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Prospect of Using ERK1/2 and p38 in Regeneration-Competent Cells of Nervous Tissue as a Drug Targets for Treating Alzheimer's Disease

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Abstract: Drugs used to treat Alzheimer's disease (AD) were developed taking into account the existing classic hypotheses of its pathogenesis (cholinergic, amyloid, and tau-protein pathological changes). However, these pharmaceuticals have shown rather low efficacy in clinical practice. Therefore, it is relevant to develop approaches for controlling neurogenesis in AD, primarily by synchronizing the activities of different cell compartments of the cellular renewal system of nerve tissue. The search for a solution to this problem is promising in the framework of the "Strategy for targeted pharmacological regulation of intracellular signal transduction in regeneration-competent cells." In this work, we studied the effects of the ERK1/2 and p38 inhibitors on nervous tissue progenitors and neuroglial cells' functioning under the conditions of modeling β-amyloid-induced neurodegeneration in vitro. We identified opposing changes in proliferation and differentiation of neural stem cells (NSCs) and neuronal-committed progenitors (NCPs) influenced by β-amyloid (Aβ). It was also found that different types of neuroglia cells' secretion of neurotrophic growth factors (astrocytes, oligodendrocytes, and microglia), when exposed to Aβ, differ in vector. The ERK1/2 and p38 inhibitors' ability to coordinate the functions of regeneration-competent cells of different types has been discovered. Under their influence, synchronization of pro-regenerative activity of NSCs, NCPs, as well as oligodendrocytes and microglial cells under conditions of β-amyloid-induced neurodegeneration was revealed. The results show the prospect of developing novel drugs to treat AD with ERK1/2 and p38 inhibitors.

Keywords: Alzheimer's disease; stem cells; neuroglia; intracellular signaling transduction; mitogen-activated protein kinases.

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1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly. Moreover, there has been a significant increase in the incidence of AD in recent years against the background of a decrease in the age of its debut [1, 2]. AD is characterized by steadily progressive cognitive impairment, loss of practical skills, and self-care ability, leading to death due to causes unrelated to the underlying disease (infections, aspiration of food, injuries, etc.). The development of described disorders occurs against the background of decompensation of adaptation mechanisms of various cellular compartments of nervous tissue [3-6]. The synthesis of neurotoxic beta-amyloid peptides (Aβ) and the formation of neurofibrillary tangles are

accompanied by the reorganization of the central nervous system and the formation of a qualitatively new - pathogenic pattern activity of many brain structures [1, 6].

The currently used drug therapy is based on the effect on the three main supposed causes of AD: cholinergic, amyloid, and tau-protein hypotheses [1, 7]. However, pharmaceuticals developed within the framework of these concepts are practically ineffective [6]. The absence of satisfactory effects from the use of these drugs indicates the relative insolvency of cholinergic, amyloidogenic, and tau-protein hypotheses, at least as "trigger" mechanisms of the AD. But the neurotoxic effect of Aβ and its critical pathogenetic role in the course of AD course is beyond doubt [7, 8].

It is known that neuronal death and destruction of synapses under the influence of Aβ are observed against the background of a loss of the nervous tissue ability for balanced neurogenesis [9, 10]. Therefore, it is advisable to find the possibility of AD therapy by stimulating the coordination of neuroregeneration with pharmacological agents - regulators of functions of regeneration-competent cells of various types (including progenitors and neuroglial cells) [12-14]. The implementation of this approach also looks promising if the data on the etiological role of somatic mutations in nerve cells (including transcriptional mutagenesis in neurons [15, 16]) in the development of AD are confirmed. The activation of dormant NSCs can possibly lead to the formation of healthy mature nerve cells (without genetic and/or biochemical defects occurring during the disease).

