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Comparison of the antinociceptive activities of physostigmine, oxotremorine and morphine in the mouse

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Summary

1. Morphine, oxotremorine and physostigmine showed antinociceptive activity in mice using the hot plate reaction time test.

2. The action of morphine, but not that of oxotremorine, was antagonized by naloxone and by nalorphine, whereas the effect of physostigmine was unaffected by naloxone and increased by nalorphine.

3. The antinociceptive effects of morphine and of physostigmine were increased by procedures reported to increase the ratio of 5-hydroxytryptamine to dopamine in the brain. It was decreased by procedures reported to cause a fall in brain 5-hydroxytryptamine or a rise in dopamine relative to 5-hydroxytryptamine.

4. The antinociceptive effect of oxotremorine was potentiated by procedures reported to decrease brain noradrenaline and was unaffected by procedures altering brain 5-hydroxytryptamine.

5. The results suggest differences in the mode of action of morphine and physostigmine on the one hand and of oxotremorine on the other.

Introduction

Antinociceptive activity has been reported for both cholinergic (Gross, Holland, Carter & Christensen, 1948; Chen, 1958) and sympathomimetic drugs (Kiessig & Orzechowski, 1940). These observations prompted an investigation of possible cholinergic and adrenergic involvement in the action of narcotic analgesic drugs rather than detailed studies of the mechanism by which these agents produce analgesia themselves. Major & Pleuvry (1971) were unable to find any differences in the mechanisms by which methylamphetamine and morphine had an antinociceptive effect in the mouse, using various pretreatments reported to induce changes in brain concentrations of 5-hydroxytryptamine, dopamine and noradrenaline.

The following experiments were designed to determine whether similar procedures could detect any differences between the mechanisms by which antinociceptive effects were produced by physostigmine, oxotremorine and morphine in the mouse.

Methods

Male and female white mice (25–35 g) of the Alderley Park strain were used. Antinociceptive effects were determined with the hot plate reaction time test as described by Bousfield & Rees (1969). The reaction times of paired groups of test and control mice were measured every 5 min for 30 min after injection and then at

10 min intervals until the reaction times of the test mice were not significantly different from that shown by control mice pretreated with the vehicle.

Results are expressed as mean maximum actual reaction times ± S.E. of the mean. A minimum of six mice and a maximum of twelve were used for each experiment. Significance was determined by the Student's *t* test except in cases where reaction times exceeded the arbitrary cut-off time of 45 seconds. For these results mean maximum values are not given and significance was determined using a non-parametric rank test, the Wilcoxon test for two samples (Diem, 1962).

Drugs

The following drugs were used: *p*-chlorophenylalanine (Koch-Light Laboratories); diethyldithiocarbamate sodium (Kodak); L-dopa (Koch-Light Laboratories); 5-hydroxytryptophan (Koch-Light Laboratories); methylamphetamine hydrochloride (Burroughs Wellcome & Co.); α-methyl-*p*-tyrosine (Sigma Chemicals Co.); morphine sulphate (Evans Medical Ltd.); nalorphine hydrobromide (Burroughs Wellcome & Co.); naloxone hydrochloride (Endo Laboratories); neostigmine methyl sulphate (Roche Products); oxotremorine (Dr. B. Cox, Department of Pharmacology, Manchester); physostigmine salicylate (T. & H. Smith) and reserpine (British Drug Houses).

