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Effect of subchronic metrifonate treatment on cerebral glucose metabolism in young and aged rats

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Abstract

The effects of subchronic administration of metrifonate, a long-lasting cholinesterase inhibitor, on local cerebral glucose utilization were assessed in 3- and 27-month old Sprague–Dawley rats, using the autoradiographic [^{14}C]2-deoxyglucose technique. Rats were treated twice daily with metrifonate (80 or 120 mg/kg) for 3 weeks. The [^{14}C]2-deoxyglucose experiment was performed 18 h after the last metrifonate administration. In 3-month old rats, metrifonate 80 mg/kg increased the average hemispheric cerebral glucose utilization by 12% ($P > 0.001$). Significant effects were observed in 19 of the 54 regions studied, including cortical and limbic regions. The higher dose induced a larger effect (average increase 17%, 24 of the 54 regions affected). In 27-month old rats, very similar effects were obtained. These results show that repeated administration of metrifonate leads to a sustained metabolic activation in rat brain, at a level comparable to the activation observed previously after a single administration of the drug.

Keywords: Cholinesterase inhibitor; Cerebral metabolism; Alzheimer's disease; Aging; (Rat)

1. Introduction

The cognitive impairments that occur in Alzheimer's disease have been attributed, at least in part, to dysfunctions of cholinergic neurotransmission (Bartus et al., 1982; Candy et al., 1988). A possible therapeutic approach in Alzheimer's disease may be to compensate for the loss of central cholinergic neurons by potentiating the activity of the remaining intact neurons. This can be achieved with inhibitors of acetylcholinesterase, the enzyme that hydrolyses acetylcholine, so that more acetylcholine is available to postsynaptic neurons. Cholinesterase inhibitors such as physostigmine and tacrine are effective in some mildly demented Alzheimer's disease patients, but the therapeutic effects of these drugs are limited in terms of both the number of responders and the quality of improvement (for review see the work of Lamy (1994)). In addition, these 'first-generation' cholinesterase inhibitors have undesirable side effects which hamper their therapeutic benefit. 'Second-generation' cholinesterase inhibitors have been developed to overcome some of these problems. One member of this class of compounds, metrifonate ((*O*,*O*-dimethyl-(1-hydroxy-2,2,2-trichloroethyl)-phosphonate), is a well tolerated, long-lasting cholinesterase inhibitor with a unique mechanism of action. Metrifonate does not inhibit cholinesterase by itself. It is slowly transformed nonenzymatically in vivo to *O*,*O*-dimethyl 2,2-dichlorvovinylphosphate (dichlorvos), a potent and long-acting cholinesterase inhibitor (Nordgren et al., 1978).

Based on results obtained for the rat, it was suggested that metrifonate might be more effective than other anticholinesterase compounds in improving cholinergic function, because of its long duration of action, low rate of side effects and ability to cause high levels of cholinesterase inhibition (Becker and Giacobini, 1988; Giacobini, 1991). Clinical evidence that metrifonate ameliorates the symptoms of Alzheimer's disease patients has been provided by a preliminary open study (Becker et al., 1990) and confirmed recently by large, double-blind studies (Cummings et al., 1998; Morris et al., 1998). Furthermore, several behavioral studies have shown that metrifonate improves the cognitive performance of aged animals (Blokland et al., 1995; Van der Staay et al., 1996; Kronforst-Collins et

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al., 1997a,b; Scali et al., 1997) or animals with memory impairment induced by scopolamine or by lesions of the medial septum or of the basal forebrain (Riekkinen et al., 1996; Itoh et al., 1997).

Metrifonate increases local cerebral glucose utilization in young and aged rats in a number of regions, including regions involved in memory (Bassant et al., 1996). This action on energy metabolism reflects probably the activation of cholinergic transmission, which might explain, at least in part, the cognitive effects of metrifonate. It has also been reported that tacrine increases local cerebral glucose utilization in Alzheimer's disease patients, in brain areas such as the parietal and temporal cortices, which show consistent alterations in the disease (Nordberg et al., 1992; Nordberg, 1993). Our first results on the metrifonate-induced increase in local cerebral glucose utilization were obtained after a single acute administration (Bassant et al., 1996). It is of interest to determine whether a metabolic effect is still observed after subchronic treatment, which would approach the clinical situation in the Alzheimer patient. It is now established that repeated administration of metrifonate to rats or rabbits leads to long-lasting cholinesterase inhibition which equals that of the 'peak' inhibition seen after a single dose (Hinz et al., 1998; Kronforst-Collins et al., 1997a). This long-lasting inhibition is not accompanied by a counterregulatory adaptation in the activity of choline-acetyltransferase, the acetylcholine-synthesizing enzyme, or in the number or affinity of acetylcholine receptors (Hinz et al., 1998). The extracellular baseline levels of acetylcholine in the cerebral cortex of rats were persistently increased after repeated administration of metrifonate, but not of tacrine (Giovannini et al., 1998). The aim of the present study was to assess whether sustained activation of brain cholinergic neurotransmission would also result in a long-lasting activation of cerebral glucose metabolism. The experiment was performed in young and aged rats after a twice daily administration of metrifonate for 3 weeks.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were obtained from IFFA CREDO, France. A total of 21 rats aged 3 months (body weight 280 ± 21 g) were used. The rats were housed at 22°C under a 12-h light/dark cycle and had free access to water and food. Aged rats (27 months, 730 ± 120 g) were obtained at 24 months of age and were housed in our animal facility for 4 months (three animals per cage). Approximately 40% survived to an age of 28 months. None of the 15 aged rats used in the experiment had tumors. The care and use of the animals was approved by the local ethics committee.

2.2. Chemicals

Metrifonate was obtained from Bayer/Troponwerke (Germany). [^{14}C]2-deoxy-D-glucose (specific activity 50–55 mCi/mmol) in ethanol was purchased from Amersham (France). The solution was evaporated under nitrogen and [^{14}C]2-deoxy-D-glucose was resuspended in isotonic saline.

2.3. Drug administration

Metrifonate (80 and 120 mg/kg) was given p.o. twice daily for 3 weeks. Control rats received the same volume (0.5 ml/100 g body weight) of vehicle (5% Na^+ citrate buffered to pH 5.5).

