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Semax-Induced Changes in Growth Factor mRNA Levels in the Rat Brain on the Third Day After Ischemia

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Abstract The peptide Semax effectively protects brain tissues against ischemic stroke. However, the molecular mechanisms that underlie its action remain unknown. We used the focal cerebral ischemia rat model with permanent middle cerebral artery occlusion (pMCAO). During the experiments, animals were given intraperitoneal injections of Semax, Pro-Gly-Pro, or saline. We studied the effect of the peptides on the expression of more than 80 growth factor genes in the cortex. As a response to Semax administration, alterations in the expression of growth factor genes were detected at 3, 24, and 72 h after pMCAO. The most pronounced effects of Semax, i.e., the down-regulation of the transcripts of 20 genes and the upregulation of 12 growth factor genes, were observed 3 days after artery occlusion. According to our data, Semax promoted the upregulation (by ≥10-fold) of the *Csf3* and *Artn* genes, as well as of the cytokine genes *Il1b* and *Il6*. The peptide products of these genes have regulatory properties and exert neuroprotective effects in injured brain tissues. We presume that Semax triggers neuroprotective mechanisms by affecting these systems via the regulation of the expression of growth factor genes.

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Abbreviations

ACTH	Adrenocorticotropic hormone
PGP	Tripeptide Pro-Gly-Pro
MCA	Middle cerebral artery
pMCAO	Permanent left middle cerebral artery occlusion
CNS	Central nervous system
VEGF	Vascular endothelial growth factor
PCR	Polymerase chain reaction
REST	Relative expression software tool
Th1 and Th2	Type 1 and 2 helper T-cells

Introduction

Ischemic brain stroke is a major contributor to mortality and disability worldwide. This condition arises as a consequence of a critical reduction in blood flow in brain tissues and leads to a massive death of neurons and the formation of the necrotic core and the penumbra zone, where the energy metabolism is retained and is functional; however, no structural changes are observed (Liu et al. 2010). The peptide Semax (Met-Glu-His-Phe-Pro-Gly-Pro), which is a synthetic peptide consisting of the ACTH(4–7) fragment and the C-terminal tripeptide Pro-Gly-Pro (PGP), is currently used effectively in the treatment of ischemic brain stroke. Semax exhibits neuroprotective properties (Storozhevykh et al. 2007) and contributes to the survival of neurons during both

hypoxia (Kaplan et al. 1992) and glutamate neurotoxicity (Storozhevykh et al. 2007). Physiological experiments have shown that Semax reduces the manifestations of neurological deficits and raises the survivability of animals with ischemic brain stroke (Miasoedov et al. 1999). The peptide effectively protects nerve tissues against diseases of the optic nerve (Polunin et al. 2000), possesses nootropic activity (de Wied 1997), and acts as an antidepressant (Vilenskiĭ et al. 2007). The effect of Semax on the expression of neurotrophins, which are the most important regulators of neuronal function and survival, was demonstrated in cell culture and confirmed in intact animals (Dolotov et al. 2006; Shadrina et al. 2001).

Concomitantly, its C-terminal tripeptide PGP is effective on its own (Badmaeva et al. 2006) and exhibits cytoprotective properties (Martynova et al. 2009). It has also been shown that PGP attenuates behavioral stress-induced abnormalities, which indicates the ability of the tripeptide to influence the CNS structures that are involved in the formation of the organism's response to stress factors (Badmaeva et al. 2006) and to alter the behavior of experimental animals (Kopylova et al. 2007). The tripeptide is the most stable fragment of Semax during its degradation in the rat brain (Shevchenko et al. 2013). Not long ago, we reported the alteration of the expression of neurotrophic factor genes (Dmitrieva et al. 2010; Stavchansky et al. 2011), of genes of the *VEGF* family (Medvedeva et al. 2012), and of several genes that participate in immune system functioning (Medvedeva et al. 2014). Notwithstanding the numerous studies published, the molecular mechanisms that underlie the action of Semax and the degree of involvement of the tripeptide PGP in these metabolic pathways remain unknown.

In parallel with the destructive ischemia-induced processes, compensatory effects aimed at restoring brain functioning have been observed. These are different processes that include neurogenesis, angiogenesis, and axonal plasticity alterations (Maćkowiak et al. 2004; Maulik and Thirunavukkarasu 2008; Schultz and Grant 1991). Growth factors are actively involved in the regulation of all of these processes (Maćkowiak et al. 2004; Naylor et al. 2005; Schultz and Grant 1991). Preclinical and clinical data have shown that growth factor therapy improves the condition of patients with ischemic stroke and reduces the damaged area of the brain (Lanfranconi et al. 2011). Previously, we showed the effect of Semax on the transcriptome in experimental ischemia conditions, and we continued studying the effect of this drug and of PGP on the expression of a considerably larger number of growth factor genes that encode proteins that play a vital role in various biological processes.

Methods

Animals

We used adult male Wistar rats (250–350 g) maintained on a 12 h light/dark cycle at a temperature of 22–24 °C with free access to food and water.

