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Antinociception from a stereospecific action of morphine microinjected into the brainstem: a local or distant site of action?

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1 Morphine (1 μg) was microinjected into rats in the midline medullary nucleus raphe magnus (NRM); 1 mm lateral into nucleus reticularis paragigantocellularis (NRPG); 2 mm lateral into the VIIth nerve nucleus and 3 mm lateral into the Vth nerve nucleus. The time course of changes in the sensitivity to noxious heat was followed by the tail flick test.

2 Significant and prolonged antinociception was seen following microinjection into NRPG. At sites 1 mm from NRPG very weak effects were seen and at 2 mm from NRPG no antinociception occurred.

3 It is concluded that 1 μg of morphine microinjected into the brainstem is unlikely to cause antinociception by entering the circulation and having effects at remote sites. The distance diffused by morphine to cause significant antinociception after microinjection of 1 μg is less than 1 mm.

4 Levorphanol (1 μg) had very similar effects to morphine but dextrorphan and saline were ineffective.

5 It is concluded that although the concentrations achieved following microinjections may be high, they are not excessive as the effects show stereospecificity. The concentrations of endogenous substances released into the synaptic cleft may also be high.

Introduction

Electrical stimulation of several discrete sites in the brainstem has been shown to cause antinociception and depress nociceptive responses of spinal neurones (Mayer & Liebeskind, 1974). The microinjection of morphine into these sites is also antinociceptive and depresses responsiveness of spinal neurones (Dickenson *et al.*, 1979). These observations have led to the postulate that an analgesic system of opiate-sensitive brainstem neurones projects to the spinal cord (Basbaum & Fields, 1978). Some of the support for this postulate arises from studies using the microinjection technique (Yeung *et al.*, 1977; Azami *et al.*, 1982). It is a difficult technique to control adequately because behavioural antinociception will only be detected when a sufficient volume of brain tissue has been affected by the drug and yet moderate volumes of drug solution cannot be administered due to the physical disruption of tissue at the injection site. Small volumes of highly concentrated drug solution have to be given. These difficulties are minimised but not overcome, by slow administration in the rat of 1 to 5 μg of drug in volumes no greater than 0.5 μl.

Two recent reports (Clark & Ryall, 1983; Clarke *et al.*, 1983) have reassessed the antinociception resulting from microinjection into cat of the very potent and lipophilic drug, etorphine. It was concluded that etorphine enters the circulation following microinjection of 10 μg to act directly upon the spinal cord. Larger amounts of morphine, 28–285 μg, diffused to enter the circulation only at the higher doses but had effects restricted to brainstem neurones at the lower doses. These antinociceptive effects were thought to result only from exceedingly high concentrations of morphine which may have nonspecific actions and were in any case higher than those resulting from systemic administration of morphine. These conclusions from neurophysiological experiments in the cat throw doubt upon the value of data obtained from microinjection of morphine during behavioural tests

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for analgesia in the rat. Some of these conclusions have been tested in the present paper by use of the tail flick response of the unanaesthetized rat to noxious heat. It is shown that moderate doses of morphine into the brainstem have stereospecific actions and that similar injections 1 mm distant do not cause antinociception. Thus although concentrations may be higher than those following systemic injection, valuable data may be obtained with the technique.

Methods

Stainless steel guide cannulae (23 gauge) were implanted into the skull of 33 female Wistar rats weighing 180–230 g and anaesthetized with sodium pentobarbitone (40 mg kg^{-1}). One week later microinjections were made into the unanaesthetized animal using the assembly described elsewhere (Azami *et al.*, 1980). A 90 μm (o.d.) glass needle protected by a system of telescoping steel tubes was inserted into the guide cannula just before the injection to project 4 mm into the brainstem. This system ensures that damage to brain tissue by implanting the guide cannula is restricted to the dorsal cerebellum and the very thin microinjection needle does not remain in brainstem tissue to cause inflammatory damage at the microinjection site. Drugs were slowly injected in a volume of 0.5 μl during a 3 min period and the needle was then removed. The following drugs were administered as 1 μg of the base equivalent weight: morphine sulphate, levorphanol tartrate and dextrorphan tartrate (Roche).

