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Inhibiting cAMP-pathways as a Potential Treatment Strategy for 'Alzheimer's Disease

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Abstract: The extremely low efficiency of existing methods of treating 'Alzheimer's disease (AD) makes it highly relevant to develop fundamentally new drugs for its therapy. Promising is the creation of approaches to stimulate neurogenesis. As part of implementing this route, it is promising to search for targets among intracellular signaling molecules, including cAMP-dependent intracellular pathways. The work aimed to study the effect of adenylate cyclase (AC) and PKA inhibitors on the functions of regeneration-competent cells (neural progenitors and neuroglial cells of various types) in the conditions of modeling β-amyloid-induced neurodegeneration (βAIN) *in vitro*. The experiments were performed on C57B1/6 male mice. We studied the effect of the AC (2′,5′-Dideoxyadenosine and PKA inhibitors (2′,5′-Dideoxyadenosine and KT 5720) on the functioning of neural stem cells (NSC), neuronal committed progenitors (NCP), and neuroglial cells of the subventricular zone of the cerebral hemispheres (SVZ). Using immunomagnetic sorting, NCP and individual types of neuroglial cells were isolated from SVZ cells. We revealed the discoordination of the activity of NSC and NCP under the influence of neurotoxic β-amyloid. The ability of the AC and PKA inhibitors to synchronize the implementation of the functions of different types of progenitors under conditions of βAIN was found. The blockade of cAMP-dependent pathways upon exposure to a neurotoxic agent also led to an increase in the production of neurotrophic growth factors by several types of neuroglial cells. Particularly pronounced was the reaction of oligodendrocytes and microglial cells during PKA inactivation. The obtained results indicate the possibility of coordinated stimulation of the functions of different types of progenitors and neuroglial cells using selective inhibitors of intracellular molecules of cAMP-dependent pathways (primarily PKA) in AD.

Keywords: Alzheimer's disease; neural stem cells; β-amyloid; adenylate cyclase; cAMP; PKA.

1. Introduction

Alzheimer's disease (AD) is a senile dementia most common in countries with long life expectancy [1]. The pharmacological methods of treating AD that exist today consist of acting on the remaining mature cells of the nervous tissue. However, this concept of pharmacotherapy is not only able to cure the disease but also to stop its progression [2, 3]. The etiology of asthma is still unknown [1, 4, 5], although a number of pathogenesis links have been revealed. Thus, the neurotoxic effect of some β-amyloid fragments on nerve cells is well known [5-7]. It has been shown that disruption of their functioning under the conditions of the formation of

amyloid aggregates occurs against the background of the loss of plasticity of the nervous tissue and its ability to neurogenesis [5, 8, 9].

At the same time, our previous studies have shown that impaired neurogenesis in AD develops as a result of impaired development of neural stem cells (NSC) and the implementation of the neurotrophin-producing function of neuroglial cells [10, 11].

Based on this, the development of pharmacological methods of AD therapy based on the principle of regulating the functions of regeneration-competent cells (RCC) of the nervous tissue and coordinating the functions of their representatives (NSC, committed precursors, neuroglial cells) looks promising [5, 10]. The implementation of this approach seems to be the most preferable within the framework of the "Strategy for targeted regulation of intracellular signal transduction in regeneration-competent cells" [3, 12-15]. The selectivity of the action is determined by the peculiarities of intracellular signaling in different types of progenitor cells and/or tissue-specific expression of certain types and isoforms of signaling molecules [3, 16]. In this regard, the most important step in the creation of new drugs for the treatment of AD within the framework of this approach is the identification of intracellular signaling molecules that determine the functioning of RCC.

cAMP-mediated signaling pathways play an important role in the control of NSC proliferation and differentiation, as well as the humoral function of neuroglial elements, including neurodegeneration caused by alcohol abuse [1, 14]. However, the significance of individual links of this cascade in the functioning of the NSC and several other elements of the nervous tissue involved in neurogenesis in AD is still not known.

