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The relationship between tremor and change in brain acetylcholine concentration produced by injection of tremorine or oxotremorine in the rat

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1. The relationship between tremor and change in brain acetylcholine concentration after the injection of tremorine or oxotremorine has been investigated in rats.

2. Tremorine produced a significant increase in whole brain acetylcholine and in tremor 5 min after injection. After this time tremor subsided but brain acetylcholine continued to increase.

3. Oxotremorine produced tremor within 30 sec. This became maximal within 5 min of injection and then declined rapidly. The brain acetylcholine concentration showed a significant increase 5 min after injection and continued to increase until 30 min afterwards.

The time course of tremorine tremor in the rat has been variously reported. Friedman, Aylesworth & Friedman (1963) noted onset of tremor within 15 min of an intraperitoneal injection of 20 mg/kg and maximum tremor at 60 min. Holmstedt & Lundgren (1966), however, found that the tremor started within 5 min of an intraperitoneal dose of 20 mg/kg and was maximum at 10 min. These latter workers also investigated the effects of tremorine on brain noradrenaline, adrenaline, dopamine and acetylcholine. They have suggested that the time course of rise in brain acetylcholine gives the best correlation with the time course of tremor. They postulated that oxotremorine (1-(2-oxopyrrolidino)-4-pyrrolidino-butyne-2), the active metabolite of tremorine (Cho, Haslett & Jenden, 1961), " does not act *per se* but acts by mobilising brain acetylcholine." The time course of the tremor produced by tremorine or oxotremorine has therefore been examined and compared with the changes produced in the concentration of acetylcholine in rat brain.

Methods

For tremor recording male Wistar rats weighing 190–210 g were used. In all the other experiments the weight range was 190–250 g.

Estimation of brain acetylcholine concentration

Acetylcholine was extracted from individual rat brains by a method previously discussed (Cox & Potkonjak, 1967). Estimations were performed on mipafox (N,N'di-isopropylphosphorodiamidic fluoride) pretreated guinea-pig ileum using a 2×2, 4 point assay, with doses given in a 4 block latin square arrangement.

Quantitative estimation of tremor

Tremor was measured by a method similar to that of Ahmed & Taylor (1959) and of Yen & Day (1965) and is fully described by Cox (1968). After injection a rat was placed in a Perspex box (18 cm × 7 cm × 7 cm) which was suspended by means of a rigid metal rod (8 cm × 1.5 mm diameter) from a gramophone pick-up head. The pick-up was clamped to a rigid metal frame through a shock absorber, which prevented vibrations of the pick-up being transmitted to three similar systems, boxes and pick-ups, attached to the same metal frame. An automatic timing device connected the output from each crystal in turn (for a period of 15 sec) to the wide band A.C. Preamplifier (7P3A) of a Grass Polygraph (Model 7). The output from this preamplifier was recorded on channel 1 (Fig. 1) to give a qualitative record of the tremor and was also led to the polygraph integrator (7P10A) on channel 2 to give an integrated value for the tremor intensity. One complete deflection of the integrator was given an arbitrary value of 100 tremor units and the integrator immediately reset itself to zero after reaching full deflection. The results for tremor are presented as the mean integrated value per 15 sec. An automatic zero was incorporated in the timing device so that each 15 sec recording period started from the base line on the integrator channel. To obtain a continuous recording from one rat at a time using the same pick-up head the timer was not used but the automatic zero was included in the circuit.

Statistical evaluation of results

Differences between mean brain acetylcholine concentrations were assessed by Student's t test (two-tailed). The Mann-Whitney U test (Siegel, 1956) was used to determine the statistical significance of the tremor results. Unless otherwise stated a significant difference between means was taken at the 95% confidence limits ($P<0.05$).

Drugs

Acetylcholine chloride and morphine hydrochloride (B.D.H. Ltd.), mipafox (L. Light & Co. Ltd.), oxotremorine as the free base and tremorine dihydrochloride. All concentrations are expressed in terms of the free base. Drug injections were freshly prepared in isotonic saline and administered intraperitoneally in a volume of 0.1 ml./100 g.

Results

Tremorine

The time courses of tremorine tremor and change in brain acetylcholine are compared in Fig. 2.