Guide cannulae were stereotaxically positioned such that microinjections were made into nucleus raphe magnus (NRM); the nucleus reticularis paragigantocellularis (NRPG) at 1.0 mm from NRM; the seventh nerve nucleus at 2.0 mm from NRM and the fifth nerve nucleus 3.0 mm from NRM. At the end of each experiment 0.5 μl of pontamine sky blue dye was microinjected to mark the injection site and the brain removed for histological examination.

Details of the tail flick test have been reported elsewhere (Azami *et al.*, 1983). Rats were placed in a ventilated glass tube for periods of about 1 min during which time their tails were laid across a nichrome wire coil which was at room temperature. An electric current was then passed through the coil which raised its temperature at an approximate rate of 9°C s^{-1}. The latency of the tail flick response was recorded. The rate of heating of the coil was adjusted before the experiment to ensure that rats flick the tail after 3 s (range ± 0.5 s). If the tail of rats displaying antinociception was left in contact with the heated coil for more than 6 s, visible damage was done to the skin and a few minutes later an inflammatory response occurred. The sensitivity of this area to further noxious stimulation was grossly abnormal and for this reason 6 s was the maximum time the tail was left in position. Using this cut off time repeated responses could be evoked with a constant latency for 2–3 h.

Rats were tested every 5 min during a 20–30 min period of familiarisation with the apparatus. Three baseline latencies were then recorded at 5 min intervals and the microinjection was given. Testing continued at 5 min intervals for 60 min and then at

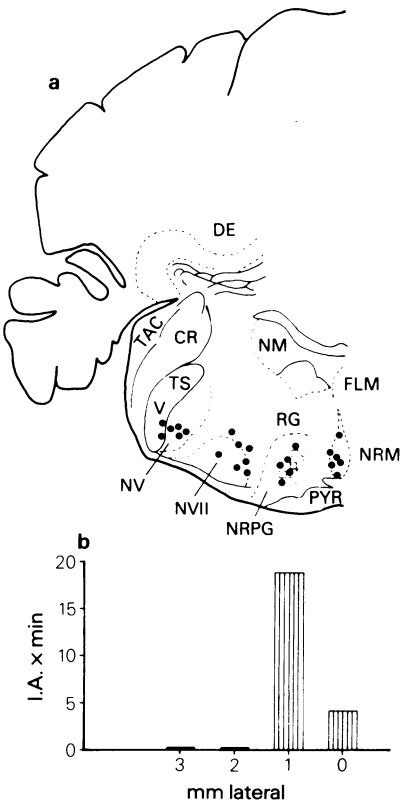

Figure 1 Cross section of the rat hindbrain at the level of the fifth nerve nucleus (NV), seventh nerve nucleus (NVII), nucleus reticularis paragigantocellularis (NRPG) and the nucleus raphe magnus (NRM). The section is traced from the atlas of Fifkova & Marsala (1967) at AP 10 and the same abbreviations used. Microinjection sites determined histologically are indicated on the section: (●) 1 μg morphine; (□) 0.5 μg saline; (○) 1 μg levorphanol and 1 μg dextrorphan.

Below the section is a histogram showing the effectiveness of 1 μg morphine injected at the sites indicated above. Zero on the abscissa scale indicates the midline (NRM) sites. The ordinate scale shows the mean index of analgesia score (I.A., see text) multiplied by the time (min) for which it was elevated. It can be seen that considerable antinociception resulted from injections of morphine into NRPG, less from NRM and none from sites 2 or 3 mm lateral to the midline.

