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Metrifonate improves spatial navigation and avoidance behavior in scopolamine-treated, medial septum-lesioned and aged rats

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Abstract

We investigated the effects of acute p.o. pretraining treatment with an indirect acetylcholinesterase inhibitor, metrifonate, on water maze spatial navigation and passive avoidance behavior. Metrifonate (10–100 mg/kg, orally, p.o.) did not improve the water maze or passive avoidance performance of young intact rats. However, in young rats metrifonate over a broad dosage range (10–100 mg/kg, p.o.) was able to alleviate the adverse effects of scopolamine (a muscarinic acetylcholine receptor antagonist; 0.4 and 2.0 mg/kg in water maze and passive avoidance study, respectively) and medial septum-lesioning on spatial reference and working memory and passive avoidance performance. In old (23-month-old) rats, a defect of water maze and passive avoidance behavior was observed. In old rats, metrifonate improved spatial reference memory function in the water maze and also passive avoidance at 10–30 mg/kg, but the 3 mg/kg dose was ineffective. Very old (27-month-old) rats had a more severe impairment of water maze performance than old rats, and metrifonate 3–30 mg/kg did not improve their spatial navigation. These results show that metrifonate may over a wide range of doses stimulate cognitive functioning, but during advanced aging neurobiological defects develop that may mask some of the therapeutic effects of metrifonate in rats.

Keywords: Scopolamine; Medial septum lesion; Aging; Metrifonate; Spatial navigation; Passive avoidance

1. Introduction

Studies conducted with brain samples from patients with Alzheimer's disease have shown a consistent and marked loss of cholinergic cells in the basal forebrain (Whitehouse et al., 1982; Bowen et al., 1983; Bartus et al., 1985; Bowen and Davison, 1986). Clinical and experimental studies support the involvement of the degeneration of cholinergic cells in the development of cognitive disorders associated with Alzheimer's disease (Bartus, 1979; Dunnett et al., 1991; Fibiger, 1991; Bowen et al., 1992; Dunnett and Fibiger, 1993). Much interest in cholinesterase inhibitors as therapeutic agents for Alzheimer's disease has been generated following the reports of cognitive facilitation after treatment with tacrine (Eagger et al., 1991; Eagger et al., 1992; Knapp et al., 1992). Tacrine acts by inhibiting the activity of acetylcholinesterase, the enzyme that hydrolyses acetylcholine. However, the clinical use of tacrine is limited, as it very frequently causes serious, dose-dependent, side-effects (Watkins et al., 1994). Furthermore, the therapeutic action of tacrine is, at best, only modest (Byrne and Arie, 1994). Therefore, novel compounds that could enhance the function of damaged cholinergic cells in the basal forebrain would be of therapeutic importance.

There are different animal cognition models available if one wishes to assess the effects of drugs on behavioral functions (Hagan and Morris, 1988; Wenk, 1993). One pharmacological model to study the efficacy of novel cholinesterase inhibitors is to investigate if novel compounds can stimulate spatial memory and avoidance behavior in scopolamine (a muscarinic acetylcholine receptor antagonist)-treated and medial septum-lesioned young rats or aged rats (Decker, 1987; Hagan and Morris, 1988; Riekkinen Jr. et al., 1991a,b; Levine, 1992). Several studies have described that drugs that activate cholinergic cells

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may stimulate test performance in these animal models of cholinergic denervation and aging. For example, we have described in our model systems that Tacrine at 3 mg/kg facilitated water maze reference memory and passive avoidance behavior of medial septum-lesioned and aged rats (Riekkinen Jr. et al., 1990, 1991a,b, 1994). However, the dose-response curve had an inverted U shape so that slightly smaller (1 mg/kg) or higher (5 mg/kg) doses of tacrine were unable to stimulate behavior. Furthermore, tacrine did not stimulate reversal learning in the water maze in young medial septum-lesioned or aged rats, suggesting that tacrine treatment may not be able to enhance working memory (Riekkinen Jr. et al., 1991a).

Metrifonate is a member of the second generation of cholinesterase inhibitors with an indirect mechanism of action, good tolerability and long duration of action (Holmstedt et al., 1978; Nordgren et al., 1978; Becker and Giacobini, 1988). The compound is hydrolyzed in aqueous solutions and rearranges to yield the long-acting cholinesterase inhibitor, dichlorvos (Hinz et al., 1996a,b). Recent studies have suggested that metrifonate treatment can enhance acetylcholine release and facilitate cognitive functioning (Blokland et al., 1996; Mori et al., 1995; Nabeshima et al., 1995). This result suggests that metrifonate may represent a potential treatment for cognitive disorders associated with Alzheimer's disease.

The aim of this study was to evaluate the efficacy of acute oral (p.o.) treatment with metrifonate on passive avoidance and water maze reference/working memory performance. We investigated the effect of metrifonate treatment at different doses on spatial and avoidance behavior of young intact, scopolamine-treated and medial septum-lesioned rats, and also of aged rats.

2. Materials and methods

2.1. Animals

Young (3-month-old; $n = 128$), old (23-month-old; $n = 32$) and very old (27-month-old; $n = 32$) male Han:Wistar rats were used in the present study. The rats were housed 3 per cage in a controlled environment (temperature: 20 ± 2°C; humidity: 50–60%; light period: 07:00–19:00 h). Food and water were available ad libitum.

