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Differential expression of calbindin D28k, calretinin and parvalbumin in the cerebellum of pups of ethanol-treated female rats

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Abstract

Three calcium-binding proteins (CaBPs), calbindin D28k, calretinin and parvalbumin, were immunohistochemically examined in the cerebellum of ten-day-old rat pups of ethanol-treated dams. Dams were treated with ethanol during pregnancy and/or lactation. In the cerebellar cortex of the pups from control groups, Purkinje cells with their processes and Golgi cells were positive for calbindin D28k, whereas interneurons (Lugaro, Golgi and unipolar brush cells) and sometimes Purkinje cells were positive for calretinin. Parvalbumin immunoreactivity was observed in Golgi and basket cells, stellate cells and in some Purkinje cells. The number of positive cells and staining intensity for calbindin D28k and parvalbumin decreased in all experimental groups, whereas the immunoreaction for calretinin was visible only in interneurons and was more intense in experimental than in control groups.

Calbindin D28k immunoreactivity in experimental groups was detected in some Purkinje cells and rarely in Golgi cells. The localization of very intense calretinin expression was visible mainly in unipolar brush cells. A parvalbumin-positive reaction was detected in single Purkinje cells and sometimes in basket cells. The results of the present study showed that immunoreactivity of the three calcium-binding proteins was found in the cells of the cerebellum of the ten-day-old pups from the control groups. In experimental groups of females treated with ethanol during pregnancy and/or lactation, we observed the most significant decrease in both the intensity and the number of immunoreactions of calbindin D28k and parvalbumin, but the intensity of the immunoreaction for calretinin was increased for interneurons. Ischaemic damage to Purkinje cells and loss of interneurons and Purkinje cells were also noted in these groups.

A possible correlation between the duration of ethanol intoxication, expression of calcium-binding proteins and pathological changes of cells in the cerebellar cortex of the pups of ethanol-treated dams is discussed.

Key words: *calbindin D28k, calretinin, parvalbumin, cerebellum, ethanol, rat pups.*

Introduction

Calcium (Ca) is a unique ion that is involved in a large number of vital physiological processes and neuronal functions [3,5,13,39,47]. Ca regulates the liberation and synthesis of neurotransmitters, hormones, axonal transport, control of enzymatic reactions, gene transcription and other processes throughout life, including the developmental period [6,42]. During deve-

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lopment, Ca controls neuronal migration, process extension, formation of neuronal networks, synaptogenesis, cell division and also the process of apoptosis [4,7,20]. A lot of intracellular calcium-responsive proteins are required to control the intraneuronal level of Ca^{2+}. These proteins are members of the so-called "EF-hand" family, also termed calcium-binding proteins (CaBPs) [26,33,42,56]. The EF-hand family of calcium-binding proteins is widely distributed in the brain. Among other well-known members of CaBPs are calbindin-D28k (CB), calretinin (CR) and parvalbumin (PV). Calbindin D28k takes several forms but only the 28 kDa molecule occurs in the neural population. CR molecular weight in the brain is about 29 kDa. PV, a small protein with molecular weight of about 12 kDa, occurs in the brain [6,49]. The distribution of these proteins in developing brain is not comparable to that observed in the adult brain, and their immunoreactivity changes during the developmental process [1,2,56].

Cerebellar development takes an exceptionally long time during the embryonic phase and the first postnatal period [36]. The cerebellum, particularly the cerebellar cortex, with the whole population of its neurons and processes, performs very important motor and autonomic functions, and participates in cognitive and emotional development [12,17,24, 29,48]. The cerebellar cortex contains Purkinje, basket, stellate, Golgi, Lugaro, unipolar brush and granule cells, which have characteristic morphological features [16,29]. The population of these cerebellar neurons demonstrates immunohistochemical localization of three calcium-binding proteins (CB, CR, PV) [44,56]. The intensity of immunoreaction and the number of positive neurons for these proteins are related to the intracellular calcium concentration [2,10,56]. Many pathological factors may change the intracellular Ca concentration and thus damage CaBPs expression.

