Available online at www.sciencedirect.com
SCIENCE DIRECT®
Neuroscience Research 51 (2005) 185–197
Neuroscience Research
www.elsevier.com/locate/neures

NADPH-consuming enzymes correlate with glucose-6-phosphate dehydrogenase in Purkinje cells: an immunohistochemical and enzyme histochemical study of the rat cerebellar cortex

Paola Ferri[a], Enrica Biagiotti[b], Patrizia Ambrogini[c], Spartaco Santi[d], Paolo Del Grande[a], Paolino Ninfali[b,*]

[a]*Institute of Morphological Sciences, University of Urbino "Carlo Bo", I-61029 Urbino, Italy*
[b]*Institute of Biological Chemistry "G. Fornaini", University of Urbino "Carlo Bo", I-61029 Urbino, Italy*
[c]*Institute of Physiological Sciences, University of Urbino "Carlo Bo", I-61029 Urbino, Italy*
[d]*Institute of Organ Transplant and Immunocytology (ITOI), Bologna Unit—CNR c/o IOR, I-40138 Bologna, Italy*

Received 19 May 2004; accepted 2 November 2004
Available online 15 December 2004

Abstract

In cerebellum of the adult rat, glucose-6-phosphate dehydrogenase (G6PD) activity is particularly localized in Purkinje cells, showing lower activity in the molecular and granule cell layers. G6PD is the first and rate-limiting step of the hexose monophosphate shunt (HMS), which has the physiological role of providing NADPH for reductive biosynthesis and detoxifying reactions.

In this study, we searched for a possible correlation between G6PD and other NADPH-consuming enzymes, such as NADPH-cytochrome P450 reductase (P450R), glutathione reductase (GR) and NADPH-diaphorase (NADPH-d). This study was performed by means of immunohistochemistry and enzyme histochemistry followed by quantitative densitometric and confocal laser scanning microscopic analyses.

Our results demonstrated that G6PD, P450R and GR have a similar distribution pattern characterized by the highest concentration of these enzymes in the somata of Purkinje cells, and by lower concentrations in the molecular and the granule cell layers. Moreover, in Purkinje cells, G6PD colocalized with both P450R and GR. NADPH-d activity showed a different distribution pattern when compared to the other enzymes, revealing the highest activity in the molecular layer and the lowest in Purkinje cells. Our results suggest a coordinated regulative mechanism of G6PD, P450R and GR based on the request of NADPH or on specific transcription factors.

Keywords: Glucose-6-phosphate dehydrogenase; NADPH-cytochrome P450 reductase; Glutathione reductase; NADPH-diaphorase; Cerebellum; Purkinje cells

1. Introduction

The cerebellum is an anatomical structure involved in maintaining balance, posture and muscle tone as well as in voluntary movement coordination. The cerebellar cortex consists of three layers, which are, from the pial surface to the innermost portion, the molecular layer, the Purkinje cell layer and the granule cell layer. The molecular layer mainly contains neurites, comprising: dendritic arborizations of Purkinje cells, granule cell axons that constitute the parallel fibers, and climbing fibers originating from the inferior olivary nucleus. Moreover, in the molecular layer, the rare presence of neurons can be observed including stellate and basket cells.

The Purkinje cell layer is made up of the Purkinje cells which are organized in a single regular row, and represent the only output cells of the cerebellar cortex; Purkinje cell's dendritic arborizations reach the molecular layer and are fan-shaped, like a flattened tree.

The granule cell layer consists of a small proportion of Golgi cells and a large number of granule cells, the spherical

* Corresponding author. Present address: Istituto di Chimica Biologica "G. Fornaini", Università di Urbino "Carlo Bo", Via A. Saffi, 2, 61029 Urbino (PU), Italy. Tel.: +39 0722 305288; fax: +39 0722 320188.
E-mail address: p.ninfali@uniurb.it (P. Ninfali).

0168-0102/$ – see front matter
doi:10.1016/j.neures.2004.11.002

cell bodies of which form densely packed clusters, separated by islands of neuropil, termed cerebellar glomeruli; more precisely, the cerebellar glomeruli are constituted by the connections between the small dendritic claw-like arborizations of granule cells, the mossy fibers' terminal rosette and rare Golgi cell axons.

Other cell types have been identified in the cerebellar cortex such as: Lugaro cells, fusiform interneurons localized just beneath the Purkinje cells in the granule cell layer (Laine and Axelrad, 1996); unipolar brush cells, which are excitatory interneurons localized in the granule cell layer, where their dendritic brush form the core of the cerebellar glomeruli (Diño et al., 2000); candelabrum cells, so called for their candelabrum-like axons, the cell bodies of which are located between the Purkinje cell somata (Laine and Axelrad, 1994).

