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Partial Anxiolytic Action of Morphine Sulphate Following Microinjection Into the Central Nucleus of the Amygdala in Rats[1]

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FILE, S. E. AND R. J. RODGERS. *Partial anxiolytic action of morphine sulphate following microinjection into the central nucleus of the amygdala in rats.* PHARMAC. BIOCHEM. BEHAV. 11(3) 313–318, 1979.—In the social interaction test of anxiety, bilateral microinjections of morphine sulphate (10 μg) into the central nucleus of the amygdala counteracted the reduction in social interaction normally seen when the test arena is unfamiliar to rats. However, these injections did not counteract the decrease in social interaction that is observed as illuminance of the arena is increased. Morphine injections into the medial site depressed social interaction below the levels shown by control animals. In the open field test, morphine produced a facilitation of peripheral activity when injected into the central nucleus whilst a decrease in rearing was observed following similar injections into the medial nucleus. Overall, these data indicate a partial anxiolytic action of morphine in the central amygdaloid nucleus. Results are discussed in relation to possible differences in opioid peptide innervation of these two amygdaloid nuclei.

Morphine Amygdala Social interaction Open field Rats

SEVERAL lines of evidence indicate that opiates may function to attenuate emotional responding in stressful situations. For example, in man, it has been argued that a major action of morphine is to reduce anxiety associated with pain or the anticipation of distress [5,20] and morphine is used as a pre-anaesthetic medication [10]. In animals, affective reactions to pain are much more readily inhibited by morphine than are non-affective responses [8, 9, 21]. In agreement with these findings are reports suggesting an anxiolytic action of morphine in various fear-motivated tasks [7, 19, 26, 28].

The recent discoveries of opiate receptors [30,36] and opioid peptides [22, 41, 42] within the CNS have led to numerous investigations of their possible functions in behavioural processes. In addition to a role in pain mechanisms [37], endogenous opioids may participate in exploration [16], sexual behaviour [6, 27, 31], parental attachment [18] and psychiatric disorders [40]. Indeed, a general role of these peptides in the mediation of stress responding has been proposed [38].

The amygdaloid complex, long implicated in the regulation of emotional behaviour [11], is an area rich in opiate receptors [3] and opioid peptides [35, 41, 42]. Electrolytic lesions of the central amygdala significantly attenuate responding in a variety of fear-motivated tasks [43] whilst damage to the medial amygdala strongly inhibits affective responses to aversive stimulation [21]. This latter effect has been equated with the action of narcotic analgesics [9]. Recently, it has been reported that microinjections of morphine into this limbic area do not alter tail-flick responses but do elevate the threshold of the more emotive jump response to electric shock and do decrease open field defaecation [33]. On the basis of this profile and the fact that the amygdala is not associated with classical pain pathways, it was argued that a possible function of the amygdaloid opiate system(s) may be to attenuate emotional responding in stressful situation [33].

In order to test this hypothesis further we examined the effects of intra-amygdaloid morphine injections in two tests of emotionality. Two injection sites within the amygdaloid complex were selected on the basis of differential opiate receptor/opioid peptide distribution [3, 35, 41, 42]. Two behavioural tests were used: the open field test which has been widely used to measure rodent emotionality; and the social interaction test of anxiety [12], in which antianxiety drugs produce a behavioural profile distinct from that found with other classes of drugs [13]. In this test, active

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social interaction between pairs of male rats is measured under different conditions of familiarity with the test arena and light level. Undrugged rats show lower levels of interaction when the test arena is unfamiliar or when the illuminance is high. Drugs that are sedative (indicated by reduced levels of spontaneous motor activity) also reduce active social interaction in all the test conditions. In contrast, anti-anxiety drugs (an acute administration of a low dose of ethanol [14], chronic (5–7 days) administration of chlordiazepoxide [12], flurazepam [13], diazepam and desmethyldiazepam [1] produce an entirely different profile: the social interaction remains high even when the test arena is unfamiliar or when the illuminance is high.

