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Halogenated (Cl-ion) songorine is a new original agonist of fibroblast growth factor receptors of neuronal-committed progenitors possessing neuroregenerative effect after cerebral ischemia and hypoxia in experimental animals

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ABSTRACT

Alkaloids, which possess a stimulating effect on the functions of progenitors of various classes, were identified. The aim of this study was to identify mechanisms underlying development neuroregenerative effects of modified (halogenated (Cl-ion)) alkaloid songorine (mSON). To reveal the participation of neuronal-committed progenitors (clonogenic PSA-NCAM + cells) subventricular area of cerebral hemispheres (SVZ) in the realization of pharmacological action of this substance and to study the mechanisms of stimulating their functions under the influence of mSON. mSON was received by halogenation (Cl-) of alkaloid and administered to the experimental animals after modeling cerebral ischemia (rats) and posthypoxic encephalopathy (mice). Therapeutic effects of mSON were assessed with the help of functional and morphological methods. To study the effect of mSON on clonogenic PSA-NCAM + cells, the role of receptors to the growth factor of fibroblasts (FGFR) and intracellular signaling molecules of neuronal-committed progenitors in their implementation. Normalization of behavior and reflexive activity on the background of significant correction of morphological pattern malfunctions in the brain of experimental animals during mSON administration after modeling of brain pathology was revealed. The increase in the number of clonogenic PSA-NCAM + cells in SVZ was determined. It was shown a direct stimulating effect of mSON on neuronal-committed progenitors, which was abolished by the addition of anti-FGFR. Significant differences in intracellular signaling in clonogenic PSA-NCAM + cells while stimulating their functions with mSON and growth factor of fibroblasts were determined. These results suggested that mSON is a new original agonist of neuronal-committed progenitors FGFR. mSON is a promising one for the development of a safe and highly effective drug for neurological practice.

Keywords: *regenerative medicine, neural stem cells, target drugs, songorine, alkaloids, signaling transduction.*

1. INTRODUCTION

Ischemic blood supply disturbances of nervous tissue and other hypoxic states are the most common pathological condition of the central nervous system (CNS). Hypoxia of brain significantly reorganizes the CNS, changes the integrative-starting activity of neurons and leads to the formation of qualitatively new pattern of interaction between individual brain structures, and in the case of de-compensation of adaptation mechanisms, triggers a chain of pathological processes resulting in progressive neurological and cognitive disorders, including the development of encephalopathy [1-6]. Pharmacological action of existing neuroprotective agents consists mainly in protection or modulation of functions of mature cells of nervous tissue surviving in the pathology. However this concept of pharmacological intervention in some cases is proved to be untenable. Available drugs are often incapable completely to restore the morphofunctional state of brain as well as prevent the development of progredient nature of pathological process in the nervous tissue [1, 6, 7].

In connection with this, the development of fundamentally new pathogenetically grounded approaches to the treatment of central nervous system diseases and creation of original cerebroprotectors with qualitatively new mechanisms of action are relevant. The search for solutions to this problem is being carried out within the framework of regenerative medicine. Rapid development of cellular technology in recent decades allowed to implement significant "breakthrough" in understanding of stem cell (SC) biology and led to the possibility of developing new direction in the treatment of many diseases - cell therapy [1, 6-13]. In this case more physiological and perspective approach to solving the problems of regenerative medicine, including treatment of central nervous system diseases, is considered to be pharmacological stimulation of endogenous SC functions by imitation of the activity of natural regulatory systems [7].

During development of "Pharmacological strategy of regenerative medicine" at the Goldberg Research Institute of Pharmacology and Regenerative Medicine the principal possibility and high effectiveness of this approach to the therapy of a number of diseases, including pathological conditions of the central nervous system, were demonstrated in the experiment [1, 6, 7, 14-19].

Alkaloids, which possess a stimulating effect on the functions of progenitor cells of various classes, were identified [16, 18, 19]. Mechanisms of action of these substances are both a direct effect on the receptor apparatus of progenitor cells, and their influence on the elements of the microenvironment of tissues.

We isolated the diterpene alkaloid of tizidine-type songorine and revealed its stimulating effect on the mesenchymal progenitors associated with the effect on receptors for the growth factor of fibroblasts (FGFR) [18, 19]. The important role of this pleiotropic early-acting growth factor in the definition of proliferative-differentiation status of cells, including NSC, is known [14, 20, 21].

At the same time, in order to reduce the toxicity of alkaloid, we obtained a sample of chemically modified songorine with the use of standard technology [22] which possesses more pronounced stimulating effect on mesenchymal progenitors *in vitro* in comparison with growth factor of fibroblasts (FGF). Taking into account the foregoing, it is of great interest to study the therapeutic properties of modified alkaloid songorine in modeling diseases of the nervous system and the role of FGFR and intracellular signal transduction of NSC in the realization of its effects.

2. MATERIALS AND METHODS

2.1. Chemicals and Drugs.

mSON was obtained at the Goldberg research Institute of Pharmacology and Regenerative Medicine. The initial substance of songorine was obtained from Aconitum baicalense herb. The extraction of substance was carried out sequentially with the use of chloroform, sulfuric acid, ether, chloroform and then it was chromatographed on deactivated aluminium oxide in hexane-acetone system (90-50%).

