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HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY OF MORPHOFUNCTIONAL STATE OF BRAIN AND BONE MARROW CELL STRUCTURES OF RATS UNDER STRESS

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Abstract

The aim of the present histochemical and immunohistochemical study was to investigate the morphofunctional states of bone marrow (BM) structures of intact and injected with hypothalamic proline rich polypeptide (PRP-1, also known as Galarmin) rats, and rats exposed to immobilization stress, as well as bilateral electro-stimulation of the hypothalamic paraventricular nucleus.

According to our morphohistochemical investigation with the method of acid phosphatase activity detection, the sinusoidal capillaries in the intact rat BM structures appear to be empty in general. On the outer side of the sinusoids, the densely stained small-sized cells are demonstrated, long and thick processes of which spread on the sinusoid wall. These fibers, perhaps, belong to BM stromal reticular cells. In BM stroma, single blood-formed elements are present and, notably, a group of cells with the short cytoplasmic extensions is revealed.

Analysis of data of all experimental rats exposed to stress and injected with PRP-1 demonstrated the hematopoiesis processes in BM; namely: in BM stroma of immobilized rats numerous round-shape or fusiform small cells resembling the mesenchimal cells with the short axon-like extensions were revealed.

Using the antiserum against PRP-1, in the immunohistochemical studies in rats under different stresses, as well as after PRP-1 administration, an increased number of PRP-immunoreactive (PRP-1-Ir) blood-formed cells is evident in BM stroma and sinusoidal capillaries. Among these cells being in different proliferation stages, the increase of single PRP-1-immunoreactive (PRP-1-Ir) varicose nerve fibers and islands of immune system PRP-1-Ir cells inside and around the sinusoids were also found.

The presence of PRP-1 in the immune system cells and nerve fibers in close contact to immune system cells suggests its synthesis and release from the hypothalamic nuclei as a result of stress stimuli of the latter and the involvement of PRP-1 in the mechanism of the hematopoiesis process. However, the synthesis of these substances in immune system cells is not excluded, as there is, for instance, an evidence of distinct neuropeptides biosynthesis in lymphocytes. To confirm this suggestion, in situ hybridization histological method and flow cytofluorimetric analysis should be performed.

In a separate series of experiments with the use of antisera against the glial fibrillary acidic protein (GFAP), neuroepithelial stem cell marker nestin, and PRP-1, we studied brain cell structures of rats subjected to prenatal immobilization stress.

The results revealed multiple nestin-Ir round cells in small arteries and cells of different sizes and shapes with very short processes and negative ectopic nuclea in the studied brain regions. Strong increase in number of GFAP-Ir astrocytes in the white matter of spinal cord and brain was notable. The nestin-Ir cell structures under study were also positive to GFAP.

Thus, adult stem cells plasticity in response to the immobilization stress is assumed in some of the studied brain regions. However, whether these cells are bone marrow-derived stem cells circulating in the blood is the question to be solved.

Keywords: rats, immobilization (IMO) stress, electrical stimulation of hypothalamic PVN, brain plasticity, proline-rich-polypeptide-1 (PRP-1), PRP-1 treatment, glial fibrillar acidic protein (GFAP), neuroepithelial stem cell marker – nestin.

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INTRODUCTION

The protection of neurons from damage and death in case of neurodegenerative disorders, such as Alzheimer disease (AD), ischemic insults, Parkinson disease (PD), is a major challenge for neuroscientists in the 21st century.

In the biochemical, immunological, and physiological studies, proline-rich peptide-1 (PRP-1), a fragment of neurophysin vasopressin associated glycoprotein isolated from bovine neurohypophysis neurosecretory granules [*Galoyan A., 1997*], has been suggested to play the role of a universal neuroprotector and neuromodulator [*Galoyan A. et al., 2000; 2001; Galoyan A., 2001*]. Our previous report on the effects of PRP-1 on SC injured rats indicated the possibility of PRP-1 involvement in the mechanisms of neuronal repair [*Abrahamyan S. et al., 2001*]. The immunohistochemical assay demonstrated that treatment with PRP-1 resulted in recovery and growth of nerve fibers, glia proliferation, and motoneuron survival. Hence, PRP-1 has been found to be a highly active neurotrophic-like substance. PRP-1-immunoreactivity (PRP-1-IR) was noticed in spinal glial cells both in the white matter and among the PRP-1-Ir motoneurons (MNs) in the spinal cord (SC) injured rats [*Abrahamyan S. et al., 2004*]. The presence of PRP-1 in glial cells was explained by us to be more likely due to its uptake from the circulating blood.

