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# Effect of metrifonate on extracellular brain acetylcholine and object recognition in aged rats

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**Abstract**

The effects of metrifonate were investigated in 4–6- and 22–24-month-old rats. Extracellular acetylcholine levels were measured by transversal microdialysis in vivo. Baseline extracellular acetylcholine levels in the cerebral cortex and hippocampus were 42% and 60% lower, respectively, in old than in young rats. Old rats did not discriminate between familiar and novel objects. In old rats, metrifonate (80 mg/kg p.o.) brought about 85% inhibition of cholinesterase activity in the cortex and hippocampus, a 4-fold increase in extracellular acetylcholine levels in the cortex only, and restored object recognition. In young rats, metrifonate caused 75% cholinesterase inhibition in the cerebral cortex and hippocampus, a 2-fold increase in cortical and hippocampal extracellular acetylcholine levels, and no effect on object recognition. The slight cholinesterase inhibition following metrifonate (30 mg/kg) in aged rats had no effect on cortical acetylcholine levels and object recognition. In conclusion, metrifonate may improve the age-associated cholinergic hypofunction and cognitive impairment.

*Keywords:* Aging; Metrifonate; Object recognition; Acetylcholine, release; Cholinesterase inhibitor

## 1. Introduction

The brain cholinergic system plays a prominent role in learning and memory (Squire and Davis, 1981), and its hypofunction is considered an important pathogenetic element of age-associated cognitive impairments, including senile dementia of the Alzheimer type (Bartus et al., 1982; Collerton, 1986). Many drugs have been proposed for the treatment of dementia (Sarter, 1991); however, the main group of compounds, targeted by a great many preclinical and clinical investigations, is constituted by cholinesterase inhibitors (Giacobini and Becker, 1994). It is assumed that these drugs may restore cholinergic neurotransmission in the brain by preventing acetylcholine hydrolysis and increasing its extracellular levels. Among the cholinesterase inhibitors, tacrine is the only drug so far officially approved by the Food and Drug Administration for the symptomatic treatment of Alzheimer's disease; unfortunately its clinical efficacy is accompanied by a relevant toxicity (Davis and Powchik, 1995). For this reason much effort is being dedicated to the development of novel cholinesterase inhibitors which diffuse mainly into the central nervous system and result in fewer side-effects. Among these second generation cholinesterase inhibitors (Giacobini, 1991), metrifonate seems of particular interest since it has been used for a long time as an anthelmintic and its safety in man is well known (Holmstedt et al., 1978; Feldmeier and Doehring, 1987; Webster, 1990). Metrifonate may be considered a prodrug since in vivo it is non-enzymatically transformed into the active cholinesterase inhibitor, dichlorvos (Nordgren and Holmstedt, 1988). It has recently been demonstrated that neither metrifonate nor dichlorvos shows any affinity for nicotinic, muscarinic and several other receptors in vitro (Hinz et al., in press). Preliminary evidence of its clinical efficacy in Alzheimer's disease has been reported (Becker et al., 1990).

Studies in rodents have repeatedly demonstrated that systemic administration of cholinesterase inhibitors such as

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physostigmine, heptylphysostigmine, tacrine (Messamore et al., 1993; Xiao et al., 1993; Cuadra et al., 1994) and, more recently, metrifonate (Mori et al., 1995) is followed by an increase in brain extracellular acetylcholine levels in young adult animals. Moreover, it has been shown that physostigmine (Mandel et al., 1989) and tacrine (Riekkinen and Riekkinen, 1995) improve cognitive performances in young rats with excitotoxic lesions of the nucleus basalis. Tacrine has been demonstrated to show beneficial effects in rats with learning and memory deficits induced by a variety of lesions and drugs causing cholinergic hypofunction, and by aging (Mohammed, 1993). The cholinesterase inhibitors, velnacrine, SDZ ENA-713 and phenserine, have also been shown to improve behavioral performances in aged animals (Tanaka et al., 1994; Jackson et al., 1995; Ikari et al., 1995).

Metrifonate facilitates task acquisition in both young and aged rats trained in the Morris water escape task (Blokland et al., 1995; Van der Staay et al., 1996), and improves Morris maze performance and passive avoidance behavior disrupted by age, scopolamine and septal lesions (Riekkinen et al., 1996). However, the effects of metrifonate administration on brain extracellular acetylcholine levels in aged rats have not been investigated and no attempt has yet been made to correlate the increase in extracellular levels of brain acetylcholine with cognitive improvement. Therefore the aim of the present work was to measure the extracellular acetylcholine levels in the cerebral cortex and hippocampus of aged rats after oral metrifonate administration, and to find whether the increase in extracellular acetylcholine levels induced by metrifonate is associated with cognitive improvement, assessed by spontaneous object recognition. According to Ennaceur and Delacour (1988) and Ennaceur and Meliani (1992) object recognition measures a form of non-spatial working memory, based on spontaneous exploratory activity, which may be considered episodic memory, and is impaired in aging rats (Scali et al., 1994). The oral route of administration was selected in order to mimic the administration route of metrifonate in humans.

## 2. Materials and methods

### 2.1. Animals

The experiments were performed on male HsdCpb:WU rats from Harlan-Winkelman (Borchen, Germany) 4–6 months (young rats) and 22–24 months of age (aged rats). The rats were randomly allocated to the different experimental groups and were housed 2 in a cage at 21–24°C, 40–60% humidity, on a 12 h light/dark cycle, with food and water ad libitum. All experiments were carried out according to the guidelines of the European Community's Council for Animal Experiments (DL 116/92).

