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ACETYLCHOLINE AND CHOLINE IN MOUSE BRAIN—INFLUENCE OF PERIPHERALLY ACTING CHOLINERGIC DRUGS

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Abstract—The effects of muscarinic drugs on levels of choline (Ch) in brain and blood and on levels and turnover of acetylcholine (ACh) in brain were studied in mice by means of a pulse injection of $^2H_6$-Ch and analysis of Ch and ACh by mass fragmentography. Oxotremorine (OT) increased the levels of Ch and ACh and reduced the turnover of ACh. Muscarine, which is supposed not to penetrate into brain increased Ch but not ACh levels and reduced ACh turnover. Methylatropine had no effect by itself but when given before OT it abolished the increase in blood Ch and counteracted the OT effect on levels and turnover of ACh. The results demonstrate, that the concentration of ACh does not solely regulate its turnover and that apart from the central actions of OT its peripheral actions play an important role for the turnover of ACh and levels of ACh and Ch in brain.

Tertiary amines with cholinergic action, e.g. oxotremorine (OT) and arecoline, increase the endogenous levels of acetylcholine (ACh) and choline (Ch) and decrease ACh turnover in the brain of mice and rats [1–3].

Such drugs, when administered systemically, also induce profound peripheral effects including haemodynamic changes. In the case of OT its haemodynamic effects alter the distribution of the drug to the brain, probably by changing its apparent volume of distribution [4]. In experiments on the effect of OT on ACh turnover we found an increased specific activity of deuterium labelled Ch in brain after i.v. injection [5]. These results suggested an increased distribution to the brain of the administered Ch. We have now carried out a series of experiments with compounds of both central and peripheral cholinergic action in order to study their effects on levels of ACh and Ch and on the synthesis rate of ACh in the mouse brain *in vivo*.

MATERIALS AND METHODS

Animals and drugs. Male NMR, albino mice weighing 20–27 g were used. (+)-Muscarine iodide† in a dose of 2.4 mg/kg was given subcutaneously, atropine methylbromide 5 mg/kg, and OT oxalate 1 mg/kg (as base) were given intraperitoneally in volumes of 5 ml/kg. *N*-2-hydroxyethyl-*N*,*N*,*N*-tri-$^2H_2$, ^{1}H-methylammonium iodide (d_6-Ch) was given intravenously (5 ml/kg) during 1 sec in a dose of 440 nmoles to each animal. *N*-(2-Hydroxyethyl)-*N*,*N*,*N*-tri-$^2H_3$-methylammonium iodide (d_9-Ch) and *N*-(2-acetoxyethyl)-*N*,*N*,*N*-tri-$^2H_3$-methylammonium iodide (d_9-ACh) were used as internal standards [5]. *N*-(2-Acetoxyethyl)-*N*,*N*,*N*-tri-$^2H_2$,^{1}H-methylammonium iodide (d_6-ACh) was used for calibration purposes. The synthesis of the deuterium labelled compounds have been described previously [5].

Procedure. Mice were treated with the drug or saline and killed by spinal dislocation. The brain was removed, weighed, and homogenized in 4 ml 0.4 *N* $HClO_4$ with an Ultra-turrax homogenizer in a small plastic scintillation flask (25 ml). The time from killing to homogenization was standardized to between 40 and 43 sec.

After addition of internal standards, 0.250 nmoles d_9-ACh (in 0.5 ml) and 0.3 nmoles d_9-Ch (in 0.5 ml), endogenous ACh and Ch together with their deuterated variants were extracted with dipicrylamine in dichloromethane as ion pairs. The Ch moieties were derivatized with propionyl chloride and the resulting mixture of ACh and propionyl choline (PrCh) derivatives was demethylated with sodium thiophenoxide [6] and analyzed by mass fragmentography according to Karlén *et al.* [5].