For many years, researchers have been seeking to understand the body's ability to repair and replace the cells and tissues of some organs, but not others. Scientists have now focused their attention on adult stem cells. It has long been known that stem cells are capable of renewing themselves and generating multiple cell types. Today, there is new evidence that stem cells are present in far more tissues and organs than once thought.

The primary functions of stem cells are to maintain the steady state functioning of a cell, called *homeostasis*, and, with limitations, to replace cells that die because of injury or disease [*Leblond C., 1964; Holtzer H., 1978*]. The list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas.

Adult stem cells behave very differently depending on their local environment. For example, hematopoietic stem cells (HSCs, blood forming cells) are constantly being generated in the bone marrow [*Domen J.,Weissman I., 1999*], where they differentiate into mature types of blood cells.

At present, some evidence indicates that neural stem/progenitor cells (NPCs) from non-neurogenic regions in the adult brain give rise to neurons mediated by the local astrocyte populations especially after injury. Adult brain cell regeneration, also known as *neurogenesis,* has been demonstrated in many species, including rodents. Adult neurogenesis is the process of generating new neurons. Bone marrow-derived stem cells are tissue-specific stem cells that are capable of self-renewal and can differentiate into cells of different tissues, including mature lineages of blood cells, neural cells both *in vitro* and *in vivo*, stromal and skeletal tissue [*Grove J. et al., 2004*].

The purpose of the present histochemical and immunohistochemical study was to examine the stress-induced response of bone marrow (BM) structures in intact and injected with hypothalamic PRP-1 rats and rats exposed to immobilization stress, as well as Bilateral electrostimulation of the hypothalamic paraventricular nucleus (PVN).

MATERIAL AND METHODS

White laboratory male rats (200-250 *g* body weight) were divided into five groups: 1) intact (n=6); 2) PRP-1-injected (n=9); 3) rats exposed to the immobilization stress (n=3); 4) rats exposed to the bilateral electrostimulation of the hypothalamic paraventricular nucleus (n=3) and 5) rats (80-100 *g* body weight) subjected to prenatal immobilization stress.

Bilateral electrostimulation with rectangular current (0.05 *ms* phase, 0.12-0.16 *mA* amplitude, 100 *Hz* frequency) was done for 1 second, 3 times with 5 second intervals, with constantan electrode by means of the steriotaxic coordinates of the hypothalamic paraventricular nucleus, lateral magnocellular part (PaLM) AP–1.8 L±0.6 DV+7.8 *mm* [*Paxinos G., Watson C., 2005*]. The sham-operated animals underwent same manipulations with implantation of the stimulating electrodes, however, without any electrical stimulation.

Following the anaesthesia with Nembutal (40-50 *mg/kg*), both the intact and experimental rats were decapitated:

- in group 2: 5 hours after the single intramuscular (*i/m*) PRP-1 injection (10 μg/100 *g*),
- in group 3: 2 hours after 2-hour immobilization stress,
- in group 4: 30 *min* after the stimulation, and
- in group 5: 45 days after the prenatal stress.

Avidin-Biotin-Complex (ABC) immunohistochemical technique [*Hsu S. et al., 1981*] and histochemical method of determining Ca^{2+}-dependent acid phosphatase activity [*Meliksetyan I., 2007*] were applied to study brain and bone marrow tissues. The tissues under study were removed and fixed in 4% paraformaldehyde prepared in 0.1M phosphate buffer (pH=7.4) during 48 hours at 4°C. Then, the tissues were cryoprotected for 24 hours in 0.1M phosphate buffered saline (PBS) containing 30% sucrose.

Histochemistry: After rinsing with distilled water, 40-50 *μm* free-floating frozen cross sections were used for histochemistry. The incubate mixture was: 20 *mL* of 0.4% lead acetate solution, 5 *mL* 1 M acetate buffer, pH=5.6, 5 *mL* 2% sodium β-glycerophosphate. This solution was brought to 100 *mL* by adding 3% $CaCl_2$ and was filtered. BM sections were incubated for 1-3 *h* in the thermostat at 37°C. After rinsing several times in distilled water, the sections were developed in 3% sodium sulfide solution for 1 minute and after rinsing mounted in balsam.

