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The Effect of Semax and Its C-End Peptide PGP on the Morphology and Proliferative Activity of Rat Brain Cells During Experimental Ischemia: A Pilot Study

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Abstract The neuropeptide preparation Semax (Met-Glu-His-Phe-Pro-Gly-Pro) has been employed successfully in clinical practice for treating patients with severe brain blood circulation disorders. In spite of numerous studies, many aspects of the therapeutic effects of this preparation remain unknown. In this context, the effects of Semax and its C-end tripeptide PGP on the functional morphology of nervous tissue cells were studied in the normal rat brain and in a model of incomplete global rat brain ischemia. In control animals, both peptides activated the capillary network and caused similar morphological changes to neurons and the neuropil regions. We show here for the first time at the histological level that Semax and PGP increased proliferation of the neuroglia, blood vessel endothelium, and progenitor cells in the subventricular zone. In these experimental conditions, only Semax abated the manifestation of ischemic damage to the nervous tissue. This was probably attributable to a decrease in vascular stasis symptoms as well as the trophic effect of the peptide.

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Abbreviations

ACTH	Adrenocorticotropic hormone
SVZ	Subventricular zone
PS	Physiological saline (0.9% NaCl)
CNS	Central nervous system
PGP	Tripeptide Pro-Gly-Pro
PCNA	Proliferating cell nuclear antigen
SO	Sham operation
H&E	Hematoxylin and eosin

Introduction

The development of new and efficient methods for rehabilitation therapy and neuroprotection in patients with ischemic brain injuries provides one of the more urgent tasks of modern medicine. Much attention has focused on studies of properties of neuropeptides, the analogs of adrenocorticotropic hormone (ACTH). Peptides of this family show neuroprotective and neurotropic properties (Strand 1999). However, the effects are transitory because of their degradation in the body by the action of endogenous proteolytic enzymes. The preparation Semax, a fragment of ACTH (4–7) linked at its C terminal with the Pro-Gly-Pro sequence (PGP), is stable and active during a prolonged period (Kaplan et al. 1996). PGP protects Semax from premature degradation; it belongs to the class of glyproline tripeptides as an active regulatory molecule. The neuroprotective properties of PGP have been demonstrated in nerve cell cultures under conditions of oxidative stress

(Martynova et al. 2009) and glutamate toxicity (Storozhevykh et al. 2007).

According to the literature, Semax has neuroprotective effects (Levitskaya et al. 2004; Grivennikov et al. 2008). Immunomodulation, deceleration of inflammatory responses, slowing the synthesis of nitrogen oxide, and limiting oxidative stress reactions are its main manifestations (Astashkin et al. 2000; Bashkatova et al. 2001). During experimentally induced global brain ischemia, Semax showed a positive effect manifested in minor neurological disorders during the first hours after surgery and in better survival of the treated animals compared with controls (Yakovleva et al. 1999). A number of studies have demonstrated that Semax activates neurotrophic genes and their receptors in cell cultures, and in the hippocampus and frontal cortex of intact animals (Shadrina et al. 2001; Dolotov et al. 2003). Both Semax and PGP stimulate the expression of some growth factor genes and their receptors following experimental ischemia in the rat brain (Dmitrieva et al. 2008).

At present, Semax is widely employed clinically for treating various central nervous system (CNS) conditions such as ischemic stroke (Gusev Eu and Skvortsova 2001), optic nerve atrophy (Polunin et al. 2000), and cerebrovascular insufficiency (Gusev et al. 2005). However, the mechanism of the neurotrophic and neuroprotective action of this polypeptide is obscure, notwithstanding numerous studies. Here, we studied the influence of both Semax and its C-end fragment PGP on the morphology and proliferative activity of rat brain cells under conditions of experimental ischemia evoked by irreversible bilateral common carotid artery occlusion.

Methods

Animals

The animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publ. no. 80–23, revised 1996). Adult male Wistar rats (200–250 g) were maintained under natural light with free access to food and water.