The substance, which refers to the diterpene alkaloids of atizine type was identified, halogenated (Cl-- ion) by standard method [22] and dissolved in distilled water to a final concentration of 0.00025%. The molecular weight of the synthesized substance was 394 Daltons, and its LD50 in mice when administered per os was 142 mg / kg. DMEM (Dulbecco's Modified Eagle's medium), fetal bovine serum (FBS), fibroblast growth factor (FGF-basic, «Sigma», USA), anti-FGFR (anti-FGFR 1, clone VBS 1, Millipore, Germany), antibodies to the fibroblast growth factor receptor (anti-FGRF) (anti-FGFR 1, clone VBS 1, «Millipore», Germany); inhibitors of signaling molecules: NF-κB «oridonin»; NF-κB, IKK and PKC «aurotiomalate»; PI3K «LY294002»; ERK1/2 «PD98059»; p38 and PKB «SB203580»; adenylate cyclase «2',5'-dideoxyadenosine»; IKK «Inhibitor IV» (all manufactured by Calbiochem, USA); JNK «SP600125» (manufactured by InvivoGen, USA); p53 (Pifithrin-α, Cyclic, Santa Cruz Biotechnology, Inc. USA); JAK1,3 «CP 690550» (InvivoGen, USA); JAK2 «AG 490» (InvivoGen, USA); STAT3 «STAT3 Inhibitor XIV, LLL12» (Calbiochem, USA), plastic plates for cultural studies («Costar», USA).

2.2. Animals and Experimental Design.

All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. The study was approved by the Institute local Ethics Committee. Experiments were carried out on outbred rats (n=96) weighting 250-300 g and on 186 CBA/CaLac mice (n=186) weighing 18-20 g. Animals of the 1st category (conventional outbred rats and linear mice) were obtained from Experimental Biological Models Department of Goldberg Research Institute of Pharmacology and Regenerative Medicine (Tomsk, Russia) (certificate available). Before the beginning of experiments (during 10 days) and over the study period, animals were contained in vivarium (air temperature 20–220C, humidity 50-60 %) in plastic cages (6-8 rats and 10-15 mice) on a normal diet (solid diet pellets (Limited Liabilily Company «Assortiment Firm», Sergiev Posad sity, Russia), water ad libitum. In order to exclude seasonal fluctuations of studied parameters, all the experiments were performed in the autumn-winter period. The animals were removed from the experiment (sacrificed) using CO2 cameras, except for the groups of rats used for histological examination (these animals were killed by decapitation).

The cerebral ischemia [16] was induced in rats under ether anesthesia by complete ligation of the left carotid artery and restriction of blood flow in the right carotid artery by 50% by means of partial ligation of artery under the control of electromagnetic flow meter MFV-1100 (Nihon Kohden, Japan). Sham-operated animals were experienced similar surgery but without coronary artery ligation and were used as control animals. Experimental rats with cerebral ischemia and sham-operated animals received 10 mcg/kg mSON peroral through a catheter once a day for 5 days. On the 3rd day in an hour after mSON administration the registration of orienting-research behavior in the open field (during the first and two subsequent minutes separately) was recorded and conditioned reflex of passive avoidance was developed.

The functional asymmetry was assessed semi-quantitatively according to the width of palpebral fissure on the side of complete stenosis of carotid artery: 0 points - palpebral fissure is completely open; 1 point - palpebral fissure on the left is less by 1/3 compared with the right one; 2 points - palpebral fissure on the left is less than on the right one by 2/3; 3 points - palpebral fissure is completely closed (in the form of a slit); 4 points - palpebral fissure on the left is completely closed. The preservation of conditioned reflex of passive avoidance was examined on the 7th day of the study [1, 6, 16]. In addition, on the 7th day histological preparations from animals brain were studied.

Posthypoxic encephalopathy was modeled in mice with the help of 500-ml hermetic chamber. The mice were placed in the hermetic chamber until an agonal seizure or stopping of the breath. These facts were determined visually, after 10 to 15 minutes. After

removing from the hermetic chamber and recovery of self-breathing after 5-10 minutes, the mice were again placed in the hermetic chamber also to the beginning of agonial state (generalized seizure or stopping of the breath). Mice with posthypoxic encephalopathy received mSON orally through a catheter at a dose of 15 mcg / kg once a day for 5 days. On the 3rd, 7th, 14th, 21st days, registration of the orienting-research behavior in the open field was carried out and a conditioned reflex of passive avoidance was developed, the safety of which was tested on the 7th, 14th, 21st days of the study [1, 6, 16].

Experiments on the study of mechanisms of action of mSON (the role of FGFR and intracellular signaling molecules of neuronal-committed progenitors in their implementation) conducted on cell cultures of cells of mice.

2.3. Estimation of the animal psychoneurological status.

Psychopharmacological effects of mSON were evaluated by functional methods. The orientation-and-exploratory activity in the open field and conditioned reflex techniques of passive avoidance response (CPAR) was recorded automatically using Ambulatory test station (Model HSFOF) and Avoidance learning and memory test station (Model HAS1000) (Lafayette Instrument Co., USA) [16].

2.4 Histological study.

On the 7th day after modeling of brain ischemia the histological preparations of the brain (preparations were fixed in 10% neutral formalin, dehydrated in a series of alcohols with rising concentration, impregnated with paraffin and cut into pieces of 4-5 microns thick, stained with hematoxylin and eosin) were studied. The state of the microvasculature of meninges and brain substance, the presence and severity of perivascular and pericellular edema, neurons with signs of degeneration, necrosis (hyperchromic nuclei, with vacuolar dystrophy) in the state of phagocytosis were assessed.