It is known that cAMP pathways are involved in the regulation of the functions of NSC and neuroglial cells, including in neurodegeneration caused by alcohol abuse [12, 17-19]. However, the role of individual protein-protein interactions within this signaling for the functioning of NSC and several other RCC of the nervous tissue involved in neurogenesis in AD is still unknown.

This work aimed to study the possibility of regulating the functions of nervous tissue RCC (neural and neuronal progenitors, astrocytes, oligodendrocytes, and microglial cells) using the AC and PKA inhibitors in modeling β-amyloid-induced neurodegeneration (βAIN) *in vitro*.

2. Materials and Methods

2.1. Chemicals and drugs.

Amyloid β 25-35 (Sigma-Aldrich, Germany); AC inhibitor (2′,5′-Dideoxyadenosine, Sigma-Aldrich, USA); PKA inhibitor (KT 5720, Sigma-Aldrich, USA); basic culture medium for nerve cells MACS Neuro Medium; reagents for magnetic immune separation (anti-PSA-NCAM MicroBeads; anti-ACSA-2 MicroBead Kit; Anti-O4 MicroBeads; Anti-CD11b MicroBeads) (all produced by MiltenyiBiotec B.V. & Co. KG, Germany); hydroxycarbamide hydurea (Calbiochem, USA); DMSO (Sigma-Aldrich, USA); Primaria Cell Culture Plate (size 96 well) (Corning, USA).

2.2. Animals and experimental design.

The experiments were carried out in accordance with the principles of the humane treatment of animals (EU Directive 2010/63/EU). Permission was previously obtained from

the local ethics committee of the Institute (protocol GRIPhRM-2022-03/11 dated January 12, 2023).

The research has been conducted on C57B1/6 male mice at the age of 8 weeks (n=30). The mice were obtained from the Department of Experimental Biological Models of the Tomsk National Research Medical Center (Tomsk, Russia). The experiments were carried out in winter. After the experiment, the mice were decapitated under anesthesia in a CO_2 chamber (M1-TSFM-1, EZ Systems Inc., USA).

In vitro experiments using cultural methods studied the effect of the AC and PKA inhibitors (30μM and 10μM respectively) on the functioning of nervous tissue progenitor cells (NSC, NCP) and the secretion of growth factors by neuroglial cells (astrocytes, oligodendrocytes, microglial cells) under conditions of βAIN simulation [5, 10]. The corresponding cell cultures with βA without inhibitors served as controls.

2.3. βAIN modeling.

In vitro simulation of neurodegeneration induced using βA fragment 25-35, which was pre-incubated (1 mM) for 7 days in a CO2 incubator for aggregation (Thermo Scientific 8000 DH, Thermo Fisher Scientific Inc., USA) at 37°C, 5% CO_2, and 100% air humidity. Then, the βA concentration in the medium was adjusted to 20 μM [5, 10].

2.4. Progenitor cells study.

The cellular material for research was isolated from the subventricular zone of the cerebral hemispheres (SVZ). The number of NSC and NPC in the cellular material was determined by the level of colony formation in cultures of unfractionated and CD56+ cells, respectively. CD56+ cells were isolated using positive immunomagnetic selection using PSA-NCAM (MIniMACS Cell Separator (Miltenyi Biotec, Germany)) [10, 15, 16]. After this, the cells were incubated in MACS Neuro Medium (10^5/ml) in a CO_2 incubator (Thermo Scientific 8000 DH, USA) under standard conditions (37^0C, 5% CO_2, and 100% air humidity) for 5 days. The content of NSCs and NPCs in the studied cellular material was assessed by its colony-forming capacity (CFU, neurospheres of more than 100 cells). The mitotic activity of CFU was also determined. For this purpose, we used hydroxyurea (at a concentration of 1 μM in the medium), which blocks the S-phase of the cell cycle. In addition, the ratio of the number of cluster-forming units (CFU, small neurospheres (30-80 cells)) to CFU was determined by the NSCs and NPCs specialization index (differentiation/maturation intensity) [5, 15].