Experimental Groups

The animals were divided into 17 groups to be compared (11 control and six experimental groups; $n=3$ each; Table 1). Control groups 1, 4, 5, 8, and 9 included intact rats, while groups 2, 3, 6, 7, 10, and 11 comprised sham-operated control animals. Rats of experimental groups 1–6 were anesthetized with ether and subjected to incomplete global ischemia of the brain induced by bilateral common carotid artery occlusion (Yakovleva et al. 1999). The dissected tissues of sham-operated animals were sewn together with no stricture of the isolated vessels. Equimolar amounts of Semax and PGP (Institute of Molecular Genetics; Russian Academy of Sciences, Moscow, Russia) were dissolved (100 μg/kg body weight of Semax or 37.5 μg/kg PGP) in saline (0.9% NaCl; PS). Fifteen minutes after surgery, either physiological saline or one of the preparations was injected intraperitoneally (0.3 ml per 100 g body weight). Thirty minutes or 24 h after the occlusion of carotid arteries (or sham operation), the animals were decapitated while under ether anesthesia. The preparations were administered three times (1, 4, and 8 h after the operation) to control groups 3, 5, 7, 9, and 11, and to experimental groups 2, 4, and 6 (Table 1).

Histology

The isolated brain was immersed in neutral formalin for 24 h and washed in 70% ethanol. Dissection was performed with orientation of the tissue segments to permit subsequent cutting in parasagittal sections from the brain's right part and coronary sections from the brain's left part at the level of the dorsal hippocampus. Tissue samples were dehydrated and embedded in Paraplast Plus (The Kendall Co., Mansfield, MA, USA). For histology, microtome sections were stained with hematoxylin and eosin. Morphological analysis was conducted with allowance for normal and pathological CNS variants (Garcia et al. 1995; Li et al. 1998; Lipton 1999). Stereotactic mapping of the damaged zones and accurate determination of the level of sections were performed according to an atlas of the rat brain (Paxinos and Watson 1997).

Immunohistochemistry

Immunohistochemical studies were carried out using murine monoclonal antibodies to proliferating cell nuclear antigen (PCNA; PC10 1:50; Calbiochem, San Diego, CA, USA) and a biotin–streptavidin–peroxidase kit for detecting mouse immunoglobulins (ImmunoO; MP Biomedicals, Aurora, OH, USA). The antibodies to PCNA and the kit had been tested first with positive control material. To eliminate masking of antigenic determinants of PCNA and to decrease background immunostaining, the sections were first treated with 0.5 M HCl (pH 0.5) at 35°C for 18 h. Endogenous peroxidase was blocked in 3% H_2O_2. The preparations were incubated overnight in a solution of primary antibodies in a humidified chamber at 4°C. In accordance with the manufacturers' recommendations, the sections were washed with phosphate-buffered saline and then treated with secondary antibodies. The substrate enzyme (peroxidase) was developed with diaminobenzidine

Table 1 General characteristics of the material used in studying the action of Semax and PGP after incomplete global ischemia of the brain induced by carotid artery clamping

No.	Group	Introduction of agent	Withdrawal from experiment
1	Control 1 (intact)	–	During anesthesia
2	Control 2 (SO)	PS, once	30 min after SO
3	Control 3 (SO)	PS, 4 times	1 day after SO
4	Control 4 (intact)	Semax, once	15 min after Semax introduction
5	Control 5 (intact)	Semax, 4 times	1 day after first Semax introduction
6	Control 6 (SO)	Semax, once	30 min after SO
7	Control 7 (SO)	Semax, 4 times	1 day after SO
8	Control 8 (intact)	PGP, once	15 min after PGP introduction
9	Control 9 (intact)	PGP, 4 times	1 day after first PGP introduction
10	Control 10 (SO)	PGP, once	30 min after SO
11	Control 11 (SO)	PGP, 4 times	1 day after SO
12	Experiment 1 (ischemia)	PS, once	30 min after occlusion
13	Experiment 2 (ischemia)	PS, 4 times	1 day after occlusion
14	Experiment 3 (ischemia)	Semax, once	30 min after occlusion
15	Experiment 4 (ischemia)	Semax, 4 times	1 day after occlusion
16	Experiment 5 (ischemia)	PGP, once	30 min after occlusion
17	Experiment 6 (ischemia)	PGP, 4 times	1 day after occlusion

(Liquid DAB+; Dako, Glostrup, Denmark) or aminoethylcarbazole (AEC+; Dako). If necessary, the cell nuclei were stained with hematoxylin. Sections using AEC processing were put in aqueous mounting medium (Faramount, Dako). Histological preparations were examined using an Olympus CX41 microscope, and photomicrography was performed with the use of a Nikon Cool Pix 4500 digital camera.

