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SUPRASPINAL MORPHINE AND DESCENDING INHIBITIONS ACTING ON THE DORSAL HORN OF THE RAT

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SUMMARY

1. Recordings were made from thirty-nine convergent neurones in the lumbar enlargement of the rat spinal cord. These neurones were activated by both innocuous and noxious stimuli applied to their excitatory receptive fields located on the extremity of the ipsilateral hind paw. Transcutaneous application of suprathreshold 2 ms square-wave pulses to the centre of the receptive field resulted in responses to A- and C-fibre activation being observed; a mean of $18{\cdot}8 \pm 1{\cdot}8$ C-fibre latency spikes was evoked per stimulus. This type of response was inhibited by applying noxious conditioning stimuli to heterotopic body areas; immersing the tail in a 52 °C water-bath caused a mean $54{\cdot}5 \pm 2{\cdot}3$ % inhibition of the C-fibre-evoked response; such inhibitory processes have been termed diffuse noxious inhibitory controls (d.n.i.c.).

2. The effects of microinjections of morphine (5 μg; 0·2 μl) on both the unconditioned C-fibre-evoked response and inhibitory processes triggered from the tail were investigated in an attempt to answer two questions: (a) does morphine increase tonic descending inhibitory processes and (b) what are the effects of morphine on descending inhibitory processes triggered by noxious stimuli?

3. The predominant effect of periaqueductal grey matter (p.a.g.) morphine on the C-fibre-evoked responses was a facilitation: 51 % of cells had their C-fibre-evoked responses increased by morphine (by roughly 50 %); 31 % of cells were not influenced while the remaining 18 % of units were depressed; however the cells classified as depressed were only marginally so. No clear relationships were found either between the microinjection sites in the p.a.g. and their corresponding effects or between the number of C-fibre-spikes evoked in the control sequences and the subsequent effect of morphine.

4. While d.n.i.c. was not altered by morphine in 56 % of cases, it was clearly reduced in the remaining cells. The effects were immediate but peaked at 40 min following the microinjection (a mean 77 % reduction) and then returned towards control values. All but three of the corresponding microinjection sites were such as to include the medio-ventral p.a.g. including the nucleus raphé dorsalis. In contrast none of the cases where d.n.i.c. was unaltered included microinjection sites in this region.

5. No relationship was found between the changes in d.n.i.c. and the number of spikes evoked in the control sequences, or the changes in the C-fibre responses.

6. Autoradiographic controls using [^{3}H]morphine showed a large diffusion of the drug within an area of about 0·75 mm around the tip of the cannula. Although the dose used (5 μg) was low, the mean concentration within the diffusion sphere was in the 10 mM range, far exceeding the concentrations reached following systemic doses of morphine.

7. It is concluded that high concentrations of morphine within the p.a.g. do not increase tonic descending inhibitory controls but rather decrease them and yet clearly depress d.n.i.c. when the injection sites are within or close to the dorsal raphe nucleus. Since the time course of these effects is related to the behavioural analgesia induced by identical procedures, these findings are discussed with reference to the functional role of d.n.i.c. in pain processes.

INTRODUCTION

The analgesic effects of morphine are mediated by at least two distinct sites in the central nervous system. The direct spinal action of opiates, demonstrated by electrophysiological studies in spinal animals, ionophoretic approaches and behavioural studies, forms the basis for the epidural or intrathecal route of administration of opiates in clinical practice (Yaksh, 1981). This spinal action has been relatively well characterized and is supported by the presence of opiate binding sites (Atweh & Kuhar, 1977*a*) and immunoreactivity to leu-enkephalin (Elde, Hökfelt, Johansson & Terenius, 1976), met-enkephalin (Hökfelt, Ljungdahl, Terenius, Elde & Nilsson, 1977) and dynorphin (Botticelli, Cox & Goldstein, 1981) within the dorsal horn.

The supraspinal action of opiates was first demonstrated by the analgesic effects of intraventricular (Tsou & Jang, 1964; Herz, Albus, Metys, Schubert & Teschemacher, 1970; Jacquet & Lajtha, 1973) and, subsequently, microinjected opiates in animals. Two major supraspinal areas seem involved: the periaqueductal grey matter (p.a.g.) of the mid-brain (Tsou & Jang, 1964; Jacquet & Lajtha, 1973, 1974; Sharpe, Garnett & Cicero, 1974; Yaksh, Yeung & Rudy, 1976; Lewis & Gebhart, 1977*a*, 1977*b*; Iwamoto, Harris, Loh & Way, 1978) and more caudally, an area of the brain stem including the nucleus raphé magnus and surrounding regions (Takagi, Satoh, Akaike, Shibata & Kuraishi, 1977; Akaike, Shibata, Satoh & Takagi, 1978; Dickenson, Oliveras & Besson, 1979; Rosenfeld & Stocco, 1980; Azami, Llewelyn & Roberts, 1982). Despite many studies on these areas the mode of action of morphine remains controversial. Since both an analgesia, in behavioural studies, and inhibitions of dorsal horn nociceptive neurones can be elicited, in a naloxone-reversible manner (Akil, Mayer & Liebeskind, 1976; Oliveras, Hosobuchi, Redjemi, Guilbaud & Besson, 1977; Rivot, Chaouch & Besson, 1979; Zorman, Hentall, Adams & Fields, 1981); see however Pert & Walter (1976), Yaksh *et al.* (1976), Gebhart & Toleikis (1978), Carstens, Klumpp & Zimmermann (1979) and Duggan & Griersmith (1979) by electrical stimulation of these areas, it has been presumed that opiates increase these descending inhibitory controls (Liebeskind, Giesler & Urca, 1976; Mayer & Price, 1976; Basbaum & Fields, 1978, 1980; Fields & Basbaum, 1978, 1984; Mayer, 1979). In previous work we have found however that morphine microinjected within the

nucleus raphe magnus did not decrease the responses of dorsal horn convergent neurones to C-fibre activation (Le Bars, Dickenson & Besson, 1980). The rat p.a.g. is relatively rich in opiate binding sites (Atweh & Kuhar, 1977*b*), predominantly of the μ subtype (Goodman, Snyder, Kuhar & Young, 1980), and contains terminals

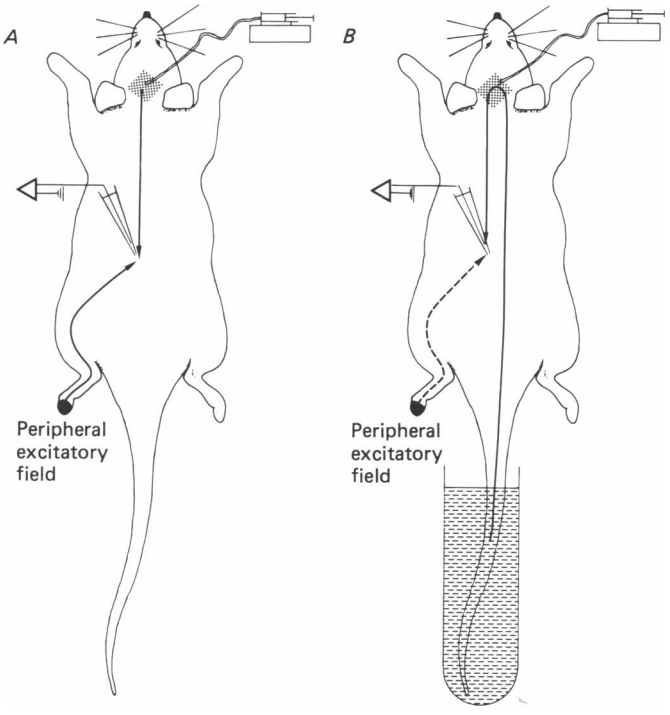

Fig. 1. Experimental design. The present work was aimed at investigating the effects of p.a.g. morphine (stippled area) on the responses of dorsal horn convergent neurones to noxious stimulation of their excitatory receptive field (*A*); if p.a.g. morphine does increase descending inhibitory processes (vertical arrow) then the responses of the neurones would be decreased, and *B*, on descending inhibitory controls (arrowed) triggered by the immersion of the tail in a 52 °C water-bath and acting on the responses of the neurone triggered from the periphery (dashed arrow).

immunoreactive to β-endorphin (Bloom, Battenberg, Rossier, Ling & Guillemin, 1978; Zakarian & Smyth, 1982), enkephalins (Elde *et al.* 1976; Hökfelt *et al.* 1977; Sar, Stumpf, Miller, Chang & Cuatrecasas, 1978; Uhl, Goodman, Kuhar, Childers & Snyders, 1979) and dynorphin (Goldstein & Ghazarossian, 1980; Hollt, Haarman, Bovermann, Jerlicz & Herz, 1980; Khachaturian, Watson, Lewis, Coy, Goldstein & Akil, 1982). The first goal of the present study was therefore a parallel study with morphine microinjected within the p.a.g.; if p.a.g. morphine does increase descending inhibitory processes (Fig. 1*A*), then the C-fibre evoked responses of dorsal horn convergent neurones would be decreased.

The second goal of the present study was to investigate the effects of p.a.g. morphine when descending inhibitory controls are triggered by a noxious stimulus, such as the immersion of the tail in a 52 °C water-bath (Fig. 1*B*). Indeed, we have

described, in a series of experiments, that on the basis of powerful modulation of convergent neurones in the spinal cord (Le Bars, Dickenson & Besson, 1979*a*, *b*) and trigeminal nucleus caudalis (Dickenson, Le Bars & Besson, 1980*a*) by diffuse noxious inhibitory controls (d.n.i.c.), the natural activation of at least some of these descending pathways is by a noxious stimulus. In this regard there is evidence for an involvement of the nucleus raphe magnus (Dickenson, Le Bars & Besson, 1980*b*) and the dorsolateral funiculus (Villaneuva, Chitour & Le Bars, 1986) in which raphe–spinal fibres travel, in the loop subserving d.n.i.c. We have recently reported that low doses of systemic morphine, lower than those required to produce direct spinal inhibitory effects on dorsal horn nociceptive neurones, dose-dependently and in a stereospecific, naloxone-reversible manner, markedly reduce d.n.i.c. (Le Bars, Chitour, Kraus, Clot, Dickenson & Besson, 1981*a*). This implies that these low doses of opiates in fact reduce descending inhibitions, at least when these are activated by a noxious stimulus.

