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STEREOSPECIFICITY OF ANTIDOTES AND THEIR MECHANISM OF
ACTION IN INTOXICATIONS WITH ORGANOPHOSPHORUS
ANTICHOLINESTERASES

ANNUAL REPORT

B. HOLMSTEDT, B. KARLEN, I. NORDGREN and L. PALMER

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Department of Toxicology, Karolinska Institutet,
Box 60400, S-104 01 Stockholm, Sweden

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Our program during the last year has mainly been concerned with studies on: - the effect of diazepam on l-hyoscyamine binding in the old stock of sick and healthy mice and new stock of healthy mice, - the usability of l-hyoscyamine binding for studies of specific receptor distribution in brain, - effect of different combinations of antagonists (l-hyoscyamine, diazepam, HI-6) on acetylcholine turnover, - duration of the effect of diazepam on acetylcholine turnover. There is a significant difference between the old and new stock of mice as far as the influence of diazepam on l-hyoscyamine binding is concerned. In order that optimal parameters can be determined, therefore, the model needs further development in the new stock of mice.					
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19. ABSTRACT

In testing the ability of the model to detect muscarinic receptors of specific subtypes, we have studied the kinetics of displacing the ligand l-hyoscyamine. So far we have performed experiments on the interaction of the unspecific ligand scopolamine with this binding. The displacement of l-hyoscyamine is time-dependent and proceeds until about half the l-hyoscyamine pool has been displaced.

l-Hyoscyamine treatment results in an increase in the synthesis rate of acetylcholine (ACh). This is probably an unfavourable feature of a protecting agent. Diazepam, on the other hand, lowers the turnover rate of ACh. The combination of diazepam and l-hyoscyamine decreases the turnover of ACh to the same extent as diazepam alone does. When l-hyoscyamine and diazepam are combined with HI-6, the dominant result is an increase of choline levels, with a slight decrease of ACh turnover.

The duration of the effect of diazepam has been followed for 4 h. After 20, 30 and 45 min, levels of acetylcholine are significantly increased, while they are returning to normal after 4 h. The fractional rate constant seems to be somewhat lowered at 4 h. The data indicate a duration of effect on acetylcholine dynamics of at least 4 h.

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SUMMARY

During the past year we have studied 1) l-hyoscyamine binding in an old and a new stock of mice; 2) conditions for displacement of l-hyoscyamine in vivo by scopolamine and size of the muscarinic receptor pool after chronic treatment with scopolamine; 3) effect of the antidotes l-hyoscyamine, diazepam and HI-6 on acetylcholine turnover separately and in combination; and 4) duration of the effect of diazepam on acetylcholine turnover.

1) In 1988 the stock of mice used in our studies contracted mouse hepatitis and our models had to be re-established in a new stock of animals. l-Hyoscyamine binding in vivo in mouse brain had been shown to be decreased by about 40% with diazepam. We were unable to repeat these results in the new stock of mice. In a preliminary study and in order to establish new experimental conditions for this model, we repeated the experiments in "old" (now healthy) and "new" stocks of mice under the same experimental conditions. The decrease in "old" healthy stock was now 25% (significant) and in "new" stock 12% only (not significant).

2) Studies of l-hyoscyamine binding as a model for assessment of receptor subpopulations (M_1 , M_2 , etc.) and receptor pool size after chronic treatments with agonists and antagonists are in progress. The feasibility of the model for the study of subpopulations was assessed by testing the ability of the known in vitro antagonist scopolamine to displace l-hyoscyamine. Doses of scopolamine (4-50 mg/kg) administered before l-hyoscyamine (2 mg/kg, i.v.) displace this ligand by about 50%. When l-hyoscyamine is given before scopolamine, 1 h is needed to displace l-hyoscyamine to the same extent.

In the experiment to measure receptor pool size, scopolamine (25 mg/kg, i.v.) was injected into mice for 12 days. After an additional 2 days, l-hyoscyamine (2 mg/kg, i.v.) was injected and then measured in brain after 2 hours. Compared to controls no difference in receptor pool size could be detected. The fact that no increased concentration was

observed may have been due to residual scopolamine still occupying receptors after 2 days. This will be evaluated by simultaneous measurement of the scopolamine concentration.

3) 1-Hyoscyamine decreases acetylcholine, increases choline and increases the rate of synthesis of acetylcholine. Diazepam increases choline and decreases acetylcholine turnover. HI-6 increases choline. The combination of the three agents increases choline and marginally decreases the turnover of acetylcholine. These experiments were conducted to provide background data for the experiments in which the protecting effect of these three antidotes against soman poisoning and their effect on acetylcholine dynamics will be studied.

4) The effect of diazepam (2 mg/kg, i.v.) on levels of acetylcholine and choline lasts for about 1 h. The effect on acetylcholine turnover lasts for about 4 h.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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INTRODUCTION

In accordance with our plans in connection with the grant we are elucidating the mechanism of the protecting effect of a combination of atropine, diazepam and an oxime against organophosphorus poisoning. For this purpose, we use different in vivo models in mice. Since intoxication by organophosphorus cholinesterase inhibitors is mediated by effects on the cholinergic nervous system, we study the effects of the protecting agents on this system. For the study of the biochemical processes, we use turnover of acetylcholine in mouse brain. We also study binding of the receptor ligand l-hyoscyamine to muscarinic receptors in brain involved in the dynamics of acetylcholine. These biochemical processes are then correlated with the signs of intoxication observed in the animals after the treatments.

