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Stereospecific enhancement of evoked release of brain acetylcholine by narcotic antagonists

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1 Electrical stimulation of nerves in the forepaw of anaesthetized rats caused an increase in the release of acetylcholine (ACh) from the cerebral cortex *in vivo*. Actions of naloxone (Nal) enantiomers and naltrexone (Ntx) were tested on this release in normal animals and those lacking pituitary gland for 3 weeks.

2 In normal animals systemic administration of (−)-Nal or Ntx, but not (+)-Nal caused a significant increase in the evoked release of ACh. Spinally administered (−)-Nal did not produce this effect. Cortical application of (−)-Nal produced a smaller increase in the evoked release of ACh.

3 In hypophysectomized rats the stimulatory action of (−)-Nal or Ntx on ACh release was significantly reduced. The ability of (−)-Nal to reverse inhibitory action of morphine or the enkephalin (FK 33,824) was not affected by hypophysectomy.

4 It is suggested that (−)-Nal and Ntx increase the stimulated release of cortical ACh by blocking the inhibitory action of an endogenous opioid at a subcortical site. An intact pituitary appears essential for a full expression of the Nal effect on evoked ACh release.

Introduction

Earlier work has shown that morphine and related narcotic analgesic agents inhibit the release of brain acetylcholine (ACh) *in vivo* through a specific action on opioid receptors (Jhamandas & Sutak, 1974; Jhamandas, Hron & Sutak, 1975). Following the discovery of two endogenous opiate pentapeptides, methionine- and leucine-enkephalin [Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975) it was demonstrated that these peptides, like the classical opioid agonists, inhibited cortical ACh release and their effects could be blocked by the opioid receptor antagonist naloxone (Nal) (Jhamandas, Sawynok & Sutak, 1976; Jhamandas & Sutak, 1980). β-Endorphin, another naturally occurring opioid peptide has been reported to inhibit the turnover of ACh in the cortex and other brain regions through a Nal-sensitive mechanism (Moroni, Cheney & Costa, 1977). These pharmacological studies with the opioid agonists have raised the possibility that the endogenous opioids may function as inhibitory modulators of cholinergic neurones. If this idea is valid, then pharmacological antagonism of the endogenous opioids should enhance activity of cholinergic neurones. Indeed, in myenteric plexus of the guinea-pig, where opioids depress ACh release, narcotic antagonists of the benzmorphan type have been shown by Waterfield & Kosterlitz (1975) to increase the release of ACh in a stereoselective fashion. Similarly, in the brain, low doses of Nal and naltrexone (Ntx) have been found to increase the release of cortical ACh provoked by electrical stimulation of the reticular formation or the medial thalamus (Jhamandas & Sutak, 1976). However, in brain experiments the pharmacological specificity of the stimulatory effect of narcotic antagonists on ACh release has not been examined. Therefore, conclusions concerning a physiological interaction between the endogenous opioids and the cholinergic neurones, based on this effect, remain in some doubt. In the present study we have studied the action of Nal and Ntx on the release of cortical ACh evoked by stimulation of peripheral nerves and have investigated the specificity of this action by testing the effect of (+)-Nal, an enantiomer with no affinity for opiate receptors.

In the past, Nal has been reported to produce hyperalgesia in rodents (Jacob, Tremblay & Colombel, 1974; Fredrickson, Burgis & Edwards, 1977; Grevert, Baizman & Goldstein, 1978), an effect presumed to result from antagonism of the endogenous opiates mobilized by the nociceptive stimulus. Systemically administered Nal has also be found to cause

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excitation of neurones in dorsal horn of the cat spinal cord (Henry, 1979). Both the hyperalgesia and the increase in neuronal firing induced by Nal have been reported to be abolished after removal of the pituitary gland (Grevert *et al.*, 1978; Henry, 1980). These observations suggest that an intact pituitary is essential for expression of the excitatory action of Nal in behavioural or electrophysiological experiments. Since narcotic antagonists augment the stimulus-evoked release of ACh from the cortex it was considered of interest to determine whether an intact pituitary gland is necessary to observe this effect. Accordingly, in this study the action of Nal and Ntx on the evoked release of ACh was examined in chronically hypophysectomized rats.

