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JNK and p53 inhibition in regeneration-competent cells of nerve tissue: a novel approach for treatment of ethanol-induced neurodegeneration

Gleb Nikolaevich ZYUZ'KOV[1*], Larisa Arkad`Evna MİROSHNİCHENKO[1], Tatyana Yur`Evna POLYKOVA[1], Larisa Alexandrovna STAVROVA[1], Elena Vladislavovna SİMANİNA[1]

[1] Russian Academy of Sciences, Tomsk National Research Medical Center, Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk, Russia

ABSTRACT

This study aimed to explore the involvement of JNK and p53 in the implementation of nervous tissue regeneration-competent cell functions in ethanol-induced neurodegeneration. The studies were conducted on the C57B1/6 mice. Ethanol-induced neurodegeneration was modeled *in vitro* and *in vivo*. The effects of the JNK and p53 inhibitors on the colony-forming capacity of NSC and neuronal-committed progenitors, their proliferative activity and intensity of specialization, as well as the neurotrophin secretion by astrocytes, oligodendrocytes, and microglial cells, were studied. The stimulating role of JNK and p53 in the mitotic activity and specialization of intact NSCs was shown. Inversion of the role of these signaling molecules in the regulation of NSC proliferation in ethanol-induced neurodegeneration has been revealed. It has been found that JNK and p53 are not involved in regulating the NCP functions. The ambiguous role of JNK and p53 in the production of neurotrophic growth factors by neuroglia. Increased secretion of neurotrophins by oligodendrocytes and microglia during the JNK and p53 blockage in the conditions of alcohol exposure was found. These results show the potential for using JNK or p53 inhibitors as novel effective drugs for alcohol encephalopathy therapy.

Keywords: Neural stem cells, neuroglia, JNK, p53, neurodegenerative diseases

*Corresponding author: E-mail: zgn@pharmso.ru
ORCIDs:
Gleb Nikolaevich Zyuz'kov: 0000-0003-0384-333X
Larisa Arkad`Evna Miroshnichenko: 0000-0002-3684-5510
Tatyana Yur`Evna Polykova: 0000-0003-0971-7844
Larisa Alexandrovna Stavrova: 0000-0002-4729-6406
Elena Vladislavovna Simanina: 0000-0003-1890-6946
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INTRODUCTION

In recent decades, the possibility of using individual intracellular signaling pathways as pharmacological targets has been actively studied. In oncopharmacology, this direction is one of the main modern trends in the creation of antitumor drugs[1]. The biggest pharmaceutical companies have developed several dozen antiblastic drugs based on intracellular signaling molecule inhibitors responsible for the growth and development of transformed cells. One of the advantages of these drugs is their selectivity, including not only to the tissue affected by the pathological process but also in some cases - to the type of tumor[2, 3].

A promising direction to solving the problems of regenerative medicine is the "Strategy of pharmacological regulation of intracellular signal transduction in regeneration-competent cells"[4-7]. This approach involves the use as targets of individual signaling molecules of progenitors and microenvironment cells of tissues (regulators of reparation) to create drugs with selective regenerative activity[8,9]. However, the development of this concept of pharmacotherapy requires a detailed understanding of the peculiarities of intracellular signaling in cells of different tissues to determine the potential targets for selective tissue-specific effects.

The implementation of this direction is particularly relevant for neurological practice to improve the effectiveness of the treatment of neurodegenerative diseases[10, 11]. Promising is the development of approaches to stimulation of neurogenesis by activation of the functions of progenitor cells of nerve tissue - multipotent neural stem cells (NSC) and neuronal-committed progenitors (NCP)[6, 12]. At the same time, it is known the importance of JNK- and p53-pathways (interacting with each other (have some "crossroads")), in regulating the processes of progenitor proliferation and differentiation[13, 14], as well as in the implementation of the functions of cytokine-producing cells[15]. However, there is no detailed understanding of their role in the functioning of nerve tissue regeneration-competent cells.

The work aimed to study the participation of JNK and p53 in the realization of the growth potential of nerve tissue precursors (NSC, NCP) and the production of neurotrophic growth factors by different neuroglial cells.