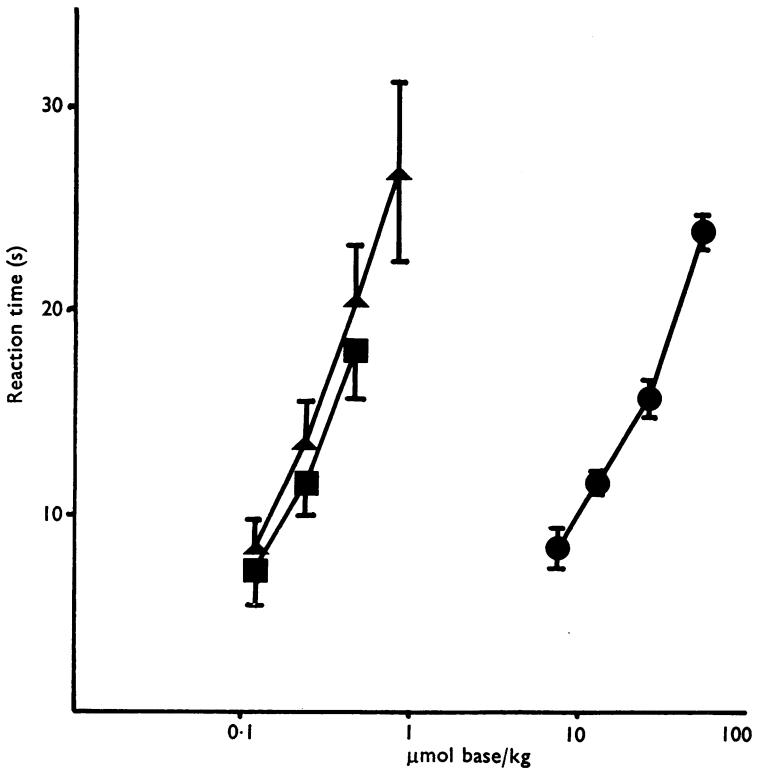

FIG. 1. Effect of morphine, intraperitoneally (●), physostigmine, subcutaneously (■) or oxotremorine, subcutaneously (▲) on the hot plate reaction time of the mouse. Ordinate, doses of drugs (µmol base/kg); abscissa, reaction time (s). Values are the means of the measured reaction times (± S.E.M.) of groups of six to twelve mice.

Reserpine was dissolved in a minimal quantity of glacial acetic acid and then diluted to 1 mg/ml with distilled water (final pH of 5·3). *p*-Chlorophenylalanine was suspended in 1% sodium carboxymethylcellulose (30 mg/ml), α-methyl-*p*-tyrosine was dispersed in 0·5 M phosphate buffer of pH 7·4 (30 mg/ml) and sufficient M sodium hydroxide added just to effect solution and L-dopa was dissolved in 0·1 N hydrochloric acid (40 mg/ml). All other drugs were dissolved in 0·9% NaCl solution (saline).

Oxotremorine, physostigmine and neostigmine were injected subcutaneously, but all other drugs were injected intraperitoneally. The dose volume injected was 0·1 ml/20 g body weight. All doses are expressed as the weights of the salts unless stated otherwise.

Results

Antinociceptive dose-response relationships

The mice injected with oxotremorine, physostigmine and morphine all had reaction times which were significantly longer than those of concurrently tested control mice injected with saline. Dose-response relationships taken at the time of maximum effect of each of the three drugs are shown in Fig. 1. These doses of oxotremorine and physostigmine did not induce marked tremor; equipotent doses of physostigmine, oxotremorine and morphine were selected for subsequent experiments.

No increase in reaction time as compared with saline treated control mice could be obtained in mice treated with up to 0·4 mg/kg of neostigmine, a dose which induced marked symptoms of peripheral cholinergic activity, that is, salivation and diarrhoea.

Effect of combining two known antinociceptive agents

Simultaneous injections of physostigmine and morphine caused an increase in reaction time consistent with a summation of the individual reaction times obtained

TABLE 1. *Effects of simultaneous injection of two antinociceptive agents on the hot plate reaction time of the mouse*

Antinociceptive agent	Reaction time (±S.E.M.) Injection of one agent	Injections of two agents
Saline	3·6±0·4	
Physostigmine (0·1 mg/kg) Morphine (10 mg/kg)	12·3±1·2 16·9±2·8	>37·9*
Physostigmine (0·1 mg/kg) Methylamphetamine (10 mg/kg)	8·0±0·4 10·4±0·4	18·6±1·1
Oxotremorine (0·05 mg/kg) Morphine (10 mg/kg)	17·3±4·7 13·2±2·3	>33·1*
Oxotremorine (0·05 mg/kg) Methylamphetamine (10 mg/kg)	20·0±2·9 8·3±0·6	11·5±1·3
Oxotremorine (0·05 mg/kg) Physostigmine (0·05 mg/kg)	12·8±1·5 10·4±0·7	19·2±3·0
Morphine (10 mg/kg) Methylamphetamine (10 mg/kg)	12·8±1·4 12·5±1·5	19·5±1·8

All values are the means of the maximum reaction times (±S.E.M.) of groups of six to twelve mice. The hot plate reaction times of the individual agents were measured concurrently with those of mice given two agents. * Some mice showed reaction times above 45 s, the arbitrary cut off time.

with each agent injected separately. Similar results were obtained for combinations of physostigmine and methylamphetamine, oxotremorine and morphine, oxotremorine and physostigmine and morphine and methylamphetamine. This was not the case, however, with combinations of oxotremorine and methylamphetamine. The actual hot plate reaction times obtained with these mixtures are shown in Table 1.