Methods

All experiments were performed on Sprague-Dawley rats (200–220 g) lightly anaesthetized with a mixture of pentobarbitone (30 mg/kg) and urethane (400 mg/kg) (Jhamandas & Sutak, 1976). The release of ACh, in the presence of neostigmine (50 μg/ml) and atropine (0.8 μg/ml) was measured from the surface of the cerebral cortex by the cup technique. Details of surgery and the method for collection of ACh and its biological estimation using the hearts of the clam, *Mercenaria*, have been described fully in previous papers from this laboratory (Jhamandas & Sutak, 1974; 1976). To induce the release of ACh from the cortex, electrical stimulation was applied to the forepaws. Biopolar electrodes (constructed from stainless steel pins) were inserted under the forepaw skin to stimulate afferent nerve fibres in the limbs. The optimal frequency of stimulation inducing release of ACh (0.5 Hz) was determined in preliminary experiments and this frequency was used in all subsequent stimulation experiments. Stimuli (square wave pulses, 10 volts, 0.3 ms duration) were delivered through a Grass stimulator (Model S-88).

Hypophysectomized rats

Hypophysectomized or sham-hypophysectomized animals used in this study (3 weeks post-surgery) were obtained from Charles River Breeding Laboratories Inc. (Willmington, Mass. U.S.A.). The animals were maintained in group cages under constant temperature and a 12 h light-dark cycle. All animals were given rat food pellets (Purina Rat Chow) and a solution containing 5% dextrose and 0.9% w/v NaCl solution (saline). At the end of experiments, the animals were decapitated. The trunk blood from each animal was collected and submitted for analysis to detect the presence of pituitary hormone prolactin by radioimmunoassay. The brain was removed and a check of the completeness of hypophysectomy was carried out by an examination of the sella turica. Absence of residual pituitary tissue and lack of prolactin in the blood was taken as evidence of the completeness of hypophysectomy.

All experiments described in this paper were performed between 09 h 00 min and 12 h 00 min.

Drugs

Drugs used were atropine sulphate, morphine sulphate, (−)-naloxone hydrochloride, (+)-naloxone hydrochloride, naltrexone hydrochloride, neostigmine bromide, pentobarbitone sodium, urethane and enkephalin analogue 33,824 (D-Ala^2-MePhe^4-Met (O)-ol-enkephalin). Drugs to be injected were dissolved in saline, and unless otherwise specified, were administered into the femoral vein in a volume of 0.2 ml. Weights of drugs refer to the salts.

Statistical tests

Student's t test (paired or unpaired) was used to evaluate the significance of differences between experiments. P values less than 0.05 were considered significant.

Results

Effects of antagonists on evoked release in normal animals

Prior to testing effects of Nal isomers and Ntx on the evoked release of ACh it was considered necessary to study their effects on release in the absence of stimulation. Actions of (−)-Nal in this regard have been studied extensively in previous work (see Introduction) but the effects of Ntx on spontaneous release have not been characterized. Figure 1 shows the effect of Ntx on the spontaneous output of ACh from the rat cortex. In the dose range 0.2–0.8 mg/kg this agent had no significant effect on the cortical release of ACh. When a low dose of Ntx (0.2 mg/kg) was administered after morphine-induced depression of cortical ACh release, it promptly reversed the inhibitory effects of the agonist (Figure 1b). At this dose, administration of (+)-Nal completely failed to reverse the inhibitory effect of morphine on ACh release (Figure 1c).