We report here a direct test of these premises, by studying the effects of morphine microinjected into the p.a.g. of the rat, under halothane anaesthesia, on both the responses of dorsal horn nociceptive neurones to peripheral stimuli, and on the inhibitory effects induced by an heterotopic nociceptive stimulus. We have also gauged the diffusion of the drug during the microinjection by autoradiographic means and so have attempted to map the p.a.g. for effects on nociceptive processing in the dorsal horn. A preliminary account of this work has appeared (Dickenson & Le Bars, 1983*b*).

METHODS

Electrophysiology

Animal preparation. Thirty-nine male Sprague–Dawley rats weighing 230–280 g were used in this series of experiments and one neurone was studied per animal. The preparation of the animals was essentially that described previously (Le Bars *et al.* 1980). Anaesthesia was induced by 2–3 % (v/v) halothane in nitrous oxide–oxygen (66–33 % mixture) and tracheal and jugular cannulae inserted. The rat was placed in a head-holder and a craniotomy made over the p.a.g. with care taken not to disturb the sagittal sinus. A laminectomy was made on vertebrae T11–L1 and the L1 lumbar spinal cord exposed. The surrounding vertebrae were clamped and stabilized and the dorsal horn zone receiving maximal input from the ipsilateral hind paw mapped using a surface ball electrode with transcutaneous electrical stimulation of the extremity of the hind paw. The level of halothane was lowered to 0·5 % and maintained at this level throughout the rest of the experiment in the same nitrous oxide–oxygen mixture. The animal was artifically ventilated and paralysed by intravenous injection of gallamine triethioide (Flaxedil). Under these anaesthetic conditions, end-expiratory carbon dioxide levels (3–4 %), are unchanged by noxious stimuli and blood pressure is in the range 100–140 mmHg with no spontaneous changes. Electrocorticograms exhibit regular slow waves and do not alter during considerably more intense noxious stimuli than those used in the present study. These stimuli produced only a transient rise in arterial blood pressure. Finally, if the gallamine is allowed to wear off there are no spontaneous flexor movements of the animal. Full details of the anaesthesia have been published (Weil-Fugazza, Godefroy & Le Bars, 1984; Schouenborg & Dickenson, 1985). Heart rate was continuously monitored and core temperature maintained at 37 ± 5 °C by means of a homeothermic blanket system. A glass micropipette filled with a mixture of 5 % sodium chloride and Pontamine Sky Blue dye (10–15 MΩ) or a glass-insulated tungsten electrode was then inserted into the dorsal horn and the laminectomy covered with 2 % Ringagel agar. A microinjection cannula was then stereotaxically placed into the p.a.g. according to the following coordinates: AP, 0; L, 0 to 1·5 mm and H, 3–6 mm (with zero corresponding to the interauricular axis). The cannula was of 230 μm external and 170 μm internal diameter and was connected to a 5 μl Hamilton syringe driven by an infusion pump.

Recordings. Once a single neurone had been isolated, the receptive field was stimulated using brush, touch, pinch and noxious heat to determine the response of the neurone. In this study all cells studied were excited by brush, touch, prod and light pressure, pinch and noxious heat. Receptive field size was mapped and the depth of the cell in the dorsal horn noted from the microdrive reading. These neurones have been designated as convergent, wide dynamic range, multi-receptive, lamina V type or class 2 and receive both innocuous and noxious, A$\alpha\beta$- and C-fibre afferent inputs. When the activity of these neurones was characterized by transcutaneous electrical stimulation (2 ms duration) via a pair of stainless-steel needles inserted at the centre of their receptive fields, they showed temporal bands of spiking very similar to those evoked by direct nerve stimulation (Menetrey, Giesler & Besson, 1977). According to their latency, and using the classification of Gasser & Erlanger (1927) and Burgess & Perl (1973) these bands were attributed to peripheral Aα- (30–100 m/s), Aδ- (4–30 m/s) and C-(0·4–2 m/s) fibre inputs. The response threshold for C fibres was determined, and the current intensity was then increased to a suprathreshold value (with a mean of 2·8 times threshold) yielding an easily reproducible neuronal response.

Experimental design. The experimental procedure consisted of sequences of 105 such suprathreshold stimuli (0·66 Hz) every 10 min. Between the 35th and the 60th stimulation (i.e. for 37 s), the distal two-thirds of the tail was immersed in hot water (52 °C).

In order to distinguish between responses due to Aα-, Aδ- and C-fibre inputs and to analyse the inhibitory effects observed during and after the application of the conditioning stimulus, a multichannel analyser (Tracor TN 1710) or Neurolog modules were routinely used for building post-stimulus histograms with the following paradigm: the first twenty responses were not considered owing to the fact that habituation or 'wind-up' phenomena were often observed at the beginning of a sequence. The post-stimulus histogram built from the 20th to the 35th responses constituted a control for the sequence. The earlier responses (35–45th) observed during immersion of the tail were discarded owing to the latency (5–10 s) of the maximal inhibitory effects; the histogram built from the 45th to the 60th responses was taken as the response conditioned by noxious heating of the tail. The post-effects were analysed for the 44 s following the cessation of noxious heating of the tail by building a post-stimulus histogram for the 60–75th (post-effects, 0–22 s) and the 75–90th (post-effects, 22–44 s) responses.

When two stable control sequences, with a variation of less than 20% in degree of inhibition of the C-fibre responses had been established, morphine sulphate was injected into the p.a.g. over a period of 2·6 min. The microinjections were 5 μg of morphine base in a volume of 0·2 μl sterile saline. The responses of the neurones and the effects of d.n.i.c. were then monitored at 10 min intervals over a period of 90 min. The results presented here are only from experiments where this exact sequence was achieved and from neurones which did not exhibit changes in spike amplitude or wave form throughout the recording period. The total length of the experiments ranged from 3 to 6 h.

Analysis of results. For each individual cell, the effects of morphine on the C-fibre responses were calculated by reference to the mean control value for the unconditioned C-fibre responses (post-stimulus histogram, 20–35th); at each particular time the unconditioned C-fibre response was expressed in terms of percentage of this mean control value. For the study of the relationship between control responses and effect of morphine (Fig. 5), the mean effect of morphine at times +30, +40 and +50 min was taken as representative of the over-all effect on a cell (see also Fig. 12).

For each individual sequence, the percentage inhibition due to d.n.i.c. was calculated by reference to the unconditioned C-fibre responses at that particular time (post-stimulus histogram, 20–35th). Inhibitions were calculated during the immersion of the tail (post-stimulus histogram, 45–60th) and following the cessation of noxious heating of the tail (post-stimulus histogram, 60–75th and 75–90th; see Fig. 10). For the study of the relationship between C-fibre responses, d.n.i.c. and morphine (Figs. 11 and 12), the mean effect of morphine at time +30, +40 and +50 min was taken as representative of the over-all effect; in addition the effect of morphine on d.n.i.c. was standardized in terms of percentage depression of inhibition: (mean inhibition at time 30–50 min × 100)/mean control inhibition. The Student's paired *t* test was used to calculate degrees of significance.

Histological controls. Following cessation of the experiment the animal was deeply anaesthetized with 5% (v/v) halothane and perfused transcardially by saline followed by 10% (v/v) formalin. Serial 30 μm sections were cut on the freezing microtome and the histological location of the

microinjection site reconstructed by reference to the atlas of Paxinos & Watson (1982) following examination of cresyl-violet-stained sections. Plate 1 provides an example of a section with the microinjection site being within the nucleus raphe dorsalis. The results presented here are only from experiments where microinjection sites were within the p.a.g. and adjacent to surrounding regions; in particular, cases where morphine was injected within the aqueduct were discarded.

Autoradiography. In order to provide information as to the diffusion of morphine at the tip of the cannulae, an additional five rats were prepared in conditions identical to that used for electrophysiological experiments.

[^{3}H]morphine with specific activity of 24 Ci/mmol (Amersham) was completed with cold morphine to achieve the concentration used in the present study, and microinjected within the p.a.g. (5 μg, 0·2 μl) over a period of 2·6 min. 30 min later, the level of halothane was increased to 3% (v/v) 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 thaw-mounted on a slide at 50 °C.

For autoradiography, we used the technique described by Young & Kuhar (1979) for localization of receptors. Emulsion-coated (Ilford K5) cover-slips were allowed to dry for at least 24 h and were sealed against one of the ends of the slide. These 'sandwiches' were then firmly tightened so that no space was left between the cover-slip and the tissue sections. After 2 months exposure at +4 °C, the autoradiograms were revealed with Kodak D19B as a developer, then fixed and thoroughly rinsed, dehydrated and put in solvent; the sections were Nissl-stained with cresyl violet; sealed slides and cover-slip were mounted with Permount.

When labelling of interest was found, a computerized analysis of the optical density of the autoradiograms was performed with a Leitz MPV3 microdensitometer coupled to a Mink 11–23 Digital computer. The region of interest was scanned; for each pixel, the computer coupled to the microdensitometer measured the optical transmission, T ($0 < T \leqslant 1$, $T = 1$ for the background), through a rectangular window aperture (100–120 μm) and the optical density was then calculated as optical density $= -\log(T)$. A digitalized representation of the optical density was printed and isodensity levels drawn.

RESULTS

Autoradiography

For autoradiographic controls, the brain and the lumbar spinal cord were removed and frozen 30–40 min following the microinjection of [^{3}H]morphine (5 μg; 0·2 μl), i.e. at the time of maximal effects both in electrophysiological (*vide infra*) and behavioural experiments (A. H. Dickenson & D. Le Bars, unpublished observations). 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 0·75 mm around the tip of the cannula. Interestingly, labelling was not found along the track of the cannula and was spherical (Pl. 2, left) or oblong (Pl. 2, right). [^{3}H]morphine diffusion was homogenous in the grey matter but appeared to be reduced by the presence of fasciculi, for example by the medial longitudinal fasciculus as shown in Pl. 2*A*.

