the microinjection of large doses of morphine into the PAG of the rat do not produce any depression of C-fibre-evoked activity of dorsal horn neurones, at least under these experimental conditions. However, since systemic morphine was effective in depressing this activity we can conclude that morphine does not increase descending inhibitions acting on these cells. This is in keeping with our previous results where 5 μg of morphine microinjected into the PAG (Dickenson & Le Bars, 1987) or the nucleus raphé magnus (NRM) (Le Bars *et al.*, 1980a) believed to be at the origins of descending inhibitory 5-hydroxytryptaminergic pathways, had similar negative effects. In a similar way we found recently that i.c.v. morphine (IIIrd ventricle) induced dose-dependently a facilitation of C-fibre-evoked responses of dorsal horn convergent neurones over a 0.6–40 μg dose range (Bouhassira *et al.*, 1986). Other authors, studying the microinjection of morphine into NRM, intracerebroventricular morphine and systemic morphine in the presence and absence of reversible block of descending controls have reached similar conclusions (Duggan *et al.*, 1980; Sinclair 1984; Llewellyn *et al.*, 1986). However, experiments in the cat with PAG and NRM morphine (Gebhart *et al.*, 1984; Du *et al.*, 1984) and another in the rat with PAG microinjection (Bennett & Mayer, 1979) have shown opiate inhibition of dorsal horn cells. Reasons for these discrepancies are not altogether clear but in the rat study only a subpopulation of cells was inhibited and the cat PAG microinjection sites were restricted to the dorsal or lateral PAG.

The location of the microinjection sites are relevant in our experiments since many studies have shown that sites in ventral PAG, particularly the nucleus raphé dorsalis are the effective sites for behavioural analgesia (Tsou & Jang, 1964; Jacquet & Lajtha, 1973; Sharpe *et al.*, 1974; Yaksh *et al.*, 1976; Lewis & Gebhart, 1977a,b). In addition, the cat does not respond to morphine in a way akin to human or rats—'feline mania' (see Villablanca *et al.*, 1984) and another complication in this species is the report that high doses (50–100 μg) of morphine are required following PAG microinjections for analgesia (Ossipov *et al.*, 1984) while the study cited above used only 10–20 μg. We deliberately chose a high dose in the rat since we wished to ensure our previous negative results with 5 μg were not due to insufficient opiate at the receptor

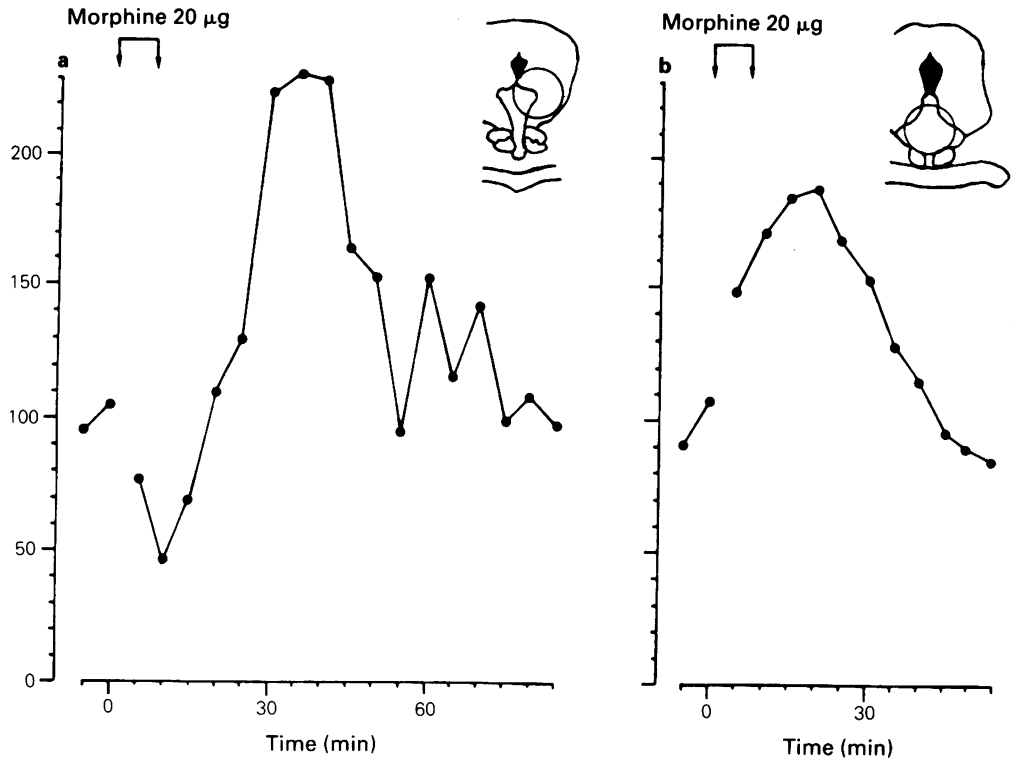

Figure 5 Individual examples of the facilitatory effects of periaqueductal grey morphine. (Presentation as in Figure 3).

