Depression of Acetylcholinesterase Synthesis Following Transient Cerebral Ischemia in Rat: Pharmacohistochemical and Biochemical Investigation

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Abstract. The effect of transient cerebral ischemia on acetylcholinesterase (AChE) synthesis was studied in rats by a modified pharmacohistochemical method The procedure involved *in vivo* irreversible inhibition of AChE by administration of the inhibitor diisopropyl fluorophosphate (DFP, 1 2 mg/kg b w , i m) 1 h before 30 min forebrain ischemia (the four-vessel occlusion model) At the onset of ischemia, 70–75% of AChE was inhibited in the brain Recirculation was followed by histochemical and biochemical investigations of newly synthesized AChE in the striatum, septum, cortex and hippocampus Control sham-operated animals were treated with the same dose of DFP For correlation, rats not treated with DFP were subjected to the same ischemic procedures and investigated simultaneously In these rats, significant decrease in AChE activity was found in the striatum, septum and hippocampus during 24 h recirculation In DFP treated rats, ischemia markedly depressed resynthesis of AChE, after 4 h recirculation, AChE activity was decreased by 45–60% in all investigated areas in comparison with controls and the AChE histochemistry showed only slightly stained neurons in the striatum and septum Twenty-four hours after ischemia, these neurons were densely stained and the increase in AChE activity indicated a partial recovery of the enzyme synthesis These results suggest that the depression of AChE synthesis after forebrain ischemia is probably transient, not accompanied by cholinergic neuron degeneration

Key words: Cerebral ischemia — Acetylcholinesterase — Pharmacohistochemistry — Choline acetyltransferase

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Introduction

Choline acetyltransferase (ChAT, EC 2 3 1 6) and acetylcholinesterase (AChE, EC 3 1 1 7) involved in the synthesis and degradation of acetylcholine (ACh) play an essential role in the process of cholinergic transmission Cholinergic cells in the brain and spinal cord have been demonstrated immunohistochemically by ChAT or histochemically by the AChE reaction (Butcher 1978, Eckenstein and Sofroniew 1983 Satoh et al 1983), and measurements of their activities have been used as an indicator of disturbed cholinergic function following ischemia (Ott et al 1975, Mrsulja et al 1978, Malatova et al 1989, Malatova and Maršala 1993)

Decreases in ChAT and AChE activities, as well as AChE histochemistry of rabbit spinal cord after infarction point to degenerative and functional changes of cholinergic neurons following ischemia (Malatova and Maršala 1993) However, a number of studies yielded controversial results concerning AChE changes in ischemic brain AChE activity was significantly reduced in gerbil brain after the occlusion of the common carotid arteries (Mrsulja et al 1978), whereas increased AChE activity has been reported in the infarcted brain cortex and basal ganglia 4 hours after the occlusion of the middle cerebral artery in baboons (Ott et al 1975) and during incomplete spinal cord ischemia in the dog (Malatova et al 1984) On the other hand, the absence of histological or biochemical changes of ChAT activity in the striatum and hippocampus was reported after transient forebrain ischemia in the rat, while the depression of glutamic acid decarboxylase activity was associated with irreversible damage of GABAergic neurons (Francis and Pulsinelli 1982) Similarly, neurons expressing AChE activity or ChAT immunoreactivity were preserved after transient ischemia in the gerbil striatum, while the immunoreactivity to enkephalin and tachykinins was markedly decreased (Chesselet et al 1990) These studies suggest that cholinergic neurons are more resistant to ischemia than other neurons

In view of existing differences we decided to investigate the changes in AChE synthesis after cerebral ischemia Since a transient forebrain ischemia is characterized by considerable changes in protein synthesis machinery (Bodsch et al 1985, Burda et al 1994), this may also concern AChE In order to study the influence of cerebral ischemia on AChE synthesis, we used the modified pharmacohistochemical method (Butcher 1978) which involves the irreversible *in vivo* inactivation of existing AChE followed by histochemical visualization of newly synthetized enzyme molecules at various time intervals In our study, AChE was inactivated by systemic administration of the irreversible inhibitor diisopropyl fluorophosphate (DFP) 1 h before transient forebrain ischemia, and the enzyme was visualized and measured during 24 h of recirculation after ischemia, when *de novo* AChE synthesis occurred AChE activity was measured in the striatum, septum, hippocampus and cerebral cortex, because severe and reproducible damage following ischemia

has been reported in these regions (Pulsinelli et al 1982) To correlate AChE and ChAT activities in the brain after ischemia, both enzymes were also investigated after equal forebrain ischemia induced in rats without DFP pretreatment

