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MORPHINE–NALOXONE INTERACTION IN THE CENTRAL CHOLINERGIC SYSTEM: THE INFLUENCE OF SUBCORTICAL LESIONING AND ELECTRICAL STIMULATION

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1 The opiate antagonist naloxone, injected or topically applied to the cerebral cortex, had no significant effect on the spontaneous output of cortical acetylcholine (ACh) in rats.

2 Morphine (2.5 mg/kg) administered intravenously inhibited the release of cortical ACh. A subsequent injection of naloxone rapidly reversed morphine-induced inhibition, and produced a sustained increase in the release of ACh. Topical application of naloxone solutions, after morphine, produced a slow and weak reversal of its inhibitory action.

3 Destruction of the medial thalamus abolished both the inhibitory effects of morphine on the cortical ACh release, and its antagonism by naloxone administered after the agonist.

4 Injection of naloxone in a low dose (0.1 mg/kg) increased the release of cortical ACh provoked by electrical stimulation of either the medial thalamus or the reticular formation in normal rats. In the morphine-dependent rat, naloxone also facilitated the evoked release and its action was greater than in control animals. The facilitatory effect of naloxone on the cortical release evoked by stimulation of the medial thalamus was greater than its effect on the release evoked by stimulation of the reticular formation in both normal and morphine-dependent rats.

5 Naltrexone, a narcotic antagonist, also facilitated the electrically stimulated release of cortical ACh.

6 It is suggested that (a) morphine and naloxone act at a subcortical site, probably the medial thalamus, to modify the cortical ACh release and that (b) naloxone may facilitate the electrically-induced release of ACh in the CNS by antagonizing the effect of the endogenous morphine-like factor, enkephalin.

Introduction

The development of a precipitated abstinence syndrome in opiate-dependent animals is characterized by an abnormal increase in the cholinergic activity. It has been suggested that a sudden increase in the release of acetylcholine (ACh) in the periphery and the central nervous system (CNS) could contribute to the excessive cholinergic activity associated with the abstinence phenomenon (Paton, 1963; Crossland, 1970). Studies reported from this and other laboratories show that the opiate antagonist naloxone, when administered to chronically morphine-treated animals, enhances the output of ACh in the cerebral cortex (Jhamandas & Sutak, 1974; Labrecque & Domino, 1974; Mullins & Phillis, 1974). Morphine itself can inhibit the release of ACh in the CNS and this effect can be antagonized by naloxone administered before or after morphine treatment (Jhamandas, Phillis & Pinsky, 1971; Matthews, Labrecque & Domino, 1973; Yaksh & Yamamura, 1975). Furthermore, the development of some signs of abstinence precipitated by naloxone, in morphine-dependent rats, is suppressed by pretreatment with ACh antagonists (Collier, Francis & Schneider, 1972; Jhamandas & Dickinson, 1973; Jhamandas, Sutak & Bell, 1973). These observations lend support to the suggestion that the ACh releasing system in the CNS could play a significant role in the development of the opiate abstinence syndrome precipitated by naloxone or related antagonists. However, the site(s) in the central cholinergic system at which naloxone, or morphine, may be acting to modify the release of ACh remain unknown.

Recently some investigators have suggested that the medial thalamus is an important brain site involved in the development of tolerance to the EEG effects of morphine (Teitelbaum, Catravas & McFarland, 1974) and the naloxone-precipitated abstinence syndrome in the rat (Wei, Loh & Way, 1973a and b). It has also been demonstrated that there is a high degree of opiate receptor binding in this area (Kuhar, Pert & Snyder,