Examination of the optical density of the labelling (Pl. 2*B*) revealed a heterogenous density of [^{3}H]morphine with a highly radioactive centre (at least 500 μm in diameter) and a progressive decay towards the edges. Plate 2*C* gives a comprehensive representation of the concentration of [^{3}H]morphine in the dorso-ventral (D–V) and latero-lateral (L–R) directions. Note the progressive decay in labelling towards the dorsal, right and left directions while in the ventral direction the more abrupt fall was probably due to the fibres of the decussation of the superior cerebellar peduncle.

An approximation can be made of the mean concentration of morphine in the brain. We have injected 5 μg morphine base in 0·2 μl (90 mM) which appeared to diffuse in

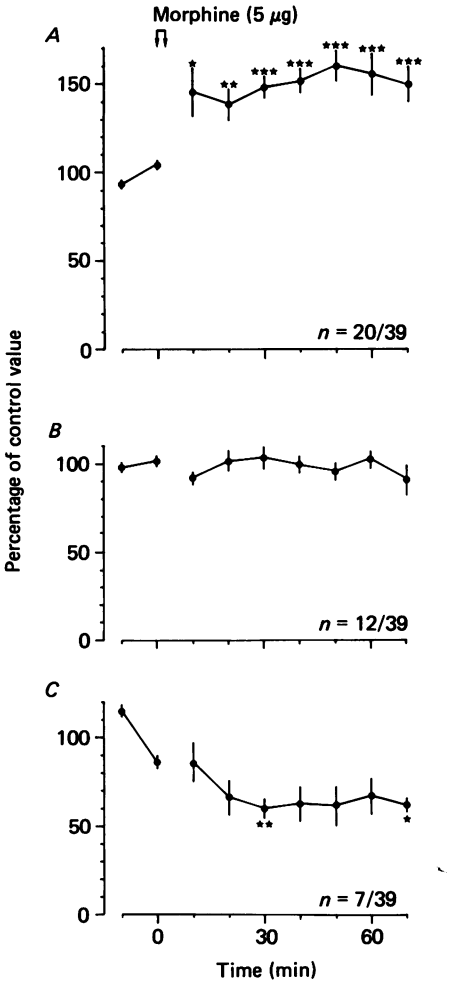

Fig. 2. Effects of p.a.g. morphine upon the C-fibre evoked responses; for each individual neurone, the pharmacological effect was calculated with reference to the mean two control pre-morphine values. Three types of effects were observed: 51 % of cells had their responses facilitated by morphine (*A*; note the plateau of the mean curve); 31 % of cells had their responses altered less than 20 % (*B*); 18 % of cells had their responses decreased by morphine (*C*; note the tendency of the control response to decline before even the microinjection and the subsequent plateau of the mean curve). $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$. The corresponding locations of the microinjection sites are shown respectively in Fig. 4*A*, *B* and *C*.

a 1·5 mm diameter sphere (a volume of 1·77 μl). The mean concentration in such a sphere was high, in the 10 mM range.

The hatched area in Pl. 2*C* represents the extent of a theoretical sphere of 0·2 μl volume (radius of 363 μm). One-half to two-thirds of the labelling was concentrated in this theoretical volume. In the analysis of data, and for clarity of presentation, we have arbitrarily chosen this theoretical volume for the drawings of the diffusion of morphine (see Figs. 3, 4, 7 and 8); it must be recognized that this is an underestimate of the real diffusion of the drug.

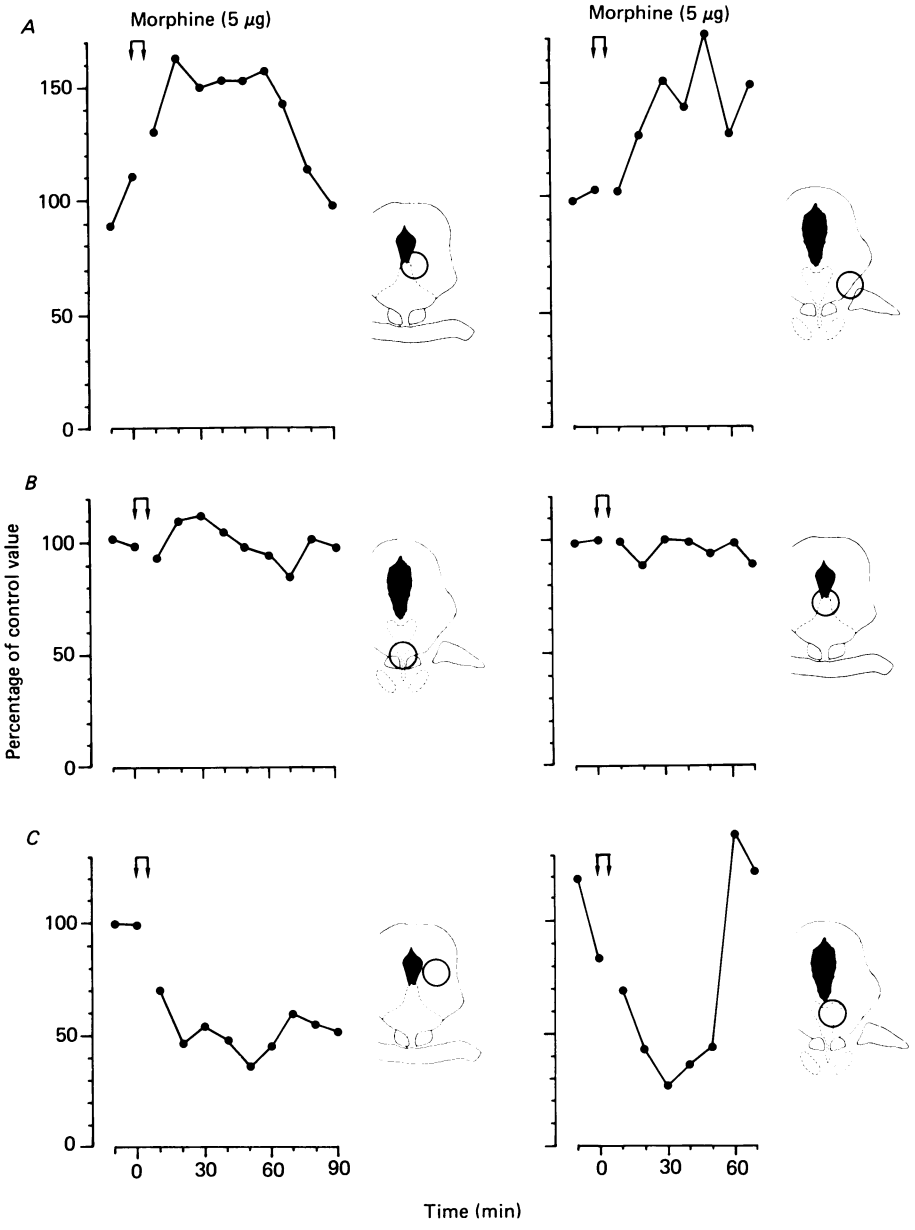

Fig. 3. Individual pairs of examples of the effect of p.a.g. morphine upon the C-fibre evoked responses. In each case the microinjection site is drawn on the right of the corresponding curve on a representative mid-brain section (see Fig. 4). *A*, facilitated responses; *B*, unaffected responses; *C*, decreased responses.

Electrophysiology

General properties of recorded units

A total of thirty-nine neurones were recorded in thirty-nine rats. Their excitatory fields were found on the extremity of the ipsilateral hind paw, included one or several toes and were sometimes larger. The cells could be activated by both noxious (pinch and radiant heat) and innocuous (hair movement, stroking and light pressure) stimuli. By applying 2 ms duration transcutaneous electrical square-wave stimuli to the centre of their excitatory receptive fields, responses due to peripheral activation of A and C fibres could be observed. The mean C-fibre threshold for these cells was $3{\cdot}8 \pm 0{\cdot}3$ mA and at a stimulation strength of a mean 2·8 times threshold, $7{\cdot}9 \pm 0{\cdot}6$ A-fibre and $18{\cdot}8 \pm 1{\cdot}8$ C-fibre latency spikes were evoked per stimulus. All the units were under the influence of d.n.i.c. During application of noxious heat to the tail a mean $54{\cdot}5 \pm 2{\cdot}3$ % inhibition of the C-fibre evoked response was produced. The A-fibre responses were also inhibited but to a lesser extent because of the supramaximal nature of the electrical shock applied on the hind paw; they were not considered in the present study. All these neurones were studied fully for at least 1 h following the microinjection of morphine.

Effects of morphine on the C-fibre evoked responses

The thirty-nine neurones studied had responses after the microinjection of 5 μg morphine within the p.a.g. and adjacent structures which were either not altered (less than 20 % variation from the control values), facilitated or inhibited. The results for these three classes of neurones are presented below.

Neurones facilitated by morphine ($n = 20/39$). Twenty of the thirty-nine cells (51 %) had their C-fibre evoked responses increased by morphine. The mean effect was apparent 10 min after the microinjection and a peak 60 % increase in responses was observed at 50 min although the general feature of the curve was a plateau (Fig. 2*A*). The time course of the effects, however, varied from one cell to another and could be divided into immediate ($n = 11/20$) or delayed ($n = 9/20$) changes (Fig. 3*A* shows examples). The plateau seen for the mean curve results from this heterogeneity.

The location of the microinjection sites corresponding to these facilitated neurones are shown in Fig. 4*A*. There was no clear relationship between a particular site in the p.a.g. and the effects since the sites were located throughout the dorso-ventral and anterio-posterior extent of the region.

Neurones not influenced by morphine ($n = 12/39$). Twelve cells (31 %) had responses which altered by less than 20 % of the control after morphine. The over-all results are shown in Fig. 2*B* and examples in Fig. 3*B*. Again there was no clear histological evidence for a relationship between site and effect (Fig. 4*B*).

Neurones depressed by morphine ($n = 7/39$). Seven cells (18 %) were depressed by the microinjection of morphine, these cells being the smallest subgroup of the whole population. As can be seen from Fig. 2*C*, at two points only (30 and 65 min) were the C-fibre evoked responses significantly depressed by morphine although the general feature of the curve was a plateau; note also that the mean control responses exhibited a tendency to decline in the absence of any pharmacological manipulation. Individual examples are shown in Fig. 3*C*.