Results

Morphology of Brain Tissue After Semax and PGP Introduction in Control Groups

After injecting PS, the histological patterns of the vascular plexus, the morphology of cortex neurons (Fig. 1a) of the diencephalon (Fig. 1b), and the mesencephalon were consistent with a normal CNS. Basophilic RNA-containing material was distributed in the form of small granules in the perikaryon of large neurons of the brainstem nuclear formations, tending to accumulate peripherally (Fig. 1c). At 15 min after Semax introduction in control groups 4 and 6, there was moderate blood filling of the vessels, more pronounced blood filling of end capillary loops in the vasoganglial ventricles, and dilation of the capillary network in the cerebral cortex (Fig. 1d) and thalamus (Fig. 1e). In the diencephalon, the morphological symptoms of capillary activation correlated well with strengthening microvesiculation of the neuropil and neuronal cytoplasm. Larger clusters of basophilic substance were observed in the perikarya of the mesencephalic nuclei (Fig. 1f). These changes reversed partially in all the above listed brain parts by 1 day after Semax introduction. Blood filling of large and mid-sized vessels of the brain cortex and subcortical centers was more marked in control groups 8–11 that were administered PGP than in the control groups 4–7. The pattern of the capillary network (Fig. 1g) did not differ from that of the rats subjected to Semax treatment. However, vacuolization of the neuropil and neuronal cytoplasm in the thalamus was marked (Fig. 1h). The basophilic material in neurons of brainstem nuclear formations appeared as large blocks. Moreover, there were numerous fusing microvesicles seen in the surrounding neuropil (Fig. 1i). The increased basophilia of the neuronal cytoplasm remained at 1 day after PGP treatment.

Morphology of Brain Tissue After Semax and PGP Introduction in Experimental Groups

Rats in experimental group 1 showed few histopathological changes at 30 min after the occlusion of the carotid arteries. Microscopy revealed blood stasis in the capillaries, the filling of widened venules with aggregated erythrocytes, and emergence of swollen neuropil areas and hyperchromic neuron bundles with pericellular edema. There was marked ischemic damage to neurons in the frontoparietal brain cortex (Fig. 2a). No gross pathological changes were detected in the lateral ventricles. Cells of the ependyma and the subependymal glia were unchanged. In experimental group 2, many neurons of the cortex (Fig. 2b), thalamus (Fig. 2c), mesencephalon, and pons (Fig. 2d) showed ischemic stress accompanied by typical consolidation of nuclei and wrinkling of the perikaryon 1 day after carotid occlusion. Wrinkled hyperchromic neurons were seen in the

