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Sustained effect of metrifonate on cerebral glucose metabolism after immunolesion of basal forebrain cholinergic neurons in rats

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Abstract

To evaluate the influence of cholinergic projections from the basal forebrain on brain metabolism, we measured the cerebral metabolic rate of glucose (CMR(glu)) after unilateral lesioning of cholinergic basal forebrain neurons with the immunotoxin 192 IgG-saporin. CMR(glu) was determined in 24 cortical and 13 sub-cortical regions using the [^{14}C]2-deoxy-D-glucose technique of Sokoloff. Average hemispheric CMR(glu) decreased by 7% ($P < 0.02$) and 5% ($P < 0.05$), 7 and 21 days after lesion, respectively. Regional effects were restricted to parietal and retrosplenial cortices, lateral habenula and the basal forebrain. We have previously shown that metrifonate increased CMR(glu) in intact rats. In lesioned rats, metrifonate (80 mg/kg, i.p.) was still active but the metabolic activation was reduced in terms of both the average hemispheric CMR(glu) and the number of regions significantly affected. Although it is reduced, the sustained effect of metrifonate in lesioned rats makes an argument for the use of this compound as treatment of cholinergic deficit in Alzheimer's disease.

Keywords: Cholinergic system; Cholinesterase inhibitor; Cerebral metabolism; Alzheimer's disease; Nucleus basalis, rat

1. Introduction

The loss of cholinergic neurons in the basal forebrain of Alzheimer's disease patients results in marked reductions in cholinergic markers in the cerebral cortex and the hippocampus (Whitehouse et al., 1982). Alzheimer's disease is also characterized by a decline in the cerebral metabolic rate of glucose (CMR(glu)) that affects initially the parietal and temporal cortices (Benson et al., 1983; Duara et al., 1986; Ibanez et al., 1998). The question of whether reductions in CMR(glu) observed in Alzheimer's disease patients result from the degeneration of cholinergic innervation has been addressed by examining the metabolic effects of lesions of the nucleus basalis magnocellularis in rats (London et al., 1984; Orzi et al., 1988; Soncrant et al., 1992; Ouchi et al., 1996) and in non-human primates (see reference in Le Mestric et al., 1998). Unilateral electrolytic or neurotoxic lesions of the nucleus basalis magnocellularis induced a transient hypometabolism, the severity of which varied markedly, depending on the study. The heterogeneity of the results might be due to the fact that lesions were not restricted to the cholinergic neurons but also damaged other neurons and fiber tracks. In the present study, we have used the immunotoxin 192 IgG-saporin for selectively lesioning the basal forebrain cholinergic neurons (Wiley et al., 1991). 192 IgG-saporin is constructed from a monoclonal antibody to the low affinity nerve growth factor receptor (NGF), p 75^{NGFR}, and saporin, a ribosome-inactivating toxin. The selectivity of 192 IgG-saporin results from the selective expression of p 75^{NGFR} by basal forebrain cholinergic neurons. Following intracerebroventricular or intratissular injections, 192 IgG-saporin binds p 75^{NGFR} and is internalized. Once internalized, the saporin component is cleaved from the antibody and disrupts the ribosomal function leading to the neuronal death (Wiley et al., 1991). Neurons that do not express 75^{NGFR} are not affected by the immunotoxin (Book et al., 1992; Heckers et al., 1994). In the first part of the present experiment, CMR(glu) was evaluated in a large number of brain regions 7 and 21 days after 192 IgG-saporin treatment.

Inhibitors of acetylcholinesterase have been used to compensate for the decrease in cholinergic transmission in

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Alzheimer's disease patients. Among them, metrifonate is a well-tolerated, long-lasting cholinesterase inhibitor with a unique mechanism of action. Metrifonate does not inhibit cholinesterase by itself; it is slowly and nonenzymatically transformed in vivo to dichlorvovinylphosphate (dichlorvos), a potent and long-acting cholinesterase inhibitor (Nordgren et al., 1978; Hinz et al., 1996). Clinical evidence that metrifonate ameliorates the symptoms of Alzheimer's disease patients has been provided by a number of large, double-blind studies (Becker et al., 1990; Cummings et al., 1998; Morris et al., 1998). Furthermore, 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 al., 1997; Scali et al., 1997) or of animals with memory impairment induced by scopolamine or by lesions of the basal forebrain (Riekkinen et al., 1996; Itoh et al., 1997).

Metrifonate increases CMR(glu) in young and aged rats in a number of regions, including those involved in memory (Bassant et al., 1996; Poindessous-Jazat et al., 1998). This action on energy metabolism probably reflects the activation of cholinergic transmission, which might in part explain the cognitive effects of metrifonate. It is important to determine whether the metabolic effects of metrifonate observed in intact rats persists in a model that mimics the cholinergic denervation occurring during Alzheimer's disease. This result was achieved in the second part of the present experiment by measuring CMR(glu) in 192 IgG saporin-treated rats after acute administration of metrifonate.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were obtained from IFFA CREDO, France. Thirty-five rats aged 3 months (body weight 280–320 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. The local ethics committee approved the care and use of the animals.

2.2. Nucleus basalis magnocellularis lesions

192 IgG-saporin (Chemicon International) was dissolved in phosphate-buffered saline (0.67 μg/ml) and stored frozen (−70°C) until used. Rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and placed in a stereotaxic apparatus. A hole was drilled in the skull for 192 IgG-saporin injection in the right side (coordinates: −1 mm posterior to bregma, 2.6 mm lateral to midline, 8 mm below the cortical surface according to the atlas of Paxinos and Watson (1986). Pressure injections were performed through a glass micropipette (tip diameter 40 μm) attached to a 10 μl Hamilton syringe. Lesioned rats ($n = 14$) were injected with 192 IgG-saporin (total amount 134 ng in 0.2 μl of 0.9% saline) at the rate of 0.01 μl/min. The micropipette remained in place an additional 10 min and was then slowly withdrawn from the brain. Control animals (sham-operated $n = 14$) received an equivalent amount of saline. All the injected rats survived without weight loss.

2.3. Chemicals

Metrifonate was obtained from Bayer/Troponwerke (Germany). Hexamethonium bromide and atropine methylbromide were obtained from Sigma (France). [^{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.

2.4. Drug administration

To block the peripheral effects of metrifonate, all the rats were pretreated with hexamethonium bromide (5 mg/kg i.p.) and atropine methylbromide (4 mg/kg s.c.) 30 and 20 min, respectively, before drug administration. Rats then received an i.p. injection of metrifonate (80 mg/kg, 0.2 ml/100 g body weight) freshly dissolved in saline (pH < 6.0) 30 min before the [^{14}C]2-deoxy-D-glucose experiment. Control rats received the same volume of saline i.p.