Materials and Methods

Forty male Wistar rats weighing 200–300 g were divided in two groups the first group, ischemia induced without AChE inhibition, and the second group, ischemia induced 1 h after a single i m injection of the AChE inhibitor DFP (diisopropyl fluorophosphate, 1 2 mg/kg), dissolved in olive oil (1 5 mg/ml) Animals of both groups were subjected to transient forebrain ischemia by the occlusion of four major arteries according to the method described by Pulsinelli and Brierley (1979) as follows On the first day, both vertebral arteries were irreversibly occluded by coagulation through the alar foramen and clasps were placed around both common carotid without interrupting carotid blood flow under pentobarbital anesthesia (50 mg/kg of body weight, i p), and clasps were placed around both common carotid arteries without interrupting carotid blood flow Rats prepared in this manner did not show any evidence of brain damage Blood flow to the brain was maintained by the carotid arteries On the following day, the carotid clasps of fully awake rats were tightened to produce 4 vessel occlusion (4-VO) In the second group the same occlusion was performed 1 h after DFP injection During the period of ischemia, rats were deeply unresponsive but they continued to breath and their body temperature was maintained by heating pads at $37\,0 \pm 0\,5$ °C Some animals stopped breathing after vascular occlusion and were excluded from further study Clasps were removed after 30 min The absence of blood flow during the occlusion and the occurrence of reflow were verified visually After the closure of the wounds, animals were returned to their cages In every group, six rats were decapitated 4 h and other six rats 24 h after recirculation Sham-operated animals subjected to the same surgery procedures without clamping of carotid arteries were used as controls, 6 of them were treated with DFP The brains were dissected and carefully frozen in liquid nitrogen

On the second day at the latest, the brains were cut in a cryostat (−12 °C) alternately into 300 μm sections for biochemical and 50 μm sections for histochemical investigations

Histochemical investigation

To correlate biochemical and histochemical results more precisely, unfixed brain sections were processed for AChE histochemistry according to the "direct-colouring" thiocholine method of Karnovsky and Roots (1964) The sections were incubated 3 h at 37 °C in a solution containing (in mmol/l) 18 acetylthiocholine iodide (Lachema, Brno, Czech Republic), 30 $CuSO_4$ 5 $K_3Fe(CN)_6$ 100 sodium citra-

te $2H_2O$ and 0 04 tetraisopropyl-pyrophosphoramide (iso-OMPA, Sigma, St Louis, USA), as an inhibitor for non-specific cholinesterase, and 100 acetate buffer, pH 5 6 One out of ten sections was counterstained by Cresylecht violet The sections were mounted in Canadian balsam and examined in a light microscope

Biochemical investigation

The striatum (caudate-putamen), septum, and the motor-sensory cortex were dissected by a microscalpel from five sections A5 4–4 2 (Fig 1) and the hippocampus from sections A2 4–1 80, according to the coronal atlas of the rat forebrain (Satoh et al 1983) Then the tissue samples were homogenized in a glass microhomogenizer in 200 μl of cooled homogenizing solution containing (in mmol/l) 200 NaCl, 40 sodium phosphate buffer (pH 7 4), 10 $MgCl_2$, and 0 5% Triton X-100 Aliquots of homogenates were taken for ChAT and AChE assays and for protein determination

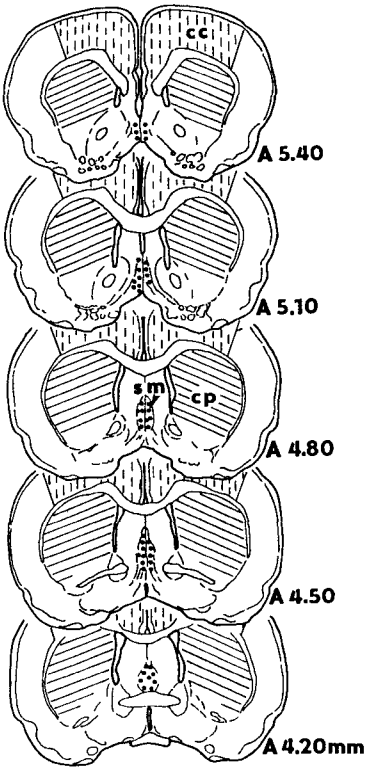


Figure 1. Diagrams presenting the forebrain sections for biochemical sampling of the striatum (caudatoputamen, cp), septum (medial septal nucleus, sm) and cerebral cortex (cc), dissected zones are marked hatched or dotted The numerical indices indicate the distance between sections in millimeters, anterior (A) from a zero plane that runs through the rostral tip of the red nucleus

AChE and ChAT activities were assayed by a slight modification of Fonnum's radiochemical method (Fonnum 1969) The ChAT activity, based on the formation of [1-^{14}C]acetylcholine (ACh), from [1-^{14}C]acetyl coenzyme A (Amersham, England) and unlabeled choline chloride, was determined twice in 35 μl of homogenates The synthesized ACh was extracted by sodium tetraphenylboron in butyl acetate and measured in LKB CompuGamma 1282 liquid scintillation counter AChE activity was measured by the formation of [^{14}C]acetate after hydrolysis of acetyl-labeled [1-^{14}C]acetylcholine (Amersham, England) in the aqueous phase The activities of ChAT and AChE were expressed in μkatals (μmoles of synthesized or hydrolyzed ACh per second) per gram of protein

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Statistical analysis was performed by one-way ANOVA followed by post-hoc Duncan's test.