Experimental Groups

Animals were divided randomly into four groups: (1) "ischemia", (2) "ischemia + Semax", (3) "ischemia + PGP" and (4) "sham-operated" groups. The permanent middle cerebral artery occlusion (pMCAO) was performed in "ischemia", "ischemia + Semax" and "ischemia + PGP" animals, whereas sham-operated animals underwent similar surgery without arterial occlusion. During the experiment, "ischemia + Semax" and "ischemia + PGP" animals were given intraperitoneal injections of Semax (100 μg/kg) or PGP (37.5 μg/kg), respectively, whereas "sham-operated" and "ischemia" animals were injected with saline (0.9 % NaCl). The first injection of Semax, PGP or saline was performed 15 min after pMCAO. The next injections were performed over 3 days: three times at 4 h intervals in the daytime. The interval between the injections delivered at night was 16 h. The peptide Semax was synthesized in the Institute of Molecular Genetics, Russian Academy of Sciences, Moscow. Commercially available in USA and Worldwide: Ceretropic (http://www.ceretropic.com/semax/), in EU and UK: SmartNootropics (http://www.smartnootropics.co.uk/semax-nootropic-peptide-30mg/).

Focal cerebral ischemia was induced as previously described (Dmitrieva et al. 2008). The irreversible electrical coagulation of the distal segment of the left middle cerebral artery was performed (Chen et al. 1986) under anaesthesia with chloral hydrate (300 mg/kg).

The rats were decapitated under anesthesia with ethyl ether 3, 24 and 72 h after the operation. Each time point included at least five animals. We isolated the frontoparietal cortex of the ischemic animals, in which, according to histological analysis of our earlier research, the damaged area was localized (Dmitrieva et al. 2008).

Real-Time RT-PCR

Total RNA was isolated from tissue samples (Chomczynski and Sacchi 1987) and complementary DNA (cDNA) was synthesized using the RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas Life Sciences), in accordance with the supplier's recommendations. Changes in the transcriptional expression patterns of genes were analyzed

using real-time RT-PCR, with RT^2 Profiler PCR Array System (PARN-041Z) designed by SABiosciences (USA), allowed to study the content of the 84 transcripts of growth factor genes. The actin (*Actb*), ribosomal protein, large, P1 (*Rplp1*), hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), ribosomal protein L13a (*Rpl13a*) and lactate dehydrogenase (*Ldha*) genes were used as internal controls. Whole gene list of RT^2 Profiler PCR Array System (PARN-041Z) see at: http://www.sabiosciences.com/rt_pcr_product/HTML/PARN-041Z.html.

The reaction mixture for PCR (25 μL) contained a sample of reverse transcription, forward and reverse primers (0.05 μM each primer), 2.5× ready-to-use PCR buffer (Synthol, Russia), consisting of Taq DNA polymerases, dNTPs, and the intercalating dye SYBR Green I. Reactions were carried out on StepOnePlus (Applied Biosystems, USA), using the following real-time PCR protocol: (1) denaturation at 95 °C for 10 min; (2) amplification with a single fluorescence measurement at 95 °C for 15 s, 60 °C for 1 min (45 cycles); (3) melting-temperature analysis of PCR products. Each time point included at least five animals and for each sample, the RT-PCR array was repeated three times. The default settings of the StepOnePlus (Applied Biosystems, USA) software were used to determine the threshold cycle (C_t). The statistical significance ($P < 0.05$) of the differences in the mRNA expression levels of the genes examined between groups was analysed via a randomization test using the Relative Expression Software Tool-384 (REST-384 ©) [details can be viewed at http://rest.gene-quantification.info/]. This software performs group-wise comparisons and statistical analyses of gene expression (target) normalized to a reference gene (ref). The expression ratio is given by:

$$R = 2^{\Delta Ct(target) - \Delta Ct(ref)}.$$

The relative expression ratio (R) of a target gene is computed, based on its real-time PCR efficiency (=2) and the crossing point (C_t) difference (Δ) of an unknown sample versus a control (Pfaffl et al. 2002).

Results

To study the effect of the peptides on the expression of interleukin genes (*Il1a*, *Il1b*, *Il2*, *Il6*, *Il7*, *Il11*, *Il12a*, and *Il18*), the family of fibroblast growth factors (*Fgf1–11*, *Fgf13–15*, *Fgf17*, *Fgf18*, and *Fgf22*), the superfamily of the transforming growth factor beta (*Tgfb*; *Bmp1–8a*, *Bmp10*, *Inha*, *InhbA, InhbB*, and *Lefty1*), and other genes, the gene panel "RTI Profiler^{TM} PCR Arrays Rat Growth Factors (PARN-041Z)" from SABiosciences was used. The expression of these genes in ischemia conditions under the action of peptides has not been studied. Data on the expression changes of the studied genes were recorded at three time points, i.e., 3, 24, and 72 h after the coagulation of the left middle cerebral artery (MCA). Sham-operated rats were used as controls in the analysis of gene expression alterations in ischemic animals. Concomitantly, the control group for Semax- or PGP-treated animals was a group of animals with ischemia. The results of gene expression variation (upregulation or downregulation) by twofold are shown in Tables 1, 2 and 3.

Analysis of Gene Expression 3 h After pMCAO

Three hours after the setup of the experiment, 17 growth factor genes exhibited altered expression under ischemia (Table 1). The expression of nine of them (*Il18*, *Gdnf*, *Bmp5*, *Bmp10*, *Inha*, *InhbB*, *Cxcl12*, *Bdnf*, and *Fgf2*) was increased compared with the expression level of the corresponding genes in sham-operated rats. The level of the *Il18* gene transcript was elevated most markedly, i.e., by fourfold, whereas the expression of eight genes (*Ereg*, *Fgf1*, *Fgf10*, *Tdgf1*, *Nodal*, *Lefty1*, *Il1b*, and *Il2*) was decreased by ≥2-fold after the occlusion of MCA compared with the control (Table 1).