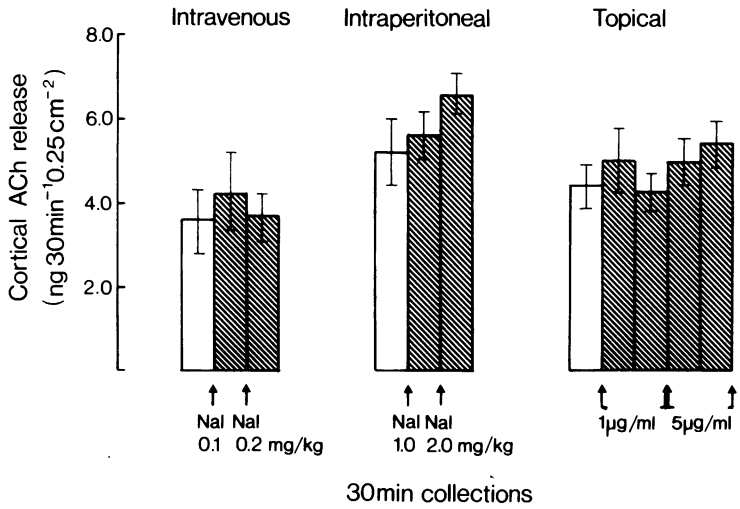

Figure 1 The effect of injected and topically applied naloxone (Nal) on the output of acetylcholine (ACh) from the cortical surface of the cerebral cortex. Naloxone was injected or applied at the points indicated by arrows. Each column represents the mean output in six experiments with s.e. mean. Open columns indicate release preceding the drug injections.

1973). The region of the medial thalamus is an integral part of the ascending reticular cholinergic system (Shute & Lewis, 1967) which projects diffusely to the cortex and releases ACh in this region. These observations make it attractive to consider that the action of naloxone in a morphine-treated animal and possibly also the effect of morphine on the cortical release of ACh, may be exerted at this region. If this idea were correct, lesioning of the medial thalamus should abolish the effects of naloxone and morphine on the release of cortical ACh. The objective of the present study was to see if this were the case.

In previous tests, naloxone has been shown to have no significant action of its own on the spontaneous output of central ACh. However, in a recent report, Waterfield & Kosterlitz (1975) demonstrated that naloxone, and other opiate antagonists, increased the electrically stimulated release of ACh in the guinea-pig myenteric plexus-longitudinal muscle preparation. A stereospecificity of this effect was also demonstrated, and it was suggested by these investigators that the opiate antagonists may be enhancing the stimulated release by antagonizing the action of an endogenous morphine-like substance, enkephalin, which is present in this tissue (Hughes, Smith, Morgan & Fothergill, 1975). The second objective of our study was to determine whether naloxone would increase the central release of ACh evoked by electrical stimulation in the CNS as it does in the periphery.

Methods

Release of cortical acetylcholine

All experiments described here were carried out on Sprague–Dawley rats weighing 250–350 g which were lightly anaesthetized with pentobarbitone (30 mg/kg) and urethane (400 mg/kg) mixture. The release of cortical ACh was investigated in the presence of neostigmine (50 μg/ml) and atropine (0.5 μg/ml) using the cup technique of MacIntosh & Oborin (1953). Experimental details of the procedure, as employed in the rat, have been described fully in a previous paper (Jhamandas & Sutak, 1974).

Lesioning and stimulation

Thermal lesions were placed bilaterally in the medial thalamus with a radio frequency lesion generator (David Kopf Instruments, Model RFG-4) using co-ordinates (AP3.4, L ± 1.0, HV−1.0) according to the rat stereotaxic atlas compiled by Pellegrino & Cushman (1967). Electrical stimulation was applied to the same area, and also to the reticular formation (AP 1.6, L 2.0, HV−2.5), by a concentric electrode 0.5 μm in diameter. Stimuli of 0.3 ms duration, rate of 10 Hz for 1 s, repeated every 10 s, were delivered through a Grass Stimulator (Model S-88). Electrode positions and sites of lesions were verified histologically.