Results

Neurological findings

After DFP injection, a slight depression of respiration and muscle shivering were observed in animals before ischemia. In spite of the intoxication, no significant difference in the neurological status was found between the DFP-treated and non-treated rats during ischemia and postischemia. After 4-VO, all animals were deeply unresponsive because of the neurological status classified as a coma, but they continued to breath. Some animals stopped breathing immediately after the vascular occlusion but their respiration was restored by mechanical stimulation of the thorax. Approximately 20% of the rats, equally in both experimental groups, were not able to breath and died. After removal of the vessel clasps (beginning of recirculation), animals remained in an unresponsive state for 1–3 h. Then, the rats woke up and maintained a characteristic posture for the next 24 h. Twenty-five percent of the animals had seizures and two of them died after 6 h recirculation.

Histochemical changes of AChE.

AChE-positive neurons were invisible in the striatum and septum of the rats which did not receive pretreatment with DFP because of a very high AChE activity in the neuropil. Brain sections displayed an intense background AChE staining and neurons were obscured (Fig. 2*A*). Under these conditions, an interesting phenomenon was observed in postischemic brain sections: during the staining procedure, the reaction product Hatchet brown, formed by AChE, completely diffused from the areas of the highest enzyme activity (striatum, diagonal band; Fig. 2*B*).

One hour after i.m. DFP injection, only slightly stained neuropil in the striatal and septal areas was found but neurons were not visible (Fig. 2*C*). Five and half-hours or 25.5 h after the administration of DFP, moderately or intensely stained AChE-positive neurons were observed in areas with less background staining (Figs. 2*D*, *F*; 3*A*, *C*; 4*A*, *C*). On the other hand, only slightly stained neurons were found after ischemia and 4 h recirculation (Figs. 2*E*; 3*B*, *D*). Striatal and septal perikarya looked devoid of the AChE reaction product, while control neurons were densely stained (Fig. 3*A*, *C*). However, after 24 h recirculation, all neurons were moderately or intensely stained (Fig. 2*G*; Fig. 4*B*, *D*) and only negligible differences were observed in comparison with the control group (Figs. 2*F*, 4*A*, *C*).