2.2. Drugs

Metrifonate was donated by Troponwerke & Co. and was dissolved in 5% sodium citrate (pH 5.5, buffered with citric acid) and injected orally (p.o.) 30 min before water maze testing. Metrifonate was administered to aged rats at 3, 10 and 30 mg/kg and to young rats at 10, 30 and 100 mg/kg. Previously aged rats have been shown to be biochemically (Hinz et al., 1996a), behaviorally (Blokland et al., 1996) and metabolically (Bassant et al., 1995) more sensitive to metrifonate than young rats. Scopolamine hydrobromide (0.4 or 2 mg/kg) (Merck) was dissolved in NaCl 0.9% and injected intraperitoneally (i.p.) 35 min before daily water maze or passive avoidance training. No drug or vehicle treatment was given on the passive avoidance testing day. Young, old and very old controls received appropriate single or combined metrifonate and scopolamine vehicle injections (p.o., p.o. + i.p.) of equal volume. Some groups of young rats were treated with scopolamine. Scopolamine 0.4 mg/kg was used in water maze and 2.0 mg/kg in passive avoidance studies, as we have earlier found that treatment at these doses disrupts behavior in these tests.

2.3. Surgery

Young rats were used for the brain-lesioning experiment. Medial septum (A: 0.0 mm, M: 0.0 mm, D: − 7.0 mm; relative to the bregma) lesions were made by passing an anodal DC current (2 mA, 5 s) via stainless steel electrodes (Riekkinen Jr. et al., 1990, 1991a,b). Controls were treated identically, but no current was applied (control-lesioned). Rats were deeply anesthetized with equithesin during the operations.

2.4. Water maze

The starting locations, which were labelled north, south, east and west, were located arbitrarily on the pool rim (Riekkinen Jr. et al., 1990). The temperature of the water was 23 ± 1.5°C The timing of the latency to find the submerged platform was started and ended by the experimenter. The swim pattern and distance were monitored by a computerized videotracking device. The computer calculated and stored the total distance swum (in cm) and also the proportion of distance swum in different quadrants or annuli of the pool. Rats were placed in the water, with their noses pointing towards the wall, at one of the starting points in a random manner.

The training schedule of the young rats consisted of 4 consecutive days of testing. Five platform trials of 70 s were assessed per day during the first 3 training days. The platform location was kept constant (in the southwest quadrant) during this period of training. During the 4th day of training, the location of the escape platform was reversed to the northeast quadrant and 6 trials of 50 s were assessed. The difference of escape distance measured during the 1st and 2nd trials of the 7th day can be used as an index of working memory (the greater the difference, the better the working memory). On each trial (days 1–4), the rats were allowed to stay on the platform for 5 s. If the rats did not find the platform during the maximum duration of the trial, the experimenter placed them on it for 5 s. A 30-s recovery period was allowed between the training trials.

The training of the old rats occurred on 7 consecutive days. During the first 5 days, 3 trials of 70 s were assessed

and the platform had a fixed location in the southwest quadrant of the pool. On the 6th day, the platform was removed from the pool and a 50-s spatial bias trial was recorded: the % time spent and distance swum in the quadrant where the platform had been during the training days were analyzed. On the 7th day, the location of the platform was reversed to the northeast quadrant and 6 trials of 50 s were assessed to analyze reversal learning ability. The difference of escape distance measured during the 1st and 2nd trials of the 6th day was as an index of working memory (the greater the difference, the better the working memory). On each trial (days 1–5 and 7), the rats were allowed to stay on the platform for 5 s. If the rats did not find the platform during the maximum duration of the trial, the experimenter placed them on it for 5 s. A 30-s recovery period was allowed between the training trials.

The water maze training schedule of the very old rats (27-month-old) rats was altered from that of the old rats because they did not learn the task as rapidly as the old rats did. The platform was located in the same quadrant on all the training days. Very old rats were trained in the water maze for 4 days (five 70-s platform trials per day, 10 s on the platform, 30 s recovery) followed by a break of 4 days. Then, 6 additional training days were assessed (eight 50-s platform trials per day, 10 s on the platform, 50 s recovery).

2.5. Passive avoidance

Passive avoidance training was started 24 h after the water maze testing sessions. The passive avoidance box consisted of a light and a dark compartment. During the training trial, the rats were placed in the light compartment and 30 s later the sliding guillotine door was opened. After the rats entered the dark compartment, the door was closed and a foot shock of 1.0 mA (3 s) was given. Then, rats were again placed in the light compartment and if they did not re-enter the dark compartment in 120 s they were removed to their home cages. If the rats re-entered the dark compartment before fulfillment of the avoidance criteria of 120 s, the shock treatment was administered again and the avoidance performance was immediately re-assessed. During the testing trial 72 h later, the rats were again placed in the light compartment and the latency to enter the dark compartment was measured (360 s maximum latency).

2.6. Biochemistry

The medial septum-lesioned and control rats were decapitated 3 days after the end of the behavioral testing. The brains of the rats were removed and dissected on ice. Hippocampi were bilaterally taken for biochemical analysis and stored at −75°C until assayed. The method of Fonnum (1975) was used to analyze choline acetyltransferase activity of 4 medial septum-lesioned rats/group. This method is based on the formation of ^{14}C-labelled acetylcholine from ^{14}C-acetyl-CoA and choline. Radiolabelled acetylcholine was extracted into the organic phase as an ion-pair with teraphenylboron; the radioactivity of the organic phase was then measured with a liquid scintillation counter.

2.7. Statistics

The one-way analysis of variance followed by Duncan's post-hoc multiple group comparison was used to analyze group differences of the data collected during testing.

3. Results

3.1. Water maze

3.1.1. Young controls

The following treatment groups were used in this study: vehicle, metrifonate 10, 30 and 100 mg/kg. Metrifonate 10, 30 and 100 mg/kg- and vehicle-treated young rats learned effectively the initial location of the hidden platform during the first 3 days and then the new location during the 4th day. No group differences were observed in the escape distance analysis during any of the training days ($F(3,27) < 0.25$, $P > 0.05$; for all individual days) (Fig. 1, part a).