METHODOLOGY

Chemicals and Drugs

MACS Neuro Medium; anti-PSA-NCAM MicroBeads; anti-ACSA-2 MicroBead Kit; Anti-O4 MicroBeads; Anti-CD11b (Microglia) MicroBeads (all manufactured by Miltenyi Biotec, Germany); JNK inhibitor «SP600125» (InvivoGen, USA); p53 inhibitor «Pifithrin-α, Cyclic» (Santa Cruz Biotechnology, Inc. USA); hydroxyurea (Calbiochem, USA); plastic plates for cultural studies (Costar, USA).

Animals and Experimental Design

All animal experiments were carried out following 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's local Ethics Committee (protocol GRIPh&RM-2020-09). Experiments were carried out on C57B1/6 mice (n=84) at the age of 2-2.5 months, weighing 20-22 g. Animals of the 1st category (conventional mice) were obtained from the 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 a vivarium (air temperature 20–22°C, humidity 50-60 %) in plastic cages (10-15 mice) on a normal diet (solid diet pellets (Limited Liability Company «Assortiment Firm», Sergiev Posad city, Russia), water ad libitum. 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 CO_2 cameras.

Using the cultural methods, we studied the direct effect of the JNK inhibitor and p53 inhibitor (at a concentration of 10 μM and 5 μM, respectively) on the realization of the growth potential of neural tissue precursor cells (NSC, NCP) and the secretion of neurotrophic growth factors (neurotrophins) by glial cells (astrocytes, oligodendrocytes, microglia) in the conditions of modeling ethanol-induced neurodegeneration *in vitro* and *in vivo*[6, 9]. The working concentrations of inhibitors of signaling molecules were determined following the instructions of the companies-developers of these reagents.

In vitro ethanol-induced neurodegeneration was obtained by adding ethanol to the culture medium at a concentration (65 mM). *In vivo* modeling of the pathological state was carried out by oral administration of a 30% C_2H_5OH solution (through a probe daily at a dose of 3 g/kg/day for 8 weeks)[6, 12]. In this case, instead of drinking water of free access, a 5% ethyl alcohol solution was used. Cellular materials for the study were taken 10 days after the end of the intro-

duction of ethanol *in vivo*. The control group in the same model was injected with distilled water in an equivalent volume (mice had free access to drinking water).

Determination of Progenitors Functional Activity

NSC were studied during the cultivation of unfractionated cells of the subventricular zone (SVZ) of the cerebral hemispheres. To study NPCs, PSA-NCAM+ cells were isolated from the SVZ cells. For this, an immunomagnetic separator MIniMACS Cell Separator (Miltenyi Biotec, Germany) was used[6, 8, 9]. PSA-NCAM+ (CD56+) cells were obtained by positive selection [16] (using appropriate antibody kits according to the methodological manufacturer's instructions). The obtained unfractionated and PSA-NCAM$^+$ cells at a concentration of 10^5 / ml were incubated in MACS Neuro Medium (Miltenyi Biotec, Germany) for 5 days in a CO_2 incubator at 37°C, 5% CO_2, and 100% air humidity. After incubation in both cases (during the cultivation of unfractionated cells and PSA-NCAM$^+$ cells), the content of clonogenic cells, their mitotic activity, and intensity of specialization were calculated. The number of NSC and NCP was determined by the yield in the respective cultures of colony-forming units (CFU, neurospheres containing more than 100 cells). The proliferative activity of the progenitors was assessed by the method of cell suicide technic using hydroxyurea (1 μM)[6]. The pool of CFU in the S-phase of the cell cycle was determined according to the formula: $N = [(a-b)/a] \times 100\%$, where a is the average for the group the number of CFU from cells not treated with hydroxyurea; b - the average for the group the number of CFU from cells treated with hydroxyurea. The intensity of the processes of specialization (differentiation/maturation) of progenitors was determined by calculating the ratio of the corresponding cluster-forming (ClFU, neurospheres of 30 - 100 cells) to CFU – the differentiation index[6, 9].