Tremorine 20 mg/kg gave a peak tremor intensity at 5 min. The tremor decreased rapidly in the following 30 min but was then maintained for the remainder of the recording period.

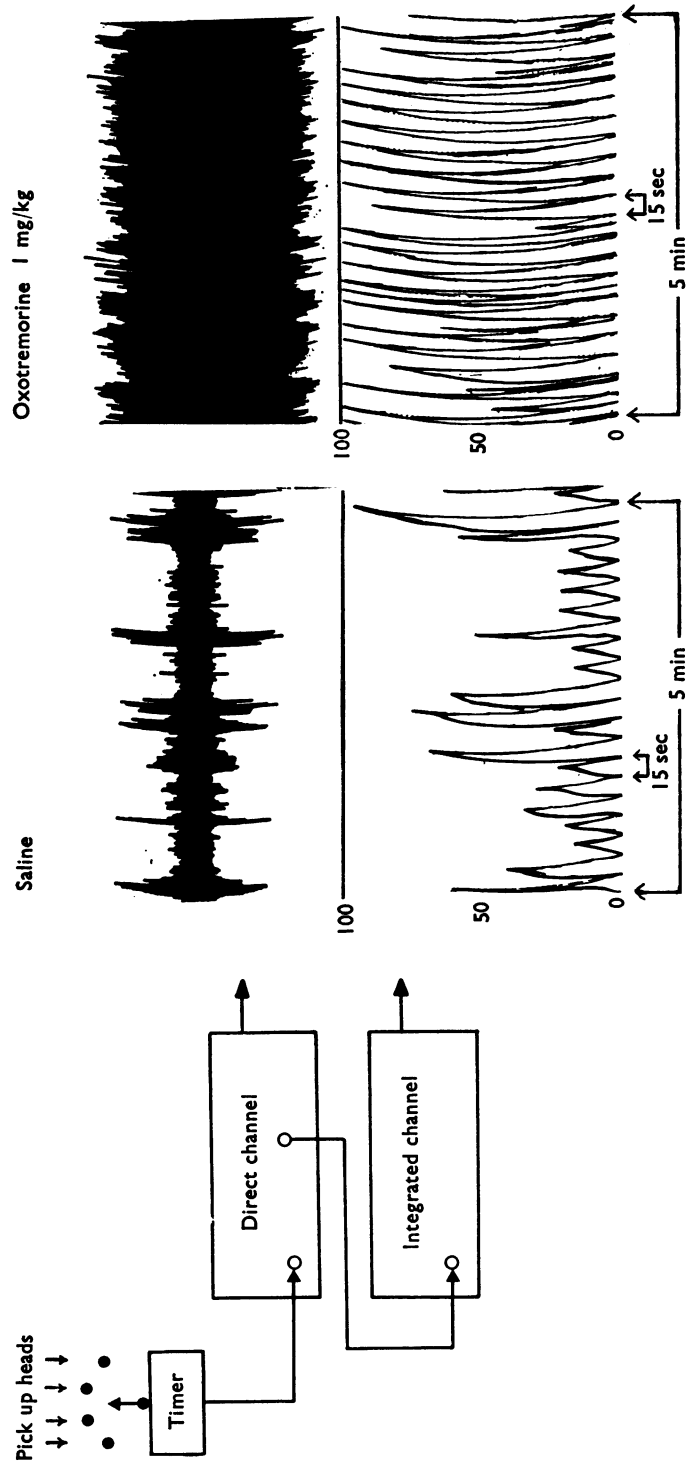

FIG. 1. Block diagram of the tremor recording apparatus. Sample traces from a saline control rat and a rat injected with oxotremorine 1.0 mg/kg are also shown.

The concentration of rat brain acetylcholine was significantly higher than the control concentration 5 min after the injection of tremorine ($P<0.001$). It continued to rise until 30 min after the injection when it reached a mean value of 5 μg/g representing an increase over the control of more than 200%. Sixty minutes after the injection of tremorine the brain acetylcholine concentration was still significantly higher than that of saline controls.

Oxotremorine tremor

The time course for oxotremorine tremor is shown in Fig. 3.