Semiquantitative estimation of muscarine in brain. To test whether any (+)-muscarine might have entered the brain and induced central effects, we analyzed the brain concentration of this drug. It could be demonstrated that (+)-muscarine was carried through the extraction procedure, was propionylated in the 5-OH position and also *N*-demethylated so that it could be gas chromatographed on the same column as ACh and PrCh, although at a higher temperature, 160°. On electron impact mass spectrometry the base peak of normuscarine was *m/e* 58. This was used for the mass fragmentographic quantitation. A calibration curve was constructed by analysing (+)-muscarine (20, 50 and 100 pmoles) added to duplicate samples of brain homogenates from untreated animals. From this curve the concentration of muscarine was calculated in brains of animals pretreated for 15 min with 2.4 mg·kg^{-1} of muscarine. At 20 pmoles the peak height of the galvanometer response was about 10 mm.

† Gift from Professor P. Waser, Zürich.

Table 1. Effect of (+)-muscarine iodide on ACh and Ch levels and synthesis of ACh

Treatment time d_6-Ch	Saline			(+)-Muscarine		
	ACh	Ch	d_6-ACh/d_6-Ch	ACh	Ch	d_6-ACh/d_6-Ch
15 sec	17.7 ± 1.8	63.2 ± 10.2	0.067 ± 0.006	19.1 ± 1.9*	65.9 ± 2.1*	0.037 ± 0.003‡
45 sec	16.9 ± 3.7	57.9 ± 6.9	0.182 ± 0.027	19.7 ± 3.4*	78.0 ± 12.7†	0.061 ± 0.006‡
2.5 min	14.6 ± 2.1	64.2 ± 10.0	0.236 ± 0.058	19.7 ± 3.2	94.9 ± 8.3‡	0.143 ± 0.021†
5 min	12.9 ± 1.7	61.0 ± 8.6	0.325 ± 0.055	19.6 ± 3.8	100.8 ± 9.7‡	0.179 ± 0.027‡
10 min	17.3 ± 1.4	60.6 ± 6.2	0.569 ± 0.036	15.9 ± 1.7*	87.3 ± 8.7‡	0.254 ± 0.015‡
20 min	16.4 ± 4.2	54.1 ± 5.7	0.707 ± 0.084	19.0 ± 1.5*	95.8 ± 10.7‡	0.319 ± 0.015‡

Mice were pretreated i.p. with saline or (+)-muscarine iodide (2.4 mg kg^{-1} s.c.) for 15 min. d_6-Ch was then given i.v. and the animals were killed 15 sec. to 20 min thereafter. The data are given as means ± S.D. (nmoles g^{-1}) of four animals.

* $P > 0.1$.
† $P < 0.05$.
‡ $P < 0.005$.

Estimation of Ch in blood. Endogenous Ch was determined in plasma of mice treated with saline or the cholinergic drug 15 min previously. The mice were killed by cervical dislocation and about 0.2–0.5 g blood was collected into 5 ml saline in 10 ml heparinized centrifuge tubes. After centrifugation at 3000 rev/min for 10 min the diluted plasma (about 4 ml) was transferred to a 25 ml centrifuge tube. After addition of d_9-Ch (10 nmoles in 1.0 ml) and 2.5 ml 1.2 *N* $HClO_4$ the samples were centrifuged at 100.000 *g* for 20 min. The supernatant was then analyzed for Ch in the same way as the brain supernatants. The results are expressed as nmoles per g whole blood.

RESULTS

Concentration of (+)-muscarine in brain. The amount of muscarine in extracts from treated animals corresponded to about 50–75 pmoles per g brain. The experiment was repeated and this time the brain was carefully rinsed with saline to remove blood before it was homogenized. The level of muscarine was now only about 10–15 pmoles per g brain.

Effect of cholinergic drugs on endogenous Ch in blood. The concentration of Ch in whole blood of saline treated mice was 13.9 ± 1.6 nmoles/g (mean ± S.D., $n = 6$). In animals treated with cholinergic drugs according to Tables 1–3 the following figures were obtained: methylatropine, 11.5 ± 2.1 ($n = 4$); methylatropine + OT, 14.5 ± 3.3 ($n = 4$); OT, 31.4 ± 4.0 ($n = 6$); muscarine, 25.1 ± 3.2 ($n = 6$).