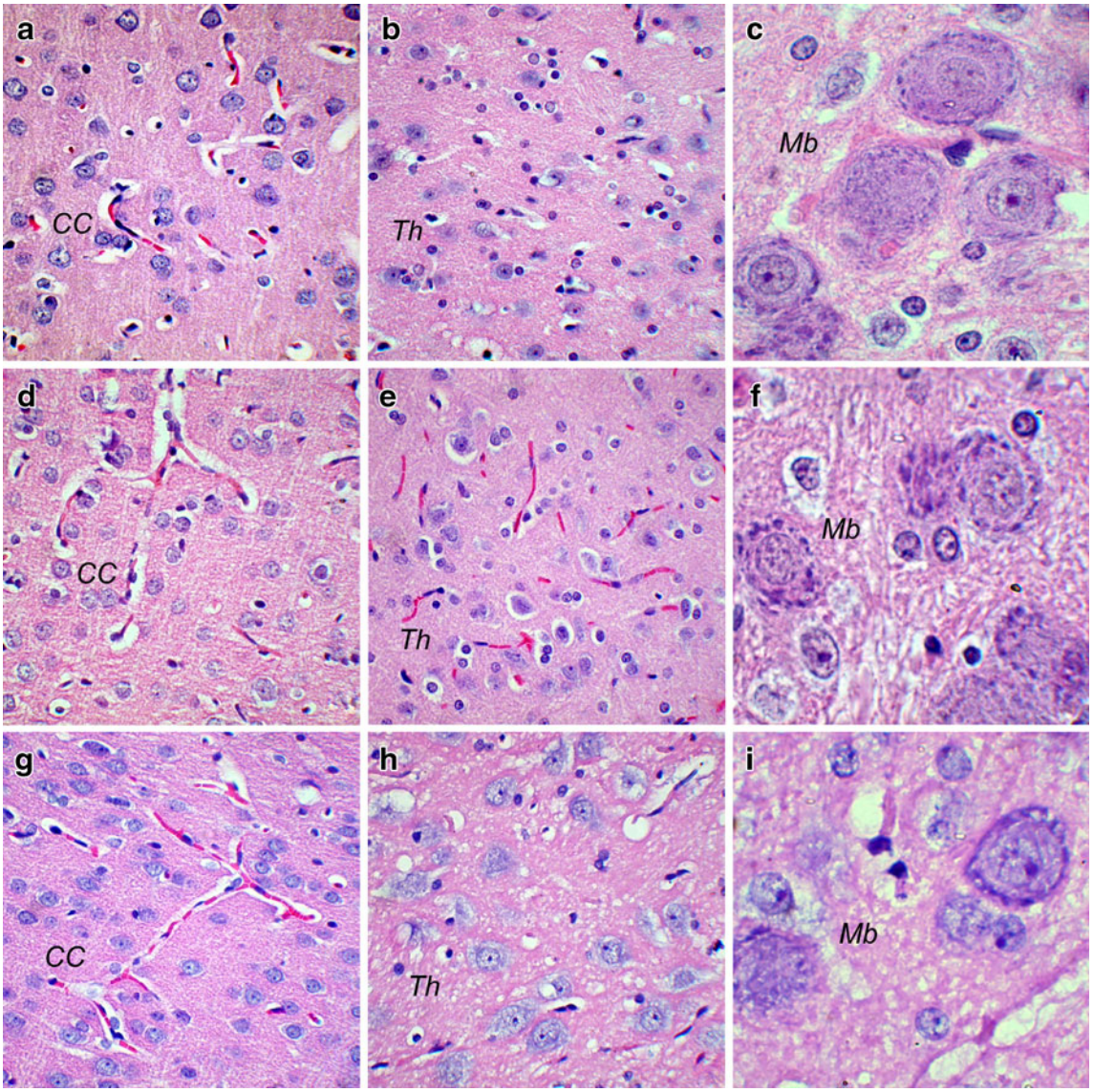

Figure 1 Morphology of cortex polymorph layer of the frontal lobe (**a**, **d**, **g**), thalamus (**b**, **e**, **h**), and of large neurons of midbrain nuclear formations (**c**, **f**, **i**) in sham-operated (**a**, **d**, **f**, **g**) and intact (**b**, **c**, **e**, **h**, **i**) rats 15 min after the introduction of physiological solution (**a**), Semax (**d**–**f**), or PGP (**g**–**i**). **d**, **e**, and **g** show dilation of the capillary network; **h** and **i** show microvesiculation of neuropil and cytoplasm of neurons; **f** and **i** show increased basophilic material in the perikarya. *CC* cerebral cortex, *Mb* midbrain, *Th* thalamus. Staining with hematoxylin and eosin. Magnification, ×280 (**a**, **b**, **d**, **e**, **g**, **h**); ×700 (**c**, **f**, **i**)

hippocampus and the cerebellum. The presence of pyknotic cells in the ischemic areas testified indirectly to possible apoptotic death of some damaged neurons. Areas of ischemic stroke with nervous tissue destruction were observed in the frontal parietal cortex, the thalamus, the hypothalamus, and the pons varolii. Brainstem white matter (substantia alba) swelling increased notably, and this was accompanied by edema.

Vascular disorders in the acute period of ischemia in Semax-administered experimental group 3 were less pronounced than they were in control animals treated with PS. Their brain cortex capillaries looked moderately dilated and were full of erythrocytes but showed no signs of stasis. Together with the pathological appearance of "dark" cells, there were small loci of ischemically damaged neurons (Fig. 2e). Microscopic changes corresponded in general to those classical symptoms of ischemic damage, regarded as potentially reversible. After 1 day, the Semax-treated rats in experimental group 4 developed broader zones of nervous tissue ischemic damage compared with experimental group 3. However, there were no signs of deep destructive changes or decomposition of cellular elements. Interestingly, the outer zones showing elevated cortical ischemia (Fig. 2f) also exhibited microvesiculation of the neuropil