Our research within the framework of this grant has so far described:

- development of a method to study binding in vivo to muscarinic receptors by measuring l-hyoscyamine in brains of mice
- study of the protecting effect of diazepam on the intoxication syndrome induced in mice by cholinergically active substances
- correlation of the protecting effect of diazepam in vivo with its effect on acetylcholine dynamics. This was studied by following choline and acetylcholine levels and acetylcholine turnover in the mouse brain using a GC-MS method developed in our laboratory
- correlation of the protective effect of diazepam with its effect on l-hyoscyamine binding in vivo after cholinergic stimulation
- development of a method to measure diazepam in brain and blood
- study of the impact of the formulation vehicle on the distribution of diazepam to mouse brain after intravenous injection of diazepam in different formulations

During the last year, our work has mainly concentrated on studies on: 1) the effect of diazepam on l-hyoscyamine binding in "old" stock of sick

and "new" stock of healthy mice (These experiments have been made to determine suitable experimental conditions for the new stock.); 2) the usability of l-hyoscyamine binding for the study of specific receptor distribution in brain (muscarine M_1 , M_2 , M_3 , etc.) (For this purpose, the ability of a second ligand to displace already-bound l-hyoscyamine after injection of scopolamine has been studied.); 3) the effect of different combinations of antagonists - i.e., diazepam, l-hyoscyamine and HI-6 - on acetylcholine turnover (These experiments represent part of the study on the protective effect of the combination of all three antidotes, which will be studied after cholinesterase inhibition.); and 4) the duration of the effect of diazepam on acetylcholine turnover.

MATERIALS AND METHODS

Animals

Male NMRI mice weighing 20-25 g were used.

Chemicals

Deuterium-labelled ACh and choline (Ch) were synthesized according to Karlén *et al.* (1). Diazepam was in the form of Diazemuls^R (KabiVitrum AB, Sweden). The reagents used for the analysis of ACh and Ch were prepared as described by Karlén *et al.* (1). l-Hyoscyamine was obtained from Sigma Chemical Company, USA. The synthesis of deuterium-labelled atropine is described in Palmér *et al.* (2). Scopolamine was obtained from Aldrich Chemie, West-Germany. HI-6 ([[(4-amino-carbonyl)pyridino]-methoxy]methyl]-2-[(hydroxyimino)methyl]-pyridinium dichloride) was a gift from Astra Meditec, Sweden. All other chemicals were of analytical grade.

Drugs and their administration

Diazepam (as Diazemuls^R) was given in doses of 1 mg/kg, i.v. l-Hyoscyamine (base) was given i.v. in doses of 2 mg/kg. HI-6 (as di-Cl monohydrate) was administered i.v. in doses of 25 mg/kg i.v. Scopolamine (base) was injected in doses of 4 or 50 mg/kg i.v. or 25 mg/kg i.p. The drugs, except diazepam, were dissolved in saline and given in a volume of 5 ml/kg.

Analysis of ACh and Ch in mouse brain

ACh and Ch were analyzed using gas chromatography-mass spectrometry (GC-MS) and deuterated internal standards, according to Karlén *et al.* (1). The mice were killed by focused microwave irradiation on the head with 2.5 kW for 0.68 sec (Metabostate, Gerling-Moore, Palo Alto, CA) in order to inactivate enzymes rapidly and to prevent postmortem changes

of ACh and Ch. The brain was homogenized in 4 ml 0.4 M HClO_4 . $[^2\text{H}_9]\text{ACh}$ (N-(2-acetoxyethyl)-N,N,N-tri $[^2\text{H}_3]$ methylammonium iodide) and $[^2\text{H}_9]\text{Ch}$ (N-(2-hydroxyethyl)-N,N,N-tri $[^2\text{H}_3]$ methylammonium iodide) were added as internal standards. The homogenates were centrifuged for 20 min at $100,000 \times g$. ACh and Ch, together with their deuterated moieties, were extracted into methylene chloride as ion pairs with dipi-crylamine (2,4,6,2',4',6'-hexanitrodiphenylamine) (DPA), and were then demethylated with sodium benzenethiolate to form the corresponding tertiary amines, which were analyzed by GC-MS using a 25-m HP-5 capillary column.

Turnover of ACh in mouse brain

The ACh turnover (TR_{ACh}) in brain was studied by following the incorporation of $[^2\text{H}_6]\text{Ch}$ (N-(2-hydroxyethyl)N,N,N-tri $[^1\text{H}_1\ ^2\text{H}_2]$ methylammonium iodide) into ACh after an i.v. injection of $[^2\text{H}_6]\text{Ch}$ (10 $\mu\text{mol/kg}$), in a volume of 5 ml/kg administered during 1 sec. The turnover rate of ACh was calculated from the specific activities of $[^2\text{H}_6]\text{ACh}$ (N-(2-acetoxyethyl)-N,N,N-tri $[^1\text{H}_1\ ^2\text{H}_2]$ methylammonium iodide) (S_{ACh}) and $[^2\text{H}_6]\text{Ch}$ (S_{Ch}) 20 and 50 sec after the $[^2\text{H}_6]\text{Ch}$ injection, according to Zilversmit (3), as described by Karlén *et al.* (4) and Nordgren *et al.* (5).

Analysis of l-hyoscyamine

The mice were killed by focused microwave irradiation, as described above. The brain tissue was homogenized in 2 ml 0.4 M HClO_4 containing $[^2\text{H}_3]$ atropine as internal standard. The homogenates were centrifuged for 20 min at $100,000 \times g$. The concentration of l-hyoscyamine was determined according to Palmér *et al.* (6), with the following modifications: to the supernatant 0.25 ml 5 M NaOH and 6 ml diethyl ether were added. After agitation and centrifugation, the ether layer was evaporated to dryness. Thirty microliters of N,O-bis(trimethylsilyl)acetamide (BSA) reagent was added to the residue, and after reaction for

30 min at 60°C, the excess of reagent was evaporated. The residue was dissolved in 25 μ l methylene chloride and analyzed by GC-MS. The gas-chromatographic column used was a 12-m SE-52 fused silica capillary column. Column temperature was 210°C.