Study of Neurotrophic Growth Factors Secretion by Neuroglial Cells

Individual fractions of glial cells (astrocytes - ACSA-2$^+$ cells, [17] oligodendrocytes - O4$^+$ cells, [18] microglia - CD11b$^+$ cells[19]) were also obtained from the SVZ using immunomagnetic positive selection (using appropriate antibody kits according to the guidelines of Miltenyi Biotec, Germany) The isolated cells at a concentration of 2×10^6 / ml were incubated in MACS Neuro Medium (Miltenyi Biotec, Germany) for 2 days in a CO_2 incubator at 37 ° C, 5% CO_2 and 100% air humidity to obtain supernatants. To determine their secretory activity (neurotrophic growth factors production), the effect of conditioned media from cells on the level of neurospheres formation (CFU) in the test system (culture of the SVZ unfractionated cells) was studied[5, 6].

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 arithmetic means. The significance level was $p < 0.05$[20].

RESULTS AND DISCUSSION

Effect of Ethanol on the Functioning of Various Types of Regeneration-Competent Cells of Nervous Tissue

The addition of a neurotoxic dose of ethanol to the culture medium did not cause a change in the level of the colony- and cluster-forming capacity of the unfractionated cells (Figure 1, A, B) and PSA-NCAM$^+$ cells (Figure 2, A, B) from the SVZ. However, there was a decrease in the NSC (CFU-N_{NSC}) and NCP (CFU-$N_{PSA\text{-}NCAM+}$) mitotic activity (up to 77.3% and 80.8% of background values, respectively) against the background of no changes in the speed of their specialization (Figure 1, C, D; 2, C, D).

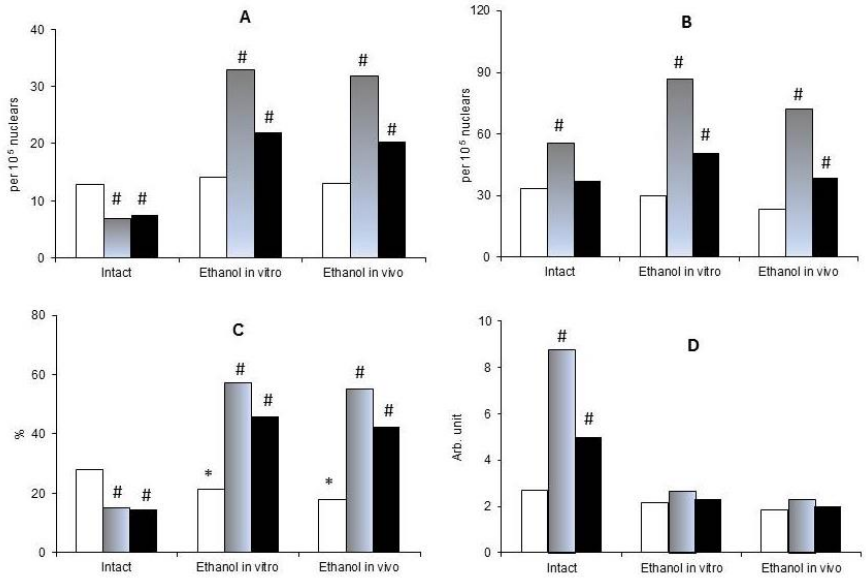

Figure 1. Number of CFU-N_{NSC} (A), CIFU-N_{NSC} (B), NSC proliferative activity (CFU-N_{NSC} in the S-phase of the cell cycle) (C), and NSC differentiation index (CIFU-N_{NSC} / CFU-N_{NSC}) (D). Here and in figures 2 and 3: cell culture without alcohol (intact); with alcohol (ethanol *in vitro*); and mice after prolonged administration of ethanol per os (ethanol *in vivo*). White bars - without signaling molecule inhibitors (white bars); gray bars - with the JNK inhibitor; black bars - with the p53 inhibitor; * - the significance of differences in indicators with intact was noted at $p < 0.05$; # - the significance of differences with the group without signaling molecule inhibitors was noted at $p < 0.05$.