2.5. Determination of the neuronal-committed progenitors content.

The content of neuronal-committed progenitors in the brain was determined on the 1st, 3rd and 7th days after the modeling of posthypoxic encephalopathy in mice. The specimens of nervous tissue were taken from subventricular area of cerebral hemispheres. To do this, use the «MiniMACS Cell Separator» (Miltenyi Biotec, Germany) by positive selection received PSA-NCAM +- cells (using the protocol, in accordance with the methodological instructions of the manufacturer) from the subventricular zone (SVZ). PSA-NCAM (CD56) is a marker for immature neuronal-committed progenitors that are permanently generated in the SVZ [23]. After that calculated the content of clonogenic PSA-NCAM + cells. For this PSA-NCAM + cells at a concentration of 105 / ml were incubated for 7 days at 37°C in humidified atmosphere of 95% air and 5% CO2 in «MACS Neuro Medium» (Miltenyi Biotec, Germany). After incubation, we counted content of neuronal-committed progenitors (clonogenic PSA-NCAM + cells) that formed neurospheres containing more than 100 cells (CFU-N) [24-27].

2.6. Study of the mSON action on neuronal-committed progenitors and secretion of growth factor by cells of nerve tissue *in vitro*. Method of cloning was used to study the *in vitro* the direct effect of alkaloid on the realization of the growth potential of neuronal-committed progenitors. For this purpose 2 ng/ml mSON was added in the liquid culture medium (see above) containing 105 / ml of PSA-NCAM + cells from the SVZ. The culture was incubated for 7 days in CO_2 incubator at 37°C in a humidified atmosphere of 100% and with the content of 5% CO_2. The similar culture containing an equimolar concentration of fibroblast growth factor (FGF-basic) ("Sigma", USA) was used as a comparison. After incubation, the number of CFU-N (see above) was counted. While determination of the secretory function of neural tissue cells the effect of conditioned media of a 2-day culture of cells from the SVZ obtained in a similar way on the level of formation of CFU-N from PSA-NCAM + cells in the test system – colony-stimulating activity (CSA) of supernatants was studied [15].

2.7. Study of the role of FGFR of neuronal-committed progenitors in the realization of mSON action *in vitro*.

In order to determine the role of FGF receptors in neurosphere formation *in vitro* in cell culture of test system containing PSA-NCAM + cells from the SVZ, the number of CFU-N, ClFU-N (neural claster-forming units), index of differentiation of CFU-N [19] was studied while adding 2 ng/ml of mSON and 2 ng/ml of mSON together with 20 μg/ml of anti-bodies to the fibroblast growth factor receptor (anti-FGFR) (anti-FGFR 1, clone VBS 1, «Milli-pore», Germany). In this case, the efficiency of mSON regulatory signal transmission through FGFR blockade was evaluated in comparison with that of fibroblast growth factor (FGF) (FGF-basic, "Sigma", USA) when it was added to the medium in an equimolar concentration with 20 μg/ml anti- FGFR .

2.8. Study of the neuronal-committed progenitors intracellular signal transduction.

The cell culture technique was employed to examine the direct effect of inhibitors of individual signaling molecules on the realization of the growth potential by neuronal-committed progenitors. The growth of CFU-N and ClFU-N was examined in cell culture in the presence of 2 ng/ml of mSON and equimolar amount of fibroblast growth factor (FGF) (FGF-basic, «Sigma», USA). Inhibitors of individual signaling molecules and their concentrations were used in the experiment: NF-κB «oridonine» (1 μM); NF-κB, IKK, PKC «aurothiomalate» (50 μM); PI3K «LY294002» (50 μM); ERK1/2 «PD98059» (100 μM); p38 and PKB «SB203580» (10 μM); adenylate cyclase «2',5'-dideoxyadenosine» (30 μM); IKK-2 «Inhibitor IV» (2 μM); JNK «SP600125» (10 μM); p53 (5 μM) (Pifithrin-α, Cyclic); JAK1.3 «CP 690550» (blocks JAK1 or JAK3, depending on the concentration of 1 nM and 100 nM, respectively); JAK2 was induced with AG 490 (50 μM); STAT3 «STAT3 Inhibitor XIV, LLL12» (5 μM). The working concentrations of the antagonists *in vitro* were determined in accordance with instructions of the developers of these chemical agents and confirmed as the optimum in own preliminary experiments on the test cultures [14, 27-31].

The specimens of nervous tissue were taken from subventricular area of cerebral hemispheres. After preliminary treatment, the specimens were placed into a special fluid culture medium containing 105 /ml viable cells; thereupon they were incubated in CO_2 incubator for 5 days at 37oC under a humidified atmosphere of 95% air and 5% CO2. After incubation, we counted clonogenic

PSA-NCAM + cells that formed neurospheres containing more than 100 cells (CFU-N) and the committed neural progenitors forming the neurospheres consisting of 30-100 cells (ClFU-N). Proliferative activity of CFU-N was assessed by the method of cell suicide using hydroxyurea. The relative content of S phase CFU-N was calculated by the formula: N=[(a-b)/a]×100%, where a is the group mean number of CFU-N grown from the cells not treated with hydroxyurea; b is the group mean number of progenitors grown from cytostatic-treated cells. Differentiation rate of CFU-N was assessed by calculating the ratio of ClFU-N to CFU-N number in tissue culture (ClFU-N/CFU-N) [28].

2.9. Statistical Analysis.

The results were analyzed with one-way ANOVA followed by Dunnett's test, Wilcoxon's test for dependent samples, and Mann–Whitney test for independent samples. The data are expressed as the arithmetic means and standard errors. The significance level was p=0.05 [32].

3. RESULTS

3.1. Correction of psychoneurological status disorders and histological changes in nervous tissue in rats with cerebral ischemia using mSON.

