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MORPHINE AND NEUROTRANSMITTER SUBSTANCES: MICRO-IONTOPHORETIC STUDY IN THE RAT BRAIN STEM

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1 The effects of microiontophoretically applied morphine and its interactions with the effects of microiontophoretic applications of either acetylcholine, (−)-noradrenaline or 5-hydroxytryptamine have been studied on single neurones in the brain stem of rats anaesthetized with urethane.

2 Morphine excited or inhibited most neurones tested and the effects, especially excitation, were often extremely powerful. However, the time course of the excitatory and inhibitory effects were somewhat different.

3 Desensitization to the excitation produced by morphine was seen after repeated or prolonged applications and it is suggested that this phenomenon may be related to the tolerance which develops after chronic administration of morphine. No desensitization was observed to inhibition of neuronal activity by morphine.

4 Morphine usually reduced the excitation of neurones by acetylcholine, noradrenaline or 5-hydroxytryptamine but sometimes potentiated the effect, although not always on the same neurones. Inhibition of neuronal activity by these compounds was never modified by morphine and neither were the effects of glutamate or D,L-homocysteic acid when used as control agonists.

5 The *in vitro* release of morphine from six micropipettes was determined and the transport number was calculated to be 0.051 (s.d. 0.021).

6 The implications of these observations in explaining the pharmacological actions of morphine are discussed.

Introduction

Morphine has a wide spectrum of pharmacological actions and numerous attempts have been made to link its central effects with neurotransmitters in the brain. Of the putative neurotransmitters, acetylcholine, noradrenaline, 5-hydroxytryptamine and dopamine have been most intensively studied with respect to their possible relationship with both the acute and chronic effects of morphine. For example, morphine has been found to alter the distribution and release of these substances in the brain, and manipulation of the levels of transmitters can modify the pharmacological response to morphine. The evidence for the involvement of one or other of these compounds has recently been reviewed (Clouet, 1971; Way & Shen, 1971; Weinstock, 1971; Way, 1972).

Although it has been established that morphine produces a number of its effects by an action on the brain stem, e.g. depression of the respiratory and vomiting centres and stimulation of the chemosensitive trigger zone, evidence from electrophysiological studies for the involvement of brain stem structures in the antinociceptive actions of morphine is controversial (Valdman, 1967; Killam, 1968; Domino, 1968).

There is substantial evidence that acetylcholine, noradrenaline and 5-hydroxytryptamine may each be involved in synaptic transmission in the brain stem (Bradley, 1968; Phillis, 1970) and these substances have also been implicated in the mechanism of action of morphine. We have therefore used the technique of microiontophoresis to investigate the effects of morphine on single neurones in the brain stem and to study its interactions with acetylcholine, noradrenaline and 5-hydroxytryptamine, applied iontophoretically to the same neurones. Some of these results have been communicated to the British Pharmacological Society (Bradley & Dray, 1973a).

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Methods

Anaesthetized albino rats (300-500 g) were used in this series of experiments. Anaesthesia was induced with halothane and maintained by intraperitoneal injections of 1.2-1.8 g/kg of urethane (BDH). This anaesthetic agent has been shown to have little effect on the sensitivity of brain stem neurones of the rat to iontophoretically applied transmitter substances (Bradley & Dray, 1973b).

Animals were partially cerebellectomized to expose the floor of the fourth ventricle. Five-barrelled glass micropipettes of external tip diameter 6-10 μm were used to record the extracellular activity of single, spontaneously active neurones in the brain stem, and to apply drug ions in the immediate vicinity of the cell.

Electrode penetrations were made between 1 and 4 mm rostral to the obex, and between 1.5 mm on either side of the midline, to a depth of 2.5 mm. Penetrations in the midline itself were avoided. Extracellular neuronal spikes were amplified and electronically counted (Bradley & Wolstencroft, 1964) as the mean firing frequency in epochs of 5 s duration.