2.4. Measurement of local cerebral glucose utilization

Local cerebral glucose utilization was measured by the quantitative [^{14}C]2-deoxy-D-glucose technique of Sokoloff et al. (1977), 18 h after the last metrifonate administration. Following 16 h of food deprivation, rats were lightly anesthetized (1% halothane in oxygen) and catheters were inserted in a femoral vein and artery. The operative sites were infiltrated with 1% xylocaine and closed with wound clips. Rats were placed in a loose-fitting plaster body cast in which the hindlimbs were restrained but which permitted free movements of the head and forelimbs. Body temperature was monitored via a rectal thermoprobe connected to a thermostatic device (Harvard, UK) which activated an external warming element when body temperature fell below 35.5°C. Mean arterial blood pressure and heart rate were assessed before and at fixed times after drug treatment by connecting the arterial catheter to a pressure transducer (Barovar, Alvar Electronic, France), which was attached to a paper chart recorder (Gould, USA). Baseline and subsequent measurements of plasma glucose concentration were made throughout the experiment (Glucose Analyzer II, Beckman, USA). Hematocrit was determined prior to the injection of [^{14}C]2-deoxy-D-glucose. Physiological variables monitored in the study were inside the following limits: mean arterial pressure 90–130 mm Hg, heart rate 350–500 beats/min, temperature 35–38°C. Plasma glucose concentrations ranged from 79 to 170 mg/dl.

After a 4-h recovery period, [^{14}C]2-deoxy-D-glucose 125 μCi/kg was administered by rapid i.v. bolus injection. Arterial blood samples (100 μl) were taken at 6, 18 and 30 s and 1, 3, 5, 7.5, 15, 25, 35 and 45 min after [^{14}C]2-deoxy-D-glucose injection. Samples were centrifuged immediately and plasma radioactivity (liquid scintillation counter 1209 LKB Wallac, USA) and glucose concentration were measured. Animals were killed 45 min after [^{14}C]2-deoxy-D-glucose injection by an overdose of pentobarbital. Brains were removed rapidly and frozen in isopentane chilled with dry ice at −45°C, then stored at

−70°C. Coronal brain sections (20-μm thick) were cut on a cryostat (Reichert-Jung, Cambridge Instrument, Germany) at −20°C. Sections were mounted on coverslips and dried immediately at 55°C on a hot plate. Autoradiographs were then prepared by exposing X-AR5 X-ray film (Kodak) to the mounted sections and [^{14}C] methylmetacrylate standards calibrated against similarly prepared 20 μm sections of brain (Amersham) for 7 days.

Autoradiograms were analyzed by quantitative densitometry with a computerized image processing device (Biocom, France). In order to identify brain structures and cortical layers, sections adjacent to those used for autoradiography were stained with Cresyl violet. Stained sections and corresponding sections in the autoradiogram were superimposed and the delineation of the structures was made according to the atlas of Paxinos and Watson (1986). The neocortex was divided into superficial layers (layers I, II and upper part of layer III), middle layers (lower part of layers III and IV) and deep layers (layers V and VI). Five separate optical density measurements for each structure were made in consecutive brain sections. For each rat and each brain region, local cerebral glucose utilization was calculated as the arithmetic mean of five independent measurements. As right–left differences were sometimes found, only measurements for the left hemisphere were considered. Tissue [^{14}C] concentrations were determined from the optical densities and a calibration curve obtained by densitometric analysis of the autoradiograms of the calibrated standards. Local cerebral glucose utilization was then calculated from the local tissue [^{14}C] concentrations, the time course of the plasma [^{14}C]2-deoxy-D-glucose and glucose concentrations and appropriate kinetic constants according to the operational equation published by Sokoloff et al. (1977). In the present experiment, the value of the lumped constant used in the calculation was 0.48 (Schuier et al., 1990).

2.5. Statistical analysis

Physiological variables were compared between age-matched control and treated rats by one-way analysis of variance (ANOVA), followed by Scheffé method. Within each experimental group, physiological variables at different times after [^{14}C]2-deoxy-D-glucose were subjected to ANOVA for repeated measures.

For each brain region, local cerebral glucose utilization was analyzed as follows: (1) 3-month old control rats and the two groups of metrifonate-treated rats (80 and 120 mg/kg) were compared by one-way ANOVA followed by Dunnett's test, for multiple comparisons; (2) 27-month old control and treated rats were analyzed in the same way; (3) local cerebral glucose utilization was analyzed by two-way ANOVA (interaction between treatment and age or mode of administration); (4) the local cerebral glucose utilization of 3- and 27-month old control rats was compared by one-way ANOVA followed by Fisher's test. Differences were considered significant if $P < 0.05$.

Table 1
Physiological variables during [^{14}C]2-deoxy-D-glucose experiment

	3 Months			27 Months		
	Controls ($n = 7$)	Metrifonate		Controls ($n = 5$)	Metrifonate	
		80 mg/kg ($n = 7$)	120 mg/kg ($n = 7$)		80 mg/kg ($n = 5$)	120 mg/kg ($n = 5$)
Body weight (g)	347 ± 23	344 ± 21	324 ± 26	765 ± 170	670 ± 95	590 ± 100
Hematocrit (%)	47 ± 2	46 ± 3	44 ± 2	41 ± 3	40 ± 3	38 ± 7
Mean arterial pressure (mm Hg)						
Before [^{14}C]DG	119 ± 5	110 ± 7	110 ± 10	118 ± 8	118 ± 11	94 ± 4[c,e]
15 min after [^{14}C]DG	110 ± 5	109 ± 7	115 ± 7	120 ± 9	117 ± 10	94 ± 4[c,e]
45 min after [^{14}C]DG	112 ± 8	104 ± 8	110 ± 14	114 ± 16	115 ± 10	96 ± 6
Heart rate (beats/min)						
Before [^{14}C]DG	487 ± 9	450 ± 31	467 ± 25	403 ± 20	439 ± 24	377 ± 24[d]
15 min after [^{14}C]DG	490 ± 12	452 ± 29	478 ± 17	419 ± 15	450 ± 18	370 ± 26[b,e]
45 min after [^{14}C]DG	485 ± 9	461 ± 32	473 ± 23	424 ± 8	444 ± 33	372 ± 17[b,e]
Body temperature (C°)						
Before [^{14}C]DG	37.2 ± 0.4	37.3 ± 0.6	37.2 ± 0.4	37.4 ± 0.4	37 ± 0.3	36.8 ± 0.6
15 min after [^{14}C]DG	37 ± 0.4	37.2 ± 0.6	37.2 ± 0.3	37.2 ± 0.4	37.4 ± 0.5	36.8 ± 0.4
45 min after [^{14}C]DG	36.9 ± 0.4	37.1 ± 0.6	37 ± 0.5	37 ± 0.7	37.5 ± 0.4	36.8 ± 0.4
Plasma glucose						
Before [^{14}C]DG	108 ± 14	104 ± 8	91 ± 10[a]	133 ± 30	111 ± 9	118 ± 33
15 min after [^{14}C]DG	106 ± 13	114 ± 20	88 ± 11	132 ± 30	109 ± 7	100 ± 36
45 min after [^{14}C]DG	111 ± 20	117 ± 21	86 ± 5[a]	138 ± 40	106 ± 9	98 ± 38

The experiment was performed 18 h after the last metrifonate administration at the end of the chronic treatment (80 or 120 mg/kg p.o. twice daily during 3 weeks).
Values are means ± S.D.
Different from age-matched controls [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.
Different from rats treated with the 80 mg/kg dose [d] $P < 0.01$, [e] $P < 0.001$ (ANOVA, Scheffé method).