Metrifonate 100 mg/kg decreased swim speed slightly ($F(3,27) = 7.7$, $P < 0.05$; for the mean of all the training days; $P < 0.05$ vs. controls) but the other doses did not significantly decrease swim speed ($P > 0.05$) (vehicle: 22 ± 3; metrifonate 10 mg/kg: 23 ± 3; metrifonate 30 mg/kg 22 ± 3; 100 mg/kg 19 ± 3; cm/s).

3.1.2. Scopolamine-treated rats

The following treatment groups were used in this study: vehicle + vehicle, scopolamine 0.4 mg/kg + vehicle, scopolamine 0.4 mg/kg + metrifonate 10, 30 or 100 mg/kg. Scopolamine 0.4 mg/kg-treated young rats had an impaired performance on all 4 training days and the swim paths were longer than the control values ($P < 0.05$) (Fig. 1, part b). A comparison between single scopolamine 0.4 mg/kg- and scopolamine 0.4 mg/kg + metrifonate 10, 30 and 100 mg/kg-treated rats showed that metrifonate dose dependently decreased swim distance values and alleviated the failure to find the platform in the water maze induced by scopolamine treatment ($F(3,27) > 6.0$, $P < 0.05$; for all daily comparisons). Metrifonate 10 mg/kg had no effect on the first 3 days of testing ($P > 0.05$), but on the 4th day it markedly alleviated the navigation failure seen in scopolamine-treated rats and decreased their escape distances ($P < 0.05$). Metrifonate 30 mg/kg did not markedly stimulate water maze navigation during the first 2 training days ($P > 0.05$), but during the 3rd and 4th days of testing a significant and marked improvement was observed ($P < 0.05$). Metrifonate 100 mg/kg had no statistically signifi-

cant effect on spatial navigation on the 1st day of testing ($P > 0.05$), but markedly stimulated water maze navigation during the remaining training days, since the escape distance values were significantly decreased when compared with those of rats treated with scopolamine alone ($P < 0.05$). The analysis of working memory index revealed that scopolamine impaired performance and that metrifonate fully restored the working memory performance of scopolamine-treated rats ($F(3,27) = 5.9$, $P < 0.05$) (vehicle: 434 ± 211; scopolamine: 76 ± 29; scopolamine + metrifonate 10 mg/kg: 327 ± 143; metrifonate 30 mg/kg: 400 ± 199; 100 mg/kg: 441 ± 201; cm)