Immunohistochemistry: After rinsing several times in PBS, the sections were treated with: a) Triton X-100, overnight, to permeabilise; b) 3% hydrogen peroxide (H_2O_2) for 60 *min* to suppress the background peroxidase activity; and c) normal goat serum (1:30) for 60 *min* to block non-specific binding of antibodies. Then the sections were incubated in (a) primary antisera (anti-PRP-1, 1:5000) for 48 *h* at 4°C; (b) secondary antiserum (biotinylated anti-rabbit immunoglobulin, 1:200) for 60 *min*; and (c) ABC (1:100) (Vector Labs, Burlingame, CA, USA) for 60 *min* at room temperature. The reaction product was visualized *via* 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) as a chromogen and 0.6% nickel-ammonium-sulfate diluted in 50 mM Tris-HCL buffer (pH=7.6) in the presence of 0.03% H_2O_2 as an oxidant. Both primary and secondary antisera were diluted in PBS containing 0.1% bovine serum albumin (BSA) and 0.01% sodium azide. All incubations, except the incubation with normal goat serum, were separated with washes in PBS. The sections were mounted on gelatin-coated slides and coverslipped in balsam. Results were analyzed using light microscopy.

Antiserum production: Antiserum to the synthetic PRP-1 was obtained according to X. Ambrosius [*Ambrosius X., 1987*]: PRP-1-BSA (bovine serum albumin) conjugate mixed with Freund's complete adjuvant to give a homogenous emulsion [*Bret-Dibat J. et al., 1994*] was injected in equal portions into the lower extremities of rabbits in the region of the popliteal lymph nodes. Immunization was repeated one month later by injection of freshly made emulsion into the region of the left popliteal lymph nodes, intramuscularly on the right side, and into the auricular vein. After the immunization, the blood samples were taken from the auricular vein on days 7, 9, and 11.

The specificity of antiserum was tested by immunodiffusion and by enzyme-linked immunosorbent assay (ELISA).

RESULTS AND DISCUSSION

According to our morphohistochemical investigation with the method of acid phosphatase activity detection, the sinusoidal capillaries in the intact rat bone marrow (BM) appeared to be most often empty (Figure 1 A).

In BM stroma, the structural elements are closely localized and the borders between them are not clearly revealed. However, single formed blood elements are present and, notably, a group of cells with the short cytoplasmic extensions (3-4 extensions) is observed (Figure 1 B). On the outer side of the sinusoids, the densely stained small-sized cells are demonstrated, long and thick processes of which spread on the sinusoid wall (Figure 1 D). These fibers, perhaps, belong to BM stromal reticular cells.

The electrical stimulation of the hypothalamic PVN resulted in the appearance of the cell structures in the sinusoids (Figure 1 C), which is more expressed after PRP-1 injection. Besides, throughout the BM, the long nerve bundles are seen (Figure 2 C), in which the single axons are noticed under higher magnification (Figure 2 D). As a result of the electrical stimulation of PVN, the processes of cells situated on the sinusoids wall are strongly stained (Figure 2 A). Moreover, some small structures resembling the spines that might be synaptic terminals were visualized on the fibers belonging to dendritic (or reticular) cells (Figure 2 B).

In the immobilized rats, cells with strongly stained nuclei and long processes are revealed in the granular background between the blood cells. These cells by their morphological characters resemble the mesenchymal cells (Figure 3 A-C).

In another series of experiments it was histochemicaly demonstrated that at detection of Ca^{2+}-dependent acid phosphatase the most changes were observed