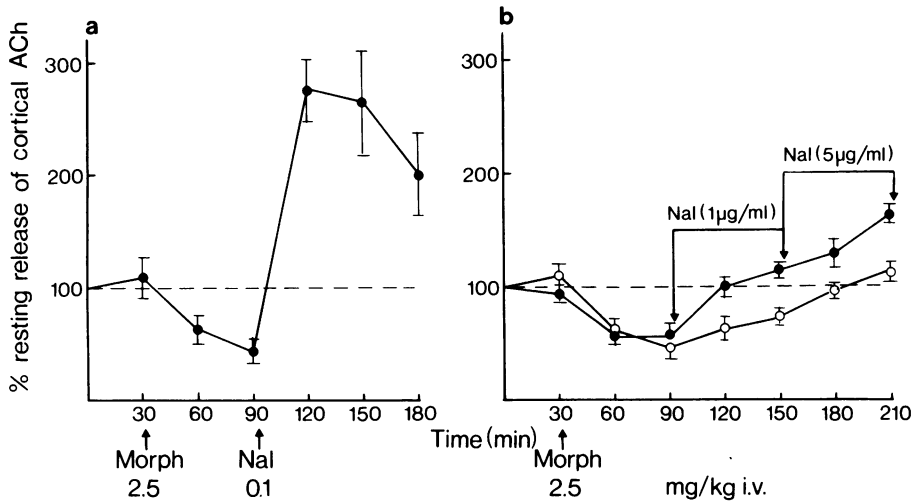

Figure 2 The reversal of morphine-induced (Morph) inhibition of acetylcholine (ACh) release by the antagonist naloxone (Nal) after (a) its injection or (b) topical application to the left cortex. In both (a) and (b) the mean results of four experiments are shown; vertical lines show s.e. mean. The average resting release in the first two collections, which is represented as 100% was 3.4 ± 0.4 and 4.1 ± 0.5 ng 30 min^{-1} 0.25 cm^{-2} in (a) and (b) respectively. (O) = Control.

Assay of acetylcholine

The solutions collected from the cerebral cortex were assayed biologically on the hearts of the clam *Mercenaria mercenaria* as described previously (Jhamandas *et al.*, 1971).

Drugs and treatment

Drugs used were atropine sulphate, naloxone hydrochloride, naltrexone hydrochloride, neostigmine bromide, pentobarbitone sodium and urethane. All drug solutions, except those applied topically, were prepared by dissolving the drugs in 0.9% w/v NaCl solution (saline). Weights of drugs refer to the salts. Chronic morphine injections were administered to rats as described in a previous paper (Jhamandas & Sutak, 1974).

Results

Effects of naloxone and morphine on the spontaneous release of acetylcholine

The histograms in Figure 1 show results of experiments in which the effects of naloxone alone were investigated on the spontaneous release of cortical ACh following its injection or topical application. Injections of the drug in doses 0.1–0.2 mg/kg intravenously, and 1.0–2.0 mg/kg intraperitoneally had no significant effect on this release. Similarly an application of two naloxone solutions (1 and 5 μg/ml) to the cortex, each for a total of 60 min, did not affect the resting output of ACh. In separate experiments, the effects of injected and locally applied naloxone were investigated following pretreatment with morphine (2.5 mg/kg i.v.). Morphine consistently reduced the output of ACh to about 50% of the pre-drug value (Figure 2). A subsequent injection of naloxone (0.1 mg/kg) not only induced a complete reversal of the morphine effect but caused the output of ACh to exceed the value of resting release by nearly 200% (Figure 2a). This effect was sustained and it did not begin to decline towards the resting value until the third post-naloxone collection period. It was reasoned that if naloxone was acting at the cortical level to reverse morphine-induced depression of release, then its local application at this site should produce a comparable degree of antagonism. In the next experiment, results of which are shown in Figure 2b, two solutions of naloxone were applied to the cortex unilaterally after depression of the release of ACh with intravenously injected morphine. Although a reversal of this depression by naloxone was discernible, as indicated by a more rapid recovery of release in the treated side, this antagonism was sluggish when compared with the large and rapid effect of the injected drug in the previous tests. Despite a prolonged application period, the release of ACh following topical naloxone was never as high as that observed after its injection. These results suggested that the