RESULTS AND DISCUSSION

l-Hyoscyamine binding

As previously reported, our suppliers of experimental animals had to change the animal stock due to a hepatitis infection. This upset the parameters of our study models. We have also reported certain problems in adjusting the experimental conditions in the receptor binding studies with l-hyoscyamine. We have now been able to compare the two stocks of mice in this respect.

Mice were pretreated for 2 min with 1 mg/kg of diazepam given as Diazemuls^R. l-Hyoscyamine, 2 mg/kg i.v., was then given and the mice were killed 2 h later by microwave irradiation. Results are compiled in Figure 1 and comprise the effect of diazepam on l-hyoscyamine binding in the new stock of mice, the old stock of healthy mice generated at the same time (a limited number of healthy progeny of the survivors were available), and data generated in 1986 in the old stock of sick mice. Comparisons have been made with animals treated with saline in all three sets of animals. Results now generated in the old healthy stock (Fig. 1; columns 1 and 2) are very similar to those generated in the same stock in 1986 (columns 5 and 6). Thus, a decrease of l-hyoscyamine binding after diazepam is clearly demonstrated. When the experiment is performed on the new stock of mice, a marginal, non-significant, decrease is observed. Therefore, and in order to find optimal parameters, the model will need further development in the new stock of mice.

In order to test the ability of the model to detect muscarinic receptors of specific subtypes, we performed experiments on the interaction of scopolamine with l-hyoscyamine binding. Thus, after establishing stable l-hyoscyamine binding in brain *in vivo* (2 mg/kg, i.v.; 2 h), it should be possible to displace this binding with different subreceptor specific ligands for mapping of regions in brain rich in such subtypes (7). In our first experiments, we have used the unspecific ligand scopolamine

for such studies in order to test the feasibility of the experimental design. When scopolamine, 4 mg/kg i.v., was given 1 min before or 1 min after l-hyoscyamine and its concentration was analyzed 2 h later, about 30 ng/g were found (compared to about 60 ng/g in controls). If, instead, scopolamine was given 1 h 50 min after l-hyoscyamine, no displacement was obtained. The same held true if the scopolamine dose was increased to 50 mg/kg. Therefore, the design of the experiment was changed and displacing doses (50 mg/kg i.v.) of scopolamine were given 1 h 50 min, 1 h 30 min and 1 h after l-hyoscyamine. The results of these experiments are depicted in Figure 2. It can be seen that the displacement is time-dependent and proceeds to about 30 ng/g when treatment is allowed to last for 1 h (column 4). This is probably the highest amount possible to displace since pretreatment with scopolamine gave the same level. This type of experiment will be carried out using receptor specific ligands.

These experiments confirm the concept that l-hyoscyamine binding in vivo represents specific binding to muscarinic receptors possible to displace with other antimuscarinic receptor ligands. The experiments were performed to enable study of, e.g., the size of this receptor pool and its dependence on chronic stimulation or blockade. In order to study the latter phenomenon, mice were treated with daily i.p. doses (25 mg/kg) of scopolamine for 12 days. They were then left untreated for 2 days, whereafter they were injected with l-hyoscyamine i.v. (2 mg/kg), sacrificed 2 h later and analyzed for brain l-hyoscyamine. Concentrations in six animals (62, 51, 51, 56, 59, and 53 ng/g; mean 55 ng/g) were marginally reduced compared to concentrations in two control animals (58, 60 ng/g). The control values are consistent with those previously reported. The experimental design was very similar to one published, in which animals (rat) treated chronically in vivo with scopolamine were analyzed for specific binding in vitro (8). The lack of demonstrable increase in receptor pool size in our experiments may depend on residual bound scopolamine masking the increase of the pool size. To investigate

this possibility, scopolamine concentrations in brain will be measured under the same conditions.

Antidotes and acetylcholine turnover

In accordance with our plans, we have studied the effect of combinations of antidotes on central cholinergic mechanisms in mice. We have studied l-hyoscyamine, diazepam and HI-6 separately and in different combinations. Data on acetylcholine turnover after administration of saline, diazepam (1 mg/kg, i.v.), l-hyoscyamine (2 mg/kg, i.v.), or HI-6 (25 mg/kg, i.v.) are collected in Table 1.

As previously shown by us and others, l-hyoscyamine decreases the endogenous level of ACh; presumably ACh output is increased (9,10). However, when diazepam is added to the injection solution, the endogenous ACh levels are normalized. Diazepam separately does not affect, or very slightly affects (increase), the ACh level. The endogenous choline (Ch) levels are increased after administration of diazepam, l-hyoscyamine, and a combination of the two.

The concentration of [$^2\text{H}_6$]-ACh is markedly decreased in animals administered diazepam and diazepam in combination with l-hyoscyamine, while l-hyoscyamine alone does not seem to have any effect. The specific activity of [$^2\text{H}_6$]-ACh (S_{ACh}) is decreased to the same extent in animals treated with diazepam and those treated with the combination diazepam/l-hyoscyamine, while an increase is seen in animals administered l-hyoscyamine alone.

The increased K_d value seen in animals pretreated with l-hyoscyamine indicates an increased synthesis rate of ACh, which is consistent with earlier findings in our laboratory and with literature data. If the increased release of ACh is a sustained effect, the synthesis rate has to be increased in order to balance the increased release and maintain the new steady state. The net TR_{ACh} is, however, kept constant since after

l-hyoscyamine a smaller amount of ACh has to be renewed. Diazepam lowers the turnover rate of ACh, as we have shown previously (11). Interestingly, the combination of diazepam and l-hyoscyamine decreases TR_{ACh} to the same extent as diazepam alone does, which is in great contrast to the effect of l-hyoscyamine alone. Thus, the effects of diazepam dominate in this combination of doses. The increased turnover of ACh after atropine is probably an unfavourable feature of a protecting agent. These results may therefore, at least partly explain the advantage of combining atropine with diazepam in cases of nerve gas poisoning.

