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Ornithine decarboxylase in reversible cerebral ischemia: an immunohistochemical study*

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Summary. Anesthetized Mongolian gerbils were subjected to 5-min ischemia and 8 h of recirculation. Vibratom sections were taken for studying changes in ornithine decarboxylase (ODC) immunoreactivity using an antiserum to ODC, and tissue samples were taken for measuring ODC activity. After 5-min ischemia and 8-h recirculation ODC activity increased 11.5-, 5.9-, and 7.9-fold in the cerebral cortex, striatum and hippocampus, respectively ($P \leq 0.05$ to 0.01). In the cortex, striatum and hippocampus of control animals immunoreactivity was low but clearly above the detection limit. The reaction was confined to neurons. After 5-min ischemia and 8-h recirculation a sharp increase in immunoreactivity was observed confined to neurons, indicating that the postischemic activation of polyamine metabolism is a neuronal response to ischemia. The immunoreactivity was markedly increased in the perinuclear cytoplasm and the dendrites. In the striatum the density of neurons exhibiting a sharp increase in immunoreactivity was more pronounced in the lateral than in the ventral part. In the hippocampus a strong reaction was present in all subfields but the CA1 subfield was particularly affected. The present study demonstrates for the first time that biosynthesis of a protein is markedly activated during the first 24 h of recirculation after 5-min cerebral ischemia of gerbils even in the vulnerable CA1 subfield, in which the overall protein synthesis is sharply reduced at the same time. Studying polyamine metabolism after ischemia may, thus, provide new information about the basic molecular mechanisms responsible for the altered gene expression after metabolic stress.

Key words: Brain – Cerebral ischemia – Gerbil – Immunohistochemistry – Hippocampus

Ornithine decarboxylase (ODC, the key enzyme in polyamine biosynthesis) catalyzes the decarboxylation of the amino acid ornithine to putrescine (diaminobutane). In the brain a rise in ODC activity has been shown to be a common response to different pathological stimuli: physical, thermal, chemical or metabolic stress induces an increase in ODC activity and thus an activation of polyamine synthesis [3]. This activation is most pronounced following severe metabolic stress as produced by reversible cerebral ischemia [3, 4, 6, 19, 25, 28]. The ischemia-induced increase in ODC activity is prevented by blocking protein synthesis [3], thus indicating that it results from de novo protein synthesis and not from an allosteric activation of the enzyme protein.

Activation of polyamine metabolism has been implicated as one of the metabolic events which may contribute to the disturbances of the blood-brain barrier after applying a cold lesion to the brain tissue [20, 21, 34] and which may be involved in the manifestation of neuronal necrosis after cerebral ischemia [25]. Under both pathological conditions a marked increase in putrescine levels has been observed, most pronounced after cerebral ischemia [20, 25]. A rise in putrescine levels may activate influx of calcium ions into and release of neurotransmitters from nerve endings [2, 15, 22]. On the other hand, evidence has been presented recently that pretreating animals with one of the polyamines (putrescine, spermidine or spermine) reduces the postischemic development of neuronal necrosis significantly [11]. It has been suggested, therefore, that postischemic activation of polyamine metabolism is necessary for the recovery of neurons from the metabolic stress produced by reversible ischemia [11].

A mandatory prerequisite for a role of polyamines in the manifestation of neuronal necrosis after cerebral

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ischemia or recovery from the metabolic stress is that the ischemia-induced activation of polyamine metabolism is a neuronal response to ischemia. ODC activity and polyamine levels are usually measured in tissue samples. The results obtained, therefore, do not permit a conclusion to be drawn about the cellular location of the observed changes (whether they take place in neurons or glial cells or both). It has been shown recently that the postischemic increase in putrescine, which has been found in the tissue homogenate, is also present in nerve endings isolated from the cerebral cortex, striatum and hippocampus [32], thus indicating that ischemia-induced activation of polyamine metabolism is also present in neurons and cannot only be explained by the proliferation of glial cells which is known to be activated by transient cerebral ischemia. ODC immunoreactivity has to the best of our present knowledge been studied after cerebral ischemia only by Dempsey et al. [6], who measured immunohistochemically the location of ODC protein in postischemic brains of Mongolian gerbils. In those experiments fluorescein-labelled antibodies were used with which immunoreactivity was absent in control animals, although in the same series of experiments ODC activity was detected in tissue homogenates prepared from the brains of control animals [6]. In addition, 40-min cerebral ischemia was used with 4-h recirculation and immunoreactivity was studied in the cerebral cortex only. In the present series of experiments the gerbil model was used, i.e., 5-min ischemia produced by bilateral common carotid artery occlusion, and ODC immunoreactivity was studied after 5-min ischemia and 8-h recirculation in the cerebral cortex, striatum and hippocampus, i.e., in both, vulnerable and non-vulnerable brain structures, using the sensitive peroxidase-antiperoxidase (PAP) technique for imaging ODC protein.