METHOD

Animals and Surgery

Male hooded rats weighing 300–350 g (Olac Ltd., Bicester) were housed in groups of six with food and water available ad lib. They were anaesthetised with Equithesin (4.0 ml/kg, Jensen-Salsbery Lab. Inc.) and bilaterally implanted with stainless steel guide cannulae aimed at sites 2 mm dorsal to the central (A/P 6.0; L ± 3.9; V 8.5) or medial (A/P 5.2; L ± 3.5; V 9.5) nucleus of the amygdala. Cannula units consisted of a 0.6 mm (OD) guide fitted with a 0.3 mm (OD) stylet. Stereotaxic coordinates were based on calculations derived from the atlas of Pellegrino and Cushman [25] and level-head surgical procedure was used. The rats were group-housed for one week after surgery and then singly-housed for five days prior to behavioural testing. They were maintained in a room with a constant light-dark cycle, with lights on at 06.00 hr and off at 17.00 hr.

Injection Technique and Drugs

During injections, stylets were replaced with 0.3 mm (OD) injection units which extended 2 mm ventral to the tips of the guide cannulae. This procedure allowed for more precise localisation of injection and prevented extensive damage to amygdaloid tissue. Whilst the animal was hand-held, injections were made via polythene tubing from 10 μl micrometer-driven Hamilton microsyringes (Model 701N). All injections were made bilaterally in a volume of 0.5 μl over, a period of 40 sec.

Morphine sulphate (20 μg/μl; May and Baker) was dissolved in sterile injection water which, alone, served for control injections. Although in previous studies [32,34], no significant behavioural effects have been observed following intra-amygdaloid injection of either saline or water, we have recently obtained motor changes with saline injection into the cortical nucleus of the amygdala (Rodgers, unpublished observations). Since this effect was not seen with water injections, the latter was specifically chosen as vehicle in an attempt to minimize the possibility of such action in the current investigation.

Apparatus

(a) Social interaction test. The test arena was 65×65×47 cm with wooden walls and floor. Infrared cells in the walls provided an automated measure of locomotor activity, a count being scored each time a beam was broken. The low and high light levels were 13 and 333 scotopic lux, respectively. A camera was mounted immediately above the test arena and the rats were observed on a video monitor in an adjacent room.

(b) Open field. This was a standard circular arena, 84 cm in diameter with walls 32 cm high. The latter were painted flat white whilst the formica floor was marked off into three concentric circles which were further subdivided to give a total of 19 segments. The open field was lit with a 100 W bulb and the illuminance at floor level was 1,167 scotopic lux.

Procedure

Rats with guide cannulae aimed at the central amygdala were randomly allocated between the drug (morphine sulphate, 10 μg) and the vehicle (sterile water) groups, such that 48 rats were in each group. Within each group the rats were assigned to pairs on the basis of weight, so that the members of a pair did not differ by more than 10 g. The pairs were then randomly allocated, six to each of four test conditions: low light, familiar (LF); high light, familiar (HF); low light, unfamiliar (LU); and high light, unfamiliar (HU).

Rats with guide cannulae aimed at the medial amygdala were likewise allocated to drug and vehicle groups and to the four test conditions.

The rats assigned to the 'familiar' test conditions were placed singly in the test arena, under the appropriate light level for a 10 min period, on the two days before the social interaction test. Those assigned to the 'unfamiliar' test conditions received two 10 min periods in the test room, but remained in their home cages. No rat was tested on more than one occasion in this test.

On the test day, injections (via 0.3 mm OD injection units which extended 2 mm beyond the guide cannulae) were made bilaterally in a volume of 0.5 μl over a period of 40 sec, immediately prior to testing. Each pair of rats was placed in the centre of the arena and their social interaction scored for 10 min by an observer who had no knowledge of the group to which they belonged or of the test condition. The following behaviours were scored as active social interaction: sniffing, following, grooming, mounting, kicking, boxing, wrestling, jumping on and crawling under or over the partner. Passive contact (when the rats sit or lie with their bodies in contact, but without interacting) was scored separately. At the end of the session the rats were removed, any boluses were removed and the floor and walls wiped. The rats were tested in an order randomised for cannula placement, drug group and test condition. Testing took place from 07.00 to 12.30 hr.