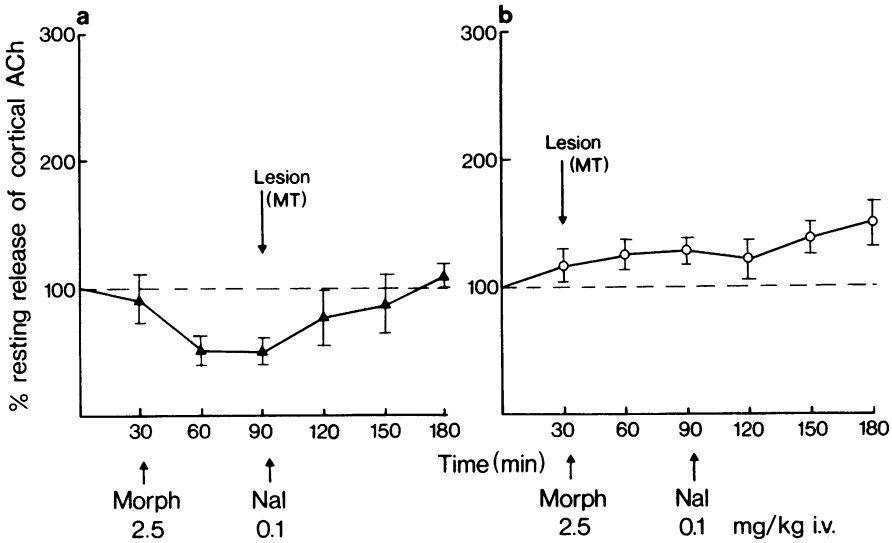

Figure 3 The effect of lesioning medial thalamus (MT) on (a) the antagonism of morphine effect on acetylcholine (ACh) release by naloxone and (b) the inhibitory effect of morphine on ACh release. Naloxone (Nal) and morphine (Morph) were injected 5 min after making the lesion in (a) and (b), respectively. Mean of 4 experiments is shown in (a) and of 6 experiments in (b); vertical lines show s.e. mean. The average resting release (100%) values were 3.8 ± 0.7 and 2.6 ± 0.2 ng 30 min^{-1} 0.25 cm^{-2}.

antagonist may have been diffusing slowly from the cortex to a subcortical site and subsequently acting there to reverse the action of the agonist.

Naloxone and morphine effect on acetylcholine release after lesion placement

The possibility that naloxone may be antagonizing the action of morphine on the cortical ACh release by acting at a subcortical site was investigated in experiments where the medial thalamus was lesioned before naloxone injection. The effect of morphine itself was also investigated in similar tests. Results of the lesion experiments are shown in Figure 3a and b. In preliminary tests it was established that the introduction of the lesion probes, or the electrodes into the brain, had no significant action on the basal output of cortical ACh. As shown in Figure 3a, a morphine injection (2.5 mg/kg) produced the expected inhibition of the cortical ACh release. When an inhibition had occurred, the medial thalamus was lesioned bilaterally and, 5 min later, an injection of naloxone (0.1 mg/kg) was administered to the animal. Naloxone did not now produce the large increase in the cortical release of ACh as it had done in the previous experiments shown in Figure 2a. The release of ACh after morphine injection and lesioning reached the control level in the next 30 min period following a lesion. This post-morphine recovery of the release of ACh to control levels was apparently more rapid than the recovery from the same dose of morphine in earlier tests which did not involve lesioning (Figure 2b, control side). This observation suggested that lesioning may have destroyed the site(s) at which morphine was exerting its inhibitory effect, and thereby terminated the agonist action. This possibility was tested in other experiments where lesions in the medial thalamus were placed before an injection of morphine (2.5 mg/kg). As shown in Figure 3b the agonist now failed to depress the cortical ACh release. A post-morphine injection of naloxone also did not modify the release.

Effects of naloxone on electrically stimulated acetylcholine release

The effects of a low dose of naloxone (0.1 mg/kg) were investigated on the cortical output of ACh evoked by electrical stimulation delivered to the medial thalamus in some tests, and the mesencephalic reticular formation in others. Experiments were carried out in normal rats, and in animals that had been chronically treated with repeated injections of morphine. Preliminary tests in the normal animals indicated that two successive periods of electrical stimulation, delivered at 10 Hz to the same region and when separated by 60–90 min, consistently provoked similar increments in the output of cortical ACh over its pre-stimulus value. This observation allowed the investigation of drug effect on the evoked output in the same animal by using one stimulation period as the

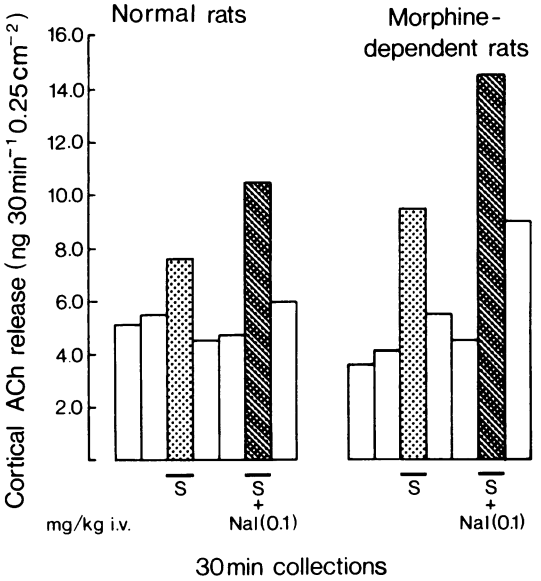

Figure 4 Effects of electrical stimulation of the medial thalamus on the release of cortical acetylcholine (ACh) before and after a naloxone (Nal) injection in a normal rat and a morphine-dependent rat (single experiments). Stimulation (S) was applied, during the periods indicated by the bar, immediately following the drug injection.