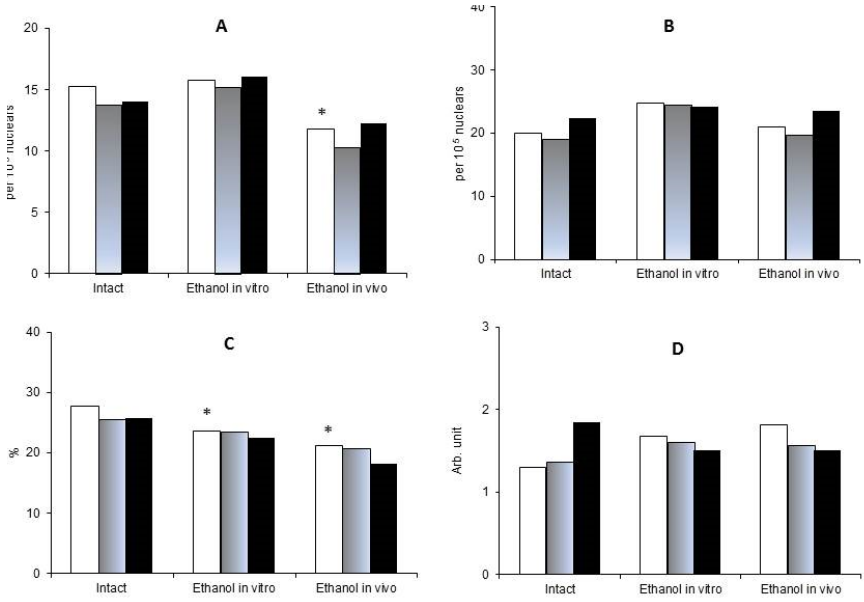

Figure 2. Number of CFU-$N_{PSA\text{-}NCAM+}$ (A), CIFU-$N_{PSA\text{-}NCAM+}$ (B), NCP proliferative activity (CFU-$N_{PSA\text{-}NCAM+}$ in the S-phase of the cell cycle) (C), and NCP differentiation index (CIFU-$N_{PSA\text{-}NCAM+}$ / CFU-$N_{PSA\text{-}NCAM+}$) (D).

The long-term introduction of ethyl alcohol *per os* was accompanied by the development of similar changes in the functioning of progenitors. However, in this case, there was a further decrease in the number of CFU-$N_{PSA\text{-}NCAM+}$ in the culture of clonogenic PSA-NCAM$^+$ cells (Figure 2, A) and the intensity of differentiation/maturation of NSC (Figure 1, D).

The ethanol addition *in vitro* was not accompanied by a change in the neurotrophin secretion by astrocytes and oligodendrocytes (Figure 3, A, B). However, there was a decrease in the value of this parameter in the conditioned media from microglial cells (up to 81.8% of the background value - a similar indicator in culture without ethanol) (Figure 3, C).

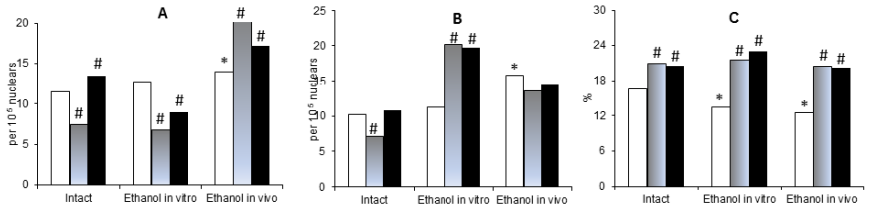

Figure 3. Effect of conditioned media of astrocytes (A), oligodendrocytes (B), and microglial cells (C) on the level of neurosphere formation in the test system.

Other changes in the production of humoral regulators were reported in cells of mice subjected to chronic alcohol intoxication. In this case, there was an increase in the neurotrophic growth factors secretions by astrocytes and oligodendrocytes (up to 121.7% and 153.7% of the initial control, respectively) against the background of the significant drop in the level of neurospheres formation in the test system containing the conditioned media from microglia (up to 75.8% of control).

Astrocytes and oligodendrocytes (primarily polydendrocytes, also called NG-2 cells and oligodendrocyte progenitor cells (OPC)) are known to produce neurotrophin family growth factors such as nerve growth factor (NGF), neurotrophic brain factor (BDNF).), neurotrophins 3 and 4 (NT3 and NT4), glial cell neurotrophic factor (GDNF), etc[21, 22]. Microglia secretes a wide range of cytokines, having both a stimulating effect on the NSC proliferation (leukemia inhibition factor (LIF), ciliary neurotrophic factor (CNTF), and others) and causing inhibition of their growth potential (interleukin-1, 6, 15 (IL-1, 6, 15), tumor necrosis factor-alpha (TNF-α)[23, 24]. Therefore, it is obvious that the detected dynamics of changes on the part of microglia reflect the parameter, which is also dependent on the products of inhibitors to realize the growth potential of progenitors - pro-inflammatory cytokines[25].