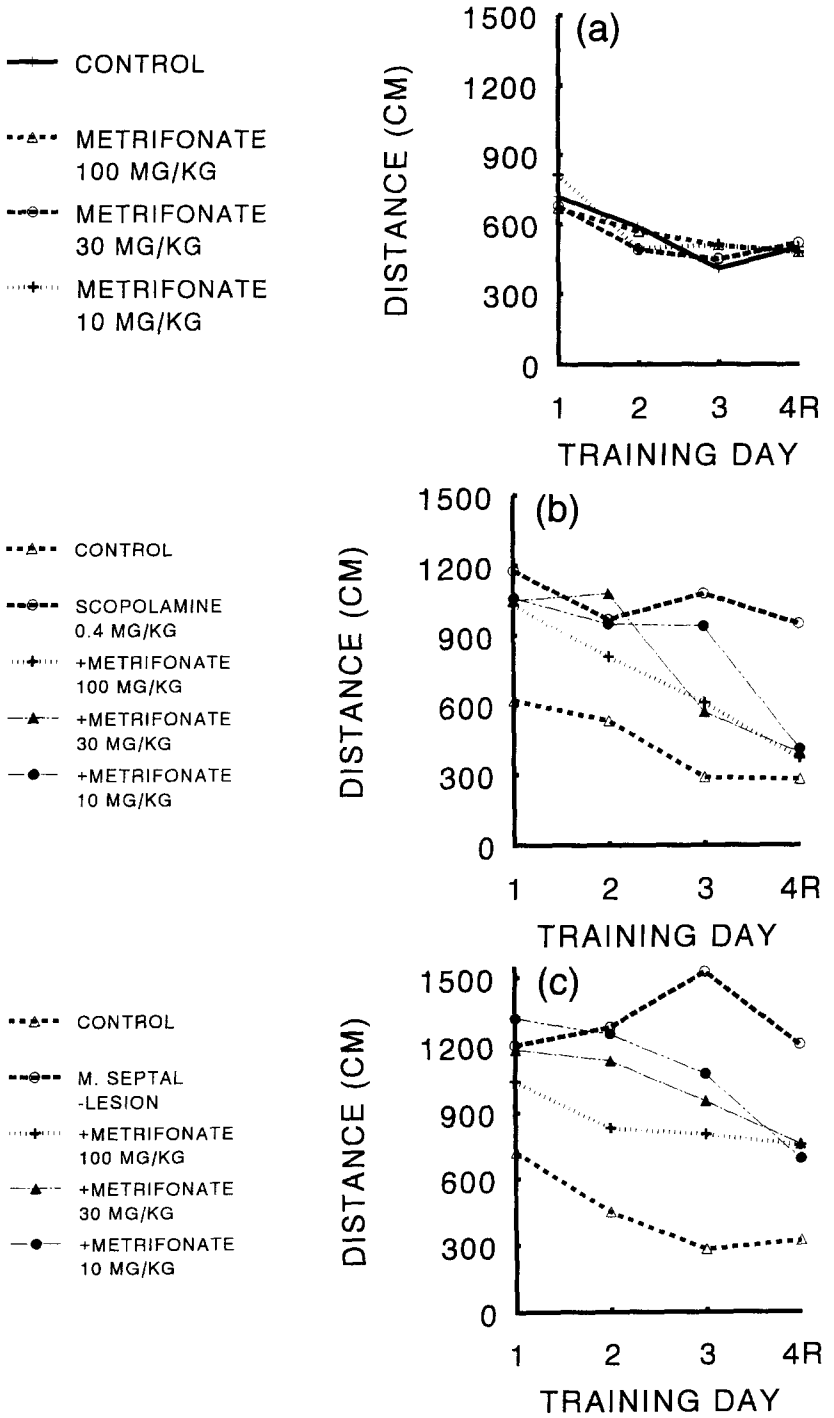

Fig. 1. Acquisition of reference (fixed platform location during days 1–3) and working (reversal of platform location during day 4) memory performance in a water maze pool by young intact (part a), scopolamine 0.4 mg/kg (i.p.)-treated (part b) and medial septum-lesioned (part c) rats treated with vehicle or metrifonate (10–100 mg/kg p.o.). Metrifonate had no effect on water maze behavior of intact rats, but stimulated reference and working memory of scopolamine-treated and medial septum-lesioned rats. Daily group means are depicted for escape distances onto a submerged platform.

Scopolamine increased swim speed significantly ($F(4,34) = 7.9$, $P < 0.05$; for the mean of all the training days) and metrifonate at the 2 highest doses completely antagonized the effect of scopolamine on swim speed ($P < 0.05$). Metrifonate 10 mg/kg did not affect the increase of swim speed seen after scopolamine treatment ($P > 0.05$) (vehicle: 22 ± 3; scopolamine: 26 ± 2; scopolamine + metrifonate 10 mg/kg: 27 ± 3; metrifonate 30 mg/kg: 22 ± 2; 100 mg/kg: 21 ± 4; cm/s)

3.1.3. Medial septum-lesioned rats

The following treatment groups were used in this study: control-lesioned + vehicle, medial septum-lesioned + vehicle, medial septum-lesioned + metrifonate 10 mg/kg, medial septum-lesioned + metrifonate 30 mg/kg, medial septum-lesioned + metrifonate 100 mg/kg. Medial septum-lesioned young rats had an impaired performance on all 4 training days and the swim paths were longer than those of control rats (Fig. 1, part c). A comparison between vehicle- and metrifonate 10, 30 and 100 mg/kg-treated medial septum-lesioned rats showed that metrifonate dose dependently decreased swim distance values and alleviated the water maze failure induced by brain lesion ($F(3,27) > 6.3$, $P < 0.05$; for all daily comparisons). Metrifonate 10 mg/kg had no effect on the first 3 days of testing ($P > 0.05$), but on the 4th day fully reversed the navigation failure present in the medial septum-lesioned rats ($P < 0.05$). Metrifonate 30 mg/kg did not markedly stimulate water maze navigation during the first 2 training days ($P > 0.05$), but during the 3rd and 4th days of testing a significant and marked improvement was observed ($P < 0.05$). Metrifonate 100 mg/kg had no statistically significant effect on spatial navigation on the 1st day of testing ($P > 0.05$), but markedly stimulated water maze navigation during the other training days ($P < 0.05$). Analysis of working memory index measured during the 4th training