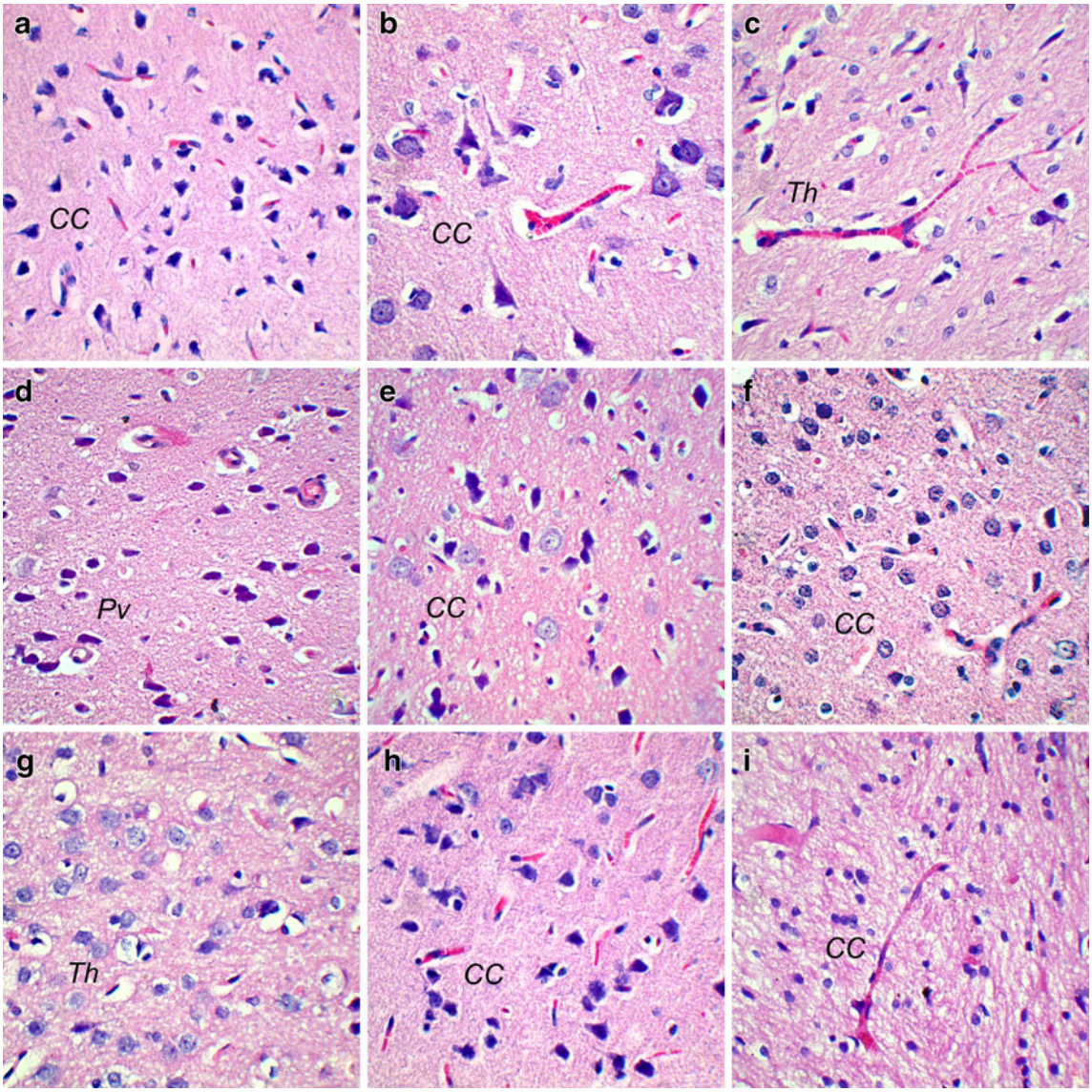

Figure 2 Ischemic damage to neurons of the external granular (**a**, **e**, **h**), interior pyramid (**b**) and polymorph (**f**, **i**) cortex layers of the frontal lobe, thalamus (**c**, **g**), and pons varolii (**d**) 30 min (**a**, **e**, **h**) and 1 day (**b-d**, **f**, **g**, **i**) after occlusion of the carotid arteries with concomitant treatment with physiological saline (**a**–**d**), Semax (**e**–**g**), or PGP (**h**, **i**). *CC* cerebral cortex, *Pv* pons varolii, *Th* thalamus. Staining with hematoxylin and eosin. Magnification, ×280

and neuronal perikarya, i.e., the same morphological changes seen in the control groups treated with Semax (Fig. 2g).

When studying H&E-stained brain preparations of PGP-treated rats from experimental groups 5 and 6, there were no exceptional pathological changes compared with animals of groups 1 and 2, respectively. By 30 min after vessel blocking and PGP administration, vascular disorders and ischemic neurons were present in the cortex of the large hemispheres (Fig. 2h, i). After 24 h, multiple centers of ischemic stroke formation showing tissue destruction and decomposition were found. The ischemic damage to neurons increased and postischemic edema developed in the white matter.

Proliferative Activity of Rat Brain Cells

In intact and in sham-operated animals of control groups 1–3, positive immunostaining for PCNA was seen in epithelial nuclei of vascular plexuses, and in ependymal (Fig. 3a) and glial (Fig. 3b) cells. Immunolabeled glial nuclei were fewest in the cerebral cortex. Typical groupings and chains of cells with immunolabeled nuclei were observed in the subventricular zone of lateral ventricles and in migration streams (Figure 3c).