The search for ways to solve this problem is rationally carried out within the framework of the "Strategy of targeted pharmacological regulation of intracellular signal transduction in regeneration-competent cells" [13, 14, 17, 18]. For this, the fundamental stage of development of this direction is the identification of the role of individual intracellular signaling molecules in the regulation of functions of various types of progenitors and neuroglial cells. These studies will determine optimal drug targets among signaling proteins. It is known that mitogen-activated protein kinases (MAPK) regulate progenitor's proliferation and differentiation, as well as cytokine production by glial cells [17, 19]. But there is no detailed understanding of the role of individual directions of MARK-pathways (including through activation of ERK1/2 and p38) in regeneration-competent cells when exposed to Aβ.

The work aimed to study the effects of the ERK1/2 and p38 inhibitors on the functioning of neural stem cells (NSCs), neuronal-committed progenitors (NCPs), and neuroglial cells (astrocytes, oligodendrocytes, and microglial cells) under the conditions of modeling β-amyloid-induced neurodegeneration in vitro.

2. Materials and Methods

2.1. Chemicals and drugs.

The serum-free 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); Amyloid β-Protein Fragment 25-35; ERK1/2 inhibitor (PD98059); p38 inhibitor (SB202190); hydroxyurea (all manufactured by Calbiochem, Germany); plastic plates for cultural studies (Costar, USA).

2.2. Animals and experimental design.

Experimental was carried out in compliance with the principles of humane treatment of experimental animals (EU Directive 2010/63/EU for animal experiments) and with the

permission of the local ethical committee (protocol GRIPh&RM-2022-01/01). Studies were carried out on 30 C57B1/6 mice at the age of 2-2.5 months. Animals of category 1 (conventional mice) were obtained from the Department of Experimental Biological Models of the Goldberg Research Institute of Pharmacology and Regenerative Medicine. After experimental studies, the animals were sacrificed with CO_2 cameras.

Using culture methods, the effect of the ERK1/2 (100 µM) and p38 (10 µM) inhibitors on the implementation of the growth potential of nervous tissue progenitors (NSCs, NCPs) [21] and the secretion of growth factors of neural progenitors by astrocytes, oligodendrocytes, microglial cells under the conditions of modeling β-amyloid-induced neurodegeneration in vitro was studied. The appropriate cell cultures with Aβ without signaling molecule inhibitors served as controls.

2.3. Modeling β-amyloid-induced neurodegeneration.

The Aβ fragment 25-35 was used in the experiment. To obtain protein aggregates, it was incubated at a concentration of 1mM for 7 days at 37°C, 5% CO2, and 100% air humidity. The aggregated Aβ was added to the culture medium in vitro to a final concentration of 20 µM [8, 22].

2.4. Progenitor cells study.

The progenitors were sampled from the lateral ventricles of the brain (subventricular zone, SVZ). NSCs were studied during the cultivation of unfractionated cells from the SVZ. NCPs were investigated in the cultivation of $CD56^+$ (PSA-$NCAM^+$) cells [13, 20]. $CD56^+$ cells were received from an SVZ using the immunomagnetic MiniMACS Cell Separator (Miltenyi Biotec, Germany) and anti-PSA-NCAM MicroBeads. Unfractionated and $CD56^+$ cells were incubated in MACS Neuro Medium at 10^5 / ml for 5 days in a CO_2 incubator (at 37°C, 5% CO_2, and 100% air humidity). After incubation of both cell types, the number of clonogenic cells, their mitotic activity, and intensity of specialization were considered. The number of NSCs and NCPs was determined by the number of colony-forming units (CFU, colonies containing more than 100 cells) in the respective cell cultures. The proliferative activity of progenitors was estimated using a cellular suicide technique using hydroxyurea (1 µM) [14]. The specialization (differentiation/maturation) intensity of NSCs and NCPs was determined by calculating the ratio of cluster-forming units (ClFU, neurospheres from 30-100 cells) to CFU [21].