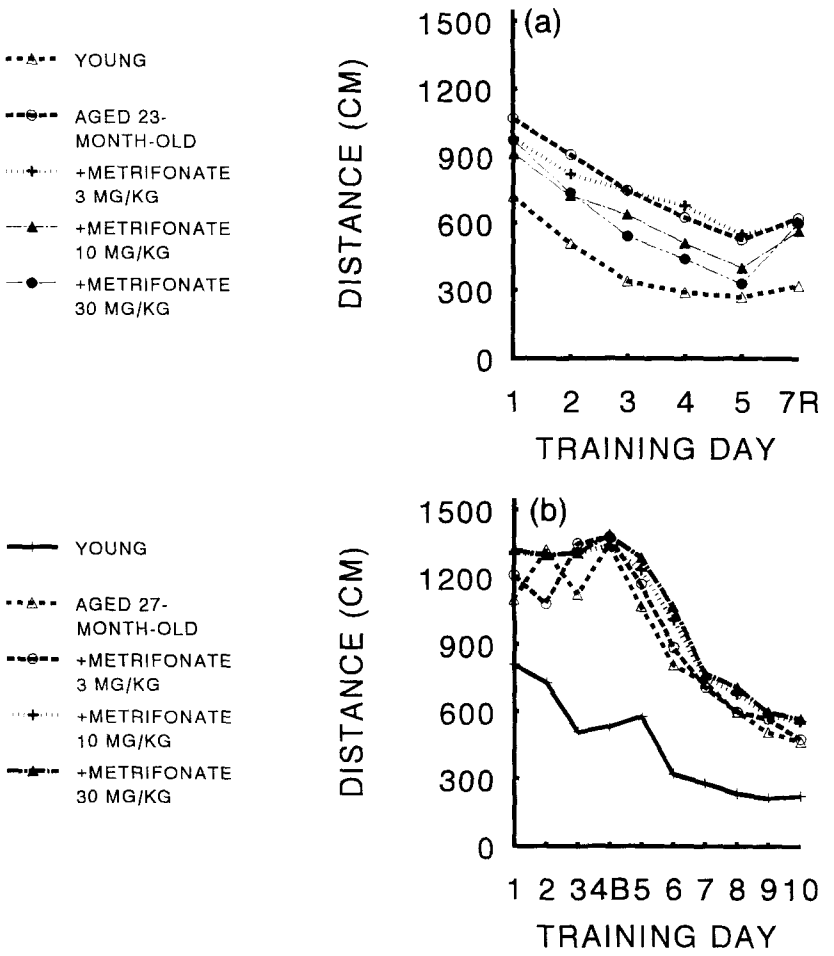

Fig. 2. Acquisition of reference (fixed platform location) and working (reversal of platform location) memory performance in a water maze pool by old (23-month-old) (part a) and very old (27-month-old) (part b) rats treated with vehicle or metrifonate (3–30 mg/kg p.o.). Metrifonate stimulated reference memory of old rats, but had no effect on water maze behavior of very old rats. Working memory performance of old and very old rats was not stimulated by metrifonate. Daily group means are depicted for escape distances onto a submerged platform. In part a, the x-axis indicates the reference memory training days 1–5 (fixed platform location), retention day 6 (no platform in the pool) and working memory training day 7R (R, reversal of the platform location). In part b, x-axis indicates the reference memory training days 1–10 (fixed platform location). There was a 4-day break between the 4th and 5th test days (B, break).

day showed a marked overall group effect ($F(4,34) = 8.9$, $P < 0.05$) that medial septum-lesioned rats had a markedly impaired performance ($P < 0.05$) and that metrifonate (10–100 mg/kg) to some extent alleviated the decrease of working memory index induced by medial septum-lesioning ($P < 0.05$) (vehicle: 362 ± 154; medial septum-lesioned: −32 ± 47; lesioned + metrifonate 10 mg/kg: 201 ± 54; +metrifonate 30 mg/kg: 221 ± 61; +metrifonate 100 mg/kg: 202 ± 34; cm).

Rats with medial septum lesions had a significantly increased swim speed ($F(4,34) = 6.9$, $P < 0.05$; for the mean of all the training days) and metrifonate at the highest dose (100 mg/kg) antagonized the effect of lesion on swim speed ($P < 0.05$). Metrifonate at 10 and 30 mg/kg did not alleviate the lesion-induced changes in swim speed ($P > 0.05$) (vehicle: 22 ± 2; medial septum-lesioned: 27 ± 3; lesioned + metrifonate 10 mg/kg: 27 ± 3; +metrifonate 30 mg/kg: 26 ± 2; +metrifonate 100 mg/kg: 22 ± 3; cm/s).

3.1.4. Old rats

The following treatment groups were used in this study: young + vehicle, old + vehicle, old + metrifonate 3 mg/kg, old + metrifonate 10 mg/kg, old + metrifonate 30 mg/kg. A comparison between young and old rats revealed an age-related failure of spatial navigation ($F(4,35) = 8.00$, $P < 0.05$; for all individual training days) to the hidden platform located at a fixed place. All the different treatment groups of aged rats had an impaired performance during this period of training, but the treatment with metrifonate 10 mg/kg decreased non-significantly ($P > 0.05$) but 30 mg/kg significantly ($P < 0.05$) swim distances during the reference memory training period. Analysis of daily escape distance values showed that metrifonate 30 mg/kg-treated rats had a better performance than aged vehicle-treated rats on the 3rd, 4th and 5th days of training ($P < 0.05$). Also the 10 mg/kg dose significantly improved performance on the 1st and 5th days of training ($P < 0.05$).

On the 6th day, the spatial bias analysis showed again an age-related failure of spatial performance ($F(4,35) = 6.4$, $P < 0.05$) and that metrifonate dose dependently improved spatial bias (data not shown). The effect of metrifonate 30 mg/kg was significant ($P < 0.05$ vs. aged controls) and this group was not impaired compared with the young controls ($P > 0.05$ vs. young controls).

On the 7th day during the platform reversal trials, old rats had an impaired performance ($F(4,35) = 10.0$, $P < 0.05$) and metrifonate 3–30 mg/kg did not stimulate water maze navigation ($P > 0.05$ vs. aged controls). Furthermore, the working memory index of aged rats was lower ($F(4,35) = 8.1$, $P < 0.05$) and metrifonate did not stimulate the performance of aged rats ($P > 0.05$) (young vehicle: 403 ± 121; old vehicle: 68 ± 80; old + metrifonate 3 mg/kg: 49 ± 57; old + metrifonate 10 mg/kg: 62 ± 39; old + metrifonate 30 mg/kg: 43 ± 42; cm).

In the statistical analysis, no marked group effect was observed in the swim speed analysis of young and old rats ($F(4,35) = 0.35$, $P > 0.05$; for the mean of all the training days; $P > 0.05$ in all group comparisons) and the group means of swim speeds were equal (young vehicle: 23 ± 3; old vehicle: 22 ± 2; old + metrifonate 3 mg/kg: 23 ± 3; old + metrifonate 10 mg/kg: 22 ± 3; old + metrifonate 30 mg/kg 23 ± 3; cm/s).

3.1.5. Very old rats

The following treatment groups were used in this study: young + vehicle, old + vehicle, old + metrifonate 3 mg/kg, old + metrifonate 10 mg/kg, old + metrifonate 30 mg/kg. Young rats learned the water task more quickly than very old rats (Fig. 2, part b); the latter had a marked impaired performance ($F(4,44) > 8.0$, $P < 0.05$; for all daily comparisons). An analysis of the navigation performance of very old vehicle and metrifonate 3, 10 and 30 mg/kg-treated very old rats showed no treatment-induced changes in any of the training days ($P > 0.05$; for all comparisons vs. very old controls).

