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Developmental pattern of calbindin D28k protein expression in the rat striatum and cerebral cortex

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We examined the protein expression of the calcium-binding protein calbindin D28k in two developing rat brain structures, the striatum and the cerebral cortex. The relative protein concentration level was quantified by means of the Western blotting method and densitometric scanning. 32 Wistar rats were used, divided according to survival period (P0–P120-postnatal days). Observations of the calbindin D28k protein expression in the rat striatum and the cerebral cortex revealed an increase in band color intensity between P0 and P10. The intensity of protein staining in older groups of animals stabilised at a similar level and in the P28 and P120 groups we observed a decrement of calbindin expression in the striatum.

Calbindin D28k stabilises the intracellular calcium level, preventing calcium-induced apoptotic cell death in neurons. Thus, changes in calbindin D28k expression might be related to its neuroprotective role in differentiation and synaptogenesis during the postnatal development of the brain.

key words: calbindin D28k, protein expression, Western blotting, development, cerebral cortex, striatum, rat

INTRODUCTION

Neurons are able to reduce Ca^{2+} accumulation via the three following mechanisms: the plasma membrane transport system, interneuronal sequestration by organelles like the endoplasmic reticulum and calcium binding proteins (CBP) such as calmodulin, parvalbumin and calbindin D28k [6].

Calbindin D28k is a major, vitamin D-dependent, calcium binding protein in the brain which is present predominantly in the cytosol and constitutes between 0.1–1.5% of the total soluble proteins in the brain [5]. The most obvious function of calbindin D28k is related to their relatively high affinity for Ca^{2+} binding, which can be important in the regulation of many neuronal processes from excitability to neuroprotection [1]. A number of previous studies have indicated direct involvement of calbindin in protecting against calcium-induced apoptotic cell death by inhibiting the adverse effects of sustained intracellular calcium on mitochondrial electron transport and on apoptotic mitochondrial alterations [10].

The appearance of apoptotic cell death in the brain areas has a role in the development of the nervous system while and calbindin D28k plays an important role in the resistance of neurons to excitotoxic damage. This protein may thus be particularly important for immature neurons because they have been shown to be especially susceptible to calcium influx [8].

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The present study, therefore, was designed to investigate differential changes in calbindin D28k expression in two developing rat brain areas, the striatum and the cerebral cortex, at the protein concentration level by means of the Western blotting method.

MATERIAL AND METHODS

The material consisted of 32 Wistar rats divided according to survival period (P0, P2, P7, P10, P14, P21, P28 and P120-postnatal days). Care and treatment of the animals were in accordance with the guidelines for laboratory animals established by the National Institute of Health as well as by the local ethical committee. All the animals were deeply anaesthetised with lethal doses of Nembutal and decapitated. The brains were removed and the samples of the striatum and cerebral cortex were rapidly microdissected under surgical microscope control.

The tissues were homogenised with buffer including protease inhibitors and an evaluation of protein concentration was carried out using colorimetric Lowry assay.

Samples containing equivalent amounts of proteins were electrophoretically separated and then transferred onto nitrocellulose membrane by semi-dry blotting. The reliability of sample loading and protein transfer was evaluated by staining nitrocellulose membrane with Ponceau S solution before immunoblotting. Non-specific binding sites on the membrane were blocked in non-fat dry milk. The membrane was incubated overnight with primary monoclonal antibody (mouse anti-calbindin-D28k, Sigma, USA) diluted 1:2000. After primary incubation, blots were incubated with biotinylated goat anti-mouse IgG (Jackson ImmunoResearch, USA) diluted 1:10000. Following incubation with alkaline phosphates, a streptavidin complex, the color development reagents (BioRad, UK) were used for visualisation. The results were documented and the relative amount of proteins in each sample was quantified by densitometric scanning (Laser Pix, Biorad, UK).

In this investigation β-actin was used as a reference protein. The antibodies used for the identification and visualisation of β-actin were as follows: mouse monoclonal anti-β-actin (Sigma, USA) diluted 1:5000 as primary IgG and biotinylated goat anti-mouse IgG (Jackson Immuno Research Laboratories, USA) diluted 1:10000 as the secondary antibody. The remaining part of the examination procedure was carried out with the same protocol under the same conditions.