Materials and methods

Reversible cerebral ischemia was produced in five female Mongolian gerbils (Meriones unguiculatus) weighing 55 to 75 g. Animals were anesthetized with halothane in 70 % N_2O and 30 % O_2 (3 % and 1.2 % halothane for induction and maintenance of anesthesia, respectively). Body temperature was kept at 37 °C by using a feedback-controlled heating pad. A skin incision was made in the neck and both common carotid arteries were exposed. Forebrain ischemia was induced by occluding both common carotid arteries with aneurysm clips. After 5-min ischemia brains were recirculated by removing the clips. The skin incision was sutured and animals were brought back to their cages. Eight hours later gerbils were reanesthetized and decapitated. In addition, five control animals were anesthetized and decapitated.

Brains were rapidly removed and divided into both hemispheres. The left hemispheres were frozen in 2-methylbutane cooled to –80 °C with liquid nitrogen for measuring ODC activity. The right hemispheres were taken for studying the location of ODC immunoreactivity. For measuring the regional pattern of ODC activity tissue samples of about 5 to 10 mg each were taken at –20 °C from the cerebral cortex, striatum and hippocampus of control and experimental animals. Samples were homogenized with 10 vol 50 μM Tris, pH 7.2, supplemented with 2 mM dithiothreitol (Tris/DTT). Analysis of ODC activity was carried out by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine as described previously [28, 29].

Location of ODC immunoreactivity was studied using a polyclonal antibody directed against mouse ODC (for purification of mouse kidney ODC and production of antibody see [16]. This antiserum has been used in the rat central nervous system [8] and has been shown to cross-react with the ODC protein from a wide range of species [16]. It has been illustrated by Isomaa et al. [16] that the application of ODC antiserum in the ODC bioassay inhibits the catalytic activity of the enzyme. It can, therefore, be assumed that the antibody binds near the catalytic center of the enzyme. In addition, it has been demonstrated that the antibody recognizes, at least partly, ODC enzyme protein covalently linked to the ODC antizyme, the macromolecular inhibitor of the enzyme [23].

Fixation of tissue was performed by overnight immersion of the hemispheres into a paraformaldehyde solution at room temperature (4 % paraformaldehyde, supplemented with 3 % sucrose). Slices were then washed with phosphate-buffered saline (PBS) solution. Endogenous peroxidase was blocked with 3 % H_2O_2, 10 % methanol in PBS, followed by extensive washing with PBS. The tissue was incubated with the primary antibody (rabbit anti-mouse ODC, dilution 1:200) at 4 °C overnight in a moist chamber. After washing with PBS sections were incubated with the secondary antibody (swine anti-rabbit IgG, dilution 1:50) and, finally, with the PAP complex (dilution 1:50). The peroxidase activity was visualized with 3,3′-diaminobenzidine according to Graham and Karnovsky [13]. Between all incubation steps sections were extensively rinsed in PBS. For negative controls the primary antibody was replaced by normal rabbit serum. In these sections immunoreactivity was absent.

Results

ODC activity

In control animals ODC activity was low and amounted to 0.40 ± 0.07, 0.29 ± 0.05 and 0.54 ± 0.31 nmol/g per h in the cerebral cortex, striatum and hippocampus, respectively (mean values ± SD, see Fig. 1). After 5-min

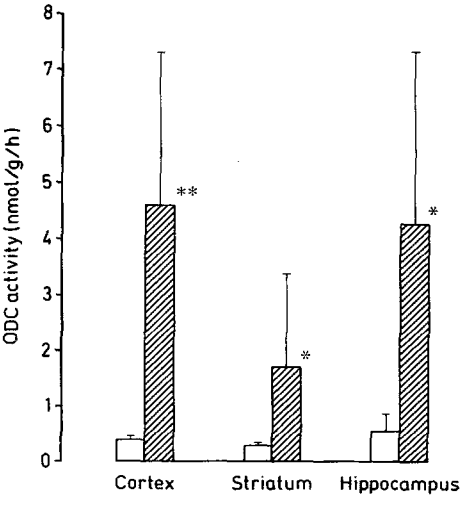

Fig. 1. Changes in ornithine decarboxylase (ODC) activity in reversible cerebral ischemia. Tissue samples of about 5 mg each were taken from the cerebral cortex, striatum and hippocampus of control gerbils and animals subjected to 5-min ischemia and 8-h recirculation. ODC activity was determined by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine. Statistically significant differences in ODC activity between control and experimental animals are indicated by * $P \leq 0.05$; ** $P \leq 0.01$. □ control, ▨ 8 h reflow