Ethanol exposure during brain development, intrauterine exposure and/or in the early postnatal period, causes significant neuronal damage to the central nervous system (CNS) [11,15,32,34,55]. Consumption of ethanol by mothers during pregnancy can lead (in humans) to fetal alcohol syndrome (FAS), or less specific fetal alcohol effects (FAE) and fetal alcohol disorders (FAD) in their offspring. These are characterized by specific facial deformation, growth retardation and CNS dysfunction. Experimental animal studies have demonstrated that the cerebellum is among the brain target sites of ethanol CNS toxigenesis [8,23,25,34]. Neuropathological studies indicate that structural and functional deficits of the cerebellum are dependent on ethanol exposure during the developmental period [23,32,34,37]. The pathogenesis of this differential vulnerability has not yet been established. In this study we report on the cerebellar cortex localization of three major calcium-binding proteins (CB, CR, PV) in ten-day-old rat pups of ethanol-treated dams. The evidenced correlation between the duration of ethanol intoxication, the expression of calcium-binding proteins and pathological changes of neurons during the developmental process of rat cerebellar cortex may help us understand the pathogenesis of ethanol teratogenesis.

Material and methods

The investigations were carried out on cerebellum collected from the ten-day-old pup offspring of outbred Wistar female rats. Females were treated with ethanol (12%, 6 g/kg body mass) during pregnancy and/or lactation. The control groups were composed of dams treated with isocaloric dextran solution with maltose, during pregnancy and/or lactation, and also untreated female rats (Table I). Solutions were administered via intragastric intubation once a day.

The animals were fed standard chow and received tap water *ad libitum*. To assess the cerebellum by light microscope, it was fixed in 4% buffered paraformaldehyde (pH 7.4) then embedded in paraffin, cut serially into 8 µm sections. Every tenth slide was stained with haematoxylin and eosin, and selected preparations were subjected to immunohistochemical reactions.

The specimens of cerebellum were stained immunohistochemically with the antibodies calbindin D-28k (Sigma, clone CB-955) at dilution of 1 : 2500, calretinin (Dako, clone DAK calret 1) at dilution 1 : 75 and parvalbumin (Sigma, clone Parv-19) at dilution 1 : 2000.

Results

The distribution of calcium-binding proteins in the cerebellar cortex in ten-day-old rat pups of control groups (ID, IID, IIID, C) and in ethanol-treated dams of three experimental groups (IA, IIA, IIIA) is summarized in Table I. The number of immunoreactive neurons and the staining intensity for CaBPs (CA, CR, PV) in control groups (ID, IID, IIID, C) were the same.

Table I. Characteristics of the studied groups

Group	Solution administered	Duration of treatment	Number of cases
	Experimental groups		
IA	ethanol	pregnancy and lactation	18
IIA	ethanol	pregnancy	18
IIIA	ethanol	lactation (days)	18
	Control groups		
ID	dextran with maltose	pregnancy and lactation	9
IID	dextran with maltose	pregnancy	9
IIID	dextran with maltose	lactation (10 days)	9
C	not treated	–	3

Calbindin D28k-positive cells in the cerebellar cortex in ten-day-old rats in the control groups were all Purkinje cells with their processes and Golgi cells (Figs. 1, 1A). The staining intensity for CB proteins was very impressive. In the pups of dams treated with ethanol during pregnancy and lactation (group IA), CB immunoreactivity was observed only in a few Purkinje cells, but in Golgi cells it was not visible (Figs. 2, 2A). In dams treated with ethanol during pregnancy (group IIA), positive Purkinje cells were occasionally observed and there was no immunoreactivity in Golgi cells (Fig. 3). In the cerebellar cortex in pups of dams

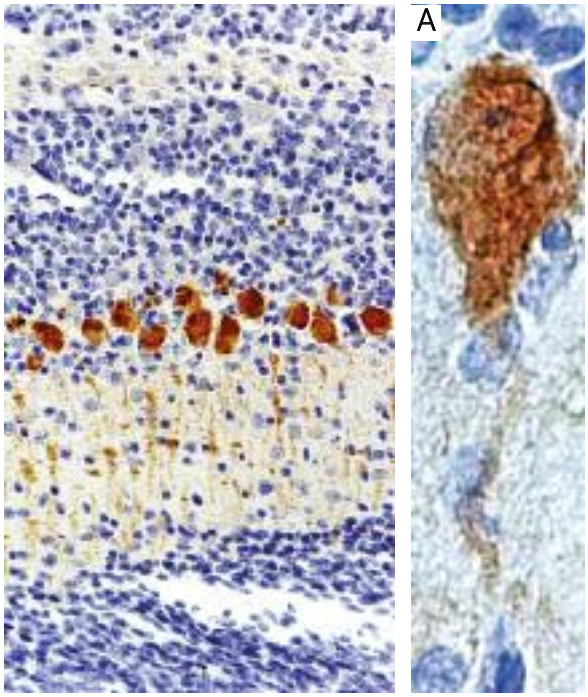

Fig. 1. Control group. Calbindin D28k immunohistochemistry in the section of a ten-day-old rat pup's cerebellar cortex. × 200. **A)** Positive Purkinje cell with process. × 630.