Oxotremorine 2 mg/kg gave a mean 15 sec tremor of 99 units for the 5 min sample. This value was significantly higher ($P=0.004$) than the corresponding control mean, but significantly lower than the mean obtained for oxotremorine 0.5 and 1.0 mg/kg. The tremor was maintained at 99 units for the first 20 min and then declined. At all times the mean 15 sec tremor was significantly higher than that recorded from saline controls. Oxotremorine 1 mg/kg gave a mean of 134 units in the 5 min sample which was significantly higher than saline control.

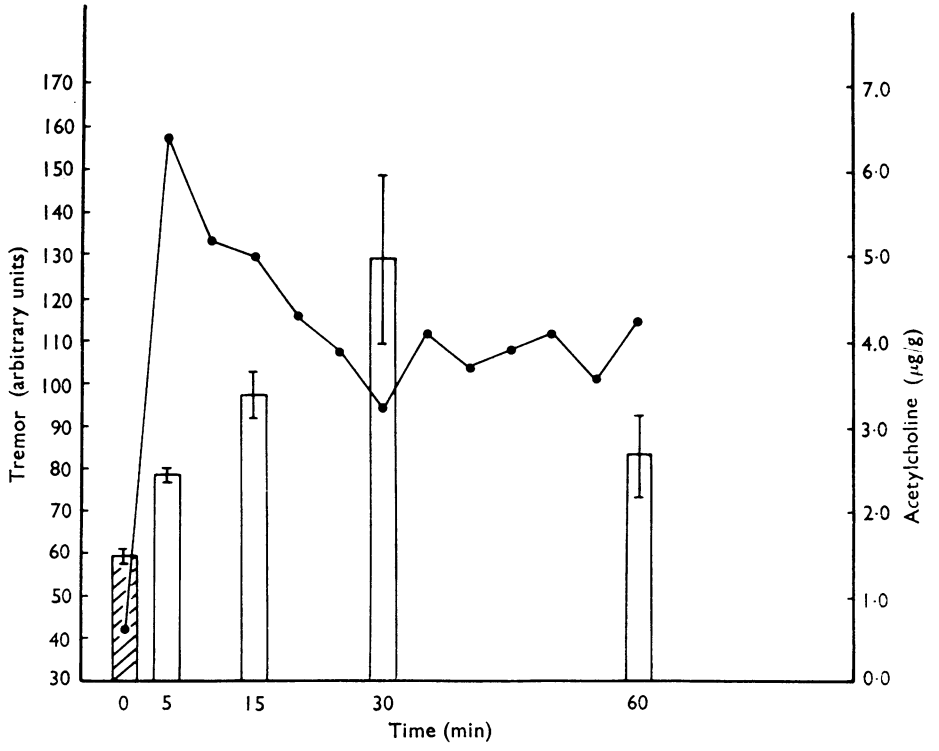

FIG. 2. Time courses of brain acetylcholine concentration and tremor induced by tremorine 20 mg/kg in the rat. Hatched column represents brain acetylcholine concentration (μg/g whole brain ± standard error) of rats pretreated with saline. Open columns represent brain acetylcholine concentration at various times after tremorine 20 mg/kg. Each value is the mean of five determinations. Mean tremor in arbitrary units (●——●). Each point is the mean for at least thirteen rats, and five separate 15 sec samples recorded at 1 min intervals for each animal.