3. Results

3.1. Physiological variables during [^{14}C]2-deoxy-D-glucose experiments

After the 80 mg/kg dose, physiological variables were not altered in metrifonate-treated rats of both ages, as compared with age-matched controls (Table 1). After the 120 mg/kg dose, arterial pressure and heart rate decreased in 27-month old rats and a number of rats had a significantly lower plasma glucose concentration than controls. However, this hypoglycemia was moderate (see Section 2) and therefore the deoxyglucose method was applied with the commonly used constants (Schuier et al., 1990).

3.2. Local cerebral glucose utilization in 3-month old rats

Subchronic treatment with metrifonate at the dose of 80 mg/kg increased the average hemispheric glucose utilization by 12% ($P < 0.001$). Values were 81.6 ± 23 and 91.2 ± 27 μmol/100 g/min for control and treated rats, respectively. In terms of regional distribution, significant effects were found in 19 of the 54 regions studied. In these regions, local cerebral glucose utilization increased by 22% as compared with controls. In cortical areas, the greatest effects were observed in the piriform (21%) and retrosplenial cortices (28%) (Table 2). Local cerebral glucose utilization increased in a large number of limbic regions, by 18% (ventral and dorsal hippocampus, medial septal complex) to 34% (preoptic area) (Table 3 and Fig. 1). In the diencephalon, marked effects were found in reticular thalamic nuclei (32%) and lateral habenula (24%) (Table 4).

At the dose of 120 mg/kg, subchronic treatment increased hemispheric glucose utilization by 17% ($P < 0.001$). Values were 81.6 ± 23 and 95.4 ± 28 μmol/100 g/min for control and treated rats, respectively. Significant effects were found in 24 of the regions studied (Tables 2–4). The average increase in local cerebral glucose utilization in these regions was 30%. In addition to the regions where changes were observed after the 80-mg/kg dose, local cerebral glucose utilization was increased in the caudate–putamen (34%), the nucleus accumbens (42%) (Fig. 1), the red nucleus (14%), the pars reticulata and compacta of the substantia nigra (30%). Although the metabolic activation appeared to be greater with the dose of 120 mg/kg, the difference with the dose of 80 mg/kg did not reach statistical significance.

Table 2
Local cerebral glucose utilization (100 μmol/100 g/min) in *cortical areas* in controls and metrifonate-treated rats aged 3 and 27 months

Brain regions	3 Months			27 Months		
	Controls ($n = 7$)	MFT 80 mg/kg ($n = 7$)	MFT 120 mg/kg ($n = 7$)	Controls ($n = 5$)	MFT 80 mg/kg ($n = 5$)	MFT 120 mg/kg ($n = 5$)
Prefrontal						
Superficial layers	89 ± 7	95 ± 10	97 ± 9	83 ± 12	84 ± 7	101 ± 14
Middle layers	105 ± 7	110 ± 8	108 ± 7	87 ± 12^{a}	104 ± 10	109 ± 17
Deep layers	81 ± 5	87 ± 8	89 ± 9	74 ± 11	80 ± 8	89 ± 9
Piriform	99 ± 13	120 ± 14^{c}	125 ± 18^{c}	94 ± 7	118 ± 15^{c}	120 ± 23^{c}
Parietal						
Superficial layers	105 ± 8	109 ± 12	113 ± 16	97 ± 19	103 ± 9	110 ± 17
Middle layers	115 ± 11	123 ± 13	128 ± 17	105 ± 18	127 ± 10^{c}	133 ± 16^{c}
Deep layers	92 ± 7	101 ± 6	94 ± 11	81 ± 13	102 ± 12	97 ± 10
Insular	112 ± 12	127 ± 13^{c}	130 ± 12^{c}	88 ± 10^{b}	110 ± 19^{c}	114 ± 15^{c}
Cingulate	104 ± 10	126 ± 9^{d}	134 ± 12^{d}	89 ± 13	117 ± 17^{c}	124 ± 22^{d}
Sensorimotor						
Superficial layers	103 ± 8	108 ± 7	107 ± 9	92 ± 13	99 ± 8	110 ± 17
Middle layers	115 ± 9	120 ± 9	119 ± 10	105 ± 11	110 ± 8	122 ± 21
Deep layers	94 ± 10	96 ± 9	96 ± 8	87 ± 10	93 ± 13	104 ± 13
Retrosplenial	103 ± 8	132 ± 12^{d}	137 ± 13^{d}	97 ± 6^{a}	120 ± 23^{c}	124 ± 26^{c}
Occipital						
Superficial layers	94 ± 8	97 ± 7	96 ± 9	79 ± 7^{a}	102 ± 11^{d}	103 ± 13^{d}
Middle layers	104 ± 11	112 ± 8	110 ± 11	87 ± 12^{a}	108 ± 14^{c}	112 ± 18^{c}
Deep layers	93 ± 8	94 ± 8	101 ± 8	84 ± 11^{a}	100 ± 16	105 ± 16^{c}
Temporal						
Superficial layers	114 ± 8	120 ± 9	121 ± 13	101 ± 14	112 ± 10	106 ± 15
Middle layers	129 ± 6	136 ± 14	137 ± 17	115 ± 15^{a}	124 ± 12	118 ± 16
Deep layers	110 ± 7	114 ± 11	118 ± 10	94 ± 15^{a}	106 ± 11	102 ± 19
Entorhinal	64 ± 4	67 ± 7	67 ± 6	56 ± 6^{a}	73 ± 6^{d}	77 ± 12^{d}
Perirhinal	66 ± 4	70 ± 7	71 ± 11	56 ± 6^{a}	69 ± 8	73 ± 16

Values are means ± S.D.
Different from 3-month old rats $^{a}P < 0.05$, $^{b}P < 0.01$ (ANOVA, Fisher's test).
Different from controls $^{c}P < 0.05$, $^{d}P < 0.01$ (ANOVA, Dunnett's test).