In subsequent tests the effect of Nal and Ntx on the release of ACh evoked by forepaw stimulation was tested using a dose (0.2 mg/kg) that had no effect on the spontaneous release but still reversed the action of morphine on ACh release. Figure 2 illustrates the type of experiment performed to test the antagonist

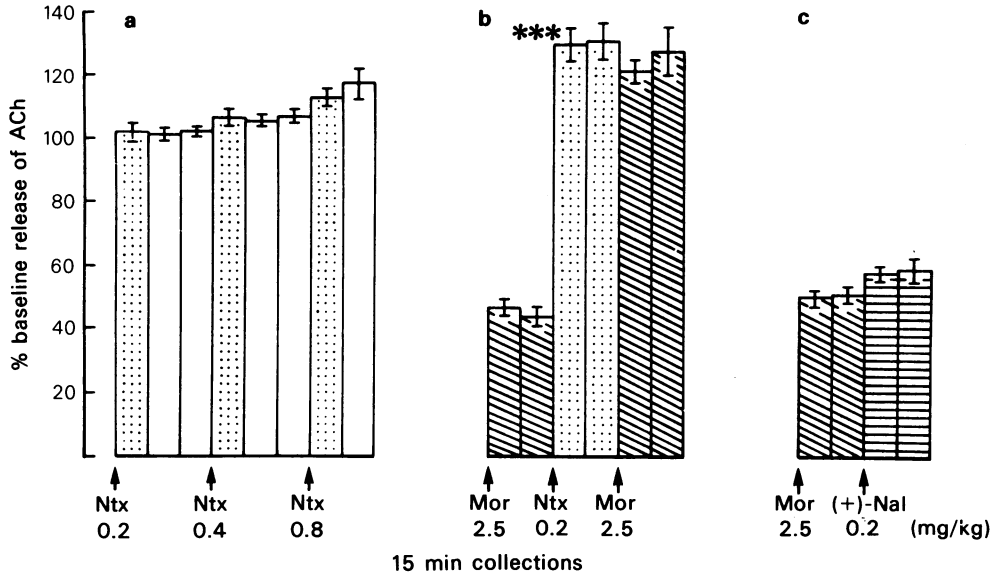

Figure 1 (a) and (b) The effect of naltrexone (Ntx) on spontaneous release of acetylcholine (ACh) from the rat cerebral cortex in the absence and presence of morphine. (c) Failure of (+)-naloxone (Nal) to reverse morphine-induced inhibition of spontaneous ACh release. The average baseline release in the first two collections, which is represented as 100%, was: (a) 2.2 ± 0.1, ($n = 4$); (b) 2.1 ± 0.1, ($n = 4$); (c) 2.4 ± 0.2, ng 15 min^{-1} 0.25 cm^{-2} ($n = 6$). Each column represents mean value; vertical lines show s.e.mean. $***P < 0.001$.

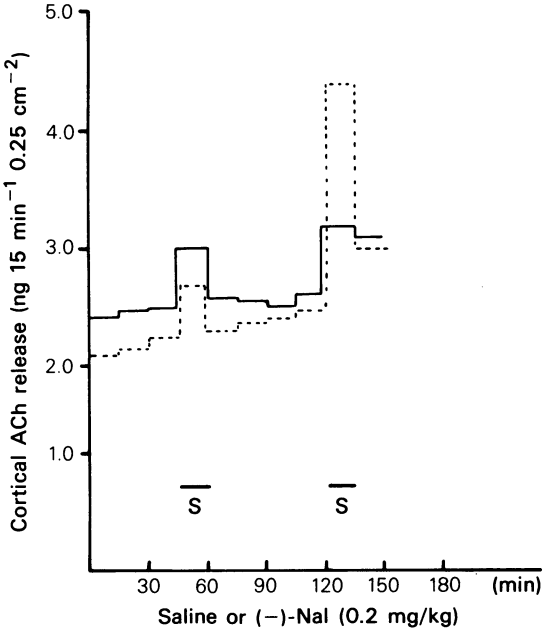

Figure 2 A representative experiment showing the effect of electrical stimulation (S) applied to the forepaw of the anaesthetized rat. Stimulation was applied bilaterally during the period indicated by horizontal bars. Saline (solid line) or (−)-naloxone (Nal) (broken line) was injected intravenously at a point indicated by the arrow.