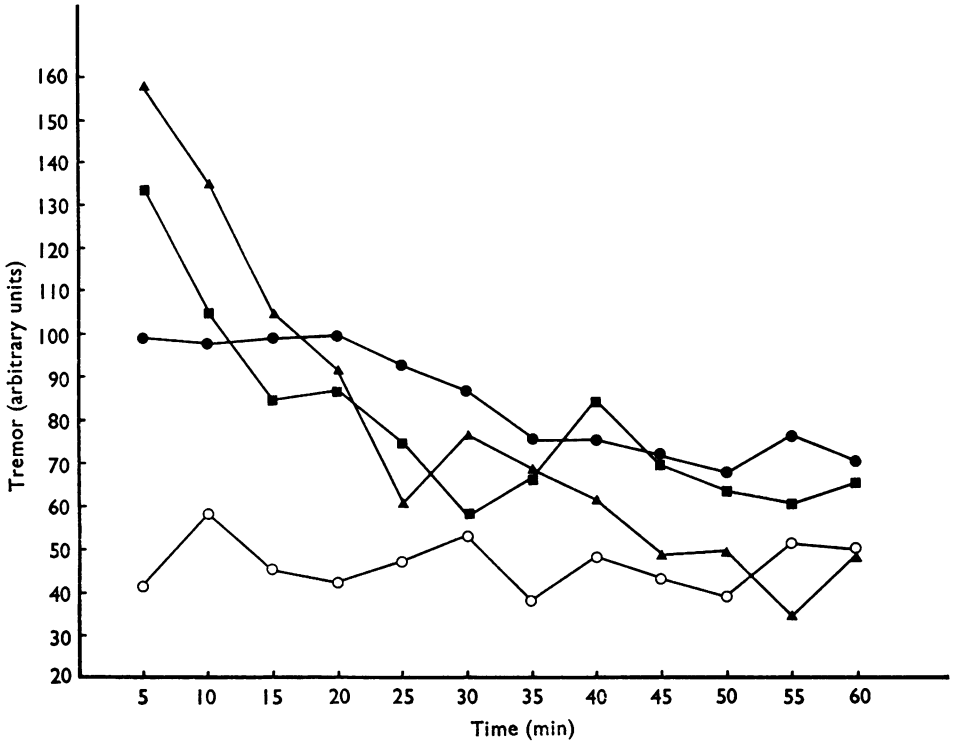

FIG. 3. Time course of oxotremorine tremor. Twenty rats were used at each dose level. Details of tremor recording as in Fig. 2. Rats pretreated with saline (○), oxotremorine 0.5 mg/kg (▲), 1.0 mg/kg (■), 2.0 mg/kg (●).

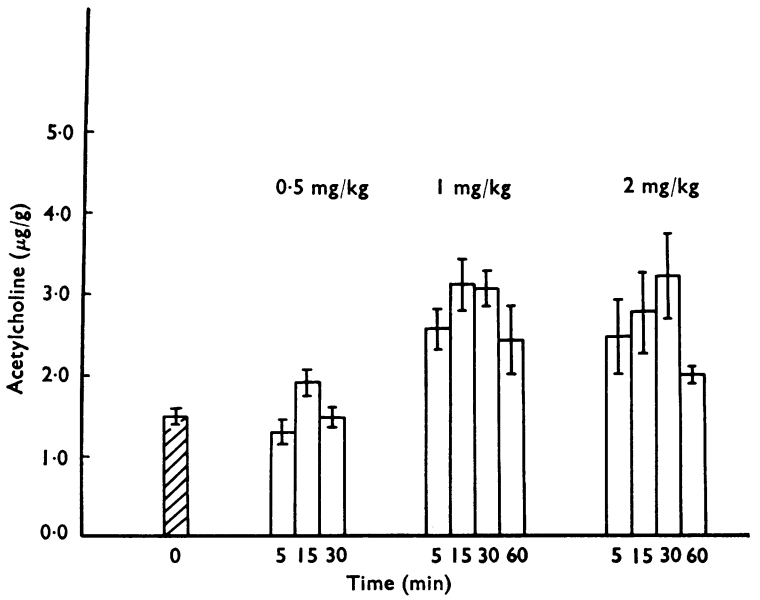

FIG. 4. Effect of oxotremorine 0.5, 1.0 and 2.0 mg/kg on rat brain acetylcholine concentration (μg/g whole brain ± standard error). Hatched column represents rats pretreated with saline. Open columns represent rats pretreated with oxotremorine for the times indicated. Each column is the mean of five determinations.

The tremor was not well maintained and had fallen to a value similar to control in the 25 min sample. The highest mean 15 sec tremor was obtained in the 5 min sample after injection of oxotremorine 0.5 mg/kg. This tremor also declined rapidly giving a value within the control range 30 min after the injection. Observation of the rats receiving oxotremorine showed that the tremor was usually present within 30 sec of the injection. No obvious differences could be seen between the responses to the 0.5 and 1.0 mg/kg doses. After oxotremorine 2 mg/kg the rats showed convulsions within 2 min of the injection before the tremor recording was made. Rigidity was also seen after injection of oxotremorine. It occurred initially between bursts of tremor activity and often outlasted the tremor, particularly after a low dose of oxotremorine.