10 min intervals to a total of 150 min after the injection. The tail flick latency (TFL) of those rats which were subsequently shown to have correctly positioned microinjections were normalised by an 'index of analgesia' (I.A.) using the formula:

$$\text{I.A.} = \frac{\text{test TFL} - \text{baseline TFL}}{6\,\text{s} - \text{baseline TFL}}$$

The mean and standard error for each time after microinjection was calculated and the significance of deviations from baseline were determined using the Mann-Whitney U test.

Results

Figure 1a shows the location of the 33 microinjection sites. The filled circles indicate the 24 sites where 1 μg morphine was given. These sites were in four groups separated by 1 mm. Six animals received morphine in or close to NRM; 5 in NRPG; 7 on the medial border of the NVII nucleus and 6 in the ventral portion of NV nucleus. The histogram below the brain section shows the relative effectiveness of morphine microinjected into the different regions. Ordinate values were obtained by multiplying the tail flick latencies in terms of 'index of analgesia' by the time during which they were significantly different from the baseline.

Morphine was most effective when given into NRPG. It was less antinociceptive when given 1 mm medially into NRM, having only one quarter of the effect seen in NRPG. This weaker effect of morphine in NRM may reflect the less potently antinociceptive actions of morphine in this nucleus but alternatively may indicate the diffusion of morphine from the injection site into the more sensitive NRPG. However, at the injection sites 1 mm lateral to NRPG, no significant increases in I.A. values were observed.

The time course of these effects of morphine are shown in Figure 2. The first tail flick test after completion of the microinjection was conducted within 30 s and at this time antinociception was not seen with any of the injections. Five minutes later however morphine into NRPG caused a significant elevation of I.A. which was maintained for 40 min. Microinjections into NRM did not cause an elevation of I.A. until 15 min after the injection. The increase was weak and only just achieved significance which reoccurred spasmodically for the next 35 min. Microinjections 1 mm lateral to NRPG did not cause a significant deviation from baseline at any time. The mean values of I.A. for this group were similar to those for NRM injections but the variances were larger. No indications of antinociception were seen following injections into the most lateral sites.

The time course of the effects of levorphanol, dextrorphan and saline microinjected into NRPG is shown in Figure 3. At no time did saline injections cause a significant deviation from baseline values. Levorphanol, 1 μg, caused a significant elevation of I.A. values which was present immediately after the injection and was maintained for 50 min. The I.A. values for morphine and levorphanol seen im-