2.5. 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). Seven days after nucleus basalis magnocellularis lesioning, experiments were performed in four experimental groups of seven rats : sham-operated and lesioned saline-injected rats; sham-operated and lesioned, metrifonate-treated rats. CMR(glu) was also measured 3 weeks after lesioning in a group of seven rats. Following 16 h of food deprivation, rats were lightly anesthetized (1% halothane in oxygen) and catheters were inserted in a femoral vein and an artery. The operative sites were infiltrated with 1% xylocaine and closed with wound clips. Rats were placed in a loose-fitting plaster body cast which restrained the hind limbs but 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 the 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 within 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, 30 min after metrifonate administration. Arterial blood samples (100 μl) were taken at 6, 18, 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] methyl-metacrylate 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 the upper part of layer III), middle layers (lower part of layer III, layer 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. Tissue [^{14}C] concentrations were determined from the optical densities and from 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 concentration, and appropriate kinetic constants according to the operational equation published by Sokoloff et al. (1977). In the present

Table 1
Physiological variables during [^{14}C] 2-deoxy-D-glucose experiment. Metrifonate injected 30 min before [^{14}C] 2-deoxy-D-glucose. Values are means ± S.D.

	Sham-lesioned		Lesioned	
	Untreated (saline) $n = 7$	Metrifonate (80 mg/kg) $n = 7$	Untreated (saline) $n = 7$	Metrifonate (80 mg/kg) $n = 7$
Body weight (g)	284 ± 29	298 ± 24	290 ± 16	299 ± 20
Hematocrit (%)	46 ± 4	45 ± 1	44 ± 3	46 ± 1
Mean arterial pressure (mm Hg)				
Before [^{14}C]DG	109 ± 10	117 ± 8	117 ± 9	119 ± 10
15 min after [^{14}C]DG	107 ± 9	112 ± 7	112 ± 10	115 ± 8
45 min after [^{14}C]DG	109 ± 6	116 ± 11	104 ± 17	120 ± 9
Heart rate (beats / min)				
Before [^{14}C]DG	467 ± 25	465 ± 20	485 ± 9	461 ± 27
15 min after [^{14}C]DG	482 ± 14	455 ± 27	482 ± 22	432 ± 37**
45 min after [^{14}C]DG	477 ± 18	465 ± 32	441 ± 60	432 ± 29
Body temperature (°C)				
Before [^{14}C]DG	36.8 ± 0.5	36.8 ± 0.5	37 ± 0.7	36.4 ± 0.6
15 min after [^{14}C]DG	36.7 ± 0.4	36 ± 0.5	36.9 ± 0.8	35.3 ± 1**
45 min after [^{14}C]DG	36.6 ± 0.5	35.8 ± 0.4^{†,a}	36.6 ± 0.5	34.9 ± 0.4***,^{b}
Plasma glucose				
Before [^{14}C]DG	116 ± 10	119 ± 17	126 ± 17	123 ± 21
15 min after [^{14}C]DG	117 ± 12	122 ± 22	127 ± 12	145 ± 23^{a}
45 min after [^{14}C]DG	116 ± 13	132 ± 20	134 ± 22	162 ± 36^{†,c}

Different from sham-lesioned untreated rats ‡ $P < 0.01$.
Different from lesioned untreated rats $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA, Scheffé Method).
Different from values before [^{14}C]DG $^{a}P < 0.05$, $^{b} < 0.01$, $^{c}P < 0.001$ (paired-sample t-test).

experiment, the value of the lumped constant used in the calculation was 0.48 (Schuier et al., 1990).

2.6. Histology

The loss of cholinergic nucleus basalis magnocellularis neurons was checked on brain sections stained for acetylcholinesterase activity according to the procedure described by Mesulam (1982) and the loss of acetylcholinesterase fiber-staining in the cortex was checked using the procedure of Karnovsky and Roots (1964). Decrease in acetylcholinesterase cortical activity was estimated by measuring the optical densities of stain product in homologous regions of the left and right sides. Rats with incomplete lesion were excluded from the study.

2.7. Statistical analysis

Physiological parameters were compared between experimental groups by one-way analysis of variance (ANOVA), followed by the Scheffé method. Within each group, physiological parameters at different time after [^{14}C]2-deoxy-D-glucose were compared using paired-sample *t*-test.