Carotid ligation resulted in a sharp asymmetry of eye fissures (up to 1.6 ± 0.3 points) and pronounced changes in the psychoneurological status. There was an increase in the number of horizontal movements of animals in the open field, pronounced more distinctly in the second period of observation (2-3 min) (Table 1). This fact indicated a predominant violation of brain cognitive function, but it didn't confirm the specific activation of research behavior [16].

In addition, a sharp deterioration in the development and reproducibility of the conditioned reflex of passive avoidance was shown (Table 2). Histological examination of brain in animals with cerebral ischemia revealed on the side with a large restriction of blood flow a significant hyperemia of pia mater, different degree of collapsed state of most of cortex vessels, perivascular and pericellular edema and neurons with hyperchromatic nuclei, vacuolar dystrophy, surrounded by phagocytes in the state of phagocytosis and neurons with pyknotic nucleus and shrunken cytoplasm in hippocampus.

Thus, the carotid artery ligation was accompanied by the development of severe ischemia with significant, but then compensated damage to the structure and functions of brain, as it is known [11, 16]. In this case, untreated rats did not show any changes (Tables 1, 2).

Administration of mSON after modeling of cerebral ischemia abolished almost completely the appearance of features of CNS activity disorder. The decrease in the asymmetry of palpebral fissure (up to 0.3 ± 0.1 points) and in the total motor activity and the number of horizontal displacements were shown. The increase in the number of animals with a preserved reflex of passive avoidance while increasing its latent time and the time of residence in the dark compartment was observed.

At that time, the restoration of brain morphology was shown to be the basis for the correction of studied behavioral and functional indices. Administration of mSON led to more uniform blood filling of the vessels of the right and left hemispheres, reduced significantly the intensity of perivascular and pericellular edema of the nervous tissue and decreased the number of neurons with a pycnotic nucleus, vacuolar dystrophy, and neurons in the state of phagocytosis in the hippocampus.

3.2. Correction of disturbances of psychoneurological status in mice after modeling of posthypoxic encephalopathy with mSON.

Hypoxia resulted in marked changes in the psychoneurological status. There was an increase in the number of horizontal displacements in the open field during the first minute of observation, and on the contrary, a decrease in this indicator while its recording at the 2nd and 3rd minutes for both on the 14th and 21st days of experience. In all cases, there was a substantial increase in the coefficient of motion asymmetry, which is considered to be a reflection of "depletion" of the motor activity in experimental animals (Table 3). The fact of a differently directed shift of horizontal activity in the first and second periods indirectly indicates a disorder of rather a cognitive function than research one of the central nervous system [15].

In addition, the modeling of encephalopathy was accompanied by the decrease in the level of reproduction of conditioned reflex of passive avoidance to 31% on the 14th and 21st days, while the values of this parameter in intact mice on the 14th and 21st days were 100% and 85%, respectively (Table 4). These changes were developed on the background of spontaneous mortality of animals which underwent hypoxia, reaching 22.4% on the 21st day of the experiment. The revealed behavioral disorders conformed fully to posthypoxic changes in CNS activity that were characteristic for this model, and reflected a marked degree of brain damage [1, 6, 15].

The administration of mSON after hypoxic exposure was accompanied by practically complete normalization of all studied parameters. The alkaloid abolished the revealing of signs of brain pathology, recorded while studying both the orienting and research behavior of mice in the open field and the reproducibility of the conditioned reflex of passive avoidance (Tables 3, 4). At the same time, no animals receiving the pharmacological agent died during the experiment.

Table 1. Indicators of oriented investigative behavior in the open field of outbred sham-operated rats (1); rats with ischemia of the brain (2); rats treated with mSON on the background of modeling cerebral ischemia (3) and sham-operated rats, treated with mSON, arb. units, (M±SEM).

Groups of animals	Total motor activity	Horizontal activity	Vertical activity	Sniffing of holes	Mink reflex	Grooming	Defecation
In the first period of the study (1 minute)							
1	18,12±2,30#	8,45±1,22 #	3,37±0,91	2,17±0,32	1,02±0,31	0,22±0,22	2,17±0,42
2	25,09±2,64	17,32±1,31	5,71±1,01	1,75±0,24	1,18±0,11	0,35±0,34	1,09±0,61

3	12,83±2,62 #	7,24±2,88 #	2,26±0,93	0,83±0,37	1,26±0,35	0,00±0,00	1,72±0,83
4	16,22±1,92 #	10,69±2,55	2,62±0,71	0,67±0,21	0,88±0,44	0,00±0,00	1,24±0,62
In the second period of the study (2-3 minute)							
1	9,48±2,11	2,43±1,21	2,67±0,55	2,45±0,71	1,22±0,76	0,63±0,32	0,60±0,60
2	32,36±3,74	16,62±2,03	6,72±1,16	3,16±0,33	2,92±0,47	1,38±0,71	0,00±0,00
3	14,16±3,05#	9,39±1,67#	2,36±0,42#	1,78±0,54	1,99±0,50	0,56±0,55	0,72±0,37
4	12,21±2,42	6,16±2,25	2,93±1,12	0,66±0,12	1,31±0,62	0,83±0,51	0,89±0,52

- the reliability of differences in indices with those in rats with cerebral ischemia (group 2) at p<0.05

Table 2. Indicators of development and reproduction of conditioned reflex of passive avoidance in outbred sham-operated rats (1); in rats with cerebral ischemia (2); in rats treated with mSON on the background of modeling cerebral ischemia (3) and in sham-operated rats.