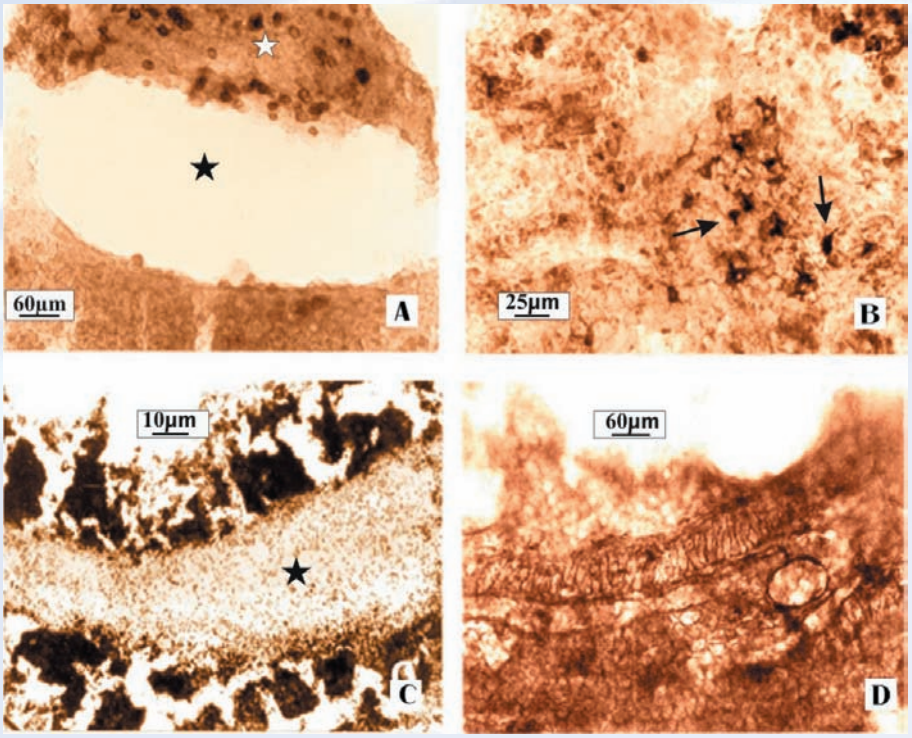

Figure 1. *BM structures in intact and exposed to the electrical stimulation of hypothalamic PVN rats. An almost empty sinusoidal capillary (asterisk) is seen (A); the scattered formed blood elements are demonstrated by the outside of the empty sinusoid. Large cells of irregular form and with the short cytoplasmic extensions (arrows) are observed in BM stroma (B). (C, D): BM structures after the electrical stimulation of the hypothalamic PVN. A sinusoid filled in with the cell structures (asterisk) (C). On the outer side of the sinusoids, the densely stained small-sized cells are demonstrated, long and thick processes of which spread on the sinusoid wall and in BM stroma. Histochemical method on detection of acid phosphatase activity.*

in the cerebral cortex, hippocampal complex, amygdaloid nuclei, LC, as well as in the cerebellum (Figure 3 D-F). On the cerebellar sections, among the granular cells dense-stained small cells with a few extensions and large unstained nuclei are revealed (Figure 3 E). On the basis of morphological characteristic they may be considered the radial glia observed in some cytoarchitectural regions of brain, including the cerebellum, directly under the *pia mater* [*Gage F., 2002*].

In BM stroma of injected with PRP-1 rats strong increase of number of blood-forming cells was observed (Figure 4 A-D). In the sinusoids, megacariocytes in the stage of blood platelets formation occur among the monocytes and macrophages (Figure 4 B).

We carried out the immunohistochemical investigation aiming to detect PRP-1- immunoreactivity (PRP-1-IR) in BM structures using the antisera to PRP-1.

The sinusoidal capillaries with the clearly observed PRP-1-immunoreactive (PRP-1-Ir) endothelial cells on the walls are found in BM of intact rats. In addition, some fine fibers accompany the vessels. PRP-1-Ir blood cells are revealed around

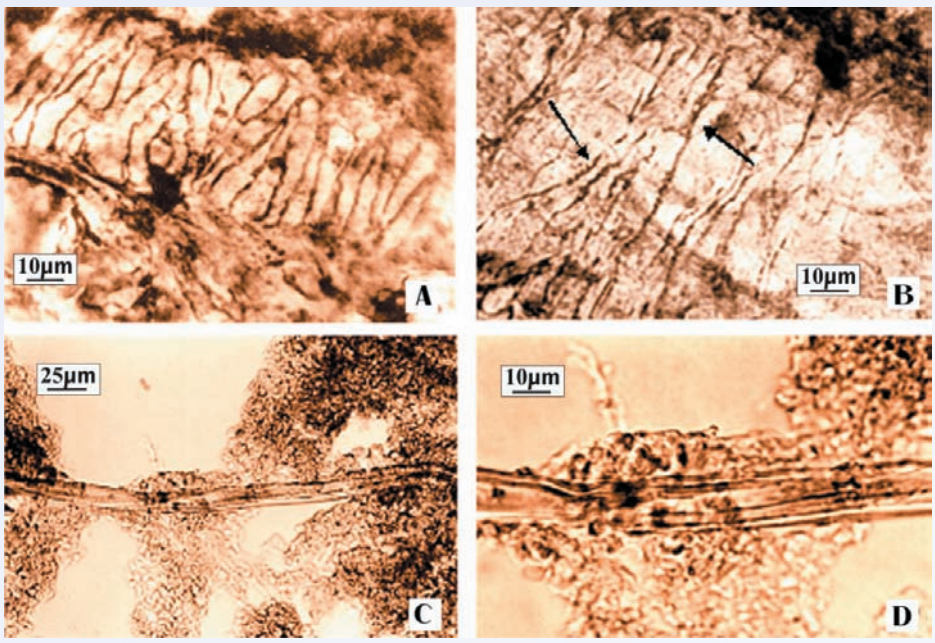

Figure 2. *BM structures in exposed to the electrical stimulation of hypothalamic PVN rats. (A-D): Increase of the acid phosphatase activity in the fibrous structures enveloped whole sinusoid (A); the small excrescences are observed on the fibers resembling the spines (arrows) (B). Throughout the BM, the long nerve bundles are seen (C), in which the single axons are noticed under the higher magnification (D). Histochemical method on detection of acid phosphatase.*