The recording barrel of the micropipette contained 4 M NaCl solution and one barrel contained 1 M NaCl solution for testing the effects of the iontophoretic current. The other barrels contained solutions of drugs adjusted to the appropriate pH as described below. Drugs were applied for one or more 5 s epochs with a current (10-50 nA) of the appropriate polarity. A retaining current of 15 nA was generally used since this current has been found to prevent the leakage of labelled compounds (Bradley & Candy, 1970) from similar micropipettes. However, higher currents of up to 40 nA were necessary to prevent leakage of amino acids.

The following drugs were used in the micropipettes at the indicated concentration (w/v) and pH: acetylcholine chloride, 5%, pH 4.0-5.0 (Sigma); (−)-noradrenaline hydrochloride, 5%, pH 5.0-6.0 (Sigma); 5-hydroxytryptamine (serotonin) bimaleinate, 5%, pH 4.5-5.5 (Koch-Light); D,L-homocysteic acid, 10% pH 8.0-9.0 (Koch-Light); monosodium glutamate, 10%, pH 8.0-9.0 (L. Light & Co.); morphine hydrochloride, 0.5-1.0%, pH 4.0-5.0 (Macfarlan Smith Ltd.).

The effects of microiontophoretic applications of morphine were studied on the spontaneous activity of single neurones in the brain stem. The effects of iontophoretically applied morphine on the responses of single neurones to iontophoretic applications of acetylcholine, (−)-noradrenaline or 5-hydroxytryptamine were also investigated. In addition, we used the method of Bradley & Candy (1970) to examine the *in vitro* release of radioactive morphine from six microelectrodes containing a 1% aqueous solution of morphine hydrochloride (pH 5.0) to which *N*-methyl-[^{14}C]-morphine hydrochloride (specific activity 57 mCi/mmol; Radiochemical Centre, Amersham) had been added. The retaining and ejecting currents used and the times for morphine release were similar to those used in the *in vivo* studies.

Results

In preliminary experiments, applications of morphine from micropipettes which contained a 10% solution produced non-selective effects, e.g. marked depression of spike height and distortion of the shape of the neuronal action potential, so that continuous recording of neuronal activity often became difficult. However, neurones that behaved in this manner were not studied further. These effects were very rarely encountered with more dilute solutions of morphine (0.5-1.0%) and in subsequent experiments a 1% solution was used in the micropipettes.

Out of 76 neurones studied, morphine increased the firing rate of 33 and depressed that of 17 when applied for periods of 0.5-10.5 minutes. The remaining 26 neurones were unaffected. Occasionally, prolonged application of morphine caused the cell under study to fire erratically or to fire with a burst pattern. This effect was observed more commonly when morphine was applied from electrodes containing high concentrations. Excitation by morphine was often very powerful and began 10-70 s after the ejecting current had been switched on. The excitation usually lasted throughout the period of application and began to decay soon after switching off the current, so that the firing rate returned to the original level 25-85 s after the current had been switched off (Figures 1a & 2).

Inhibition by morphine occurred 10-90 s after the current had been switched on. Usually the neuronal firing rate was only gradually reduced during the application and continued to fall after switching off the current (Figure 1b). Return to the original firing rate took from 10 s to over 30 min and some neurones never showed complete recovery.

When more than one application of morphine was made to the same neurone, the excitation produced was often diminished in magnitude or a longer period of morphine application was needed to produce the same magnitude of response as did a previous application (Figure 2a). This effect was studied in 13 neurones, to which morphine was applied three or more times. Most neurones showed this desensitization or tachyphylaxis to

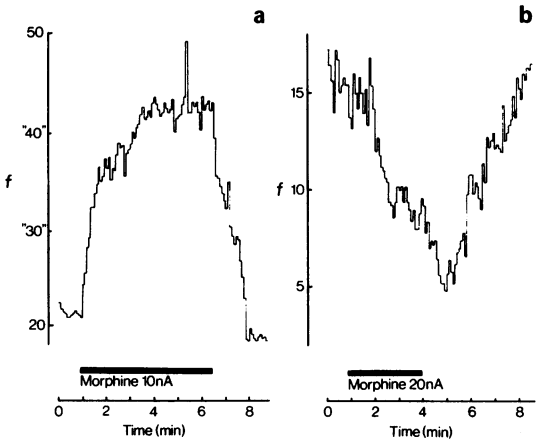

Fig. 1 The effects of microiontophoretic applications of morphine on the activity of spontaneously firing brain stem neurones. The mean firing frequency (f, impulses s^{-1}) in successive 5 s epochs is plotted against the time (min). Iontophoretic applications are indicated by the horizontal bars and the ejecting currents also shown. (a) Excitation of a neurone by prolonged application of morphine (10 nA); (b) inhibition of a neurone by morphine (20 nA).