The analysis of swim speed values revealed that very old rats swam slower than young rats ($F(4,35) = 9.9$, $P < 0.05$; for the mean of all the training days; $P < 0.05$ for all group comparisons between young and very old rats). Furthermore, metrifonate 10 and 30 mg/kg-treated

Table 1
The effects of pretraining trial p.o. metrifonate (10, 30 and 100 mg/kg) treatment on passive avoidance testing trial entry latencies (s) of young controls, scopolamine 2.0 mg/kg-treated and medial septum-lesioned rats. Also the effect of p.o. metrifonate 3, 10 and 30 mg/kg was investigated on the passive avoidance behavior of 23- and 27-month-old rats

	Treatment					
	Age (month)	Vehicle	3 mg/kg	10 mg/kg	30 mg/kg	100 mg/kg
Young control	3	337 ± 13	N.D.	319 ± 41	314 ± 27	323 ± 23
Scopolamine	3	178 ± 36 [a]	N.D.	274 ± 48 [a]	327 ± 18 [b]	346 ± 16 [b]
Medial septum lesion	3	233 ± 58 [a]	N.D.	278 ± 46 [a]	327 ± 48 [b]	346 ± 16 [b]
Old	23	194 ± 55 [a]	190 ± 59 [a]	244 ± 56 [a]	261 ± 61 [a,b]	N.D.
Very old	27	161 ± 102 [a]	180 ± 80 [a]	214 ± 67 [a,b]	250 ± 74 [a,b]	N.D.

[a] $P < 0.05$ vs. vehicle-treated young controls; [b] $P < 0.05$ vs. vehicle-treated controls of the metrifonate-treated groups (scopolamine-treated or medial septum-lesioned young rats, old or very old controls); Duncan's post-hoc group comparison. The value of young control group is the mean of the young controls in all the 5 different experiments. Mean ± S.D. is shown. N.D., not determined.

very old rats swam slower than the vehicle-treated very old rats ($P < 0.05$) (young vehicle: 22 ± 2; very old vehicle: 18 ± 3; old + metrifonate 3 mg/kg: 18 ± 4; very old + metrifonate 10 mg/kg: 15 ± 3; very old + metrifonate 30 mg/kg 15 ± 2; cm/s)

3.2. Passive avoidance

Table 1 shows the passive avoidance treatment groups and testing trial entry latency values.

In young neurologically intact controls, metrifonate treatment did not modulate the number of foot shocks required to avoid entering the chamber for 120 s during the training trial and had no effect on the entry latency during the testing trial ($F(3,27) < 1$, $P > 0.05$; for both comparisons; $P > 0.05$ for all group comparisons) (Table 1).

All the experimental groups receiving scopolamine 2.0 mg/kg in combination with either vehicle or metrifonate 10, 30 or 100 mg/kg had learned to avoid re-entrering the dark compartment after the first shock treatment during the training trial. During the testing trial, scopolamine 2.0 mg/kg-treated rats had an impaired performance and all the doses of metrifonate 10–100 mg/kg alleviated this defect ($F(4,34) = 8.3$, $P < 0.05$) (Table 1).

The training trial performance of control-lesioned and different groups of medial septum-lesioned rats was accurate after the 1st shock trial. During the testing trial, medial septum-lesioned rats had an impaired performance, but metrifonate at 30 and 100 mg/kg stimulated avoidance behavior ($F(4,34) = 10.1$, $P < 0.05$) (Table 1).

Old and very old rats also did not re-enter the dark compartment after they had received a foot shock following their initial entry during the training trial. However, during the testing trial the old and very old rats had an impaired performance ($F(4,35) > 10.0$, $P < 0.05$; for both comparisons) and metrifonate dose dependently improved passive avoidance behavior. The old rats treated with metrifonate 10 mg/kg had a non-significantly better performance ($P > 0.05$) than the vehicle-treated group of old rats, but the group of old rats receiving 30 mg/kg had a significantly ($P < 0.05$) better performance than old controls. Similarly, metrifonate 10 and 30 mg/kg increased entry latencies of very old rats during the testing trial and the effect was significant at both of these doses ($P < 0.05$).

3.3. Biochemistry

Medial septum lesions decreased choline acetyltransferase activity in the hippocampi of all the groups equally and metrifonate treatment, which had been terminated 3 days before decapitation had no effect on enzyme activity (control-lesioned: 1.21 ± 0.11, medial septum-lesioned + vehicle: 0.39 ± 0.08; medial septum-lesioned-lesioned + metrifonate 3 mg/kg: 0.43 ± 0.10; medial septum-lesioned + metrifonate 10 mg/kg: 0.38 ± 0.12; medial septum-lesioned + metrifonate 30 mg/kg: 0.41 ± 0.10; nmol/mg protein/min) ($F(4,14) = 7.7$, $P < 0.05$; control-lesioned vs. any of the medial septum-lesioned group: $P < 0.05$;).

4. Discussion

We observed that metrifonate markedly stimulated water maze reference memory (i.e. when there was fixed location of the hidden escape platform) and reversal learning (i.e. reversal of the location of hidden escape platform) as well as passive avoidance performance in scopolamine-treated and medial septum-lesioned rats. In 23-month-old aged rats, metrifonate treatment improved spatial reference memory and passive avoidance, but had no effect on navigation performance during the platform reversal stage. In contrast, in young neurologically normal rats metrifonate had no effect on navigation or avoidance behavior and also in advanced age (27-month-old rats) the therapeutic effect of metrifonate was no longer apparent. These results demonstrate that metrifonate is able to improve cognitive function in different animal models and that the therapeutic effect is observed in the dose range of 10–100 mg/kg.