HI-6 alone induces a marginally increasing effect on the acetylcholine level, a statistically significant increasing effect on choline levels and a slight increasing effect on acetylcholine turnover.

When the three treatments are combined in the same injection, the dominant result is an increase of choline levels, with a slight decrease of the acetylcholine turnover. In this last combination, the differing effects of diazepam and l-hyoscyamine on acetylcholine level seem to be balanced. The choline level is increased and the mole ratio of labelled choline is reduced, reflecting the effects of diazepam, l-hyoscyamine and HI-6. Consequently, the acetylcholine turnover is somewhat decreased. The aim of these experiments has been to collect background data on the protective effect of this three-drug combination on soman poisoning and the correlation of the effect with acetylcholine dynamics.

An important issue for the mechanism of protection with antidotes is the duration of effect and how this correlates with the dynamics of acetylcholine. We have already found that l-hyoscyamine concentration persists for a long time (up to at least 30 h). We know from our experiments that the concentration in brain of diazepam is much more short-lived. When high doses of diazepam (25-35 mg/kg) are given, the levels of diazepam are down to a few $\mu\text{g/g}$ after 30 min. Thus, the half-life is very short, which is in accordance with previous findings (12).

In the above experiments, diazepam was given 10 min before [$^2\text{H}_6$]-Ch and its effect on acetylcholine turnover evaluated 20 and 50 sec later. Experiments have also been performed to study the duration of this effect (Table 2). After 20, 30 and 45 min, levels of acetylcholine are significantly increased, while they are returning to normal after 4 h. The tendency for an increase seen at 10 min is, thus, fully developed at 20 min and persists for at least 45 min. The levels of choline also stay elevated for 45 min, but have become normalized after 4 h. The fractional rate constant is maximally decreased after 10 min and seems to be somewhat lowered, still, at 4 h. The data tend to indicate a duration of effect on the acetylcholine dynamics (K_a) of at least 4 h.

CONCLUSIONS

Studies of specific binding of l-hyoscyamine to muscarinic receptors in the mouse brain have been made in stocks of old healthy and new healthy mice. Displacement of l-hyoscyamine with diazepam seen in old "sick" stock was repeated in old healthy stock. Optimal experimental conditions for new stock have yet to be found.

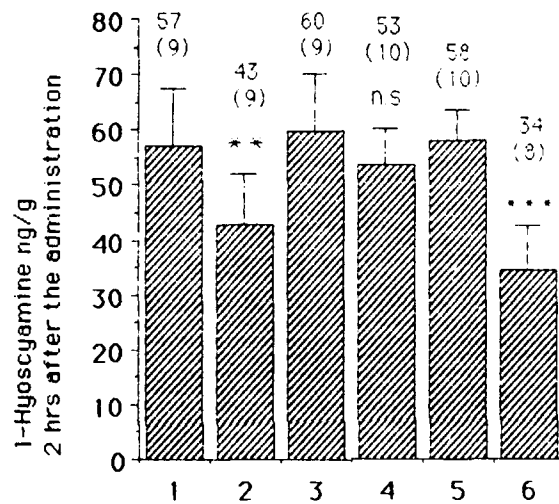
The study of muscarinic subreceptor pools in mouse brain has started. It has been shown possible to displace about 50% of l-hyoscyamine either by pretreatment with scopolamine or by displacement ("chase") of an established concentration by treatment with scopolamine for 1 h.

The effect of l-hyoscyamine, diazepam and HI-6 on acetylcholine turnover has been studied separately after each antidote and after combinations of these blockers. The effect of l-hyoscyamine and diazepam given separately has been reported earlier. HI-6 increases endogenous choline levels and slightly increases the turnover of acetylcholine. The combination of the three antidotes increases choline and slightly decreases the turnover of acetylcholine.

The effect of diazepam on acetylcholine turnover lasts for about 4 h. The effect on choline and acetylcholine levels lasts for about 1 h.

FIGURE 1

Effect of diazepam on specific binding of l-hyoscyamine in brain of old and new mouse stocks. The animals were pretreated with saline or diazepam (1 mg/kg, i.v.) 2 min before the injection of l-hyoscyamine (2 mg/kg, i.v.). Diazepam is given as Diazemuls^R.



- 1) Old mouse stock saline (healthy progeny)
- 2) Old mouse stock Diazemuls (healthy progeny)
- 3) New mouse stock saline
- 4) New mouse stock Diazemuls
- 5) 1986 values saline (old stock of sick mice)
- 6) 1986 values Diazemuls (old stock of sick mice)

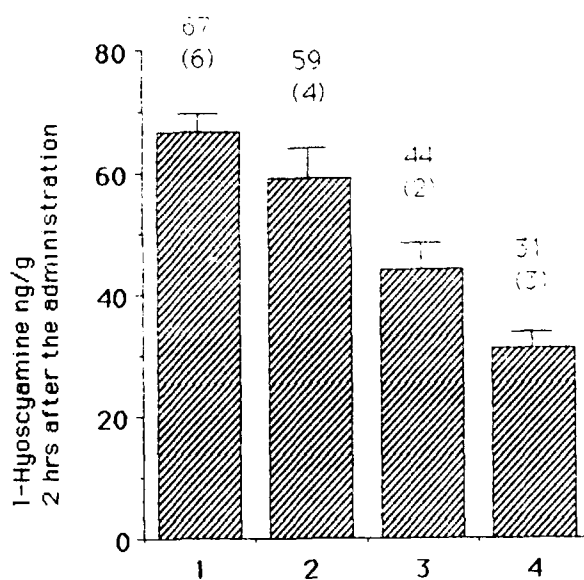
Two-tailed Student's t-test of saline treatment means in comparison to diazepam treatment means.

*** $2p < 0.001$, ** $2p < 0.01$, n.s. = not significant.

Figures are mean values, with number of animals within parentheses. The bars show the standard deviation.