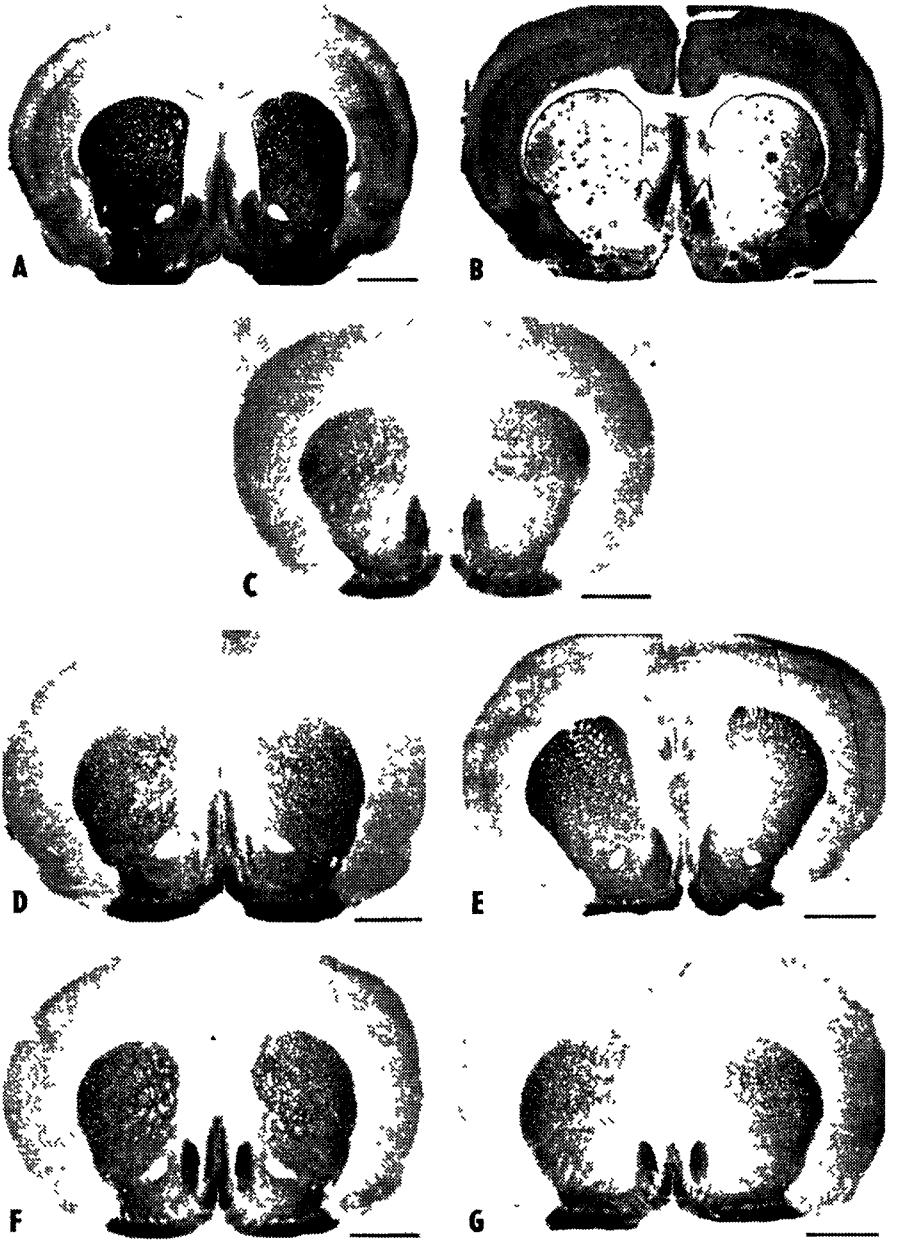


Figure 2. The overview of histochemical (*A*, *B*) and pharmacohistochemical (*C*, *D*, *E*, *F*, *G*) visualization of acetylcholinesterase in unfixed frontal sections of the rat forebrain at the level of the caudatoputamen (see Fig 1) in the following experimental groups

Biochemical changes of AChE and ChAT activities

AChE activity was measured in microsamples of the striatum, septum, hippocampus and cerebral cortex in all animals; results are graphically illustrated in Fig. 5. Additionally, ChAT activity was simultaneously measured in the same tissue samples from rats subjected to ischemia without DFP pretreatment, and the values are presented in Table 1.

In rats without DFP pretreatment, a significant decrease in AChE activity following ischemia was found in the striatum, septum and hippocampus. However, no changes were detected in the cerebral cortex. ChAT activity was significantly decreased only in the striatum and hippocampus 4 h after ischemia (Tab 1). After 24 h recirculation, no significant differences in ChAT activity between ischemic and control groups were found.

One hour after DFP administration, i.e at the onset of ischemia, AChE activity was inhibited by 70–75% in all investigated areas (after cauterization of vertebral arteries) and sham-operated rats. After 4 h recirculation, AChE activity in the striatum, septum and hippocampus of DFP-pretreated animals decreased

Table 1. Choline acetyltransferase (ChAT) activity in different areas of the brain after 30 min forebrain ischemia in rats non-treated with DFP

Experimental group	Striatum	Septum	Hippocampus	Cerebral cortex
Sham-operated Control	54 70 ± 2 18	39 88 ± 1 89	14 34 ± 0 81	13 64 ± 0 67
Ischemia 30 min Recirculation 4h	41 97 ± 2 12***	38 45 ± 1 96	11 56 ± 0 66*	12 59 ± 0 57
Recirculation 24h	49 79 ± 4 16	43 52 ± 2 19	14 16 ± 0 43	13 33 ± 0 48

Values of ChAT activity, expressed in nkatals/g protein, represent arithmetical mean ± S E M ($n = 6$) Statistical significance ***$p < 0\ 001$, *$p < 0\ 05$

(*A*) Sham-operated control without DFP pretreatment Brain sections display an intense background AChE staining and neurons are obscured (*B*) Four hours after 30 min ischemia without DFP pretreatment The diffusion of the reaction product Hatchet brown, formed by AChE, from the areas of the highest enzyme activity (striatum, diagonal band (*C*) One hour after DFP injection (1 2 mg/kg, i m) The inhibition of AChE shows slightly stained neuropil without staining of neurons (*D*) Sham-operated control group, 5 5 h after DFP injection, (*E*) 4 hours after 30 min of ischemia (performed 1 h after DFP injection), (*F*) sham-operated control group, 25 5 h after DFP injection, (*G*) 24 hours after 30 min ischemia (performed 1 h after DFP injection) AChE-positive neurons visible in less background staining, for details, see in Figs 3 and 4 Scale bar = 2 mm