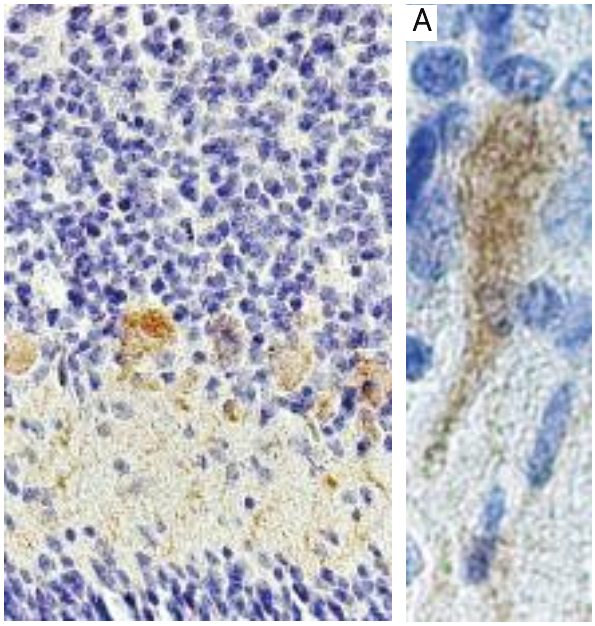

Fig. 2. Experimental group IA. Calbindin D28k with weak immunoreaction observed in a few Purkinje cells. × 200. **A)** Immunostaining in Purkinje cell with process. Hypoxic changes in Purkinje cell. × 630.

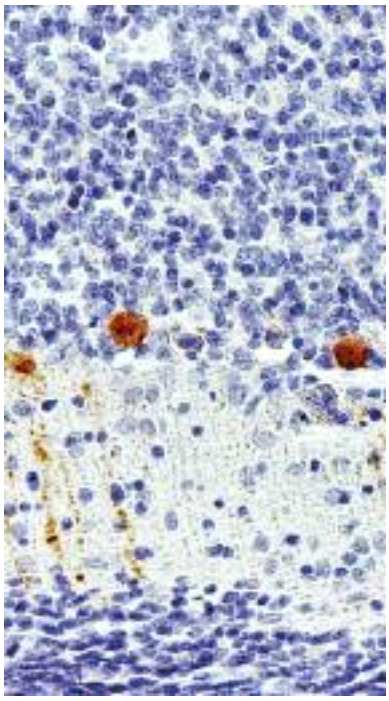

Fig. 3. Experimental group IIA. Calbindin D28k-positive immunoreaction observed in several Purkinje cells and their processes. × 200.

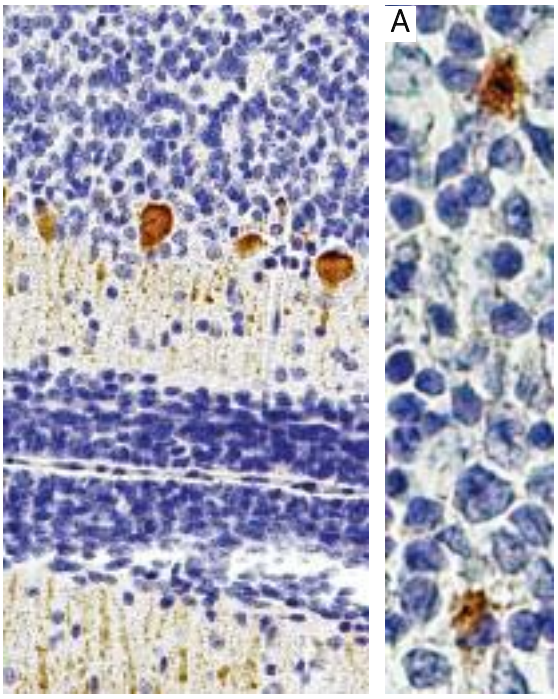

Fig. 4. Experimental group IIIA. Calbindin D28k-positive immunoreaction noted in Purkinje cells and their processes. × 200. **A)** Positive reaction in Golgi cells. × 400.

treated with ethanol during lactation (group IIIA), expression of CB proteins in Purkinje and Golgi cells was slightly visible (Figs. 4, 4A).