Table 3
Local cerebral glucose utilization (100 μmol/100 g/min) in *limbic areas* and *white matter* in controls and metrifonate-treated rats aged 3 and 27 months

Brain regions	3 Months			27 Months		
	Controls ($n = 7$)	MFT 80 mg/kg ($n = 7$)	MFT 120 mg/kg ($n = 7$)	Controls ($n = 5$)	MFT 80 mg/kg ($n = 5$)	MFT 120 mg/kg ($n = 5$)
Medial septum	64 ± 5	76 ± 6^{c}	76 ± 9^{c}	52 ± 6^{b}	68 ± 9^{d}	71 ± 9^{d}
Diagonal band of Broca (vertical limb)	68 ± 5	79 ± 7^{c}	84 ± 11^{d}	56 ± 10	73 ± 9^{c}	76 ± 11^{d}
Diagonal band of Broca (horizontal limb)	73 ± 6	89 ± 12^{c}	96 ± 15^{d}	74 ± 12^{a}	78 ± 8	90 ± 17
Dorsal hippocampus CA1 (stratum radiatum)	59 ± 5	72 ± 7^{d}	73 ± 7^{d}	53 ± 6	72 ± 9^{c}	75 ± 13^{d}
Dorsal hippocampus CA3 (stratum radiatum)	71 ± 5	84 ± 10^{c}	88 ± 10^{d}	63 ± 8	78 ± 9^{c}	80 ± 10^{c}
Dentate gyrus (multiform layer)	61 ± 5	72 ± 7^{c}	78 ± 10^{d}	54 ± 11	63 ± 8	75 ± 9^{d}
Ventral hippocampus CA1 (stratum radiatum)	61 ± 4	71 ± 6^{d}	73 ± 8^{d}	53 ± 7^{a}	66 ± 6^{d}	71 ± 7^{d}
Ventral hippocampus CA3 (stratum radiatum)	71 ± 4	88 ± 7^{d}	94 ± 12^{d}	67 ± 6	76 ± 5^{c}	79 ± 7^{d}
Subiculum	85 ± 7	109 ± 17^{d}	116 ± 12^{d}	76 ± 9	99 ± 14^{c}	104 ± 16^{d}
Basolateral amygdala	82 ± 6	104 ± 10^{d}	111 ± 8^{d}	78 ± 10	87 ± 8	92 ± 29
Preoptic area (magnocellularis)	76 ± 6	102 ± 12^{d}	114 ± 10^{d}	73 ± 13	81 ± 6	96 ± 19
Fimbria fornix	27 ± 3	28 ± 4	31 ± 7	25 ± 3	27 ± 4	32 ± 5
Corpus callosum	30 ± 5	31 ± 5	35 ± 8	23 ± 3	26 ± 3	27 ± 6

See Table 2 for legend.

3.3. Local cerebral glucose utilization in 27-month old rats

Subchronic treatment with metrifonate (80 mg/kg) increased the average hemispheric glucose utilization by 15% ($P < 0.001$). Values were 73.3 ± 23 and 84.5 ± 26 μmol/100 g/min for control and treated rats, respectively. In terms of regional distribution, significant effects were found in 20 of the 54 regions studied. In these regions, local cerebral glucose utilization increased by 26% as compared with controls. In cortical areas, the greatest effects were observed in the piriform and insular (25%), occipital, cingulate and entorhinal cortices (30%) (Table 2 and Fig. 2). Consistent effects were observed in limbic regions such as medial septum and diagonal band of Broca (30%), dorsal and ventral hippocampus (13 to 36%) and subiculum (30‰) (Table 3 and Fig. 2).

The higher dose of metrifonate (120 mg/kg) induced a greater metabolic effect in 27-month old rats ($P < 0.05$ as compared to 80 mg/kg). Average hemispheric glucose utilization increased by 23% (90.1 ± 28 μmol/100 g/min as compared with 73.3 ± 23 μmol/100 g/min in age-matched controls). Significant effects were found in 26 of the regions studied (Tables 2–4). The average increase in local cerebral glucose utilization in these regions was 31%. In addition to the regions where changes were observed after the 80 mg/kg dose, local cerebral glucose utilization increased in the substantia innominata (26%), the dentate gyrus (39%), the pars compacta of the substantia nigra (20%) and the ventral thalamus (34%) (Fig. 2).

3.4. Comparison of 3- and 27-month old rats

Age-related decreases in local cerebral glucose utilization occurred in 20 regions (Tables 2–4 and Figs. 2 and 3). Overall, metrifonate not only maintained its activity in the majority of these regions, but also tended to be more effective in some regions in the older rats. Thus, in the occipital and entorhinal cortex, globus pallidus and ventral tegmental area, aged rats showed a significant increase in local cerebral glucose utilization while this was not the case in young rats. The interaction between age and treatment on average hemispheric and regional values was estimated by a two-way ANOVA. There was no significant main treatment/age effect, indicating a similar effect of metrifonate in both age groups.

3.5. Comparison with acute treatment

Results obtained in the present experiment with the dose of 80 mg/kg were compared with those of a previous experiment performed 30 min after a single i.p. injection of metrifonate at the dose 80 mg/kg to rats of the same strain (Bassant et al., 1996). Subchronic treatment had a greater effect than single administration especially in aged rats (Table 5) but the difference did not reach statistical significance (two-way ANOVA). The topography of the increases in local cerebral glucose utilization differed somewhat according to the mode of administration. Subchronic treatment increased local cerebral glucose utilization in several limbic regions (medial septum, vertical limb of the diagonal band of Broca, dorsal and ventral hippocampus, subiculum) which were not affected by acute treatment. The opposite was observed for the superior colliculus, temporal cortex and substantia nigra (pars compacta) and interpositus nucleus of 3-month old rats and for the ventral thalamus and interpeduncular nucleus of 27-month old rats.

4. Discussion

In the present work, we determined whether repeated administration of metrifonate to rats stimulated local cerebral glucose utilization as previously observed in rats