action. In control tests two successive periods of stimulation, separated by a 60 min interval, consistently raised the output of ACh by 15–20% over the pre-stimulus resting level. In drug experiments the second period of stimulation was combined with an injection of the antagonist under study. The bar graph in Figure 3 shows cumulative results obtained in several experiments of the type that are represented in Figure 2. In control tests, a saline injection, given before the second stimulation, produced no significant change in the evoked release of ACh. Administration of (−)-Nal on the other hand increased the stimulus evoked release of ACh by about 100% over baseline release. In contrast (+)-Nal at the same dose failed to affect ACh release. Injection of Ntx, like Nal, caused a significant rise in the evoked release of ACh but its facilitatory effect was slightly less than the effect of (−)-Nal. Application of stimulation in combination with these antagonists, was associated with piloerection, hyperventilation, muscle twitching and mydriasis. These signs were most intense during the 5–7 min period which immediately followed stimulation. By contrast, saline or (+)-Nal administration did not elicit signs of comparable intensity.

To test whether (−)-Nal was facilitating the evoked release of ACh by acting at the spinal cord or the cerebral cortex, experiments were performed in which the antagonist was administered intrathecally or applied locally to the cortex. Results of such

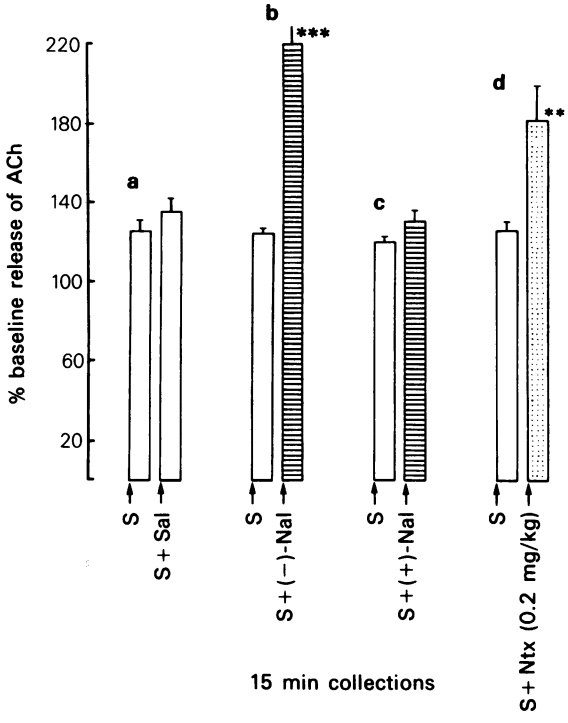

Figure 3 Effect of saline (Sal), (−)-naloxone (Nal), (+)-naloxone and naltrexone (Ntx) on the stimulus (S) evoked release of cortical acetylcholine (ACh). Open column represents release during the first period of stimulation in absence of the drug and filled columns represent release during the second period of stimulation in combination with the drug. The results shown here were obtained from experiments illustrated in Figure 2, and these have been normalized by expressing release values as a percentage of the pre-stimulus baseline value in each case. The average baseline values, represented as 100%, were: left to right, 1.9 ± 0.2 ($n = 8$); 2.4 ± 0.2 ($n = 6$); 2.2 ± 0.2 ($n = 8$), 2.2 ± 0.1 ng, 15 min^{-1} 0.25 cm^{-2}, ($n = 6$). Significance of drug effects is indicated by $^{***}P < 0.001$; $^{**}P < 0.01$. Vertical bars show s.e.mean.

experiments are shown in Figure 4. Spinally injected (−)-Nal (10 μg dose) produced no significant change in the evoked release of ACh. Cortical application of (−)-Nal significantly increased this release but the magnitude of the effect was less than that observed after intravenous injection of the antagonist.