### 2.2. Cholinesterase activity

Brain cholinesterase activity was measured by the method of Ellman et al. (1961). Forty-five minutes after drug or vehicle administration, the rats were killed by decapitation. The brain structures were dissected out on ice and frozen immediately. On the day of testing, samples were homogenized at 4°C in 0.9% NaCl solution (1:10, w/v). Cholinesterase was solubilized by adding a 2.5-fold volume of 0.7% Triton X-100 solution. 10 μl of the mixture was added to 1 ml of a buffered solution containing 0.25 mM dithiobisnitrobenzoate and 1 mM acetylthiocholine. The reaction was stopped by adding tacrine (final concentration 25 μM) and quantified photometrically at 412 nm.

### 2.3. Microdialysis and determination of extracellular acetylcholine levels

Extracellular acetylcholine levels were measured in the cerebral cortex and hippocampus by a microdialysis technique, as previously reported (Giovannini et al., 1991). Briefly, the rats were anesthetized with ketamine (150 mg/kg i.p.) and placed in a stereotaxic frame. A microdialysis tube (AN 69 membrane, Hospal Dasco, Italy, molecular mass cut-off $>15$ kDa), covered with Super-Epoxy glue except for a region corresponding to the length of parietal cortices and dorsal hippocampi (8 and 6 mm long, respectively), was inserted transversely through either of the two brain structures as described below. After sagittal cutting the overlying skin and temporal muscles were retracted and folded away, and holes were drilled bilaterally at the level of the dorsal hippocampi (AP $= -3.3$, DV $= -3.5$ for aged and DV $= -3.3$ for young rats) or parietal cortices (AP $= -0.5$, DV $= -2.7$ for aged rats and DV $= -2.3$ for young rats); all coordinates (Paxinos and Watson, 1982) were taken over the bone and referred to bregma, with bregma and lambda on a horizontal plane. The microdialysis tube was then gently inserted through the holes using the micromanipulator of the stereotaxic instrument. The ends of the dialysis tube were bent upward, secured to the parietal skull with acrylic dental cement and the skin was sutured. The rats were housed with free access to food and water to recover from surgery (one rat per cage). On the following day, the membrane was perfused at a constant flow rate (3 μl/min) with Ringer solution (NaCl 147 mM, $CaCl_2$ 1.2 mM, KCl 4.0 mM). No cholinesterase inhibitors or other drugs were added to the Ringer solution. After a 1-h settling period, the dialysate was collected at 20-min intervals for 3 h in minitubes containing 5 μl of 0.05 mM HCl to prevent hydrolysis of acetylcholine and directly assayed for acetylcholine. After three samples had been collected under basal conditions the rats were treated either with metrifonate or with appropriate vehicle and samples were collected for the next 2 h after drug treatment. At the end of

the experiment, the rats were anesthetized and killed by decapitation and their brains were removed and fixed in 10% sodium phosphate-buffered formaldehyde. The brains were cut with a microtome and placement of the tube was checked by histological examination on 50 μm thick coronal slices. Results obtained from rats in which the dialysis membrane was positioned outside the structure under investigation were discarded (about 5%).

Acetylcholine levels in the dialysates were assayed directly with a high-performance liquid chromatographic method with an enzyme reactor and an electrochemical detector as described by Damsma et al. (1987) and Giovannini et al. (1991). To evaluate the amount of acetylcholine in the samples, a linear regression curve was made with acetylcholine standards and the peak heights of this compound in the samples were compared with those of the standards by means of an integrator (P.E. Nelson, model 1020). The detection limit was 50 fmol of acetylcholine.

### 2.4. Object recognition

Object recognition was evaluated according to Ennaceur and Delacour (1988) and Bartolini et al. (1996). Briefly, the rats were placed in a white colored polyvinylchloride arena ($70 \times 60 \times 30$ cm) illuminated by a 75 W lamp suspended 50 cm above the arena. The objects to be discriminated were cubes, pyramids and cylinders made of gray plastic. The day before testing, the rats were allowed to explore the arena for 2 min. On the day of the test, a session of 2 trials separated by an intertrial interval of 60 min was carried out. In the first trial (T1) two identical objects were presented in two opposite corners of the arena. The rats were left in the arena until criterion was reached. The criterion was reached at 20 s of total exploration of the objects and at this point the rat was removed and placed in the home cage. Exploration was defined as directing the nose at a distance $< 2$ cm to the object and/or touching it with the nose. During the second trial (T2) one of the objects presented in T1 was replaced by a new (differently shaped) object and the rats were left in the arena for 5 min. The times spent exploring the familiar ($F$) and the new object ($N$) were recorded separately and the difference between the two exploration times was taken as the discrimination index ($D = N - F/N + F$). From one rat to the next, care was taken to avoid object and place preference by randomly changing the role of the objects (familiar or new object) and their position in the two opposite corners of the box during T2. Furthermore, in order to avoid olfactory stimuli the objects to be discriminated were cleaned carefully.