In conditions of ischemia, Semax altered the expression level of 12 genes if the effect of ischemia itself was taken into consideration. Although the peptide exerted no action on genes that were upregulated in response to ischemia, it significantly raised the level of the transcripts of four genes (*Artn*, *Bmp8a*, *InhbA*, and *Spp1*). In ischemia conditions, the hexapeptide was effective in decreasing the expression of eight genes. The level of the transcripts of five of these genes (*Fgf3*, *Fgf13*, *Hgf*, *Lep*, and *Ntf3*) was not affected by the occlusion, whereas the expression of three of these genes (*Ereg*, *Tdgf1*, and *Fgf10*) was downregulated to an even greater extent (Table 1).

Three hours after pMCAO, and relative to the effect of ischemia, PGP significantly increased the expression of five genes: *Bdnf*, *Fgf2*, *Igf2*, and *Zfp9* were upregulated by twofold, while the expression of *InhbA* was upregulated by fivefold. Moreover, the expression of only one gene, *Csf3*, was reduced by threefold compared with the control. We must note that, at this time point, the effect of PGP on the gene that encodes the protective protein Spp 1 was close to the effect of Semax; however, the mean value of the change in its level of expression did not reach 2.0 (Table 1).

Analysis of Gene Expression 24 h After the Occlusion of the MCA

Twenty-four hours after MCA coagulation, ischemia affected the expression of 26 growth factor genes (Table 2), and the expression level of five genes (*Spp1*,

Table 1 Alteration in the expression of growth factor genes during focal ischemia in the presence of physiological solution and under the action of Semax or of the tripeptide PGP in the rat brain 3 h after pMCAO

Refseq ID	Gene Simbol	Ischemia 3ч	Ischemia + Semax 3ч	Ischemia + PGP 3ч
NM_019165	*Il18*	**4.08*** ↑	0.67	1.37
NM_001031824	*Bmp10*	**2.05*** ↑	1.07	1.30
NM_001108168	*Bmp5*	**2.40*** ↑	0.66	1.96
NM_022177	*Cxcl12*	**2.09*** ↑	0.78	1.21
NM_019139	*Gdnf*	**2.48*** ↑	0.63	1.62
NM_012590	*Inh a*	**2.11*** ↑	1.25	1.43
NM_080771	*Inh bB*	**2.06*** ↑	0.72	1.34
NM_012513	*Bdnf*	**2.11*** ↑	1.39	**2.21*** ↑
NM_019199	*Fgf2*	**2.07*** ↑	1.76	**2.14*** ↑
NM_001169120	*Zfp91*	1.11	1.10	**2.10*** ↑
NM_031511	*Igf2*	1.06	1.26	**2.55*** ↑
NM_017128	***InhbA***	1.27	**3.28*** ↑	**5.73*** ↑
NM_053397	*Artn*	1.11	**3.56*** ↑	1.81
NM_001109432	*Bmp8a*	0.99	**3.33*** ↑	1.30
NM_012881	*Spp1*	1.61	**2.17*** ↑	1.82
NM_001106394	*Nodal*	**0.47*** ↓	0.86	1.52
NM_001109080	*Lefty1*	**0.47*** ↓	1.02	1.19
NM_053836	*Il2*	**0.43*** ↓	0.54	0.59
NM_031512	*Il1b*	**0.38*** ↓	1.29	1.42
NM_012952	*Fgf1*	**0.45*** ↓	0.80	1.24
NM_021689	*Ereg*	**0.48*** ↓	**0.28*** ↓	0.69
NM_012846	*Fgf10*	**0.42*** ↓	**0.19*** ↓	0.77
XM_001056317	*Tdgf1*	**0.39*** ↓	**0.35*** ↓	1.18
NM_130816	*Fgf13*	1.47	**0.46*** ↓	0.89
NM_130751	*Fgf3*	0.41	**0.43*** ↓	1.16
NM_017017	*Hgf*	1.21	**0.42*** ↓	0.98
NM_031073	*Ntf3*	0.56	**0.30*** ↓	1.02
NM_013076	*Lep*	1.71	**0.27*** ↓	0.73
NM_017104	*Csf3*	0.59	0.54	**0.28*** ↓

Gene expression analysis was performed using real-time RT-PCR. Sham-operated animals provided a control for the "ischemia group" animals, and ischemized animals were controls for the "ischemia + Semax" and "ischemia + PGP" groups. Statistically significant alterations in gene expression, i.e., ≥2-fold compared with control animals, are shown in bold type. The gene symbols of the genes that exhibited altered expression by ≥5-fold are also indicated in bold type. *Statistical significance was set at $P < 0.05$. The ↑ and ↓ *arrows* indicate up- or downregulation of gene expression, respectively

Igf2, *Lep*, *Clcf1*, and *S100a6*) was upregulated by several fold compared with the expression level of the same genes in sham-operated animals; the remaining 21 growth factor genes were downregulated by 2–4-fold (Table 2).

Twenty-four hours after the operation, Semax affected the expression of 11 growth factor genes, three of which (*Spp1*, *Lep*, and *Lif*) exhibited significantly altered expression levels under the effect of ischemia. Ischemia resulted in an increase in the level of transcripts of the genes *Spp1* (8.43) and *Lep* (5.99) and a decrease in the level of *Lif* (0.40) transcripts (Table 2). Compared with the elevated expression levels observed during ischemia, Semax significantly increased the expression of *Spp1* to an even greater extent (2.28), whereas the expression of *Lep* and *Lif* was, under the same conditions, decreased by 3- and 4-fold, respectively (Table 2). Although Semax produced no effect on the majority of the genes that exhibited a low expression during ischemia, it downregulated the transcription level of eight genes, namely, *Cxcl1*, *Fgf2*, *Fgf3*, *Fgf8*, *Ereg*, *Tdgf1*, *Ntf3*, and *Il3* (Table 2).