Effect of antagonist on evoked release in hypophysectomized animals

Experiments performed on 3 week hypophysectomized animals were similar in design to those illustrated in Figure 2. The baseline release of ACh in this group of animals (2.1 ± 0.1 ng 15 min^{-1}; $n = 28$, pooled experiments) was not significantly different from the baseline release in normal animals (2.3 ± 0.2 ng 15 min^{-1}; $n = 20$, pooled experiments). Forepaw stimulation in hypophysectomized animals increased ACh release by 15–20% over the pre-stimulus baseline value. A statistical comparison of the values for evoked release between normal and the hypophysectomized rats did not reveal a significant difference. The effects of Nal and Ntx on the stimulus-induced release of ACh in hypophysectomized rats is shown in Figure 5a and b. Both antagonists produced a small but significant increase in this release but when compared with their effects in normal animals (see Figure 3) the facilitatory effect of these agents in hypophysectomized rats was marked-

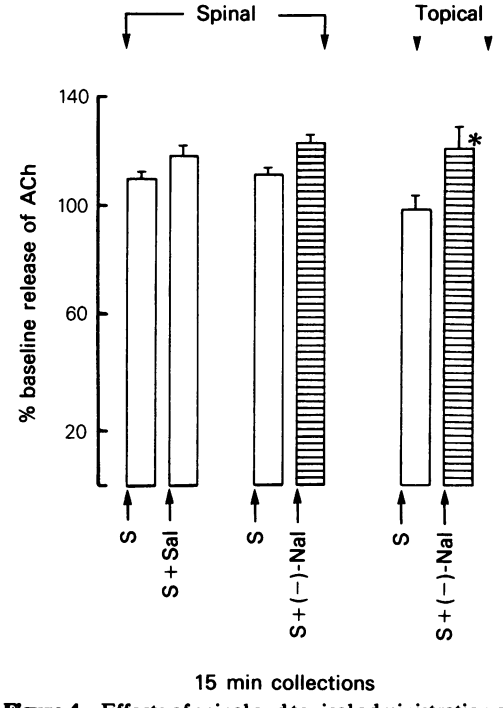

Figure 4 Effects of spinal and topical administration of (−)-naloxone (Nal) on the stimulus evoked release of cortical acetylcholine (ACh). Spinal injection was performed via a cetheter (PE.10 polythene tubing, 8 cm length) introduced into the intrathecal space through a cut in the cisterna magna. Normal saline (Sal, 5 μl) or (−)-Nal (10 μg in 5 μl) was injected and subsequently the catheter flushed with 5 μl of saline. Topical application of (−)-Nal was made by including drug (10 μg) in the solution contained in the cortical cup. Baseline release values, represented as 100%, are average release values in two collections preceding the first stimulation. These values were: left to right, 2.4 ± 0.2 ($n = 6$), 2.2 ± 0.1 ($n = 8$), 2.2 ± 0.2 ng 15 min^{-1} 0.25 cm^{-2} ($n = 6$). Significance of drug effect is indicated by $^{*}P < 0.05$; vertical lines show s.e.mean.