2.5. Neuroglial cells study.

From the SVZ using the immunomagnetic MiniMACS Cell Separator (Miltenyi Biotec, Germany), anti-ACSA-2 MicroBead, Anti-O4 MicroBeads, and Anti-CD11b MicroBeads received astrocytes, oligodendrocytes and microglial cells were obtained, respectively. Isolated cells at a 2×10^6 / ml concentration were incubated to obtain supernatants in MACS Neuro Medium for 2 days in a CO_2 incubator (at 37°C, 5% CO_2, and 100% air humidity). To determine their secretory activity (production of neurotrophins, a combination of growth factors active against CFU), the effect of conditioned media on the level of neurosphere formation (neurosphere-stimulating activity, NSA) in the test system was studied. The test system was a culture of intact unfractionated cells of the SVZ (at a concentration of 10^5/mL) in MACS Neuro Medium [14, 17].

2.6. Statistical analysis.

The data were analyzed with one-way ANOVA followed by Dunnett's test, Wilcoxon's test for dependent samples, and the Mann-Whitney test for independent samples. The data are expressed as arithmetic means. The significance level was $p < 0.05$ (Curtis et.al., 2015) [23].

3. Results and Discussion

3.1. Aβ effect on the functioning of progenitors.

The addition of the Aβ neurotoxic fragment to the culture medium of unfractionated cells was accompanied by a decrease in the level of colony formation. The amount of CFU_{NSCs} was 78.7% of the baseline in the Aβ-free cell culture (Figure 1). These changes reflected a drop in the NSCs mitotic activity (up to 71.5% of the corresponding parameter in the medium without Aβ). However, an increase in the number of $ClFU_{NSCs}$ (up to 141.1% of the baseline) was observed, which was a logical result of an increase in the intensity of NSCs specialization. The progenitor cell differentiation index reached 181.1% in the medium containing no neurotoxic agent.

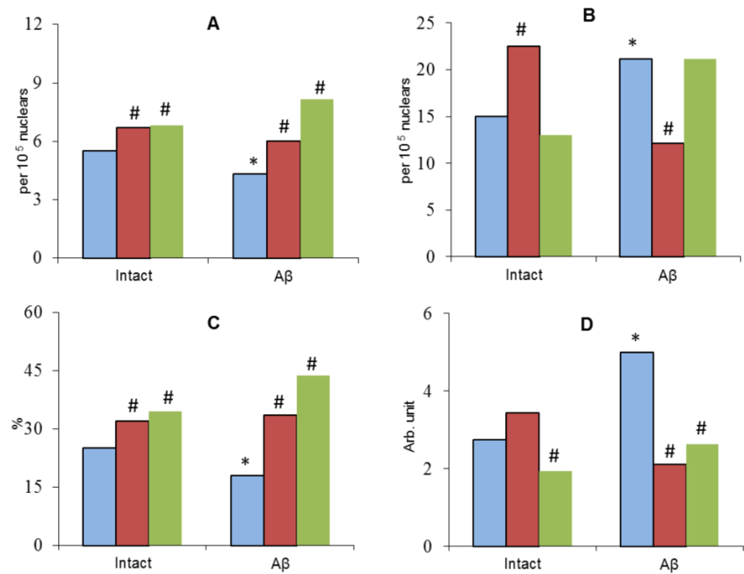

Figure 1. Amount of (**A**) CFUNSC, (**B**) ClFUNSC, (**C**) NSCs proliferative activity, and (**D**) their differentiation index. Here and in Fig. 2 and 3: cell culture without βA (intact); with βA. Blue bars - without inhibitors (blue bars); red bars – with the ERK1/2 inhibitor; green bars – with the p38 inhibitor;* - differences with intact; # - differences with the group without inhibitors at $p < 0.05$.

The committed-neuronal progenitors responded differently to Aβ. Exposure to $CD56^+$ cells was accompanied by an increase in the number of CFU_{NCPs} (up to 341.6% of baseline), proliferating CFU_{NCPs} (up to 206.1% of baseline), and $ClFU_{NCPs}$ (up to 200.1% of baseline). However, there was a decrease in the rate of NCPs specialization (up to 55.6% of that in a medium without Aβ) (Figure 2).