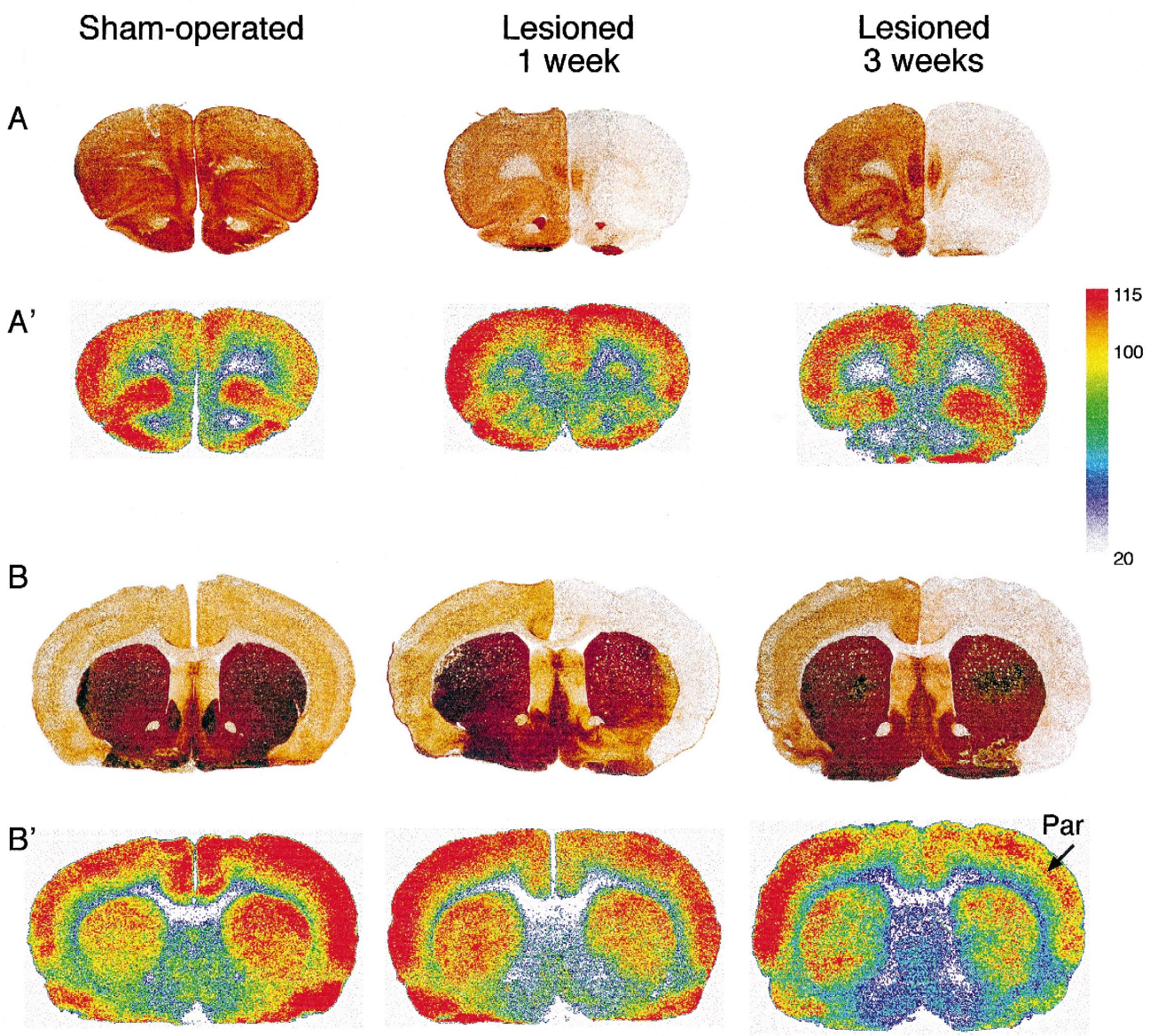

Fig. 1. Effect of lesioning cholinergic neurons of the nucleus basalis magnocellularis (right side) on acetylcholinesterase activity and local cerebral glucose utilization. (A–B) Coronal sections of rat brain through the prefrontal (A) and the parietal (B) cortex, processed for acetylcholinesterase activity (Karnovski and Roots' method). (A′–B′) Pseudocoloured autoradiograms obtained from adjacent brain sections (scale: μmol/100 g/min). Notice that 1 week after lesion, the marked reduction in acetylcholinesterase activity in the lesioned side (A) is not accompanied by change in local cerebral glucose utilization (A′). Three weeks after lesion, a decrease in local cerebral glucose utilization is observed (B′), limited to the parietal cortex (Par).

For each region, acetylcholinesterase activity from homologous left (intact) and right (lesioned) sides were compared by paired *t*-test. CMR(glu) values from intact and lesioned sides were also compared by paired *t*-test (interhemispheric asymmetry). In addition, left and right CMR(glu) values from different groups were compared by one- and two-way ANOVA followed by Scheffé's method for multiple comparison. Differences were considered significant if $p < 0.05$.

3. Results

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

The lesion of the nucleus basalis magnocellularis did not induce changes in physiological variables in saline-injected rats (Table 1). During the course of the experiment, body temperature decreased in sham-operated and lesioned rats treated with metrifonate. This effect was significantly greater in lesioned rats. Metrifonate decreased heart rate and increased plasma glucose concentration in lesioned rats. However, physiological variables monitored in the study were within the required limits of the method of Sokoloff et al., (1977).

3.2. Effect of nucleus basalis magnocellularis lesioning on CMR(glu)

No significant right–left difference in acetylcholinesterase activity between homologous cortical regions was observed in sham-operated rats (paired *t*-test, $t_{34} = 1.12$, $P = 0.26$) (Figs. 1 and 2). In lesioned rats, the loss of