One week after the social interaction test 10 rats from each group were selected at random from those that had been tested in the LU and HU test condition (five from each). Those that had previously received morphine were allocated to vehicle injection groups and those from previous vehicle groups were now allocated to the morphine groups. Injections were made as before immediately prior to testing. Each rat was placed in the centre of the open field and observed for a four minute trial. The following behaviours were recorded: peripheral activity (number of segments entered in the outer circle), central activity number of segments entered in the inner two circles), rearing (frequency of vertical activity), grooming (time spent, sec) and defaecation (number of faecal boluses deposited). Following each test session, the apparatus was thoroughly cleansed.

The day following behavioural testing, the animals were overdosed with sodium pentobarbitone, and 0.5 μl trypan blue was infused bilaterally in order to aid localisation of

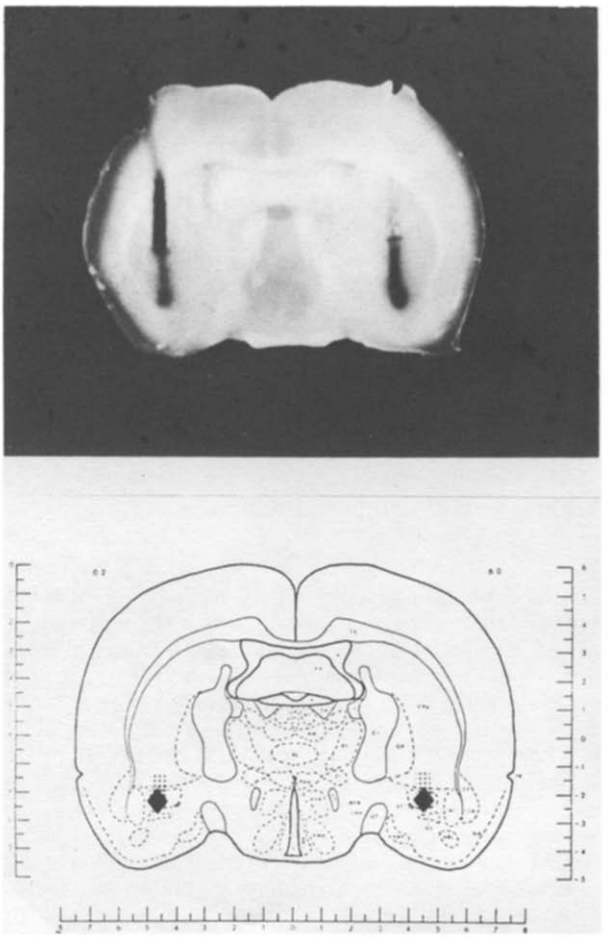

FIG. 1. TOP: Representative histological section (unstained) illustrating microinjection sites in the central amygdaloid nucleus. BOTTOM: Schematic representation of microinjection sites, after the atlas of Pellegrino and Cushman [29].

