Duration of action of naloxone subcutaneous pellets in antagonizing the eeg and operant behavioural effects of morphine in the rat

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Naloxone pellets, as a depot preparation, blocked relapse in morphine-treated post addict rats (Moreton, Young & others, 1975) and prevented changes in rapid eye movement (REM) sleep produced by morphine self-injections (Young, Moreton & others, 1975). While relapse to morphine was prevented by subcutaneously implanted naloxone pellets (Moreton & others, 1975; Young & others, 1975), the duration of the action of these pellets was not determined. Using eeg and behavioral techniques, we have assessed the duration of action of this pellet depot formulation of naloxone.

In the naive rat, a 10 mg kg^{-1} (i.p.) injection of morphine produces cortical eeg slow waves (eeg slow bursts) and blocks occurrences of slow-wave sleep and REM sleep for approximately 3 h (Khazan & Colasanti, 1971; Colasanti & Khazan, 1973). These effects of morphine were blocked by a 2 mg kg^{-1} (i.p.) dose of naloxone administered 5 min before the morphine injection (Colasanti & Khazan, 1973). We have now examined the duration of action of naloxone subcutaneous pellets in blocking REM sleep suppressant effects of morphine.

Morphine and heroin decrease the operant lever pressing of rats (Rhodus, Elsmore & Manning, 1974). Narcotic antagonists, including naloxone, blocked the rate-decreasing effects of morphine in operant key pecking studies employing pigeons (McMillan, Wolf & Carchman, 1970; Dykstra, McMillan & Harris, 1974). In the present study the duration of action of naloxone subcutaneous pellets in blocking the rate-decreasing effects of morphine upon operant lever pressing was also assessed.

Adult female Sprague-Dawley rats, 250–300 g, under ketamine anaesthesia (100 to 150 mg kg^{-1}, i.p.) had stainless steel screws implanted over the frontal and parietal cortices for bipolar eeg recordings. For electromyographic (emg) recordings, stainless steel wires were inserted into the left and right temporalis muscles. Electrodes were soldered to a female miniature connector which was attached to the skull with dental acrylate.

The rats were maintained in individual cages that permitted free movement and the recording of eeg and emg activity and were on a schedule giving light from 6 am to 10 pm.

Naloxone base pellets were prepared by mixing 100 mg of naloxone base with inert fillers, 100 mg of Avicel (FMC Corp., Marcus Hook, Pa.) and 2 mg of Syloid 63 (Davison Chemical, Balitmore, Md.). The mixture was compressed into a pellet using a hand pellet press (Parr Instrument Co., Moline, Ill.) and implanted subcutaneously in the dorsal region between the shoulders. Placebo pellets consisted of inert fillers. Morphine sulphate was dissolved in physiological saline and administered intraperitoneally.

The rats were allowed to adjust to the cages for one week. Six rats were then implanted subcutaneously with 2 × 100 mg naloxone pellets at 3 pm, while two others were implanted with placebos. On the next day, the rats were divided among the following three groups: (1) four rats with naloxone pellets and receiving one injection of morphine (10 mg kg^{-1}, i.p.) each day at 1 pm; (2) two rats with naloxone pellets and receiving one injection of isotonic saline (i.p.) each day at 1 pm; and (3) two rats with placebo pellets and receiving one injection of isotonic saline (i.p.) each day at 1 pm. The dependent variable was the time from each injection to the onset of the first REM sleep episode. These daily injections were continued for approximately three weeks.

Adult female Sprague-Dawley rats, initially 250–300 g, were maintained at 80% of their free-feeding weight. Each rat was trained in a BRS-LVE Test Cage to lever press for 45 mg Noyes food pellets on a variable interval 1 min schedule of reinforcement. Daily sessions lasted for 45 min. Five rats were used. After stabilized patterns of responding were established, three of the rats were implanted subcutaneously with 2 × 100 mg naloxone pellets as in the first experiment, while two others were implanted with placebos. Beginning on the next day and

Table 1. *Days of antagonism by naloxone pellets of morphine REM sleep suppression as indicated by REM sleep onsets.* Values are given as the mean time ± standard error from each daily injection until the occurrence of the first REM sleep episode. Each mean was calculated for the indicated number of days in parenthesis after pellet implantation.