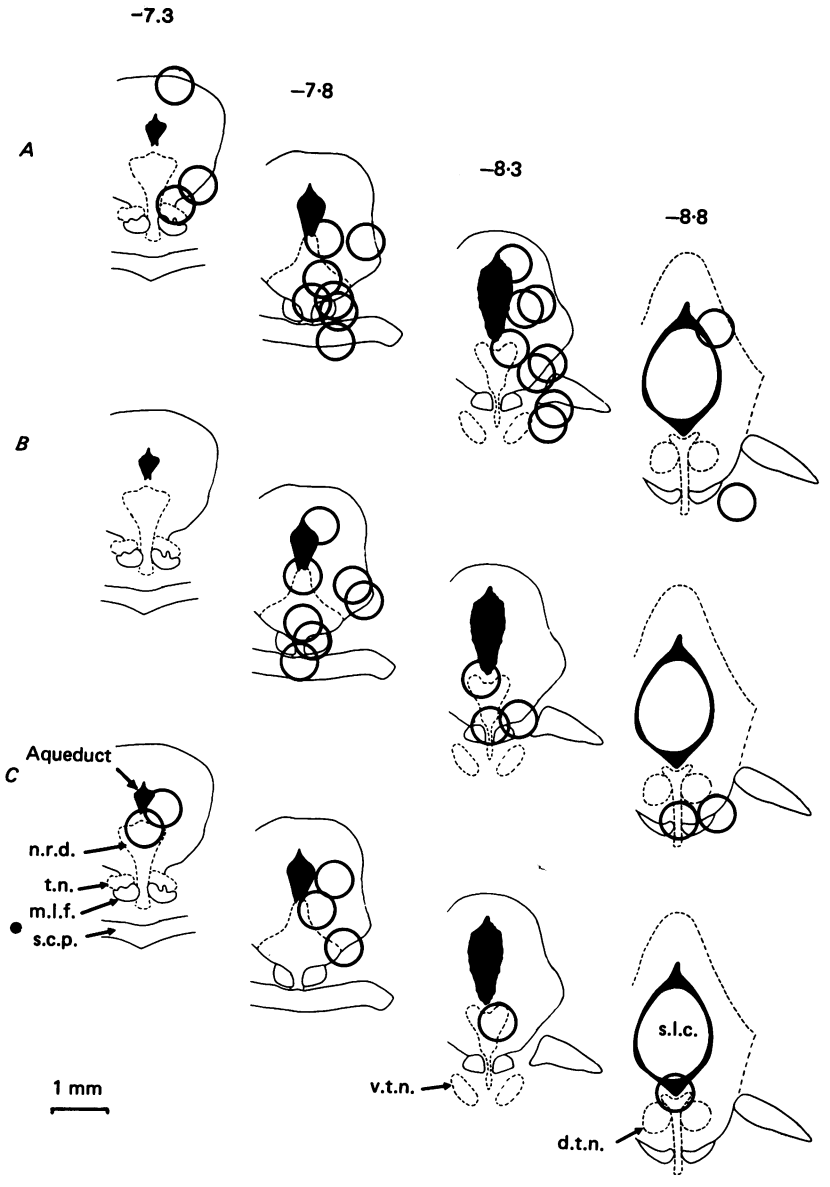

Fig. 4. Location of microinjection sites with reference to the three types of effects of p.a.g. morphine observed on C-fibre evoked responses. The corresponding mean curves are respectively shown in Fig. 2 *A*, *B* and *C*. *A*, facilitated responses ($n = 20/39$); *B*, unaffected responses ($n = 12/39$); *C*, decreased responses ($n = 7/39$). Drawing from the atlas of Paxinos & Watson (1982); anterior-posterior coordinates are indicated on the upper part of the figures with reference to bregma. Abbreviations: n.r.d., nucleus raphe dorsalis; t.n., troclear nucleus; m.l.f., medial longitudinal fasciculus; s.c.p., superior cerebellar peduncle; v.t.n., ventral tegmental nucleus; d.t.n., dorsal tegmental nucleus; s.l.c., second lobule of the cerebellum.

The location of the microinjection sites (Fig. 4*C*) again reveals a degree of spread within the p.a.g. although six of the seven sites were immediately adjacent to the aqueduct and the fourth ventricle.

Relationship between control responses and effect of morphine. The cells variously influenced by morphine had similar physiological characteristics, receptive fields and were all subject to d.n.i.c., and, furthermore, there was no clear relationship between

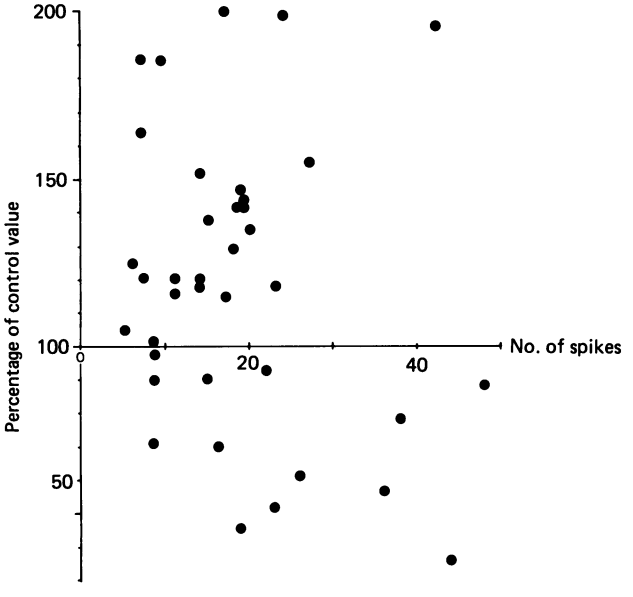

Fig. 5. Relationship between control C-fibre responses and subsequent effect of p.a.g. morphine. Abscissa: mean initial C-fibre response in terms of mean number of spikes per electrical shock; ordinate: mean percentage of control value observed at time +30, +40 and +50 min following p.a.g. morphine (see text).

the histology and direction of the effect of microinjection. Hence we attempted to relate the mean initial C-fibre response (spikes per stimulus) with the effect of morphine. This is illustrated in Fig. 5 and it is clear that there was no relationship between the number of C-fibre spikes evoked in the control sequences and the effect of morphine measured 30–50 min following the microinjection.

Effects of morphine on d.n.i.c.

As previously stated the thirty-nine neurones studied were all subject to d.n.i.c. (mean 54·5±2·3% inhibition). Morphine microinjection either had no effect on d.n.i.c. (less than 20% change) or clearly reduced d.n.i.c.

Lack of effect on d.n.i.c. (n = 22/39). The effect of d.n.i.c. on twenty-two cells (56%) was not altered by the microinjection of morphine. The over-all results for this population are illustrated in Fig. 6*A* where it can be seen that despite a tendency for d.n.i.c. to decline after morphine there was no significant difference from the controls; note the variability of the inhibitory effects over the 30–60 min following the microinjection. Two individual examples are shown in Fig. 7*A*, one cell

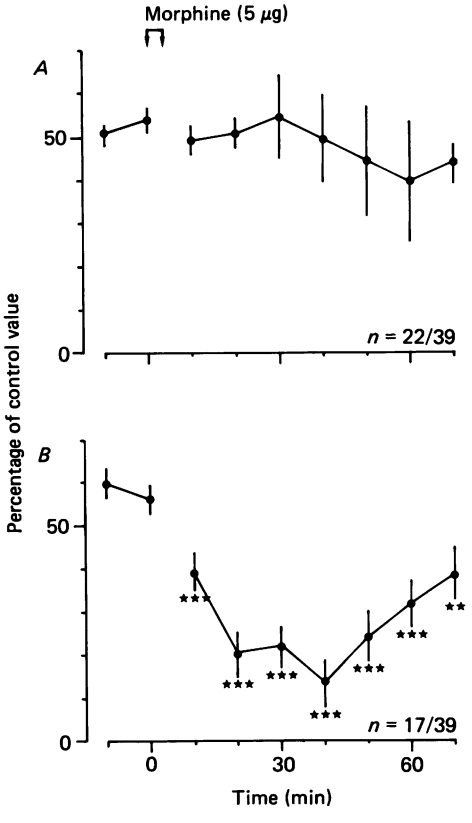

Fig. 6. Effects of p.a.g. morphine upon d.n.i.c. *A*, for 56% of cells, the effect of d.n.i.c. was not altered by p.a.g. morphine. *B*, for 44% of cells, a highly significant reduction of d.n.i.c. was seen following p.a.g. morphine. Note the time course of this effect (statistics as for Fig. 2). The locations of the corresponding microinjection sites are shown in Fig. 8*A* and *B*.

illustrating the cases where d.n.i.c. did not alter at all, the other a case where despite some fluctuations in effect there was no clear direction of change. The location of the microinjection sites where morphine did not influence d.n.i.c. are given in Fig. 8*A*. It is obvious that these sites differ from those in Fig. 8*B* (sites effective in reducing d.n.i.c.) since the former only marginally extend into the nucleus raphe dorsalis and are mainly concentrated laterally either dorsal or alongside the nucleus raphe dorsalis. However, certain sites located dorsally alongside the aqueduct were in the same zone as some of the sites from which morphine elicited a reduction in d.n.i.c. although the former tended to be more caudal (see below).

Reduced effects of d.n.i.c. (n = 17/39). Seventeen neurones (44%) were subject to a reduction in d.n.i.c. following morphine. The over-all results are shown in Fig. 6*B* from which it can be seen that the effects were immediate and peaked at 40 min following the microinjection. At this time d.n.i.c. were reduced from a mean control inhibition of 57·9±3·4 to 13·4±5·6% (77% reduction). Subsequently the effects wore off and the responses returned towards control values. An individual example in the form of post-stimulus histograms is shown in Fig. 9 illustrating these findings.

