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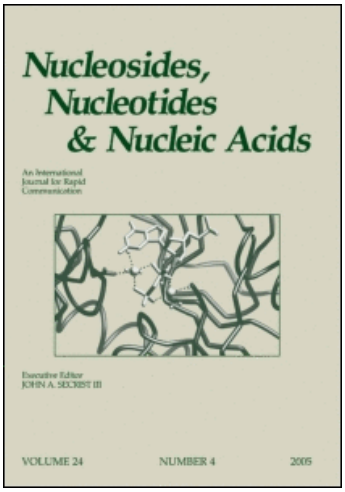

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REGION-SPECIFIC DISTRIBUTION OF DIHYDROOROTATE DEHYDROGENASE IN THE RAT CENTRAL NERVOUS SYSTEM POINTS TO PYRIMIDINE DE NOVO SYNTHESIS IN NEURONS

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□ *An increasing body of evidence has suggested a role of pyrimidine nucleotides not only in metabolic pathways in the developing nervous system but also in adult brain functions. There is still little known about the cellular sources of pyrimidine synthesis and the distribution of enzymes involved in the biosynthetic pathway of pyrimidines in the central nervous system (CNS) of mammals. Therefore, we investigated the activity of dihydroorotate dehydrogenase (DHODH), the fourth enzyme in the pyrimidine de novo synthesis, by catalytic enzyme histochemistry in adult rat brain. Its distribution was confirmed by Western blot analysis of dissected brain regions and by immunohistochemical analysis of paraffin-embedded formalin fixed sections employing an affinity-purified highly specific antibody. Microscopic analysis of brain sections revealed the presence of enzymatically active DHODH in many CNS regions, albeit at different intensities. High levels of both DHODH activity and immunoreactivity were observed in the neocortex, hippocampus, spinal cord and choroid plexus; lower levels were seen in the cerebellum, and only marginal expression in brainstem. The prominent staining of neuronal cell bodies in these regions suggests a neuronal location of DHODH. Neuronally derived pyrimidine de novo synthesis in the rodent CNS, independent of exogeneous sources, would provide pyrimidines for dynamic processes of membrane assembly, rearrangement and neuronal plasticity, as well as supplying uridine nucleotides as neuronal signalling molecules.*

Keywords Brain; dihydroorotate dehydrogenase; neurons; pyrimidines

INTRODUCTION

A role of pyrimidine nucleotides in various metabolic processes important for the proper functioning of the nervous tissue has been suggested for some time. In addition to their role as building blocks for DNA and RNA, pyrimidines are a prerequisite for the synthesis of phospholipids, glycolipids, and glycoproteins of neuronal membranes.[1,2] Nucleotides, in

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particular ATP, but also UTP and UDP are known to act as neurotransmitter in the sympathetic nervous system, both within ganglia and at the level of target organs.[3,4] Some metabotropic P2Y receptor subtypes are sensitive to uridine nucleotides.[5] Therefore, a continous supply of pyrimidine nucleotides should be essential not only for the developing CNS, but equally for plasticity, regeneration, and neurotransmission of the adult brain. However, compared with purines, much less is known about pyrimidine synthesis in rodent brain. The occurrence of the complete de novo pyrimidine biosynthesis in adult brain was originally deduced from measurements of ^{14}C bicarbonate incorporation in orotate and RNA of rat brain slices and minced tissues.[6] Almost two decades later, rodent astrocytes were immunostained for the CAD enzyme, which performs the first three steps of UMP synthesis.[7] However, more recently it was concluded that the adult brain's requirement for uridine is predominantly satisfied from uridine transport from the blood in rats as well as in humans.[8] Since there is still very limited knowledge of pyrimidine metabolism in the many different regions of the CNS, we investigated adult rat brain for the expression and regional activity pattern of mitochondrially associated dihydroorotate dehydrogenase (DHODH), the fourth enzyme in the pyrimidine de novo synthesis, by enzyme histochemistry, and by Western blot analysis of dissected brain regions and immunohistochemical analysis of formalin fixed paraffin-embedded (FFPE) sections employing an affinity-purified highly specific antibody.

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MATERIALS AND METHODS

Histochemistry. Catalytic enzyme histochemistry of DHODH on 14 μm cryostat sections of adult rat brain and spinal cord was performed as