### 2.5. Drugs

Metrifonate, supplied by Bayer/Troponwerke (Cologne, Germany), was freshly dissolved in 5% Na-citrate buffer adjusted to pH 5.5–6 and a volume of 2 ml/kg was administered by gavage. Appropriate vehicle (Na-citrate, 2 ml/kg) was administered to separate control groups. Drug and vehicle were administered per os (p.o.) as a single dose 30 min before the first trial of object recognition test or 45 min before cholinesterase activity determination. In microdialysis experiments the drug or vehicle was administered orally after collections of 3 samples in basal conditions and samples were collected for the next 2 h after treatment.

### 2.6. Statistical analysis

Acetylcholine release was expressed as percent variation over the mean of the first three samples, which was taken as baseline acetylcholine release (see figure legends). Differences in drug effects on acetylcholine release were evaluated using the mean percent changes calculated for each animal between 40 and 140 min after drug or vehicle treatment. Statistical analysis was performed using the NCSS 5.0 program. Significance was calculated by means of one-way analysis of variance (ANOVA) followed by least significant differences (LSD) Fisher post-hoc analysis.

The behavioral data were analyzed by means of nonparametric Kruskal-Wallis ANOVA and multiple comparison $Z$-values or Student's $t$-test, where appropriate.

Cholinesterase activity data were analyzed by means of one- and two-way ANOVA with the factors age (young, old), dose (0, 30, 80 mg/kg) and brain region (cortex, cerebellum, hippocampus, striatum, midbrain), where regions were considered as levels of a repeated measures factor. The level of significance was set at $P < 0.05$. To assess the effects of metrifonate treatment in more detail, individual ANOVAs per age and brain region were performed, supplemented with Fisher LSD post-hoc analysis. Because of multiple analyses, only effects with associated probabilities $< 0.01$ were considered. Prior to ANOVA, the data were checked for extreme values defined separately in each treatment group by a Dixon test at $P < 0.01$ (Dixon, 1959a,b). Animals showing an extreme value in one of the brain regions were excluded from further analysis. This applied to three young animals, one in each dose group (0, 30, 80 mg/kg). In addition, the data from one of the aged vehicle-treated groups were excluded from the analysis due to a missing value for one of the brain regions (cerebellum). The final group sizes were $n = 7$ for each of young treatment groups and the aged control group, and $n = 8$ for the aged groups treated with 30 and 80 mg/kg metrifonate, respectively.

## 3. Results

No gross adverse effects or behavioral symptoms were observed after the administration of metrifonate at the doses of 10 and 30 mg/kg. Slight signs of cholinergic

Table 1
Effect of metrifonate (MTF) administration on cholinesterase activity in different brain areas of young and aged rats

| | Young rats | | | Aged rats | | |
|---|---|---|---|---|---|---|
| | Vehicle | MTF 30 mg/kg | MTF 80 mg/kg | Vehicle | MTF 30 mg/kg | MTF 80 mg/kg |
| Cortex | 89 ± 2.9 | 77 ± 2.9 [a] | 21 ± 1.8 [a] | 77 ± 2.4 [b] | 57 ± 5.8 [a] | 12 ± 1.6 [a] |
| Cerebellum | 53 ± 3.4 | 40 ± 5.4 | 18 ± 3.0 [a] | 57 ± 3.7 | 36 ± 5.3 [a] | 7.6 ± 0.9 [a] |
| Hippocampus | 81 ± 5.7 | 73 ± 4.0 | 22 ± 2.4 [a] | 74 ± 3.2 | 52 ± 5.7 | 11 ± 1.5 [a] |
| Striatum | 296 ± 21 | 308 ± 19 | 99 ± 14 [a] | 262 ± 21 | 201 ± 25 | 38 ± 8.1 [a] |
| Midbrain | 141 ± 6.5 | 110 ± 7.2 [a] | 40 ± 2.5 [a] | 101 ± 4.3 [b] | 67 ± 6.4 [a] | 14 ± 1.8 [a] |

Cholinesterase activity, calculated 45 min after oral drug or vehicle administration, is expressed as nmol/mg protein per min (mean ± S.E.M.; $n = 7–8$). For statistical analysis, see details in Sections 2 and 3.
[a] $P < 0.01$ vs. appropriate vehicle controls (two-way ANOVA supplemented with Fisher LSD post-hoc comparisons).
[b] $P < 0.02$ vs. young rats (one-way ANOVA).

activation (tremor, defecation and fasciculation) were observed for the first 15–30 min after the administration of metrifonate 80 mg/kg.

### 3.1. Cholinesterase activity

Cholinesterase activity levels (Table 1) in the cortex and midbrain of aged rats were significantly lower than in those of young rats ($F(1,14) = 11.75$ and 14.03, respectively; $P < 0.02$). The enzyme was inhibited by metrifonate (Table 1), irrespective of age (General mean for Dose: $F(2,38) = 168.36$; $P < 0.001$). There was no interaction between Age and Dose ($F(2,38) = 1.84$; n.s.), indicating that metrifonate mediated a similar level of inhibition in aged and young rats.

Cholinesterase activity clearly differed in various brain regions, as confirmed by repeated measures ANOVA (Regions: $F(4,152) = 442.94$; $P < 0.001$), striatum showing the highest level of activity, and cerebellum the lowest, respectively. The profile across regions was differentially influenced by age (Regions by Age interaction: $F(4,152) = 15.85$; $P < 0.001$). The various regions also showed a differential sensitivity toward metrifonate-induced cholinesterase inhibition (Regions by Dose interaction: $F(8,152) = 54.49$; $P < 0.001$). However, this profile of drug sensitivity was similar in aged and young rats, as there was no interaction between Regions, Age, and Dose ($F(8,152) = 1.45$, n.s.).