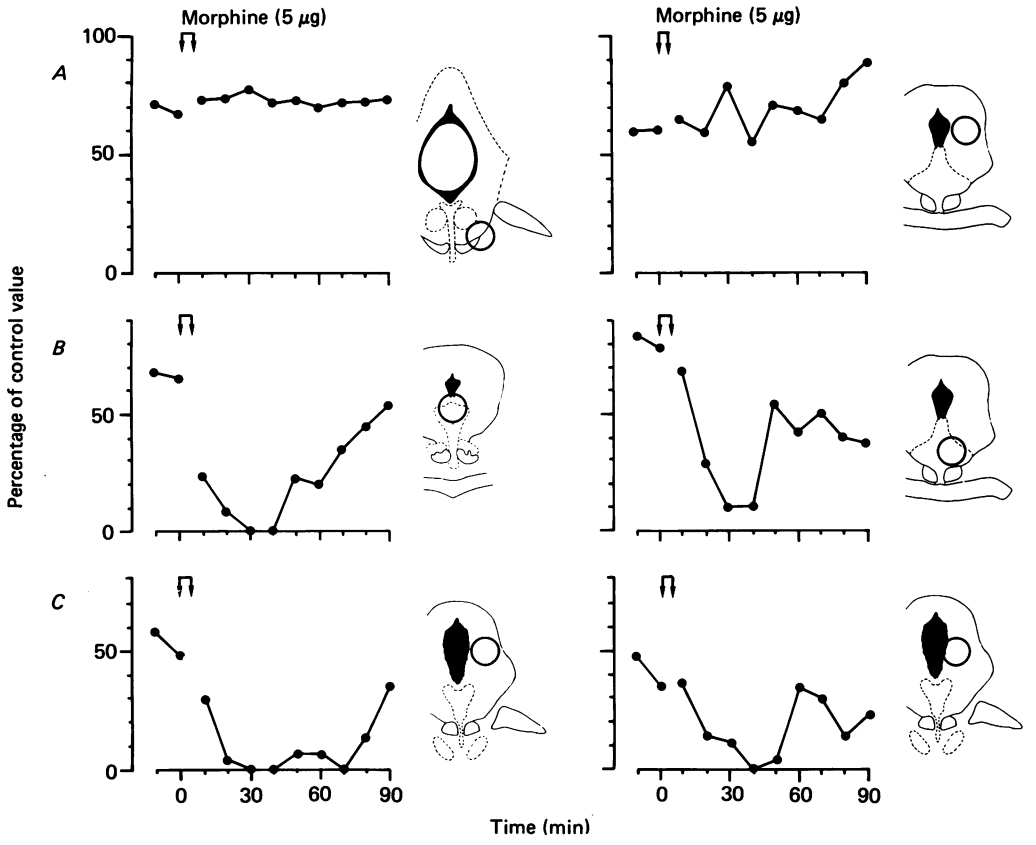

Fig. 7. Individual pairs of examples of the effects of p.a.g. morphine upon d.n.i.c. In each case, the microinjection site is drawn on the right of the corresponding curve on a representative mid-brain section. *A*, no change in inhibition; *B*, decreased inhibition with microinjection sites within the nucleus raphe dorsalis; *C*, decreased inhibition with microinjection sites within the lateral p.a.g.

Examination of the location of the extremity of the microinjection cannula in these seventeen cases (Fig. 8*B*) reveals that the injection sites were either located within or immediately adjacent to the nucleus raphe dorsalis or more dorsolaterally, just adjacent to the aqueduct. Since there seemed to be two sites producing these reductions in d.n.i.c. the four examples given in Fig. 7 *B* and *C* include two from each site. As can be seen from these and indeed from the over-all results, there was no real difference in the effects from either site in terms of onset, time course or degree of reduction. Over-all, all but three of the microinjection sites were such as to include the nucleus raphe dorsalis.

The post-conditioning effects of d.n.i.c. were also considerably reduced after morphine. Fig. 10 illustrates that there was a clear and profound reduction in the post-effects of d.n.i.c. in the immediate 22 s following conditioning and in those with clear control post-effects. The post-effects in the epoch 22–44 s were completely abolished and on occasions even some excitations were observed.

Relationship between C-fibre responses, d.n.i.c. and morphine. With the purpose of analysing the relationship between C-fibre responses, d.n.i.c. and p.a.g. morphine,

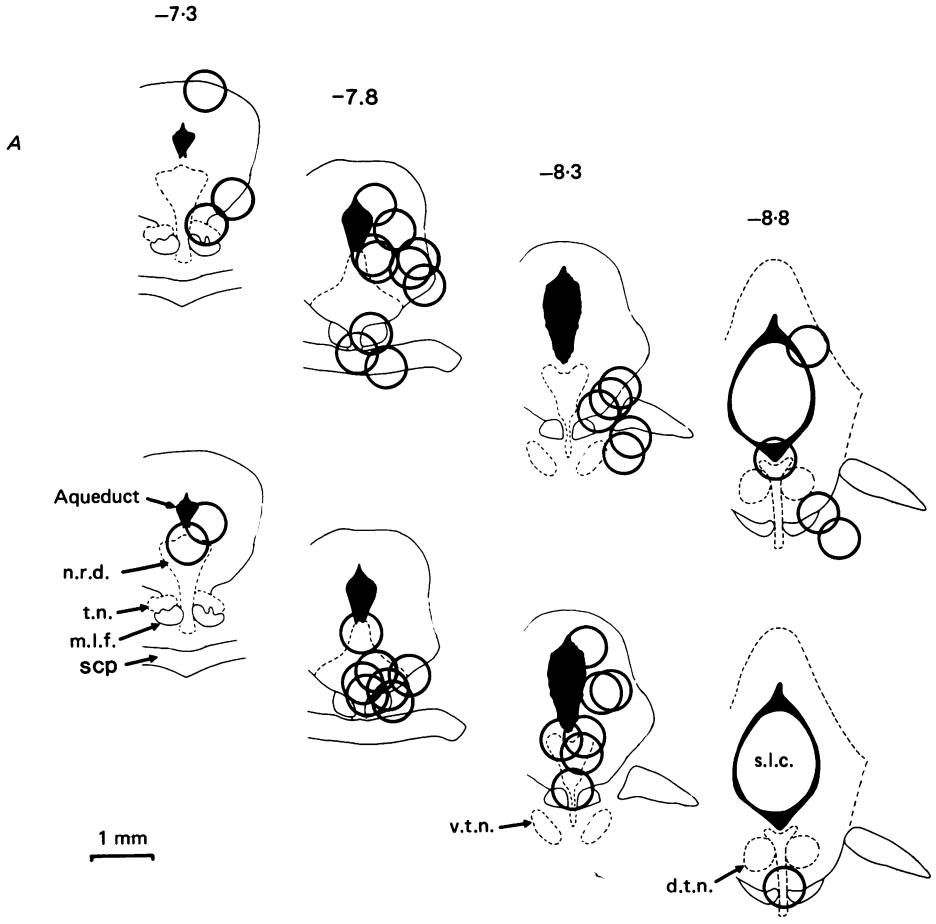

Fig. 8. Location of microinjection sites with reference to the two types of effects of p.a.g. morphine upon d.n.i.c. The corresponding mean curves are shown respectively in Fig. 6*A* and *B*. *A*, no change in inhibition ($n = 22/39$); *B*, decreased inhibition ($n = 17/37$). Abbreviations and symbols as in Fig. 4.

the effects of morphine upon d.n.i.c. were standardized in terms of percentage depression of inhibition observed 30–50 min following the microinjection (see Methods); for each individual cell, this value was plotted against the mean initial C-fibre response (Fig. 11) and against the percentage of the response calculated 30–50 min following morphine microinjection (Fig. 12). As shown in Fig. 11 the initial C-fibre responses of the cells showed no correlation with the direction and strength of the subsequent effect of morphine on d.n.i.c.; this Figure also illustrates the preponderant effect of morphine in reducing the inhibitory effects of d.n.i.c.. Fig. 12 is an analysis of the relation between the direction of changes of the C-fibre responses and the direction of the changes in d.n.i.c., elicited by the microinjection. There is clearly no relationship between the changes in the C-fibre responses and the changes in d.n.i.c. so that marked reductions in d.n.i.c. could be observed for neurones with either enhanced or reduced C-fibre responses. Thus the effect on d.n.i.c. of morphine is not likely to be secondary to changes in C-fibre responses.