Enzymatic activities of the cerebellar neurons have been the object of extensive research and several physiological and comparative considerations have been formulated (Bertoni-Freddari et al., 2001; Bronzetti et al., 1988; Guennoun et al., 1995; Lawrence et al., 1990; Moreno et al., 1995; Ukena et al., 1998; Voogd et al., 2003). Our attention has been focused on the hexose monophosphate shunt (HMS), and in particular on the role of glucose-6-phosphate dehydrogenase (G6PD). G6PD is a housekeeping enzyme, which transforms glucose-6-phosphate (G6P) into 6-phosphoglucono-δ-lactone (6PGD) with NADPH production. This enzyme is the first and rate-limiting step of the HMS, which provides pentose phosphates for nucleic acid synthesis and NADPH for reductive biosynthesis and detoxification reactions (Luzzatto, 1995). Moreover, G6PD is considered to have a pivotal role in antioxidant defence mechanisms (Pandolfi et al., 1995). This role is very important considering that the cerebellum, like other areas of the central nervous system (Reiter, 1995), is exposed to oxidative stress (Romero et al., 1991), mostly due to the high rate of O_2 metabolism and the high content of auto-oxidizable neurotransmitters (Baquer et al., 1988).

In cerebellum, G6PD activity was 55 ± 4 IU/g protein as measured by spectrophotometric assay, and histochemical analysis showed that G6PD activity was mainly localized in Purkinje cells (Biagiotti et al., 2001).

In this study, we examine the distribution of G6PD protein in cerebellar structures more in detail by means of immunohistochemical as well as enzyme histochemical methods. Moreover, we focus on the presence and distribution of some NADPH-dependent enzymes in order to establish a possible correlation between G6PD and these enzymes. For this purpose, we examined: NADPH-cytochrome P450 reductase (P450R), necessary to regenerate the reduced form of cytochrome P450 (Anzenbacher and Anzenbacherova, 2001; Tsutsui et al., 2000); glutathione reductase (GR), involved in maintenance of reduced glutathione (GSH) (Dringen, 2000); NADPH-diaphorase (NADPH-d), the physiological function of which is not well clarified, but it catalyzes the reduction of nitro-blue tetrazolium salt (NBT) to insoluble blue formazan in the presence of NADPH (Stoward et al., 1991). The relative concentrations of these enzymes in different cerebellar structures have been investigated through the densitometric analysis of staining intensities. To verify possible colocalization of G6PD and P450R or GR, double labeling of the enzymes was performed followed by confocal laser scanning microscopy.

2. Materials and methods

2.1. Animals and histological procedures

Five-month-old male Sprague–Dawley rats were used for this study. All animal use was conducted in accordance with the European Union guidelines and Italian laws.

The rats were anaesthetized with sodium thiopental via i.p. (45 mg/kg body weight). For G6PD histochemistry, the rats ($n = 3$) were killed immediately after anaesthesia with an intracardiac injection of the same anaesthetic, the cerebella were removed and immediately frozen in liquid nitrogen and stored at −80 °C. For G6PD, P450R and GR immunohistochemistry, the animals ($n = 3$, for each enzyme) were anaesthetized and killed with an intracardiac injection of the same anaesthetic. The cerebella were then removed, fixed with Carnoy for 48 h and embedded in Paraplast Plus paraffin (Sigma; melting point = 56–58 °C). For NADPH-d histochemistry, the rats ($n = 3$) were anaesthetized as described above and killed by an intracardial perfusion with normal saline followed by 4% paraformaldehyde and 15% saturated picric acid solution in phosphate buffer saline (PBS; 0.01 M, pH 7.4). Cerebella were immediately excised, postfixed in the same fixative for 3 h, cryoprotected in 30% sucrose overnight and frozen in liquid nitrogen. The samples were stored at −80 °C.

All specimens were cut both in coronal and in sagittal planes.

In order to investigate a possible colocalization of G6PD with other enzymes, some paraffin-embedded sections were also utilized for double labeling immunofluorescence, followed by confocal microscopic analysis.

2.2. G6PD histochemistry

The samples were cut into 8 μm thick sections with a cryostat at −25 °C. Sections were incubated with the reaction mixture, containing 10 mM G6P, 0.8 mM NADP, 4 mM $MgCl_2$, 5 mM NaN_3, 0.45 mM 1-methoxyphenazine methosulfate (m-PMS) and 5 mM nitro-blue tetrazolium (NBT) dissolved in 18% polyvinyl alcohol (PVA, average MW 70,000–100,000, Sigma) prepared with phosphate buffer Na/K (0.1 M, pH 7.0), for 10 min at 37 °C. Afterwards, sections were rinsed with phosphate buffer Na/K (0.1 M, pH 5.3) at 60 °C and mounted with glycerol-