Groups of animals	Control, 21th day		
	Latent time of reflex, sec	Total time of residence in the dark compartment, sec	Percentage of animals with reflex,%
1	168,04±4,17	11,32±2,41 #	90,32 #
2	70,33±10,72	108,44±19,79	25,47
3	157,04±3,81 #	24,99±5,84 #	80,69 #
4	152,75±12,93 #	28,66±12,33	80,54 #

- the reliability of differences in indices with those in rats with cerebral ischemia (group 2) at p <0.05

Table 3. Indices of oriented investigative behavior in the open field in the open field of CBA/CaLac mice (1); in mice after hypoxic exposure (2); in mice trated with mSON on the background of modeling posthypoxic encephalopathy (3), in arb. units, (M±SEM).

Groups of animals	Total motor activity	Horizontal activity	Vertical activity	Sniffing of holes	Mink reflex	Grooming	Coefficient of asymmetry,%
14th day							
In the first period of the study (1 minute)							
1	12,82 ± 2,24	4,42 ± 1,03	0,33 ± 0,10	5,14 ± 0,31	2,81 ± 0,35	0,10 ± 0,10	37,91 ± 2,62
2	9,76 ± 2,55	8,67 ± 0,90*	0,32 ± 0,10	0,73 ± 0,42*	0,74 ± 0,50*	0,22 ± 0,10	85,43 ± 5,95*
3	10,43 ± 1,70	3,06 ± 1,15#	0,62 ± 0,21	3,22 ± 0,51#	3,06 ± 0,32#	0,22 ± 0,10	30,80 ± 4,22#
In the second period of the study (2-3 minute)							
1	35,37 ± 5,61	17,52 ± 3,05	0,62 ± 0,20	10,10 ± 1,79	8,55 ± 1,51	0,37 ± 0,21	42,67 ± 5,32
2	9,05 ± 6,43*	5,77 ± 2,71*	0,75 ± 0,31	0,80± 1,11*	1,23 ± 2,47*	0,55 ± 0,27	63,92 ± 2,41*
3	27,26 ± 3,21#	14,32 ± 1,26#	1,57 ± 0,64	6,01 ± 0,67#	7,72 ± 0,51#	1,39 ± 0,31	46,83 ± 1,98#
21th day							
In the first period of the study (1 minute)							
1	14,35 ± 3,71	6,38 ± 1,11	0,82 ± 0,23	3,43 ± 0,61	3,21 ± 0,21	0,34 ± 0,12	34,82 ± 2,07
2	18,99 ± 2,64	11,25 ± 0,52	0,10 ± 0,10	0,22 ± 0,22	0,22 ± 0,22	0,26 ± 0,13	79,19 ± 6,36*
3	13,50 ± 2,93	6,08 ± 0,92	0,81 ± 0,62	3,71 ± 0,38	4,46 ± 0,53	0,24 ± 0,11	35,99 ± 2,72
In the second period of the study (2-3 minute)							
1	26,93 ± 2,34	11,22 ± 1,00	0,43 ± 0,16	7,47 ± 1,11	7,89 ± 2,37	1,87 ± 0,43	42,53 ± 6,34
2	5,77 ± 1,34*	4,91 ± 0,72*	0,0 ± 0,0*	1,02 ± 0,53*	2,15 ± 0,42*	0,85 ± 0,27*	87,56 ± 5,61*
3	25,54 ± 3,67#	12,42 ± 1,15#	0,52 ± 0,10 #	6,85 ± 0,10#	7,10 ± 0,41#	0,29 ± 0,24*#	41,87 ± 6,73#

* - the reliability of differences in indices with those in intact mice (group 1) at p <0.05

- the reliability of differences in indices with those in mice with posthypoxic encephalopathy (group 2) at p <0.05

3.3. Mechanisms of mSON cerebroprotective effects.

Modeling of posthypoxic encephalopathy was accompanied by an increase in the content of neuronal-committed progenitors (clonogenic PSA-NCAM + cells, CFU-N) in the SVZ (up to 433.3% and 217.3% of baseline level on the 3rd and 7th days of the experiment, respectively). These changes were developed on the background of an increase of the factors secretion stimulating their functions by cells of the microenvironment of nervous tissue. CSA levels reached 600.0% and 169.5% from similar parameters in animals without pathology (Figure 1).

mSON increased the number of CFU-N from PSA-NCAM + cells up to 258,4% и 593,2% of that in control mice (which did not receive the drug after hypoxia) on the 3rd and 7th day of the experiment, respectively (Figure 1, A). Moreover, the secretory function of glial cells increased markedly. The secretion of active toward CFU-N neurotrophic growth factors by glial cells increased up to 270.1% and 911.2% of the control mice on the 3rd and 7th day, respectively (Figure 1, B).

The direct action of alkaloid on CFU-N from PSA-NCAM + cells was detected in *in vitro* experiments. The addition of mSON in the PSA-NCAM + cells culture from SVZ resulted in a sharp increase in the formation of neurospheres (up to 246.8% of the baseline value) (Figure 2, A).

The study of the transmission pathway of stimulatory signal by mSON to the effector cells revealed the key role of their receptors to the growth factor of fibroblasts (FGF) in the realization of this

phenomenon. The addition of antibodies to the FGF receptor together with studied alkaloid in the culture of intact brain PSA-NCAM + cells was accompanied by the almost complete abolishment of the enhancement of the neurosphere formation process (Figure 2, A).