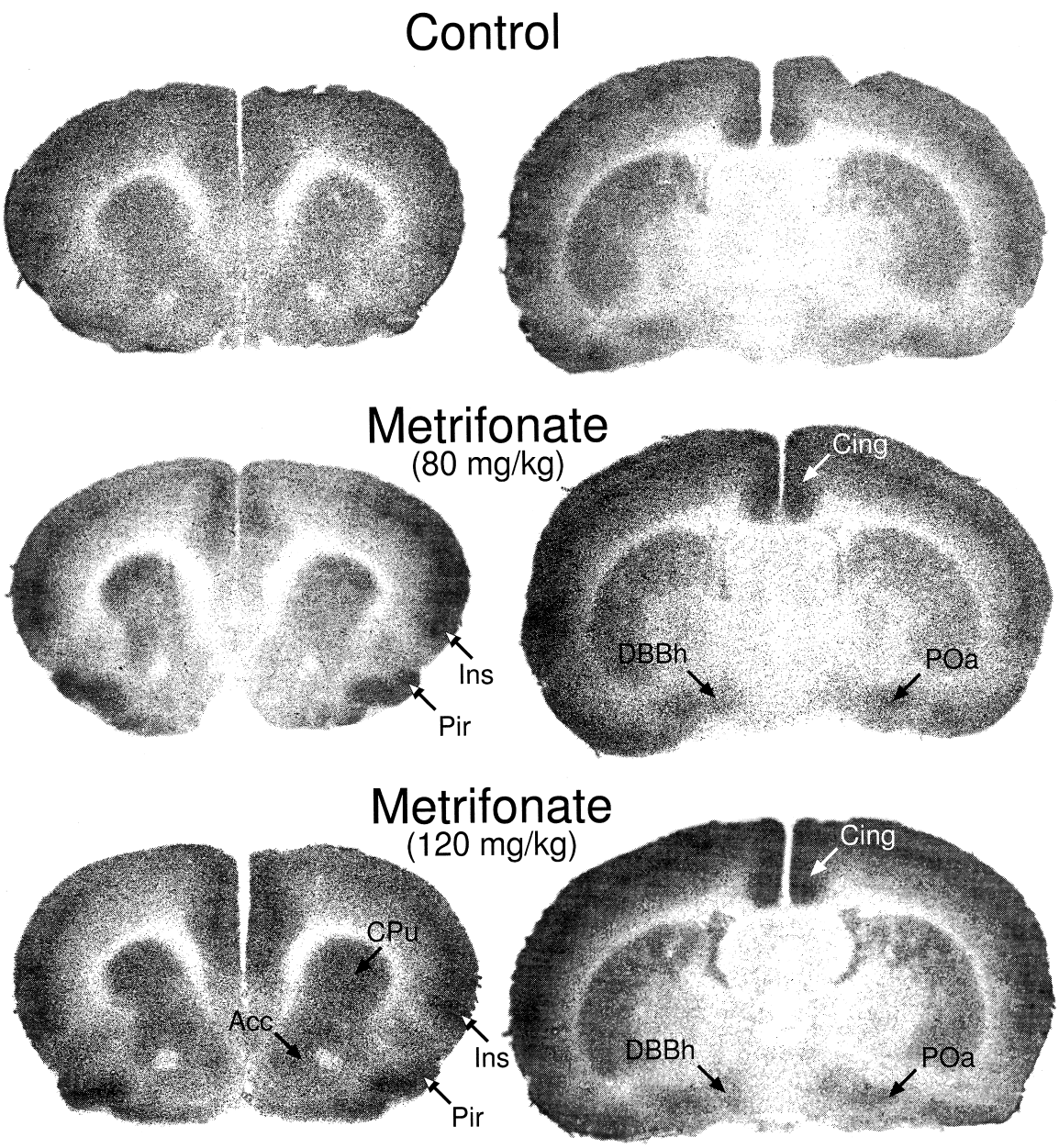

Fig. 1. Autoradiograms from control and metrifonate-treated 3-month old rats. *Control*: local cerebral glucose utilization under basal conditions. *Metrifonate* (80 or 120 mg/kg, twice daily during 3 weeks): regions with significant increases in local cerebral glucose utilization are indicated by arrows. Notice that the metabolic activation was greater with the higher dose. *Abbreviations*: Ins: insular cortex, Pir: piriform cortex, Cing: cingulate cortex, POa: preoptic area, DBBh: diagonal band of Broca (horizontal limb), CPu: caudate–putamen, Acc: accumbens nucleus.

acutely treated with the drug (Bassant et al., 1996). Our data show that subchronic administration of metrifonate indeed increased local cerebral glucose utilization in 3- and 27-month old rats. The amplitude of the effect was similar in both age groups.

The persistence of the effect on repeated treatment indicates that the activation of local cerebral glucose utilization induced by metrifonate does not undergo desensitization upon prolonged treatment. This finding is in agreement with other data demonstrating that prolonged inhibition of acetylcholinesterase during subchronic oral administration of metrifonate is not accompanied by changes in other cholinergic parameters such as acetylcholine synthesis and muscarinic or nicotinic receptor binding (Hinz et al., 1998). The absence of adaptive changes in cholinergic receptor binding during subchronic treatment with metrifonate is at variance with the counterregulatory effect of tacrine, another inhibitor of acetylcholinesterase, which down-regulates the number but not the affinity of muscarinic receptors and upregulates nicotinic receptor binding in rats (Flynn and Mash, 1989; Nilsson-Hakansson et al., 1990).

Table 4
Local cerebral glucose utilization (100 μmol/100 g/min) in *basal ganglia, diencephalon, brainstem and cerebellum* in controls and metrifonate-treated rats aged 3 and 27 months

Brain regions	3 Months			27 Months		
	Controls ($n = 7$)	MFT 80 mg/kg ($n = 7$)	MFT 120 mg/kg ($n = 7$)	Controls ($n = 5$)	MFT 80 mg/kg ($n = 5$)	MFT 120 mg/kg ($n = 5$)
Caude–putamen (anterior)	85 ± 8	92 ± 11	114 ± 15[d]	75 ± 7[a]	82 ± 6	86 ± 14
Globus pallidus (central part)	62 ± 8	59 ± 4	63 ± 5	43 ± 7[b]	54 ± 5[c]	56 ± 5[c]
Nucleus accumbens	69 ± 7	78 ± 7	98 ± 20[d]	71 ± 12	81 ± 8	85 ± 9
Substantia innominata	57 ± 6	59 ± 4	60 ± 5	49 ± 7	53 ± 6	62 ± 8[c]
Ventral thalamus	89 ± 6	108 ± 12[d]	113 ± 10[d]	90 ± 12	99 ± 15	121 ± 7[c]
Reticular thalamic nucleus	69 ± 5	91 ± 14[d]	104 ± 12[d]	67 ± 11	93 ± 9[d]	96 ± 11[d]
Anterior hypothalamic area	51 ± 4	56 ± 7	57 ± 8	46 ± 6	46 ± 7	50 ± 13
Ventral hypothalamus	50 ± 7	54 ± 8	52 ± 10	48 ± 5	51 ± 5	57 ± 4
Substantia nigra (pars reticulata)	56 ± 5	61 ± 5	74 ± 11[d]	45 ± 7	58 ± 6[c]	62 ± 8[d]
Substantia nigra (pars compacta)	81 ± 6	87 ± 9	104 ± 17[d]	68 ± 5[b]	76 ± 12	82 ± 11[c]
Lateral habenula	107 ± 15	148 ± 14[d]	133 ± 12[d]	106 ± 13	123 ± 11[c]	124 ± 11[c]
Red nucleus	79 ± 5	83 ± 7	90 ± 9[c]	67 ± 7	83 ± 10[c]	87 ± 12[d]
Ventral tegmental area	58 ± 5	64 ± 7	66 ± 11	47 ± 8[b]	52 ± 10	59 ± 7[c]
Interpeduncular nucleus	101 ± 12	125 ± 15[c]	122 ± 16[d]	90 ± 6[a]	94 ± 15	115 ± 25
Superior colliculus	83 ± 7	93 ± 13	93 ± 9	70 ± 10[a]	74 ± 9	98 ± 30
Central grey matter	57 ± 6	61 ± 8	59 ± 8	54 ± 7	53 ± 5	55 ± 7
Raphe dorsalis	81 ± 8	87 ± 9	90 ± 13	73 ± 10	76 ± 8	75 ± 13
Peduncular tegmental nucleus	57 ± 3	61 ± 6	59 ± 5	58 ± 3	61 ± 9	60 ± 8
Laterodorsal tegmental nucleus	101 ± 14	103 ± 12	97 ± 13	91 ± 18	90 ± 15	103 ± 7
Interpositus nucleus	92 ± 10	107 ± 16	112 ± 8	97 ± 14	101 ± 14	105 ± 15