FIGURE 2

Influence of scopolamine on specific binding of l-hyoscyamine in mouse brain. The animals were administered l-hyoscyamine (2 mg/kg, i.v.) and injected scopolamine (50 mg/kg, i.v.) 1 h 50 min, 1 h 30 min or 1 h later.



- 1) Controls
- 2) Scopolamine 1 h 50 min after l-hyoscyamine
- 3) Scopolamine 1 h 30 min after l-hyoscyamine
- 4) Scopolamine 1 h after l-hyoscyamine

Figures are mean values, with number of animals within parentheses. The bars show the standard deviation.

TABLE 1

Effect of diazepam, l-hyoscyamine and HI-6 on dynamics of acetylcholine in mouse brain. Concentration (nmol/g) of endogenous and [$^2\text{H}_6$]-substituted ACh and Ch, specific activity (mole ratio, S) of [$^2\text{H}_6$]-ACh and [$^2\text{H}_6$]-Ch, fractional rate constant (K_a , min^{-1}) and turnover rate of ACh (TR_{ACh} , nmol/gxmin) in whole brain of mice injected intravenously with 10 $\mu\text{mol/kg}$ [$^2\text{H}_6$]-Ch and sacrificed 20 and 50 sec later. Prior to the [$^2\text{H}_6$]-Ch injection, the mice were pretreated for 10 min with saline, diazepam 1 mg/kg i.v., l-hyoscyamine 2 mg/kg i.v., a combination of diazepam and l-hyoscyamine, HI-6 25 mg/kg i.v. or a combination of diazepam, l-hyoscyamine and HI-6.

TREATMENT									
Saline i.v., 10 min prior to [$^2\text{H}_6$]-Ch	Diazepam 1 mg/kg i.v., 10 min prior to [$^2\text{H}_6$]-Ch	l-Hyoscyamine 2 mg/kg i.v., 10 min prior to [$^2\text{H}_6$]-Ch	l-Hyoscyamine 2 mg/kg i.v., Diazepam 1 mg/kg i.v., 10 min prior to [$^2\text{H}_6$]-Ch	HI-6 25 mg/kg i.v., 10 min prior to [$^2\text{H}_6$]-Ch	HI-6 25 mg/kg i.v., l-Hyoscyamine 2 mg/kg i.v., Diazepam 1 mg/kg i.v., 10 min prior to [$^2\text{H}_6$]-Ch				
23.5 $\pm$ 3.3 (10)	26.0 $\pm$ 4.4 (10) n.s.	17.3 $\pm$ 2.5 (10)***	22.9 $\pm$ 1.9 (9) n.s.	26.3 $\pm$ 1.9 (9)*	24.2 $\pm$ 1.7 (9) n.s.				
22.8 $\pm$ 3.1 (10)	24.2 $\pm$ 2.5 (10) n.s.	18.4 $\pm$ 3.6 (10)**	21.1 $\pm$ 1.5 (10) n.s.	23.5 $\pm$ 2.1 (10) n.s.	22.6 $\pm$ 2.3 (10) n.s.				
29.5 $\pm$ 3.5 (10)	40.0 $\pm$ 6.5 (9)***	40.4 $\pm$ 3.7 (10)***	40.6 $\pm$ 1.9 (9)***	35.9 $\pm$ 3.5 (9)***	41.9 $\pm$ 4.2 (9)***				
29.5 $\pm$ 4.9 (10)	38.6 $\pm$ 3.3 (10)***	39.1 $\pm$ 4.2 (10)***	40.2 $\pm$ 5.1 (10)***	34.6 $\pm$ 2.5 (10)**	41.8 $\pm$ 4.6 (10)***				
0.261 $\pm$ 0.033 (10)	0.168 $\pm$ 0.042 (9)***	0.226 $\pm$ 0.058 (9) n.s.	0.145 $\pm$ 0.019 (9)***	0.217 $\pm$ 0.027 (7)*	0.128 $\pm$ 0.020 (8)***				
0.525 $\pm$ 0.088 (9)	0.287 $\pm$ 0.045 (8)***	0.510 $\pm$ 0.150 (10) n.s.	0.272 $\pm$ 0.065 (10)***	0.532 $\pm$ 0.060 (8)	0.278 $\pm$ 0.058 (10)***				
3.51 $\pm$ 0.69 (10)	3.78 $\pm$ 1.82 (9) n.s.	4.64 $\pm$ 1.94 (8) n.s.	3.65 $\pm$ 1.53 (9) n.s.	4.15 $\pm$ 0.76 (7) n.s.	2.58 $\pm$ 0.66 (8)*				
2.69 $\pm$ 0.76 (9)	2.96 $\pm$ 1.01 (8) n.s.	3.28 $\pm$ 1.14 (10) n.s.	3.29 $\pm$ 1.09 (10) n.s.	3.01 $\pm$ 0.64 (8) n.s.	2.69 $\pm$ 0.76 (10) n.s.				
0.0114 $\pm$ 0.0020 (10)	0.00642 $\pm$ 0.00130 (9)***	0.0133 $\pm$ 0.0032 (9) n.s.	0.00620 $\pm$ 0.00071 (9)***	0.00856 $\pm$ 0.00158 (7)**	0.00546 $\pm$ 0.00139 (8)***				
0.0237 $\pm$ 0.0065 (9)	0.0120 $\pm$ 0.0022 (8)***	0.0267 $\pm$ 0.0059 (10) n.s.	0.0125 $\pm$ 0.0014 (10)***	0.0220 $\pm$ 0.0030 (8) n.s.	0.0122 $\pm$ 0.0024 (10)***				
0.107 $\pm$ 0.025 (10)	0.0873 $\pm$ 0.0425 (8) n.s.	0.108 $\pm$ 0.039 (9) n.s.	0.0832 $\pm$ 0.0332 (9) n.s.	0.104 $\pm$ 0.022 (7) n.s.	0.0611 $\pm$ 0.0130 (8)***				
0.0910 $\pm$ 0.0285 (9)	0.0710 $\pm$ 0.0240 (8) n.s.	0.0762 $\pm$ 0.0212 (10) n.s.	0.0770 $\pm$ 0.0238 (10) n.s.	0.0820 $\pm$ 0.0150 (8)	0.0594 $\pm$ 0.0119 (10)**				
0.30	0.16	0.37	0.18	0.35	0.26				
6.95	4.02	6.60	3.96	8.72	6.08				

with number of animals within parentheses.