Oxotremorine and brain acetylcholine

The effect of oxotremorine 0.5, 1 and 2 mg/kg on rat brain acetylcholine concentration is shown in Fig. 4. After oxotremorine 0.5 mg/kg, brain acetylcholine concentration was unchanged at 5 min. It became significantly higher ($P<0.05$) than the control concentration 15 min after the injection, but had returned to the control level after 30 min.

Oxotremorine 1 mg/kg produced a significant increase in brain acetylcholine concentration after 5 ($P<0.01$), 15 ($P<0.01$) and 30 ($P<0.001$) min. The highest mean value of 3 μg/g was recorded at 15 min after the injection. This dose also produced an increase in brain acetylcholine after 60 min, but the difference did not

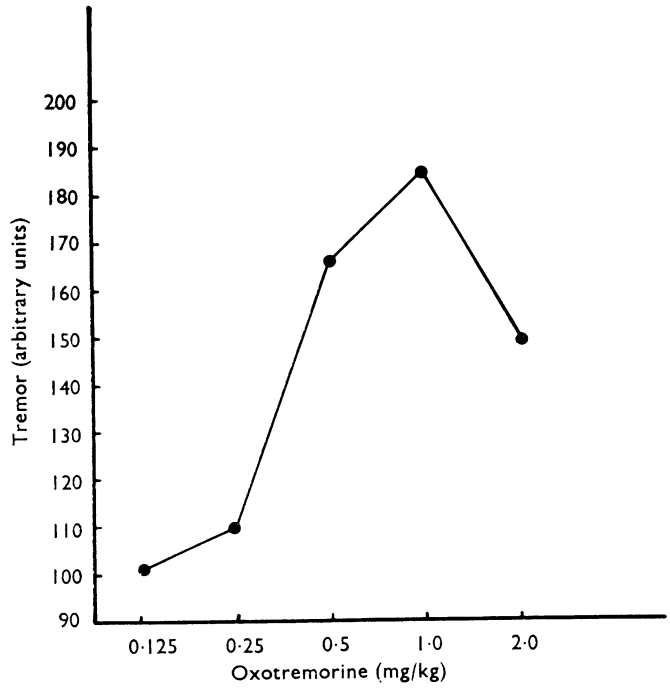

FIG. 5. Log dose response curve for oxotremorine-induced tremor. Each point is the mean 15 sec tremor from five rats recorded continuously for 5 min (30 sec to 5 min, 30 sec after injection). The corresponding control mean was 42 units from seven rats.

achieve the accepted level of statistical significance ($0.1>P>0.05$). These increases in rat brain acetylcholine concentration after 5, 15 and 30 min were also significantly higher than the corresponding mean values obtained after oxotremorine 0.5 mg/kg.

A dose of oxotremorine 2 mg/kg gave an increase in rat brain acetylcholine concentration. The values were higher than controls after 5 ($0.1>P>0.05$), 15 ($P<0.05$), 30 ($P<0.02$) and 60 ($P<0.01$) min. The maximal mean value of 3.2 μg/g was recorded at 30 min after the injection. All mean values after this dose of oxotremorine showed a significant difference when compared with the mean values after oxotremorine 0.5 mg/kg for the times indicated. The increases produced by oxotremorine 2 mg/kg were not significantly different from the corresponding mean values recorded after oxotremorine 1 mg/kg.

Oxotremorine tremor, log dose response curve

The dose response curve for oxotremorine-induced tremor is shown in Fig. 5. The tremor was recorded for a period of 5 min (30 sec to 5 min 30 sec after the injection). The graph shows that the mean tremor increased as the dose of oxotremorine increased from 0.125 to 1 mg/kg. After oxotremorine 2 mg/kg there was a decrease in the tremor produced. Control rats recorded for the same time period gave a mean tremor value of 42 units. All doses of oxotremorine used gave mean tremor values significantly higher than this control mean.