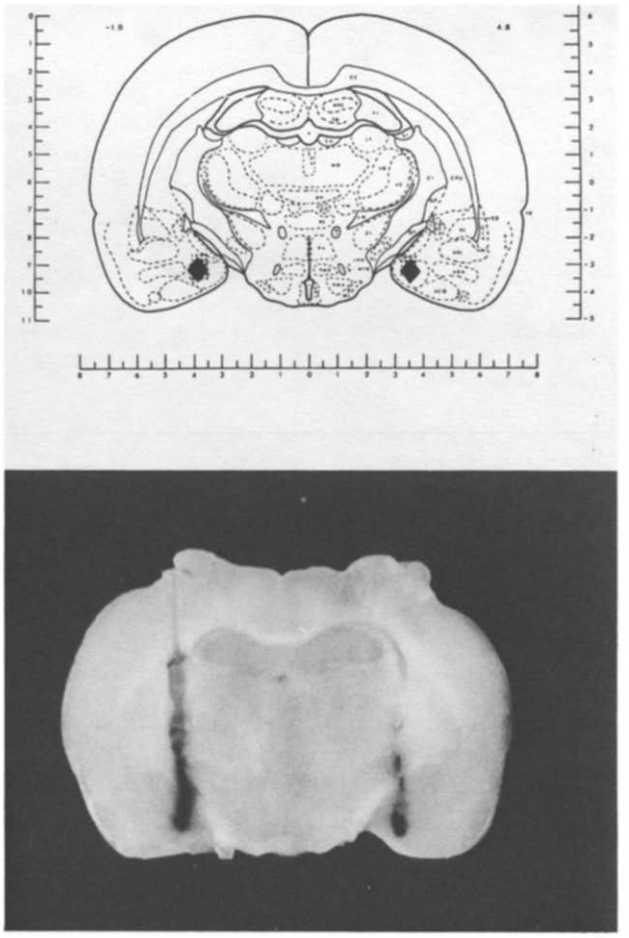

FIG. 2. TOP: Schematic representation of microinjection sites in the medial amygdaloid nucleus, after the atlas of Pellegrino and Cushman [29]. BOTTOM: Representative histological section (unstained) illustrating microinjection sites in the medial amygdaloid nucleus.

injection sites. They were then intracardially perfused with formal saline and their brains retained for histological examination [28]. The data from animals with placement errors were excluded.

Statistics

The social interaction scores were subjected to a three way analysis of variance with the drug treatment (at two levels: vehicle and morphine), the light level (at two levels: low and high) and familiarity (at two levels: familiar and unfamiliar) as the three factors.

Pair scores were used (a maximum score of 1200 sec) as the score from one rat cannot be assumed to be independent of its partner's score. A drug may change the overall level of social interaction; or it may result in less change in the level of social interaction with the manipulation of light level and unfamiliarity, in which case there would be a significant drug×familiarity and/or drug×light level interaction, as is seen with anxiolytic drugs [11].

The open field scores were analysed by the non-parametric Mann-Whitney U test, as they did not meet the requirements for parametric statistics.

RESULTS

(a) Histology

Figures 1 and 2 illustrate representative injection sites in each placement group with an indication of the extent of diffusion to adjacent structures. In the medial amygdaloid groups, trypan blue dye was observed in maximum concentration towards the ventral aspect of the nucleus with a largely ventro-dorsal diffusion pattern. In the central amygdaloid groups, maximal dye concentration was located dorsomedially with minimal diffusion to overlying caudate-putamen tissue. In no instance, was there any evidence of dye diffusion between central and medial amygdaloid nuclei.

(b) Social Interaction

The left-hand panel of Fig. 3 shows the mean time spent in active social interaction by rats receiving injections of morphine or of water into the central amygdala. The control rats show the usual decrease in social interaction with an increase in light level and when the test arena was unfamiliar. Both the manipulation of light level and of familiarity pro-

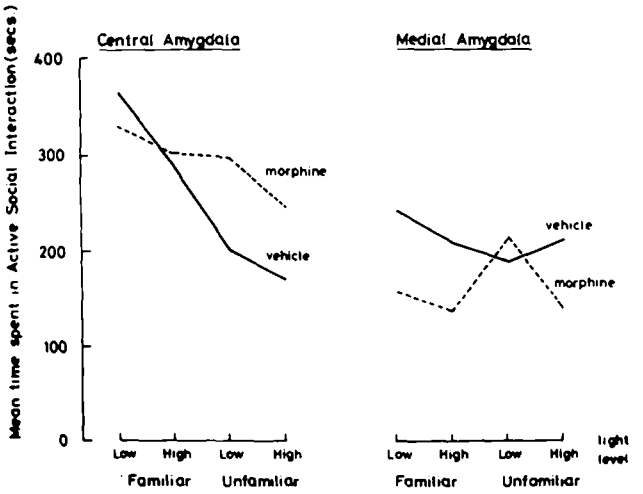

FIG. 3. Mean time spent in active social interaction by rats with injections of vehicle (———) and morphine 10 μg (----) into the central amygdala (left-hand graph) or into the medial amygdala (right-hand graph).

duced significant decreases in social interaction in the rats with central placements, $F(1,34)=4.9$, $p<0.05$ and $F(1,34)=21.2$, $p<0.001$, respectively. Morphine did not significantly alter the overall level of social interaction, but it did significantly reduce the change occurring between the familiar and unfamiliar test conditions (drug×familiarity interaction, $F(1,34)=4.95$, $p<0.05$.