The obtained results revealed opposite changes in proliferation and differentiation of NSCs and NSCs under conditions of β-amyloid-induced neurodegeneration. This explains the inconsistency of data [6, 8, 22] on divergent changes in progenitors' functioning (stimulation or inhibition) under the influence of Aβ fragment 25-35. Likely, the response to the toxic action

of the pathogenic peptide of progenitors depends on their initial proliferative and differentiated potential.

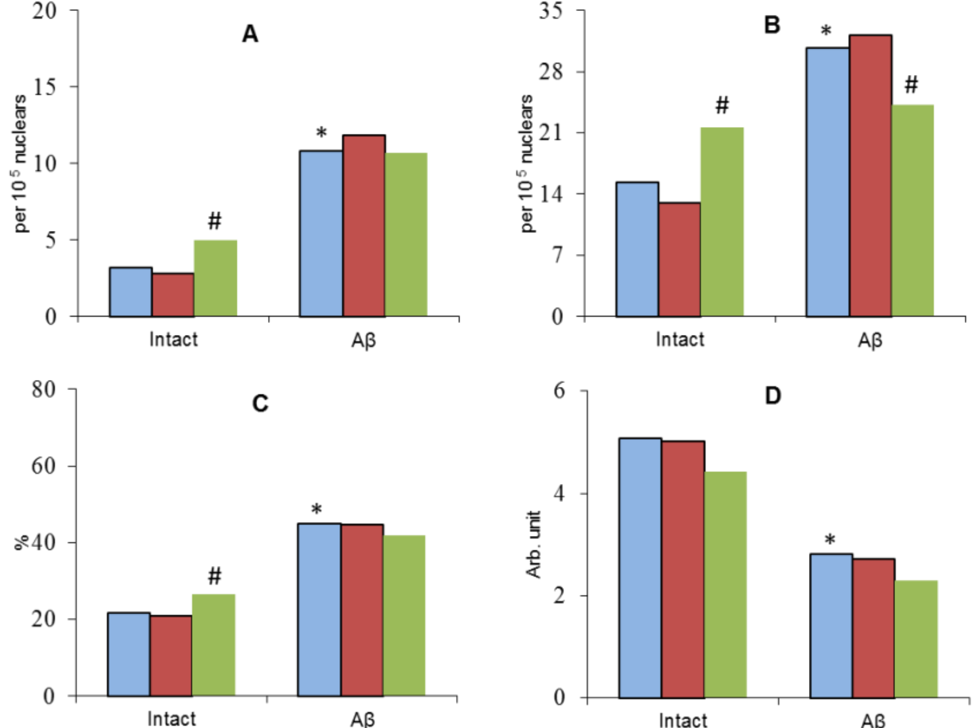

Figure 2. Amount of (**A**) CFU_{NCPs}, (**B**) $ClFU_{NCPs}$, (**C**) NCPs proliferative activity, and (**D**) their differentiation index of.

3.2. Aβ effect on the secretory function of neuroglial cells.

Toxic exposure increased growth factor secretion by astrocytes (up to 200.0% of baseline) and microglial cells (up to 123.3% of baseline) (Figure 3, A, C). In contrast, oligodendrocytes did not change their secretory activity under the influence of Aβ (Figure 3B).