See Table 2 for legend.

Although the increase in local cerebral glucose utilization was strikingly similar after subchronic administration of metrifonate and single administration (Bassant et al., 1996; see also Table 5), it is noteworthy that the large increases in local cerebral glucose utilization in this study were observed 18 h after the last administration of metrifonate. At such a late time point, local cerebral glucose utilization in rats would have recovered to baseline values after a single administration of metrifonate, due to the transient nature of the acute effect (Bassant et al., 1996). Hence, the effect of the drug is not only maintained but also prolonged upon repeated administration. This therapeutically interesting effect is explained by the fact that repeated administration of metrifonate establishes stable and long-lasting acetylcholinesterase inhibition in rats and rabbits, which achieves a steady-state after 10–15 doses and shows very slow recovery kinetics (Hinz et al., 1998; Kronforst-Collins et al., 1997b). Not only does the inhibition of acetylcholinesterase activity switch from transient to prolonged, but so does the resulting increase in extracellular acetylcholine levels in rat cerebral cortex (Giovannini et al., 1998) and the facilitation of cognitive performance of medial septum-lesioned rats in a Morris water escape task (Riekkinen et al., 1997).

4.1. Effects of metrifonate on local cerebral glucose utilization in 3-month old rats

As anticipated, most of the regions in which metrifonate increased cerebral glucose utilization have a strong cholinergic innervation (Mash and Potter, 1986; Paxinos and Watson, 1986). This finding indicates that the metrifonate-induced metabolic activation is probably due to its anticholinesterase properties or, more exactly, to the action of its active metabolite, dichlorvos. A relationship between the level of acetylcholinesterase activity and responsiveness to metrifonate has been already observed in the majority of regions after acute treatment (Bassant et al., 1996). There was, however, no strict correlation, as acute metrifonate did not affect local cerebral glucose utilization in some regions with a high level of acetylcholinesterase activity, such as striatum, medial septal area and dorsal hippocampus (Bassant et al., 1996). It is interesting to note that significant increases in local cerebral glucose utilization were observed in these regions after subchronic treatment, so that the topography of the effects more closely reflects the distribution of changes in acetylcholinesterase activity seen after subchronic treatment as compared to acute treatment. In contrast, the previously observed increases in local cerebral glucose utilization in the interpositus nucleus, the superior colliculus and the temporal cortex after a single dose were not found in the present study. When the [^{14}C]2-deoxy-D-glucose experiment was performed at the peak of acetylcholinesterase inhibition, i.e., 30 min after i.p. injection of metrifonate (acute regimen), it is possible that the metabolic activation observed in the cerebellum, the visual and auditory systems is due to side effects such as tremors and to an exacerbated sensitivity to sensory messages. This assumption is supported by the observation that the frequency and