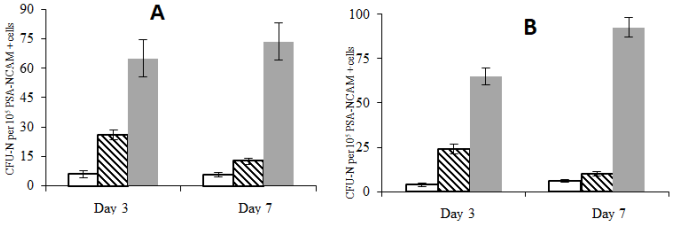

Figure 1. CFU-N count in the PSA-NCAM + cells culture (A) and formation of neurospheres in the test system (B) after addition of conditioned medium from cells of the subventricular zone of lateral ventricle of the brain intact CBA/CaLac mice (white bars); mice with posthypoxic encephalopathy (shaded bars); after administration of mSON mice with posthypoxic encephalopathy (gray bars). Whiskers indicate the confidence interval at p=0.05.

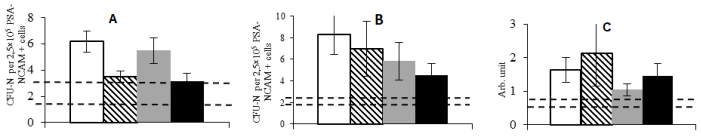

Figure 2. CFU-N count (A); ClFU-N count (B) and index of differentiation of CFU-N (C) in the PSA-NCAM + cells culture of the subventricular zone CBA/CaLac mice after influence mSON *in vitro* (white bars), mSON with antibodies to fibroblast growth factor receptor (anti-FGF-R) *in vitro* (shaded bars), with fibroblast growth factor (FGF) *in vitro* (gray bars) and FGF with c anti-FGF-R *in vitro* (black bars). Whiskers indicate the confidence interval at p=0.05
The area between the dotted lines corresponds to the confidence interval of the given characteristic in intact culture of CFU-N p=0.05.

The yield of CFU-N was 56.7% from the similar parameter in the test system without anti-FGFR. However, the number of ClFU-N did not change (Figure 2, B). The revealed activation of the earliest (CFU-N) progenitors is indicative for predominant effect of mSON through the receptors to FGF on the neuronal-committed progenitors proliferative activity. This fact was confirmed by the similarity of described dynamics of yields of CFU-N, ClFU-N and maturation of precursors in the presence of anti-FGFR in the culture when FGF was used as a stimulator of the progenitor elements (Figure 2). Specific effects of anti-FGFR are known to be connected mostly with the mitogenic activity of cytokine [21, 22]. At the same time, mSON influenced more the differentiation of CFU-N from PSA-NCAM + cells (up to 56.2%) (Figure 2, B).

3.4. Comparative study of intracellular signal transduction in neuronal-committed progenitors under the influence of mSON and FGF.

In the course of the experiment, the addition of mSON to the culture of PSA-NCAM + cells increased the number of CFU-N and ClFU-N in the medium: it reached the value of 309.1% and 262.8% of the baseline, respectively. It was followed by a significant increase in the ratio of CFU-N at S-phase of the cell cycle. At the same time, the index of differentiation of neuronal-committed progenitors was at the level of that observed in cell culture without alkaloid (Table 5).

The addition of FGF to the culture medium led to a comparable increase in the number of CFU-N and a less significant increase in the ClFU-N (up to 276.6% and 194.6% from the baseline, respectively) (Table 5). In this case, the index of differentiation of neuronal-committed progenitors was at the level of that in the culture of cells without addition of FGF. The obtained results corresponded to the literature data about the properties of the investigated early-growth factor which help to stimulate the functions of progenitors [14, 19].

The study of the participation of individual signaling molecules in the realization of proliferative-differentiating potential of clonogenic PSA-NCAM + cells under the influence of mSON and FGF revealed a number of specific characteristics of intracellular signaling for each of FGFR agonists. Blockade of individual signaling molecules in the conditions of stimulating realization of growth potential of progenitor cells by FGF resulted in the following changes. There was a significant decrease in the amount of CFU-N and their mitotic activity (cells in the S-phase of the cell cycle) during the blockade of NF-κB, IKK, PKC, PI3K, ERK1/2, JAK1, JAK2, JAK3 and STAT3 (Fig.). At the same time, only the blockade of NF-κB, IKK, PI3K, JAK1, JAK2, JAK3 and STAT3 reduced the number of ClFU-N. Inactivation of NF-κB together with IKK, PKC (by aurotrimalate) and ERK1/2 did not affect the yield of precursors.

Absence of participation of p38 and PKB in the cytokine-stimulated realization of progenitors functions was an important characteristic of signal transduction [33]. In contrast to the above-described changes, the blockade of JNK and p53 was accompanied, on the contrary, by an increase in the number of CFU-N and ClFU-N from PSA-NCAM + cells in the culture medium against a background of increased proliferative activity of neuronal-committed progenitors cells. In this case, direct dependence of the rate of progenitor cells differentiation from PI3K and STAT3 activity was found, and the inverse dependence of this index from the activity of adenylate cyclase, JAK1, JAK2, JAK3 (Table 5).

The blockade of individual signaling molecules while injecting mSON cells in the culture, was accompanied by other changes in the functioning progenitor cells in some cases. Thus, the blockade of NF-κB together with IKK, PKC (aurotrimalate) and inactivation of ERK1/2 resulted in a decrease in the number of both CFU-N and ClFU-N. Moreover, the blockade of adenylate cyclase and p38 and PKB was accompanied by a decrease in the number of neuronal-committed progenitors of both types. In addition, in this case, the inactivation of IKK, ERK1/2, p38, together with PKB, JNK and p53 resulted in a significant decrease in the rate of progenitor elements differentiation. The dynamics of changes in the other parameters being studied while using different inhibitors of signaling molecules generally corresponded to that studied when FGF was used as a stimulant of progenitors (Table 5).