Twenty-four hours after the operation, PGP affected the expression of only four growth factor genes compared with the animals in the "ischemia" group, who had been administered physiological solution (Table 2). Compared

Table 2 Alteration in the expression of growth factor genes during focal ischemia in the presence of physiological solution and under the action of Semax or of the tripeptide PGP in the rat brain 24 h after pMCAO

Refseq ID	Gene simbol	Ischemia 24 h	Ischemia + Semax 24 h	Ischemia + PGP 24 h
NM_012881	***Spp1***	**8.43*** ↑	**2.28*** ↑	**2.05*** ↑
NM_031511	***Igf2***	**7.76*** ↑	1.04	0.95
NM_013076	***Lep***	**5.99*** ↑	**0.31*** ↓	1.19
NM_207615	***Clcf1***	**5.36*** ↑	0.52	0.89
NM_053485	*S100a6*	**2.17*** ↑	0.97	0.56
XM_343148	*Gdf11*	**0.48*** ↓	0.67	1.09
NM_030859	*Mdk*	**0.40*** ↓	1.02	0.62
NM_017128	*InhbA*	**0.36*** ↓	0.76	1.56
NM_131908	*Fgf7*	**0.34*** ↓	0.67	0.48
NM_133519	*Il11*	**0.39*** ↓	0.59	**0.42*** ↓
NM_022196	*Lif*	**0.40*** ↓	**0.25*** ↓	0.62
NM_031073	*Ntf3*	0.77	**0.46*** ↓	0.94
NM_019199	*Fgf2*	0.88	**0.45*** ↓	0.75
NM_030845	*Cxcl1*	0.54	**0.45*** ↓	0.57
XM_001056317	*Tdgf1*	0.61	**0.41*** ↓	0.77
NM_021689	*Ereg*	0.68	**0.37*** ↓	1.37
NM_031513	*Il3*	0.83	**0.32*** ↓	0.93
NM_022182	*Fgf8*	1.86	**0.31*** ↓	1.13
NM_130751	*Fgf3*	0.80	**0.27*** ↓	1.02
NM_013110	*Il7*	**0.50*** ↓	1.86	1.19
XM_001066344	*Gdf5*	**0.49*** ↓	1.48	0.60
NM_012671	*Tgfa*	**0.49*** ↓	0.80	1.10
NM_031512	*Il1b*	**0.48*** ↓	1.05	0.74
NM_013107	*Bmp6*	**0.47*** ↓	0.93	0.90
NM_022223	*Fgf15*	**0.47*** ↓	0.39	0.68
NM_031588	*Nrg1*	**0.47*** ↓	0.71	0.99
NM_022177	*Cxcl12*	**0.42*** ↓	0.89	0.90
NM_053428	*Fgf14*	**0.41*** ↓	0.70	1.20
NM_024375	*Gdf10*	**0.41*** ↓	1.01	0.96
NM_019124	*Rabep1*	**0.37*** ↓	0.86	1.39
NM_012827	*Bmp4*	**0.35*** ↓	0.72	0.65
NM_019139	*Gdnf*	**0.30*** ↓	0.84	1.97
NM_130816	*Fgf13*	**0.26*** ↓	0.79	0.87
NM_178866	*Igf1*	**0.24*** ↓	0.96	1.49
NM_017104	*Csf3*	1.04	0.56	**0.46*** ↓
NM_012589	*Il6*	0.92	0.59	**0.28*** ↓

See the note in Table 1

with the control, the tripeptide upregulated the *Spp1* gene by twofold, thus producing an effect that was similar to that of Semax (Table 2). PGP also exerted a downregulating effect on the transcription levels of *Il6* and *Csf3* and on the expression of *Il11*, which was already decreased by ischemia (Table 2). A reliable PGP-induced downregulation of *Lif* expression was also recorded, but the average value of change in its expression did not reach twofold (0.62) (Table 2; Fig. 1).

Analysis of the Expression of the Genes 72 h After pMCAO

Seventy-two hours after pMCAO in the rat brain, 24 genes exhibited altered expression levels (Table 3). Half of these genes were upregulated by 2–9-fold, whereas the remaining genes were downregulated compared with the levels observed in the sham-operated animals. The transcription level of *Spp1* was elevated most markedly, by ninefold,

Table 3 Alteration in the expression of growth factor genes during focal ischemia in the presence of physiological solution and under the action of Semax or of the tripeptide PGP in the rat brain 72 h after pMCAO

