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Age-dependent changes of pyridoxal phosphate synthesizing enzymes immunoreactivities and activities in the gerbil hippocampal CA1 region

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Abstract

In the present study, age-related changes of pyridoxal 5′-phosphate (PLP) synthesizing enzymes, pyridoxal kinase (PLK) and pyridoxine 5′-phosphate oxidase (PNPO), their protein contents and activities were examined in the gerbil hippocampus proper. Significant age-dependent changes in PLK and PNPO immunoreactivities were found in the CA1 region, but not in the CA2/3 region. In the postnatal month 1 (PM 1) group, PLK and PNPO immunoreactivities were detected mainly in the stratum pyramidale of the CA1 region. PLK and PNPO immunoreactivities and their protein contents were highest in the PM 6 group, showing that many CA1 pyramidal cells had strong PLK and PNPO immunoreactivities. Thereafter, PLK and PNPO immunoreactivities started to decrease and were very low at PM 24. Alterations in the change patterns in protein contents and total activities of PLK and PNPO corresponded to the immunohistochemical data, but their specific activities were not altered in any experimental group. Based on double immunofluorescence study, PLK and PNPO immunoreactive cells in the strata oriens and radiatum were identified as GABAergic cells. Therefore, decreases of PLK and PNPO in the hippocampal CA1 region of aged brains may be involved in aging processes related with γ-aminobutyric acid (GABA) function.

Keywords: Aging; Hippocampus; Pyramidal cells; GABA; Pyridoxal 5′-phosphate; Pyridoxal kinase; Pyridoxine 5′-phosphate oxidase; Gerbil

1. Introduction

It has been reported that microtubule associated protein 2 immunoreactivity is significantly decreased in the hippocampal CA1 region of 59-week-old mice. This result means that dendrites and axons in the hippocampal CA1 neurons are particularly susceptible to aging processes (Himeda et al., 2005). The balance of glutamate and γ-aminobutyric acid (GABA) in the neurons is very important in maintenance of hippocampal neurons. An abnormal GABA function is related with various neurological disorders such as depression (Tunnicliff and Malatynska, 2003), epilepsy (Houser, 1991; Kang et al., 2001a,b, 2002a,c), schizophrenia (Reynolds et al., 2001; Heckers et al., 2002), Parkinsonism (Calabresi et al., 2000) and cerebral ischemia (Arabadzisz and Freund, 1999; Kang et al., 2002b). Moreover, it has been suggested that GABA is involved in a number of cerebral functions that become altered with age, such as learning and memory, emotion and motivation, and motor functions (Cook and Sepinwall, 1975; Marczynski et al., 1994). Hence, the metabolic pathway of GABA is of much importance in terms of aging processes.

GABA is formed from glutamate by the action of two glutamate decarboxylase (GAD) isoforms, GAD65 and GAD67

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(Esclapez and Houser, 1999; Fukuda et al., 1998; Kang et al., 2001a). And, GAD activity is regulated by a cycle of activation and inactivation determined by the binding and release of its cofactor pyridoxal 5′-phosphate (PLP), and PLP is catalyzed by pyridoxal kinase (PLK) or pyridoxine 5′-phosphate oxidase (PNPO) (Choi et al., 1987; Erlander et al., 1991; Erlander and Tobin, 1991).

The metabolism of PLP has been studied extensively in normal tissues in several laboratories (Bahn et al., 2002; Choi et al., 1987; Kwok and Churchich, 1980). PLP is formed in mammalian cells by the phosphorylation of pyridoxal by PLK (EC 2.7.1.35) or by the oxidation of PNP or by the oxidation of pyridoxamine-5-phosphate (PMP) by the cytosolic enzyme PNPO (EC 1.4.3.5).

The Mongolian gerbil provides an ideal opportunity for studying the mechanisms of epileptogenesis (Kang et al., 2001a) and delayed neuronal death after transient forebrain ischemia (Kang et al., 2002b). Recently, we reported that PLK and PNPO immunoreactivities were significantly lower in a 30 min postictal group than that in a pre-seizure group (Kang et al., 2002a,c). In addition, we found that GAD isoform immunoreactivities were elevated markedly in the hippocampal CA1 region at 30 min after 5 min of transient forebrain ischemia. Moreover, at 3–12 h after transient ischemia, their immunoreactivities recovered to the sham level (Kang et al., 2001b). Thus, our findings suggest that the differential alterations of GABA metabolism might be one of the important factors of the neuronal damage induced by ischemia. Because we used only 3–4 months gerbils in these studies, we were not able to determine whether aging influences GABA metabolism. This issue must be resolved by studying the relationships between GABA metabolism and age-related dysfunction in the hippocampus at various ages.