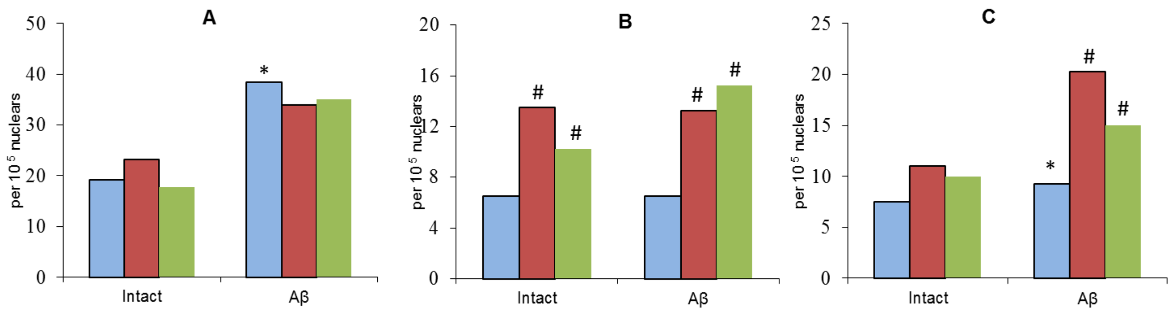

Figure 3. Effect of conditioned media of (**A**) astrocytes, (**B**) oligodendrocytes, and (**C**) microglial cells on the level of neurosphere formation in the test system.

However, it should be borne in mind that the NSA of glial cell conditioned media in these experiments is an integral indicator. Its changes may be associated with changes not only in the production of progenitor growth stimulants (leukemia induction factor, ciliary neurotrophic factor, etc. [14. 24]) but also in inhibitors (for example, pro-inflammatory cytokines: interleukins-1, 6. 15, tumor necrosis factor-alpha, etc. [3. 17]).

3.3. Effects of the ERK1/2 and p38 inhibitors on the functioning of NSCs.

The study of the participation of MAPK-pathways in implementing the growth potential of the NSCs made it possible to identify some interesting phenomena. The introduction of ERK1/2 and p38 inhibitors into the culture medium in both cases (in the presence of and without Aβ) increased the level of colony formation of unfractionated cells (Figure 1, A). The amount of CFU_{NSCs}, when cultured in an amyloid-free medium, increased to 121.3% and 124.2% when the blockade of ERK1/2 and p38 of the control levels. These changes resulted from an increase in NSCs proliferative activity (up to 127.1% and 137.1% of control at the blockade of ERK1/2 and p38, respectively) (Figure 1, C).

The selective inactivation of signal molecules under the conditions of modeling β-amyloid-induced neurodegeneration was accompanied by the development of similar reactions of the functioning of NSCs. But these changes were more pronounced. The increase in CFU_{NSCs} and their proliferative activity reached 138.6% and 187.1% (with blockade of ERK1/2), and 188.7% and 243.5% (with inactivation of p38) of the corresponding control levels (Figure 1). Besides, the ERK1/2 and p38 inhibitors in the presence of Aβ reduced the progenitor differentiation index to that of cells under optimal living conditions (Figure 1, D).

Thus, the ERK1/2 and p38 inhibitors leveled the negative effect of Aβ on the progression of the NSCs functions.

3.4. Effects of the ERK1/2 and p38 inhibitors on the functioning of NCPs.

Unsimallar patterns of changes in functioning were observed when MAPKs inhibitors were exposed to $CD56^+$ clonogenic cells. Both in the conditions of optimal vital activity and the modeling β-amyloid-induced neurodegeneration, ERK1/2 blockade in the NCPs did not affect the indicators studied (Figure 2). The addition of the p38 inhibitor to an Aβ-free medium with $CD56^+$ cells was accompanied by an increase in CFU_{NCPs}, their proliferative activity, and the number of $ClFU_{NCPs}$ (up to 157.7%, 121.4%, and 141.2% of the control values). But there was no change in these indicators during the p38 blockade in the culture of $CD56^+$ clonogenic cells with Aβ. However, in this case, as well as in the inactivation of ERK1/2 in the NCPs, it should be noted that there is a high level of NCPs proliferative activity caused by Aβ.

Thus, the ERK1/2 and p38 inhibitors did not adversely affect the realization of the growth potential of NCPs under the β-amyloid-induced neurodegeneration simulation.

3.5. Effect of the ERK1/2 and p38 inhibitors on the functioning of neuroglial cells.

In both cases, the blockade of ERK1/2 and p38 in astrocytes (during incubation in the medium with and without Aβ) did not change the NSA of the conditioned media (Figure 3, A). In contrast, impaired signal transduction through ERK1/2 and p38 in oligodendrocytes and microglial cells, regardless of their culture conditions (in the presence of Aβ and without it), was accompanied by a pronounced increase in growth factor production (Figure 3, A, B). The increase in NSA in supernatants obtained from $O4^+$ and $CD11b^+$ cells in the Aβ-medium reached 203.8% and 218.9% at ERK1/2 blockade and 234.6% and 162.1% at p38 blockade from control levels, respectively.