morphine excitation (10 out of 13), but with three neurones, excitation by morphine was unaltered after repeated applications (Figure 2b). In six neurones where morphine produced inhibition, tachyphylaxis was not observed.

The effects of iontophoretically applied morphine were examined on neurones which responded submaximally yet consistently to iontophoretically applied acetylcholine, (−)-noradrenaline or 5-hydroxytryptamine. One of these neurotransmitters sometimes acted as a control agonist when its effects were not modified by morphine. Normally, however glutamate or homocysteic acid were used as control agonists. Morphine was applied for various periods ranging from 0.5 min to a total of 14 min and the effects of the transmitter substances retested.

Microiontophoretically applied acetylcholine excited most neurones studied and inhibition by acetylcholine was rarely seen. These effects on brain stem neurones were qualitatively similar to those previously described (Bradley & Dray, 1972). The excitation produced by acetylcholine was reduced in 18 neurones (Fig. 3a), unaffected in 24 and potentiated in four neurones (Fig. 4a) following microiontophoretic application of morphine. There appeared to be little correlation between the effects produced by morphine and those of acetylcholine when both compounds were applied to the same neurone (Table 1). Thus, 22 neurones were excited by both compounds, five were excited by acetylcholine, but inhibited by morphine, and 18 were excited by acetylcholine, but unaffected by morphine.

Four types of response to microiontophoretically applied (−)-noradrenaline have been reported for cat (Boakes, Bradley, Brookes, Candy & Wolstencroft, 1971) and rat (Bradley & Dray, 1973b) brain stem neurones, and similar responses were observed in the present series of experiments. These were: short- or long-lasting inhibition, a simple excitatory response and a biphasic response which consisted of excitation preceded by a short inhibitory phase.

Excitation by (−)-noradrenaline was antagonized in 14 neurones (Fig. 3b), unaffected in eight, and potentiated in five (Fig. 4b) by microiontophoretically applied morphine. Inhibition by noradrenaline was never affected by morphine (nine neurones). Little correlation was observed between the effects of noradrenaline and those of morphine when applied to the same neurone (Table 1). Both compounds excited 10 neurones whereas in another seven, noradrenaline was excitatory and morphine inhibitory. Ten neurones were excited by noradrenaline but unaffected by morphine, and of nine neurones inhibited by noradrenaline, four were inhibited, two excited and three unaffected by morphine.

Table 1 Comparison of the effects of morphine with those of acetylcholine, noradrenaline or 5-hydroxytryptamine, when applied microiontophoretically to the same neurone.

	Acetylcholine			*Noradrenaline*			*5-Hydroxytryptamine*		
	+	○	−	+	○	−	+	○	−
Morphine +	22	3	1	10	1	2	20	0	1
,, ○	18	2	0	10	3	3	15	5	0
,, −	5	0	1	7	1	4	12	0	0

The figures refer to the number of cells tested; + = excitation; ○ = no effect; − = inhibition. There was no correlation (χ^2 test) between the effects produced by morphine and those of the neurotransmitter substances.