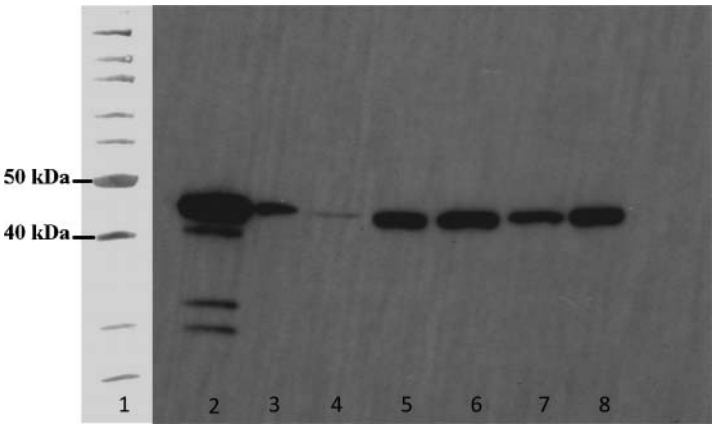
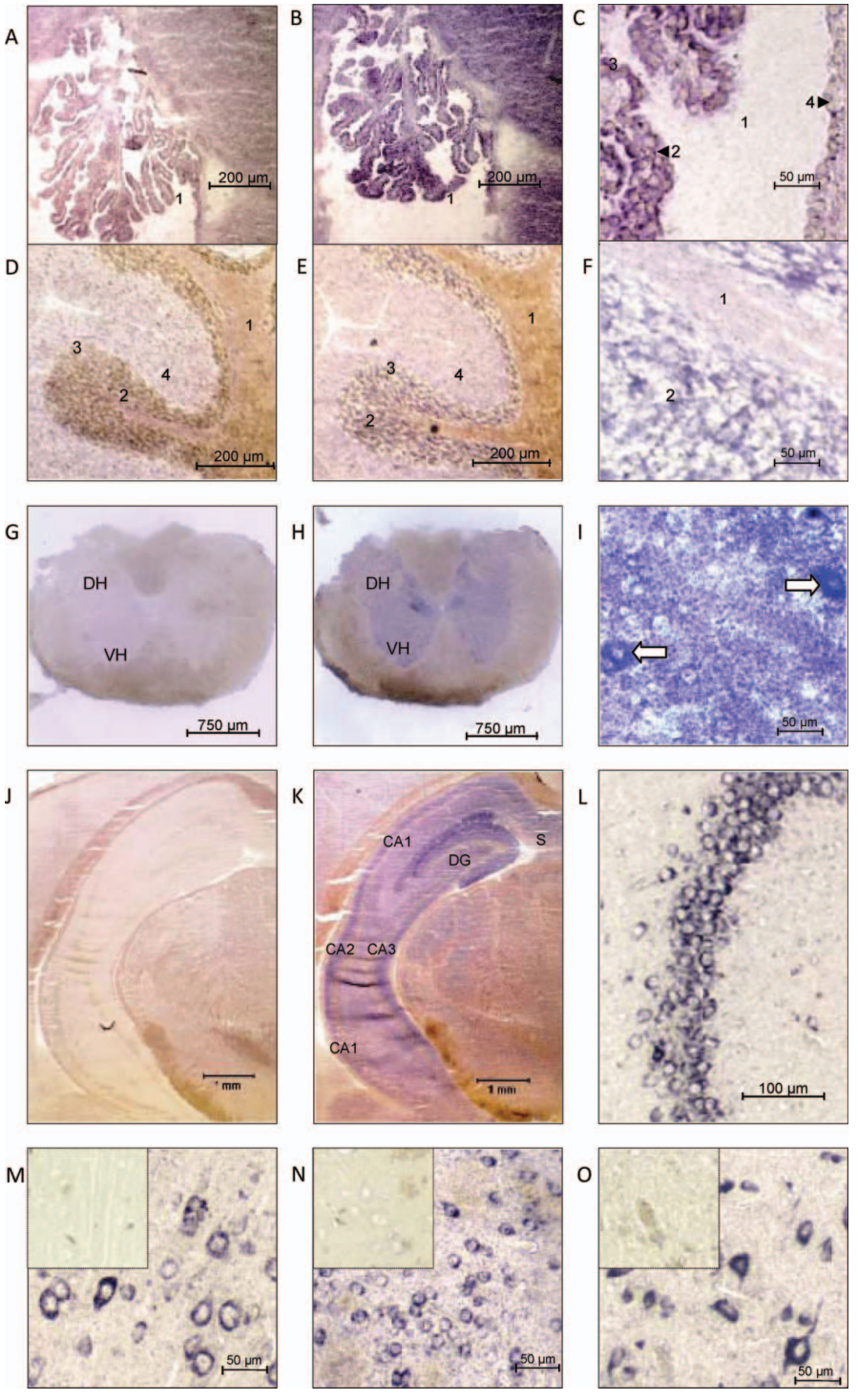


FIGURE 1 Western blot detection of DHODH: 13 μg mitochondrial protein were analysed on each lane. 1: marker proteins (10–200 kDa); 2: DHODH (46 kDa) 0.4 μg; 3: cerebellum; 4: brainstem; 5: hippocampus; 6: cortex; 7: striatum; 8: spinal cord.