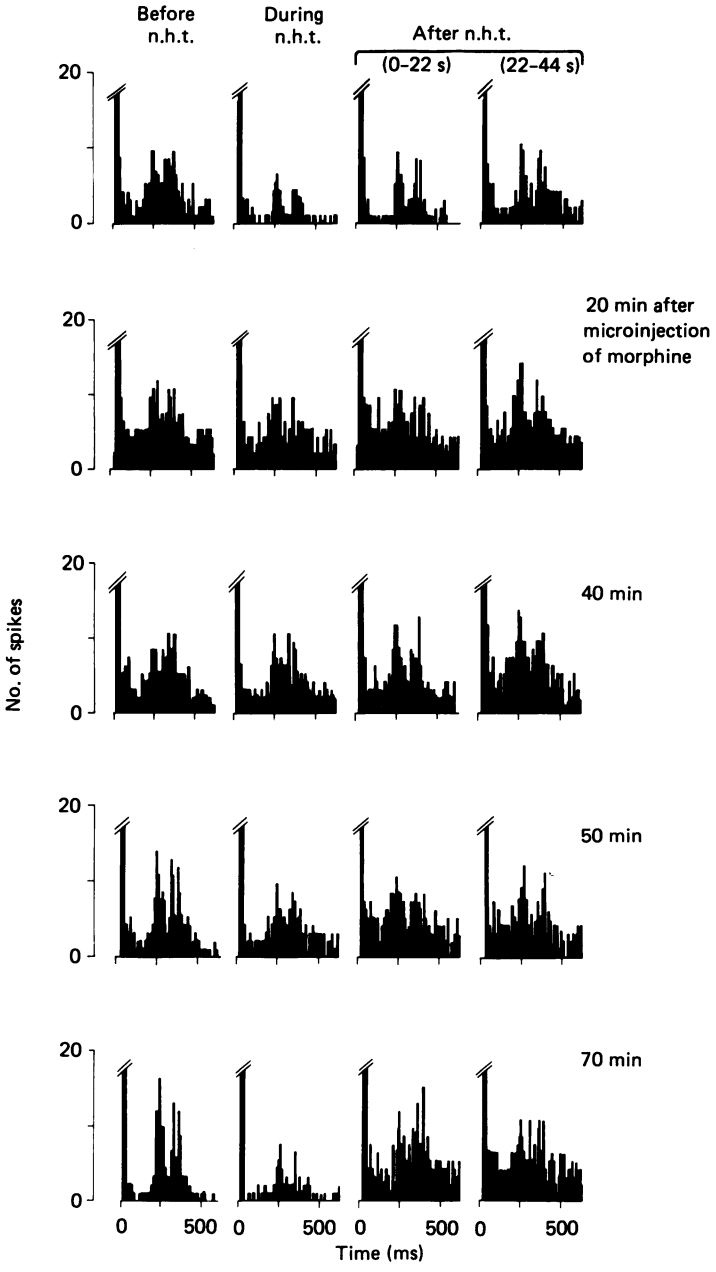

Fig. 9. Individual example of the effects of p.a.g. morphine (5 μg; 0·2 μl) upon the inhibition triggered by noxious heat applied on the tail (n.h.t.). Post-stimulus histograms (ordinate: number of spikes obtained with fifteen trials and a bin width of 5 ms) built during the periods defined in Methods for each horizontal series: before n.h.t. (control), during n.h.t. (conditioned response), and after n.h.t. (after-effects). The earlier Aα responses are truncated for clarity of presentation. P.a.g. morphine resulted in only a slight modification of the control responses but a reduction of the n.h.t.-induced inhibition. Note the total recovery 70 min following the microinjection of morphine.

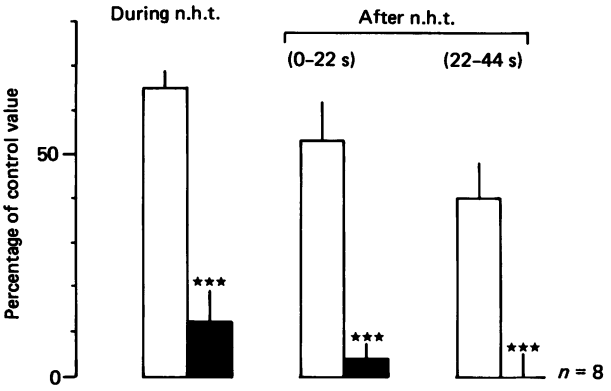

Fig. 10. Effects of p.a.g. morphine upon the inhibition triggered by noxious heat applied on the tail (n.h.t.) and the subsequent after-effects. Open bars: control inhibitions; filled bars: inhibitions observed 40 min after p.a.g. morphine. Statistics as for Fig. 2.

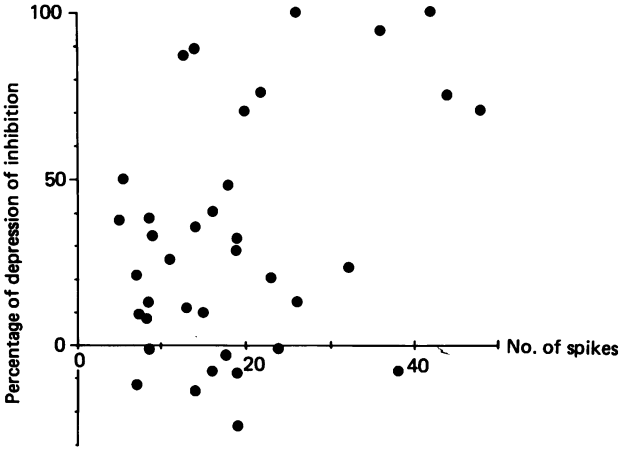

Fig. 11. Relationship between control C-fibre responses and subsequent effects of p.a.g. morphine on d.n.i.c. Abscissa: mean initial C-fibre response in terms of mean number of spikes per electrical shock; ordinate: percentage of depression of inhibition,

$$\left(\frac{\text{mean inhibition at time } +30,\ +40,\ 50 \text{ min}}{\text{mean control inhibition}}\right) \times 100.$$

DISCUSSION

Over-all, the results indicate that a supraspinal action of morphine will reduce the descending inhibitions evoked by a noxious stimulus whilst producing no clear change in the response of dorsal horn neurones to noxious stimuli. The interpretation of this finding for the analgesia produced by morphine is discussed later; we first wish to comment on the autoradiographic results.

We used a small amount (5 μg) and volume (0·2 μl) for the microinjections of morphine base (90 mM). However, as shown by our autoradiographic study, the diffusion of morphine was large, although a gradient of concentration was obvious from the centre to the periphery. As an approximation, one can consider that

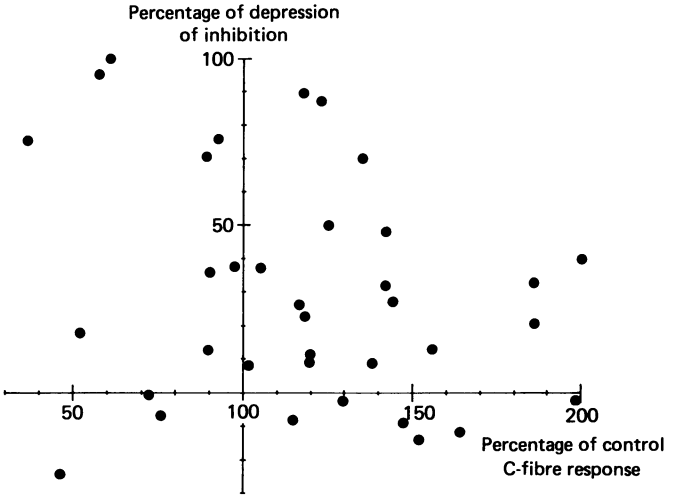

Fig. 12. Relationship between the effects of p.a.g. morphine on the unconditioned C-fibre responses and on d.n.i.c. (mean values at time +30, +40, +50 min). Abscissa: percentage of control value of the C-fibre responses; ordinate: percentage of depression of inhibition (see legend of Fig. 11).

morphine diffused in a 1·5 mm diameter sphere; and the mean concentration in such a theoretical volume was in the 10 mM range. Bolander, Kourtopoulos, Lundberg & Persson (1983) have estimated the concentration of morphine in the brain following 10 mg/kg subcutaneous injection; the highest concentration was found in the cerebellum and, 30 min after the administration, approximately 100 ng of morphine chloride/g was found in this structure, which corresponds to a concentration of 0·3 μM. We can therefore conclude that our microinjection technique led to a very high level of concentration, at least 10^4 fold higher than that observed following a large dose of systemic morphine. As has already been pointed out by Clark, Edeson & Ryall (1983) whose conclusions are nearly identical to ours, this must be kept in mind when interpreting the present work, and more generally, data involving microinjection techniques. For clarity of presentation we have used the extent of a theoretical sphere of 0·2 μl in our Figures, while it is obvious from our radiographic study that morphine diffused in a 1·75 μl sphere (radius of 0·75 mm) where the mean concentration reached 10 mM. Thus a large diffusion and a very high concentration are the two main characteristics of the spread of morphine. However, it is important to note that the extent of diffusion is clear cut and did not include other structures, in particular the spinal cord where no labelling was found.

Earlier behavioural studies have generally taken the point of the cannula as the injection site and have made extensive mapping studies of the parts of the p.a.g. eliciting hypoalgesic effects (most commonly using the tail-flick test). The fact that neighbouring points could produce either analgesia or negative results suggests that very high concentrations of morphine were necessary for an effect to be found. There is a general agreement that the ventro-caudal part of the p.a.g. contains the most effective sites (Tsou & Jang, 1964; Jacquet & Lajtha, 1973; Sharpe *et al.* 1974; Yaksh

et al. 1976; Lewis & Gebhart, 1977*a*, *b*; Iwamoto *et al.* 1978). Interestingly, some authors (Sharpe *et al.* 1974; Lewis & Gebhart, 1977*a*, *b*) have emphasized the medial part of this region as being the more sensitive to morphine, as indeed we found in this study. It is obvious that we have largely covered this region in the present work (see Figs. 4 and 8), and taken together, these data lead us to believe that we would have observed an effect, if one was to be found.

We have chosen to record convergent neurones for two main reasons. First, they are most probably involved in the transmission and integration of nociceptive information at the spinal level; the most convincing argument in this respect being their clear ability to be influenced by converging excitatory and inhibitory mechanisms in a fashion which can be related to clinical observations (see references in Le Bars, Dickenson, Besson & Villaneuva, 1986). For example, viscero-somatic convergence could explain clinical referred pain and most of the manipulations which result in hypoalgesia or analgesia in humans also result in a reduction of 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 and periventricular structures and heterotopic noxious stimulation. The second reason is the fact that they are strongly modulated by supraspinal structures; in particular p.a.g. electrical stimulation induces strong inhibitory effects upon dorsal horn convergent neurones (Guilbaud, Besson, Liebeskind & Oliveras, 1972; Liebeskind, Guilbaud, Besson & Oliveras, 1973; Oliveras, Besson, Guilbaud & Liebeskind, 1974; Carstens, Yokota & Zimmermann, 1979; Duggan & Griersmith, 1979; Hayes, Price, Ruda & Dubner, 1979; Carstens, Klumpp & Zimmermann, 1980; Carstens, Bihl, Irvine & Zimmermann, 1981; Yezierski, Wilcox & Willis, 1982). Thus this type of neurone appears appropriate for an effect to be found following p.a.g. morphine.

With regard to identification of the neurones, d.n.i.c. influences all lumbar dorsal horn and trigeminal nucleus caudalis covergent neurones (Le Bars *et al.* 1979*a*; Dickenson *et al.* 1980*a*) including the one-third of these cells which are at the origin of the spinothalamic and trigeminothalamic tracts (Dickenson & Le Bars, 1983*a*). Furthermore, in a series of experiments recording the activity of these neurones simultaneously with flexor peroneal nerve activity, d.n.i.c. depressed both the neuronal and motor nerve activity evoked by C-fibre stimulation (Schouenborg & Dickenson, 1985). Thus d.n.i.c. acting on these dorsal horn neurones can modulate both ascending sensory information and reflex activity in the rat. Distant noxious stimuli in man have also been shown to reduce both the sensory and motor responses to pain (Willer, Roby & Le Bars, 1984).