Fifteen minutes after Semax introduction, the intensity of PCNA immunostaining of cell nuclei increased markedly in control groups 4 and 6. This increased

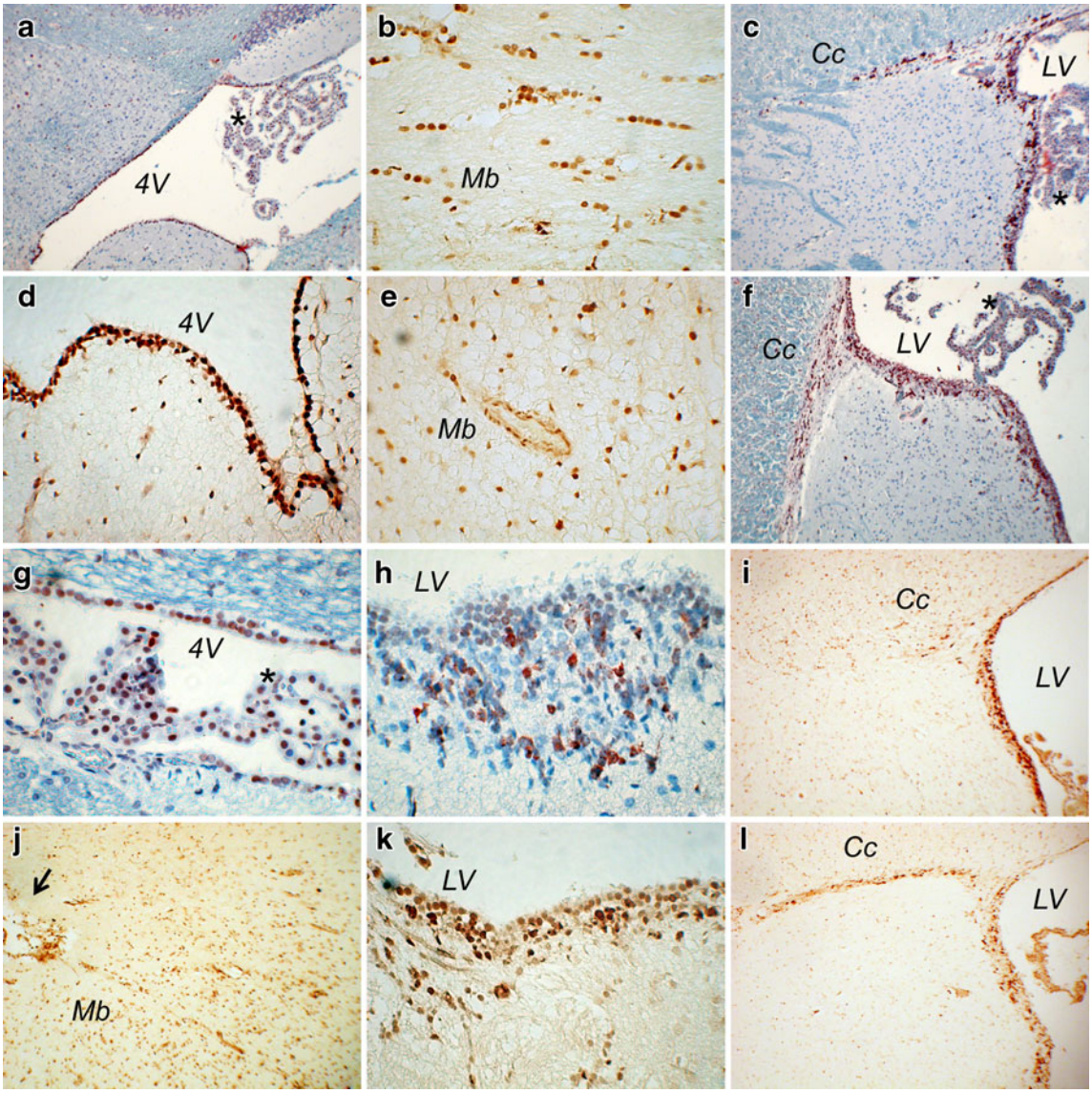

Figure 3 Proliferation of ependymal cells of the fourth ventricle (**a**, **d**, **g**), endothelium of vascular plexuses (**a**, **g**), neuroglia and vessel endothelium of the midbrain (**b**, **e**, **j**), progenitor cells in the subventricular zone (**c**, **f**, **i**, **l**), subependymal glia (**h**, **k**) in intact animals (**a**, **c**, **f**) 30 min after sham operation (**b**, **d**, **e**) with the introduction of physiological saline (**b**) or Semax (**d**, **e**, **f**) at 30 min (**j**) and 1 day (**g**–**i**, **k**, **l**) after carotid artery occlusion. Treatments included physiological saline control (**g**–**i**), Semax (**j**, **k**), and PGP (**i**). *4V* 4th ventricle, *Cc* corpus collosum, *LV* lateral ventricle, *Mb* midbrain; *asterisks* mark vascular plexuses; an *arrow* shows a local center of ischemia. Immunostaining for PCNA in cell nuclei using the biotin–streptavidin–peroxidase complex method. **a**, **c**, and **f**–**h** show aminoethylcarbazole (red nuclei) and hematoxylin staining; **b**, **d**, **e**, and **i**–**l** show diaminobenzidine staining (brown nuclei). Magnification, ×70 (**a**, **c**, **f**, **i**, **j** and **l**); ×280 (**b**, **d**, **e**, **g**, **h** and **k**)

response was extremely marked in cells of the ependyma and subependymal glia (Fig. 3d), in the neuroglia and in the endothelium of midbrain vessels (Fig. 3e) and vascular plexuses, and in the repopulating cells in the rostral section of the lateral ventricles (Fig. 3f). One day after the start of Semax treatment, the PCNA response of proliferating cells decreased and was a little different from that observed in animals of control groups 5 and 7. With PGP treatment, no major changes in neuronal proliferative activity were seen, except for a more intense PCNA immunostaining of glial cell nuclei in the brain stem portion of control group 8.

At 30 min after carotid occlusion in experimental group 1, decreased PCNA immunostaining intensity was observed in swelling zones, probably caused by a rapid progression of hypoxia in ischemic regions. However, positive PCNA immunostaining was virtually unchanged in the nuclei of cells of the subependymal glia, in the endothelium of vascular plexus, and outside the zones of pathological changes in the mesencephalon and the