Effect of (+)-muscarine on endogenous ACh and Ch levels and synthesis of ACh in brain. The time course of the effect of muscarine iodide (2.4 mg·kg^{-1}, s.c.) on ACh and Ch levels was studied. Muscarine at this dose induced profound peripheral effects, e.g. salivation, lacrimation and diarrhea. A small increase in the concentration of ACh was observed (Table 1). The concentration of Ch was increased significantly after 17.5 min and remained elevated for the time period studied (35 min).

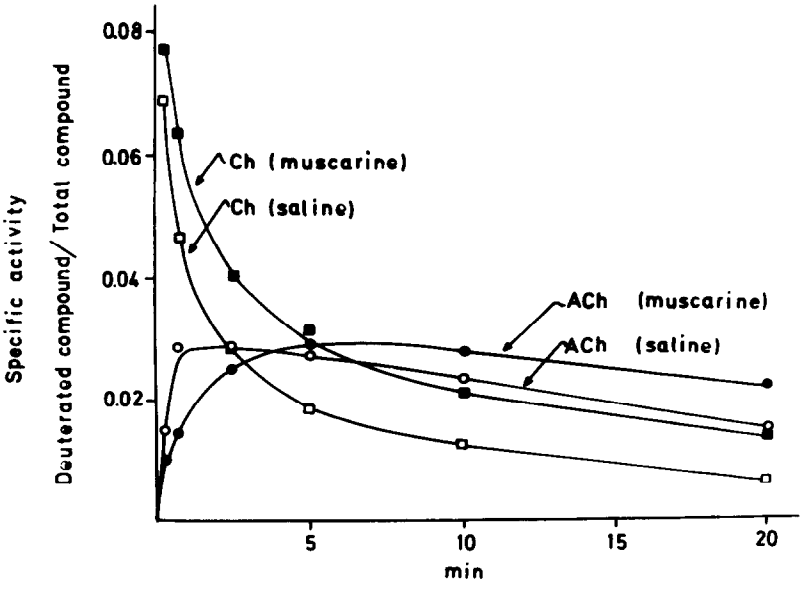

Fig. 1. Sp. act. of ACh and Ch in brains of mice 15 sec to 20 min after an i.v. pulse injection of 440 nmoles d_6-Ch. The mice were pretreated with either saline or (+)-muscarine for 15 min.

Table 2. Effect of OT on ACh and Ch levels and synthesis of ACh

Pretreatment time (min)	Exp. no.	Treatment time (sec) d_6-Ch	Saline ACh nmoles/g	Saline Ch nmoles/g	Saline d_6-ACh/d_6-Ch	OT ACh nmoles/g	OT Ch nmoles/g	OT d_6-ACh/d_6-Ch
15	1	15	19.7 ± 2.8	75.2 ± 9.2	0.053	32.1 ± 4.8‡	86.5 ± 17.3*	0.016
		45			1.100			0.035
45	2	15	17.3 ± 3.3	71.1 ± 3.7	0.060	32.0 ± 1.7‡	103.3 ± 8.5‡	0.013
		45			0.159			0.021
90	3	15	16.0 ± 1.3	74.3 ± 5.8	0.056	30.6 ± 3.4‡	176.5 ± 45.5†	0.015
		45			0.161			0.018
120	4	15	14.5 ± 1.5	63.1 ± 3.8	0.037	26.3 ± 2.6‡	120.0 ± 14.7‡	0.009
		45			0.122			0.017
150	5	15	15.2 ± 2.0	64.6 ± 5.7	0.043	27.7 ± 2.6‡	121.9 ± 30.1†	0.018
		45			0.134			0.015
180	6	15	18.2 ± 4.1	70.2 ± 4.4	0.033	21.6 ± 3.8*	80.6 ± 26.1*	0.039
		45			0.159			0.079

Mice were pretreated i.p. with saline or OT (1 mg kg^{-1}, base) for 15–180 min. d_6-Ch was then given i.v. and the animals were killed 15 or 45 sec. later. The figures for ACh and Ch concentrations are means ± S.D. (nmoles kg^{-1}) of four animals and the figures for the ratio of d_6-ACh and d_6-Ch are the means of two animals.

* $P > 0.1$.
† $P < 0.02$.
‡ $P < 0.005$.