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described previously for other tissues using the nitroblue tetrazolium/formazan technique.[9] DHODH is associated only with the inner membrane of mitochondria and uses ubiquinone of the respiratory chain as electron acceptor. Therefore, the oxidation of the substrate dihydroorotate (10 mM) can be easily determined by trapping the electrons with tetrazolium (1 mM) in the presence of cyanide (10 mM) to prevent their further flow along the chain to oxygen, in phosphate buffer pH 8, 37°C, 60 minutes. The insoluble blue formazan product which becomes deposited on the subcellular site of the enzyme activity can be localized by light microscopy. The specificity of the histochemical method for DHODH was confirmed by incubation of the brain sections with DHODH-specific inhibitors, for example, brequinar or redoxal, which reduced the blue precipitate (data not shown), as described previously.[9]

Western Blot Analysis

Mitochondria from dissected regions of the brain[9] were transferred to Laemmli buffer and further processed for SDS-PAGE/Western blotting using an affinity-purified antibody raised in rabbits against DHODH and peroxidase conjugated anti-rabbit IgG with the ECL detection kit.[10]

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Immunocytochemistry

Paraformaldehyde-fixed paraffin embedded brain tissues were cut into 8 μm sections which were deparaffinised and stained with the same anti-DHODH IgG. Immunocytochemical detection was performed using a donkey anti-rabbit IgG and the ABC-method as described previously with tyramide-signal amplification according to the manufacturer's protocol (Applied Biosystems Deutschland GmBH, Darmstadt, Germany).[11]

FIGURE 2 Light microscopic visualization of DHODH in frozen and fixed sections of rat brain. (A–K) Dehydrogenase activity was localized by the blue staining of the formazan deposit on incubation with dihydroorotate and NBT; controls were incubated without dihydroorotate. (L–O) For immunostaining, fixed sections were incubated with anti-DHODH IgG; controls were incubated without anti-DHODH. (A) Choroid plexus control; (B) with DHO; (C) with DHO at higher magnification. 1, liquor cerebrospinalis; 2, cuboidal plexus epithelium; 3, tufts of capillaries; 4, ventricular ependyma. (D) Cerebellum control; (E) with DHO; (F) with DHO, at higher magnification. 1, white matter; 2, granular layer; 3, Purkinje cells; 4, molecular layer. (G) Spinal cord control; (H) with DHO; (I) with DHO at higher magnification. VH, ventral horn; DH, dorsal horn. ⇐, motoneurons of VH. (J) Hippocampus control; (K) with DHO; CA1-CA3, fields of hippocampus; DG, dentate gyrus; S, subiculum; (L) immunostaining of hippocampus DHODH at higher magnification. (M) Immunohistochemical localization of DHODH in nerve cell bodies of frontal cortex; (N) of striatum; (O) of pars reticulata of the substantia nigra; inserts, control without anti-DHODH IgG.

RESULTS AND DISCUSSION

The immunoblotting of mitochondrial protein from rat brain tissues, with recombinant rat DHODH protein as reference,[6] revealed the presence of DHODH in most of the dissected regions of the rat brain, albeit at different levels. In comparison to hippocampus and cortex, where the strongest bands were observed, less enzyme protein was detected in the cerebellum, and even less in brain stem extracts (Figure 1).

Some of these regions were investigated using the catalytic enzyme histochemistry. Figure 2 shows the light-microscope visualisation of the insoluble blue-violet formazan precipitate as the result of active DHODH in the different regions of the rat brain. The choroid plexus epithelium (Figures 2A–2C), which is responsible for the formation and excretion of cerebrospinal fluid, exhibited a high activity staining in comparison to the ependyma cells, which completely cover the ventricles. This is in accordance with previous findings on high DHODH activities in cells of tissues with known high excretory or absorptive activities.[6] The sections of the cerebellar cortex, with its characteristic layers (Figures 2D–2F), revealed a strong formazan deposit on incubation with dihydroorotate, predominantly in the granular layer of the graymatter, where a great number of cell bodies of granule cells are known to be in close vicinity to each other. Likewise, a characteristic blue staining can be seen in the graymatter but not in the white matter of the spinal cord (Figures 2G–2I). At higher magnifications we located prominent DHODH activity in motoneurons of the ventral horn (Figure 2I). The photomicrographs of the hippocampus (Figures 2J and 2K) show a characteristic pattern of DHODH activity in the fields of the Ammonshorn. From the control sections incubated without the substrate dihydroorotate (DHO), it can be deduced that completely negative controls could not be obtained with some tissues (Figures 2A, 2D, 2G, and 2J). This non-DHODH specific staining may be due to other dehydrogenases which can still use endogenous substrates, and NBT as final electron acceptor.

Immunocytochemical staining of the hippocampal formation with anti-DHODH IgG confirmed the presence of the enzyme. Magnification of the CA3 regions illustrates a bright blue immunoreactive staining of the cell bodies of pyramidal neurons (Figure 2L). Similar cellular staining patterns were observed in frontal cortex, striatum and pars reticulata of the substantia nigra (Figure 2M–2O).

Our analyses of the DHODH distribution in different regions of the adult rat brain provide evidence that—besides the choroid plexus—neurons especially have the capacity for pyrimidine de novo synthesis. Lack of DHODH immunoreactivity in astrocytes suggests absence of DHODH or levels below the detection limit in astroglia. With respect to dynamics of membranes and plasticity processes in neurons, it can be assumed that pyrimidine de novo synthesis is important not only for RNA synthesis, but equally for the

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marked glycolipid and glycoprotein turnover in these cells. Moreover, the ad hoc requirement for uridine nucleotides as signalling molecules for cell-surface receptors could be excellently met by de novo synthesis activities independent from the salvage of preformed pyrimidine nucleosides.

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