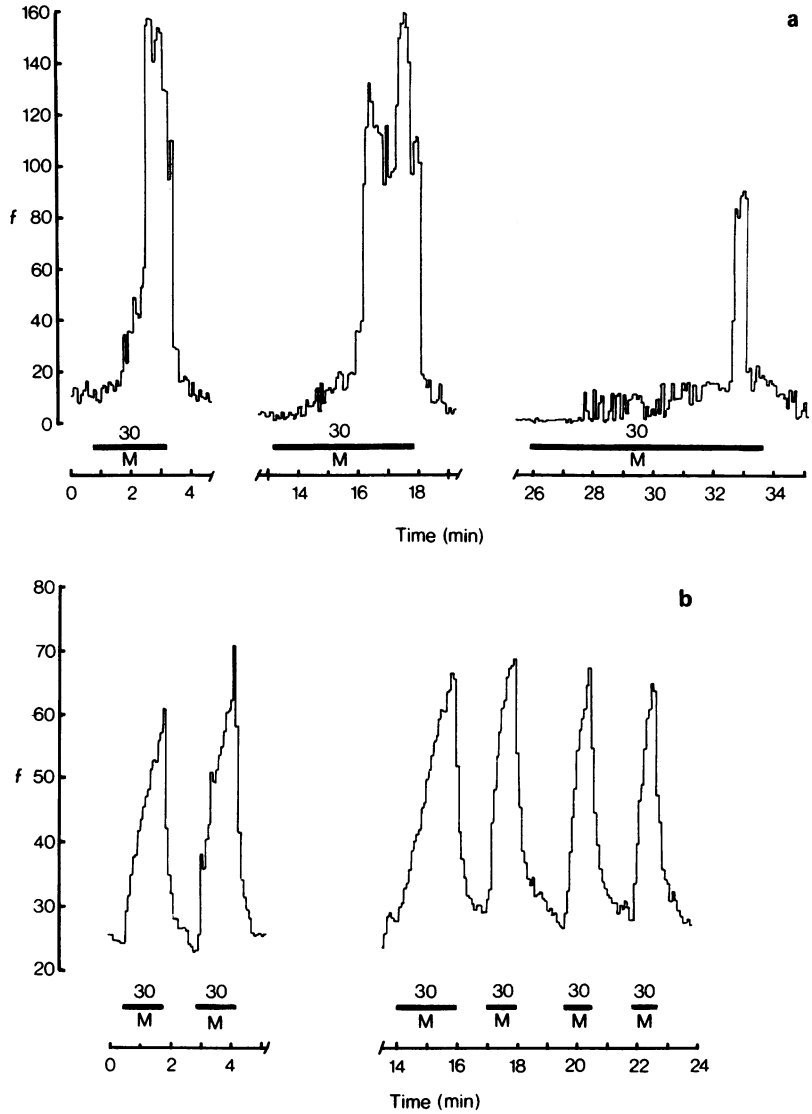

Fig. 2 Excitatory responses to repeated microiontophoretic application of morphine (M) to the same neurone. (a) Reduction of the excitatory response with consecutive applications of morphine (M); (b) a neurone showing no desensitization to repeated morphine applications.

The responses of brain stem neurones to 5-hydroxytryptamine were qualitatively similar to those seen in the cat (Boakes, Bradley, Briggs & Dray, 1970) and in the rat (Dray, 1971; Bradley & Dray, 1973b) and consisted of a short- or long-lasting inhibitory response, a simple excitation and an excitatory response preceded by a brief period of inhibition.

Out of 48 neurones studied, morphine reduced the excitatory response to 5-hydroxytryptamine in 17 (Fig. 3c) and potentiated it in four (Figure 4c). Inhibition of one neurone by 5-hydroxytryptamine was unaffected by morphine. There was no correlation between the effects produced by morphine and 5-hydroxytryptamine when applied to the same neurone (Table 1). Thus, 20 neurones were excited by both compounds, 12 were excited by 5-hydroxytryptamine but inhibited by morphine, and 15 were excited by 5-hydroxytryptamine but unaffected by morphine.