site. Estimates of the local concentration from the autoradiography give a mean local concentration in the 25–50 mM range within a 2.5–3 mm diameter sphere. Two conclusions could thus be drawn: (1) The diffusion of morphine was large, far exceeding the theoretical spheres of 0.5 μl drawn on Figure 1, and including a large portion of the PAG and the adjacent reticular formation; (2) The concentration was far higher than that achieved in the CNS following high doses of systemic morphine; for instance, 30 min following a subcutaneous injection of 10 mg kg^{-1}, the highest concentrations found in the brain are in the 0.3 μM range (Bolander *et al.*, 1983). We can therefore conclude that our microinjection technique led to a very high concentration, at least five thousand fold higher than that observed following 10 mg kg^{-1}, s.c. However, it is important to note that the extent of diffusion was clear cut and did not include other structures, in particular the spinal cord where no labelling was found.

There is a general agreement that the ventro-caudal part of the PAG contains the most effective sites (Tsou & Jang, 1964; Sharpe *et al.*, 1974; Jacquet & Lajtha, 1975; Yaksh *et al.*, 1976; Lewis & Gebhart, 1977a, b). It is obvious that we have largely covered this region in the present work (see Figure 1). We therefore feel that there is no evidence in the rat that morphine increases descending inhibitions, but rather the converse.

The basis for the decrease in descending inhibitory controls from the brainstem may well be the reduction in diffuse noxious inhibitory controls (DNIC) observed following microinjection of 5 μg of morphine into the PAG, 0.6–10 μg intracerebroventricular morphine and low doses of systemic morphine, the latter being doses with no effect on the evoked C-fibre responses (Le Bars *et al.*, 1981; Bouhassira *et al.*, 1986; Dickenson & Le Bars, 1987). DNIC are predominantly 5-hydroxytryptaminergic controls descending in the dorsolateral funiculus and involving the NRM, (see Le Bars *et al.*, 1986) and the PAG is held to exert an influence on the spinal cord via interconnections with the NRM. It is conceivable that the surgery induced a certain degree of DNIC, possibly tonically active during the recording sessions. Thus any lifting of the DNIC by PAG morphine will tend to favour facilitation of the neuronal responses, as we have reported here, and as has been described by others. Thus we believe that in the rat at least, although evidence for

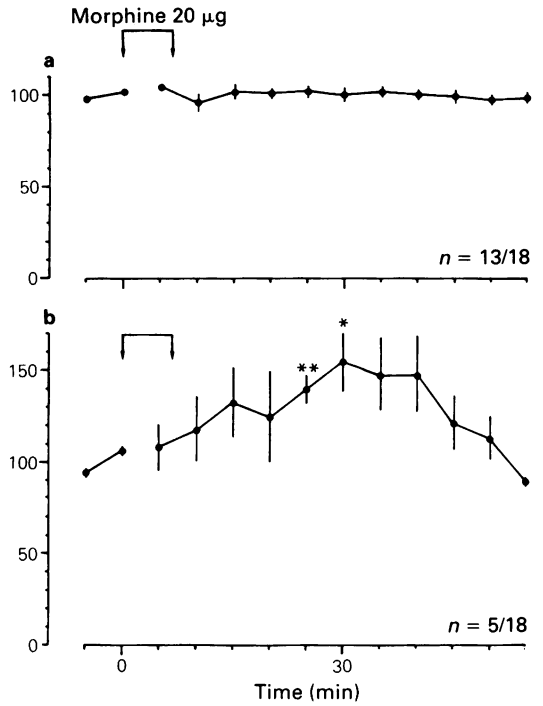

Figure 6 Mean effects of periaqueductal grey (PAG) morphine upon the C-fibre-evoked responses; for each individual neurone, the effect of morphine was calculated with reference to the mean two control pre-morphine values. Two types of effects were observed: (a) 72% of cells had their responses altered by less than 20% by morphine; (b) 28% of cells had their responses facilitated by morphine ($*P<0.05$; $**P<0.01$; paired t test). The corresponding location of the microinjection sites are shown respectively in Figures 1a and b.

and against exists in the cat, supraspinal morphine does not increase descending inhibitions and hence the resultant analgesia is unlikely to be produced by this action. A premise as to how analgesia can be elicited via the reduction in DNIC has been discussed elsewhere (Le Bars *et al.*, 1981, 1983; Dickenson & Le Bars, 1987); the present results support this hypothesis.

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