ischemia and 8-h recirculation ODC activity was significantly increased in all brain structures studies: to 4.60 nmol/g per h in the cortex ($P \leq 0.01$ cf. control animals); to 1.70 ± 1.66 nmol/g per h in the striatum ($P \leq 0.05$) and to 4.27 ± 3.04 nmol/g per h in the hippocampus ($P \leq 0.05$).

ODC immunoreactivity

Ischemia-induced regional changes in ODC immunoreactivity are illustrated in Fig. 2–5. In control animals a low immunoreactivity could be found in all brain structures studied. Immunoreactivity was clearly confined to neurons in both control and experimental animals. The highest density of the reaction product was present in the perinuclear cytoplasm.

In the cerebral cortex of experimental animals a sharp increase in ODC protein content was observed which was, however, not clearly confined to specific layers of the cortex (Fig. 2b). In animals exhibiting high ODC activity serveral dark dots were present particularly in layers 2 and 3 of the cortex (Fig. 2b, arrows). Using a higher magnification and changing the focus these sharply stained dots seemed to be dendrites (Fig. 2c, arrows).

In the striatum of control animals ODC immunoreactivity was low and neurons stood out only slightly from the background (Fig. 3a) without showing any preference for the lateral or medial part of this structure. In animals exhibiting high ODC activity the content of ODC protein was sharply increased in small- and medium-sized neurons (Fig. 3b,c). In these brains the density of neurons with high immunoreactivity was clearly more pronounced in the lateral (Fig. 3b) than in the medial part of the striatum (Fig. 3c).

In control animals ODC immunoreactivity was clearly detectable in the hippocampus, both in the CA1- and CA3-subfield (Figs. 4a, 5a). However, neuronal cell bodies did not stood out from the surrounding tissue. Following cerebral ischemia ODC immunoreactivity was markedly increased, particularly in the hippocampal CA1 subfield: in the brain of the gerbil illustrated in Fig. 4b hippocampal ODC activity (decarboxylation of ornithine to putrescine) was only about twice that of control levels but ODC immunoreactivity (binding of ODC antibody to ODC protein) was markedly increased in comparison to that found in control animals. In the CA3 subfield, by contrast, ODC immunoreactivity was still similar to controls in an animal in which ODC activity was six times above that of controls (Fig. 5b). In animals exhibiting high ODC activity in the hippocampus ODC immunoreactivity was sharply increased in both the CA1 and CA3 subfield. The content of the reaction product was, however, more pronounced in the CA1-subfield (Fig. 4c, in comparison to Fig. 5c).