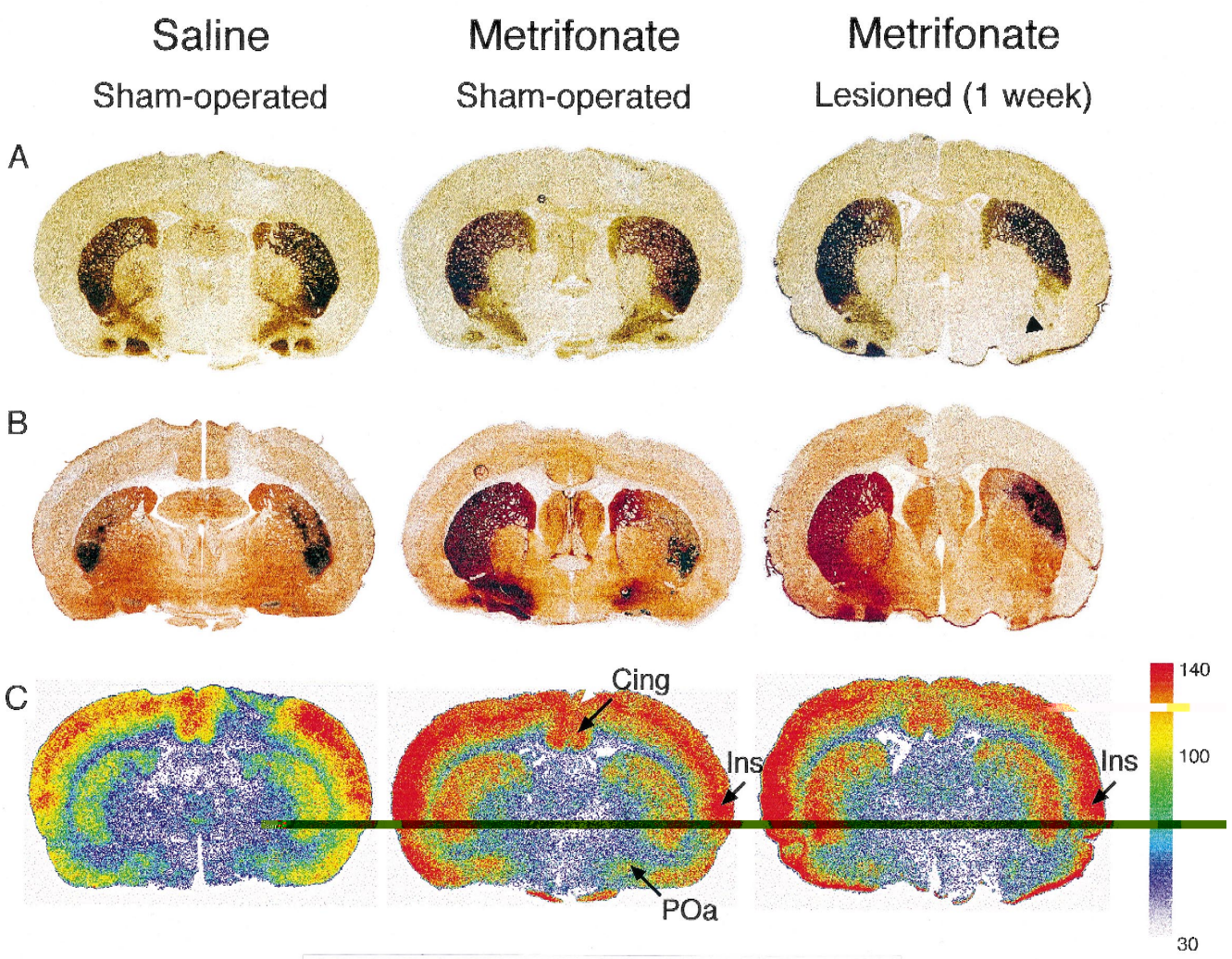

Fig. 2. Effect of metrifonate (80 mg/kg) on local cerebral glucose utilization in sham-operated and lesioned rats. (A) Coronal brain sections showing the loss of acetylcholinesterase-positive neurons in the nucleus basalis magnocellularis (arrowhead) (Mesulam's method). (B) Coronal brain sections (close to the sections shown in (A) processed for acetylcholinesterase activity in cholinergic terminals. (C) Pseudocoloured autoradiograms obtained from adjacent brain sections. Arrows indicate regions with significant increases in glucose utilization. Notice that the effect of metrifonate is preserved in the insular cortex (Ins) but lost in the cingulate cortex (Cing) and the magnocellular preoptic area (POa).

cholinergic neurons in the right nucleus basalis magnocellularis (Fig. 2A) induced ipsilateral decreases in acetylcholinesterase activity in the cortical areas which ranged from 70% to 90% ($t_{34} = 14.79$, $P = 0.0001$) (Figs. 1A–B and 2B). Consistent with previous findings (Heckers et al., 1994), acetylcholinesterase activity in the other regions, including the basolateral amygdala, the lateral habenula and the thalamic nuclei, was unaffected in lesioned rats ($t_{23} = 1.64$, $P = 0.154$).

CMR(glu) was measured in 24 regions innervated by the nucleus basalis magnocellularis and in 13 subcortical regions in which a metabolic effect of inhibitors of acetylcholinesterase had been found in previous studies (Bassant et al., 1993, 1996). One week after sham-lesioning, there was no difference in CMR(glu) values between right and left hemispheres in the control group (mean values compared by ANOVA, $F = 0.06$, $P = 0.8$; right–left values in homologous regions compared by paired *t*-test, all values of $t_6 < 1.8$). One week after lesioning, the average hemispheric CMR(glu) in the right projection regions (cortical areas, basolateral amygdala, reticular thalamic nucleus, lateral habenula) and the nucleus basalis magnocellularis

Table 2
Cerebral metabolic rate of glucose (CMR(glu)) in regions innervated by the nucleus basalis magnocellularis and in subcortical regions in sham-operated and lesioned rats 1 and 3 weeks after lesioning. For each region, differences between the left side (intact) and the right side (sham-lesioned or lesioned) are estimated by paired *t*-test. CMR(glu) = μmol/100 g/min. Values are means ± S.D. (S) = superficial layers, (M) = middle layers, (D) = deep layers

Brain regions	Sham-operated $n = 7$		Lesioned (1 week) $n = 7$		Lesioned (3 weeks) $n = 7$	
	Left	Right	Left	Right	Left	Right
Projection regions						
Frontal cortex (S)	88 ± 15	92 ± 16	97 ± 9	93 ± 6	92 ± 7	88 ± 7
Frontal cortex (M)	95 ± 15	97 ± 14	105 ± 12	95 ± 8	103 ± 5	98 ± 7
Frontal cortex (D)	77 ± 14	80 ± 10	86 ± 12	84 ± 11	84 ± 3	85 ± 14
Piriform cortex	106 ± 10	110 ± 11	113 ± 6	103 ± 11	104 ± 9	107 ± 14
Parietal cortex (S)	100 ± 12	102 ± 15	98 ± 12	89 ± 9	100 ± 6	85 ± 12[a]
Parietal cortex (M)	109 ± 13	107 ± 13	114 ± 18	96 ± 13	110 ± 8	96 ± 9[c]
Parietal cortex (D)	88 ± 9	87 ± 14	90 ± 17	80 ± 12	94 ± 9	84 ± 11[c]
Insular cortex	107 ± 14	104 ± 11	106 ± 14	101 ± 9	111 ± 5	106 ± 9
Cingulate cortex	102 ±	98 ± 15	109 ± 6	104 ± 7	104 ± 7	100 ± 7
Sensorimotor cortex (S)	97 ± 9	99 ± 13	101 ± 8	97 ± 9	95 ± 7	93 ± 9
Sensorimotor cortex (M)	110 ± 10	110 ± 13	109 ± 13	104 ± 4	113 ± 12	103 ± 7
Sensorimotor cortex (D)	89 ± 8	92 ± 10	88 ± 6	85 ± 5	89 ± 12	88 ± 10
Entorhinal cortex	61 ± 6	64 ± 10	61 ± 5	60 ± 7	63 ± 8	66 ± 3
Retrosplenial cortex	113 ± 12	109 ± 10	109 ± 8	98 ± 9[c]	106 ± 7	102 ± 9
Occipital cortex (S)	98 ± 12	102 ± 16	99 ± 9	97 ± 14	95 ± 6	86 ± 7
Occipital cortex (M)	107 ± 13	108 ± 17	107 ± 8	106 ± 11	111 ± 7	106 ± 11
Occipital cortex (D)	95 ± 10	94 ± 16	96 ± 10	91 ± 11	95 ± 10	89 ± 11
Temporal cortex (S)	108 ± 13	111 ± 13	105 ± 6	98 ± 8	108 ± 5	107 ± 7
Temporal cortex (M)	123 ± 11	128 ± 10	121 ± 8	112 ± 13	123 ± 7	119 ± 10
Temporal cortex (D)	104 ± 15	100 ± 15	101 ± 5	93 ± 8	96 ± 10	93 ± 8
N. basalis magnocellularis	55 ± 7	55 ± 7	52 ± 5	42 ± 9[c]	53 ± 5	39 ± 11[c]
Basolateral amygdala	85 ± 9	85 ± 10	83 ± 8	77 ± 7	82 ± 9	81 ± 7
Reticular thalamic nucleus	67 ± 6	65 ± 4	66 ± 7	67 ± 7	70 ± 4	70 ± 9
Lateral habenula	100 ± 13	97 ± 9	112 ± 10	91 ± 18	101 ± 15	83 ± 15[c]
Other regions						
Preoptic area	80 ± 11	81 ± 12	87 ± 11	73 ± 6[b]	88 ± 11	71 ± 17[b]
Diagonal band of Broca (horiz.)	75 ± 8	75 ± 9	70 ± 10	62 ± 8[b]	75 ± 7	63 ± 10[b]
Ventral thalamus	85 ± 6	84 ± 5	87 ± 6	86 ± 9	93 ± 12	92 ± 9
Dorsal hippocampus (CA1)	57 ± 10	55 ± 10	58 ± 3	57 ± 6	57 ± 5	58 ± 6
Dorsal hippocampus (CA3)	68 ± 9	64 ± 11	71 ± 6	72 ± 7	67 ± 3	71 ± 6
Dentate gyrus	62 ± 10	60 ± 14	62 ± 4	62 ± 2	61 ± 5	59 ± 4
Subiculum	88 ± 13	88 ± 16	89 ± 10	85 ± 11	79 ± 9	76 ± 8
Ventral hippocampus (CA1)	58 ± 7	59 ± 9	61 ± 5	57 ± 4	56 ± 4	55 ± 4
Ventral hippocampus (CA3)	70 ± 4	73 ± 7	75 ± 10	72 ± 9	75 ± 6	73 ± 6
Substantia nigra (pars c.)	78 ± 8	77 ± 9	75 ± 5	76 ± 7	78 ± 6	77 ± 5
Interpeduncular nucleus	104 ± 10	id	103 ± 7	id	105 ± 7	id
Superior colliculus	81 ± 5	80 ± 8	86 ± 6	80 ± 5	85 ± 7	83 ± 6
Interpositus nucleus	96 ± 7	100 ± 11	95 ± 6	96 ± 16	104 ± 14	105 ± 12