The microinjection of 5 μg of morphine into various zones of the caudal p.a.g. which covered the ventral and mediolateral part and included much of the dorsal part had a predominant facilitatory effect on the dorsal horn neurones since 51 % of the C-fibre responses were facilitated, 31 % not influenced and only 10 % inhibited. Furthermore, the cells we classified as inhibited were only marginally so. These results are not in accord with the two other studies on this subject, in that depressive effects on some neurones have been reported in the rat (Bennett & Mayer, 1979) and clear reductions in the responses of spinal neurones to noxious heat stimuli have been reported following p.a.g. morphine in the cat (Gebhart, Sandkühler, Thalhammer & Zim-

mermann, 1984). In the former study, morphine (4–16 μg in 0·5 μl) depressed the radiant-heat-evoked responses by 25 % or more in only nine out of twenty cases while seven neurones were unaffected and four facilitated; unfortunately no precise quantification was provided in this paper, making comparison difficult. In the latter study the sites of injection (10–20 μg in 0·5–1 μl) were all in the dorsal or lateral p.a.g., and never in its medioventral aspect where morphine has been reported to be the most effective for producing behavioural antinociception (see above). In addition, such doses of morphine in the ventrolateral p.a.g. have not been found to elicit antinociceptive reactions in the cat; 50–100 μg are needed for a behavioural effect to be observed (Ossipov, Goldstein & Malseed, 1984).

In any case, the predominant facilitatory effect could be interpreted in two ways: (1) as suggested by Duggan, Griersmith & North (1980), morphine could have decreased tonic descending inhibition which potentially can modulate the output of convergent neurones, and (2) such facilitation could be the result of the lifting of d.n.i.c. as discussed below; indeed the surgical preparation of the animal could be the source of nociceptive input, consequently triggering the d.n.i.c. system in a 'tonic' way during the experiments (Clarke & Matthews, 1983). However, this latter hypothesis appeared unlikely because there was no relationship between facilitatory effects on unconditioned C-fibre response and reduction of d.n.i.c. (see Fig. 12); in addition the time courses of the two effects were found to be completely different (compare Figs. 2*A* and 6*B*). The former hypothesis appears therefore more likely; however, its physiological significance still remains obscure.

The effect of morphine on the inhibitions produced by noxious heat applied to the tail, to activate d.n.i.c., was either no change or a marked reduction in d.n.i.c. (44 % of cells). The reduction in d.n.i.c. was clear and powerful, reaching, at peak effect, a 77 % reduction. Furthermore, the reduction in d.n.i.c. was clearly unrelated to the effects of morphine on the unconditioned C-fibre responses or to the magnitude of the initial C-fibre response d.n.i.c. was tested against.

The time course of the effects of morphine on d.n.i.c. matches, both in terms of onset and duration, the time course of the behavioural analgesia we found with an identical microinjection technique using the threshold to vocalization following electric shock of the tail to gauge the antinociception (A. H. Dickenson & D. Le Bars, unpublished observation).

The zones in the p.a.g. where morphine microinjection produced reductions in d.n.i.c. tended to be either the region of nucleus raphe dorsalis underlying the aqueduct and ventricle or less often, just lateral to the aqueduct. Examination of the sites producing behavioural antinociception in the rat reveal that the medioventral and to a lesser extent the ventrolateral p.a.g. are the effective sites (see above). Indeed, injection into the dorsal p.a.g. frequently elicits aversive and arousal reactions (Jacquet & Lajtha, 1973, 1974; Sharpe *et al.* 1974). Interestingly, p.a.g. sites from which electrical stimulation produced 'pure analgesic effects', i.e. without behavioural side-effects, are confined to the ventromedial and ventrolateral parts of this structure (Fardin, Oliveras & Besson, 1984*a*, *b*). In addition, cell bodies containing serotonin in the p.a.g. are confined to the ventromedial and to a lesser extent in the ventrolateral regions of the caudal p.a.g. (Steinbusch, 1981; Clements, Beitz, Fletcher & Mullet, 1985) where they constitute one-third of the total number

of neurones (Descarries, Watkins, Garcia & Beaudet, 1982). Finally, the μ subtype of opiate receptors are predominant in the ventral regions by comparison to the dorsal regions of the p.a.g. (Moscovitz & Goodman, 1984).

Thus, from these results, morphine microinjected in those zones which elicit antinociception in both reflex and more integrated tests in animals, produces no clear effect on C-fibre-evoked activity yet markedly reduces descending inhibitions produced by noxious stimuli. This finding is in good agreement with the effects of low doses of systemic morphine on these activities. We have previously shown that doses of morphine without effect on the C-fibre-evoked activity of dorsal horn cells reduce d.n.i.c. in a stereospecific, dose-dependent and naloxone-reversible fashion (Le Bars *et al.* 1981*a*). The lack of effect of systemic morphine on the C-fibre activity implies a lack of effect at the spinal level and hence a supraspinal site of action. This premise that the supraspinal action of morphine does not include inhibitions of dorsal horn cells is supported by our previous results (Le Bars *et al.* 1980) in rats following microinjection into sites within nucleus raphe magnus which support analgesia. In this study we found that morphine injected at sites within the nucleus raphé magnus either did not influence dorsal horn neurones, or indeed, produced a facilitation of their responses, the time course of which paralleled the behavioural analgesia seen in previous testing of the same animals. Finally, the depressive effect of intravenous morphine on C-fibre responses was found to be remarkably similar in intact and spinal rats in the 1–10 mg/kg range (Le Bars, Guilbaud, Chitour & Besson, 1980), again arguing against an increase by the drug of tonic descending inhibitions. Three studies in the cat, two using reversible cold block to temporarily abolish descending inhibitions (Duggan *et al.* 1980; Soja & Sinclair, 1983) and the other intraventricular injections (Sinclair, 1984) all concluded that morphine does not increase descending inhibitions. However, a fourth study (Du, Kitahata, Thalhammer & Zimmermann, 1984), using microinjections within the nucleus raphe magnus, concluded the converse. It should be noted, however, that the cat is a species which does not respond to morphine in a way akin to humans or rats: low systemic doses induce stereotyped behaviour while higher doses (> 5 mg/kg) result in 'feline mania' (see Villablanca, Harris, Burgess & De Andres, 1984). To our knowledge there is no available behavioural data in the cat regarding the effects of morphine microinjections in the nucleus raphe magnus.

Thus, the conclusions would seem to be that opiates, acting supraspinally, decrease descending inhibitions, at least when these are triggered by a noxious stimulus. The evidence that d.n.i.c. involve descending inhibitory controls is now considerable. Both spinalization (Le Bars *et al.* 1979*b*) and injection of local anaesthetic in the cord (Cadden, Villaneuva, Chitour & Le Bars, 1983) at the C1–C2 level block the inhibitions acting on lumbar nociceptive neurones. Furthermore, manipulations of the descending serotoninergic projections from nucleus raphe magnus of the brain stem, the terminals of which synapse in the dorsal horn of the spinal cord (Skagerberg & Bjorklund, 1985) interfere with d.n.i.c. Hence lesions of the nucleus (Dickenson *et al.* 1980*b*), section of the dorsolateral funiculus (Villanueva *et al.* 1986) in which the descending fibres travel, depletion of serotonin (Dickenson, Rivot, Chaouch, Besson & Le Bars, 1981) and serotonin receptor antagonism (Chitour, Dickenson & Le Bars, 1982) all markedly reduce d.n.i.c. Since direct spinal projections from the

p.a.g. have been only rarely described it is held that the p.a.g. exerts its effects on spinal cord function via the nucleus raphe magnus.

Thus, a test of our findings would be that morphine, in low doses, reduces raphe–spinal serotonin function. Unfortunately, many have studied this interaction in the absence of a noxious stimulus and so these studies have little relevance to the antinociceptive effects of the opiate. However, it has been shown that the increased synthesis of serotonin in the dorsal horn produced by a noxious stimulus is markedly reduced by morphine (Weil-Fugazza *et al.* 1984). In addition we have preliminary unpublished data suggesting that the increase in serotonin release from the cord induced by noxious stimulation is, indeed, countered by 1 mg/kg of systemic morphine.

We did not attempt to test the effects of naloxone on the changes produced by morphine in the present study. The reasons for this are the possible difficulties in interpretation of any effects of naloxone. Local naloxone would be expected to reverse the effects of morphine at the p.a.g. level but we feel that due to the size of the rat mid-brain any second microinjection into the p.a.g. of naloxone would, first, produce tissue damage and, secondly, be almost impossible to localize to the same site as the morphine. In addition, the lipophilic nature of naloxone (Misra, Pontani, Vadlamani & Mule, 1976) would result in a much larger diffusion than the hydrophilic morphine and, furthermore, vascular resorption could result in a diffusion to the whole body, including the spinal cord (Clark & Ryall, 1983). Systemic naloxone would be expected to increase the unconditioned C-fibres responses, but also reverse the effects of morphine both on the C-fibre responses and d.n.i.c., leading over-all to a reduced C-fibre response and an increase in the inhibitions, respectively. However, systemic naloxone alone has been shown to reduce d.n.i.c. (Le Bars, Chitour, Kraus, Dickenson & Besson, 1981*b*). With this complexity we felt it would be impossible to interpret any effect of naloxone. However, on the other hand, the time course of any effects of morphine would seem to be a valuable gauge of specificity (see above).