In this study, we first identified age-dependent changes in PLK and PNPO immunoreactivities, in their protein contents and in their activities in the hippocampus proper at various ages in a gerbil model.

2. Materials and methods

2.1. Experimental animals

We used the progeny of Mongolian gerbils (*Meriones unguiculatus*) obtained from the Experimental Animal Center, Hallym University, Chunchon, South Korea, at postnatal month 1 (PM 1) ($n = 14$), PM 3 ($n = 14$), PM 6 ($n = 14$), PM 12 ($n = 14$) and PM 24 ($n = 14$). The animals were housed at constant temperature (23 °C) and relative humidity (60%) with a fixed 12-h light:12-h dark cycle and free access to food and water. Procedures involving animals and their care were conformed to the institutional guidelines, which are in compliance with current international laws and policies (*NIH Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 85-23, 1985, revised 1996) and were approved by the Hallym's Medical Center Institutional Animal Care and Use Committee. All the experiments were conducted to minimize the number of animals used and suffering.

2.2. Confirm of antibody specificity

Animal brains from rats, mice and gerbils were removed and homogenized in a 10 mM phosphate buffer (PB) containing 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM 2-mercaptoethanol and 1 mM phenylmethyl sulfonyl fluoride (PMSF). The individual 25% (w/v) homogenates were centrifuged at 10,000 × *g* for 1 h. Five microliters of each supernatant was mixed with an equal volume of a 2× SDS-sample buffer and boiled for 3 min. The cooled samples were applied to a SDS-PAGE and transferred to nitrocellulose membranes. The blots were processed by the procedures described in Western blot.

In order to establish the specificity of the immunostaining, a negative control test was carried out with pre-immune serum instead of the primary antibody. The negative control test was conducted in all groups.

2.3. Tissue processing and immunohistochemistry

Seven gerbils in each group were anesthetized by injecting sodium pentobarbital (40 mg/kg body weight, i.p.) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 0.1% sodium nitrite and 1 U/100 ml of heparin, followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4). After perfusion, forebrains with the hippocampus were removed, post-fixed for 4–6 h in the same fixative at 4 °C and stored in a 30% phosphate-buffered sucrose solution for 12–24 h. Serial coronal sections of 30 μm thickness were cut with a freezing microtome, and immunohistochemically processed by using the free floating method (Hwang et al., 2004a,d).

The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min, incubated in 10% normal horse serum in PBS for 30 min, and in diluted mouse anti-PLK (Hwang et al., 2004c; Kang et al., 2002a) and anti-PNPO (Bahn et al., 2002; Kang et al., 2002c) for 48 h at 4 °C. Thereafter, the sections were exposed to biotinylated anti-mouse IgG (diluted 1:200; Vector, USA) and streptavidin peroxidase complex (diluted 1:200; Vector). They were then visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Fluka, USA) and hydrogen peroxide in 0.1 M Tris buffer (pH 7.4).

2.4. Double immunofluorescence study

To confirm the neuronal type containing PLK and PNPO immunoreactivities, double immunofluorescence staining for both the mouse anti-PLK antiserum (1:25) or mouse anti-PNPO antiserum (1:25) and the rabbit anti-GAD (diluted 1:200; Chemicon, USA) was performed. Sections were incubated in the mixture of antisera overnight at room temperature. After washing three times for 10 min with PBS, the sections were also incubated in a mixture of both Cy3-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch, USA) and FITC-conjugated goat anti-rabbit IgG (1:600; Jackson ImmunoResearch) for 2 h at room temperature. The immunoreactions were observed under the Axioscope microscope attached HBO100 (Carl Zeiss, Germany).

2.5. Western blot analysis

Rat, mouse and gerbils in each group, which are mentioned above, were used in this immunoblot study. After sacrifice and removal of the hippocampus, the tissues were homogenized in 50 mM Tris containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 0.1 mM ethylene glycol bis (2-aminoethyl ether)-*N*,*N*,*N*′,*N*′ tetraacetic acid (EGTA) (pH 8.0), 0.2% NP-40, 10 mM EDTA (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthvanadate, 1 mM PMSF and 1 mM dithiothreitol (DTT). After centrifugation at 10,000 × *g*, the protein concentration was determined in the supernatants by using the Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, USA). Aliquots containing 20 μg total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. Then, each aliquot was loaded onto a 10% polyacryamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Schleicher and Schuell, USA). To reduce background staining, the filters were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with mouse anti-PLK antiserum (1:500) or anti-PNPO antiserum (1:500), with peroxidase conjugated anti-mouse IgG (Sigma, USA), and then with ECL kit (Amersham, USA).