[a] $P < 0.05$.
[b] $P < 0.02$.
[c] $P < 0.01$.

decreased by 7% ($P < 0.02$). Values were 97 ± 20 and 90 ± 18 μmol/100 g/min for the intact side and for the lesioned side, respectively. The hypometabolism found in the lesioned side reflected a trend to lower CMR(glu) values in several brain areas such as the parietal and temporal cortices, the lateral habenula, the basolateral amygdala but left–right differences were statistically significant only in the retrosplenial cortex ($t_6 = 5.58$, $P < 0.01$) and the nucleus basalis magnocellularis ($t_6 = 3.73$, $P < 0.01$, paired *t*-test)) (Table 2).

In subcortical regions, the average decrease in CMR(glu) in the lesioned side was not statistically significant 1 week after lesioning (3.8%). Values were 78 ± 15 and 75 ± 16 μmol/100 g/min for the intact side and for the lesioned side, respectively. However, significant left–right differences occurred in two regions: the magnocellularis preoptic area ($t_6 = 3.06$, $P < 0.02$) and the nucleus of the diagonal band of Broca (horizontal limb) ($t_6 = 3.41$, $P < 0.05$, paired *t*-test) (Table 2).

Three weeks after lesioning, the average hemispheric CMR(glu) remained lower in the right projection regions (5.5% decrease, $P < 0.05$). Values were 96 ± 18 and 91 ± 19 μmol/100 g/min for the intact side and for the lesioned side, respectively. In the subcortical regions, the

Table 3
Cerebral metabolic rate of glucose in sham-operated and lesioned rats treated with metrifonate (80 mg/kg i.p.) 1 week after lesioning or sham-lesioning. CMR(glu) = μmol/100g/min. Values are means ± S.D. (S) = superficial layers, (M) = middle layers, (D) = deep layers

Brain regions	Sham-operated Saline $n = 7$		Sham-operated Metrifonate $n = 7$		Lesioned Metrifonate $n = 7$	
	Left	Right	Left	Right	Left	Right
Projection regions						
Frontal cortex (S)	88 ± 15	92 ± 16	91 ± 11	90 ± 9	96 ± 8	94 ± 6
Frontal cortex (M)	95 ± 15	97 ± 14	103 ± 10	103 ± 9	107 ± 8	104 ± 8
Frontal cortex (D)	77 ± 14	80 ± 10	85 ± 8	87 ± 6	86 ± 8	86 ± 8
Piriform cortex	106 ± 10	110 ± 11	112 ± 14	115 ± 10	125 ± 10	114 ± 10
Parietal cortex (S)	100 ± 12	102 ± 15	103 ± 9	101 ± 5	107 ± 7	92 ± 6
Parietal cortex (M)	109 ± 13	107 ± 13	118 ± 7	114 ± 7	119 ± 11	102 ± 6
Parietal cortex (D)	88 ± 9	87 ± 14	86 ± 7	91 ± 7	91 ± 9	86 ± 5
Insular cortex	107 ± 14	104 ± 11	138 ± 12	138 ± 9[c]	138 ± 19	125 ± 10[c]
Cingulate cortex	102 ±	98 ± 15	117 ± 12	118 ± 12[b]	114 ± 12	106 ± 15
Sensorimotor cortex (S)	97 ± 9	99 ± 13	97 ± 9	97 ± 5	101 ± 9	96 ± 11
Sensorimotor cortex (M)	110 ± 10	110 ± 13	111 ± 11	114 ± 3	114 ± 10	104 ± 6
Sensorimotor cortex (D)	89 ± 8	92 ± 10	85 ± 9	90 ± 4	88 ± 9	86 ± 8
Entorhinal cortex	61 ± 6	64 ± 10	65 ± 4	65 ± 5	64 ± 7	61 ± 3
Retrosplenial cortex	113 ± 12	109 ± 10	132 ± 11	129 ± 12[b]	115 ± 7	109 ± 7
Occipital cortex (S)	98 ± 12	102 ± 16	89 ± 7	99 ± 6	97 ± 7	92 ± 8
Occipital cortex (M)	107 ± 13	108 ± 17	107 ± 17	105 ± 8	109 ± 8	105 ± 9
Occipital cortex (D)	95 ± 10	94 ± 16	93 ± 10	95 ± 10	98 ± 8	94 ± 8
Temporal cortex (S)	108 ± 13	111 ± 13	123 ± 6	125 ± 9[c]	113 ± 10	113 ± 8[c]
Temporal cortex (M)	123 ± 11	128 ± 10	141 ± 7	139 ± 9[b]	139 ± 7	122 ± 13[b]
Temporal cortex (D)	104 ± 15	100 ± 15	114 ± 8	118 ± 12[b]	110 ± 9	101 ± 9
Nucleus basalis magnocellularis	55 ± 7	55 ± 7	60 ± 8	63 ± 10	52 ± 4	45 ± 11
Basolateral amygdala	85 ± 9	85 ± 10	95 ±	96 ± 11[a]	86 ± 9	85 ± 9
Reticular thalamic nucleus	67 ± 6	65 ± 4	90 ± 15	92 ± 13[c]	85 ± 9	82 ± 14[c]
Lateral habenula	100 ± 13	97 ± 9	121 ± 14	123 ± 17[b]	132 ± 21	92 ± 21
Other regions						
Preoptic area (magnocellularis)	80 ± 11	81 ± 12	95 ± 14	96 ± 12[a]	95 ± 13	73 ± 17
Diagonal band of Broca (horiz.)	75 ± 8	75 ± 9	82 ± 7	83 ± 10	80 ± 7	68 ± 14
Ventral thalamus	85 ± 6	84 ± 5	115 ± 14	114 ± 13[c]	110 ± 17	110 ± 17[c]
Dorsal hippocampus (CA1)	57 ± 10	55 ± 10	58 ± 6	59 ± 6	54 ± 5	53 ± 5
Dorsal hippocampus (CA3)	68 ± 9	64 ± 11	71 ± 5	72 ± 8	67 ± 8	64 ± 7
Dentate gyrus	62 ± 10	60 ± 14	63 ± 4	64 ± 4	58 ± 7	57 ± 8
Subiculum	88 ± 13	88 ± 16	89 ± 12	89 ± 14	82 ±	82 ±
Ventral hippocampus (CA1)	58 ± 7	59 ± 9	62 ± 4	64 ± 6	59 ± 8	60 ± 3
Ventral hippocampus (CA3)	70 ± 4	73 ± 7	84 ± 5	81 ± 7[b]	83 ± 9	77 ± 7
Substantia nigra (pars c.)	78 ± 8	77 ± 9	90 ± 8	89 ± 12[b]	85 ± 8	81 ± 9
Interpeduncular nucleus	104 ± 10	id	116 ± 4	id[a]	120 ± 9	id[a]
Superior colliculus	81 ± 5	80 ± 8	117 ± 19	117 ± 19[c]	123 ± 21	122 ± 20[c]
Interpositus nucleus	96 ± 7	100 ± 11	109 ± 11	109 ± 13[b]	117 ± 18	113 ± 14[c]