Table 2
Baseline acetylcholine levels from the cortex and hippocampus of young and aged rats (pmol/20 min ± S.E.M.)

| | Young rats | Aged rats | % Decrease |
|---|---|---|---|
| Cortex | 0.33 ± 0.07 (12) | 0.19 ± 0.03 [a] (17) | −42% |
| Hippocampus | 0.30 ± 0.07 (11) | 0.12 ± 0.03 [b] (17) | −60% |

The baseline extracellular level of acetylcholine was calculated as follows: three dialysate samples were obtained from each animal and averaged, then the mean value for each animal was pooled with those of other rats in order to obtain the final mean ± S.E.M. [a] $P < 0.05$ and [b] $P < 0.02$ vs. young rats (Student's $t$-test). Number of rats in parentheses.

### 3.2. Extracellular acetylcholine levels

Extracellular acetylcholine levels measured in the cortex and hippocampus of young and aged rats before the administration of metrifonate and without cholinesterase inhibitor in the perfusion Ringer solution are shown in Table 2. The levels in the aged rats were significantly lower than in the young rats (−42% in the cortex and −60% in the hippocampus), and showed only small variations throughout the experiment, as can be seen in Figs. 1 and 2.

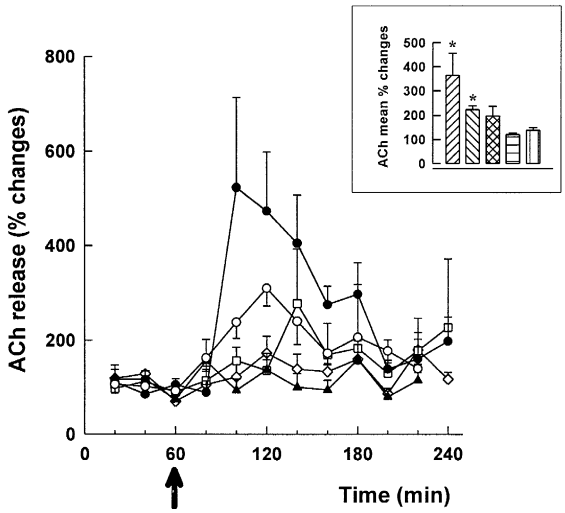


Fig. 1. Effect of metrifonate administration on cortical acetylcholine release in young and aged rats. Metrifonate or vehicle was administered after collection of three baseline samples, as shown by the arrow. Acetylcholine release is expressed as percent change over the mean of the first three samples and normalized to 100% (mean ± S.E.M.; $n = 4–6$ independent experiments). Aged rats: vehicle, ▲; metrifonate 30 mg/kg, □; metrifonate 80 mg/kg, ●. Young rats: vehicle, ◇; metrifonate 80 mg/kg, ○. Inset: Each bar in the inset represents the mean percent changes calculated from 100 to 200 min for each curve. Aged rats: vehicle, 5th bar (from left); metrifonate 30 mg/kg, 3rd bar; metrifonate 80 mg/kg, 1st bar. Young rats: vehicle, 4th bar; metrifonate 80 mg/kg, 2nd bar. Significance of differences among the five experimental groups was calculated using a one-way analysis of variance (ANOVA) ($F(4,20) = 4.49$; $P < 0.01$) followed by Fisher LSD test (* at least $P < 0.05$ vs. appropriate controls).

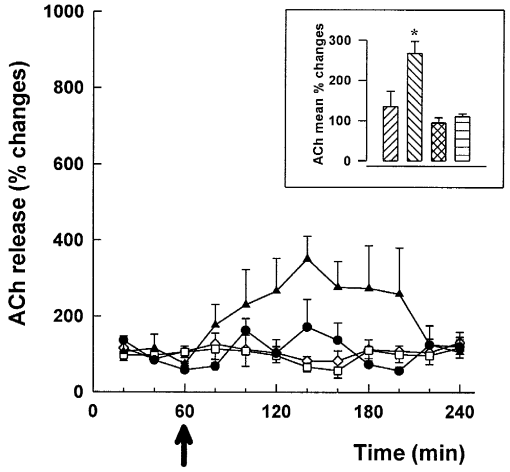


Fig. 2. Effect of metrifonate administration on hippocampal acetylcholine release in young and aged rats. Metrifonate or vehicle was administered after collection of three baseline samples, as shown by the arrow. Acetylcholine release is expressed as percent change over the mean of the first three samples and normalized to 100% (mean ± S.E.M.; $n = 4–6$ independent experiments). Aged rats: vehicle, □; metrifonate 80 mg/kg, ●. Young rats: vehicle, ◇; metrifonate 80 mg/kg, ▲. Inset: Each bar in the inset represents the mean percent changes calculated from 100 to 200 min for each curve. Aged rats: vehicle, 3rd bar (from left); metrifonate 80 mg/kg, 1st bar. Young rats: vehicle, 4th bar; metrifonate 80 mg/kg, 2nd bar. Significance of differences among the four experimental groups was calculated using one-way ANOVA ($F(3,13) = 7.66$; $P < 0.01$) followed by Fisher LSD test (* at least $P < 0.05$ vs. appropriate controls).