Calretinin-positive cells in the cerebellar cortex of pups in the control groups were interneurons – Lugaro, Golgi, unipolar brush cells – and some weak Purkinje cells (Figs. 5A-C). In the experimental group IA, the localization of CR expression was visible only in interneurons (Fig. 6). All unipolar brush cells showed very positive immunoreactivity (Figs. 6, 6A). Calretinin-positive interneurons showed lower intensity in the remaining two groups (IIA, IIIA) (Figs. 7, 8). Positive Purkinje cells were found in experimental group IIIA (Fig. 8).

Parvalbumin-positive neurons were found in the cerebellar cortex in the control group in some Purkinje cells and in Golgi, basket and stellate cells (Figs. 9A-C). In the IIA and IIIA experimental groups, a PA-positive reaction was visible in a few Purkinje cells and sometimes also in basket cells. (Figs. 10, 11). The slightest immunoreaction in interneurons and no immunoreaction in Purkinje cells was observed in group IA (dams treated with ethanol during pregnancy and lactation) (Fig. 12).

In all the experimental groups (IA, IIA, IIIA), a fall in the number of Purkinje cells and their hypoxic/ischaemic damage and loss of interneurons were also noted (Figs. 2, 7, 8, 10-12).

Discussion

The expression of calbindin D28k, calretinin and parvalbumin, three important calcium-binding proteins, observed in distinct neuronal populations, was examined in ten-day-old neonatal rat pups of both control and experimental groups. Immunoreactivity for these CaBPs has been detected in similar populations of neurons in mammals (including humans) and rodents or birds [18,22,30,53,54]. The distribution of three CaBPs in the rat cerebellar cortex including Purkinje cells, granule cells and interneurons, as well as their processes, has been documented previously [21,46,56]. Our study also showed the distribution of calbindin D28k immunoreactivity in the cerebellar cortex of control pups virtually in all Purkinje cells with their processes and in Golgi cells. All CB immunoreactive Purkinje cells have a regular surface. Lugaro, Golgi, and unipolar brush cells and some Purkinje cells were positive for calretinin. The majori-

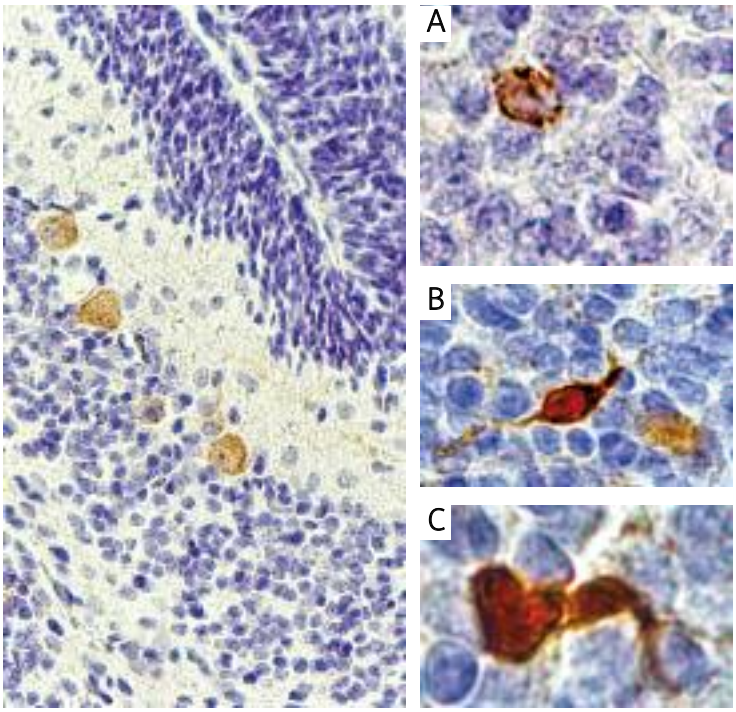

Fig. 5. Control group. Calretinin immunohistochemistry in the section of a ten-day-old rat pup's cerebellar cortex. × 200. **A)** Positive Golgi cell. × 400. **B)** Positive Lugaro cell. × 400. **C)** Positive unipolar brush cell. × 400.