[a] $P < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.

hemispheric difference was not significant (3.8%). The left–right comparisons showed that statistically significant decreases occurred in the parietal cortex ($t_6 = 4.38$, $P < 0.01$) (Fig. 1B′), the lateral habenula ($t_6 = 4.96$, $P < 0.01$), the nucleus basalis magnocellularis ($t_6 = 4.52$, $P < 0.01$), the magnocellularis preoptic area ($t_6 = 3.34$, $P < 0.02$) and nucleus of the diagonal band of Broca ($t_6 = 4.42$, $P < 0.01$) (Table 2).

The interaction between sides and groups (sham-operated and lesioned rats) was estimated by means of two-way ANOVA. There was a significant main side/group effect confirming the tendency towards lower CMR(glu) in the lesioned side 1 week ($F = 5.33$, $P = 0.02$) but not 3 weeks ($F = 3.47$, $P = 0.06$) after lesioning.

3.3. Effects of metrifonate on CMR(glu) in sham-operated rats

In sham-operated rats, metrifonate (80 mg/kg i.p., 30 min before [^{14}C]2-deoxy-D-glucose injection) increased the average CMR(glu) by 9.3% and 8.4% in the projection regions (left and right hemispheres, respectively, $P < 0.01$ for the both sides). In the subcortical regions, the metrifonate-induced increase in CMR(glu) reached 16% in the left and right hemispheres ($P < 0.001$). There was no significant main side/treatment effect, indicating a similar effect of metrifonate in both sides ($F = 0.08$, $P = 0.77$).

In terms of regional distribution, significant effects were found in 16 of the 37 regions studied. In cortical areas, the greatest effects were observed in the insular cortex (33%) and in the superficial and middle layers of the temporal cortex (18%), as seen in Table 3 and Figs. 2 and 3. In sub-cortical regions, marked effects were observed in the thalamic nuclei (40%), the superior colliculus (46%) and the lateral habenula (27%), as seen in Table 3 and Figs. 2 and 3. These results are in agreement with those obtained previously (Bassant et al., 1996).

3.4. Effects of metrifonate on CMR(glu) in lesioned rats

Comparing the lesioned sides (right) of saline and metrifonate-treated rats showed that average CMR(glu) in the projection regions increased by 6.4% ($P < 0.02$) after treatment. Values were 95.8 ± 19 and 90 ± 18 μmol/100 g/min for the metrifonate-treated vs. the saline-treated rats, respectively. In the sub-cortical regions, average CMR(glu) increased by 10.3% ($P < 0.01$). Values were 83 ± 26 and 75.3 ± 16 μmol/100 g/min for the metrifonate-treated vs. the saline-treated rats, respectively.

In terms of regional distribution, significant effects were found in 8 of the 37 regions studied. CMR(glu) increased in the insular (24%) and temporal cortices (15%),