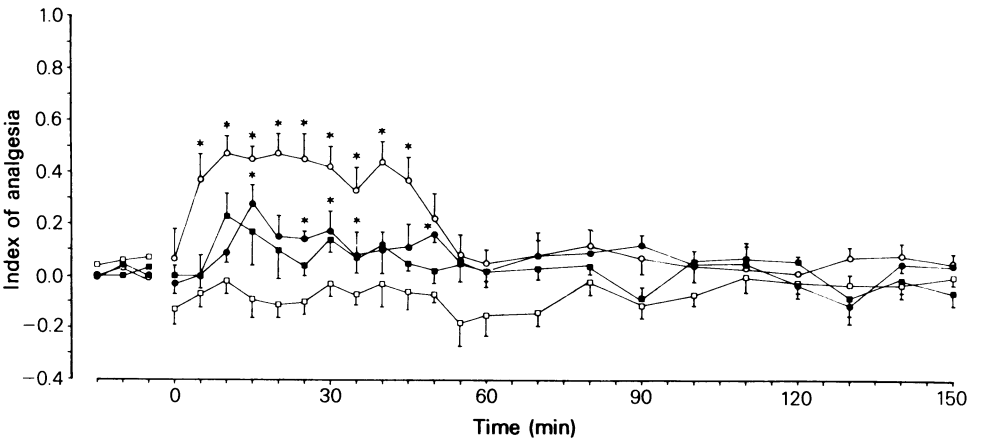

Figure 2 The effect of microinjecting 1 μg morphine into midline sites (NRM ●) and sites lateral to the midline by 1 mm (○, NRPG), 2 mm (■) and 3 mm (□). The microinjections were given at time 0 min. The mean index of analgesia (±s.e.) is shown for each group of animals. Injections into NRPG (1 mm lateral) caused significant elevation of tail flick latency 5 min after the injection which was maintained for 45 min. Injections into NRM were much less effective and significance was only achieved occasionally. Injections 2 mm lateral to the midline (1 mm from NRPG) had no significant effects. $n = 6$ for NRM injections and injections 3 mm lateral to midline; $n = 5$ for 1 mm and $n = 7$ for 2 mm lateral to midline.

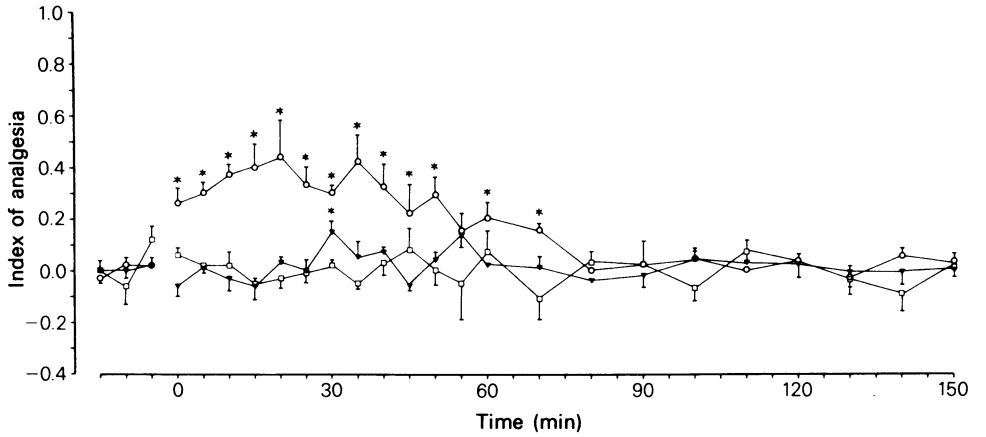

Figure 3 The effects of 1 μg of levorphanol (○, $n = 5$), dextrorphan (▼, $n = 5$) and saline (□, $n = 4$) microinjected into NRPG at 0 min. Levorphanol caused a rapid elevation of the I.A. which was maintained for 70 min. Dextrorphan did not alter the tail flick latency significantly other than a single point after 30 min. Saline was without effect.

mediately after the microinjection were not significantly different from each other and no importance can be attached to the apparent difference in latency to onset. The graph shows that the effect of levorphanol continued to increase until 20 min after the injection but there is no significant difference between the I.A. scores at 0 and 20 min. Dextrorphan, 1 μg, had very little effect. One score, 30 min after the microinjection, appeared to be increased but this may well be spurious.

Discussion

This study confirmed the delayed onset of the antinociceptive effects of microinjected morphine that were observed in many earlier reports (Yaksh *et al.*, 1976; Azami *et al.*, 1982). In NRPG, 1 μg of microinjected morphine had no effect 30 s after injection and caused peak antinociception 10 min later. The calculations of Clark *et al.* (1983) show that at this time, peak concentrations would be achieved at distances of 1 mm from the injection site. There are two implications of this observation. Firstly, the modification of behavioural responses reasonably requires the involvement of a minimal volume of tissue and the delayed antinociception may reflect the time taken for the drug to diffuse sufficiently to affect this minimal volume. Secondly however, the relevant effects of morphine on cellular function in brain are not well established. Apart from short latency excitation (Davies & Dray, 1978) and depression of neurones (Bradley & Bramwell, 1977) longer latency changes in neurotransmitter release are reported (Jessel & Iversen, 1977) and it is unclear if the delayed onset reflects slow metabolic effects of morphine.