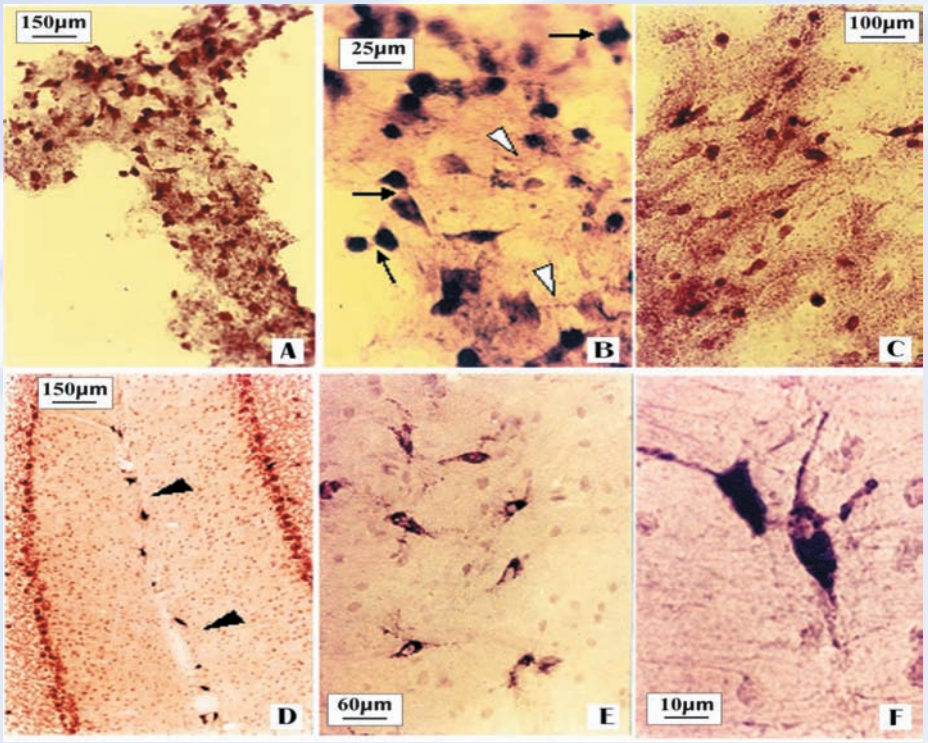

Figure 3. *Cell structures in immobilized rats brain and bone marrow. A-C: numerous round in shape or fusiform small cells with the short axon-like extensions resembling the mesenchimal cells are demonstrated in BM stroma of immobilized rats; B: among these cells being in different proliferation stages (arrows) some varicose fibers (arrow heads) are well seen. D-F: cell structures in the cerebellum of the newborn rats exposed to the prenatal immobilization stress. D: small cells with very short processes (arrow heads) situated in the cerebellar white matter between the fornix. E: darkly stained cells with round light nuclei and short extensions under the cerebellar dura mater. F: among the granule cells some darkly stained fusiform cells with the processes and ectopied nuclei are located. Histochemical method on detection of acid phosphatase activity.*

the sinusoids, but not inside. Some fibers are demonstrated on the vessels wall, perhaps, representing the fibers of BM reticular cells.

In rats under the different stresses, as well as after PRP-1 administration the increased number of PRP-1-Ir cell structures as islets was revealed both in BM stroma (Figure 5 B-D), and in the sinusoids (Figure 5 A). Besides, some PRP-1-Ir capillaries are well demonstrated among the blood cells (Figure 5 A). The appearance of PRP-1-Ir fine nerve fibers on the sinusoids wall and in the BM stroma (Figures 5 C) is also evident. The network of nerve fibers is also revealed.

The groups of PRP-1-Ir small cells of yet unknown origin are seen in the close vicinity to the nerve fibers (not demonstrated). The size and shape of these cells and the localization of their nuclei resemble the small-sized neurons.

Thus, analysis of data obtained for all experimental rats exposed to stress and injected with PRP-1 demonstrated the processes of hematopoiesis in BM.