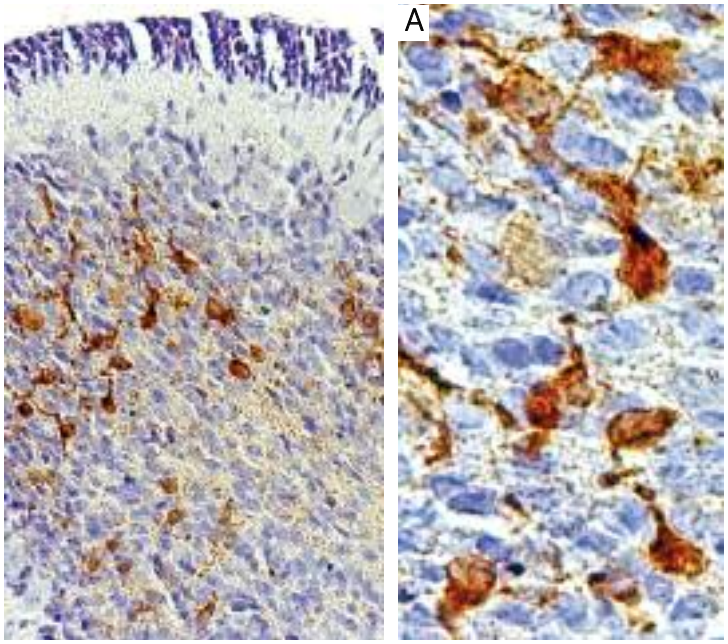

Fig. 6. Experimental group IA. Very intense calretinin immunoreaction observed in interneurons. × 200. **A)** Unipolar brush cells showed very intense reaction. × 400.

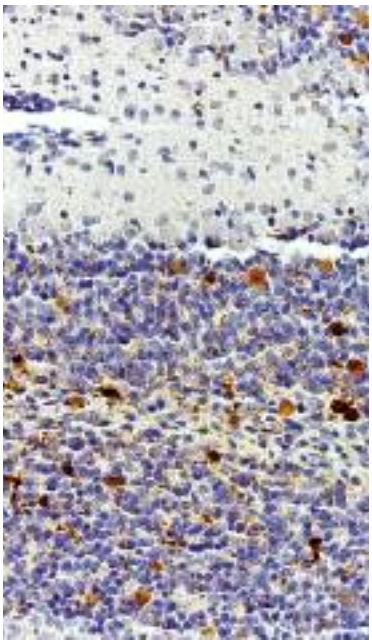

Fig. 7. Experimental group IIA. Intense calretinin reaction observed in interneurons. Purkinje cell deficiency. × 100.

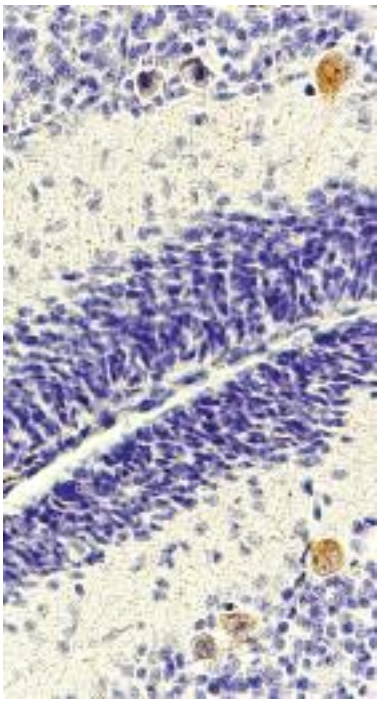

Fig. 8. Experimental group IIIA. Calretinin immunoreaction observed in a few Purkinje cells. Decrease in the number and ischaemic damage were observed in Purkinje cells. × 200.

ty of Purkinje cells showed a strong PV reactivity. In addition, a PV-positive reaction was observed in Golgi, basket and stellate cells.

The localization of expression of CaBPs in the cerebellum of ten-day-old pups of ethanol-treated dams underwent changes. The ethanol-induced cerebellar degeneration not only in adults but also in children whose mothers have been drinking during pregnancy and lactation has been described previously [15,31,38,41,55]. Unfortunately, ethanol-induced cerebellar degeneration causes neuropsychological deficits observed in children with FAS, FAE and FAD [9,32,40]. A lot of mechanisms underlying ethanol-induced cellular degeneration and functional alterations have been documented. Hoffman *et al.* (1995) suggested that excitotoxicity (excessive stimulation of neurotransmitters) is an important mechanism contributing to ethanol-induced neuropathology in the cerebellum [28]. Pathophysiological mechanisms such as glial abnormalities, increase in pro-apoptosis gene expression, decreased mitochondrial enzyme activities, change in growth factors, oxidative stress and many others, cause cerebellar degeneration with cognitive disorders observed in offspring whose mothers have been drinking ethanol [19,27,43,50,51].