What then could be the basis for the antinociception produced by supraspinal morphine, if indeed these descending inhibitions are reduced? On the basis of the existence of d.n.i.c. and hence the powerful inhibitions of trigeminal and dorsal horn convergent neurones by a distant noxious stimulus we have suggested (Le Bars *et al.* 1979*b*) that the significant nociceptive signal from these neurones is not simply a function of their afferent input or even the efferent output of these neurones. Indeed, if this were the case, since these neurones can be excited to a greater extent by innocuous as compared to noxious stimuli, under certain conditions of peripheral stimuli, both in the spinal and intact anaesthetized rat (Le Bars & Chitour, 1983) it is difficult to envisage how unambiguous messages could emanate from these neurones. Thus we proposed that a local restricted noxious stimulus whilst producing excitation of appropriate nociceptive neurones segmentally also induces d.n.i.c.. The activation of these descending inhibitions will reduce activity in all other spinal and trigeminal neurones located outside the segmental excitatory pool. These cells would presumably be active due to hair, touch, pressure and other innocuous inputs. The contrast message between the excited and large inhibited pool of cells may well then signal pain, so that a reduction in d.n.i.c. will reduce this contrast and so reduce the painful sensation.

This premise that the supraspinal action of opiates involves mechanisms that do not include inhibition of spinal nociceptive neurones local to the noxious stimulus has bearing on the clinical descriptions of morphine analgesia. Therapeutic doses of morphine result in a state where although the pain can still be located and recognized as nociceptive, affective aspects are altered whereupon it is no longer unpleasant. This would seem relevant to our hypothesis. The spinal action of opiates, requiring either direct application (intrathecal or epidural) or high systemic doses such as those used in anaesthesiology, would result in a block of transmission with a resultant inability to locate or recognize the stimulus, which is in fact the case. Since animal studies have demonstrated a synergy between supraspinal and spinal opiates (Yeung & Rudy, 1980) over a range of doses of systemic morphine, the low doses would interfere with d.n.i.c. which would be reinforced by increasing spinal inhibitory effects as doses increased. A profound analgesia would then result.

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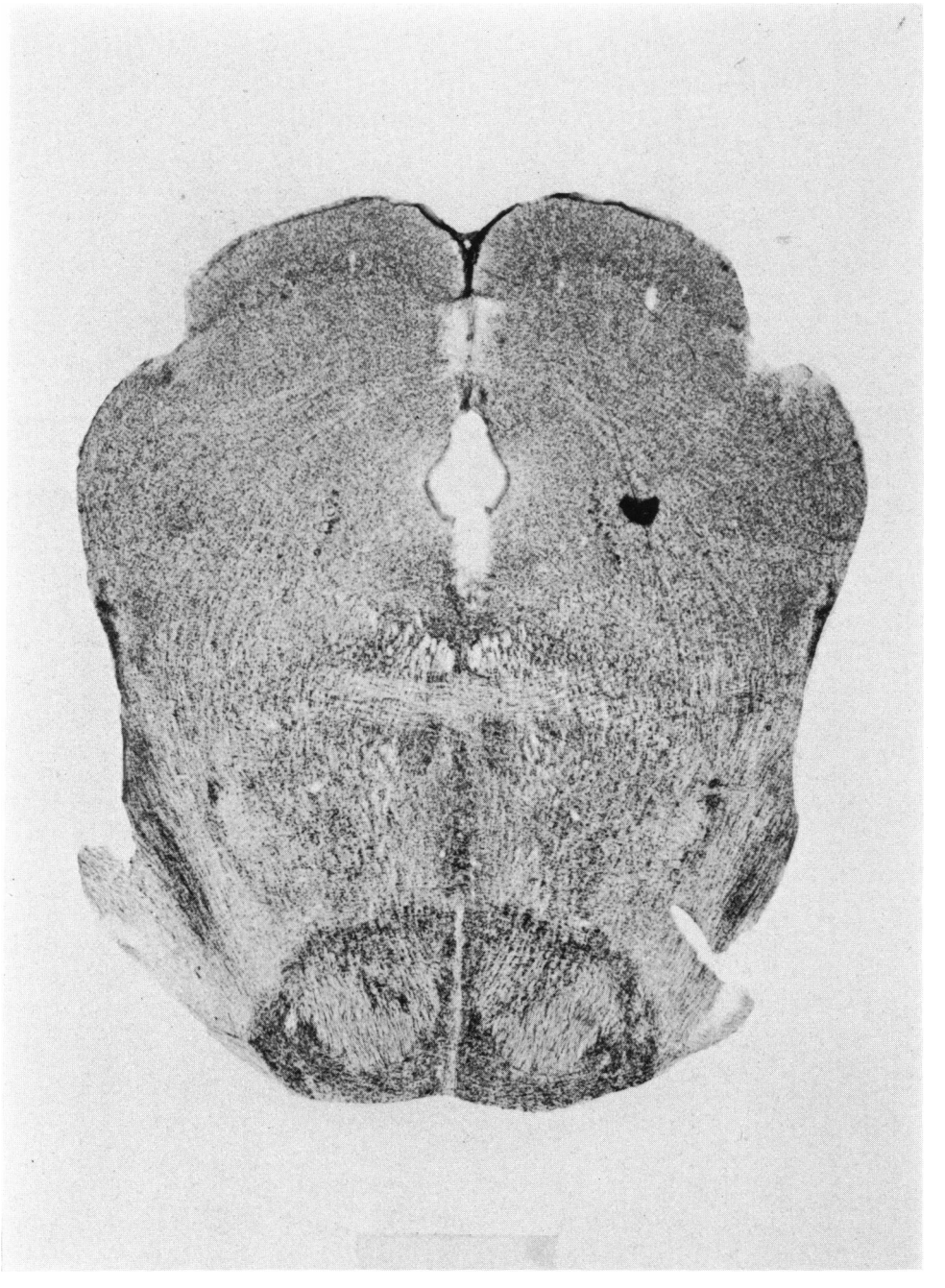

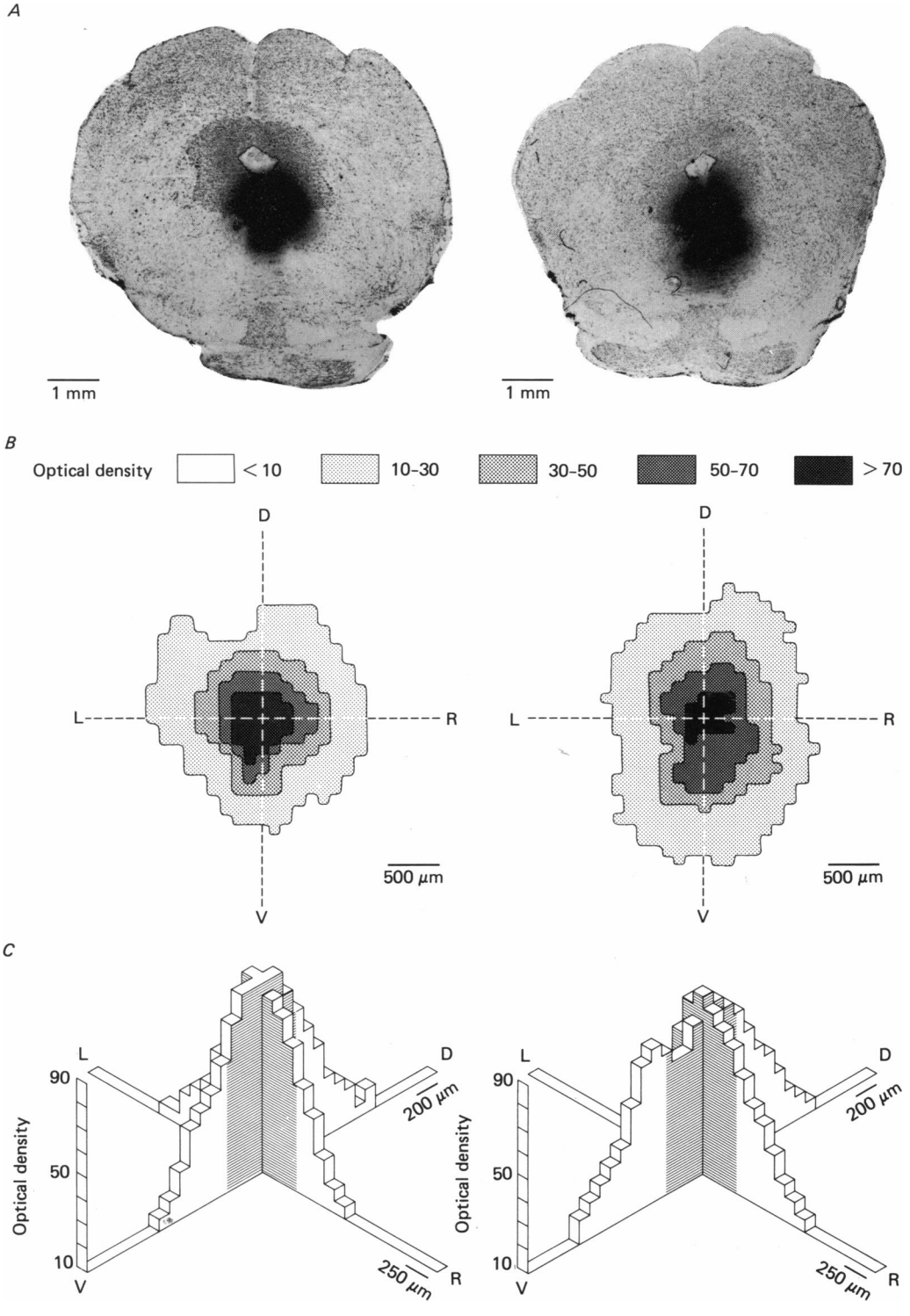
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EXPLANATION OF PLATES

PLATE 1

Example of a cresyl-violet-stained section of the mid-brain showing the trace of the cannula used for the microinjection of morphine. In this case the microinjection site was located within the nucleus raphe dorsalis.

PLATE 2

Two examples (right and left) of autoradiographic controls of the diffusion of [^{3}H]morphine (5 μg; 0·2 μl) injected within the p.a.g. (see text). *A*, bright-field photomicrograph of the section, counter-stained with cresyl violet, and the superimposed autoradiogram. *B*, computer reconstruction of the optical densities of the autoradiograms shown in *A*. (D, dorsal; V, ventral; L, left; R, right.) *C*, corresponding histograms of optical density along the dashed lines drawn in *B*.