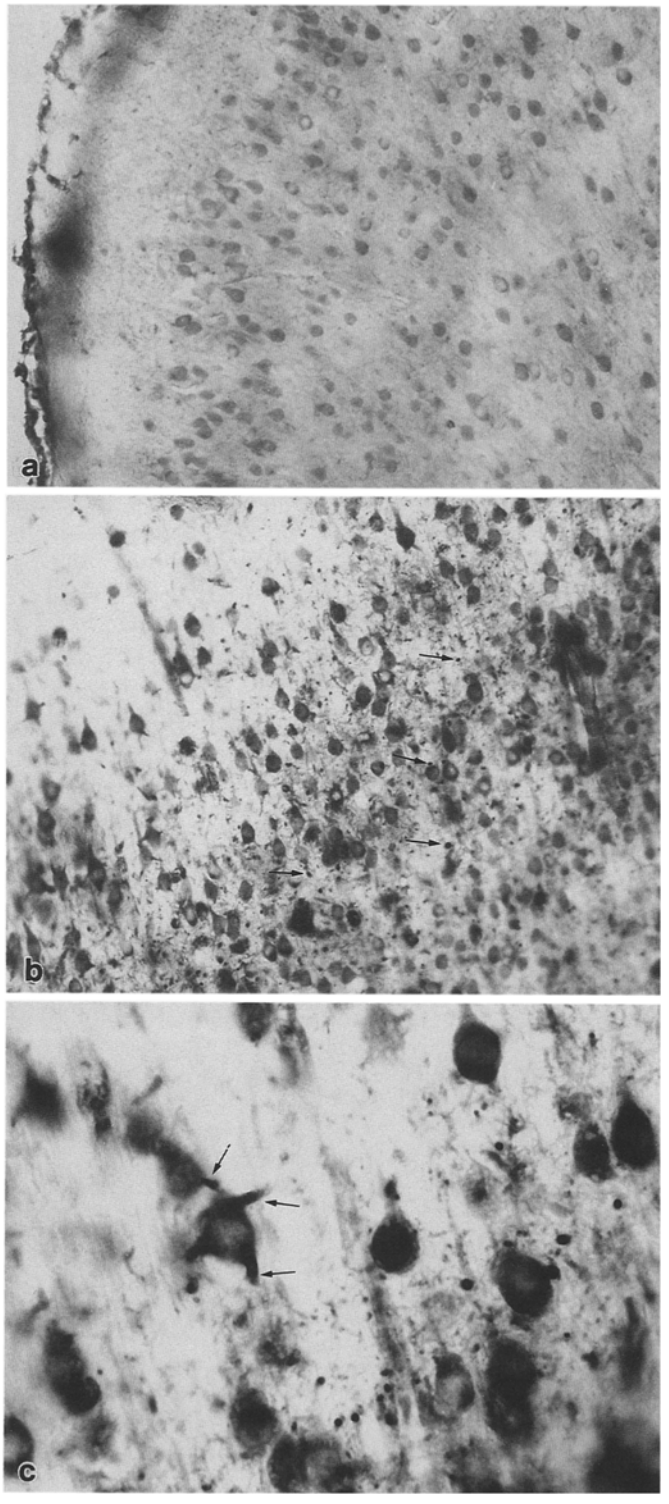

Fig. 2a–c. Ischemia-induced changes in ODC immunoreactivity in the cerebral cortex of gerbils. **a** Control animal. Neurons stand out only slightly from the surrounding brain tissue. **b, c** Animal subjected to 5-min ischemia and 8 h of recirculation in which ODC activity amounted to 6.2 nmol/g per h. In this animal several dark dots were detectable, particularly in layers 2 and 3 of the cortex (**b,** *arrows*). Using a higher magnification these dots could be identified as dendrites (**c**)