These findings were largely in line with the previously obtained data[6, 12] on the violation of implementation, primarily the proliferation of the nerve tissue progenitors under the influence of neurotoxic doses of ethanol. Therefore, it should be considered that the most vulnerable, especially with prolonged exposure to alcohol on the body, are precursors determined in the neuronal direction of development. Thus, the results indicate the development of ethanol-induced neurodegeneration of compensatory response from of astroglia and oligodendroglia, aimed at stimulation of neurogenesis in the conditions of violation of the NSC proliferation[12, 23]. At the same time, the detected decrease of the neurospheres formation under the influence of the microglial cells conditioned media is not so unambiguous. This circumstance was due, first of all, to the increase in

the secretion of the pro-inflammatory cytokines[25], rather than to a decrease in the neurotrophins production. It is known that the intensity of the inflammatory reaction in the nervous tissue in chronic alcohol consumption, in many respects, determines the development further of the pathological process. Expressed inflammation may not only not contribute to tissue repair, but also damage it[26, 27].

Role of JNK and p53 in Implementation Functions of Nervous Tissue Progenitors Under Conditions of Their Optimal Vital Activity

The addition of the signaling molecule inhibitors to the culture with the SVZ unfractionated cells was accompanied by a significant decrease in the level of colony formation. The number of CFU-N_{NSC} was 54.6% and 58.5% of the background value under the blockade of JNK and p53, respectively (Figure 1, A). The state of the ClFU-N_{NSC} (which are precursors with a lower self-renewal capacity and proliferating potential[6, 12]) was characterized by less unambiguous changes. The violation of JNK phosphorylation led to an increase in the number of ClFU-N_{NSC} in culture (up to 166.7% of the background), while the inactivation of p53 did not affect the value of this parameter (Figure 1, B). In both cases, however, there was a significant increase in the intensity of progenitor specialization processes. The differentiation index was 327.5% and 185.5% of control with the JNK and p53 inhibitors respectively (Figure 1, D).

Other phenomena were observed in the study of the role of JNK and p53 in the functioning of the neuronal-committed progenitors. The blockade of signal transduction via JNK and p53 did not change the values of the studied indicators in the culture of PSA-$NCAM^+$ cells (Figure 2).

Thus, JNK- and p53-pathways are essential in regulating the cell cycle of only NSC. They are responsible for maintaining their multipotency and self-renewal capacity. That is, JNK and p53 play one of the key roles in the maintaining the "deep reserve" of the CNS regeneration – resident stem cells[5, 28].

Role of JNK and p53 in Growth Factors Secretion by Neuroglial Cells Under Conditions of Their Optimal Vital Activity

It is known that the functioning of progenitor cells depends on the state of the microenvironment of tissue cells. They can exert their influence by secretion of humoral factors and direct intercellular communications that provide, among other things, the provision of cytokines in a biologically active form[4, 23]. In this regard, when choosing the targets among the intracellular signaling molecules in the progenitors should take into account their role in regulating the functions of individual populations of neuroglia[12].

Studies of JNK and p53 participation in the growth factors productions by different fractions of glial cells have revealed ambiguous changes. Thus, the JNK and p53 inhibitors caused a significant reduction in the neurotrophins secretion by astrocytes (ACSA-2$^+$ cell) (Figure 3, A), reaching 54.3% and 76.1% of the background, respectively. The capacity of microglia (CD11b$^+$ cells) to influence the realization of the growth potential of precursors (due to the production of humoral factors) during the blockade of JNK and p53, on the contrary, increased (up to 116.7% and 122.7% of the baseline, respectively) (Figure 3, C). At the same time, there were no changes in the functioning of oligodendrocytes (O4$^+$ cells) in any of the cases (Figure 3, B).