2.5. Neuroglial cells study.

The fractions of astrocytes, oligodendrocytes, and microglial cells were isolated from SVZ cells using the MIniMACS Cell Separator and anti-ACSA-2 MicroBead, anti-O4 MicroBead, and anti-CD11b MicroBead, respectively. The cells were incubated in MACS Neuro Medium in the CO_2 incubator (Thermo Scientific 8000 DH, Thermo Fisher Scientific Inc., USA) under standard conditions at a concentration of 2×10^6/ml for 2 days to obtain supernatants. The production of neurotrophins (a complex of all humoral factors active against CFU) was studied by the effect of conditioned media (supernatants) on the level of neurosphere formation (neurosphere stimulating activity, NSA) in the test system [9]. The test system was a culture of intact unfractionated cells of the SVZ (at a concentration of 10^5/mL in MACS Neuro Medium) [5, 14].

2.6. Statistical analysis.

The results were analyzed with one-way ANOVA followed by Dunnett's test, Wilcoxon's test, and Mann-Whitney test for independent samples. The data are expressed as arithmetic means at $p \leq 0.05$ (Curtis et al., 2015) [20].

3. Results and Discussion

3.1. NSC and NCP functioning under the influence of Aβ.

The toxic effect of the Aβ 25-35 fragment on progenitor cells was manifested in a decrease in the yield of CFU_{NSC} (up to 77.1% of the initial level) and their actively dividing forms (Figure 1, A). At the same time, the NSC specialization accelerated up to 180.2% of that in cell culture without a neurotoxic agent. The increase in the number of $ClFU_{NSC}$ was observed in the medium containing Aβ (up to 138.7% of the control) (Figure 1, B). The Aβ, at the same time, stimulated the clonogenic activity of $CD56^+$ cells. The number of CFU_{NCP} and their proliferative activity increased to 351.3% and 207.9% of the corresponding control values under conditions of optimal vital activity (Figure. 2C). The specialization index of multipotent progenitors increased (up to 187.6% of the initial level), while in committed precursors, on the contrary, it significantly decreased (up to 53.3% of the same indicator in the control) (Figure 2, D). The results were consistent with our earlier data on the uncoupling of the functioning of NSC and NCP under the influence of neurotoxic fragments of Aβ [8, 21]. Moreover, the important reflection of the dysfunction of the regeneration mechanisms of the "deep reserve" should be considered an excessively high rate of the NSC maturation, which can be the cause of aberrant cell development [15, 22, 23].

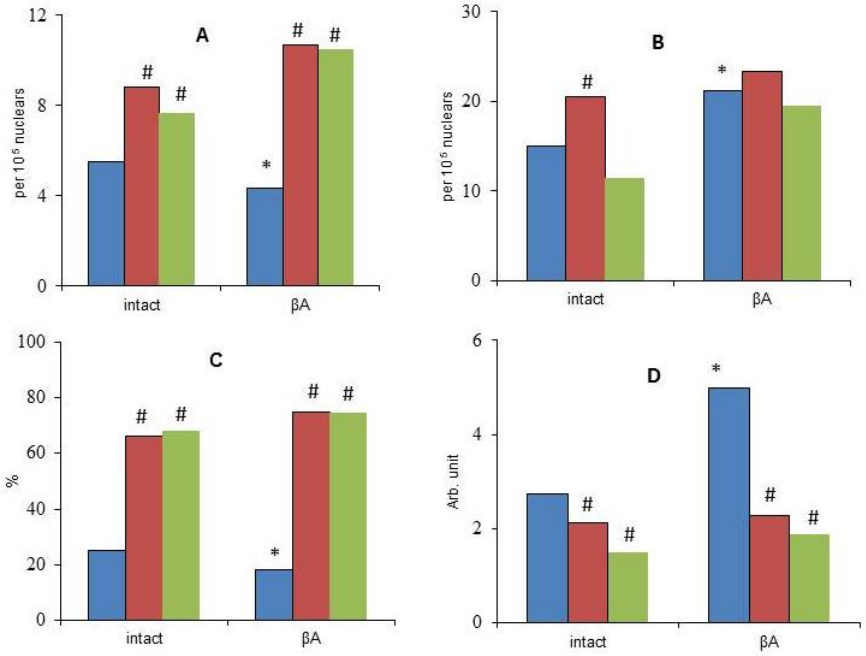

Figure 1. Number of **(A)** CFU_{NSC}; **(B)** $ClFU_{NSC}$; **(C)** NSC mitotic index; **(D)** NSC differentiation index.