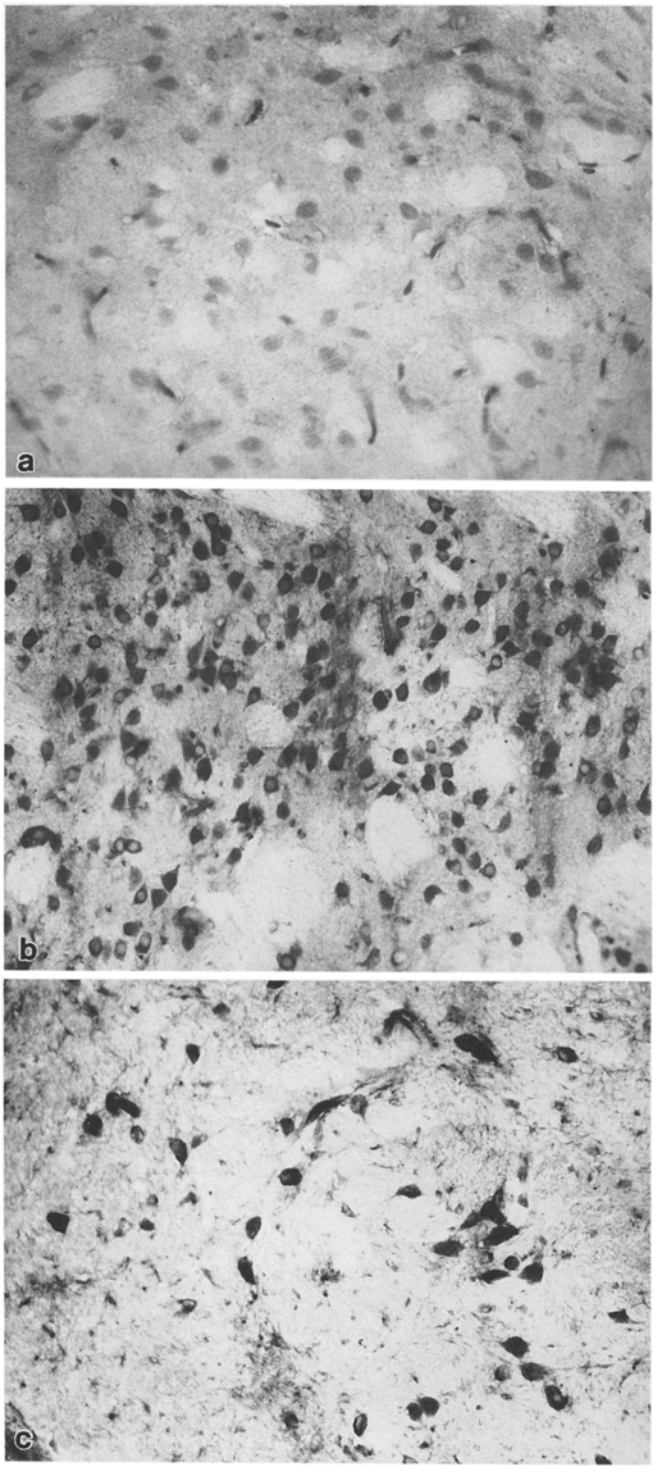

Fig. 3a–c. Ischemia-induced changes in ODC immunoreactivity in the striatum of gerbils. **a** Control animal, **b, c** Animal subjected to 5-min ischemia and 8 h of recirculation (ODC activity amounted to 4.6 nmol/g per h). The density of neurons exhibiting a sharp increase in ODC immunoreactivity was much higher in the lateral (**b**) than in the ventral part of the striatum (**c**)