Discussion

Tremor induced by tremorine in rats has been reported to begin within 5 min of the injection (Holmstedt & Lundgren, 1966). The only change noted in the concentration of the substances occurring naturally in the central nervous system, which they assayed, during that time was an increase in rat brain acetylcholine. It was this fact which led Holmstedt & Lundgren to postulate that the increase in brain acetylcholine and tremor produced by tremorine were causally related. Our results confirm that for tremorine 20 mg/kg both tremor and increase in brain acetylcholine can be observed 5 min after the injection. Figure 2 shows, however, that whereas the tremor reaches a maximum within the first 10 min of the injection and then decreases, the brain acetylcholine concentration continues to rise after 5 min and reaches a maximum at 30 min. Therefore, although the onset of tremor and the initial increase in brain acetylcholine coincide, the time courses of these two effects are different. Tremorine requires a metabolic change before exerting its effects (Cho, Haslett & Jenden, 1961) so interpretation of the time course results is complicated. This objection does not hold for oxotremorine.

Oxotremorine in a dose of 0.5 mg/kg produced tremor within 30 sec of the injection but there was no measurable change in brain acetylcholine 5 min after the injection. The only significant increase was observed 15 min after the injection when the tremor symptoms were subsiding. Higher doses of oxotremorine (1 and 2 mg/kg) which also produced tremor within 30 sec, gave increases in brain acetylcholine 5 min after injection. As in the tremorine experiments, however, the brain acetylcholine continued to rise as the tremor symptoms subsided. Hammer, Karlen, Rane & Sjöqvist (1968) showed that 65% of an intraperitoneal dose of tremorine disappeared from the rat during the first hour after injection. Oxotremorine was cleared more rapidly; 74% was eliminated in 20 min and 91% within 60 min.

The disappearance of the tremor after tremorine and oxotremorine compares well with the reported percentages of each drug remaining in the rat at 60 min. It seems, however, that brain acetylcholine can continue to rise as the amount of oxotremorine in the rat is decreasing.

The oxotremorine time course results do not provide any good evidence of a causal relationship between tremor and increase in brain acetylcholine concentration in the rat. Further, the log dose response curve to oxotremorine shows that doses lower than 0.5 mg/kg produce tremor within 5 min of injection, but that whole brain acetylcholine changes are only observed at this time with doses higher than 0.5 mg/kg. It is not possible, on the basis of this evidence, to support the claim that the change in whole brain acetylcholine and tremor are causally related, but this does not necessarily mean that oxotremorine tremor is not acetylcholine mediated. Small changes in brain acetylcholine concentration in restricted areas would not be detected in whole brain acetylcholine estimations. Also changes in turnover of acetylcholine could occur without alteration of whole brain levels. It is also possible that a high concentration of acetylcholine at a receptor site could make it refractory to further impulses, thus distorting the tremor time course.

The mechanism of the oxotremorine-induced rise in brain acetylcholine is uncertain. *In vitro* studies have been unable to show any significant effect of oxotremorine on either choline acetylase or cholinesterase (Holmstedt, Lundgren, Schuberth & Sundwall, 1965). At present little work has been reported concerning the origin of the increased acetylcholine after oxotremorine. Crossland & Slater (1968) have reported that 30 min after a high dose of tremorine (75 mg/kg) there was an increase in both free and bound acetylcholine in rat brain.

Thus although oxotremorine produces an increase in whole brain acetylcholine it does not seem likely that its tremorgenic action is related to this increase. The tremorgenic action might be related to its reported action on muscarinic receptors. (Cho, Haslett & Jenden, 1962 ; Lévy & Michel-Ber, 1965, 1967.)

Oxotremorine was kindly supplied by Dr. A. K. Cho, Department of Pharmacology, U.C.L.A., Los Angeles, California, U.S.A. and Dr. R. W. Brimblecombe, Ministry of Defence, Chemical Defence Experimental Establishment, Porton Down, Wiltshire. Tremorine dihydrochloride was kindly supplied by May & Baker Ltd., Dagenham, Essex, and Lilly Ltd., Research Laboratories, Speke, Liverpool.

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