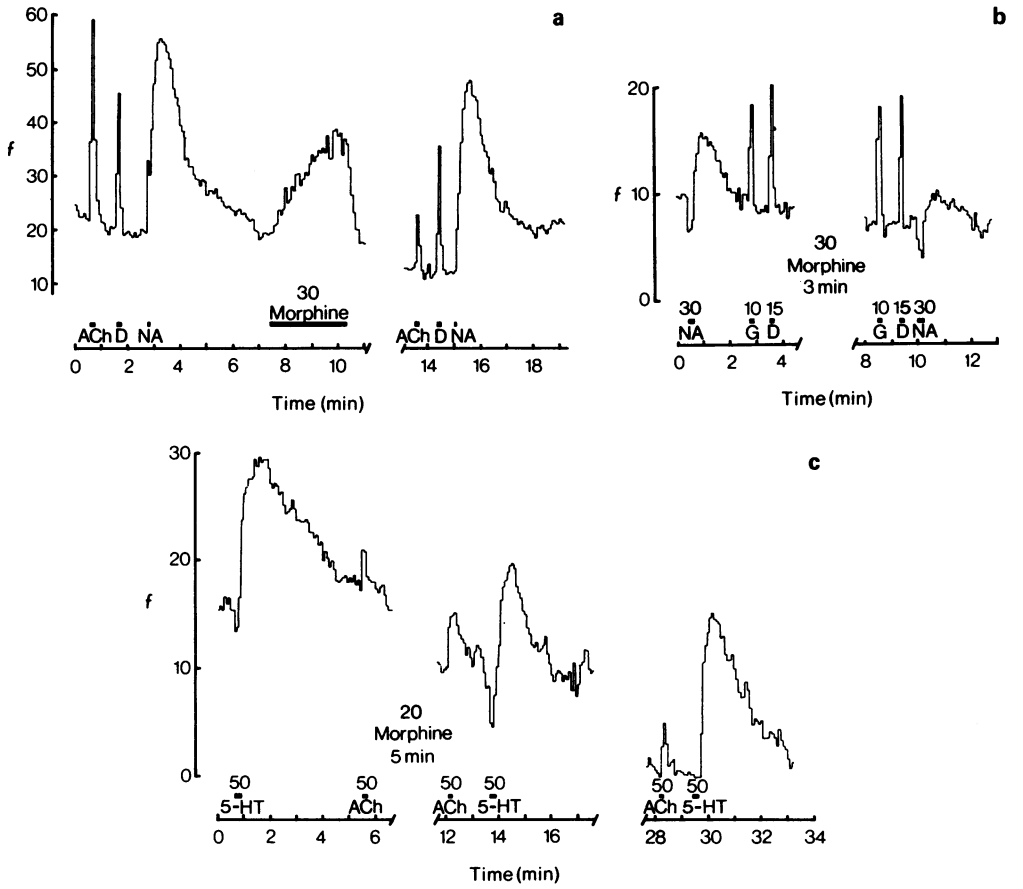

Fig. 3 Antagonism by morphine of excitatory responses to microiontophoretically applied acetylcholine, noradrenaline or 5-hydroxytryptamine. (a) An application of morphine (30 nA) reduced the excitatory response to acetylcholine (ACh), but not that to either D,L-homocysteic acid (D) or noradrenaline (NA). The ejecting current was 50 nA, unless otherwise indicated; (b) excitation by noradrenaline (NA) but not that by glutamate (G) or D,L-homocysteic acid (D) was reduced by applying morphine to this cell for 3 min at 30 nA; (c) a 5 min application of morphine (20 nA) reduced excitation by 5-hydroxytryptamine (5-HT) but not excitation by acetylcholine (ACh). Partial recovery of the 5-HT response occurred 12 min later.

Release of morphine from micropipettes

The release of morphine from each of the six microelectrodes tested, using the same parameters as those used in the *in vivo* experiments, was linearly related to the charge passed through the micropipette barrel and each regression line calculated from the data for the individual electrodes, gave a highly significant regression coefficient. The slope of the calculated regression lines, expressed as mol per coulomb multiplied by Faraday's Number, gave the transport number of morphine for each electrode. The mean transport number for the six electrodes was 0.051 (s.d. 0.021).