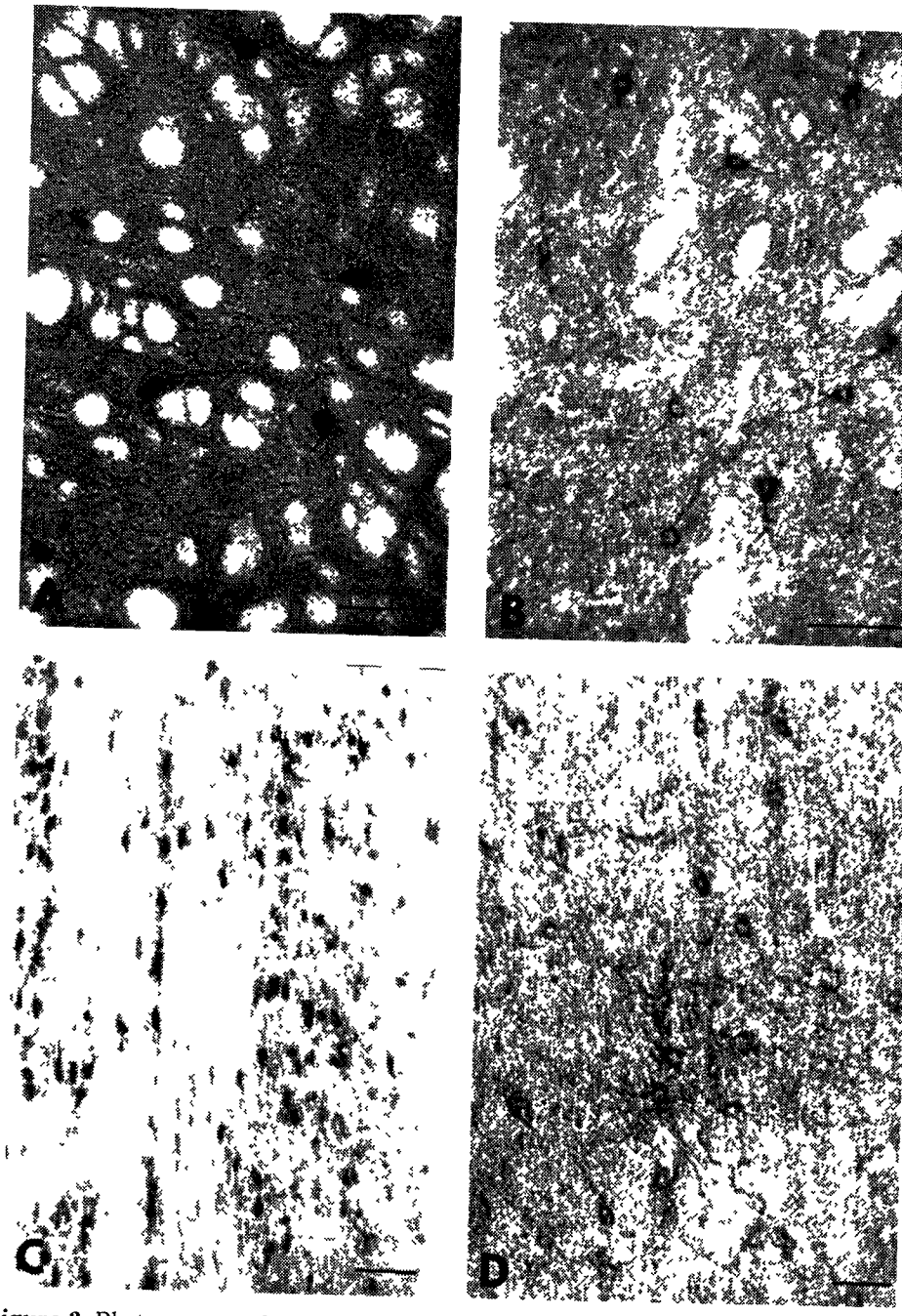


Figure 3. Photomicrograph of the dorso-lateral area of the rat striatum (A, B) and medial septal nucleus (C, D) in unfixed sections processed for DFP-AChE pharmacohistochem-

approximately by 50% and in the cerebral cortex by 39% in comparison with the sham-operated control group (Fig. 5). After 24 h recirculation, a significant increase (< 0.05) in AChE activity was recorded in comparison with the activity at 4 h, but differences between experimental and control groups persisted in all investigated areas.

Discussion

In this study, Butcher's pharmacohistochemical procedure (Butcher 1978) in combination with radiochemical measurement of AChE activity enabled to observe AChE-producing neurons and evaluate changes in AChE synthesis after transient forebrain ischemia. Preliminary experiments with AChE inhibition before ischemia have shown that the most suitable dose of DFP is 1.2 mg/kg body weight i.m., without any evident influence on the neurological status of rats during ischemia-recirculation period. A renewal of AChE activity after DFP injection in the control group corresponded to data on *de novo* AChE synthesis (Austin and James 1970; Butcher 1978). It has been established that complete AChE regeneration takes days or weeks (Austin and James 1970), but the appearance of newly synthesized AChE in the synaptic regions is apparent within several hours after DFP injection (Mailly and Bouchaud 1986).

Our biochemical measurements of AChE activity and the corresponding histochemical observations suggested that AChE synthesis in the striatum and septum/diagonal band significantly decreased after 4-VO ischemia. AChE-positive neurons in these areas were only slightly stained or almost devoid of the reaction product, while the control neurons were densely stained. The hippocampus and cerebral cortex represent mainly synaptic regions where AChE activity may reflect changes in the axonal transport (Malatová et al. 1989).