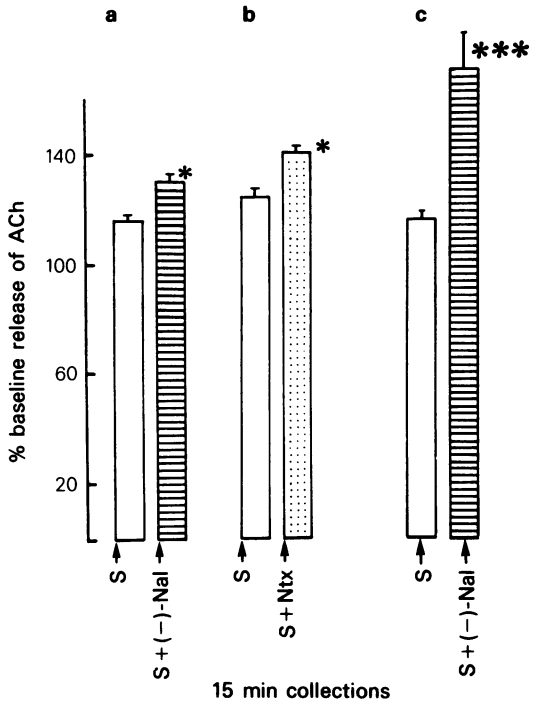

Figure 5 (a) and (b) Effect of narcotic antagonists (−)-naloxone (Nal) and naltrexone (Ntx) on the stimulus (S)-evoked release of acetylcholine (ACh) from the cortex in 3 week-hypophysectomized rats. (c) Effect of (−)-Nal on this release in 3 week sham-hypophysectomized rats. The results, obtained in experiments of type shown in Figure 2, have been normalized by expressing release as a percentage of pre-stimulus baseline value. The average baseline values, represented as 100%, were: (a) 1.9 ± 0.2, ($n. = 6$); (b) 1.9 ± 0.1 ($n = 8$); (c) 2.2 ± 0.2 ng $15\,min^{-1}$ $0.25\,cm^{-2}$ ($n = 6$). Significance of drug effect is indicated by $^{***}P < 0.001$, $^{*}P < 0.05$. Vertical lines show s.e.mean.

ly reduced. Because of this attenuation of the antagonist action, the effect of Nal was tested in a group of 3 week, sham-hypophysectomized rats. As shown in Figure 5c, administration of (−)-Nal in these animals produced an increase in evoked ACh release which was similar in size to that seen in normal rats.

To determine whether the reduced effect of narcotic antagonists in hypophysectomized rats observed here was due to impaired ability of (−)-Nal to antagonize opioid action, or to its impaired penetration into the brain, its effects were tested against two exogenous opioid agonists, morphine and an enkephalin, in ACh release experiments on these rats. The experiments performed in this part of the study were similar to those represented in Figure 1b. Results are shown in Figure 6. In both normal and hypophysectomized animals, morphine and a systemically active enkephalin, FK 33,824, depressed the release of ACh, and Nal promptly and fully reversed their inhibitory action. In the morphine experiments Nal produced a characteristic rebound release of ACh that has been observed in previous tests (Jhamandas & Sutak, 1980). Both the magnitude of the agonist-induced depression of ACh release and the degree of (−)-Nal-induced reversal in the normal and hypophysectomized rats were similar. Thus hypophysectomy did not affect the antagonistic action of (−)-Nal against exogenously administered opioid agonists.

Discussion

In this study the two narcotic antagonists, (−)-Nal and Ntx produced an increase in the *in vivo* release of cortical ACh induced by sensory stimulation without affecting the spontaneous release. Two observations