cerebellum. After 1 day, cell proliferation marked by PCNA immunostaining decreased in almost all brain parts, simultaneously with increased ischemic damage. Less intense PCNA immunostaining was seen in the nuclei of ependyma cells, in endothelium cells of ventricular vascular plexuses, and in the subependymal glia (Fig. 3g, h). Proliferation of cells in the subventricular zone and migration streams (Fig. 3i) was reduced notably.

Proliferation of neural cells was greater in experimental group 3 than in experimental group 1. Distinct zones of proliferation of neuroglia and vessel endothelium were seen along the periphery of local ischemic centers (Fig. 3j). One day after inducing ischemia in experimental group 4, the proliferative activity of cells had reduced (Fig. 3k), although it remained higher than in experimental rats not administered Semax.

At 30 min after the occlusion of brain vessels (experimental group 5) and against the background of PGP administration, decreased PCNA immunostaining was only found in the brain loci showing ischemic damage. Experimental group 6 displayed higher focal proliferation of glia and vessel endothelium, and stronger PCNA immunostaining in subependymal glia nuclei and cells in the rostral migration stream (Fig. 3l), than did rats in experimental group 2 who only received PS.

Discussion

Comparative morphological and functional studies of the effect produced by Semax and PGP on the brain of control group animals testify that both peptides activate the capillary network and equally affect the morphology of neurons. We have discovered no principal differences in the effect of Semax and its C-end peptide. They both acted in one direction, and this was accompanied by elevated amounts of basophilic substance in neuronal cytoplasm and similar microstructural changes in the neuropil. Both Semax and PGP appeared to affect certain metabolic and secretory processes in the brain irreversibly. However, Semax affected PCNA expression more effectively in the short term, whereas PGP exerted a more prolonged action on neuropil changes and on the accumulation of basophilic material in large neurons.

Immunohistochemical detection of nuclear proteins involved in cell division is widely employed for studying the functional morphology and evaluation of cell proliferation. PCNA is used reliably to investigate repair and neurogenesis in the nervous system (Chen et al. 2003; Johnson et al. 2004; Tsyb et al. 2009). Judging by the strengthened expression of PCNA in the control animals' brain cell nuclei, Semax appears to have stimulated proliferation in the nervous tissue's repopulating elements.