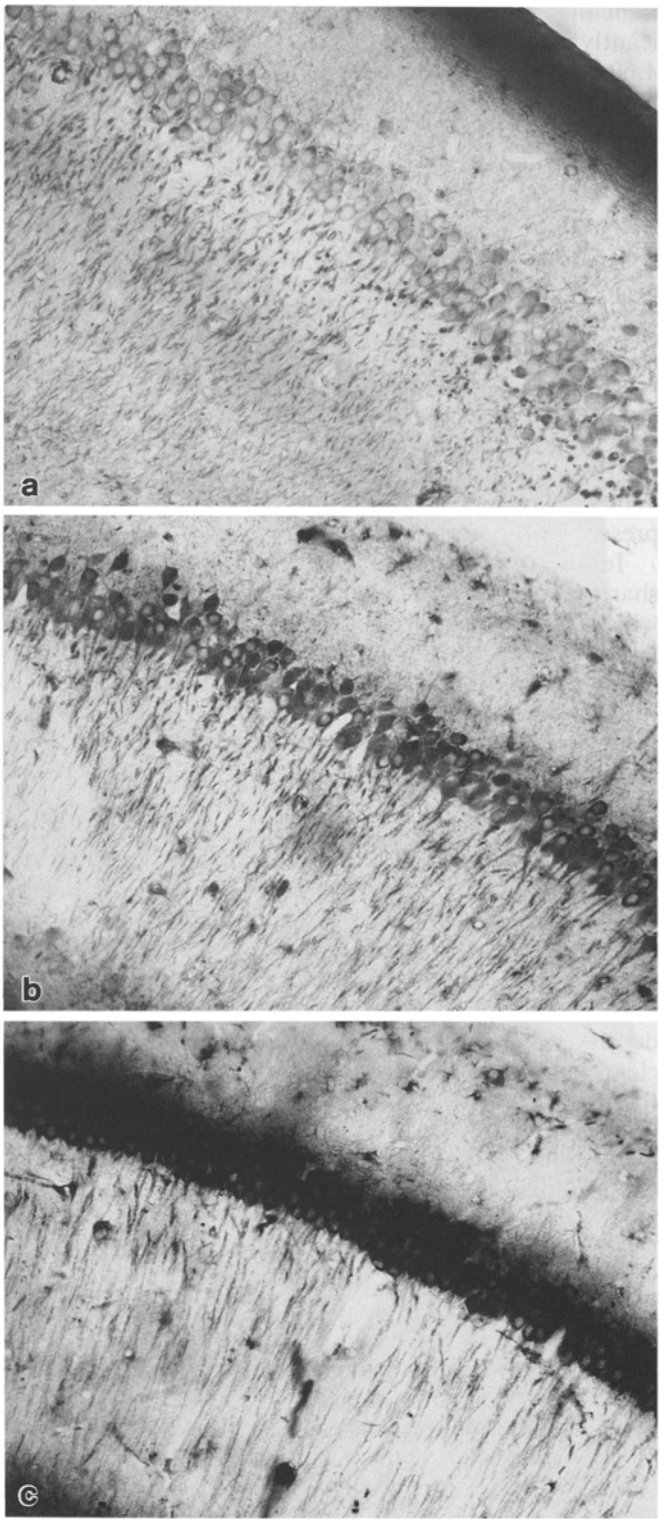

Fig. 4a–c. Ischemia-induced changes in ODC immunoreactivity in the hippocampal CA1 subfield of gerbils. **a** Control animal, neurons of the stratum pyramidale were clearly visible but ODC immunoreactivity was not markedly above that of the surrounding tissue. **b, c** Animals subjected to 5-min ischemia and 8 h of recirculation. **b** Animal in which hippocampal ODC activity amounted to 1.2 nmol/g per h (i.e., only about two fold above controls). The immunoreactivity of neuronal cell bodies was markedly enhanced. **c** Animal in which hippocampal ODC activity amounted to 4.1 nmol/g per h (i.e., about eight fold above controls). In this animal a sharp increase in ODC immunoreactivity was present, particularly in the perinuclear cytoplasm

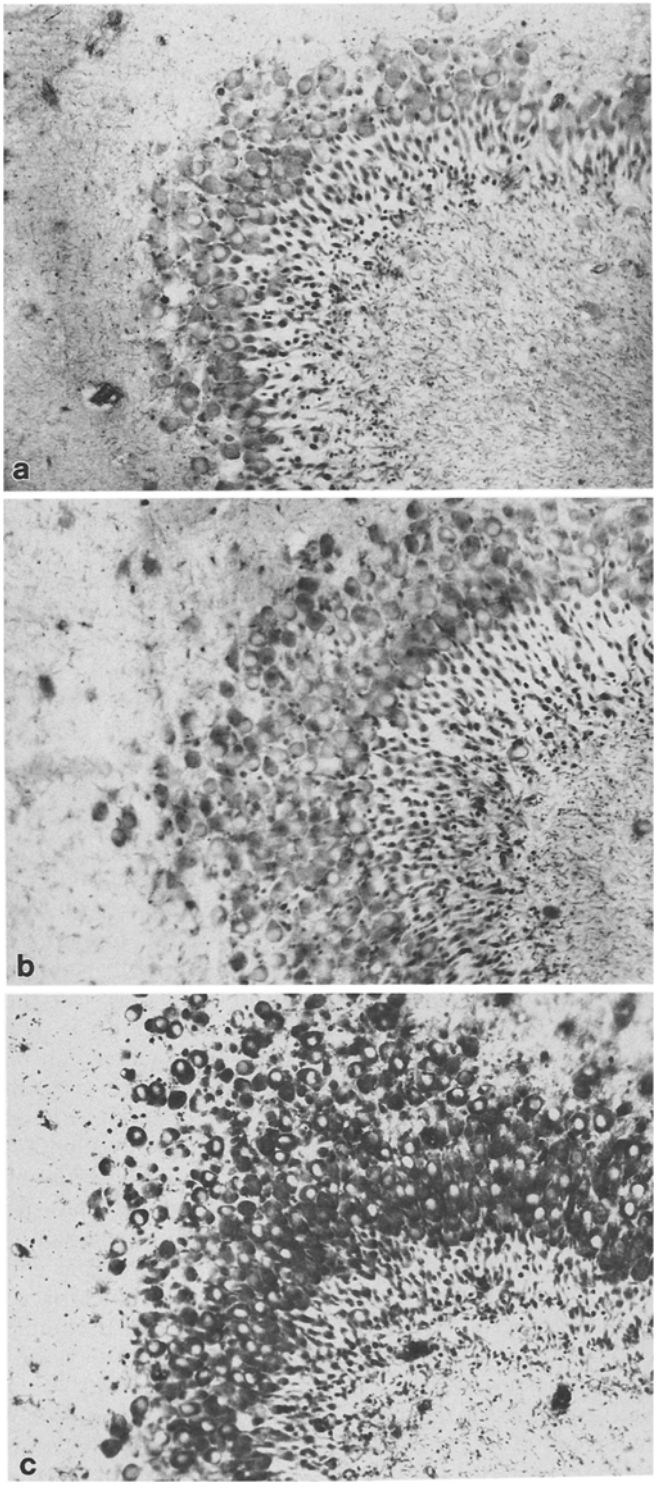

Fig. 5a–c. Ischemia-induced changes in ODC immunoreactivity in the hippocampal CA3 subfield of gerbils. **a** Control animal; **b, c** Animals-subjected to 5-min ischemia and 8 h of recirculation. In the animal illustrated in **b** (hippocampal ODC activity amounted to 3.3 nmol/g per h) ODC immunoreactivity was similar as that found in the control animal. However, in animal illustrated in **c** (hippocampal ODC activity 4.1 nmol/g per h) ODC immunoreactivity was markedly increased in the neurons of the CA3 subfield