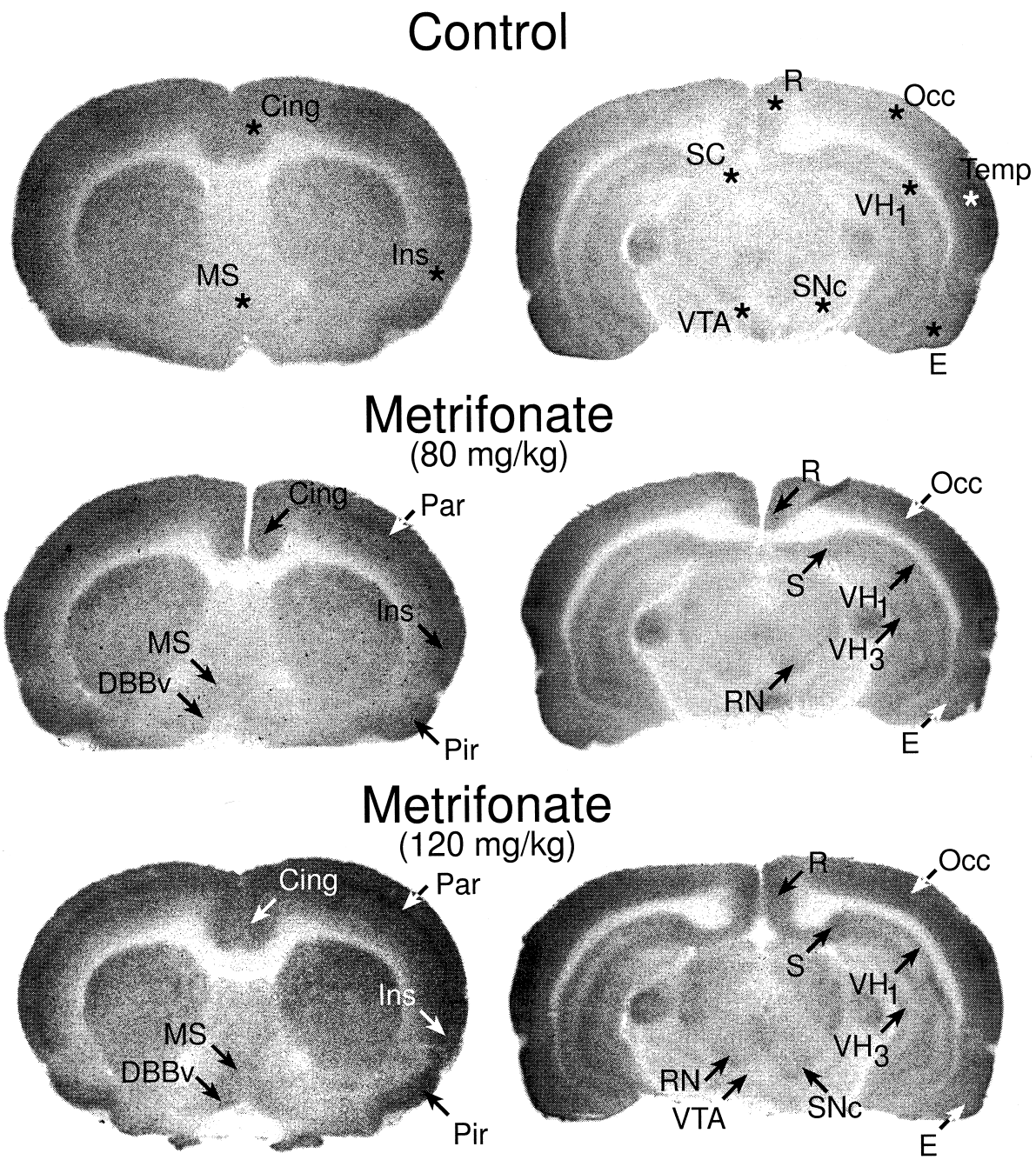

Fig. 2. Autoradiograms from control and metrifonate-treated 27-month old rats. *Control*: local cerebral glucose utilization under basal conditions. Regions with significant age-related hypometabolism are indicated by an asterisk. *Metrifonate* (80 or 120 mg/kg, twice daily during 3 weeks): regions with significant increases in local cerebral glucose utilization are indicated by arrows. Notice that the metabolic activation was greater with the higher dose and that the metrifonate was also active in regions with age-related hypometabolism. *Abbreviations*: *left side*: Cing: cingulate cortex, Ins: insular cortex, MS: medial septum, DBBv: diagonal band of Broca (vertical limb), Par: parietal cortex; *right side*: R: retrosplenial cortex, Occ: occipital cortex, Temp: temporal cortex, SC: superior colliculus, S: subiculum, VH1: ventral hippocampus (CA1 field), VH3: ventral hippocampus (CA3 field), SNc: substantia nigra (pars compacta), RN: red nucleus, VTA: ventral tegmental area, En: entorhinal cortex.

intensity of adverse cholinergic effects induced by acute overdosing of metrifonate decrease upon repeated administration of the drug and are absent 18 h after drug administration (Blokland et al., 1995; Giovannini et al., 1998)

To our knowledge, this is the first study of the effect of chronic administration of a cholinesterase inhibitor on local cerebral glucose utilization in animal models. Some studies have investigated the effects of chronic treatment with cholinergic agonists. Controversial results have been found after chronic nicotine treatment. London et al. (1990) did not observe an effect on basal glucose utilization in any of the cerebral regions examined. However, chronic

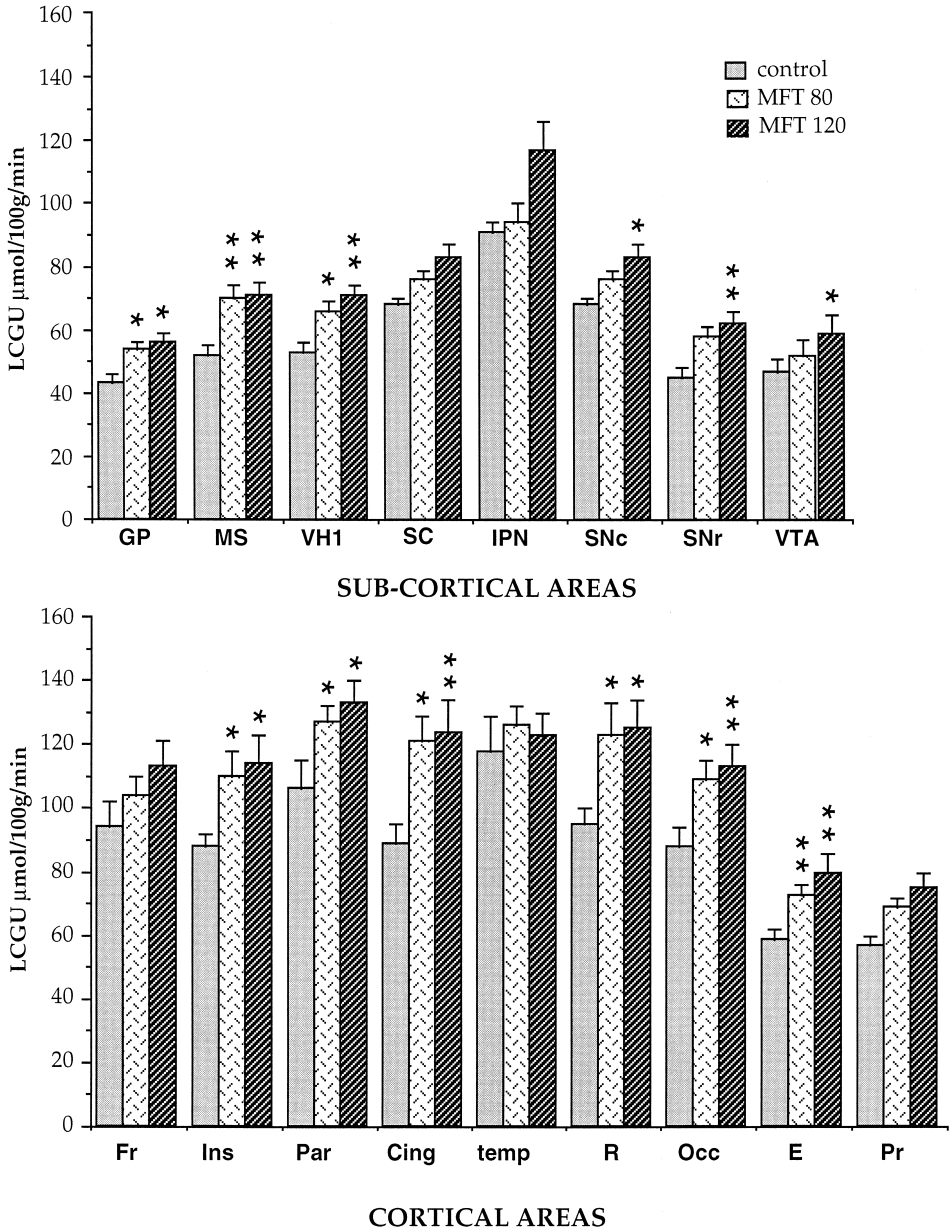

Fig. 3. Effects of subchronic administration of metrifonate on local cerebral glucose utilization in aged rats. These diagrams show local cerebral glucose utilization in the *regions where age-related hypometabolism occurs*. Notice that metrifonate increased local cerebral glucose utilization in a large number of these regions. *Abbreviations*: GP: globus pallidus, MS: medial septum, VH1: ventral hippocampus (CA1 field), SC: superior colliculus, IPN: interpeduncular nucleus, SNc: substantia nigra (pars compacta), SNr: substantia nigra (pars reticulata), VTA: ventral tegmental area, Fr: frontal cortex, Ins: insular cortex, Par: parietal cortex, Cing: cingulate cortex, Temp: temporal cortex, R: retrosplenial cortex, Occ: occipital cortex, E: entorhinal cortex, Pr: perirhinal cortex.