In animals with occluded carotid arteries, ischemic damage to the nervous tissue develops against a background of vascular disturbance. Brain damage progresses from small loci of affected neurons to diffuse areas of ischemic stroke, accompanied by profound destructive changes and the death of neurons. The pathology and pathophysiology of ischemic brain damage are well known (Ginsberg 2003; Cheng et al. 2004). Abrupt interruptions to the oxygen and glucose supplies of the CNS trigger a number of pathological cascades, leading to extended death of neurons. The secondary mechanisms of this damage include complex biochemical and physiological processes. Many factors entailing secondary autodestructive reactions are involved in the mechanisms of delayed damage and death of neurons. These include damaging free radicals, exciting amino acids, eicosanoids, lipid degradation products, tissue cations, inflammation, and immune responses. These secondary factors can act either consecutively or in concert, and evoke delayed or slowly progressing dystrophic changes and cell death (Yakovlev and Faden 2004). Among many identified mechanisms, the following factors play a critical role in ischemic brain injury: excessive activation of glutamate receptors, accumulation of intracellular calcium cations, excessive production of free radicals, and the initiation of apoptosis. Perfusion changes in the brain tissue during ischemic stroke also determine changes in patterns of gene expression (Iadecola 1999). There are grounds to believe that interruption of this pathological cascade would allow the protection of at least a part of the brain tissue (Cheng et al. 2004). Theoretically, a neuroprotector should be the antagonist of several damaging factors at once (Ginsberg 2003).

In these experimental conditions, only Semax abated the manifestation of ischemic damage to the nervous tissue. In our view, the protective action of Semax on nervous tissue is explicable by several mechanisms. First, alleviation of the symptoms of vascular stasis during disturbances in brain blood circulation provides a histologically verifiable neuroprotective action. According to the available data (Cherkasova et al. 2001), the antithrombotic and anticoagulant action of Semax might play a significant role under conditions of ischemic damage. By preventing the aggregation and formation of erythrocyte debris in the microcirculatory channels, leading to reduced brain blood perfusion, Semax is likely to contribute to nervous tissue oxygenation and to hamper the development of secondary destructive reactions. Second, increased PCNA expression in the cell nuclei of ependymocytes, neuroglia, and blood vessel endothelium after Semax administration to animals of both control and experimental groups testifies to the stimulatory effect of this peptide on cells directly involved in the trophic supply of the CNS. Remarkably, increased PCNA expression was recorded as soon as 15 min after Semax

treatment, both in control rats and during the acute period of blood circulation disturbance. There are grounds to believe that Semax activates immediate early genes and thereby triggers the signal transduction cascade that stimulates cell proliferation. Studies on the effects of Semax on the expression of the immediate early gene c-fos, whose protein product plays an important role in cell proliferation and differentiation, support this hypothesis. This protein product was increased in the rat brain in response to the intraperitoneal introduction of Semax (Umriukhin et al. 2001; Dmitrieva et al. 2008). Finally, the trophic effect of this peptide might involve the expression of neurotrophic genes directly involved in homeostatic regulation in the CNS. Thus, Semax stimulates the transcription of mRNA for neurotrophins and their receptors in the CNS of animals subjected to experimental ischemia (Dmitrieva et al. 2010).

According to our data, proliferation of a pool of progenitor cells in the area of the lateral ventricles is activated soon after Semax treatment. The subventricular zone (SVZ) of the lateral ventricles is believed to constitute one of the main stem cell niches in the adult brain (Abrous et al. 2005; Faiz et al. 2005; Tsyb et al. 2009). This subependymal zone stretching along the entire wall of the lateral ventricles is a reservoir for the largest population of dividing cells in the adult mammalian brain. Cells migrate from here through the rostral migration stream to the olfactory bulb. New neurons that leave the neurogenesis region of the SVZ can also migrate to other destinations where they get integrated into the nervous network. Pathological events occurring during adult brain damage and ischemic trauma stimulate neurogenesis in such germination zones. However, repopulating nerve stem cells show limited possibilities for the generation of new neurons in response to damage. Possibly, Semax stimulation of the proliferative activity of germination zone cells creates prerequisites for regeneration of the brain's damaged cellular and tissue structures under ischemic conditions.

Despite the unidirectional effect of Semax and its C-end fragment on the capillary network, the proliferative activity of cells, and the morphology of neurons, we discovered no neuroprotective action of PGP on brain tissues in the early phase of ischemia. Unlike Semax, PGP induced the proliferation of progenitor cells during later periods. Therefore, we cannot rule out a delayed or prolonged effect of this peptide on repair processes in nervous tissue.

Thus, among two peptides studied here, only Semax exerted a neuroprotective effect in the acute period of ischemia by reducing the ischemic damage and preventing the local tissue destruction. The effects of Semax revealed here and evident differences between animal groups argue for more extended morpho-functional research of Semax action on mobilization of adaptive repair processes in CNS compared with this pilot-sized study.

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