Role of JNK and p53 in Implementation Functions of Nervous Tissue Progenitors in Ethanol-Induced Neurodegeneration

The study of the effect of alcohol on the participation of JNK- and p53-pathways in realizing the growth potential of neural tissue precursors revealed some interesting phenomena. The addition into the culture medium with ethanol the JNK and p53 inhibitors led to a significant increase in the NSC proliferative activity and neurosphere formation (Figure 1, A, C). At the same time, the JNK blockade accelerated the NSC specialization to 123.6% of control (medium with ethanol without the signaling molecules inhibitors) (Figure 1, D). The inactivation of JNK and p53 in the neuronal-committed progenitors in the simulation of ethanol-induced neurodegeneration *in vitro* did not cause changes in their functioning (Figure 2).

Similar patterns were revealed in the cultivation of nerve tissue cells in alcoholized mice. The inactivation of JNK and p53 led to an increase in the content of CFU-N_{NSC} and their pro-proliferative activity in the culture of unfractionated cells of the SVZ (Figure 1, A, C). Besides, the JNK inhibitor also saw an increase in the NSC specialization index (Figure 1, D). However, there was no change in JNK and p53 involvement in regulating the NCP functions. The blockage of these signaling molecules in neuronal-committed progenitors of mice, which had long been treated with ethanol, did not affect the realization of their growth potential (Figure 2).

The results indicate an inversion of the role of JNK and p53 in the regulation of the NSC proliferation in ethanol-induced neurodegeneration.

Role of JNK and p53 in Growth Factors Secretion by Neuroglial Cells in Ethanol-Induced Neurodegeneration

Changes in the functioning of different types of neuroglial cells depending on their living conditions under the JNK and p53 blockade were ambiguous. The

inactivation of JNK and p53 in the incubation of astrocytes in the presence of C_2H_5OH *in vitro* was accompanied by a drop in the neurotrophic growth factors production (up to 45.1% and 78.4% of control values, respectively - in a medium with ethanol without the signaling molecules inhibitors). However, in mice that received ethyl alcohol *per os*, JNK and p53 blockade in astrocytes did not affect the neurotrophin secretion (Figure 1, A).

Another phenomenology was observed in the study of the functioning of oligodendrocytes. Disruption of signal transduction via JNK and p53 in oligodendrocytes in exposure to ethanol *in vitro*, on the contrary, led to an increase in their growth factors production, especially expressed in the use of the JNK inhibitor (Figure 3, B). At the same time, the blockade of JNK in the oligodendrocytes of animals, which were treated with alcohol for a long time, caused, on the contrary, a significant reduction in the neurotrophin secretion (up to 84.1% of similar parameters in cells without signaling modification). The p53 inhibitor, in this case, did not affect the production of growth factors by oligodendrocytes.

One-way changes were observed on the part of the secretory activity of microglial cells. In all cases (the addition of C_2H_5OH *in vitro* and the introduction of ethanol to mice *per os*), inactivation of signaling molecules led to an increase in the secretion of growth factors by microglial cells. A particularly pronounced increase in this indicator was with the use of the JNK inhibitor (up to 150.0% and 156.2% of the control values respectively) (Figure 3, C).

Thus, JNK and p53 played an ambiguous role in regulating the secretory function of different types of neuroglial cells when modeling ethanol-induced neurodegeneration *in vitro* and *in vivo*.

The findings confirm the evidence that there are significant peculiarities of the role of JNK and p53 in the regulation of the cell cycle of different types of regeneration-competent cells[5, 8, 13, 14]. It was found that JNK- and p53-pathways are responsible for maintaining the "deep reserve" of the plasticity of the CNS - for maintaining the self-renewal, rapid proliferation, and multiple differentiation of NSC. In the functioning of the neuronal-committed progenitors (the most mobile department of tissue-specific regeneration[5, 9]) these signaling molecules do not take significant participation.

However, it is known that balanced neurogenesis can occur through the implementation of the functions of exclusively multipotent NSC (without the participation of NCP)[5, 28, 29]. Therefore, the important role of JNK and p53 in the progression of the NSC cell cycle indicates the potential for their use as pharmacological targets (Figure 4).