Refseq ID	Gene simbol	Ischemia 72 h	Ischemia + Semax 72 h	Ischemia + PGP 72 h
NM_053397	***Artn***	1.63	**12.33*** ↑	**3.55*** ↑
NM_053428	*Fgf14*	**3.23*** ↑	**0.26*** ↓	**0.26*** ↓
NM_022177	*Cxcl12*	**3.99*** ↑	**0.46*** ↓	0.66
NM_013107	*Bmp6*	**3.80*** ↑	**0.42*** ↓	0.60
NM_001108168	*Bmp5*	**2.32*** ↑	**0.23*** ↓	1.00
NM_012881	***Spp1***	**9.56*** ↑	0.91	0.73
NM_023981	***Csf1***	**5.05*** ↑	1.40	0.82
NM_017105	*Bmp3*	**4.09*** ↑	0.55	0.73
NM_012951	*Fgf11*	**3.92*** ↑	0.74	0.60
NM_019305	*Fgf22*	**2.86*** ↑	0.53	0.54
NM_012827	*Bmp4*	**2.58*** ↑	0.61	1.54
XM_342591	*Bmp7*	**2.24*** ↑	1.32	0.77
NM_001106394	*Nodal*	**0.49*** ↓	0.43	1.05
XM_001056317	*Tdgf1*	**0.50*** ↓	0.72	1.03
NM_022223	*Fgf15*	**0.49*** ↓	1.01	0.75
NM_012590	*Inha*	**0.45*** ↓	1.02	0.75
NM_130751	*Fgf3*	**0.40*** ↓	0.78	1.01
NM_017019	*Il1a*	**0.32*** ↓	2.98	1.55
NM_201270	*Il4*	**0.19*** ↓	2.24	1.51
NM_031512	***Il1b***	**0.48*** ↓	**28.95*** ↑	1.52
NM_017104	***Csf3***	**0.40*** ↓	**23.05*** ↑	0.51
NM_012952	*Fgf1*	**0.45*** ↓	**2.10*** ↑	0.76
NM_012589	***Il6***	0.70	**9.78*** ↑	0.58
NM_021578	*Tgfb1*	**2.04*** ↑	**3.28*** ↑	0.98
NM_053485	*S100a6*	1.06	**2.92*** ↑	1.64
NM_013174	*Tgfb3*	0.83	**2.37*** ↑	1.42
NM_207615	*Clcf1*	0.81	**2.33*** ↑	1.62
NM_019198	*Fgf18*	1.06	**2.19*** ↑	1.17
XM_227525	*Ngfb*	0.54	**2.11*** ↑	1.32
NM_133519	*Il11*	0.92	**2.52*** ↑	**0.46*** ↓
NM_017178	*Bmp2*	1.21	**0.36*** ↓	**0.34*** ↓
NM_019139	***Gdnf***	4.67	**0.35*** ↓	**0.09*** ↓
NM_012842	*Egf*	1.89	**0.31*** ↓	**0.36*** ↓
XM_343148	*Gdf11*	2.38	**0.29*** ↓	**0.31*** ↓
NM_017017	*Hgf*	3.77	**0.26*** ↓	**0.39*** ↓
NM_021843	*Kitlg*	1.49	**0.21*** ↓	**0.24*** ↓
NM_031511	*Igf2*	**0.49*** ↓	**0.31*** ↓	**0.40*** ↓
NM_012846	*Fgf10*	**0.48*** ↓	**0.31*** ↓	1.11
NM_080771	*InhbB*	2.84	**0.50*** ↓	0.64
NM_031513	*Il3*	0.62	**0.49*** ↓	1.45
NM_012671	*Tgfa*	1.16	**0.37*** ↓	0.68
NM_031073	*Ntf3*	0.74	**0.32*** ↓	0.73
NM_019124	*Rabep1*	1.93	**0.30*** ↓	0.58
NM_053836	*Il2*	0.52	**0.30*** ↓	0.45
NM_130816	*Fgf13*	1.63	**0.26*** ↓	0.52
NM_019151	*Gdf8*	1.83	**0.29*** ↓	**2.10*** ↑
NM_131908	*Fgf7*	1.65	0.53	**0.48*** ↓
NM_001169120	*Zfp91*	1.59	0.60	**0.41*** ↓

Table 3 continued

Refseq ID	Gene simbol	Ischemia 72 h	Ischemia + Semax 72 h	Ischemia + PGP 72 h
NM_012513	*Bdnf*	0.68	1.09	**0.41*** ↓
NM_022196	*Lif*	1.60	1.34	**0.40*** ↓