Naloxone (N) or placebo (P) pellets: saline treatment		Naloxone pellets: morphine treatment*		
Rat No.	REM sleep onset (min)	Rat No.	REM sleep onset (min)	
N-1	41·9 ± 4·2 (21) Days 1–21	M-1	63·4 ± 9·1 (10) Days 1–10	174·0 ± 9·8 (4) Days 11–14
N-2	25·6 ± 5·7 (20) Days 1–20	M-2	67·0 ± 10·9 (12) Days 1–12	161·0 ± 7·0 (7) Days 13–19
P-3	44·0 ± 8·9 (21) Days 1–21	M-3	51·5 ± 7·3 (16) Days 1–16	143·5 ± 0·6 (2) Days 17–18
P-4	40·7 ± 1·9 (24) Days 1–24	M-4	63·4 ± 7·2 (16) Days 1–16	175·5 ± 11·1 (5) Days 17–21

Mann-Whitney U test: *$P < 0.005$.

* Correspondence.

thereafter, a single daily injection of isotonic saline (0·25 ml, i.p.) was alternated with an injection of morphine (10 mg kg^{-1}, i.p.). Injections were given immediately before each session.

Naloxone pellets were effective in antagonizing morphine produced REM sleep suppression for about two weeks (See Table 1). This was demonstrated in the naloxone-treated group receiving daily morphine injections by a significant shift in REM sleep onsets following the injections (Mann-Whitney U = 2, $P < 0.005$). For example, REM sleep episodes occurred within 51·5 to 67·0 min after the administration of morphine during the first 10 to 16 days and, then, shifted to averages from 143·5 to 175·5 min thereafter. In contrast, latencies to REM sleep onset averaged from 25·6 to 44·0 min with no changes in rats receiving daily intraperitoneal injections of isotonic saline.

Naloxone pellets were also effective in antagonizing the morphine-induced operant response suppression for 10 to 13 days (See Table 2). This was demonstrated in the naloxone pellet group receiving alternate day injections (i.p.) of morphine by a significant shift in the amount of response suppression (Mann-Whitney U = 7, $P < 0.005$). For example, rat No. M-5 averaged 115·0% of control for 10 days during morphine treatment, and, then, shifted abruptly to an average of 55·1% of control during the next five days. Similar data are indicated for rats Nos M-6 and M-7. In contrast, the same rats averaged from 77·0 to 99·9% of control with no abrupt changes during saline treatment.

In the rats implanted with placebo pellets, morphine treatment significantly suppressed operant responding to 10·7 and 49·9% of control over a period of three weeks (Mann-Whitney U = 0, $P < 0.005$), while saline treatment during the same period had no effect upon operant responding as compared to control values.

Previous studies have shown that morphine affects eeg and suppresses REM sleep in the naive rabbit (Khazan & Sawyer, 1964), rat (Khazan, Weeks & Schroeder, 1967; Khazan, 1975), and cat (Echols & Jewett, 1969; 1972). Colasanti & Khazan (1973) and Moreton & others (1974) found that injections of naloxone blocked the suppressive effect of morphine on REM sleep in the naive rat. Furthermore, it has been demonstrated that morphine decreases the rate of operant responding in pigeons (McMillan & others, 1970; Dykstra & others, 1974) and rats (Rhodus & others, 1974), while naloxone blocked the rate-decreasing effect of morphine upon operant responding in the pigeon (McMillan & others, 1970). Naloxone pre-treatment has also been shown to eliminate opiate-seeking behaviour in rats self-administrating doses of morphine that were probably not high enough to establish tolerance and physical dependence (Davis & Smith, 1974).

Table 2. *Days of antagonism by naloxone pellets of morphine operant response suppression.* Values are given as the mean number of variable interval responses (% control) ± s.e. Each mean was calculated for the indicated days in parentheses after pellet implantation for those days on which saline or morphine treatment was given.

Rat No.	Placebo pellets* Saline	Morphine	
P-5	99·5 ± 6·4 (11) Days 1–21	10·7 ± 2·9 (10) Days 1–21	
P-6	94·5 ± 3·1 (11) Days 1–22	49·9 ± 3·9 (11) Days 1–22	
	Naloxone pellets Saline	Morphine	
M-5	99·9 ± 3·7 (8) Days 1–16	115·0 ± 11·2 (5) Days 1–10	55·1 ± 3·0 (3) Days 11–16
M-6	77·0 ± 4·9 (8) Days 1–17	105·0 ± 8·3 (6) Days 1–11	55·2 ± 11·6 (3) Days 12–17
M-7	79·8 ± 5·2 (10) Days 1–20	74·8 ± 3·9 (7) Days 1–13	53·6 ± 2·4 (3) Days 14–20

Mann-Whitney U test: *$P < 0.005$.