Discussion

In the present series of experiments ischemia-induced changes in both ODC enzymatic activity (decarboxylation of ornithine to putrescine) and ODC immunoreactivity (binding of ODC antibody to ODC protein) were studied in different brain structures. The most prominent findings were the observations that(a) reversible cerebral ischemia causes an increase in ODC immunoreactivity in all brain structures studied; (b) ODC immunoreactivity was confined to neurons; and (c) in the striatum and hippocampus the increase in immunoreactivity was most pronounced in vulnerable areas, namely the lateral striatum and the hippocampal CA1 subfield. These topics will be discussed in detail below.

It has been shown in several studies that reversible cerebral ischemia induces an increase in ODC activity [4, 6, 7, 19, 27, 28]. However, by measuring ODC activity no conclusion can be drawn as to whether the observed change in enzyme activity results from an activation of the enzyme by allosteric mechanisms or from de novo protein synthesis. The observation that the postischemic rise in ODC activity can be blocked with anisomycine [4] indicates that indeed protein synthesis is involved. This observation is confirmed by results from Dempsey et al. [7] who found an increase in the ODC immunoreactivity after cerebral ischemia in the cerebral cortex, and the results of the present study illustrating a marked increase in both ODC activity and immunoreactivity in all brain structures studied. Thus, the postischemic increase in ODC activity results from an active process, namely the activation of ODC gene expression. This assumption is corroborated by recent results of Dempsey et al. [7], who observed in ischemia-induced increase in ODC mRNA.

It has been proposed previously that the postischemic activation of polyamine metabolism is involved in the manifestation of neuronal necrosis [25]. A mandatory prerequisite for a role of polyamines in the manifestation of ischemia-induced neuronal necrosis or in the recovery process following ischemia is that the activation of polyamine synthesis is taking place in neurons. This question can, however, not be answered by measuring ODC activity in tissue samples because using this approach it is not possible to differentiate between neuronal and glial cells. The results of the present study clearly indicate that the postischemic increase in ODC immunoreactivity is indeed a neuronal response to the metabolic stress produced by cerebral ischemia. This is somewhat surprising because an increase in ODC synthesis plays an important role in cell proliferation [14] and glial proliferation is a well-known response to cerebral ischemia [30]. However, ODC activity peaks at two different recirculation times after cerebral ischemia, namely at about 8 h and a second time at about 2 to 3 days of recirculation [4]. The increase in ODC activity observed after 8 h of recirculation seems to be a neuronal response (see results of the present study) but the smaller increase found after 2 to 3 days of recirculation may result from the proliferation of glial cells [5].

In the present study the postischemic increase in ODC immunoreactivity was found in all brain structures studied, these changes were most pronounced in vulnerable regions such as the lateral striatum and the hippocampal CA1 subfield, particularly in experimental animals exhibiting high ODC activity. It is a well-known phenomenon that the overall protein synthesis is markedly suppressed after cerebral ischemia [1, 33]. At the same time certain proteins are preferentially expressed, such as the heat-shock proteins (hsp70) and the proto-oncogene c-fos [17, 35]. However, the regional distribution and temporal profiles of the expression of ODC, c-fos and hsp70 are completely different: as illustrated in the present study, expression of ODC is an early event and a sharp increase in immunoreactivity is found already after 8 h of recirculation. Prominent staining of neurons for c-fos mRNA or hsp70, in contrast, is not found before 24 to 48 h of recirculation [17, 35]. In addition, in the hippocampus most prominent staining for ODC protein was present in the CA1 subfield, but staining for hsp70 protein was most pronounced in the CA3 subfield, whereas the CA1 subfield was spared. C-fos gene expression is markedly activated at the transcriptional level in the hippocampal CA1 subfield following prolonged recirculation but there is no information available up to now as to whether the c-fos protein is synthesized in the CA1 subfield after ischemia. These observations indicate that the activation of ODC, c-fos or hsp70 expression may represent different molecular mechanisms.