4.1. Metrifonate and cognitive functioning in young rats

Previous studies have described that metrifonate, at selected doses, may have a beneficial effect on memory and learning. For example, Kronforst et al. (1995) reported that chronic 7 days treatment with metrifonate at doses of 6, 12 and 24 mg/kg facilitated eyeblink conditioning in aging rabbits. They reported that (Kronforst et al., 1995) eyeblink facilitation occurred within a range of 30–80% steady state cholinesterase inhibition. In addition, Van der Staay et al. (1996) described that acute p.o. metrifonate treatment at 12.5 and 25 mg/kg facilitated two-way active avoidance behavior of young rats. They (Van der Staay et al., 1996) also tested metrifonate on reference memory of young and aged rats in a water maze test and reported that treatment with metrifonate at the single dose tested (12.5 mg/kg) decreased the escape distance to the hidden platform in both sets of rats. In contrast to the study of Van der Staay et al. (1996), we did not observe any improvement in spatial reference memory or passive avoidance after metrifonate treatment in neurologically normal rats. One possible explanation for the lack of an effect of metrifonate on behavior in our behavioral tests is the accurate performance of young controls that may have imposed a ceiling effect that masked any performance improving effect of metrifonate. Indeed, the latency of controls during the passive avoidance testing trial was near-perfect (336 s group mean, 360 maximum latency). This issue could be investigated by modulating the training paradigm to produce less accurate performance. Therefore, it is possible that manipulation of the mnemonic demands

of the tests may be necessary to reveal the possible effects of metrifonate to improve avoidance and spatial behavior. A second possibility is that a strain difference in the rats used in the present and the earlier (Van der Staay et al., 1996) study may explain the variability in the sensitivity to the effects of metrifonate on behavior.

We observed that metrifonate could block the defect in water maze navigation induced by the muscarinic acetylcholine receptor antagonist, scopolamine 0.4 mg/kg. Scopolamine 0.4 mg/kg-treated rats also swam faster than the controls and metrifonate fully blocked the scopolamine treatment-induced increase of swim speed. Interestingly, metrifonate also normalized the increased swim speed of scopolamine-treated rats at a dose (30 mg/kg) that had no effect on the swim speed of control rats, further suggesting that the effect of metrifonate on motor activity is mediated via cholinergic systems. Finally, metrifonate 30 and 100 mg/kg also reversed the passive avoidance defect produced by scopolamine at 2 mg/kg. These results further support previous biochemical studies showing that metrifonate may potentially elevate synaptic acetylcholine levels by inhibiting cholinesterase activity (Reiner et al., 1975; Nordgren et al., 1978; Becker and Giacobini, 1988; Mori et al., 1995; Nabeshima et al., 1995). We also found that metrifonate stimulated water maze reference memory and reversal learning, and the passive avoidance behavior of young medial septum-lesioned rats, but none of the doses tested fully reversed the defect in performance. It is not likely that the failure to completely reverse the lesion-induced behavioral defect results from an inappropriate dose range of metrifonate used, since higher doses of metrifonate caused side-effects and the lowest tested dose was the least effective. Nevertheless, it is possible that at some untested dose a more complete alleviation of lesion-induced defect could have been detected. A more probable explanation for the incomplete reversal of the medial septum lesion-induced water maze and passive avoidance defect is that the non-specific lesion method disrupts behavior by damaging not only cholinergic but also non-cholinergic cells and fibers in the lesioned brain tissue (Dunnett et al., 1991; Dunnett and Fibiger, 1993). Thus, metrifonate may reverse only that part of the reference memory, reversal learning and passive avoidance defect that results from cholinergic cell loss (Parson et al., 1987). Medial septum-lesioned rats swam faster than the controls in the water maze and metrifonate 100 mg/kg slowed the swimming speed of lesioned rats to the same extent as in the control rats. However, the dose-response relation of metrifonate on swim speed was different in scopolamine-treated and medial septum-lesioned rats. The smaller dose of metrifonate (30 mg/kg) that fully reversed scopolamine-induced locomotor hyperactivity had no effect on swim speed of medial septum-lesioned or control rats. Thus, it is likely that treatment with metrifonate does not stabilize medial septum lesion-induced hyperactivity per se and that the locomotor depressing action of a high dose of metrifonate (100 mg/kg) is mediated independently of the medial septum area. Therefore, it is possible that destruction of medial septum cholinergic cells may not be responsible for the increased swim speed induced by electrolytic lesions and that loss of non-cholinergic cells as a result of the lesion (e.g. GABA cells) or fibers passing through the lesioned area may contribute to the locomotor hyperactivity.

4.2. Metrifonate and age-related cognitive defects

We also tested the effects of metrifonate treatment on the aging-induced spatial reference/working memory and passive avoidance failure. Interestingly, the effect of metrifonate was to some extent age dependent. Metrifonate 10 and 30 mg/kg enhanced spatial reference memory and passive avoidance behavior in 23-month-old aged rats, but had no effect on spatial working memory and reversal learning performance. Old rats did not decrease their escape distance values as effectively as young rats did after the 1st training trial or during later trials on the 7th training day, and metrifonate failed to modulate these performance measures. Importantly, the present study found that metrifonate prevented the reversal learning failure induced by either scopolamine or medial septum-lesioning. Furthermore, metrifonate treatment effectively increased the working memory index of scopolamine-treated and medial septum-lesioned rats, as the metrifonate-treated scopolamine and medial septum-lesioned rats were capable of 1-trial learning. Furthermore, the acquisition of a new reference memory strategy (seen as a decrease of escape distance values during the rest of the trials measured on the 4th day) by scopolamine-treated and medial septum-lesioned rats was improved by metrifonate treatment. Thus, the failure of metrifonate to improve spatial working memory and reversal learning in old rats may result from the development of neuropathological defects that cannot be alleviated by cholinergic drugs (Decker and McGaugh, 1991; Miettinen et al., 1993). Indeed, previously Sirviö et al. (1992) have shown that tacrine fails to stimulate spatial working memory in an operant chamber delayed non-matching to position test, indicating that cholinesterase inhibitors may not be effective at enhancing working memory in old rats. However, metrifonate treatment given on all the platform training days may have differently affected the ability of old vs. scopolamine-treated and medial septum-lesioned young rats to acquire a strategy to deal with the platform reversal by modulating the pace of extinction of a previously reinforced search strategy. Therefore, we are now presently investigating if metrifonate can increase working memory index of medial septum-lesioned rats treated with no drug prior to the platform reversal stage. Our study also described that 27-month-old aged rats were more severely impaired in water maze navigation than 23-month-old rats, and that metrifonate at 3–30 mg/kg had no effect on the spatial