Here and in figures 2 and 3: cell culture without (intact); with Aβ. Blue bars - without signaling molecule inhibitors; red bars - with the AC inhibitor; green bars - with the PKA inhibitor; $p<0.05$ compared to *intact cells (no inhibitors), # values without signaling molecule inhibitors.

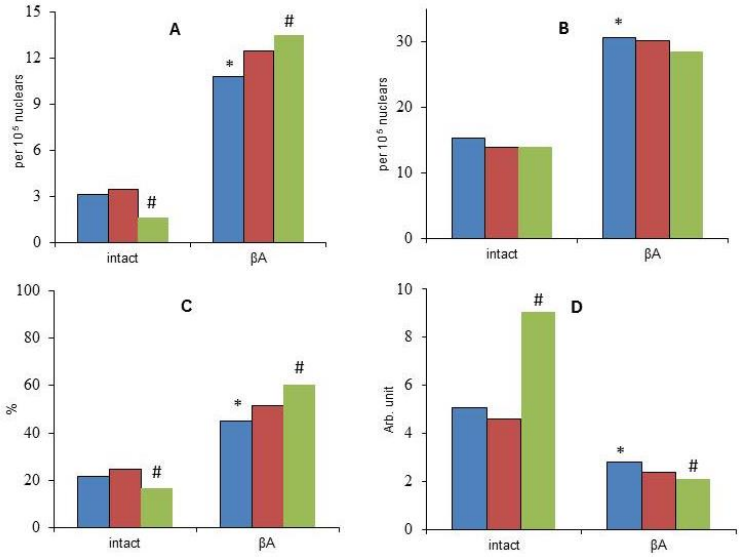

Figure 2. Number of **(A)** CFUNCP; **(B)** ClFUNCP; **(C)** NCP mitotic index; **(D)** NCP differentiation index.

3.2. Functioning of neuroglial cells under the influence of Aβ.

The neurotoxic effect of Aβ on neuroglial cells was accompanied by an increase in the secretion of neurotrophic growth factors by ACSA-2^+ cells and $CD11b^+$ cells (up to 207.3% and 126.2% of the corresponding levels in the Aβ-free medium). The NSA of $O4^+$ cells did not change under the influence of Aβ (Figure 3, A-C).

However, it should be taken into account that the registered changes in the NSA of the conditioned media of neuroglial cells were an integrative indicator of the production of both cytokines that have a stimulating effect on neural progenitors and the secretion of factors - inhibitors of their functions (primarily pro-inflammatory cytokines) [24-26]. Amyloid is known to initiate an excessive inflammatory response, which aggravates brain damage in AD [2, 4].

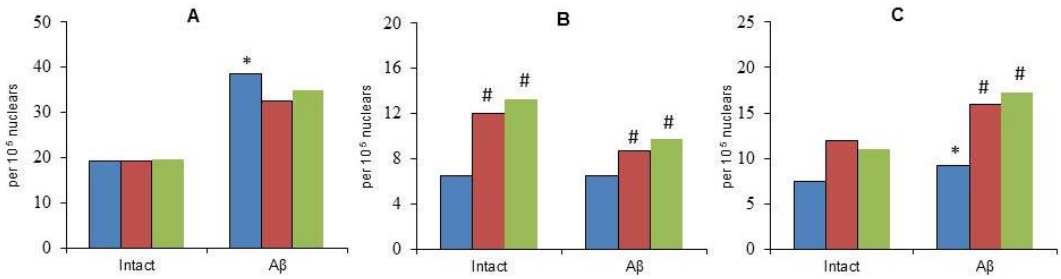

Figure 3. NSA of conditioned media of **(A)** astrocytes; **(B)** oligodendrocytes; **(C)** microglial cells.