At the same time, it has been found that ethanol-induced neurodegeneration leads to the inversion of the role of JNK and p53 in the regulation of the proliferation of multipotent NSC. The blockage of these signaling molecules in the conditions of ethanol intoxication can significantly increase the degree of realization of the growth potential of NSC and thus, obviously, stimulate neurogenesis. But it should be taken into account that such therapy can lead to the transformation of the ethanol-induced pattern of intracellular signaling into the «original» (normal) state in the *de novo* formed progenitors[6, 9, 12]. That is, to cause a "reverse" inversion of the role of JNK and p53 in the regulation of the NSC functions. In such conditions, the JNK and p53 inhibitors will, on the contrary, inhibit the implementation of the functions of these intact progenitor cells.

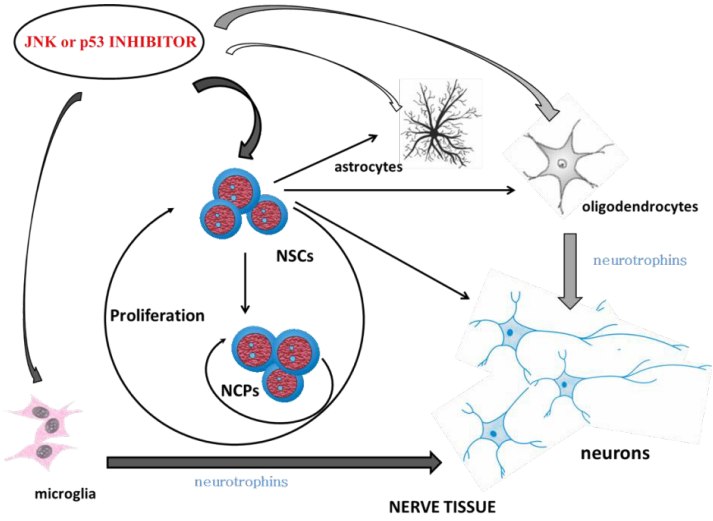

Figure 4. Effect of JNK or p53 inhibitor on the regeneration-competent cells of the nerve tissue in ethanol-induced neurodegeneration. NSCs - neural stem cells; NCPs - neuronal-committed progenitor cells; grey arrows are a stimulating influence; white arrows are an inhibitory effect.

Therefore, the use of JNK and p53 inhibitors in alcoholic encephalopathy may be promising only during the manifestation of the disease (and the cancellation of their use when there are clinical signs of reconvalescence)[30].

The detected increase in the secretion of neurotrophic growth factors by oligodendrocytes and microglia when blockaded in them JNK and p53 in the conditions of exposure to ethanol is an additional reason in favor of the possibility of accelerating the reparation of the CNS by inactivating these signal molecules.

But it should be taken into account that JNK and p53 inhibitors have an ambiguous effect on the functioning of astrocytes in the modeling of ethanol-induced neurodegeneration *in vitro* and *in vivo*. Further research should determine how much this factor can be neglected.

Besides, the anti-mutagenic properties of the p53 protein (p53 - "guardian of the genome"[31]) are known. This determines the need for further consideration of the carcinogenic safety of the potential use of its potency modifiers as drugs. The p53 can also be targeted via JNK-pathway[13]. However, it is only one of the directions of signal transduction via JNK[32, 33], which in some cases is not implemented. Besides, even the anti-blastic properties of JNK inhibitors are known[4, 34]. Therefore, the potential for drug (carcinogenic) safety in JNK activity/expression inhibitors is higher.

The results reveal the feasibility of further study of the possibility of creating novel effective drugs for the therapy of alcoholic encephalopathy based on JNK or p53 inhibitors[34]. The development of pharmacotherapy approaches using JNK activity/expression inhibitors is especially promising.

AUTHOR CONTRIBUTION

Conceptualization: Gleb N. Zyuz`kov; Methodology: Gleb N. Zyuz`kov, Larisa A. Miroshnichenko; Animal caring: Tatyana Yu. Polyakova, Larisa A. Stavrova and Elena V. Simanina; Writing: Gleb N. Zyuz`kov.

ETHICS APPROVAL

The study was approved by the Institute`s local Ethics Committee (Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Center, Russian Academy of Sciences) (protocol GRIPh&RM-2020-09). No humans were used in this research. All animal experiments were carried out following the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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