The specific activity of i.v. injected d_6-Ch was elevated between 15 sec and 20 min in animals pretreated with muscarine compared to controls (Fig. 1). In the case of ACh a reduction in sp. act. was seen for the first 4 min followed by a slight elevation from 4 up to 20 min. The synthesis rate of ACh obtained from the ratio between d_6-labelled ACh and Ch was reduced in animals treated with muscarine compared to controls (Fig. 3).

Effect of OT on endogenous levels of ACh and Ch and synthesis of ACh in brain. The time course of the effect of OT on levels of ACh and Ch was studied between 15 and 180 min after administration of OT (Table 2). The values of the controls in different experiments ranged between 14.5 and 19.7 nmoles·g^{-1} for ACh and 63.1 and 75.2 nmoles·g^{-1} for Ch. After OT treatment ACh levels increased to above 30 nmoles·g^{-1} after 15 min and decreased slowly to almost normal after 180 min. The time course of the increase in endogenous Ch was delayed compared to ACh with a peak concentration appearing at about 90 min after OT. At 180 min the levels of Ch were almost back to normal.

The effect of varying the time of pretreatment on the initial synthesis rate of ACh was also studied (Table 2). The fate of pulse injected d_6-Ch was determined after 15 and 45 sec. OT drastically reduced the mole ratio between d_6-ACh and d_6-Ch and the effect was maximal between 15 and 120 min. After 180 min the synthesis rate had almost returned to normal.

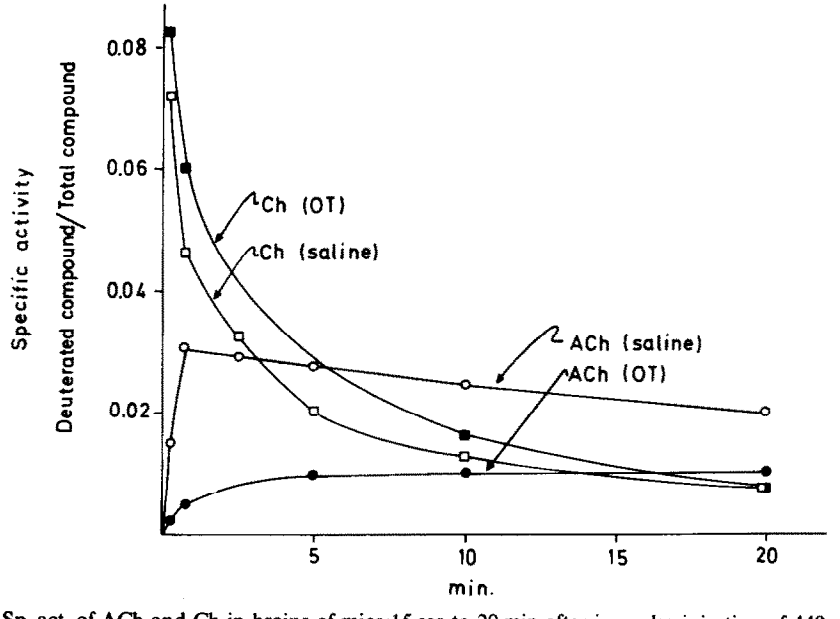

Fig. 2. Sp. act. of ACh and Ch in brains of mice 15 sec to 20 min after i.v. pulse injection of 440 nmoles d_6-Ch. The mice were pretreated with either saline or OT for 15 min.