It is interesting to note that after cerebral ischemia the expression of ODC is sharply activated even in the vulnerable CA1-subfield, i.e., a structure in which the overall protein synthesis is severely suppressed during the same recirculation time [1, 33]. These results indicate that the protein synthesis machinery is not completely broken after ischemia: after ischemia the protein synthesis machinery may be switched from the physiological state to a state during which specific proteins are preferentially synthesized. If this interpretation is valid the inability of protein synthesis in vulnerable brain structures to switch back the synthesis machinery to the physiological state may play the prominent role in the development of neuronal necrosis. It is interesting to note that the inability of regional cerebral protein synthesis to recover from metabolic stress is not region-specific but strongly depends on the vulnerability of the respective region to different kinds of stress: following cerebral ischemia recovery of protein synthesis is least in the hippocampal CA1 subfield [1, 33], the region most vulnerable to ischemia, whereas following reversible hypoglycemia recovery of protein synthesis is least in the lateral striatum [18], the region most vulnerable to hypoglycemia.

It has been suggested that the postischemic overshoot in putrescine formation plays a role in the manifestation of neuronal necrosis [25]. This overshoot in putrescine formation results most probably from the postischemic increase in ODC and decrease in *S*-adenosylmethionine decarboxylase activity [4, 28]. On the other hand, the suggestion has been made that activation of polyamine metabolism plays an important role for the recovery of neurons from axonal injury [9, 10]. This suggestion has been made because after axonal injury ODC activity increases and polyamines have been shown to accelerate recovery. Recently, Gilad and Gilad [11] presented results indicating that pretreating animals with putrescine, spermidine or spermine reduced significantly the density of ischemia-induced neuronal necrosis, thus implying that the postischemic activation of polyamine synthesis may play a role in the recovery of neurons from the metabolic stress. However, it has to be established whether pretreating animals with polyamines accelerates or reduces the postischemic changes in polyamine profiles, particularly the sharp increase in putrescine levels. Indeed, it has been shown previously that barbiturate treatment of gerbils inhibited the postischemic rise in putrescine levels in the hippocampal CA1 subfield significantly but did not reduce the postischemic increase in ODC activity [28].

Postischemic putrescine levels correlate closely with the density of neuronal necrosis [26]. Because of these results and the known activities of putrescine in mediating calcium fluxes at the cell membrane and neurotransmitter release from nerve endings (see above), we feel that these changes are involved in the manfestation of ischemic cell damage. This interpretation is corroborated by the observation that in tissue culture the development of neuronal necrosis produced by the neurotoxin *N*-methyl-*D*-aspartate can be completely prevented by blocking ODC with the specific inhibitor α-difluoromethylornithine [24], thus implying that an activation of ODC may play a role in the development of neuronal necrosis under all those pathological conditions which are triggered by an activation of the *N*-methyl-*D*-aspartate receptor. In addition, it has recently been shown that ischemic cell damage can be markedly reduced by inhibiting protein synthesis with cylcoheximide [12]. It has, therefore, been speculated that abnormal proteins may be responsible for ischemia-induced delayed neuronal death in the hippocampus [12]. An alternative explanation would be that the observed overactivation of polyamine metabolism is involved in the pathological process of ischemic cell damage which then can be prevented by blocking of ODC synthesis with cylcoheximide. The postischemic activation of polyamine metabolism and the development of neuronal necrosis can then be viewed as an active process triggered during ischemia (presumably mediated by activation of the *N*-methyl-*D*-aspartate receptor, [31, 36] and manifested following recirculation when the protein synthesis machinery recovers at least partially [33].

In conclusion, the results of the present study indicate that reversible cerebral ischemia induces a sharp activation of ODC synthesis as evidenced by the rise in ODC immunoreactivity, most pronounced in vulnerable brain structures such as the lateral striatum and the hippocampal CA1 subfield. Thus, activation of ODC synthesis takes place during the 1st day of recirculation even in those brain structures where the overall protein synthesis is severely suppressed (hippocampal CA1 subfield).

This is in contrast to the ischemia-induced activation of heat-shock protein synthesis where the CA1 subfield is spared. ODC is the first and rate-limiting enzyme in the biosynthesis of polyamines, the functions and activities of which are largely known. Studying polyamine metabolism may, therefore, provide new information about the molecular mechanisms involved in the development of neuronal necrosis after severe metabolic stress as produced by cerebral ischemia.

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