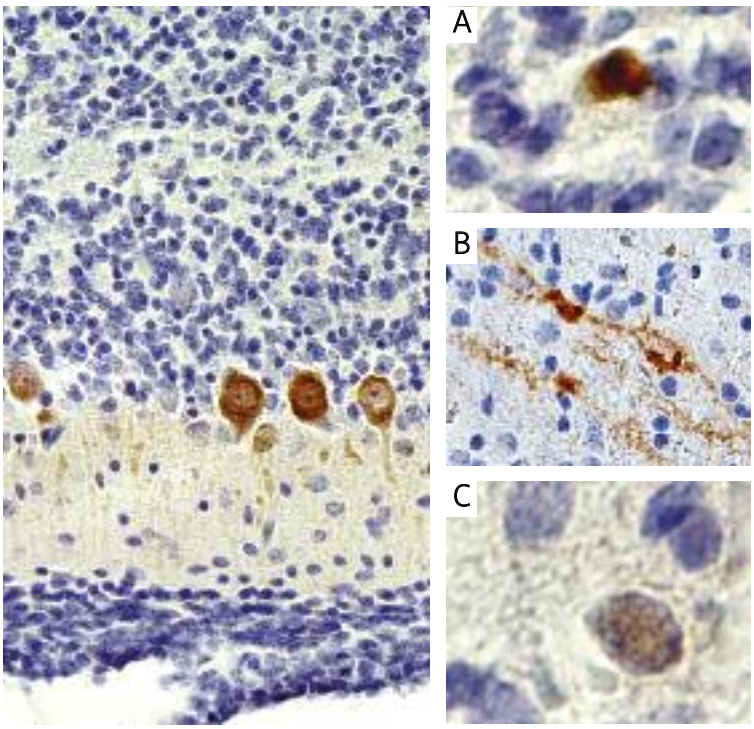

Fig. 9. Control group. Parvalbumin immunohistochemistry in a section of ten-day-old pups' cerebellar cortex. × 200. **A)** Positive Golgi cell. × 400. **B)** Positive stellate cells. × 400. **C)** Positive basket cell. × 400.

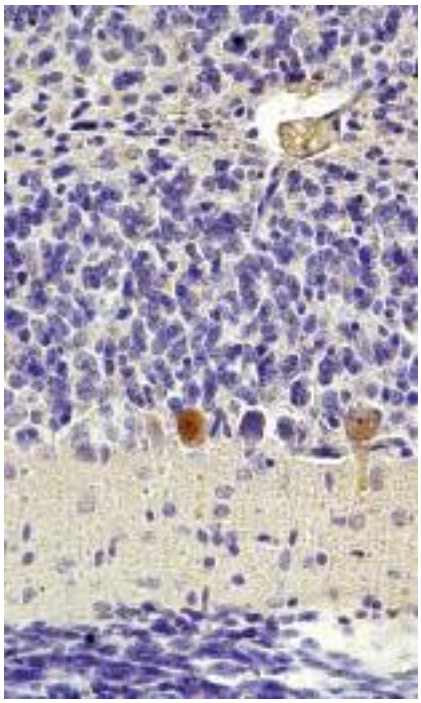

Fig. 10. Experimental group IIA. Parvalbumin-positive reaction in several Purkinje cells was observed. Hypoxic changes in Purkinje cells were noted. × 200.

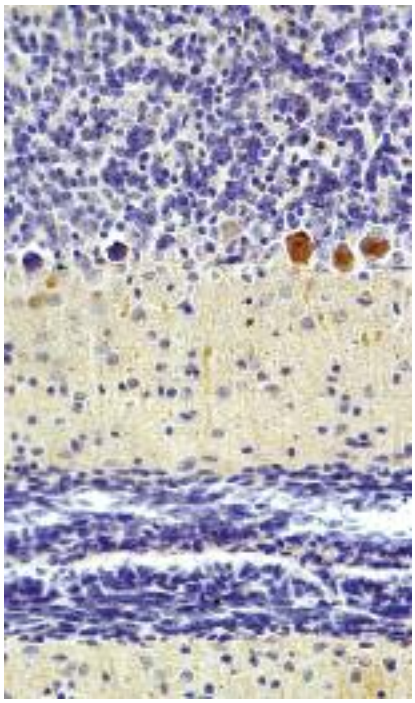

Fig. 11. Experimental group IIIA. Parvalbumin-positive reaction visible in some Purkinje cells. Hypoxic changes were observed in Purkinje cells. × 200.