Fig. 1 illustrates the changes in extracellular acetylcholine levels in the cerebral cortex after metrifonate administration. In aged rats, the administration of metrifonate 80 mg/kg brought about a sharp, almost 4-fold increase (mean percent changes) in extracellular acetylcholine levels which peaked 40 min after administration and disappeared within the next 100 min. In young rats the administration of the same dose of metrifonate resulted in a statistically significant 2-fold increase (mean percent changes) in extracellular acetylcholine levels, with a peak 60 min after administration and a return to baseline levels in the next 80 min. Interestingly, the absolute maximal acetylcholine level after metrifonate treatment in aged rats was $1.1 \pm 0.29$ pmol/20 min, a value close to that of $0.89 \pm 0.24$ found in young rats while the absolute maximal acetylcholine levels in control animals after vehicle administration were $0.29 \pm 0.08$ pmol/20 min and $0.47 \pm 0.09$ for aged and young rats, respectively. In aged rats, the administration of 30 mg/kg of metrifonate was followed only by a small, not statistically significant increase.

Fig. 2 shows that metrifonate 80 mg/kg p.o. administered to young rats brought about a 3-fold increase in extracellular acetylcholine levels in the hippocampus, reaching a maximum of $1.0 \pm 0.25$ pmol/20 min 80 min after administration and lasting 140 min. Conversely, in the aged rats the effect of metrifonate was slight and irregular and reached a maximum of $0.16 \pm 0.07$ pmol/20 min (30% over the baseline level) 80 min after administration. Absolute maximal acetylcholine levels in control animals after vehicle administration were $0.13 \pm 0.04$ pmol/20 min and $0.33 \pm 0.10$ for aged and young rats, respectively.

### 3.3. Object recognition

Table 3 summarizes the effect of metrifonate on object recognition in young and aged rats. Neither aging nor treatment influenced the time necessary to reach criterion in the first trial (T1 in Table 3). When placed in the arena 60 min after the first exploration period, the young rats were able to discriminate between the familiar and novel objects as shown by the longer exploration time of the latter and the greater discrimination index. The aged rats spent more time than the young in exploring the familiar object and lost the ability to discriminate between the two objects. Discrimination was restored by the administration of metrifonate 80 mg/kg administered 30 min before the first trial. Metrifonate at 10 mg/kg and 30 mg/kg was ineffective to improve object recognition. Metrifonate administration did not modify object recognition in young rats.

Table 3
Effect of metrifonate administration on object recognition in the rat

| Treatment (mg/kg p.o.) | Age (months) | T1 exploration (s ± S.E.M.) | T2 objects | | Discrimination index |
|---|---|---|---|---|---|
| | | | Familiar (s ± S.E.M.) | Novel (s ± S.E.M.) | |
| Vehicle (10) | 6 | 199.3 ± 15.8 | 4.8 ± 1.0 | 9.3 ± 0.9 [a] | 0.37 ± 0.5 [b] |
| Metrifonate 80 (8) | 6 | 198.8 ± 25.7 | 3.3 ± 0.3 | 8.0 ± 0.8 [a] | 0.40 ± 0.06 [b] |
| Vehicle (10) | 22 | 206.1 ± 18.4 | 7.4 ± 0.7 | 8.7 ± 0.6 | 0.08 ± 0.03 |
| Metrifonate 10 (5) | 22 | 247.2 ± 9.70 | 6.6 ± 2.3 | 7.3 ± 3.2 | 0.11 ± 0.06 |
| Metrifonate 30 (8) | 22 | 212.5 ± 24.3 | 7.4 ± 1.7 | 9.3 ± 2.3 | 0.12 ± 0.05 |
| Metrifonate 80 (10) | 22 | 198.2 ± 19.7 | 7.6 ± 1.4 | 14.1 ± 1.4 [a] | 0.33 ± 0.08 [b] |

Discrimination index calculated as: $N - F/N + F$, where $N$ = exploration time of the novel object, and $F$ = exploration time of the familiar object. No significant difference was observed in T1 values (non-parametric Kruskal-Wallis ANOVA ($5 = P < 0.254$). The drugs were administered 30 min before the first trial; time between the two trials 60 min. Number of animals in parentheses.

[a] $P < 0.05$ $N$ versus $F$ (two-tailed paired $t$-test).

[b] $P < 0.05$ versus saline 22 months (non-parametric Kruskal-Wallis ANOVA ($5 = P < 0.003$) and multiple comparison $Z$-value).

## 4. Discussion

Our present experiments demonstrated that metrifonate, given p.o. to aged and young rats at the dose of 80 mg/kg: (1) inhibits cholinesterase activity in aged and young rats; (2) increases cortical acetylcholine release 4- and 2-fold in aged and young rats, respectively; (3) increases hippocampal acetylcholine release in young rats only; (4) improves object recognition in aged rats. Lower doses of metrifonate did not improve object recognition, and no significant effect on acetylcholine levels was found with 30 mg/kg.