See the note in Table 1

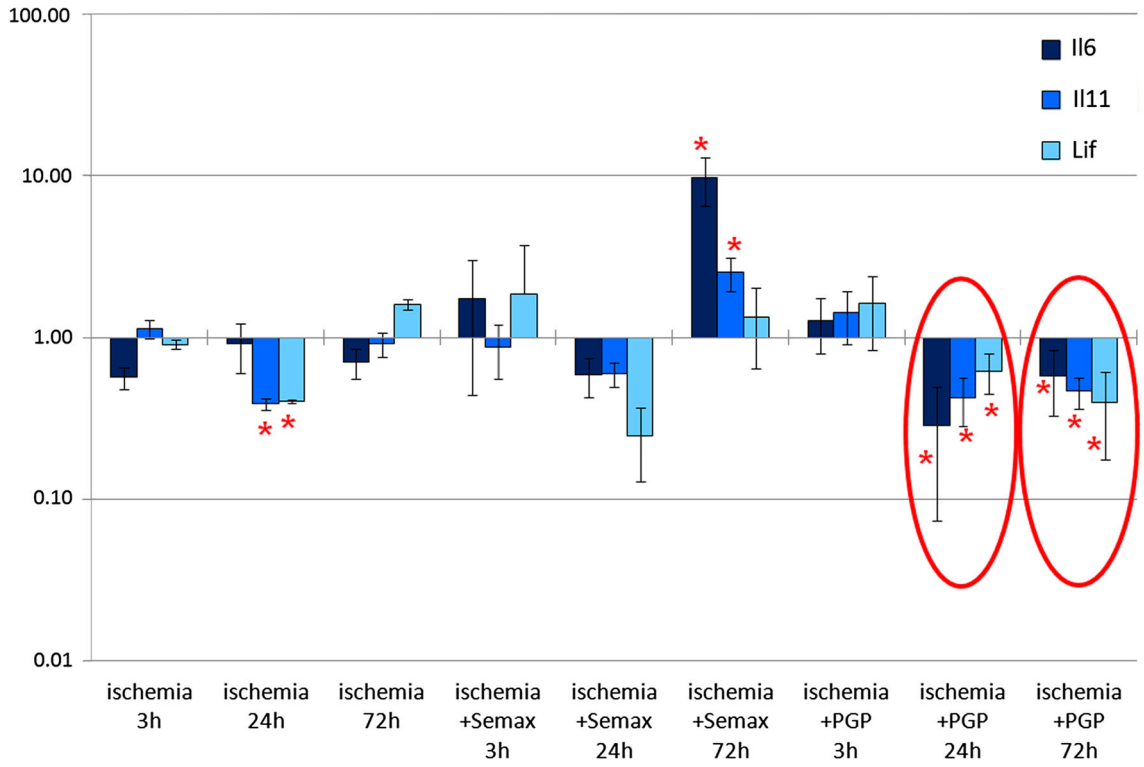

Fig. 1 Expression analysis of the Il-6-like cytokine gene group in ischemia upon administration of Semax and tripeptide PGP. Gene expression analysis was conducted by the real-time RT-PCR method. Sham-operated animals were the control for the animals of the "ischemia" group, the group of ischemized animals were the control for the "ischemia + Semax" and "ischemia + PGP" groups. The level of mRNA of the genes studied was normalized to the internal control genes *Rplp1, Rpl13a, Hprt1, Ldha* and *Actb.* The data are presented in relative units with standard deviation (±); *statistically significant difference ($p < 0.05$)

whereas the largest decrease in expression (by fivefold) was observed for transcripts of *Il4* (Table 3).

Under the action of Semax, the expression of 32 genes was altered 72 h after occlusion (Table 3). Periodic administration of the peptide upregulated the expression of 12 growth factor genes, with the largest variation in expression (by ≥10-fold) detected in four genes: *Il6*, *Artn*, *Csf3*, and *Il1b* (Table 3). We observed the Semax-induced downregulation of 20 growth factor genes (Table 3). The effect of Semax on seven of these genes (*Il1b*, *Csf3*, *Fgf1*, *Fgf14*, *Cxcl12*, *Bmp5*, and *Bmp6*) was opposite to that of ischemia (Table 3).

PGP changed the levels of the transcripts of 15 genes 72 h after ischemia onset (Table 3). At this time point, the effect of the tripeptide overlapped significantly with that of Semax (Table 3). Under the action of PGP, increased expression of growth factor genes compared with the expression of the genes in sham-operated animals was found for only two genes, *Artn* and *Gdf8*; in contrast, *Gdnf* was downregulated by more than tenfold (Table 3). The effect of Semax and PGP on the *Il11* and *Gdf8* genes was opposite: Semax increased the level of *Il11* gene transcripts (by 2.52-fold) and decreased the expression of the *Gdf8* gene (by 0.29-fold); the action of the tripeptide on these two genes was inverse (Table 3). PGP also acted differently compared with Semax on several genes that encode Il-6-like cytokines (*Il6*, *Il11*, and *Lif*) represented on the panel used here (Fig. 1).

Discussion

Selection of Time Points for the Analysis of Gene Expression

We studied the expression of growth factor genes under the action of Semax and its C-terminal tripeptide (PGP) 3, 24, and 72 h after the occlusion of the left MCA. Brain ischemia initiates a complicated cascade of events that occur at the molecular and cellular levels. According to the literature, excitotoxicity, mitochondrial derangements, emergence of active oxygen forms, and apoptosis occur during the first 3 h after occlusion (Amantea et al. 2009). We used this time point to study gene expression in the early stage of ischemia progression. Up to 24 h, the area of the brain stroke reaches its maximal size; moreover, the formation of the penumbra, which is a zone with a conserved energy metabolism and a low level of intracellular potassium, ends at this time (Astrup et al. 1981). The brain stroke area continues growing during the period from 24 to 72 h postischemia, albeit more slowly compared with the growth observed during the first day: the damage expands to the penumbra area because of inflammation processes induced by microglia and astroglia (Centonze et al. 2007; Lipton 1999). Thus, the time points that were selected here for experimentation allowed the analysis of the effect of the peptides studied on gene function at significant stages of postischemia events.

Impact of Ischemia on the Expression of Growth Factor Genes

Growth factor genes have long been the object of research in models of cerebral ischemia. Data on the expression alteration of growth factor genes during cerebral ischemia are essentially different. These differences depend on the model chosen (methods and duration of vessel occlusion, anesthetics used, etc.), the method used for sample preparation, and the time interval after the onset of the experiment (Durukan and Tatlisumak 2007).

According to our data, the expression of 17 genes was reliably altered 3 h after pMCAO. Genes with enhanced transcription included a gene that encodes an anti-inflammation mediator (*Il18*) (Braeuninger et al. 2010; Hedtjärn et al. 2002) and growth factor genes with neuroprotective properties (*Gdnf*, *Bdnf*, and *Fgf2*) (Alzheimer and Werner 2002; Jiang et al. 2011; Wang et al. 2002).

As ischemia progressed, at 24 h after pMCAO we observed a predominant downregulation of growth factor genes: 21 out of the 26 genes that exhibited a significantly altered expression were downregulated (Table 2). However, the expression level of several genes (*Igf2*, *Spp1*, and *Lep*) that encode proteins that are involved in the regulation of postischemic pathogenic and immune processes, according to the literature, was considerably increased (Johnsen et al. 2005; Wang et al. 1998).