Thus, the inactivation of ERK1/2 and p38 in oligodendrocytes and microglial cells under conditions of β-amyloid-induced neurodegeneration significantly stimulated their ability to increase the functional activity of progenitors. At the same time, MAPKs do not play a significant role in regulating the secretory function of astroglia.

The results of the studies indicate a pronounced discoordination of the functioning of NSCs and a violation of the implementation of secretory function by neuroglial cells under the influence of β-amyloid toxic fragments [6, 8, 22]. Exposure to Aβ is accompanied by inhibition of the progression of the cell cycle of NSCs and their very rapid differentiation. While NCPs, on the contrary, actively proliferate against the background of low intensity of their specialization processes. The detected changes (desynchronization of functions of regeneration-competent cells) can cause the formation of mature neural tissue cells having certain functional "defects" [19, 25]. Moreover, it should be borne in mind that under the influence of phosphorylated tau-proteins and disorders of the cholinergic system, these abnormalities in the functioning of progenitors in AD in situ will only be more manifested and aggravated [26].

The experiments data showed that ERK1/2 and p38-pathways have an important role in developing the detected mechanisms of disadaptation [17, 19, 20]. For the first time, the possibility of interfacing the proliferation and differentiation of NSCs and NCPs, as well as the activation of the compensatory response of different types of neuroglia in β-amyloid-induced neurodegeneration with selective the ERK1/2 and p38 inhibitors was demonstrated (Figure 4).

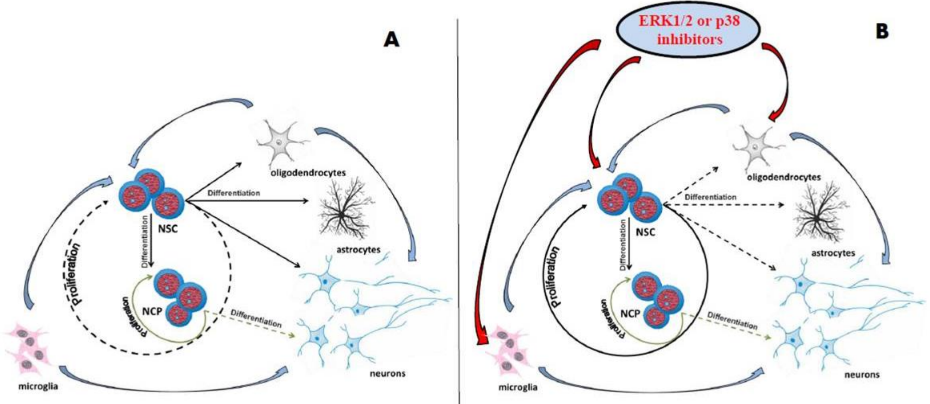

Figure 4. (**a**) Desynchronization of the activity NSCs and NCPs in β-amyloid-induced-induced neurodegeneration; **(b)** Synchronizing the activity of regenerative-competent cells under the influence of ERK1/2 and p38 inhibitors. Continuous lines - stimulation; dashed lines - inhibition; wide blue arrows are the neurotrophic influence of glial cells; wide red arrows are the stimulating effect of inhibitors of signaling molecules.

4. Conclusions

The obtained results indicate the feasibility of finding a solution to the problem of neurogenesis disorders in Alzheimer's disease within the framework of the "Strategy of targeted pharmacological regulation of intracellular signal transduction in regeneration-competent cells" [13, 14, 27]. Particularly promising is developing approaches to promote coordinated neuroregeneration based on ERK1/2 and p38 inhibitors.

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Conflicts of Interest

The authors declare no conflict of interest.

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