Morphine in the central amygdala resulted in a significant overall increase in motor activity, $F(1,34)=4.27$, $p<0.05$; this was due to the drugged rats showing significantly higher levels of locomotor activity across all the test conditions. This resulted in a significant drug×familiarity interaction, $F(1,34)=11.9$, $p<0.002$.

The right-hand panel in Fig. 3 shows the social interaction scores for the rats that received injections into the medial amygdala. In the medial amygdala injections of both water and morphine resulted in a steady level of social interaction across all the test conditions, precluding any conclusions about morphine's effects on social interaction, except that the overall level was significantly lower in the drugged animals compared with the controls, $F(1,37)=4.8$, $p<0.05$. However injections of morphine into the medial amygdala did not significantly change the level of locomotor activity.

Passive contact was rare and was observed in three pairs only (in one pair with vehicle injections into the central amygdala, in one with morphine into the central amygdala and in one pair with morphine into the medial amygdala), and the greatest duration was only eight seconds. Defaecation was also low in all groups, it occurred in 33% of the pairs with central placements and in 42% of the pairs with medial placements, and was unaffected by morphine.

(c) Open Field

The open field data are summarised in Table 1. Bilateral morphine injections into the central amygdaloid nucleus resulted in increased peripheral ambulation ($U=15$, $p<0.05$) when compared with control values. In the medial nucleus, morphine significantly reduced rearing behaviour ($U=23$, $p<0.05$). No other morphine versus vehicle comparisons (at either site) yielded statistical significance.

DISCUSSION

The present data provide at least partial support for the hypothesis that opiate mechanisms within the amygdala are involved in the modulation of emotional reactivity in stressful situations [33]. Morphine injections into the central nucleus of the amygdala produced a partially anxiolytic profile, i.e., they counteracted the reduction in social interaction that is normally seen when the test arena is unfamiliar to the rats. Although, unlike the benzodiazepines, morphine did not counteract the decrease in social interaction that is seen when the light level is increased. It might be argued that the morphine-treated rats were not exploring the unfamiliar arena, whereas the vehicle-treated rats were, and that decreased social interaction in the latter was due to response competition between exploration and social interaction. We feel that this is unlikely as it has previously been shown that undrugged rats show less, rather than more, exploratory behaviour in the unfamiliar test arena [12]. Also, in this experiment there was no change in the locomotor activity of the control rats across the four test conditions, whereas the morphine-injected rats showed increased activity in the unfamiliar arena. These changes are incompatible with a response-competition interpretation of our results. Rats with morphine injections into the central nucleus showed increased locomotor activity in the periphery of the open field,

TABLE 1

EFFECTS OF INTRA-AMYGDALOID MORPHINE INJECTIONS (10μg BILATERAL) ON OPEN FIELD BEHAVIOURS (X ± SEM) DURING A FOUR MIN OBSERVATION PERIOD.

Injection	N	Peripheral Activity (Squares entered)	Central Activity (Squares entered)	Rearing (frequency)	Grooming (time, sec)	Boluses (number)
Central						
Vehicle	8	64.3 ± 5.2	19.6 ± 2.4	25.9 ± 4.1	13.4 ± 5.3	1.1 ± 0.6
Morphine	8	87.3 ± 7.7*	18.1 ± 2.0	26.9 ± 4.5	13.0 ± 5.0	2.4 ± 0.7
Medial						
Vehicle	10	74.2 ± 7.8	21.3 ± 3.3	37.4 ± 3.9	14.8 ± 4.9	0.6 ± 0.3
Morphine	10	82.3 ± 4.1	16.6 ± 2.1	26.5 ± 2.8*	11.7 ± 2.8	1.3 ± 0.6

*$p<0.05$

a result compatible with increased activity seen in the unfamiliar arena in the social interaction test. Interestingly, lesions of the central amygdala have been reported to increase all behavioural measures in a more complex open-field situation [43] and the authors strongly argued for a fear-reduction explanation of their results. However, it seems unlikely that a functional lesion could account for current results since control-treated animals exhibited the normal decrease in social interaction across test conditions. Instead, these findings together suggest that the central amygdaloid nucleus may participate in the facilitation of fear-motivated behaviours and that, under normal conditions, its activity may be modulated by opioid release.