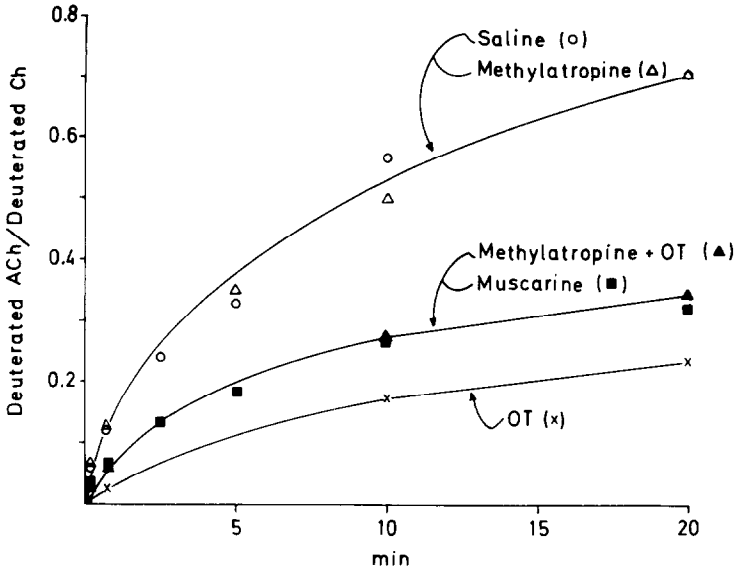

Fig. 3. Time course of the incorporation of i.v. pulse injected d_6-Ch into ACh following pretreatment with OT, muscarine, atropine methylbromide and the combination of atropine methylbromide and OT.

In separate experiments animals were pretreated with OT and 15 min later d_6-Ch was injected i.v. and its incorporation into ACh was studied for the following 20 min (Fig. 2). The sp. act. of Ch was increased and this increase was most marked for the first minutes. After 20 min the sp. act. was about the same as in controls. The sp. act. of ACh was drastically reduced during the whole period. The mole ratio between deuterium labelled ACh and Ch was reduced compared to controls and also compared to the animals treated with muscarine (Fig. 3).

Pretreatment with methylatropine abolished the salivation but not the tremor induced by OT. Methylatropine alone did not change the concentrations of ACh and Ch. The combination of methylatropine and OT increased both Ch and ACh significantly (Table 3) and the rate of incorporation of injected d_6-Ch into ACh was reduced (Fig. 3).

DISCUSSION

The results confirm earlier findings that the concentration of ACh is increased in brains of mice treated with the centrally acting muscarinic drug OT. The central origin of this effect is further established by the fact that the peripherally acting drug, methylatropine, which is not supposed to penetrate into the brain to any significant degree fails to counteract the increase in ACh levels induced by OT. The increase of ACh following OT can, however, be blocked by the centrally acting drug atropine [1].

The effect of OT on brain ACh lasts for about 3 hr. During this time the synthesis rate of ACh is decreased (Table 2). During the onset of the OT syndrome, i.e. for the first 15 min [7] and during the decline of symptoms, between 120 and 180 min, a causal relation between levels and synthesis rate of ACh

Table 3. Effect of atropine methylbromide alone and in combination with OT on levels of ACh and Ch and on synthesis of ACh

Pretreatment drug and time (min) Atropine methylbrom.	OT	Treatment time (sec) d_6-Ch	Exp. no.	ACh	Ch	d_6-ACh/d_6-Ch
—	—	15	1	17.2 ± 3.0	64.9 ± 8.7	0.058 ± 0.017
—	—	45	2	16.7 ± 3.9	69.3 ± 9.3	0.165 ± 0.040
25	—	15	3	15.6 ± 2.8	61.0 ± 11.5	0.071 ± 0.022
25	—	45	4	14.5 ± 3.7	55.6 ± 7.9	0.131 ± 0.042
10	15	15	5	27.1 ± 1.5	77.2 ± 9.3	0.026 ± 0.005
10	15	45	6	28.3 ± 2.2	73.7 ± 3.2	0.054 ± 0.003

Mice were pretreated s.c. with atropine methylbromide (5 mg kg^{-1}) alone or with atropine methylbromide (5 mg kg^{-1}) followed by OT (1 mg kg^{-1}, i.p.). d_6-Ch was then given i.v. and the animals were killed after 15 and 45 sec.

The data are given as means ± S.D. (nmoles g^{-1}) of four animals.

ACh, $P < 0.001$: 1–5, 2–6, 3–5, 4–6.