In the present experiments, the baseline extracellular acetylcholine levels, measured in the absence of a cholinesterase inhibitor, were 42% lower in the cerebral cortex and 60% lower in the hippocampus of aged than of young rats. The levels were more than 10 times lower in both young and old rats than those measured in the presence of a cholinesterase inhibitor added to the perfusing Ringer solution (Wu et al., 1988; Scali et al., 1994, 1995), and were comparable to those found by Messamore et al. (1993) and Cuadra et al. (1994) in young rats without cholinesterase inhibitor. The lower level of acetylcholine release found in the aging rats is consistent with previous findings from our and other laboratories (Wu et al., 1988; Scali et al., 1994, 1995; Quirion et al., 1995) and is an indication of cholinergic hypofunction associated with brain aging. This suggestion is further supported by the finding of an age-related decrease in brain cholinesterase activity. The alteration in diurnal variation of acetylcholine release and lack of correlation between locomotor activity and acetylcholine release observed in old rats (Mitsushima et al., 1996) may be the reason why aging is not always associated with a decrease in acetylcholine release in the rat, as shown by Fischer et al. (1991) in the hippocampus of 24-month-old female rats.

Oral administration of metrifonate is followed by inhibition of brain cholinesterase activity. After the administration of 80 mg/kg of metrifonate about 85% cholinesterase inhibition was found in both the cortex and hippocampus of old rats, whereas in young rats 72% cholinesterase inhibition in the cortex and 77% in the hippocampus were found. In young rats this degree of inhibition was accompanied by a 2-fold increase in extracellular acetylcholine levels in both the cerebral cortex and hippocampus. Conversely, in aged rats the 85% inhibition induced by metrifonate (80 mg/kg) resulted in a 4-fold increase in extracellular acetylcholine levels in the cortex but only a slight increase in the hippocampus. In the cortex, metrifonate administration abolished the difference in extracellular acetylcholine levels between aged and young rats. Similarly, tacrine has been shown to restore stimulated acetylcholine release from brain tissue of Alzheimer patients to its control levels (Nilsson et al., 1987). Taken together, our results indicate that, in aged rats, regional differences in the regulation of extracellular acetylcholine levels become apparent, which may region dependently alter the physiological consequences of cholinesterase inhibition by metrifonate or other cholinesterase inhibitors. In this regard, Cuadra et al. (1994), comparing cholinesterase inhibition and the increase in acetylcholine extracellular levels in young rats after the administration of physostigmine and heptylphysostigmine, concluded that extracellular acetylcholine levels are not directly related to cholinesterase inhibition. Furthermore, the same authors demonstrated that cholinesterase inhibitor treatment also affects other neurotransmitter systems (Cuadra et al., 1994; Mori et al., 1995). It is possible that the regional differences observed in response to cholinesterase inhibitor treatment might be due to a different interplay among neurotransmitter systems.

The increase in extracellular acetylcholine levels evoked by metrifonate in young rats in our experiments was smaller than that observed by Mori et al. (1995). However, these authors administered metrifonate subcutaneously and differences in absorption and distribution, as well as rat strain differences, may explain this discrepancy.

Consistent with previous observations (Soininen et al., 1990; Blokland et al., 1995), acute administration of cholinesterase-inhibiting doses of metrifonate to rats led to transient peripheral cholinergic activation, as evidenced by behavioral symptoms such as tremor, defecation and fasciculation. Overall, these effects were mild and did not interfere with the outcome of behavioral testing in the object recognition test. All animals, irrespective of age or treatment, showed an active exploratory behavior as reflected by similar times to reach criterion during the first exposure to the objects.

The object recognition test measures the non-spatial memory, with the characteristic of episodic memory in the rat (Ennaceur and Delacour, 1988; Ennaceur and Meliani, 1992), assessed in non-human primates by the visual recognition test, which depends on the activity of cortical circuitry (Funahashi et al., 1989). It has been demonstrated (Scali et al., 1994; Bartolini et al., 1996) that, in young rats either treated with scopolamine or lesioned in the nucleus basalis and in aged rats, object discrimination requires the integrity of the cortical cholinergic system. Accordingly, we observed that object recognition in aged rats is restored by metrifonate at a dose that strongly inhibits cholinesterase and increases cortical acetylcholine release to levels comparable to those of young rats. No object recognition recovery takes place after a dose of metrifonate which causes only slight changes in cortical acetylcholine levels and cholinesterase activity. These results suggest that, in aging rats, there is a relation between the effects of metrifonate on cholinesterase inhibition, the increase in cortical acetylcholine levels and object recognition recovery. It should be noted that object recognition in young rats, evaluated 60 min after the first session, is neither improved nor impaired by the large increase in cortical acetylcholine. A longer intertrial period, making retention more difficult, may have been needed to detect the metri-

fonate effect in young rats also, as observed with other cognition enhancers (Bartolini et al., 1996). Object recognition appears to depend on the integrity of the cortical cholinergic system, as shown by the finding that the behavioral effect is seen even if hippocampal acetylcholine levels remain low. Previous studies (Blokland et al., 1995; Van der Staay et al., 1996; Riekkinen et al., 1996) demonstrated that metrifonate improves acquisition and memory in old rats tested in the Morris-maze and active avoidance tests, even at doses lower than those found effective in the object recognition test. In recent years much evidence has confirmed that activation of brain cholinergic functions is accompanied by an improvement of cognitive functions in aging rodents (Fischer et al., 1987; Vannucchi et al., 1990; Riekkinen et al., 1991; Ikari et al., 1995). The possibility that some behaviors might be more sensitive than object recognition to discrete cholinergic activation should be considered.