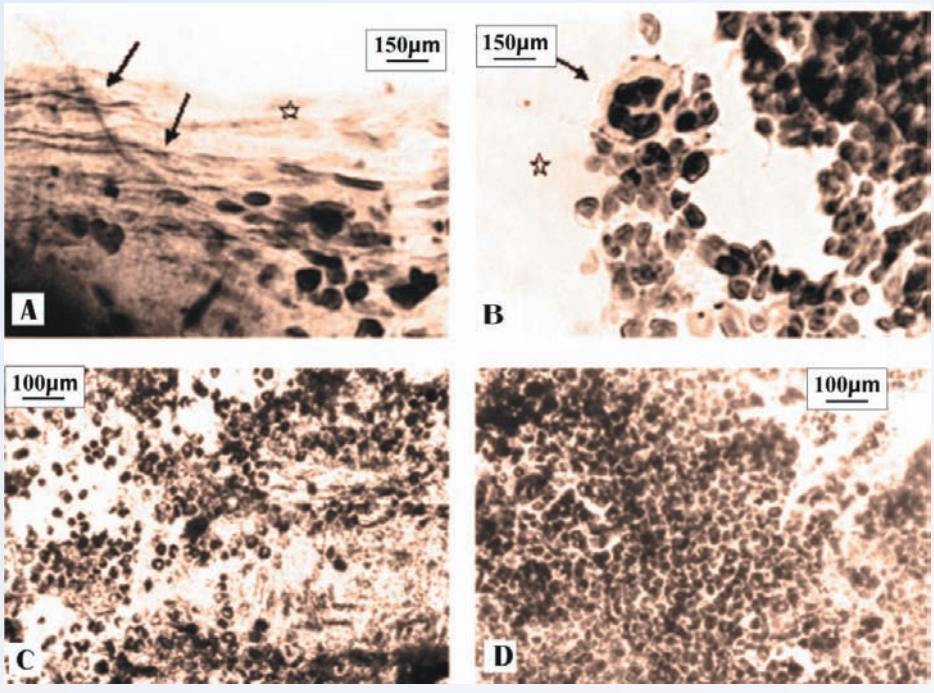

Figure 4. *BM structures in the PRP-1 injected rats. (A-D): Hematopoiesis process obtained in BM as a result of PRP-1 i/m injection. (A): Sinusoid (asterisks) filled in with the blood-forming cells; fibers (arrows) crossing the sinusoid are also seen. Increased number of immune system cells both in stroma (C, D) and sinusoid (asterisks) (A, B). In the sinusoid, a megakaryocyte (arrow) with the homogenously and densely stained nuclei and being in the stage of platelets release is also demonstrated (B). Histochemical method on detection of acid phosphatase activity.*

The presence of PRP-1 in the immune system cells and nerve fibers in close contact to immune system cells suggest its synthesis and release from the hypothalamic nuclei as a result of stress stimuli of the latter and the involvement of PRP-1 in the mechanism of the hematopoiesis process. However, the synthesis of these substances in the immune system cells is not also excluded, as there is, for instance, evidence of distinct neuropeptides biosynthesis in the lymphocytes. To confirm this suggestion, *in situ* hybridization histological method and flow cytofluorimetric analysis are required.

P Bossolasco and co-workers [*Bossolasco P. et al., 2004*] find that certain populations of human bone marrow cells express neural genes even before treatment. The native (untreated) bone marrow-derived mesenchymal stem cells (MSCs) express many neural specific genes at the mRNA or protein level. By cytofluorimetric analysis, MSCs contain the highest percentage of cells expressing neuronal and glial genes compared to other cell populations: one marker that is actually detected at the immunocytochemical level in MSCs is a neuroepithelial stem cell marker nestin [*Vogel W. et al., 2003; Lu P. et al., 2004*]. However, nestin is expressed not only in early CNS tissue, but also in other developing cells, including muscle and myocardium [*Lendahl U. et al., 1990; Sjoberg G. et al., 1994*]. Most *in vivo* studies have relied only on immunocytochemical labeling of a few neuronal marker genes and have not demonstrated generation of mature functional neurons from bone marrow-derived stem cell transplants. In two key studies published in 2000 [*Brazelton T. et al., 2000; Mezey E. et al., 2000*], however, many of these donor cells expressing neuronal marker genes were round in shape and had very few axon-like extensions [*Brazelton T. et al., 2000*]. Similarly, a later study also demonstrated neuronal marker gene expression in human MSCs transplanted into the ischemic brain of rats; however, these cells were spherical in morphology with few processes [*Zhao L. et al., 2002*].