Neostigmine (up to 0·4 mg/kg) caused no significant changes in the reaction time obtained with morphine and methylamphetamine.

Effect of narcotic antagonists

Nalorphine (10 mg/kg) had no significant effect on the reaction times of saline-treated control mice or mice injected with 0·05 mg/kg oxotremorine.

The maximum mean hot plate reaction time obtained in mice injected with a mixture of 10 mg/kg of morphine and 10 mg/kg of nalorphine, each drug by a separate injection, was $4·7 \pm 0·6$ seconds. This was significantly less ($P<0·001$) than the reaction time obtained with morphine alone ($10·8 \pm 0·7$ s). In contrast, the maximum mean reaction time obtained with physostigmine (0·1 mg/kg) and nalorphine (10 mg/kg) was $16·6 \pm 1·0$ s which was significantly greater ($P<0·001$) than the reaction time obtained with physostigmine alone ($10 \pm 0·7$ s).

Naloxone (1 mg/kg), a narcotic antagonist with no agonist activity, had no significant effects on the reaction times obtained for mice injected with saline, oxotremorine or physostigmine. It significantly reduced the reaction time obtained with morphine.

Effect of reserpine

The effects of pretreatment for 4 h with 5 mg/kg of reserpine on the antinociceptive effects of morphine, physostigmine and oxotremorine are shown in Fig. 2. The increases in reaction time induced by morphine and physostigmine were reduced,

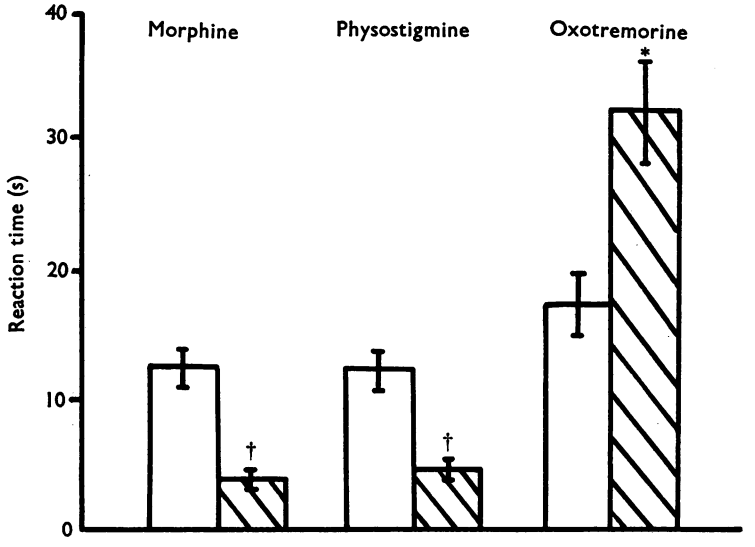

FIG. 2. Effect of reserpine on the hot plate reaction times after injection of morphine (10 mg/kg), physostigmine (0·1 mg/kg) and oxotremorine (0·05 mg/kg). No pretreatment □; 4 h after intraperitoneal injection of reserpine (5 mg/kg), ▩. Columns indicate the means of the maximum actual reaction times (±S.E.M.) of groups of six to ten mice. *$P<0·01$; †$P<0·001$ when compared with mice without reserpine pretreatment.