Our present study demonstrated that the naloxone subcutaneous pellets that effectively prevented relapse to morphine self-administration in post-addict rats (Moreton & others, 1975) blocked both the REM sleep suppressant effects and the operant behaviour suppressant effects of morphine for approximately two weeks. It is suggested that the two experimental models used in the present study to assess the duration of action of naloxone pellets in antagonizing morphine effects may contribute to the delineation of duration of action of long-acting preparations of morphine antagonists.

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Structure-activity relationships of methionine-enkephalin

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The amino acid sequence of methionine–enkephalin has been found to correspond to residues 61–65 of pituitary β-lipotropin (Hughes, Smith & others, 1975b). More recently, it has been shown that not only methionine–enkephalin but also the lipotropin fragments 61–68, 61–69, 61–76, 61–87, 61–89, 61–91 interact with the opiate receptors in brain homogenates, guinea-pig ileum or mouse vas deferens (Bradbury, Smyth & others, 1976; Cox, Goldstein & Li, 1976; Gráf, Rónai & others, 1976; Guillemin, Ling & Burgus, 1976; Waterfield, Hughes & Kosterlitz, unpublished observations).

$Lipotropin_{61-64}$ has only 3% of the potency of methionine–enkephalin to inhibit [^{3}H]dihydromorphine binding in brain homogenates (Bradbury & others, 1976) and only 1% of its potency to depress the electrically induced contractions of the guinea-pig ileum and mouse vas deferens (Table 1). Methionine–enkephalin, together with leucine–enkephalin (Hughes & others, 1975b), therefore appears to be the smallest peptide capable of interacting significantly with opiate receptors. Although it is not a potent antinociceptive agent, even after injection into the cerebral ventricles (Belluzzi, Grant & others, 1976; Feldberg & Smyth, 1976), its possible physiological significance is indicated by its ability to reduce the rate of spontaneous and evoked firing of neurons in the brain stem of the rat and cat (Bradley, Briggs & others, 1976; Gent & Wolstencroft, 1976) and the cortex, thalamus and medulla of the rat (Hill, Pepper & Mitchell, personal communication). Since this effect has a fast onset of action and declines rapidly after turning off the iontophoretic current, degradation to a much less potent tetrapeptide may play an important role in bringing about the termination of action after the release of methionine–enkephalin, and also of leucine–enkephalin. It was therefore important to study the structure-activity relationship of methionine–enkephalin.

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Table 1. *Structure-activity relationships of methionine–enkephalin.* The peptides have been synthesized by the solution method (Bower, Guest & Morgan, in preparation). The agonist activities of the compounds were determined on the mouse vas deferens (Hughes, Kosterlitz & Leslie, 1975a) and the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum (Kosterlitz & Watt, 1968; Kosterlitz, Lydon & Watt, 1970). None of the compounds showed antagonist activity in the guinea-pig ileum (dose ratio <2). The ID50 values of methionine–enkaphalin were 100 nM in the guinea-pig ileum and 12·5 nM in the mouse vas deferens.

Compound	Relative agonist activity (Methionine–enkephalin = 1) Mouse vas deferens	Guinea-pig ileum
Tyr–Gly–Gly–Phe–Met	1	1
Tyr–Gly–Gly–Phe–Gly	0·03	0·02
Tyr–Gly–Gly–Phe	0·01	0·01
Des–NH_2–Tyr–Gly–Gly–Phe–Met	0	0
Phe–Gly–Gly–Phe–Met	0·0003	0·002
Tyr–Gly–Gly–Tyr–Met	0·001	0·003

Removal of methionine at the C-terminus causes a loss of 99% of activity. Since replacement of methionine by glycine also decreases activity by 97–98% (Table 1), it would appear that an amino acid with a hydrophobic side chain at the C-terminus is essential for activity of the pentapeptide. This view is supported by the fact that replacement of methionine by leucine does not decrease the potency in the mouse vas deferens although activity is reduced by 60% in the guinea-pig ileum (Waterfield, Hughes & Kosterlitz, unpublished observations).

Another essential feature is the need for an intact