Table 4. Indices of development and reproduction of conditioned reflex of passive avoidance in CBA/CaLac mice (1); in CBA/CaLac mice after hypoxic exposure (2); in mice treated with mSON on the background of modeling posthypoxic encephalopathy (3), (M±SEM).

Groups of observation, doses	Control					
	14th day			21th day		
	Latent time of reflex, sec	Total time of residence in the dark compartment, sec	Percentage of animals with reflex,%	Latent time of reflex, sec	Total time of residence in the dark compartment, sec	Percentage of animals with reflex,%
1	180,00 ± 0,00	0,00 ± 0,00	100,0	175,32 ± 11,07	14,62 ± 9,03	92,45
2	138,72 ± 21,07*	37,91 ± 6,45*	31,25*	124,36 ± 11,85	42,31 ± 10,52	34,49*
3	166,16 ± 15,95	12,79 ± 9,77	72,37 #	172,71 ± 6,43	7,07 ± 6,15	87,76 #

* - the reliability of differences in indices with those in intact mice (group 1) at $p < 0.05$

- the reliability of differences in indices with those in mice with posthypoxic encephalopathy (group 2) at $p < 0.05$

Table 5. Content of CFU-N, ClFU-N, proliferative activity and intensity of differentiation of clonogenic PSA-NCAM + cells from SVZ of CBA/CaLac mice under exposure of mSON and FGF together with inhibitors of individual signal molecules, (M±SEM).

Inhibitors	CFU-F, per $2,5\times10^5$ cells			CLFU-F, per $2,5\times10^5$ cells			Proliferative activity (ratio of S-phase CFU-F), %			Intensity of differentiation, arb. units		
	intact	mSON	FGF	intact	mSON	FGF	intact	mSON	FGF	intact	mSON	FGF
Without inhibitors	1,87±0,17	5,78±0,34	5,17±0,22	3,17±0,28	8,33±0,42	6,17±0,37	29,5±3,13	75,17±2,54	74,33±2,97	2,17±0,19	1,96±0,17	1,22±0,10
NF-κB	0,83±0,17 *	1,83±0,41 #	2,83±0,32&	1,0±0,16 *	2,83±0,31 #	2,83±0,17 &	14,17±1,54 *	15,33±1,21 #	15,83±1,01 &	1,0±0,26 *	1,11±0,12 #	1,21±0,17
NF-κB, IKK, PKC	1,67±0,21	2,33±0,67 #	3,63±0,31&	2,50±0,75	**4,50±0,50 #**	5,17±0,65	29,33±2,69	21,0±2,34 #	29,33±2,70 &	2,0±0,34	2,,01±0,23	1,28±0,14
IKK-2	1,67±0,17	3,17±0,31 #	1,67±0,56&	1,33±0,42 *	2,67±0,32 #	2,67±0,32 &	31,33±3,02	32,33±1,17 #	36,33±1,21 &	0,67±0,17 *	**1,0±0,15 #**	2,08±0,45
PI3K	0,67±0,33 *	1,5±0,43 #	3,83±0,31&	1,33±0,22 *	2,67±0,42 #	2,0±0,26 &	15,33±2,26 *	12,50±1,12 #	16,33±2,30 &	1,72±0,37	2,08±0,52	0,73±0,26
ERK1/2	0,67±0,21 *	3,51±0,22 #	3,57±0,14&	2,87±0,33	2,67±0,36 #	3,83±1,14 &	18,33±3,07 *	17,62±2,11 #	18,67±2,91 &	2,33±0,33	**0,78±0,15 #**	1,90±0,36
p38, PKB	1,83±0,38	**2,0±0,53 #**	5,2±0,37	3,50±0,67	**1,33±0,21 #**	6,04±1,05	29,01±1,79	**16,17±3,79 #**	72,50±2,14 &	2,08±0,34	**0,87±0,16 #**	1,20±0,24
Adenylate cyclase	1,42±0,25	**2,33±0,26 #**	5,25±0,75	2,67±0,21	**3,83±0,42 #**	7,51±1,44	33,33±2,79	**31,36±2,79 #**	71,33±6,79	2,33±0,42	2,33±0,34	1,96±0,15&
JNK	6,03±0,91 *	28,97±2,56#	12,3±1,38&	12,14±2,3*	36,97±4,56 #	11,83±1,58&	61,67±2,67 *	97,21±4,36 #	97,44±2,58 &	2,26±0,45	**1,06±0,10 #**	1,63±0,37
p53	6,59±1,22 *	39,51±6,47#	9,63±1,47&	9,16±2,34 *	29,64±3,69 #	11,17±1,78&	66,50±4,10 *	92,5±10,56 #	87,63±2,59 &	1,75±0,27	**0,75±0,11 #**	1,34±0,12
JAK1	1,77±0,24	2,36±0,34 #	2,51±0,27&	3,26±0,34	3,97±0,58 #	4,16±0,14 &	25,83±3,52	15,83±3,41 #	27,83±2,36 &	1,79±0,23	1,63±0,25	1,74±0,19 &
JAK2	0,62±0,11 *	0,24±0,10 #	1,26±0,27&	1,12±0,24 *	2,44±0,29 #	3,01±0,27 &	13,36±4,67 *	31,0±1,95 #	17,2±2,36 &	1,86±0,33	3,64±0,37 #	2,97±0,36 &
JAK3	0,63±0,12 *	2,34±0,08 #	2,41±0,06&	3,02±0,15	5,63±0,17 #	4,03±0,23 &	15,26±3,12 *	18,26±3,09 #	19,78±3,27 &	3,28±0,39 *	2,31±0,37	1,88±0,10 &
STAT3	0,63±0,09 *	0,2±0,2 #	0,1±0,1 &	0,57±0,21 *	0,1±0,1 #	0,18±0,18 &	12,33±2,41 *	9,08±1,23 #	10,86±2,73 &	0,97±0,36 *	0,50±0,22 #	0,52±0,14 &

* - the reliability of differences in indices in the culture of cells without stimulators (intact) at $p < 0.05$

- the reliability of differences in indices in the culture of cells with mSON at $p < 0.05$

& - the reliability of differences in indices in the culture of cells with FGF at $p < 0.05$

The effects of mSON, which are absent in FGF, are highlighted by black-faced type.