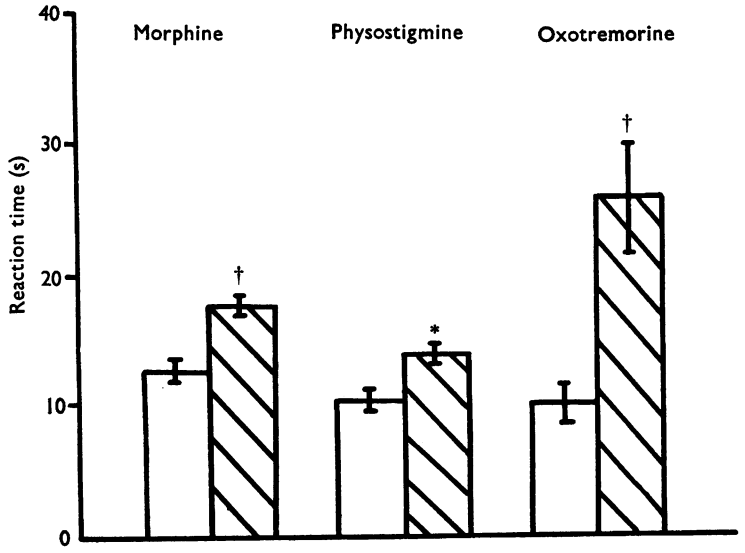

FIG. 3. Effect of α-methyl-*p*-tyrosine on the hot plate reaction times after injection of morphine (10 mg/kg), physostigmine (0·1 mg/kg) and oxotremorine (0·05 mg/kg). No pretreatment, □; 4 h after intraperitoneal injection of α-methyl-*p*-tyrosine (150 mg/kg), ▨. Columns indicate maximum mean reaction times (±S.E.M.) of groups of six to twelve mice. **P*<0·01; †*P*<0·005 when compared with mice without pretreatment with α-methyl-*p*-tyrosine.

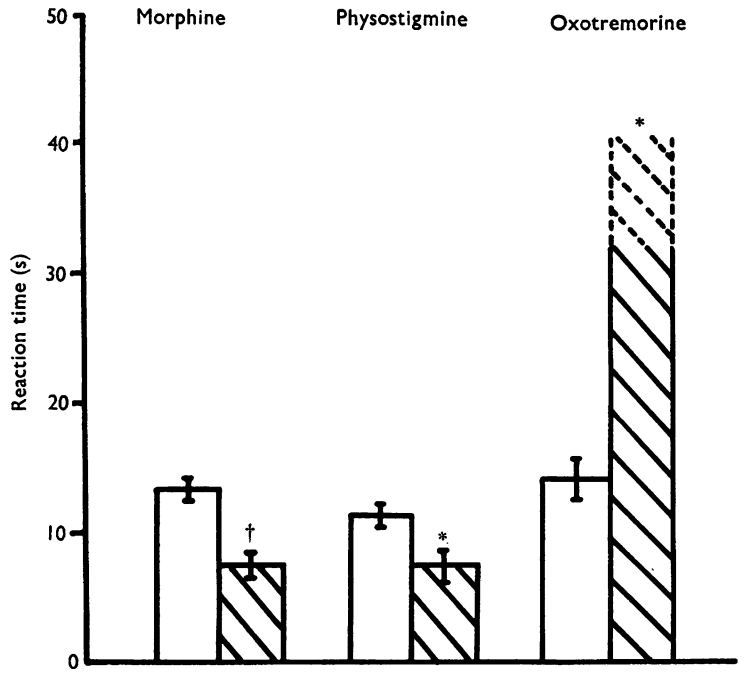

FIG. 4. Effect of sodium diethyldithiocarbamate on the hot plate reaction times after injection of morphine (10 mg/kg), physostigmine (0·1 mg/kg) and oxotremorine (0·05 mg/kg). No pretreatment, □; 4 h after intraperitoneal injection of diethyldithiocarbamate (400 mg/kg), ■. Columns indicate maximum mean reaction times (±S.E.M.) of groups of nine to twelve mice. **P*<0·01; †*P*<0·001 when compared with mice without pretreatment with diethyldithiocarbamate. Some mice exhibited reaction times exceeding the arbitrary cut off time of 45 s, ▩.

but the increase obtained with oxotremorine was potentiated. Qualitatively similar results were obtained with pretreatment with 5 mg/kg of reserpine for 18 h and with pretreatment with 2 mg/kg of reserpine for 4 and 18 hours. Reserpine (5 mg/kg and 2 mg/kg alone) had no significant effects on the reaction times 4 and 18 h after injection.

*Effect of α-methyl-*p*-tyrosine, diethyldithiocarbamate and* L-*dopa*

The effects of pretreatment for 4 h with α-methyl-*p*-tyrosine (150 mg/kg) or sodium diethyldithiocarbamate (400 mg/kg) on the effects of morphine, physostigmine and oxotremorine are shown in Figs. 3 and 4. The effects of all three drugs were increased in animals pretreated with α-methyl-*p*-tyrosine, but only oxotremorine was potentiated in mice pretreated with diethyldithiocarbamate. Both morphine and physostigmine were less active in the latter group of mice. Neither α-methyl-*p*-tyrosine nor diethyldithiocarbamate alone had significant effects on reaction times 4 h after injection.