The adult rodent CNS contains small numbers of multipotential progenitor cells, most of which are situated in the periventricular zone of the forebrain, but which also exist in other parts of the nervous system, such as surrounding the central canal of the spinal cord [*McKeon R. et al., 1995; Canoll P. et al., 1996; Powell E. et al., 1997; Zuo J. et al., 1998*]. In the normal brain, the main function of these cells is to migrate into the olfactory lobe to produce new neurones and glial cells [*McKeon et al., 1995*]. These cells express the intermediate filament nestin. There are two sources of nestin-positive cells in the injured brain [*Johansson C. et al., 1999*]. Since nestin appears in CNS injuries, the possibility arises that progenitors could be its source. It can be shown in the spinal cord that nestin-expressing periventricular cells start to migrate to the site of injury and start to express GFAP [*Keirstead H. et al., 1995*]. Whether this also applies to other areas of the CNS is not established. However, astrocytes around the lesion

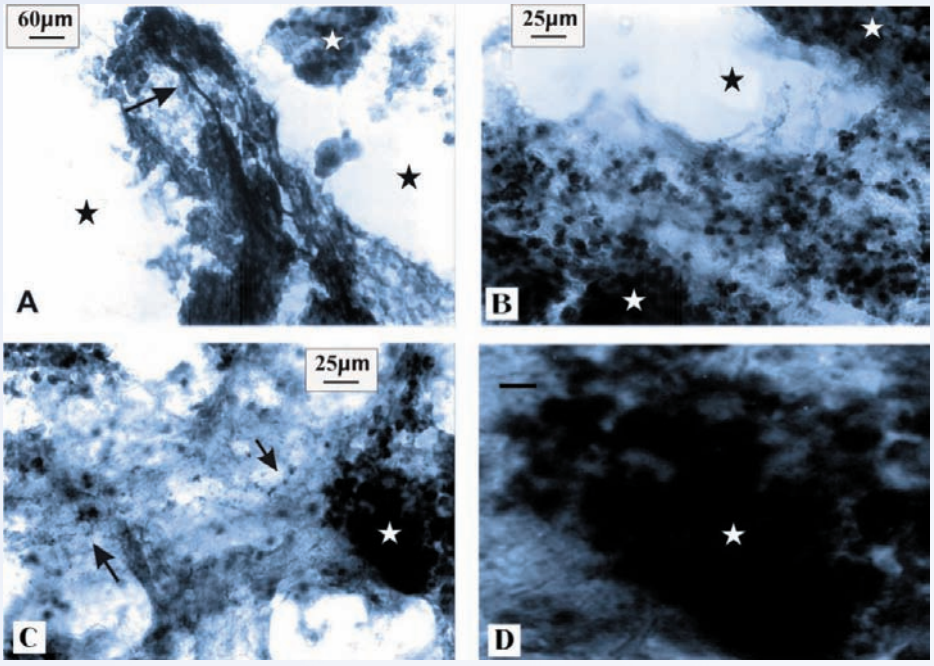

Figure 5. *PRP-1-Immunoreactive (PRP-1-Ir) cell structures in the immobilized rats bone marrow. Islands of immune system PRP-1-Ir cells (white asterisks) inside (A) and around the sinusoids (black asterisks) in BM stroma (B, C). A PRP-1-Ir capillary (arrow) (A) and few number of single PRP-1-Ir varicose nerve fibers (arrows) in the BM stroma (C). (D): fragment of 5C. ABC Immunohistochemical method.*

also produce nestin following injury. It is appropriate to mention here that by our data, in response to Central Asian Cobra *Naja naja oxiana* snake venom (NOX) administration to the trauma-injured rats the increased number of PRP-1-Ir nerve fibers and astrocytes, as well as lined up PRP-1-Ir cells migrating towards the injury area in the SC lesion region were found out [*Abrahamyan A. et al., 2007*]. PRP-1 have been established to be involved in the underlying mechanisms of promotion of neuronal recovery processes following NOX venom treatment.

There are also experiments, in which axons have clearly been able to regenerate *in vivo* on astrocyte processes [*Krueger S. et al., 1986*].

Taking into account both our and literature data, using the antisera against the nestin, GFAP и PRP-1, we immunohistochemicaly studied brain structures of rats exposed to the prenatal immobilization stress.

Results obtained demonstrate a number of nestin-Ir cells of different sizes and forms in the SC: dense nestin-Ir cells of roundish shape in the arterioles (Figure 6 A), with very short processes and negative nucleus (Figure 6 B); with an axon and densely stained ectopied nucleus (Figure 6 C).

GFAP-Ir cell structures of different size are found in the SC dorsal horn (Figure 6 D). Besides, GFAP-Ir astrocytes are observed in close proximity with the blood vessels (Figure 6 E) everywhere, mainly in the white matter. Some GFAP-Ir, roundish in morphology structure (Figure 6 E), are also demonstrated inside of vessels.

As for PRP-1 localization in various brain regions, PRP-1-Ir round in morphology small cell structures occur inside (Figure 6 F) and next to blood vessels in the SC white matter; PRP-1-Ir capillaries and varicose nerve fibers in many brain areas are also well seen.