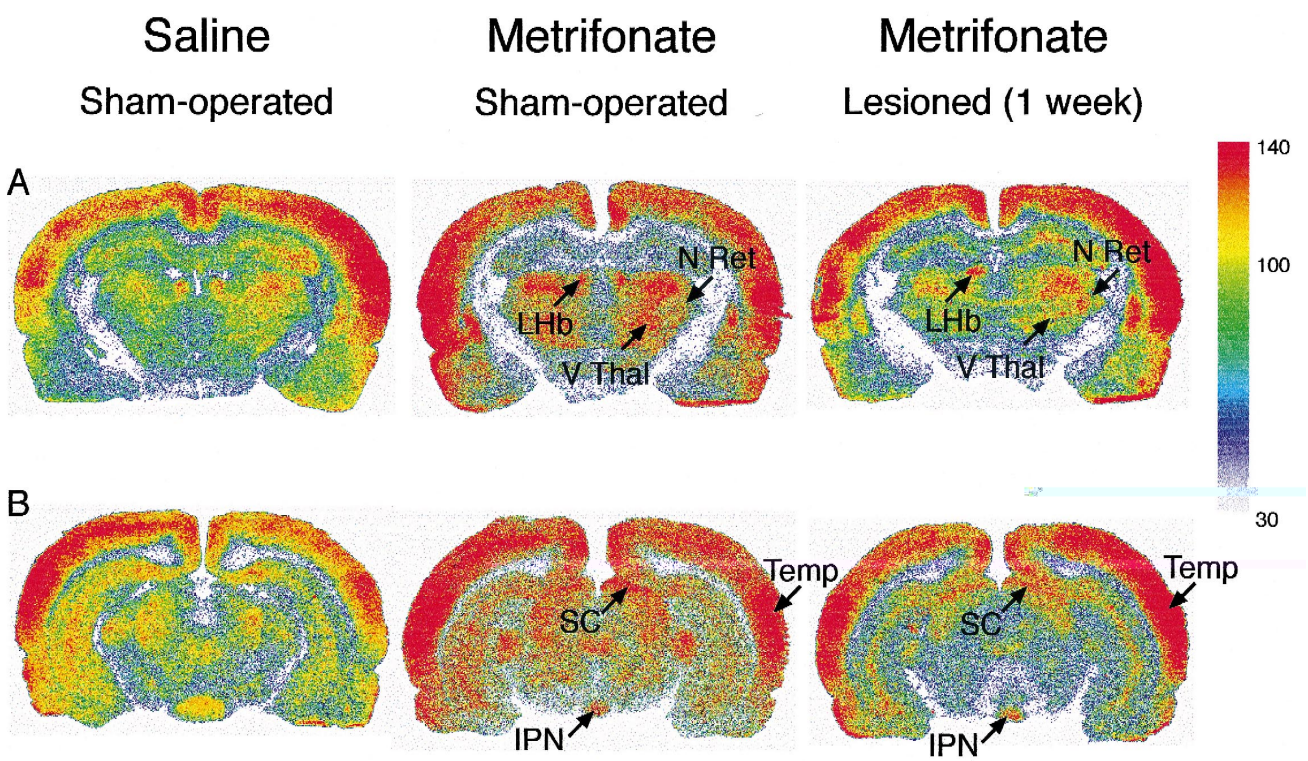

Fig. 3. Effect of metrifonate (80 mg/kg) on local cerebral glucose utilization in sham-operated and lesioned rats. (A) Pseudocoloured autoradiograms obtained from brain sections through the level of the lateral habenula (LHb). Notice that the effect of metrifonate remains both in the ventral thalamus (V Thal) and the reticular nucleus (N ret), but is no longer observed in the LHb. (B) Pseudocoloured autoradiograms obtained from brain sections more caudal than in (A). Notice that the effect of metrifonate is preserved in the superior colliculus (SC), the temporal cortex (Temp) and the interpeduncular nucleus (IPN) in the lesioned rat.

in the ventral (28%) and reticular thalamic nuclei (22%), in the superior colliculus (52%), and in the interpeduncular (16%) and interpositus nuclei (18%), as seen in Table 3 and Figs. 2C and 3.

3.5. Comparison of the effects of metrifonate in sham-operated and lesioned rats

On the left side, the effect of metrifonate was similar in sham-operated and lesioned rats ($P = 0.94$). On the right side, however, the effect was significantly smaller in the projection regions of lesioned rats ($P < 0.02$) and was also smaller in the other regions, but the difference did not reach statistical significance ($P = 0.29$).

There was a significant main side/group effect in the region of projection ($F = 7.89$, $P = 0.01$), indicating that the treatment increased the differences between the sides. This main side/group effect was not found in sub-cortical regions ($F = 0.64$, $P = 0.42$). The metrifonate-induced increase in CMR(glu) was less in the lesioned than in the intact side. However, CMR(glu) in the lesioned side was greater in metrifonate-treated rats than in saline-injected ones.

Regional distribution of the responses in lesioned rats showed that metrifonate-induced increases in CMR(glu) were no longer observed in eight regions. The metabolic activation was either absent (cingulate cortex, lateral habenula, preoptic area) (Table 3) or not sufficient to reach statistical significance (retrosplenial cortex and the deep layer of the temporal cortex, basolateral amygdala, CA3 field of the dorsal hippocampus, substantia nigra pars compacta). The regions where metrifonate was still significantly active were those where the largest effects were observed in sham-operated treated rats such as insular and temporal cortex, thalamic nuclei, superior colliculus (Figs. 2 and 3)

In a number of regions, the left–right differences were not significant in basal conditions (saline-treated lesioned rats, paired *t*-test) but reached the statistical significance after treatment. This effect occurred in the parietal ($t_6 = 3.79$, $P < 0.01$), the sensorimotor ($t_6 = 2.67$, $P < 0.05$) and the cingulate cortices ($t_6 = 2.79$, $P < 0.05$), in the lateral habenula ($t_6 = 4.54$, $P < 0.01$) and the CA3 field of the ventral hippocampus ($t_6 = 5.50$, $P < 0.01$).

4. Discussion

4.1. Metabolic effect of lesioning the cholinergic projections from the basal forebrain

Lesioning cholinergic neurons of the basal forebrain with 192 IgG-saporin induced an almost complete loss of the cholinergic innervation in the target areas. Our results show that this denervation is associated, at best, with a moderate decrease in glucose utilization. For example, 7 days after lesioning, CMR(glu) is significantly reduced in one cortical region, the retrosplenial cortex, out of the 24 regions studied. Furthermore, in terms of average hemispheric values, CMR(glu) tends to normalize with time. The effect observed in the basal forebrain itself (nucleus basalis, preoptic area, horizontal limb of the diagonal band of Broca) is probably due to the loss of cholinergic neurons per se. In spite of the differences that exist among the experimental procedures (species, time scales of the experiments, nature of the lesions), a comparison of the data obtained by various groups can be attempted. Our results are at variance with those reporting marked reductions in CMR(glu) in the frontal, parietal and temporal cortex 4 days (Orzi et al., 1988) or 2 weeks (Eguchi et al., 1995) after excitoxic lesioning of nucleus basalis magnocellularis. In the study by Orzi et al. (1988), CMR(glu) was still reduced 2 weeks after lesioning, but the effect was less and limited to the frontal cortex. The discrepancies between our results and others might be due to the fact that kainic or ibotenic acids damage not only cholinergic neurons but, in addition, a major fraction of the gamma-aminobutyric acid (GABA) projecting neurons located in the nucleus basalis magnocellularis (Lindefors et al., 1992; Gritti et al., 1997).

On the other hand, our results confirm those of Lamarca and Fibiger (1984), who showed that the [^{14}C]2-deoxy-D-glucose uptake was not modified 7 days after lesioning the nucleus basalis magnocellularis with ibotenic acid in rats. Our results confirm also that CMR(glu) returns progressively to normal while the cholinergic markers in the projection regions are still reduced (London et al., 1984; Soncrant et al., 1992; Ouchi et al., 1996). Finally, excitotoxic lesions of the entire basal forebrain cholinergic system in baboons, performed with an improved methodology eliminating non-specific effects, induced only mild and transient CMR(glu) declines in the posterior hippocampus and the anterior temporal cortex (Le Mestric et al., 1998).