* $P < 0.001$, n.s. = not significant, two-tailed Student's t-test of treatment means.

TABLE 1

Effect of diazepam, l-hyoscyamine and HI-6 on dynamics of acetylcholine in mouse brain. Concentration (nmol/g) of endogenous and [$^2\text{H}_6$]-substituted ACh and Ch, specific activity (mole ratio, S) of [$^2\text{H}_6$]-ACh and [$^2\text{H}_6$]-Ch, fractional rate constant (K_a , min^{-1}) and turnover rate of ACh (TR_{ACh} , nmol/gxmin) in whole brain of mice injected intravenously with 10 $\mu\text{mol/kg}$ [$^2\text{H}_6$]-Ch and sacrificed 20 and 50 sec later. Prior to the [$^2\text{H}_6$]-Ch injection, the mice were pretreated for 10 min with saline, diazepam 1 mg/kg i.v., l-hyoscyamine 2 mg/kg i.v., a combination of diazepam and l-hyoscyamine, HI-6 25 mg/kg i.v. or a combination of diazepam, l-hyoscyamine and HI-6.

TREATMENT

	Time sec	Saline i.v. 10 min prior to [$^2\text{H}_6$]-Ch	Diazepam 1 mg/kg i.v. 10 min prior to [$^2\text{H}_6$]-Ch	l-Hyoscyamine 2 mg/kg i.v. 10 min prior to [$^2\text{H}_6$]-Ch	l-Hyoscyamine 2 mg/kg i.v. Diazepam 1 mg/kg i.v. 10 min prior to [$^2\text{H}_6$]-Ch	HI-6 25 mg/kg i.v. 10 min prior to [$^2\text{H}_6$]-Ch
ACh	20	23.5 $\pm$ 3.3 (10)	26.0 $\pm$ 4.4 (10) ^{n.s.}	17.3 $\pm$ 2.5 (10) ^{***}	22.9 $\pm$ 1.9 (9) ^{n.s.}	26.3 $\pm$ 1.9
	50	22.8 $\pm$ 3.1 (10)	24.2 $\pm$ 2.5 (10) ^{n.s.}	18.4 $\pm$ 3.6 (10) ^{**}	21.1 $\pm$ 1.5 (10) ^{n.s.}	23.5 $\pm$ 2.1
Ch	20	29.5 $\pm$ 3.5 (10)	40.0 $\pm$ 6.5 (9) ^{***}	40.4 $\pm$ 3.7 (10) ^{***}	40.6 $\pm$ 1.9 (9) ^{***}	35.9 $\pm$ 3.5
	50	29.5 $\pm$ 4.9 (10)	38.6 $\pm$ 3.3 (10) ^{***}	39.1 $\pm$ 4.2 (10) ^{***}	40.2 $\pm$ 5.1 (10) ^{***}	34.6 $\pm$ 2.5
[$^2\text{H}_6$]-ACh	20	0.261 $\pm$ 0.033 (10)	0.168 $\pm$ 0.042 (9) ^{***}	0.226 $\pm$ 0.058 (9) ^{n.s.}	0.145 $\pm$ 0.019 (9) ^{***}	0.217 $\pm$ 0.027
	50	0.525 $\pm$ 0.088 (9)	0.287 $\pm$ 0.045 (8) ^{***}	0.510 $\pm$ 0.150 (10) ^{n.s.}	0.272 $\pm$ 0.065 (10) ^{***}	0.532 $\pm$ 0.060
[$^2\text{H}_6$]-Ch	20	3.51 $\pm$ 0.69 (10)	3.78 $\pm$ 1.82 (9) ^{n.s.}	4.64 $\pm$ 1.94 (8) ^{n.s.}	3.65 $\pm$ 1.53 (9) ^{n.s.}	4.15 $\pm$ 0.76
	50	2.69 $\pm$ 0.76 (9)	2.96 $\pm$ 1.01 (8) ^{n.s.}	3.28 $\pm$ 1.14 (10) ^{n.s.}	3.29 $\pm$ 1.09 (10) ^{n.s.}	3.01 $\pm$ 0.64
S _{ACh}	20	0.0114 $\pm$ 0.0020 (10)	0.00642 $\pm$ 0.00130 (9) ^{***}	0.0133 $\pm$ 0.0032 (9) ^{n.s.}	0.00620 $\pm$ 0.00071 (9) ^{***}	0.00856 $\pm$ 0.00158
	50	0.0237 $\pm$ 0.0065 (9)	0.0120 $\pm$ 0.0022 (8) ^{***}	0.0267 $\pm$ 0.0059 (10) ^{n.s.}	0.0125 $\pm$ 0.0014 (10) ^{***}	0.0220 $\pm$ 0.0030
S _{Ch}	20	0.107 $\pm$ 0.025 (10)	0.0873 $\pm$ 0.0425 (8) ^{n.s.}	0.108 $\pm$ 0.039 (9) ^{n.s.}	0.0832 $\pm$ 0.0332 (9) ^{n.s.}	0.104 $\pm$ 0.022
	50	0.0910 $\pm$ 0.0285 (9)	0.0710 $\pm$ 0.0240 (8) ^{n.s.}	0.0762 $\pm$ 0.0212 (10) ^{n.s.}	0.0770 $\pm$ 0.0238 (10) ^{n.s.}	0.0820 $\pm$ 0.0150
K_a		0.30	0.16	0.37	0.18	0.35
TR _{ACh}		6.95	4.02	6.60	3.96	8.72

The data are mean $\pm$ SD, with number of animals within parentheses.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant, two-tailed Student's t-test of treatment means in comparison to saline means.