Earlier experimental studies demonstrated that Purkinje and granule cells and interneurons of the cerebellar cortex are the neurons vulnerable to ethanol exposure [31,40,52]. The specific distribution pattern of calcium-binding proteins in the cerebellar cortex suggests that they may be involved in cell activities, and the alteration of their immunohistological presentation may lead to neurodegenerative conditions. In our study, calbindin D28k-positive immunoreaction was not observed in Golgi cells and only sometimes in Purkinje cells in the experimental IA and IIA groups. The staining intensity of CB decreased in all experimental groups. CB-expressing neurons are resistant to migration, differentiation and excitotoxic insults; thus the decrease in CB reaction may cause developmental and functional disturbances [7,36,56].

Parvalbumin immunoreactivity was not observed in Purkinje, basket and stellate cells in the experimental IA group. In two other experimental groups (IIA and IIIA), PV-positive weaker staining intensity reaction was visible only in a few Purkinje and basket cells. As has been postulated, PV regulates the liberation and synthesis of the neurotransmitter γ-aminobutyric acid (GABA), and in the after-hyperpolariza-

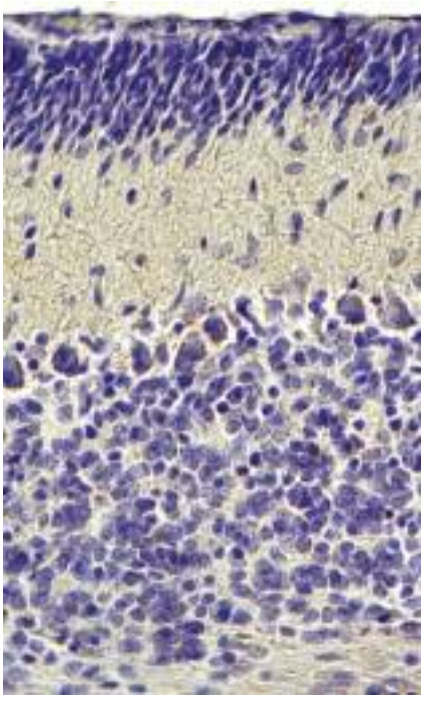

Fig. 12. Experimental group IA. Parvalbumin-positive reaction was not detected in Purkinje cells. Hypoxic/ischaemic change in Purkinje cells. × 200.

tion period the lack of or decrease in PV immunoreactivity may suggest the alteration of cerebellar function in these mechanisms [1,7,42].

Only the calretinin immunoreaction was more intense in all interneurons, above all, in unipolar brush cells in the experimental groups as compared with the control groups. The highest intensity of CR was visible in group IA. No immunoreactivity of CR in Purkinje cells was observed. Excessively high CR immunoreactivity in interneurons may cause functional alteration and death of neurons in mechanisms of excessive activity of neuronal metabolism [1,14].

In our experimental groups, the loss of or ischaemic changes in Purkinje cells and loss of interneurons in the cerebellar cortex suggest that the disturbed immunoreactivity of CaBPs after ethanol intoxication may induce neuronal death by necrosis after a period of ischaemia or apoptosis [35,57]. Disturbances of CaBPs are able to prevent the neuronal death by more than one mechanism, as well as mitochondrial dysfunction [35]. CB probably inhibits the activity of caspase-3, while the increase of CR may indicate mitochondrial calcium accumulation with enhanced production of reactive oxygen species (ROS), whereas PV may provoke the decrease in mitochondrial membrane potential [45].

Therefore, too high or too low level of intracellular calcium causes both neuronal and functional disturbances.

The results of our present study showed that ethanol intoxication during pregnancy and/or lactation causes alteration in the immunoreaction of calcium-binding proteins (CB, CR, PV) in neurons of the cerebellar cortex of ten-day-old rat pups. Changes of immunoreactivity of these CaBPs may indicate an important role in calcium-dependent ethanol-induced developmental and functional alterations.

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