After 72 h, ischemia significantly affected the level of the transcripts of 24 genes; among them, equal numbers of growth factor genes were up- or downregulated (Table 3). At this time point, both active resident cells (microglia and astrocytes) and immune cells are involved in the secretion of growth factors (Amantea et al. 2009). The expression level of *Spp1* and *Csf1*, which was considerably enhanced at this time point, is likely to be involved in regeneration processes that occur in damaged brain tissues (Table 3) (Amantea et al. 2009; Luo et al. 2013; Meller et al. 2005; Sawada et al. 2009; Schäbitz et al. 2003; Wang et al. 2010; Zhang et al. 2009). Table 4 shows the data obtained and reported by a large number of researchers; these data testify to the neuroprotective properties (stimulation of angiogenesis, reduction of the size of the damaged area, and improvement of patients with brain stroke) of some growth factors encoded by the genes analyzed here.

Semax-Mediated Alteration in the Expression of Growth Factor Genes After pMCAO

It is well known that, 3 days after pMCAO and during the slowing down of the growth of the penumbra zone (Centonze et al. 2007; Lipton 1999), the activated microglia induce and maintain the inflammatory process at the core of ischemia. The downregulation of a large number of genes, which was observed after Semax administration at 72 h after pMCAO, probably reflected the inhibition of microglia and of glial inflammatory reactions. Interestingly, during the periods mentioned, we found no noticeable effect of the preparation on the expression of the genes that exhibited an altered response to ischemia in animals who received physiological solution. The peptide predominantly affected the expression of genes that showed no pronounced effect of ischemic damage (Tables 1, 2, 3). We presume that the effect of Semax triggers additional protective mechanisms in damaged cells or activates groups of cells that had not been exposed to ischemia.

Seventy-two hours after pMCAO, a Semax-induced response was observed for *Csf3* and *Artn* and for the cytokine genes *Il1b* and *Il6*, which were upregulated by $\geq$10-fold. According to the literature, the products of the *Csf3* and *Artn* genes exert a neuroprotective effect (Table 4). In our experiment, the expression level of the *Il1b* gene was considerably increased under the action of Semax during the first three postischemia days. A large number of studies devoted to this interleukin have described its effects in a contradictory manner. Several studies

Table 4 Growth factor effects on brain tissue in CNS lesions

Gene	Effects	References
Il1b	By affecting intracellular signaling pathways, Il1b induces the production of secondary mediators of inflammation: cytokines, chemokines, prostaglandins, and growth factors;	(Pinteaux et al. 2009) (Boutin et al. 2001) (John et al. 2004)
	Mice lacking both forms of Il1 (*Il1a* and *Il1b*) exhibited dramatically reduced ischemic infarct volumes compared with wild-type animals (total volume, 70 %; cortex, 87 % reduction);	(Argaw et al. 2006) (Wang et al. 2010)
	Il1b regulates astrogliosis after CNS damage;	(Carlson et al. 1999a)
	Il1b induces the neuronal secretion of Vegfa by astrocytes (Vegfa is a powerful inductor of BBB permeability and angiogenesis);	(Huang and Sheng 2010) (Naldini et al. 2011)
	Il1b modulates neuronal glutamate release and protects against glucose-oxygen-serum deprivation;	
	treatment of cortical neuronal cultures with recombinant Il1b confers a concentration-dependent neuroprotective effect against excitotoxic challenge with NMDA;	
	Il1b affects cognitive function (learning and memory);	
	via monocyte activation, Il1b is the principal regulator of Spp1-induced angiogenesis.	
Csf1	Csf1 regulates the survival, proliferation, and chemotaxis of macrophages, and supports their activation;	(Pixley and Stanley 2004) (Lampron et al. 2013)
	After preventive treatment of the tissues, Csf1 enhances the level of circulating bone-marrow-derived cells that migrate into the central nervous system in models of neurodegeneration, which leads to neuroprotection;	(Luo et al. 2013) (Erblich et al. 2011)
	Csf1 provides powerful neuroprotective and survival signals in forebrain neurons injured by kainic acid-induced excitotoxicity;	
	Csf1 and its receptor Csf1r are required for the development of microglia, for a fully functional olfactory system, and for the maintenance of a normal brain structure.	
Csf3	Csf3 has an antiapoptotic effect after focal cerebral ischemia in the rat;	(Solaroglu et al. 2007)
	Csf3 significantly reduces lesion size in transient, but not permanent, models of ischemic stroke;	(England et al. 2009) (Pan et al. 2010)
	Csf3 protects primary cortical neurons against excitotoxicity induced by glutamate;	(Schäbitz and Schneider 2006; Schäbitz et al. 2003; Solaroglu et al. 2006)
	Csf3 is a neuroprotective factor, as demonstrated by cerebral ischemia studies in vitro and in vivo;	(Lanfranconi et al. 2011)
	Csf3 reduces lesion size in rodent stroke models.	
Artn	Artn is a survival factor in sensory and sympathetic neurons and supports the survival of dopaminergic midbrain neurons in culture;	(Baloh et al. 1998) (Park and Hong 2006)
	the transcriptional regulation of Artn is related to neurite outgrowth and actin polymerization in mature DRG neurons;	(Sawada et al. 2009)
	presumably, Artn is able to reduce the size of the brain stroke area after MCAO.	
Il6	The expression of the gene that encodes the Il6 cytokine correlates with brain infarct volume, stroke severity, and long-term outcome;	(Smith et al. 2004) (Clark et al. 2000)
	Il6 does not have a direct effect on acute ischemic injury;	(Amantea et al. 2009; Carlson et al. 1999; Suzuki et al. 2009)
	Il6 has a neuroprotective action;	
	Il6 is an important endogenous inhibitor of neuronal death during permanent focal cerebral ischemia in the rat.	(Loddick et al. 1998)
Spp1	Incubation of cortical neuronal cultures with Spp1 protects against cell death from oxygen and glucose deprivation; the intracerebral ventricular administration of Spp1 caused a marked reduction in infarct size after transient MCAO in a murine stroke model;	(Meller et al. 2005) (Yan et al. 2009) (Mazzali et al. 2002)
	Spp1 is a mediator of the lateral migration of neuroblasts from the subventricular zone after focal cerebral ischemia;	
	Spp1 regulates inflammation and cell survival and inhibits *iNOS* gene expression.	