Ch, $P < 0.1$: 1–3, 2–6, P 0.05: 1–5, 3–5, $P < 0.01$: 4–6, 2–4.

d_6-ACh/d_6-Ch, $P < 0.01$: 4–6, 3–5; $P < 0.001$: 1–5, 2–6.

seems to exist (Table 2). However, it is interesting to note that the quaternary drug muscarine, which in our experiments can be found only in trace amounts in the brain, does not increase the ACh levels, yet decreases its synthesis rate (Table 1, Fig. 3). The diminished synthesis rate after muscarine is also reflected in the displacement of the sp. act. curve of ACh in which the activity develops slower, peaks at a later time (about 5 min compared to about 2.5 min in controls) and also prevails for a longer time (Fig. 1). Schubert *et al.* [2] have suggested that the effects of OT on endogenous levels and synthesis rate of ACh might be explained by the fact that OT is a muscarinic agonist and that activation of muscarinic receptors would lead to a decreased release of ACh by a negative feedback mechanism. This would result in an increased concentration of ACh, whereupon its synthesis would decrease. However, this hypothesis cannot explain the reduced synthesis following muscarine treatment since the concentration of ACh is not significantly changed by this drug. The simplest explanation for the central effect of muscarine is that the small but measurable concentration achieved in the brain (about 10 pmole·g^{-1}) is enough to reduce the synthesis of ACh but not to affect its endogenous level. This difference in action may be dependent upon concentration but may also be due to qualitative differences in action between OT and muscarine, in which case OT but not muscarine would have the property to block the release of ACh from nerve endings. Another possible explanation for the action of muscarine may be its peripheral haemodynamic effects including hypoxia. It has been shown that hypoxia may decrease the activity of Ch acetylase by 20 per cent [8]. On the other hand Carson *et al.* [9] have concluded after experiments with a choline acetylase inhibitor, that cholinacetylase seems to be in great excess in the brain and that a marginal inhibition of this enzyme's activity does not affect the synthesis of ACh.

The elevation of endogenous Ch seen in brain and blood after OT can also be produced with muscarine. It has been shown earlier that the peripheral effects of OT can alter its own distribution and when these effects were blocked with methylatropine a smaller proportion of OT was distributed to the brain [4]. OT has been shown also to increase the concentration of injected [^{3}H]dextrane in blood [10]. The increased concentration of endogenous Ch in blood and brain of OT treated mice could be explained by the lowered apparent volume of distribution caused by the peripheral haemodynamic changes induced by the drug. The increased sp. act. of d_6-Ch in brain for the first 15 min after OT or muscarine (Figs 1 and 2) can be explained in a similar way. The return towards normal sp. act. 20 min after OT may be due to the subsequent increase in endogenous Ch (Fig. 2). Haubrich *et al.* [11] have shown that the half life of Ch in plasma of guinea pigs is less than 1 min and that uptake into peripheral tissues is the principal mechanism for its rapid removal.

When the peripheral cholinergic effects of OT were blocked with methylatropine the central action of OT increased ACh levels and decreased its synthesis rate (Table 3, Fig. 3). However, somewhat unexpectedly the endogenous Ch levels in brain were also increased and this finding suggests another mechanism than a decreased apparent volume of distribution. The ratio between blood and brain concentrations of Ch appears to have been changed. It is unlikely that decreased utilization of Ch due to reduced synthesis of ACh is responsible for this increase. Haubrich *et al.* [11] have demonstrated that neither the concentration of ACh nor its rate of synthesis was correlated with the concentration of free, endogenous Ch. Both ACh and Ch in brain therefore seem to be influenced by OT in two ways. An indirect peripheral effect on Ch that can possibly be explained by haemodynamic changes, and one of central origin that may be caused by intracellular mobilization [12]. On ACh the effects of central and peripheral origin may be visualized by the difference in its maximal concentrations obtained following OT alone and OT in combination with methylatropine. In addition, a slower synthesis rate of ACh is evident with OT alone. This difference may, however, be explained by the higher concentration of OT in the brain in animals treated with OT alone compared to animals in which the cholinergic effects have been blocked peripherally with methylatropine [4]. Another explanation for this difference is offered by the experiments with muscarine in which the peripheral effects on ACh synthesis in brain are demonstrated. Thus, the peripheral effects including hypoxia may add to the decreased synthesis rate of ACh caused by the central action of OT.

In conclusion, the experiments in this paper have demonstrated that the peripheral effects of cholinergic drugs may play a role in the metabolism of ACh and Ch in the brain.

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