4. CONCLUSIONS

Creation of drugs - regulators of SC functions, in the framework of the developing "Pharmacological strategy of regenerative medicine" based on the principle of imitation to natural regulatory systems, is the especially urgent task in respect of neurodegenerative diseases [6, 7]. In this case, the potential use of genetic engineering analogs of growth factors and other cytokines that stimulate the functions of neural stem cells is significantly hampered in neurological practice due to a number of objective circumstances. These substances are the pleiotropic and multifunctional factors in one way or another. This fact in some cases does not meet the principles of selectivity of pharmacological effects. They also do not possess the necessary pharmacokinetic characteristics. Virtually they unable to penetrate through the blood-brain barrier, and their protein nature predetermines high immunogenicity and the only possible parenteral way of administration [7, 15-17, 34-37]. Although the most appropriate in regenerative medicine is the oral use of medicines since they are expected to be used by long-lasting, repeatedly recurrent courses [1].

At the same time, there is a group of substances - alkaloids, which are distinguished by high selectivity of action and by pharmacokinetic characteristics required in this case [15, 16, 18, 38-40]. There are many drugs based on alkaloids of different pharmacological groups (analgesics, antiarrhythmics, anticholinergic drugs, sympathomimetics and others). In all cases, they are considered to be highly effective agents possessing most often the receptor mechanism of action [39].

Proceeding from abovementioned, the development of neuroprotective agent with regenerative activity based on the originally modified plant alkaloid songorine with permissible toxicity (LD50 = 142 mg/kg, which is 9467 times higher than the maximum effective therapeutic dose) is very promising.

In the course of studies on models of cerebral ischemia and posthypoxic encephalopathy, pronounced neuroprotective effects of mSON were demonstrated. At the same time, significant stimulating neuronal-committed progenitors activity of agent played a significant role in the process of restoring the structure and functions of central nervous system [1, 6, 33, 45, 46].

Moreover, in the increase of regenerative potential of progenitors both the direct action of mSON on them and the rise of growth factors production by cells of nervous tissue microenvironment under the influence of this agent are of great importance [15, 47, 49].

Thus, the key role in the stimulation of neuronal-committed progenitors transfer functions under the influence of mSON belongs to the receptors to FGF. The involvement of microenvironment elements nervous tissue - glial cells, in the process of compensating CNS injury is obviously determined by the expression of receptors for fibroblast growth factor with glial cells (including astrocytes) [15, 19]. Moreover, it is the secretion of neurotrophic factor that can be the basis of the detected phenomenon of direct anti-ischemic activity of the alkaloid in the early periods after hypoxia.

At the same time, a number of significant differences in intracellular transduction happened under the influence of mSON on cells in comparison with FGF was revealed [14, 33]. Unlike FGF, the modified alkaloid, in addition to proliferation, significantly stimulates the differentiation of progenitor cells. In this case, mSON leads to the activation of cAMP-dependent and p38-mediated (alternative MAP-kinase pathways) signaling, as well as to the involvement of protein kinase B in signaling process via NF-κB (as "SB203580" inhibitor, in addition to p38 inhibition, inhibits the phosphorylation of protein kinase B) [45, 46].

In addition, mSON increases the degree of participation of ERK1/2 (classical MAP-kinase pathway) [28] in the regulation of a cell cycle of neuronal-committed progenitors, and also there is an involvement of IKK, ERK1/2, JNK and p53 in the process of differentiation of progenitors. Thus, during the stimulation of neuronal-committed progenitors functions under the influence of mSON a maximum number of basic elements of intracellular cascades of signal transduction is involved [33].

Despite the revealed common target of exposure - FGFR, the determined differences in signal transmission under the action of mSON and FGF can be explained by: 1) the effect of mSON and FGF on different subtypes of receptors to FGF, or 2) different affinities of these ligands to receptors, or 3) the possibility of alkaloid penetration into the cell and its additional direct effect on the elements of intracellular signal transduction.

Additional experiments are required to answer the question about the reasons of revealed differences in the mechanisms of mSON and FGF action. In the case of confirmation of the direct effect of modified alkaloid on intracellular signaling molecules, the results obtained can be considered as one of substantiations for the perspective of a new direction of targeted therapy in regenerative medicine proposed by us - "The strategy of pharmacological control of intracellular signal transduction in regenerative-competent cells" [7, 28, 29]. This concept supposes a selective effect on relatively specific parts of the signal cascade (both cytoplasmic and nuclear signaling molecules) which are involved in the processes of cell cycle progression and the development of progenitor cells of different classes. Therefore, the revealed key role of FGFR and the peculiarities of intracellular signaling in neuronal-committed progenitors under the influence of modified songorine, which has the pronounced neuroregenerative effect, allow considering this agent as a new original agonist of FGFR. Moreover, this substance is a promising one for the development of a safe and highly effective drug for neurological practice, including the therapy of neurodegenerative diseases.

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