In conclusion, a single oral administration of metrifonate at the dose of 80 mg/kg to aged rats is followed by marked cholinesterase inhibition, a marked increase in cortical extracellular acetylcholine levels to levels comparable to those of young rats, and object recognition recovery to the level of performance of young adult rats. These findings support the possibility that metrifonate might be useful for correcting the memory impairment that is attributed to cholinergic hypofunction and is a characteristic feature of Alzheimer's disease.

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## References

Bartolini, L., F. Casamenti and G. Pepeu, 1996, Aniracetam restores object recognition impaired by age, scopolamine, and nucleus basalis lesions, Pharmacol. Biochem. Behav. 53, 277.

Bartus, R.T., R.L. Dean, B. Beer and A.S. Lippa, 1982, The cholinergic hypothesis of geriatric memory dysfunction, Science 217, 408.

Becker, R.E., J. Colliver, R. Elble, E. Feldman, E. Giacobini, V. Kumar, S. Markwell, P. Moriearty, R. Parks, S.D. Shillcutt, L. Unni, S. Vicari, C. Womack and R.F. Zec, 1990, Effects of metrifonate, a long-acting cholinesterase inhibitor, in Alzheimer disease, Drug Dev. Res. 19, 425.

Blokland, A., V. Hinz and B.H. Schmidt, 1995, Effects of metrifonate and tacrine in the spatial Morris task and modified Irwin test: evaluation of the efficacy/safety profile in rats, Drug Dev. Res. 36, 166.

Collerton, D., 1986, Cholinergic function and intellectual decline in Alzheimer's disease, Neuroscience 19, 1.

Cuadra, G., K. Summers and E. Giacobini, 1994, Cholinesterase inhibitor effects on neurotransmitters in rat cortex in vivo, J. Pharmacol. Exp. Ther. 270, 277.

Damsma, G., D. Lammerts Van Bueren, B.H.C. Westerink and A.S. Horn, 1987, Determination of acetylcholine in the femtomole range by means of HPLC, a post-column enzyme reactor, and electro-chemical detection, Chromatographia 24, 827.

Davis, K.L. and P. Powchik, 1995, Tacrine, Lancet 345, 625.

Dixon, W.J., 1959a, Analyses of extreme values, Ann. Math. Stat. 21, 488.

Dixon, W.J., 1959b, Ratios involving extreme values, Ann. Math. Stat. 22, 68.

Ellman, G.L., K.D. Courtney, V. Andres and R.M. Featherstone, 1961, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7, 88.

Ennaceur, A. and J. Delacour, 1988, A new one trial test for neurobiological studies of memory in rats. 1: Behavioral data, Behav. Brain Res. 31, 47.

Ennaceur, A. and K. Meliani, 1992, A new one-trial test for neurobiological studies of memory in rats. III: Spatial versus non spatial working memory, Behav. Brain Res. 51, 83.

Feldmeier, R. and E. Doehring, 1987, Clinical experience with metrifonate, Acta Trop. 44, 357.

Fischer, W., K. Wictorin, A. Björklund, L.R. Williams, S. Varon and F.H. Gage, 1987, Amelioration of cholinergic neurons atrophy and spatial memory impairment in aged rats by nerve growth factor, Nature 329, 65.

Fischer, W., O.J. Nilsson and A. Björklund, 1991, In vivo acetylcholine release as measured by microdialysis is unaltered in the hippocampus of cognitively impaired aged rats with degenerative changes in the basal forebrain, Brain Res. 556, 44.

Funahashi, S., C.J. Bruce and P.S. Goldman-Rakic, 1989, Mnemonic coding of visual space in the monkey's dorsolateral prefrontal cortex, J. Neurophysiol. 61, 814.

Giacobini, E., 1991, The second generation of cholinesterase inhibitors: pharmacological aspects, in: Cholinergic Basis for Alzheimer Therapy, eds. R. Becker and E. Giacobini (Birkhäuser, Boston, MA) p. 247.

Giacobini, E. and R. Becker, 1994, Development of drugs for Alzheimer therapy: a decade of progress, in: Alzheimer Disease: Therapeutic Strategies, eds. E. Giacobini and R. Becker (Birkhäuser, Boston, MA) p. 1.

Giovannini, M.G., F. Casamenti, A. Nistri, F. Paoli and G. Pepeu, 1991, Effect of thyrotropin releasing hormone (TRH) on acetylcholine release from different brain areas investigated by microdialysis, Br. J. Pharmacol. 102, 363.

Hinz, V.C., A. Blokland, F.J. Van der Staay, I. Gebert, T. Schurman and B.H. Schmidt, Receptor interaction profile and CNS general pharmacology of metrifonate and its transformation product dichlorvos in rodents, Drug Dev. Res. (in press).

Holmstedt, B., I. Nordgren, M. Sandoz and A. Sundwall, 1978, Metrifonate: summary of toxicological and pharmacological information available, Arch. Toxicol. 41, 3.

Ikari, H., E.L. Spangler, N.H. Greig, P. Xue-Feng, A. Brossi, D. Speer, N. Patel and D.K. Ingram, 1995, Maze learning in aged rats is enhanced by phenserine, a novel anticholinesterase, NeuroReport 6, 481.

Jackson, W.J., J.J. Buccafusco, A.V. Terry, D.J. Turk and D.K. Rush, 1995, Velnacrine maleate improves delayed matching performance by aged monkey, Psychopharmacology 119, 391.