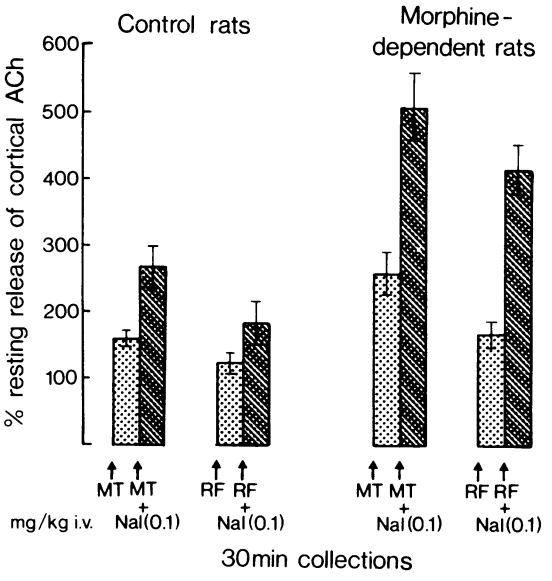

Figure 5 Effect of electrical stimulation of the medial thalamus (MT) or the reticular formation (RF) before and after naloxone injection in normal and morphine-dependent rats. The results obtained from four to six experiments, of the type shown in Figure 4, have been normalized by expressing the stimulated release as a percentage of the pre-stimulus resting release. In experiments involving MT and RF stimulation the average values, expressed as ng 30 min^{-1} 0.25 cm^{-2}, for resting release were: control rats, 4.7 ± 0.3 ($n=6$) and 3.7 ± 0.9 ($n=4$), respectively; morphine-dependent rats, 3.0 ± 0.2 ($n=4$) and 3.3 ± 0.8 ($n=4$).

control, and delivering the other in combination with the drug under investigation.

The results of representative tests with naloxone in normal and morphine-dependent animals are shown in Figure 4, while the combined results of several tests are shown in Figure 5. As shown in Figure 4 (left histogram) the electrical stimulation of the medial thalamus alone in the normal rat increased the cortical ACh output by 50% over the pre-stimulus resting level. When the stimulation was delivered immediately after naloxone the release of ACh rose to about 120% over the resting value, indicating a clear facilitation of the electrically evoked release by the antagonist. The low dose of naloxone used here was without effect on the spontaneous release as observed earlier (Figure 1). In a comparative test on the morphine-dependent animal, also shown in Figure 4 (right histogram), the stimulation alone increased the release by 150% and its combination with naloxone treatment caused the output to rise to nearly 300% over the resting value. This indicated that pretreatment with morphine greatly intensified the stimulatory effect of naloxone on the electrically induced release of ACh from the cortex. The morphine-dependent animals, although under the influence of the anaesthetic, in these tests exhibited mild classical signs of opiate withdrawal soon after receiving naloxone. These signs did not appear in the normal rats but piloerection and salivation was often observed in these animals.

The results of several experiments in which naloxone effects were tested on the electrical stimulation of either the medial thalamus, or the reticular formation are shown in Figure 5. As shown here, naloxone facilitated the electrically evoked release when both subcortical sites were stimulated. However, its action on the evoked release following medial thalamus stimulation were greater in magnitude, in both normal and morphine-treated animals, suggesting a possibly greater sensitivity of this site to the antagonist's action.

In order to investigate whether the actions of naloxone were shared by the other opiate antagonists its effects were compared with those of a related agent, naltrexone. Initial tests with a low dose of naltrexone (0.1 mg/kg) indicated a lack of significant effect on the spontaneous release when administered by itself, and a rapid reversal of morphine effect when administered after the agonist. The effects of naltrexone on the release of ACh evoked by the stimulation of medial