TABLE 2

Duration of effect of diazepam on dynamics of acetylcholine in mouse brain. Concentration (nmol/g) of endogenous and [^3H]-substituted ACh and Ch, specific activity (mole ratio, S) of [^3H]-ACh and [^3H]-Ch, fractional rate constant ($k_a \cdot \text{min}^{-1}$) and turnover rate of ACh (TR_{ACh} , nmol/gxmin) in whole brain of mice injected intravenously with 10 $\mu\text{mol/kg}$ [^3H]-ACh and sacrificed 20 and 50 sec later. Prior to the [^3H]-ACh injection, the mice were pretreated with saline (10 min) or diazepam (10, 20, 30, 45 min or 4 h).

TREATMENT

Saline i.v. 10 min prior to [^3H]-Ch	Diazepam 1 mg/kg i.v. 10 min prior to [^3H]-Ch	Diazepam 1 mg/kg i.v. 20 min prior to [^3H]-Ch	Diazepam 1 mg/kg i.v. 30 min prior to [^3H]-Ch	Diazepam 1 mg/kg i.v. 45 min prior to [^3H]-Ch	Diazepam 1 mg/kg i.v. 4 h prior to [^3H]-Ch
23.5 $\pm$ 3.3 (10)	26.0 $\pm$ 4.4 (10) ^{n.s.}	29.3 $\pm$ 2.3 (5)**	29.0 $\pm$ 1.1 (6)**	28.4 $\pm$ 1.1 (7)**	26.9 $\pm$ 2.9 (6) ^{n.s.}
22.8 $\pm$ 3.1 (10)	24.2 $\pm$ 2.5 (10) ^{n.s.}	29.3 $\pm$ 1.8 (5)**	29.4 $\pm$ 2.6 (6)**	29.4 $\pm$ 1.3 (5)**	28.3 $\pm$ 2.0 (6)**
29.5 $\pm$ 3.5 (10)	40.0 $\pm$ 6.5 (9)**	40.3 $\pm$ 2.9 (6)**	38.0 $\pm$ 2.6 (6)**	45.9 $\pm$ 7.0 (7)**	32.5 $\pm$ 3.7 (6) ^{n.s.}
29.5 $\pm$ 4.9 (10)	38.6 $\pm$ 3.3 (10)**	42.6 $\pm$ 1.7 (5)**	38.4 $\pm$ 3.6 (6)**	45.2 $\pm$ 7.1 (5)**	32.4 $\pm$ 3.1 (6) ^{n.s.}
0.261 $\pm$ 0.033 (10)	0.168 $\pm$ 0.042 (9)**	0.144 $\pm$ 0.017 (5)**	0.176 $\pm$ 0.059 (6)**	0.143 $\pm$ 0.015 (6)**	0.194 $\pm$ 0.042 (6)**
0.525 $\pm$ 0.088 (9)	0.287 $\pm$ 0.045 (8)**	0.304 $\pm$ 0.042 (5)**	0.357 $\pm$ 0.074 (6)**	0.292 $\pm$ 0.011 (5)**	0.381 $\pm$ 0.091 (6)**
3.51 $\pm$ 0.69 (10)	3.78 $\pm$ 1.82 (9) ^{n.s.}	2.28 $\pm$ 1.04 (5)*	2.73 $\pm$ 0.46 (6)*	2.14 $\pm$ 0.51 (6)**	2.60 $\pm$ 0.38 (6)*
2.69 $\pm$ 0.76 (9)	2.96 $\pm$ 1.01 (8) ^{n.s.}	2.33 $\pm$ 0.26 (5) ^{n.s.}	1.89 $\pm$ 0.49 (6)*	1.94 $\pm$ 0.78 (5) ^{n.s.}	2.18 $\pm$ 0.43 (6) ^{n.s.}
0.0114 $\pm$ 0.0020 (10)	0.00642 $\pm$ 0.00130 (9)**	0.00486 $\pm$ 0.00066 (5)**	0.00594 $\pm$ 0.00169 (6)**	0.00503 $\pm$ 0.00062 (6)**	0.00731 $\pm$ 0.00209 (6)**
0.0237 $\pm$ 0.0065 (9)	0.0120 $\pm$ 0.0022 (8)**	0.0106 $\pm$ 0.0011 (5)**	0.0122 $\pm$ 0.0027 (6)**	0.00982 $\pm$ 0.00122 (5)**	0.0140 $\pm$ 0.0045 (6)**
0.107 $\pm$ 0.025 (10)	0.0873 $\pm$ 0.0425 (8) ^{n.s.}	0.0620 $\pm$ 0.0068 (6)**	0.0661 $\pm$ 0.0125 (6)**	0.0461 $\pm$ 0.0086 (6)**	0.0753 $\pm$ 0.0975 (6)**
0.0910 $\pm$ 0.0285 (9)	0.0710 $\pm$ 0.0240 (8) ^{n.s.}	0.0513 $\pm$ 0.0044 (5)**	0.0470 $\pm$ 0.0150 (6)**	0.0413 $\pm$ 0.0090 (5)**	0.0651 $\pm$ 0.0138 (6) ^{n.s.}
0.30	0.16	0.23	0.26	0.26	0.22
6.95	4.02	6.74	7.59	7.51	6.20

*The number of animals within parentheses.
n.s., n.s. = not significant, two-tailed Student's t-test of treatment means.