reported its role in the pathogenesis of brain ischemic damage (Boutin et al. 2001; Caso et al. 2007; Viviani et al. 2003). As an important mediator of the inflammatory response, Il1b participates in the activation of such processes as proliferation, differentiation, and apoptosis. Experimental models helped show that the blocking of its

binding to receptors considerably reduces CNS damage (Allan and Pinteaux 2003) and Il1b increases the sensitivity of neurons to hypoxia and excitotoxicity (Viviani et al. 2003). In parallel with the negative effects identified, a neuroprotective action of this interleukin was also detected (Table 4) (Fogal et al. 2005; Pinteaux et al. 2009; Strijbos and Rothwell 1995). Possibly, the activation of this cytokine at day 3, at which time the growth of the penumbra slows down and the postischemic regeneration of tissues starts (Centonze et al. 2007), produces, to a larger degree, a positive, rather than a negative, effect in cooperation with several neuroprotective growth factors (Table 4) (Carlson et al. 1999; Naldini et al. 2011).

At this time, the action of Semax considerably increased the level of the transcript of another cytokine, Il6 (Table 3; Fig. 1) (Centonze et al. 2007). The expression of the gene that encodes this cytokine is well correlated with the core size and clinical outcome of the cerebral stroke (Smith et al. 2004); however, this interleukin has no direct effect on CNS tissue damage in acute brain stroke (Clark et al. 2000). Moreover, some authors showed that the introduction of Il6 exerted a neuroprotective action (Table 4) (Amantea et al. 2009; Suzuki et al. 2009).

We must note that cytokine genes constitute a considerable part of the growth factor genes studied here. The growth factors Il11 and Lif, which, as well as Il6, are members of the family of neurotrophic Il-6-like cytokines (Guk and Kuprash 2011). Depending on cell type, Il11 contributes to both cellular death and survival (Zhang et al. 2011). The leukemia-inhibitory factor (Lif) is a pleiotropic cytokine that is involved in hematopoyesis and in endocrine system functioning (Auernhammer 2000). We did not observe any essential impact of Semax on *Il11* or *Lif* 3 and 24 h after pMCAO. However, the preparation increased the expression of the *Il11* gene (Table 3; Fig. 1) at 72 h, which was probably a consequence of the significant upregulation of the *Il1b* gene (Zhang et al. 2011).

PGP Impact on the Expression of Growth Factor Genes After pMCAO

As revealed in our study, the effect of PGP coincided only partially with the action of Semax on growth factor expression in ischemized tissues of the cerebral cortex. Special attention must be paid to the action of PGP, which was similar to that of Semax, on the *Spp1* gene 1 day after pMCAO. Interestingly, Spp1 expression was enhanced by ischemia, and Semax administration led to the maintenance of a high level of the Spp1 transcript after pMCAO at all time points of the experiment (Tables 1, 2, 3). This growth factor is a multifunctional regulator of inflammatory processes (Table 4) that triggers the expression of a great number of pro- and anti-inflammatory mediators (Mazzali et al. 2002) and is involved in the formation of Th1 and Th2 cells via Il6 (Diehl and Rincón 2002).

Concomitantly, the effect of the tripeptide on Il6-like cytokines was essentially different from the effect of Semax. PGP lowered the expression of Il6 family-like cytokine genes during the acute phase of brain stroke (up to 24 h) and 3 days after it. Taking into consideration the fact that this expression alteration was statistically significant, the tripeptide exerted a suppressive effect on the expression of *Il6*, *Il11*, and *Lif* at both 24 and 72 h postocclusion (Tables 2, 3; Fig. 1).

Conclusion

The list of growth factor genes that exhibited altered expression under the effect of Semax might point to diverse events involving these genes: neurogenesis, the vascularization of damaged tissues, and the immune response (Table 4). All of these processes exhibit cross talk. Angiogenesis and neurogenesis show similarities, and these two processes involve common growth factors (Font et al. 2010). Immune system cells participate in the formation of new blood vessels (Silvestre et al. 2008). Further research will show whether the neuroprotective effect observed is conditioned by the effect of Semax on these systems or whether it is predominantly caused by the action of the peptide on only one of them.

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Compliance with Ethical Standards

Conflict of Interest Authors E. V. Medvedeva, V. G. Dmitrieva, V. V. Stavchansky, O. V. Povarova, S. A. Limborska, N. F. Myasoedov and L. V. Dergunova declare that they have no conflict of interest.

Ethical approval All experimental protocols were approved by Bioethics Comission of Lomonosov Moscow State University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 80–23, revised 1996). This article does not contain any studies with human participants performed by any of the authors.

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