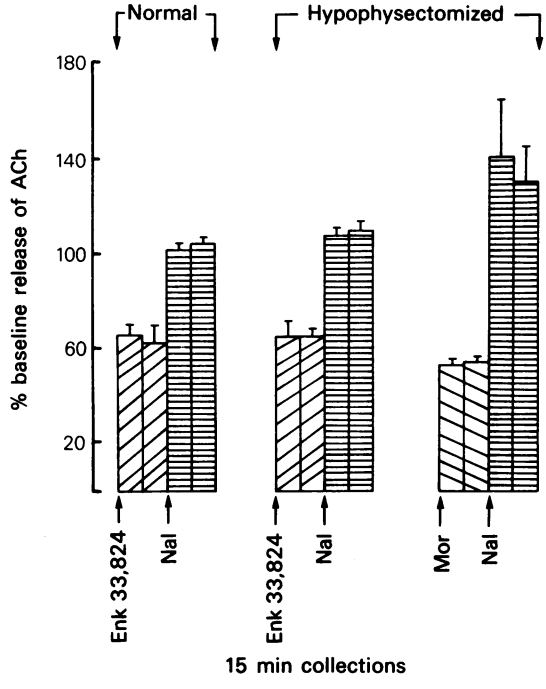

Figure 6 Reversal of the inhibitory effect of exogenously administered enkephalin (Enk) 33,824 and morphine (Mor) on the spontaneous cortical release of acetylcholine (ACh) by systemically administered (−)-naloxone (Nal) in normal and in 3 week-hypophysectomized rats. Morphine effect in normal rats, not shown here, is shown in Figure 1. The average baseline release of two collections preceding the first drug injection and represented as 100%, was: left to right, 1.90 ± 0.2 ($n = 8$), 2.2 ± 0.2 ($n = 6$), 2.2 ± 0.3 ng $15\,min^{-1}\,cm^{-2}$. Vertical bars show s.e.mean.

made here suggest that this action results from antagonism of an endogenous opioid released during stimulation. First, the excitatory effect of antagonists was seen at a low dose, which, in previous tests was shown to block the inhibitory action of exogenously administered methionine- and leucine-enkephalin on cortical ACh release (Jhamandas *et al.*, 1976). Second, administration of (+)-Nal at this dose failed to facilitate the evoked release of cortical ACh. This stereoselectivity of action shows that the excitatory action of Nal and Ntx observed here, and in a previous study (Jhamandas & Sutak, 1976), involves an interaction with opiate receptors. It is conceivable that an endogenous opioid released during stimulation inhibits activity in cholinergic neurones releasing ACh at the cerebral cortex and that narcotic antagonists remove this inhibition by blocking opioid receptors. This explanation is supported by a recent study showing that in the cat, stimulation of high threshold primary afferent fibres leads to the release of methionine-enkephalin-like immunoreactivity in perfusates of the spinal cord and the cerebral ventricles (Yaksh & Elde, 1981). Furthermore, electrical stimulation of forepaws in anaesthetized rats, as employed in the present study, previously has been found to provoke spinal release of a substance that shows morphine-like properties in the opiate receptor binding assay (Jhamandas, Yaksh, Bergstrom, Wang & Terenius, 1981).

The site at which narcotic antagonists act to exert their disinhibitory action on cortical ACh release is not known. A spinal site of action would appear plausible on the basis of neurochemical studies showing that sensory stimulation causes spinal release of enkephalins (see above), and electrophysiological studies showing that Nal increases discharge of spinal neurones excited by noxious stimulation (Henry, 1979). Surprisingly, spinal injection of (−)-Nal at a dose known to block the analgesic action of intrathecal morphine (Yaksh & Rudy, 1977), did not facilitate the evoked release of ACh in the present study. This observation suggests that opioid antagonists may be acting at a supraspinal site to exert their effect on this release. Cortically applied (−)-Nal did augment the release of ACh, but since its stimulatory effect was considerably weaker than that of systemic Nal, it is unlikely that the cerebral cortex is a major site of its facilitatory action. In the past, experiments on isolated slices of rat cortex have failed to reveal an opiate specific inhibition of ACh release by morphine or enkephalins (Szerb, 1974; Jhamandas *et al.*, 1975; Jhamandas & Elliott, 1980). In view of the above facts it would seem that Nal and Ntx act mainly at a subcortical site in the brain to block an enkephalinergic inhibition of cholinergic neurones. Although the location of such a site is unknown at the moment some speculation may be made in this regard. Recent studies have shown that a part of the cholinergic projection to the frontal cortex originates from large neurones located at the base of the globus pallidus (Johnston, McKinney & Coyle, 1979; Lehmann, Nagy, Atmadja & Fibiger, 1980). The globus pallidus is known to be rich in enkephalin (Rossier, Vargo, Minick, Ling, Bloom & Guillemin, 1977; Kobayashi, Palkovits, Miller, Chang & Cautrecasas, 1978) and this region is believed to receive an enkephalinergic projection from the caudate nucleus (Cuello & Paxinos, 1978). It is possible that an interaction between enkephalins and cholinergic neurones occurs at the level of the globus pallidus and that narcotic antagonists block this interaction by acting at this region. This possibility remains to be investigated in future experiments.