Discussion

When applied iontophoretically to brain stem neurones in the rat, morphine produced a number of complex effects. Thus, it increased or reduced the spontaneous firing of a number of neurones although the time course of these effects was different. Excitation of neurones by iontophoretically applied morphine was frequently observed and could be demonstrated without any apparent changes in the shape of the action potential or in the regularity of the firing pattern. The effect was often powerful, although relatively slow in onset, and began to diminish soon after the end of the application. On the other hand, inhibition by

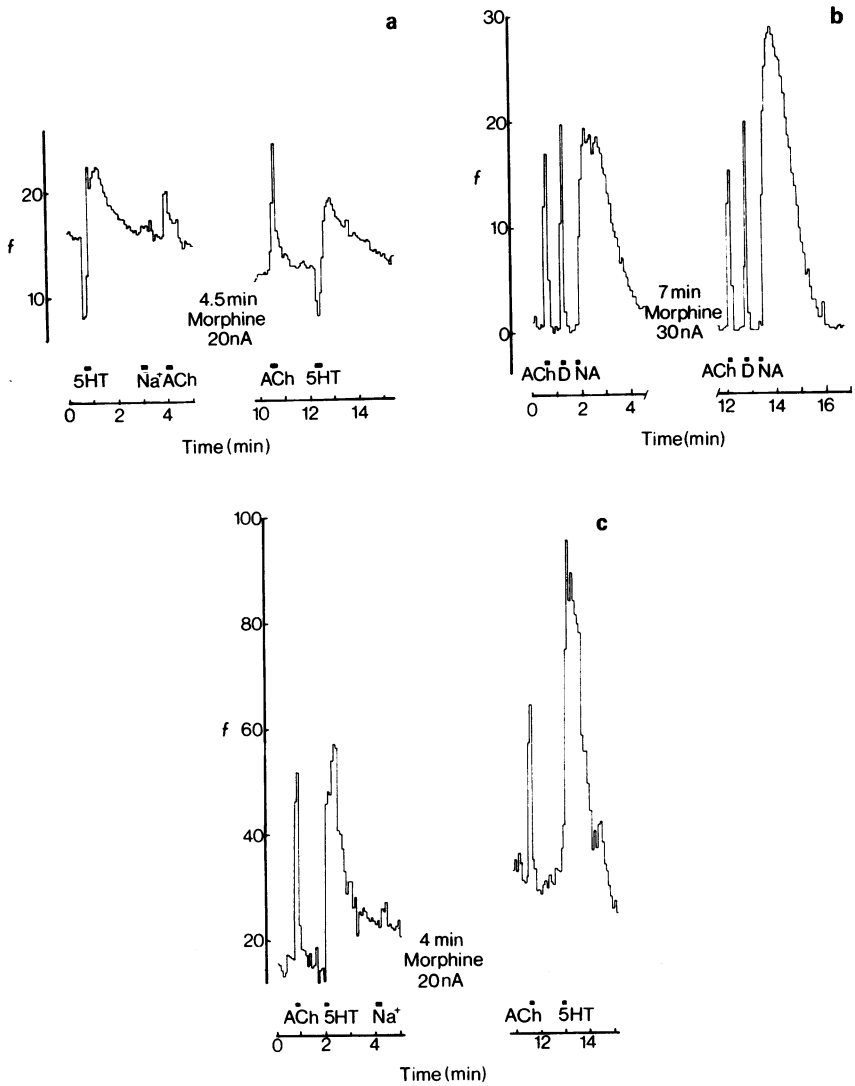

Fig. 4 Potentiation, by microiontophoretically applied morphine, of excitatory responses to acetylcholine, noradrenaline or 5-hydroxytryptamine. Unless otherwise indicated, the ejecting current was 50 nA. (a) The excitatory response to acetylcholine (ACh) was potentiated after a 4.5 min application of morphine (20 nA). Excitation by 5-hydroxytryptamine (5-HT) was unaffected; (b) potentiation of the excitatory response to noradrenaline (NA) but not that to acetylcholine (ACh) or D,L-homocysteic acid (D) after an application of morphine (30 nA) for 7 minutes; (c) morphine, applied for 4 min, potentiated excitation by 5-hydroxytryptamine (5-HT) but not that by acetylcholine (ACh).

morphine was found less frequently and its onset was more gradual than the excitatory effect; also, recovery of the inhibitory effect was often prolonged. The low value (0.051) found for the transport number of morphine suggests that relatively small amounts released from the micropipettes are capable of producing these powerful effects. This is in keeping with the finding that intraventricular injections of morphine were 500-1000 times more effective than intravenous injections when its antinociceptive activity to stimulation of the rabbit tooth-pulp was measured (Herz, Albus, Metyš, Schubert & Teschemacher, 1970). In fact, only a small proportion of an injected dose of morphine reaches the brain (Mulé, 1971; Bullock, unpub-

lished observations) and this implies that the *in vivo* concentration in the vicinity of the neurones must be very small. Since microiontophoretically applied morphine did not affect the activity of all the neurones tested, it would be of interest to determine whether morphine-sensitive neurones were localized to any particular part of the brain stem.