Table 2 shows the effects of pretreatment for 15 min with L-dopa on the antinociceptive effects of morphine, physostigmine and oxotremorine. The effectiveness of all three antinociceptive agents was reduced. In this experiment the variability of reaction times obtained with oxotremorine made it necessary to use 0·1 mg/kg oxotremorine, instead of 0·05 mg/kg, to obtain significant differences in animals pretreated with L-dopa. L-dopa caused a slight fall in the reaction time when injected alone. Fifteen minutes after injection of L-dopa (200 mg/kg) mice had a mean reaction time of $2{\cdot}4 \pm 0{\cdot}6$ s compared with a saline control value of $4{\cdot}2 \pm 0{\cdot}8$ seconds.

Effects of 5-hydroxytryptophan and p-*chlorophenylalanine*

The antinociceptice effects of morphine, physostigmine and oxotremorine in mice pretreated with 75 mg/kg 5-hydroxytryptophan for 10 min or with 150 mg/kg *p*-chlorophenylalanine twice daily for 3 days are shown in Table 3. The antinociceptive activities of morphine and physostigmine were increased by 5-hydroxytryptophan, but decreased by *p*-chlorophenylalanine. Oxotremorine, however, was unaffected by either pretreatment. Mice pretreated with 5-hydroxytryptophan or *p*-chlorophenylalanine alone had reaction times not significantly different from saline control mice.

Discussion

The present study indicates that when the hot plate reaction time is used to measure antinociceptive activity in the mouse, oxotremorine, physostigmine and

TABLE 2. *Effect of L-dopa on the variation of the hot plate reaction time induced by antinociceptive agents in mice*

Antinociceptive agent	Reaction time (±S.E.M.)	
	No pretreatment	Pretreated with L-dopa (200 mg/kg)
Morphine (10 mg/kg, i.p.)	$12{\cdot}1 \pm 1{\cdot}1$	$6{\cdot}7 \pm 1{\cdot}0$*
Physostigmine (0·1 mg/kg, s.c.)	$11{\cdot}7 \pm 1{\cdot}0$	$7{\cdot}8 \pm 0{\cdot}7$*
Oxotremorine (0·1 mg/kg, s.c.)	$36{\cdot}6 \pm 5{\cdot}1$	$7{\cdot}6 \pm 1{\cdot}4$†

The results are expressed as the means of the reaction times measured at the time after injection when the maximum reaction time was obtained in the 'No pretreatment' group. L-dopa was injected 15 min before the antinociceptive agent. * $P<0{\cdot}01$; † $P<0{\cdot}001$.

morphine are active, but the mechanisms by which they exert their effects differ. The antinociceptive activity of neither physostigmine nor oxotremorine was antagonized by doses of naloxone and nalorphine which completely antagonized the action of morphine. This indicates that physostigmine and oxotremorine do not mediate their antinociceptive activity via the narcotic analgesic receptor. Similar observations were made by Ireson (1970) using the electroshock and writhing tests in mice. However, using the tail flick test in mice, Harris, Dewey, Howes, Kennedy & Pars (1969) found that naloxone caused some diminution of the antinociceptive activity of physostigmine and oxotremorine.

The action of nalorphine, the narcotic antagonist with marked agonist activity, showed that the mechanisms of action of oxotremorine and physostigmine are different. Whereas oxotremorine was unaffected by nalorphine, the effect of physostigmine was significantly increased. Both Harris *et al.* (1969) and Ireson (1970) reported potentiation of physostigmine by nalorphine in their test systems. The former suggested that the results could be indicative of cholinergic mechanisms being involved in the actions of narcotic antagonist analgesics, whilst the latter suggested that both physostigmine and nalorphine could be partial agonists at analgesic receptor sites.

Further differences between the mechanisms of action of physostigmine and oxotremorine can be seen in reserpine pretreated mice. The antinociceptive activity of morphine and physostigmine was reduced whereas that of oxotremorine was potentiated. Somerville & Whittle (1967) showed that reserpine pretreatment in the mouse (2 mg/kg for 4 h) induced significant depletion of noradrenaline, dopamine and 5-HT; thus changes in any or all of these amines could be responsible for the findings reported in the present paper.

Koe & Weissman (1966) demonstrated that 5-hydroxytryptophan raised the 5-HT content of brain and *p*-chlorophenylalanine, a tryptophan hydroxylase inhibitor, reduced the 5-HT content of mouse brain. Pretreatments with these two agents induced no changes in the effects of oxotremorine. This is in marked contrast to the results obtained with physostigmine and morphine; 5-hydroxytryptophan increased antinociceptive activity and *p*-chlorophenylalanine decreased it.