treatment reduced the response to a single injection of nicotine given 2 min before the deoxyglucose experiment. Grünwald et al. (1988) found that chronic administration of nicotine increased cerebral glucose utilization in a limited number of regions of the visual and limbic systems and in the globus pallidus. According to the authors, the number of regions affected was too small to support the hypothesis of an up-regulation of nicotinic receptors induced by long-term treatment. The effects of both acute and chronic administration of the muscarinic agonist arecoline on cerebral blood flow and metabolism have been compared in conscious rats (Maiese et al., 1994). A larger increase in average hemispheric cerebral glucose utilization was found after chronic treatment. The distribution of the effects was somewhat different depending on the treatment. For example, increased local cerebral glucose utilization values were observed in the superior colliculus and the interpositus nucleus only after acute treatment, the

Table 5
Comparison of the effects of single and repeated doses of metrifonate (80 mg/kg)

Experimental groups	3-Month old rats		27-Month old rats	
	Single dose (i.p.)	Repeated doses (p.o.)	Single dose (i.p.)	Repeated doses (p.o.)
Average hemispheric cerebral glucose utilization (μmol/100 g/min)	86 ± 30	91.2 ± 27	77 ± 25	84.5 ± 26
Percent (%) of increase (whole brain)	10.5%	12%	10%	15%
Number of regions affected (out of 54 regions)	17	19	18	20
Average increase in local cerebral glucose utilization in the affected regions	23%	22%	25%	26%

(Single dose measurements are those reported in the work of Bassant et al., 1996).

opposite being found in the dorsal hippocampus. It is interesting to note that similar results were obtained in the present experiment.

4.2. Effects of metrifonate on local cerebral glucose utilization in 27-month old rats

Moderate age-related reductions of brain metabolism have been observed in humans (Horwitz et al., 1986; Khul et al., 1996) and in rats (Smith et al., 1980; Wree et al., 1991; Bassant et al., 1994). Severe hypometabolism is a constant finding in the brain of Alzheimer's disease patients (Benson et al., 1983; De Leon et al., 1983; Foster et al., 1984). Alterations of local cerebral glucose utilization might be due to deficits in neurotransmitter systems, including the cholinergic system. It was, therefore, important to determine whether the activating effect of repeated metrifonate on brain glucose metabolism observed in young rats was still present in aged rats, particularly in regions where age-related hypometabolism occurs. This question was also addressed in the previous study which showed that a single dose of metrifonate increased local cerebral glucose utilization in several brain regions in the aged rat. The response of aged and young rats was similar in terms of average hemispheric cerebral glucose utilization, number of regions affected and amplitude of regional effect. It was interesting to note that metrifonate was still significantly active in regions that show age-related hypometabolism. For example, in the insular and cingulate cortices, the ventral hippocampus, the thalamus and the superior colliculus, the balance between the effect of metrifonate and the age-related hypometabolism resulted in an increased local cerebral glucose utilization which exceeded that of young controls (Bassant et al., 1996). In the present experiment, subchronic treatment with metrifonate in aged rats induced a metabolic activation which was slightly greater than that observed in young rats. Following acute treatment, two other acetylcholinesterase inhibitors tacrine and physostigmine, were found to be less active in aged rats than in young rats, in terms of both the number of regions affected and the magnitude of metabolic activation (Bassant et al., 1995). In agreement with these findings, a recent study of the effect of subchronic administration of metrifonate and tacrine on cortical acetylcholine release in aged rats has shown that metrifonate but not tacrine induces a significant and long-lasting increase in cortical acetylcholine release (Giovannini et al., 1998).

It is conceivable that the ability of metrifonate to produce a longer-lasting inhibition of acetylcholinesterase than tacrine, especially after repeated administration (Soininen et al., 1990), might partly explain the greater effect of metrifonate on local cerebral glucose utilization and acetylcholine release in the aged brain, where the cholinergic transmission is reduced. Measurement of extracellular acetylcholine levels by microdialysis in vivo has shown, indeed, that the baseline release of acetylcholine is lower in aged rats than in young rats (Wu et al., 1988; Scali et al., 1997). A single oral dose of metrifonate given to aged rats increased the cortical acetylcholine release four-fold in aged rats but and only two-fold in young rats (Scali et al., 1997). The tendency for a greater effect of metrifonate in aged vs. young rat brain is consistent with our finding that metrifonate at least maintains its effect on local cerebral glucose utilization in aged rats, both after acute (Bassant et al., 1996) and repeated administration (this study).

4.3. Increased regional metabolic activity and therapeutic effect of metrifonate

The present study shows that long-term treatment with metrifonate, unlike acute treatment, increased metabolic activity in a number of regions of the limbic system (cingulate, retrosplenial and piriform cortex; subiculum, medial septum, hippocampus). Our results are consistent with those of a microdialysis study showing that acetylcholine release in the hippocampus of aged rats was increased only during subchronic administration of metrifonate (Giovannini et al., 1998). How can we explain that the hippocampal response to metrifonate is achieved only after repeated administration? One possible explanation is that cholinesterase inhibitors decrease acetylcholine release via an acetylcholine-mediated inhibitory feedback mechanism at the presynaptic level. The sustained increase in acetylcholine levels induced by subchronic treatment with

metrifonate might depress this autoregulation by altering the functionality of presynaptic receptors. The effect of metrifonate on cholinergic transmission would be reinforced upon long-term treatment. Further studies are required to show whether this presynaptic mechanism occurs and why it affects preferentially the hippocampus. It is likely that the sustained cholinergic activation induced by metrifonate enhances neuronal activity, as shown by increased local cerebral glucose utilization, in regions involved in memory processing. Such an increase in local cerebral glucose utilization might occur in Alzheimer's disease patients receiving metrifonate and contribute to the therapeutic effect of the compound. Support for this assumption comes from the finding that subchronic administration of metrifonate not only leads to a permanent activation of the cholinergic system as discussed above, but also mediates a persistent cognitive activation, as demonstrated in medial septum-lesioned rats (Riekkinen et al., 1997), neurologically intact adult rats (Schmidt and Heinig, 1998) and aged rabbits (Kronforst-Collins et al., 1997b). Therefore, the results of the present study support the use of metrifonate as a symptomatic once-daily treatment of mild to moderate Alzheimer's disease.

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