To establish with behavioural studies of a nociceptive spinal reflex in the rat whether diffusion distances greater than 1 mm were involved, we have microinjected morphine into the brainstem at sites separated by 1 mm. Significant antinociception was obtained only from NRPG and NRM. The nonsignificant and transient effect of morphine injected 1 mm lateral to NRPG indicates that it is unlikely that morphine caused antinociception by affecting tissues at distances greater than 1 mm from the injection site. These weak effects may reflect the weak actions of morphine at these sites or may reflect diffusion into the sensitive NRPG (Akaike *et al.*, 1978; Azami *et al.*, 1982). The effects are so small that the distance of 1 mm probably reflects the limits of diffusion to achieve an effect upon the tail flick test. The microinjection sites 2 mm lateral to NRPG failed to elevate tail flick latency and confirms that diffusion distances of 2 mm or entry of morphine into the circulation following injection of 1 μg and an action at spinal or other distant sites cannot explain the results.

Although the analgesia following 1 μg morphine into NRPG seems to be a localised effect, the possibility that nonspecific actions of morphine may result from excessive concentrations was examined by microinjecting the stereoisomers levorphanol and dextrorphan into NRPG. Levorphanol, 1 μg, elevated the index of analgesia but saline and dextrorphan did not. It may be concluded that the receptor site for the antinociceptive effects of morphine microinjected into NRPG shows stereospecificity and the antinociception does not result simply from nonspecific

actions due to excessively high local concentrations. These data do not support the possibility raised by Clark *et al.* (1983) that morphine nonspecifically releases an endogenous opioid which diffuses to the spinal cord where its action is blocked by naloxone.

The present study has not attempted to establish experimentally the local concentration of morphine at the injection site. The assumptions and calculations of Clark *et al.* (1983) may be correct in indicating that the local concentration of morphine achieved following microinjection exceeds that averaged throughout the body after systemic injection. Conclusions may not be drawn therefore that systemic morphine exerts its action at a particular site simply because microinjected morphine is effective at that site. Azami *et al.* (1982) concluded that NRPG was more sensitive to the effects of microinjected morphine but demonstrated that NRM was more important to the effects of systemically injected morphine. They observed that naloxone microinjected into NRM significantly reduced the antinociceptive effects of systemic morphine but that giving naloxone to progressively more lateral sites reduced its effectiveness to zero. Naloxone is extremely lipid-soluble and like etorphine readily diffuses and enters the circulation. By injecting naloxone into lateral sites these factors were adequately controlled and its lack of effect laterally showed that diffusion to distant sites was not a likely explanation.

Systemic morphine may be antinociceptive due to simultaneous actions at several sites in the CNS (Kitahata *et al.*, 1974; Yaksh & Rudy, 1976). Local microinjections attempt to activate just one of these sites and a stronger action of morphine on the smaller number of neurones may be necessary before antinociception will be seen. This may explain why comparatively high local concentrations of morphine are necessary. In their evaluation of the microinjection technique Clark *et al.* (1983) concluded that concentrations of putative neurotransmitters applied by microinjection may be excessively high. However, although the concentration of neurotransmitters released into the synaptic cleft is not known, in the neuromuscular junction it may well be extremely high (3×10^{-4} M, Hartzell *et al.*, 1975; 1×10^{-5} M calculated from Potter, 1970). The local concentrations achieved by microinjection may not be excessive in these terms, especially when moderate doses of 1–5 μg of morphine are microinjected or the putative neurotransmitters 5-hydroxytryptamine, glutamic acid or neurotensin are given. Indeed probably the most important information gained with the microinjection technique concerns neurotransmission.

It may be concluded from these studies that although the calculated concentration of morphine following microinjection may be higher than that achieved systemically, the drug exerts restricted and stereospecific actions within the brainstem to cause antinociception. There is no support therefore for the conclusions of Clark *et al.* (1983) that the concentrations achieved are exceedingly high. The results obtained with the microinjection technique may well have relevance to the action of neurotransmitters within discrete areas of the brain.

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