Data from the present study correspond to reported inhibition of protein synthesis in the same model of ischemia from our laboratory. It was shown (Burda et al. 1994) that this inhibition during ischemia is due to the depletion of energy substrates, and the following post-ischemic recirculation phase was associated with considerable changes in protein synthesis machinery. The initiation of protein synthesis was blocked by the phosphorylation of the initiation factor 2 (eIF-2), polysomes were disaggregated and the rate of protein synthesis decreased by 70%.

istry 5 5 h after DFP administration In sham-operated control group, 5 5 h after injection of DFP, densely stained neurons in the striatum (A) and septum (C) in less background staining show the activity of *de novo* synthesized AChE After 30 min ischemia (induced 1 h after DFP injection), and 4 h recirculation, slightly stained and hardly distinguishable AChE-positive neurons (B, D) show the depression of AChE synthesis Scale bar = 100 μm

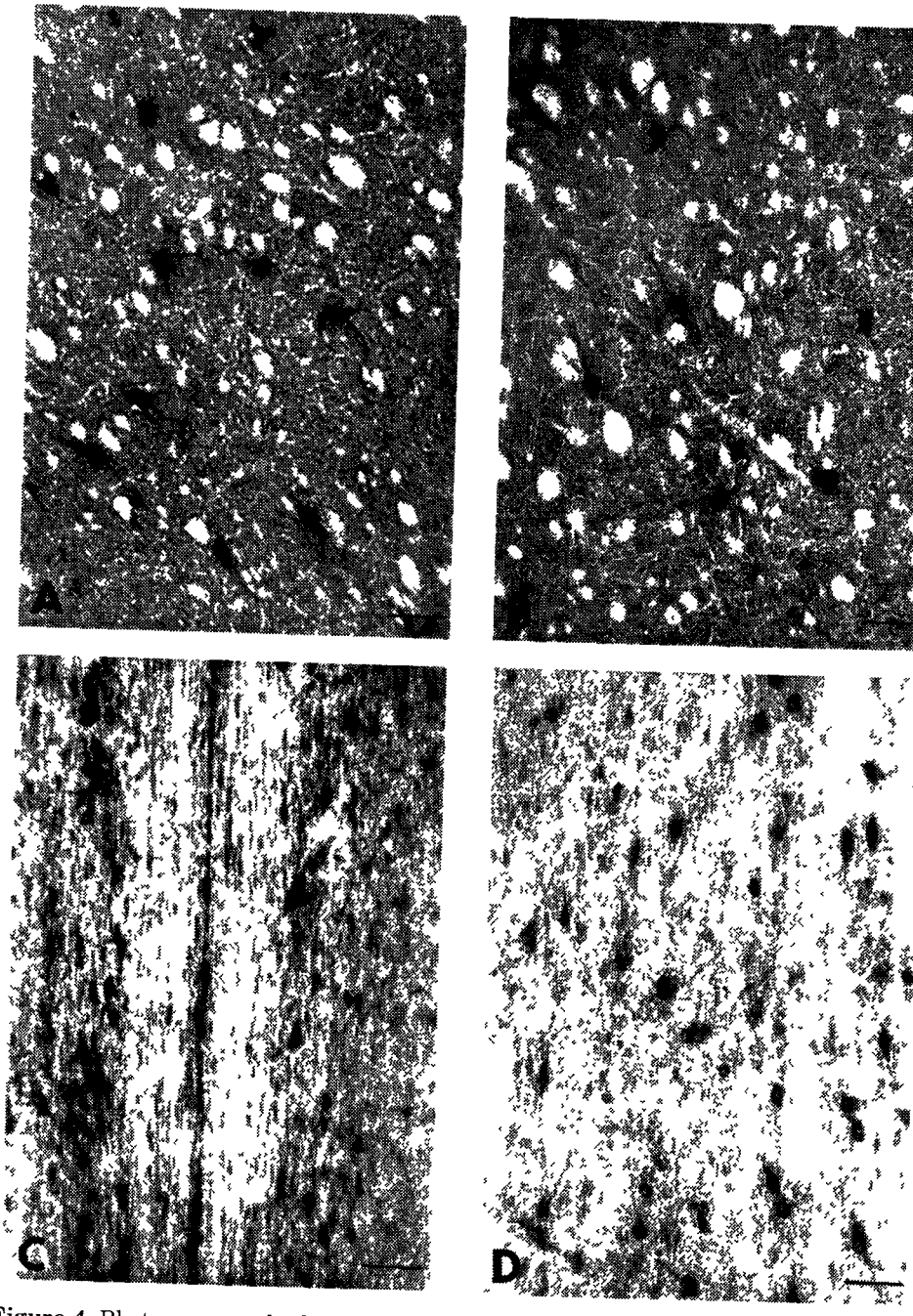
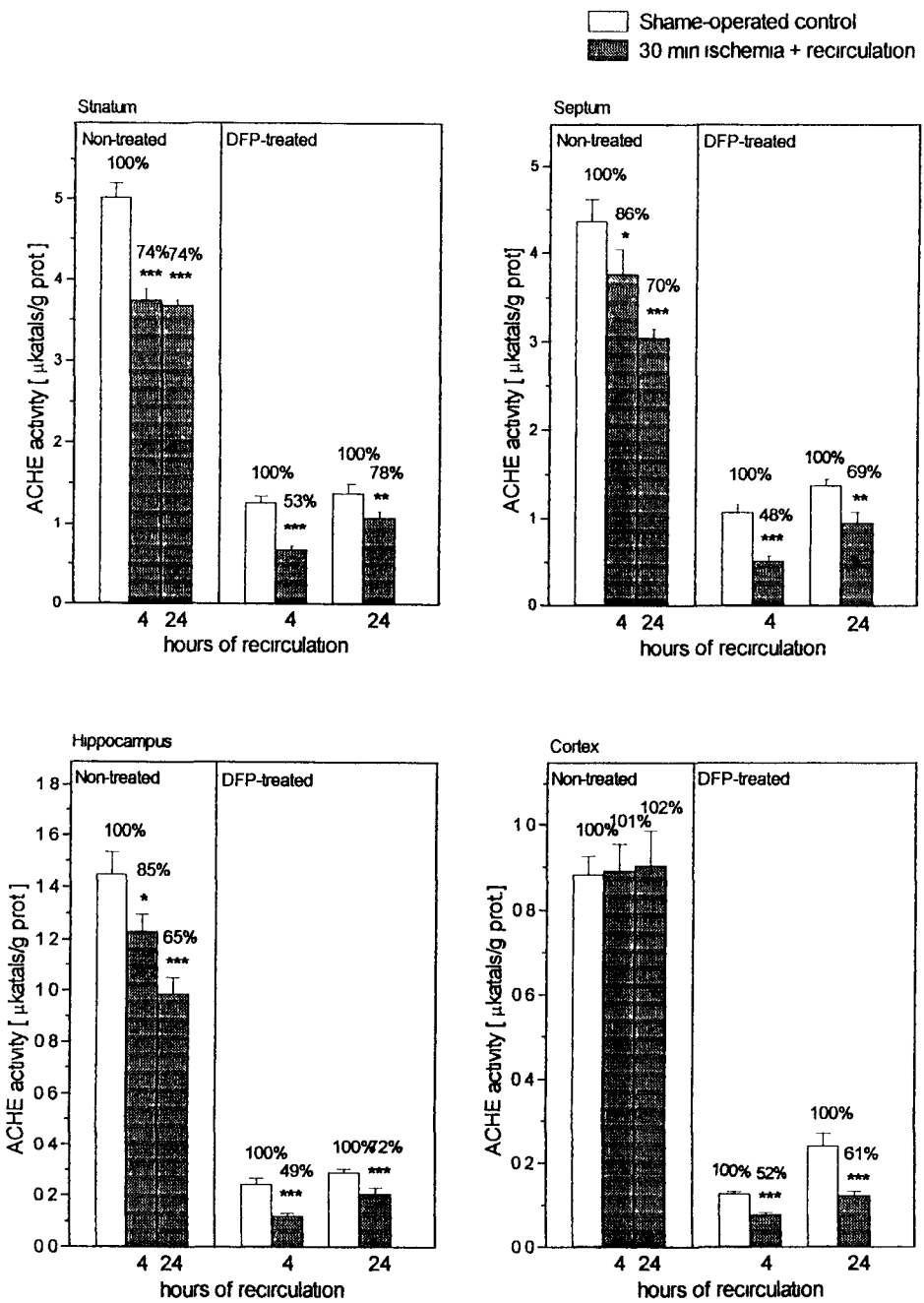


Figure 4. Photomicrograph of the dorso-lateral area of the rat striatum (A, B) and medial septal nucleus (C, D) in unfixed sections processed for DFP-AChE pharmacohistochem-

Since the turnover of AChE is quite rapid when compared to average turnover of brain proteins (Wenthold et al 1974), the depression of AChE synthesis may have an influence on the cholinergic function after ischemia Functional recovery of the brain is tightly associated with the restoration of protein synthesis The inhibition of AChE synthesis after forebrain ischemia was probably transient, because intense staining of AChE-producing neurons and a significant elevation of AChE activity after 24 h recirculation in comparison with 4h recirculation suggested a tendency of AChE synthesis to recover