TABLE 2

Duration of effect of diazepam on dynamics of acetylcholine in mouse brain. Concentration (nmol/g) of endogenous $[^3\text{H}_6]$ -substituted ACh and Ch, specific activity (mole ratio, S) of $[^3\text{H}_6]$ -ACh and $[^3\text{H}_6]$ -Ch, fractional rate constant (λ_a , min^{-1}) and turnover rate of ACh (TR_{ACh}, nmol/gxmin) in whole brain of mice injected intravenously with 10 $\mu\text{mol/kg}$ $[^3\text{H}_6]$ -Ch and sacrificed 20 and 50 sec later. Prior to the $[^3\text{H}_6]$ -Ch injection, the mice were pretreated with saline (10 min) or diazepam (10, 20, 30, 45 min or 4 h).

TREATMENT

	Time sec	Saline i.v. 10 min prior to $[^3\text{H}_6]$ -Ch	Diazepam 1 mg/kg i.v. 10 min prior to $[^3\text{H}_6]$ -Ch	Diazepam 1 mg/kg i.v. 20 min prior to $[^3\text{H}_6]$ -Ch	Diazepam 1 mg/kg i.v. 30 min prior to $[^3\text{H}_6]$ -Ch	Diazepam 1 mg/kg i.v. 45 min prior to $[^3\text{H}_6]$ -Ch
ACh	20	23.5 $\pm$ 3.3 (10)	26.0 $\pm$ 4.4 (10) ^{n.s.}	29.3 $\pm$ 2.3 (5) ^{**}	29.0 $\pm$ 1.1 (6) ^{**}	28.4 $\pm$ 1.1 (6) ^{**}
	50	22.8 $\pm$ 3.1 (10)	24.2 $\pm$ 2.5 (10) ^{n.s.}	29.3 $\pm$ 1.8 (5) ^{***}	29.4 $\pm$ 2.6 (6) ^{***}	29.4 $\pm$ 1.3 (6) ^{***}
Ch	20	29.5 $\pm$ 3.5 (10)	40.0 $\pm$ 6.5 (9) ^{***}	40.3 $\pm$ 2.9 (6) ^{***}	38.0 $\pm$ 2.6 (6) ^{***}	45.9 $\pm$ 7.0 (6) ^{***}
	50	29.5 $\pm$ 4.9 (10)	38.6 $\pm$ 3.3 (10) ^{***}	42.6 $\pm$ 1.7 (5) ^{***}	38.4 $\pm$ 3.6 (6) ^{***}	45.2 $\pm$ 7.1 (6) ^{***}
$[^3\text{H}_6]$ -ACh	20	0.261 $\pm$ 0.033 (10)	0.168 $\pm$ 0.042 (9) ^{***}	0.144 $\pm$ 0.017 (5) ^{***}	0.176 $\pm$ 0.039 (6) ^{***}	0.143 $\pm$ 0.015 (6) ^{***}
	50	0.525 $\pm$ 0.088 (9)	0.287 $\pm$ 0.045 (8) ^{***}	0.304 $\pm$ 0.042 (5) ^{***}	0.357 $\pm$ 0.074 (6) ^{***}	0.292 $\pm$ 0.011 (6) ^{***}
$[^3\text{H}_6]$ -Ch	20	3.51 $\pm$ 0.69 (10)	3.78 $\pm$ 1.82 (9) ^{n.s.}	2.28 $\pm$ 1.04 (6) [*]	2.73 $\pm$ 0.46 (6) [*]	2.14 $\pm$ 0.51 (6) [*]
	50	2.69 $\pm$ 0.76 (9)	2.96 $\pm$ 1.01 (8) ^{n.s.}	2.33 $\pm$ 0.26 (5) ^{n.s.}	1.89 $\pm$ 0.49 (6) [*]	1.94 $\pm$ 0.78 (6) [*]
S _{ACh}	20	0.0114 $\pm$ 0.0020 (10)	0.00642 $\pm$ 0.00130 (9) ^{***}	0.00486 $\pm$ 0.00066 (5) ^{***}	0.00594 $\pm$ 0.00169 (6) ^{***}	0.00503 $\pm$ 0.00062 (6) ^{***}
	50	0.0237 $\pm$ 0.0065 (9)	0.0120 $\pm$ 0.0022 (8) ^{***}	0.0106 $\pm$ 0.0011 (5) ^{***}	0.0122 $\pm$ 0.0027 (6) ^{***}	0.00982 $\pm$ 0.00122 (6) ^{***}
S _{Ch}	20	0.107 $\pm$ 0.025 (10)	0.0873 $\pm$ 0.0425 (8) ^{n.s.}	0.0620 $\pm$ 0.0068 (6) ^{***}	0.0661 $\pm$ 0.0125 (6) ^{***}	0.0461 $\pm$ 0.0086 (6) ^{***}
	50	0.0910 $\pm$ 0.0285 (9)	0.0710 $\pm$ 0.0240 (8) ^{n.s.}	0.0513 $\pm$ 0.0044 (5) ^{**}	0.0470 $\pm$ 0.0150 (6) ^{**}	0.0413 $\pm$ 0.0090 (6) ^{**}
K _a		0.30	0.16	0.23	0.26	0.26
TR _{ACh}		6.95	4.02	6.74	7.59	7.51

The data are mean $\pm$ SD, with number of animals within parentheses.
^{*} p<0.05, ^{**} p<0.01, ^{***} p<0.001, n.s. = not significant, two-tailed Student's t-test of treatment means in comparison to saline means.

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ABBREVIATIONS

Ch, Choline

GC-MS, Gas chromatography - mass spectrometry

DPA, 2,4,6,2',4',6'-Hexanitrodiphenylamine

TR_{ACh}, Acetylcholine turnover

S_{ACh}, Specific activity of [²H₆]ACh

S_{Ch}, Specific activity of [²H₆]Ch

BSA, N,O-Bis(trimethylsilyl)acetamide

HI-6, [[[4-amino-carbonyl]pyridino]methoxy]methyl]-2-
[(hydroxyimino)methyl]-pyridinium dichloride

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