Mandel, R.J., A.D. Chen, D.J. Connor and L.J. Thal, 1989, Continuous physostigmine infusions in rats with excitotoxic lesions of the nucleus basalis magnocellularis: effects on performance in the water maze and cortical cholinergic markers, J. Pharmacol. Exp. Ther. 251, 612.

Messamore, E., U. Warpman, N. Ogane and E. Giacobini, 1993, Cholinesterase inhibitor effects on extracellular acetylcholine in rat cortex, Neuropharmacology 32, 745.

Mitsushima, D., T. Mizuno and F. Kimura, 1996, Age-related changes in diurnal acetylcholine release in the prefrontal cortex of male rats as measured by microdialysis, Neuroscience 72, 429.

Mohammed, A.H., 1993, Effects of cholinesterase inhibitors on learning

and memory in rats: a brief review with special reference to THA, Acta Neurol. Scand. Suppl. 149, 13.

Mori, F., G. Cuadra and E. Giacobini, 1995, Metrifonate effects on acetylcholine and biogenic amines in rat cortex, Neurochem. Res. 20, 1081.

Nilsson, L., A. Adem, J. Hardy, B. Wimbland and A. Nordberg, 1987, Do tetrahydroaminoacridine (THA) and physostigmine restore acetylcholine release in Alzheimer brains via nicotinic receptors?, J. Neural Neurotransm. 70, 357.

Nordgren, I. and B. Holmstedt, 1988, Metrifonate: a review, in: Current Research in Alzheimer Therapy, eds. E. Giacobini and R. Becker (Taylor and Francis, New York, NY) p. 281.

Paxinos, G. and G. Watson, 1982, The Rat Brain in Stereotaxic Coordinates (Academic Press, New York, NY).

Quirion, R., A. Wilson, W. Rowe, I. Aubert, J. Richard, H. Doods, A. Parent, N. White and M.J. Meaney, 1995, Facilitation of acetylcholine release and cognitive performance by an M2-muscarinic receptor antagonist in aged memory-impaired rats, J. Neurosci. 15, 1455.

Riekkinen, P. and M. Riekkinen, 1995, Effects of tetrahydroaminoacridine and nicotine in nucleus basalis and serotonin-lesioned rats, Eur. J. Pharmacol. 279, 65.

Riekkinen, P., J. Sirviö, M. Riekkinen and P. Riekkinen, 1991, Tetrahydroaminoacridine improves passive avoidance retention defects induced by aging and medial septal lesion but not by fimbria-fornix lesion, Brain Res. Bull. 27, 587.

Riekkinen Jr., P., B. Schmidt, R. Stefanski, J. Kuitunen and M. Riekkinen, 1996, Metrifonate improves spatial navigation and avoidance behavior in scopolamine-treated, medial septal-lesioned and aged rats, Eur. J. Pharmacol. 309, 121.

Sarter, M., 1991, Taking stock of cognition enhancers, Trends Pharmacol. Sci. 12, 456.

Scali, C., F. Casamenti, M. Pazzagli, L. Bartolini and G. Pepeu, 1994, Nerve growth factor increases extracellular acetylcholine levels in the parietal cortex and hippocampus of aged rats and restores object recognition, Neurosci. Lett. 170, 117.

Scali, C., M.G. Vannucchi, G. Pepeu and F. Casamenti, 1995, Peripherally injected scopolamine differentially modulates acetylcholine release in vivo in the young and aged rats, Neurosci. Lett. 197, 171.

Soininen, H., L. Unni and S. Shillcutt, 1990, Effect of acute and chronic cholinesterase inhibition on biogenic amines in rat brain, Neurochem. Res. 15, 1185.

Squire, L.R. and H.P. Davis, 1981, The pharmacology of memory: a neurobiological perspective, Annu. Rev. Pharmacol. Toxicol. 21, 326.

Tanaka, K., N. Ogawa, M. Asanuma, Y. Kondo and A. Mori, 1994, Chronic administration of acetylcholinesterase inhibitor in the senescent rat brain, Neurobiol. Aging 15, 721.

Van der Staay, F.J., V.C. Hinz and B.H. Schmidt, 1996, Effects of metrifonate on escape and avoidance learning in young and aged rats, Behav. Pharmacol. 7, 56.

Vannucchi, M.G., F. Casamenti and G. Pepeu, 1990, Decrease of acetylcholine release from cortical slices in aged rats: investigations into its reversal by phosphatidylserine, J. Neurochem. 55, 819.

Webster Jr., L.T., 1990, Chemotherapy of parasitic infections, in: Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edn., eds. A. Goodman Gilman, T.W. Rall, A.S. Nies and P. Taylor (Pergamon Press, New York, NY) p. 964.

Wu, C.W., R. Bertorelli, M. Sacconi, G. Pepeu and S. Consolo, 1988, Decrease of brain acetylcholine release in aging freely-moving rats detected by microdialysis, Neurobiol. Aging 9, 357.

Xiao, W.B., A. Nordberg and X. Zhang, 1993, Effect of in vivo microdialysis of 1,2,3,4-tetrahydro-9-aminoacridine (THA) on the extracellular concentration of acetylcholine in the striatum of anaesthetized rats, J. Pharmacol. Exp. Ther. 265, 759.