Both excitation and inhibition of the activity of single neurones has been reported for the reticular formation and for other areas of the brain after either microiontophoretic (Krnjević, 1965; Duggan & Curtis, 1972) or intravenous administration of morphine (Eidelberg & Bond, 1972). Although morphine generally depresses structures in the brain stem, it has been shown to increase the reactivity of the reticular formation following stimulation of the hippocampus (Gangloff & Monnier, 1957) and, in low doses, to exert a facilitatory action on the lower brain stem (Satoh & Takagi, 1971).

Two possibilities, either that morphine produces its effects by a direct action on central neurones or that the effects are a consequence of interactions with endogenous transmitter substances (see Table 2), must be considered. It is conceivable that microiontophoretically applied morphine might act on specific neuronal receptors and it would therefore be of interest to know whether the excitation or inhibition of neurones could be mimicked by other opiates and blocked by morphine antagonists. Acute administration of morphine has been shown to release noradrenaline from the brain (Gunne, 1959), increase noradrenaline turnover in the brain stem (Sugrue, 1973) and inhibit its uptake (Ciofalo & Lucero, 1972).

Morphine has been shown to increase the turnover of 5-hydroxytryptamine in the brain (Yarbrough, Buxbaum & Sanders-Bush, 1971) and to block the effects of 5-hydroxytryptamine on ganglia (Gyermek & Bindler, 1962). Finally, acute administration of morphine has been found to increase brain levels of acetylcholine (Crossland & Slater, 1968) and to depress the release of acetylcholine from the guinea-pig ileum (Paton, 1957) and mammalian brain (Beleslin & Polak, 1965).

In addition, acetylcholine, noradrenaline and 5-hydroxytryptamine have all been found to produce excitation or inhibition of brain stem neurones when applied by microiontophoresis. From the results presented here, it seems that morphine-induced excitation or inhibition is unlikely to be due to a release of endogenous acetylcholine, noradrenaline or 5-hydroxytryptamine, since little correlation was observed between the effects of these three substances applied microiontophoretically and the effects of morphine, applied to the same neurone. However, depletion of the central stores of these neurotransmitters and the retesting of the effects of morphine will be necessary before the release hypothesis can be definitely discarded.

In the present experiments, repeated application of morphine usually led to a reduction in the magnitude and duration of the excitatory response. However, some neurones did not show this desensitization to morphine excitation and none showed it to morphine-induced inhibition. Although the time scale for this effect is shorter than that normally considered for the development of morphine tolerance, we would like to suggest, in view of the fact that tolerance to morphine has been found to develop rapidly after a single injection in man (Martin & Fraser, 1961) and animals (Collier, Francis & Schneider, 1972), that desensitization to microiontophoretic morphine may be regarded as a form of 'acute tolerance' and may reflect, at the level of the single neurone, the phenomenon which occurs

Table 2 Interactions of iontophoretically applied morphine with effects of excitatory and inhibitory agents.

		Blocked	*Not blocked*	*Potentiated*
Acetylcholine	+	18	24	4
	−	0	2	0
Noradrenaline	+	14	8	5
	−	0	9	0
5-hydroxytryptamine	+	17	26	4
	−	0	1	0
Glutamate	+	1	17	0
D,L-homocysteic acid	+	0	18	0

The figures refer to the number of neurones tested: + = excitation; − = inhibition.

during the development of central tolerance to repeated systemic administration of morphine.

Thus, although morphine has complex effects when applied microiontophoretically to brain stem neurones, particularly in relation to effects of neurotransmitters, the fact that it has a powerful excitatory action on many neurones, and that this effect shows 'tolerance' with repeated applications, is probably worthy of further investigation, particularly with specific antagonists.

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