The lack of marked decreases in CMR(glu) in the brain region deprived of the cholinergic innervation might be explained, at least in part, by the small density of cholinergic terminals in the neocortex (Richardson, 1981; Descarries et al., 1997). Using an in vivo marker of vesicular acetylcholine transporters, Kuhl et al., (1996) evaluated the presynaptic cholinergic terminal density in association with measurements of glucose utilization in normal aging and Alzheimer's disease. This study showed that the coupling between cortical cholinergic terminals and CMR(glu) was far from consistent but varied in function with the disease onset and with the brain structures. In the medio-temporal and in the cingulate cortice, the significant decline in cholinergic terminal density observed in early-onset Alzheimer's disease was not accompanied by a decrease in CMR(glu). Our results are in agreement with this finding and tend to rule out a cholinergic basis for the neocortical hypometabolism observed in Alzheimer's disease patients.

4.2. Metabolic effect of metrifonate in lesioned rats

Cholinesterase inhibitors increased CMR(glu) in young and aged rats (Bassant et al., 1995, 1996). This metabolic activation might result from an increased activity of the cholinoceptive neurons, in response to the prolonged presence of acetylcholine in the synaptic cleft. This explanation seems questionable in view of the present results, which indicate that the cholinergic inputs have, at best, a marginal effect on brain metabolism. But CRM(glu) was measured while rats were in a resting state. We must take into account that differences between intact and lesioned rats would have been more marked in cases of physiological stimulation. This hypothesis is consistent with the fact that nucleus basalis magnocellularis lesions alter stimulus-evoked metabolic activity in the barrel field of the mouse sensory cortex whereas the basal CMR(glu) is not affected (Ma et al., 1989).

The effect of metrifonate is partly maintained after nucleus basalis magnocellularis lesion. The regional analysis shows that the response to the compound is either persistent, lost or reduced. These differences might result from differential effects of the lesion due to cytoarchitectural particularities. However, anatomical data do not provide consistent explanations. For example, although the cholinergic innervation does not differ in the cingulate and the retrosplenial cortices (Saper, 1984), the response was lost in the former and was only reduced in the later. The effect of metrifonate was lost in the lateral habenula, which receives sparse cholinergic fibers, and was only reduced in the basolateral amygdala, which is densely innervated (Woolf, 1991). It has been shown that a number of cholinergic neurons in the nucleus basalis do not express p75 NGF receptors and are not lesioned by 192 IgG-saporin. These neurons innervate the amygdala (Heckers et al., 1994) and might be responsible for the preservation of the response in this region.

4.3. Plausible explanations of the sustained effect of metrifonate

In view of the drastic cholinergic denervation induced by 192 IgG-saporin, the sustained effect of metrifonate on CMR(glu) is an unexpected result. Several explanations might be offered.

4.3.1. Incomplete lesioning

Due to the dispersion of the cholinergic neurons throughout a number of cell groups in the basal forebrain, it cannot be ruled out that a few cholinergic neurons were not lesioned. It is questionable that the marked effect of metrifonate in insular and temporal cortices after lesion might be due to some residual cholinergic neurons. However, it is possible that compensatory changes occur in these few neurons. Indeed, after deafferentation, remodelling of synaptic contact leads to an enlargement of the remaining synaptic boutons and larger postsynaptic sites (Hillman and Chen, 1985). Such reorganization might occur in cholinergic synapses, allowing metrifonate to have a preserved although reduced effect.

4.3.2. Receptor upregulation

Data on changes in cholinergic receptors after nucleus basalis magnocellularis ablation are controversial; the number of receptors have been found to be reduced, increased or unchanged (see reference in De Micheli et al., 1993). More recently, it was found that immunolesion by 192 IgG-saporin did not change the level of cortical nicotinic receptors. In contrast, the binding levels of muscarinic receptors (M_1 and M_2 types) increased in a number of cortical areas (Roßner et al., 1995). These increases could be regarded as an indication of receptor supersensitivity. However, the metabolic responses to muscarinic and nicotinic receptor agonists were not enhanced after basal forebrain lesions with ibotenic acid (De Micheli et al., 1993). Similarly, we have not observed greater CMR(glu) in lesioned vs. intact sides in the metrifonate-treated rat. It cannot be ruled out, however, that upregulation of postsynaptic muscarinic receptor might work to sustain the response to metrifonate.

4.3.3. Intrinsic cholinergic cortical neurons

The sustained CMR(glu) responsiveness to metrifonate might be due to an effect on intrinsic cholinergic cortical neurons. The presence of cholinergic neurons in the rat cortex is unequivocally demonstrated (Lauterborn et al., 1993). They could contribute to the effect of metrifonate in normal conditions and be responsible, at least in part, for the remaining response after cholinergic denervation. Such a mechanism might occur in patients since neurons with choline acetyltransferase imunoreactivity are present in a number of human cerebral cortical areas (Kasashima et al., 1999).

4.3.4. Effects other than inhibition of acetylcholinesterase

Beneficial effects of metrifonate on memory impairments induced by scopolamine or basal forebrain lesioning have been observed after doses which do not inhibit acetylcholinesterase activity (Van der Staay et al., 1996; Itoh et al., 1997). This indicates that metrifonate might affect the impairment of learning by mechanisms other than acetylcholinesterase inhibition. However, there is no direct evidence in favor of this explanation. In vitro studies do not reveal any affinity with a large number of biological targets including monoaminergic and glutamatergic receptors (Hinz et al., 1996). The action of metrifonate on neurotransmitters other than acetylcholine have been investigated. An increase in the extracellular level of noradrenaline was observed in the rat cortex after a single injection of metrifonate (Mori et al., 1995). It is possible that the activation of noradrenergic transmission plays a role, probably minor, in the metabolic effect of metrifonate in the

intact animal. On that account, it could contribute to the sustained response in lesioned rat since it has been shown that cholinergic immunolesion with 192 IgG-saporin does not affect the tissue levels of noradrenaline in cortical or hippocampal regions (Waite et al., 1994) and does not reduce the binding of the postsynaptic α1-adrenoreceptor (Heider et al., 1997).

4.4. Conclusions

Metrifonate is still able to increase CMR(glu) in a limited number of regions after cholinergic denervation. Although the effect of the compound is less than in normal conditions, it is interesting to note that a metabolic activation persists in regions such as the temporal cortex, in which there is decreased metabolism in patients with Alzheimer's disease. The mechanisms that may account for the sustained response to metrifonate remain to be elucidated but the possibility exists that the drug can continue to affect intrinsic cholinergic neurons.

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