behavior of 27-month-old rats. However, metrifonate treatment did improve passive avoidance performance in 27-month-old aged rats. Thus, it is possible that, during advanced aging, neuropathological defects develop that aggravate spatial navigation failure and mask the therapeutic effect of metrifonate observed on spatial reference memory in 23-month-old rats (Decker and McGaugh, 1991; Miettinen et al., 1993).

4.3. Correlation between the behavioral and biochemical effects of metrifonate

An important point is to compare the metrifonate doses that stimulated water maze and passive avoidance behavior with the doses of metrifonate that affected parameters of cholinergic transmission. In the present study, we found that the therapeutic effect was dose dependent in the range of 3–100 mg/kg. Earlier ex vivo studies have shown that the lowest behaviorally active dose of metrifonate (10 mg/kg) may cause only a weak inhibition of acetylcholinesterase activity (Hinz et al. 1995). Hinz et al. (1996a) described that a marked cholinesterase inhibition in rat brain was induced at doses of metrifonate of 30 mg/kg p.o. or above, aged rats being more sensitive than young adult rats: oral ED_{50} values for 3- and 19-month-old rats being 90 mg/kg and 60 mg/kg, respectively. Furthermore, the in vivo microdialysis study of Mori et al. (1995) found that metrifonate 80 mg/kg induced a robust (1800%) increase of acetylcholine levels. The specificity of the biochemical and behavioral effects of metrifonate for the cholinergic system is supported by the study of Mori et al. (1995), who found no or only weak increases in monoamine levels in samples collected with in vivo microdialysis system. Therefore, the present behavioral and previous biochemical studies indicate that metrifonate dose dependently facilitates water maze and passive avoidance behavior at doses which augment cholinergic transmission.

4.4. Comparison of tacrine and metrifonate

The behavioral effects of metrifonate on spatial navigation and avoidance are to some extent similar to the changes on water maze and passive avoidance behavior observed after treatment with tacrine. First, tacrine (1–5 mg/kg i.p.) (Riekkinen Jr. et al., 1990, 1991a,b) also did not improve the water maze or passive avoidance behavior of young neurologically intact rats under the same test conditions. Second, tacrine at a single active dose (3 mg/kg i.p.) (Riekkinen Jr. et al., 1990) and metrifonate in a dose range of 10–100 mg/kg (p.o.) stimulated water maze reference memory and passive avoidance performance in medial septum-lesioned and aged rats. Third, tacrine and metrifonate failed to improve spatial working memory in aged rats (Riekkinen Jr. et al., 1991a,b). Therefore, it is possible that tacrine and metrifonate may alleviate the defect in water maze and passive avoidance performance resulting from the damage to cholinergic neurons induced by brain-lesioning or aging. However, some important differences exist between tacrine and metrifonate in their action on spatial navigation. We found that metrifonate significantly decreased escape distance values during water maze working memory trials, but tacrine did not facilitate the water maze working memory of young medial septum-lesioned rats. A comparison of the dose-response curves of metrifonate and tacrine revealed that the therapeutic window of metrifonate was considerably broader than that of tacrine. Metrifonate over a range of doses (10–100 mg/kg) facilitated reference and working memory and passive avoidance functioning in young rats, but the dose-response curve of tacrine had a narrow inverted U shape with only a single effective dose being found. The broader therapeutic window of metrifonate and greater efficacy on working memory behavior may result from the better efficacy/safety ratio of metrifonate compared to that of tacrine (Blokland et al., 1996). Indeed, we found that metrifonate decreased the swim speed of young controls only at the highest dose (100 mg/kg) tested, but the rats treated with this dose could swim appropriately and locate the platform. Further, metrifonate at doses up to 30 mg/kg, which effectively stimulated water maze navigation in old rats, had no adverse effects on swim speed. Interestingly, very old rats that had a motor defect (the swim speed was slow) were more sensitive to the motor performance depressing effect of metrifonate. We found that 10 and 30 mg/kg doses decreased slightly the swim speed of very old rats. In contrast, our earlier data indicate that tacrine may more severely inhibit the motor activity of the Wistar rat strain used in our earlier and present behavioral studies. First, tacrine 3 mg/kg markedly reduced the swim speed of young rats and a dose of 5 mg/kg inhibited swim performance. Second, in aged rats, the only therapeutically active dose of tacrine (3 mg/kg) also markedly slowed swim speed. Therefore, the present and previous data of Blokland et al. (1996) and Van der Staay et al. (1996) showing that metrifonate produces only mild or no motor side-effects in water maze at a therapeutically active dose range suggest that metrifonate is tolerated better than tacrine and is more effective in stimulating cognitive functioning in animal models.

In conclusion, the present and a previous (Blokland et al., 1996) study may be taken as further evidence that metrifonate stimulates effectively spatial navigation and avoidance behavior over a considerably broader dosage range than tacrine does. This result further supports the development of metrifonate as a potential therapy for the treatment of cognitive disorders associated with Alzheimer's disease (Becker and Giacobini, 1988).

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