Comparable measurements of both cholinergic enzyme activities after ischemia without DFP-pretreatment have shown that changes in AChE activity were more pronounced than those in ChAT activity Decrease in ChAT activity in the striatum and hippocampus was found only 4 h after ischemia, while the loss of AChE activity persisted during the whole 24 h of recirculation A decrease in total enzyme activities after ischemia may involve not only the depression of enzyme synthesis, but also the enzyme inhibition and increased enzyme degradation following ischemia/recirculation injury Since our previous results in the spinal cord ischemia suggested that ChAT is more susceptible to ischemia than AChE (Malatova et al 1984, Malatová and Maršala 1993) the results from the brain were surprising This discrepancy between changes of ChAT and AChE after ischemia could be associated with their different location and function in the brain In the striatum, both enzymes are located in large, intrinsic neurons, which constitute about 1% of all striatal neurons (Fibiger 1982, Satoh et al 1983) In cholinergic transmission, functional ChAT is localized presynaptically, while AChE is present postsynaptically ChAT is localized only in cholinergic neurons while AChE occurs also in non-cholinergic (cholinoceptive) neurons which receive cholinergic afferents (Kaiya et al 1980, Eckenstein and Sofroniew 1983) Ultrastructural investigation of the striatum showed the localization of AChE also in GABAergic middle-sized spiny neurons which may synthesize and release this enzyme into the synaptic cleft (Kaiya et al 1980, Mailly and Bouchaud 1986) Then, the loss of AChE activity may reflect changes not only in cholinergic neurons, but also in more vulnerable cholinoceptive neurons, e g GABAergic, peptidergic or dopaminergic ones Francis and Pulsinelli (1982) at 5 8 days after the same forebrain ischemia did not found any signifi cant changes in the striatal and hippocampal ChAT activity while a depletion of glutamic acid decarboxylase activity was accompanied with irreversible damage to striatal GABAergic neurons Selective preservation of cholinergic neurons was also

istry 25 5 hours after DFP administration In sham-operated control group, intensely stained neurons in the striatum (*A*) and septum (*C*) show the activity of *de novo* synthesized AChE 25 5 h after injection of DFP After 30 min ischemia (induced 1 h after DFP injection), and 24 h recirculation, densely stained neurons in the striatum (*B*) and septum (*D*) show recovery of the AChE synthesis Scale bar = 100 μm


Shame-operated control
30 min ischemia + recirculation
Striatum
Non-treated
DFP-treated
ACHE activity [μkatals/g prot]
hours of recirculation
Septum
Hippocampus
Cortex

observed using the pharmacohistochemical method for AChE in the gerbil striatum 4 and 7 days after transient ischemia (Chesselet et al 1990) According to these results, cholinergic neurons have been regarded less vulnerable to ischemia than other neurons (Pulsinelli 1985) However, Ishimaru et al (1994) reported that despite the morphological persistance of cholinergic neurons and unaffected ChAT activity, the presynaptic terminals of the hippocampal cholinergic neurons were vulnerable to ischemia and that cholinergic dysfunction preceded postsynaptic CA1 pyramidal cell death Induced release of ACh at the cholinergic terminals has been found as the most reliable index of the functioning cholinergic system We suppose that postsynaptically located AChE might be downregulated by the decrease of synaptic ACh release

The diffusion of the AChE reaction product Hatchet brown from areas of the highest activity (striatum, nucleus of the diagonal band), observed during histochemical processing of unfixed postischemic brain sections (without DFP pre treatment), was probably due to ischemic injury of membranes In our previous study (Malatova and Maršala 1993), a similar effect, manifesting the ischemia recirculation injury in the spinal cord, has been found and explained as follows Since only AChE bound on the membranes might be visualized, by the direct thio choline method without fixation, the diffusion of the reaction product would reflect an alteration of the enzyme solubility (Malatova and Maršala 1980) and increased membrane permeability owing to the membrane lipid peroxidation following ischemia (Lukačova et al 1998)

In conclusion the present DFP pharmacohistochemical investigation of AChE, supplemented with radiochemical measurement of enzyme activity, provide an experimental demonstration of the downregulation of AChE synthesis in the striatal and septal neurons following ischemia The factors contributing to the decrease in AChE synthesis might be the depletion of mitochondrial oxidative phosphorylation and inhibition of proteosynthesis (Bodsch et al 1985, Burda et al 1994) The results suggest that the inhibition of AChE synthesis after forebrain ischemia was probably transient, not accompanied by a degeneration of cholinergic neurons

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Figure 5 Acetylcholinesterase (AChE) activity in the striatum, septum, hippocampus and cerebral cortex of rats subjected to 30 min forebrain ischemia and subsequent for 4 or 24 h recirculation (dark columns) and from corresponding control sham-operated rats (white columns) Ischemia was induced in two groups of animals non-treated and pretreated with DFP (1 2 mg/kg i m) 1 h before ischemia The values represent arithmetical means ± S E M ($n = 6$) Changes in AChE activity after ischemia are evaluated against the corresponding sham operated control groups percentage values are added Statistical significance * = $p < 0\ 05$ ** = $p < 0\ 01$ *** = $p < 0\ 001$

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