In the medial amygdala injections of both water and morphine resulted in a steady level of social interaction across all the test conditions, although the overall level was significantly lower in the drugged animals compared with the controls. This was the only behaviour on which water injections had any effect. They were without effect on locomotor activity and open field activity (this study), exploration [15] and there was no difference between pre- and post-injection shock thresholds [15]. There was no evidence of water injections into the central amygdala having any effect in any of the tests.

Our current inability to detect an anxiolytic action of intra-amygdaloid morphine on the open field test is largely in agreement with previous results [33]. However, whilst in the earlier report no effects of morphine injection into the central nucleus were found and in the current study we detected an increase in peripheral activity. This discrepancy may be due to the failure of the earlier study to differentiate between peripheral and central ambulation, only total activity being measured. Also, in the earlier report, a significant decrease in defaecation was observed following medial injections of morphine, in contrast to the present negative findings on this parameter. This inconsistency, plus the new finding of decreased rearing following such treatment, may relate either to strain differences between the studies or, more likely, to factors associated with the precise site of injection. The earlier study involved injections into the corticomedial area of the amygdala whilst our injections were much more precisely localized within the medial nucleus. Overall, these results, together with criticisms of the open field test [2], suggest that the social interaction test of anxiety may be a more sensitive index of drug-induced changes in emotional reactivity.

A problem frequently encountered in the interpretation of microinjection studies is that of site specificity. Thus the possibility exists that the behavioural effects observed in the current study reflect morphine action in areas other than the actual sites of injection. However, histological verification of injection sites revealed maximal staining (trypan blue) within the desired amygdaloid nuclei without significant diffusion to adjacent sites. Whilst dye injections can only provide a crude estimate of drug diffusion, it should be noted that, when intracerebrally-administered, over 90% of labelled morphine remains at the site of injection for at least one hour [25]. Together, these data strongly argue against a diffusion effect and suggest that the differential behavioural effects in the present investigation relate directly to morphine action at the sites of injection. The different pattern of results seen with morphine injections into central and medial amygdaloid nuclei may relate to the differential distribution of opioid peptides within this limbic complex. Although moderate-high densities of opiate receptors have been found in both nuclei [3], regional localization studies suggest that the natural ligand at these sites may differ. Specifically, the medial amygdaloid nucleus has been shown to receive a β-endorphinergic input from the medial-basal hypothalamus [4] whilst the central nucleus displays high levels of enkephalin cell bodies, fibres and terminals [35]. Although this latter pattern is consistent with the presence of enkephalinergic interneurons in the central nucleus, a specific enkephalin-containing neuronal pathway has been found to project from this area to the bed nucleus of the stria terminalis [33]. Thus the behavioural differences observed following morphine injections into these two amygdaloid sites may reflect differences between the behavioural modulatory roles of the enkephalinergic and β-endophinergic systems.

Whilst the results of the present experiment suggest that morphine injections into the central amygdaloid nucleus exert a partial anxiolytic effect, we are unable to claim opiate specificity for these effects. In order to do this it is necessary to demonstrate stereo-specificity of the morphine effects and/or their antagonism by naloxone. Until this can be done the relationship with enkephalinergic and β-endophinergic pathways must remain speculative. It has recently been suggested that morphine may interact with more than one type of receptor to produce its behavioural effects [23] and, indeed, both we and other workers have reported that only some of the behavioural effects of intracerebral morphine injections can be reversed or blocked by opiate antagonists [15, 17, 24]. We therefore propose to determine whether the morphine effects found in this experiment are opiate specific, and to study the effects of enkephalins and larger opioid peptides in the social interaction test.

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