RESULTS

Observations of calbindin protein expression in the rat striatum during postnatal period (P0–P120) revealed an increase in band color intensity between the P0 and P10 groups. After the increment period, the intensity of protein staining in older groups of animals (P14, P21) stabilised at a similar level (Fig. 1). However, the maximum level of relative protein concentration in the striatum was recorded in the P21 group. We observed a decrement of calbindin expression in the P28 and P120 groups.

On studying the band color intensity occurring in the cerebral cortex, we also observed an increase between the P0 and P10 groups. After the 10th day of postnatal life, the relative amount of calbindin D28k protein calculated in older groups of animals (P14-P120) stabilised (Fig. 2). However, the maximum intensity of protein staining in the cerebral cortex was recorded in the P21 group.

To summarise, the results of this investigation showed that a period of rising calbindin D28k protein expression is observeable both in the striatum and the cerebral cortex until the 10th day of postnatal life. In both these brain areas we recorded no differences in the relative protein concentration levels of the P14 and P21 groups and the intensity of protein staining in these groups stabilised at a similar level. Observation of calbindin D28k protein ex-

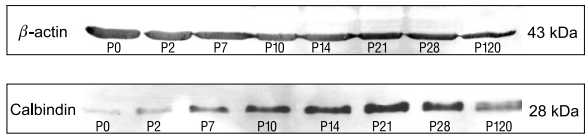

Figure 1. Age-related comparative analysis of calbidin D28k expression in the striatum. Upper panel: Western blots of β-actin carried out in 8 groups (P0–P120 days of postnatal life). Bottom panels: Western blots of calbindin at different ages. Equal amounts of proteins were used both in β-actin and calbindin detection.

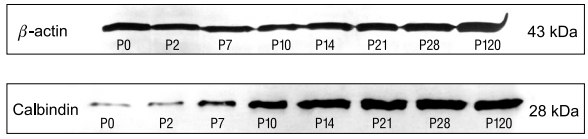

Figure 2. Age-related comparative analysis calbidin D28k expression in the cerebral cortex. Upper panel: Western blots of β-actin carried out in 8 groups (P0–P120 days of postnatal life). Bottom panels: Western blots of calbindin at different ages. Equal amounts of proteins were used both in β-actin and calbindin detection.

pression in the P28 and P120 groups revealed differences between the striatum and the cerebral cortex. In the striatum we recorded a decrement in band color intensity in contrast to the cerebral cortex where we observed no change in relative protein expression after the 10th day of postnatal life.

DISCUSSION

The connections between the *globus pallidus*, *substantia nigra*, lateral complex of the thalamus and the striatum are formed mainly during prenatal life and the appearance of apoptotic cell death is observed in these structures during the first postnatal days. In the development of the cerebral cortex and subcortical white matter in the rat the establishment of the final cell population takes place between P3–P7 and is also associated with apoptotic death of neurons [7, 11]. Apoptosis in these areas during the first postnatal week may be a consequence of the elimination of neurons that failed to make appropriate connections [7].

Many studies directly implicate calbindin D28k in protection against calcium-induced apoptotic cell death. A dramatic rise in free cytosolic calcium causes excitotoxin — mediated neurodegeneration. The effects of cytosolic calcium accumulation include activation of proteases, phospholipases and endonucleases, generation of reactive oxygen species, inhibition of protein synthesis and derangement of the cytoskeleton [4].

On the other hand neurons use calcium as a second messenger to integrate numerous cellular pathways. These include neuronal development and maturation, gene expression, synaptic plasticity, transmitter release, excitability and cell death [3]. It has, therefore, been suggested that an optimal calcium level is needed for the functioning of neurons and astrocytes and that either an elevation or marked reduction in calcium can result in degeneration.

Previous investigations have revealed that the calcium increases are remarkably enhanced from the end of the second to the beginning of the third postnatal week [2, 9], which may be confirmation of our results. We proved that calbindin D28k expression rises in the striatum and cerebral cortex area during first week of postnatal life in groups P0 to P10. After this increase, a stabilisation in calbindin protein concentration both in the striatum and cerebral cortex is observed. These data suggest that the calbindin participate in the early postnatal maturation both in the striatum and the cerebral cortex. The simultaneous appearance of calbindin in comparable concentration in these areas may indicate the involvement of this protein in the maturation of corticostriatal connections and the differential pattern of calbindin expression during first weeks of postnatal life is probably the result of age-dependent modifications in calcium buffering.

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