TABLE 3. *Effects of drugs reported to induce changes in brain 5-hydroxytryptamine content on the antinociceptive effects of morphine, physostigmine and oxotremorine*

Drug	Maximum mean reaction time (±S.E.M.) No pretreatment	Pretreatment with 5-HTP
Saline	6·8±0·5	6·9±0·8
Morphine (10 mg/kg, i.p.)	12·5±0·9	21·5±1·6†
Physostigmine (0·1 mg/kg, s.c.)	11·4±0·9	15·6±1·1*
Oxotremorine (0·05 mg/kg, s.c.)	13·5±1·8	15·4±1·8
	No pretreatment	Pretreatment with *p*-CPA
Saline	7·3±0·9	7·6±0·2
Morphine (10 mg/kg, i.p.)	15·3±0·7	7·3±0·9†
Physostigmine (0·1 mg/kg, s.c.)	11·6±0·6	6·9±0·5†
Oxotremorine (0·05 mg/kg, s.c.)	11·3±0·9	11·3±1·0

All values are the means of the maximum reaction times (±S.E.M.) in groups of six mice for saline and nine–twelve mice for the antinociceptive agents. 5-Hydroxytryptophan (5-HTP) (75 mg/kg) was injected 10 min before the antinociceptive agent and mice were pretreated with 150 mg/kg *p*-chlorophenylalanine (*p*-CPA) twice daily for 3 days. * $P<0.01$; † $P<0.001$.

Reduction of brain noradrenaline and dopamine without concurrent reduction in 5-HT can be obtained by pretreatment with the tyrosine hydroxylase inhibitor, α-methyl-*p*-tyrosine (Spector, 1966). The antinociceptive activity of all three agents was potentiated in mice pretreated with this drug. Conversely pretreatment with L-dopa, which raises brain content of dopamine and noradrenaline (Lotti & Porter, 1970), reduced their activity.

However, diethyldithiocarbamate, a dopamine β-hydroxylase inhibitor which reduces brain noradrenaline without concurrent reduction in dopamine (Carlsson, Lindqvist, Fuxe & Hökfelt, 1966), reduced the activities of morphine and physostigmine whilst that of oxotremorine was potentiated.

Thus it appears that the antinociceptive activity of oxotremorine is consistently potentiated by procedures which have been reported to lower the brain noradrenaline content and unaffected by procedures which change the brain 5-HT content. Involvement of noradrenaline in antinociceptive activity of oxotremorine may explain the mutual antagonism of oxotremorine and methylamphetamine, since methylamphetamine releases noradrenaline from neuronal stores.

Sethy, Naik & Sheth (1971) reported changes in tremorine analgesia by drugs which influence amine synthesis. The changes reported for the effects of diethyldithiocarbamate, reserpine and *p*-chlorophenylalanine are at variance with the results obtained in the present study for oxotremorine, the active metabolite of tremorine. The evidence available at present is not sufficient to explain this discrepancy. There may be differences in technique and dosages which are responsible for it.

Comparison of the antinociceptive activities of physostigmine, oxotremorine or morphine may be confused by the use of the intraperitoneal route for morphine and the subcutaneous route for the other two agents. The absorption of a drug injected subcutaneously is more readily influenced by other drugs, body temperature and movement than is injection by the intraperitoneal route. Reserpine reduced the antinociceptive activity of morphine, injected intraperitoneally, but potentiated the effects of oxotremorine, injected subcutaneously. Fennessy & Lee (1970) reported similar findings for morphine when injected subcutaneously; unpublished observations of the present authors have shown that although the duration of the antinociceptive activity of oxotremorine was reduced when injected intraperitoneally, it was still potentiated in reserpine-pretreated animals. Furthermore, differences seen between oxotremorine and morphine were also seen between oxotremorine and physostigmine, both of which were injected subcutaneously. Thus, although the use of different routes of injection may affect the results quantitatively, it is unlikely to produce the major qualitative differences reported in the present study.

Major & Pleuvry (1971) found that the antinociceptive activity of morphine or methylamphetamine was increased by procedures reported to cause a rise in the ratio of 5-HT to dopamine in the brain and decreased by procedures reported to cause a fall in 5-HT or a fall in the ratio of 5-HT to dopamine. The antinociceptive activity of physostigmine fits in with this pattern.

Thus, provided the different courses of pretreatment produced their effects on the activity of the three antinociceptive agents by modification of brain amine content, then the present study indicates that morphine and physostigmine act on systems involving catecholamines and 5-HT, whilst oxotremorine acts on systems involving catecholamines but not 5-HT.

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