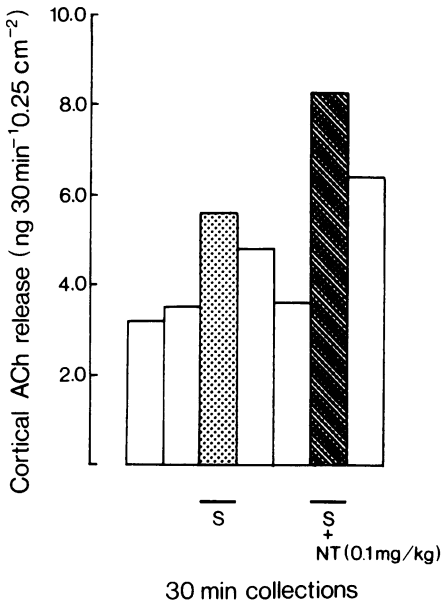

Figure 6 Effect of naltrexone (NT) on the release of cortical acetylcholine (ACh) evoked by the electrical stimulation of the medial thalamus in a normal rat (single experiment). Stimulation (S) was applied during the period indicated by the bars, immediately following the drug injection.

thalamus are shown in Figure 6; like naloxone, it markedly increased the stimulated output of ACh in three of the five experiments on normal animals. Its effect in the morphine-dependent animals was not tested.

Discussion

The inhibitory effects of morphine on the release of cortical ACh could result from its action at the cholinergic nerve terminals in the cortex, or at some subcortical site in the ascending system of cholinergic neurones projecting to the cortex. Evidence from previous investigations with morphine favours the latter possibility. These studies have shown that application of morphine solutions to one side of the cortex depresses the release of ACh on both sides of the cortex, possibly by diffusion of the drug to a subcortical site (Jhamandas, Pinsky & Phillis, 1970). Szerb (1974) has reported that the release of labelled ACh from isolated slices of the cortex and other regions was not inhibited in the presence of morphine. In a recent study of two narcotic agonists, methadone and levorphanol, and their isomers, it was found that a clear stereospecificity of inhibitory action of the cortical ACh release could be observed *in vivo* but not *in vitro* (Jhamandas, Hron & Sutak, 1975). The experiments described here, in which lesions placed in a lower level resulted in a loss of morphine effect, provide further evidence for a subcortical site of action. The fact that both the depressant effect of morphine on release of ACh and its antagonism by naloxone were abolished following lesioning of the medial thalamus suggests that this region could be an important site for an interaction between the opiate agonist and its antagonist occurring in the ascending cholinergic system. The slow antagonism of injected morphine by cortically applied naloxone observed here could have resulted from a slow diffusion of the antagonist to this region. Since the medial thalamus is interposed between the brainstem reticular formation, where the outflow of the ascending cholinergic system originates, and the cerebral cortex, where ACh is released, the possibility of an even lower site of action cannot be excluded on the basis of the present experiments. In this context, Garau, Mulas & Pepeu (1975) have reported that a loss of morphine action on the cortical ACh release results following lesions of the raphe nuclei in the brainstem.

Although naloxone has been shown to have no significant action on the spontaneous release, the experiments described here show that it greatly increases the electrically stimulated release of ACh. This finding in the CNS is similar to that of Waterfield & Kosterlitz (1975) in the guinea-pig myenteric plexus, where naloxone and two benzomorphan antagonists were found to enhance the electrically induced release of ACh. Since a stereospecificity of this action with antagonists of the benzomorphan series could be demonstrated, these authors suggested that the opiate antagonists may be enhancing the stimulated release by antagonizing the action of enkephalin which is present in the guinea-pig ileum and inhibits the release of ACh in this preparation (Hughes *et al.*, 1975). Although a stereospecificity of the facilitatory action of antagonists on the evoked release was not investigated in the present tests, the results obtained could be interpreted in similar terms. The facilitation of central ACh release by naloxone may reflect the antagonism of enkephalin or a morphine-like factor (Hughes, 1975; Pasternak, Goodman & Snyder, 1975) which is perhaps released following electrical stimulation. The possibility of the facilitatory effect being simply a non-specific action of naloxone is diminished by the fact that it was (a) apparent at a low dose of the antagonist, (b) intensified by making the animals morphine-dependent and (c) observed with another selective antagonist, naltrexone. It is also apparent that the facilitation of stimulated release was greater following the thalamic stimulation than the reticular stimulation. The reason for this difference is not clear, however, it is attractive to suggest that the difference could be due to a greater release of enkephalin by the thalamic stimulation, and

consequently a more effective antagonism by naloxone. These observations suggest that the action of enkephalin on central release of ACh, and its antagonism by naloxone, should be evaluated in future experiments.

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