It was recently reported that transplanted bone marrow cells in the brain predominantly preserve their hematopoietic identity [*Ono K. et al., 2003; Hudson J. et al., 2004; Walczak P. et al., 2004*] or differentiate into parenchymal microglial cells and perivascular cells [*Corti S. et al., 2002*; *Hess D. et al., 2004*].

Presence of PRP-1-Ir cell structures in the perivascular areas could be, most probably, the microglial or perivascular cells. It is not expected that proliferation and differentiation of brain stem cells could be influenced by the neuroprotective proline

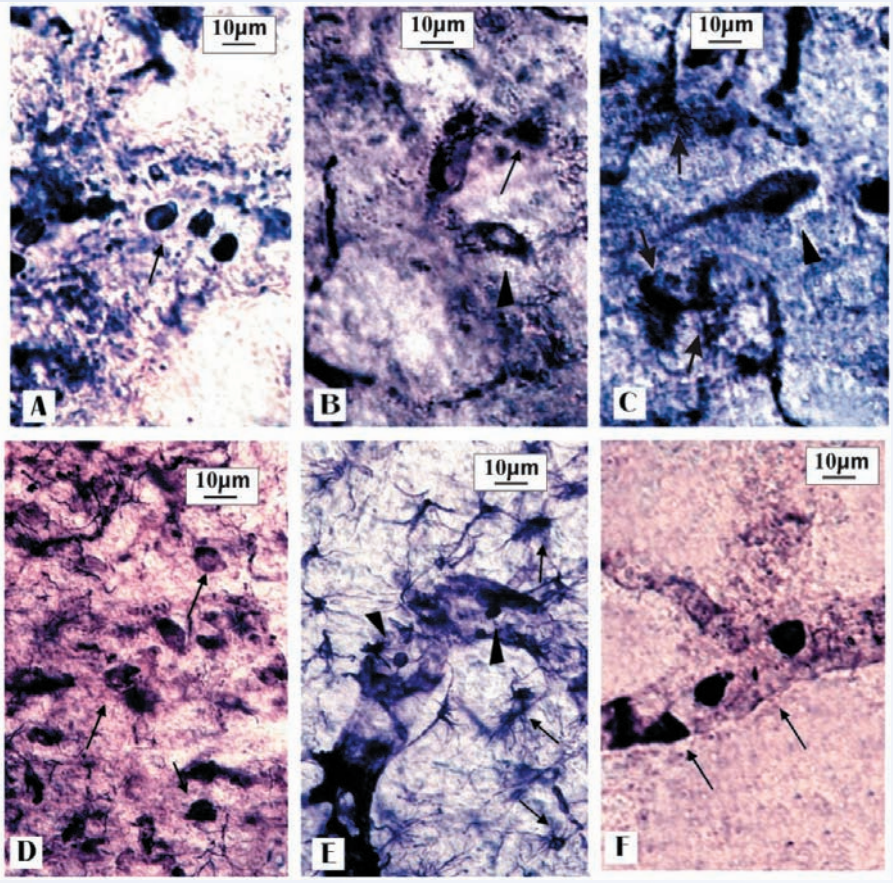

Figure 6. ***A-F:*** *Nestin-, GFAP- and PRP-1-immunoreactive brain cell structures in exposed to prenatal immobilization stress rats. (A-C): nestin-Ir structures in the SC. A number of nestin-Ir cells of different size and form are revealed. (A): dense nestin-Ir cells of roundish shape in the arteriole. (B): with very short processes and negative nucleus (arrow head). (C): with an axon and densely stained ectopied nucleus (arrow head). (D, E): GFAP-Ir cell structures in the SC. (D): GFAP-Ir cell structures of different size are found in the SC dorsal horn (arrows). (E): GFAP-Ir astrocytes are well seen in close proximity with the blood vessels (arrows) everywhere, mainly in the white matter, and GFAP-Ir roundish in morphology structures (arrow heads) are demonstrated inside of vessels. (F): PRP-1-Ir round cells are seen inside (arrows) of capillaries in the SC white matter. ABC Immunohistochemical method.*

rich polypeptide similar to various growth factors.

A recent report indicates that the astrocytes that occur in the subventricular zone of the rodent brain act as neural stem cells. The cells with astrocyte markers appear to generate neurons *in vivo*, as identified by their expression of specific neuronal markers.

In the present study, the astrocyte-like cells identified with the glial fibrillary acidic protein were positive for neural stem cells marker nestin as well. Thus, adult stem cells plasticity in response to the immobilization stress is assumed in some of the studied brain regions. However, whether these cells are bone marrow-derived stem cells circulating in the blood is the question to be solved.

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