In hypophysectomized animals the excitatory action of (−)-Nal and Ntx on ACh release evoked by sensory stimulation was significantly reduced. The ability of (−)-Nal to reverse the action of the exogenously administered opioid agonists was not affected in these animals. Therefore factors such as altered opioid receptor reactivity or altered Nal penetrability in the brain apparently do not account for the observed attenuation of antagonist action following pituitary removal. The decreased effect of Nal might be due to a weak enkephalin-mediated inhibition of cholinergic neurones resulting from a diminished release of endogenous opioids. While the status of the enkephalin or endorphin release in the brain of hypophysectomized animals is unknown, the steady state brain levels of these peptides have been shown in some studies to remain unchanged in such animals (Rossier *et al.*, 1977; Kobayashi *et al.*, 1978; Gibson, Ginsburg, Hart & Kitchen, 1980). However, in a recent study it was shown that removal of the anterior lobe of rat pituitary lowers β-endorphin levels in the hypothalamus and in the periventricular tissue (Przewlocki, Millan, Gramsch, Millan & Herz, 1982). Interestingly in the latter study, levels of methionine-enkephalin in the striatum (which includes globus pallidus) were also reduced following long term hypophysectomy. In view of the latter study, the lower levels of certain opioid peptides in brain of the hypophysectomized animals could account for the weak stimulatory action of (−)-Nal on ACh release. A difficulty with this interpretation is that in hypophysectomized rats, the application of electrical stimulation alone, while it provoked cortical ACh release, did not exert a stronger effect in such animals when compared with normal animals. A stronger effect of the stimulus would be anticipated if an enkephalin-mediated inhibition was either weak or was totally absent in animals lacking the pituitary. It should be pointed out that in the present study, an extensive comparison of the effects of sensory stimulation in two groups of animals was not undertaken.

A detailed comparison may reveal differences that are not readily apparent under conditions of present experiments.

The findings of Nal action in hypophysectomized rats in this study are similar to those of Grevert *et al.* (1978), who observed that in hypophysectomized mice the hyperalgesic action of Nal was abolished but the baseline latency of reaction to the pain stimulus was unchanged. They suggested that the abolition of the action of Nal in these mice was possibly due to lack of a factor (of unknown identify) that facilitates the action of this agent. Hypophysectomy in the rat has been reported to reduce brain content of several non-opioid peptides such as somatostatin, thyrotrophin releasing hormone (TRH) and α-melanotropin (Bassiri & Utiger, 1974; O'Donohue, Holmquist & Jacobowitz, 1979; Terry & Crowley, 1980). Somatostatin is of some interest because in the opiate receptor binding assay it has been reported to have a partial antagonist-like profile (Terenius, 1976). In certain brain areas somatostatin has been shown to increase the turnover of ACh, an effect which is opposite to that of the enkephalins (Malthe, Sørenssen, Wood, Cheney & Costa, 1978). Interestingly, somatostatin also occurs in the globus pallidus and is released from this region following depolarization (Lee & Iversen, 1981). It would be of interest therefore to test whether somatostatin, or other neuropeptides whose content is influenced by hypophysectomy, can restore the excitatory action of Nal in animals lacking the pituitary.

In conclusion, the present study showing a stereoselective effect of antagonists on evoked cortical ACh release provides firmer evidence for the notion that endogenous opiates interact with brain cholinergic neurones under conditions of sensory stimulation. The failure of antagonists to influence spontaneous release of ACh argues against a tonic regulation of cholinergic neurones by endogenous opioids. The importance of endogenous opioids in numerous brain functions that are thought to be mediated by cholinergic mechanisms remains to be evaluated.

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