3.3. Effect of the AC and PKA inhibitors on the NSC functioning.

The study of the role of cAMP-mediated pathways in implementing the functions of neural progenitors revealed several interesting phenomena. The introduction of the AC and PKA inhibitors into the medium, both in the presence of Aβ and without it, was accompanied by an increase in the level of colony formation and the CFU_{NSC} rate mitosis. The most pronounced were the changes in the cell culture in the medium with Aβ. The number of CFU_{NSC} reached 246.4% and 242.5% of the control value (medium with Aβ without inhibitors) when using the AC and PKA blockers, respectively (Figure 1, A). Moreover, the achieved values of this parameter in this case significantly exceeded those in the Aβ-free medium. Similar changes were observed concerning the mitotic activity of NSC. (Figure 1, C). Besides, in all cases, there

was a decrease in the specialization rate of progenitor cells. Thus, under the conditions of modeling AβIN, the decrease in the NSC differentiation index was 45.4% and 37.8% of the control level under the influence of the AC and PKA inhibitors, respectively (Figure 1, D). These changes most likely reflect an increase in neuroregeneration potential due to the prevention of aberrant development of nerve cells [10, 14].

Thus, the selective blockade of AC and PKA stimulated the progression of the cell cycle of multipotent NSC. Moreover, under conditions of neurotoxic exposure to Aβ, these changes were most pronounced.

3.4. Effect of the AC and PKA inhibitors on the NCP functioning.

Other regularities were revealed when studying the effect of inhibitors of various signaling molecules of the cAMP-signaling pathway on the functioning of committed neuronal precursors. The violation of cAMP synthesis did not affect the NCP functioning under intact conditions of their vital activity *in vitro* and under the influence of Aβ. However NCP reacted to adding the PKA inhibitor to the cell culture. Moreover, in the Aβ-free medium, the number of CFU_{NCP} and their proliferative activity decreased (up to 56.4% and 78.3% of the initial levels, respectively) (Figure 2, A-C). At the same time, their maturation accelerated (up to 179.1% of the initial level). Under modeling AβIN, the NCP response to the PKA inhibitor was different. The blockade of this signaling molecule caused an increase in the number of CFU_{NCP} and their mitotic rate in the culture of CD56+ cells (up to 123.5% and 131.7% of those in the control, respectively). Simultaneously, a decrease in the intensity of their specialization was recorded to 74.9% of the control (medium with Aβ without inhibitors of signaling molecules) (Figure 2, D).

Thus, the reaction of committed neuronal precursors to the PKA inhibitor depended on the initial conditions of their vital activity. However, during neurodegeneration, this pharmacological agent stimulated their proliferation.

3.5. Effect of the AC and PKA inhibitors on the functioning of neuroglial cells.

The experiments have shown the ambiguous effect of the selective AC and PKA blockers on implementing the secretory function of neuroglial cells. The violation of cAMP synthesis and inactivation of PKA in astrocytes ($ACSA\text{-}2^+$ cells) did not change the NSA of their conditioned media obtained from cells in the Aβ-free medium and with a toxic agent (Figure 3, A).

The blockade of AC and PKA in $O4^+$ and $CD11b^+$ cells was accompanied by a pronounced increase in the NSA of their supernatants in both cases (cell culture with and without Aβ) (Figure 3, B-C). The increase in the number of NSC in the test system when using supernatants of oligodendrocytes and microglial cells reached 183.2% and 158.7% with the AC blockade and 201.4% and 149.2% with the PKA blockade from control levels, respectively. (Fig. 3). The AβIN modeling somewhat reduced the intensity of stimulation of neurotrophin production by $O4^+$ cells and, conversely, significantly increased it in $CD11b^+$ cells in response to the AC and PKA inactivation (Figure 3).

Thus, disruption of cAMP synthesis, as well as blockade of PKA, stimulated the ability of oligodendrocytes and microglial cells to activate the functioning of progenitors, including in AβIN.

The findings confirm the appearance of discoordination in the activity of multipotent SC and committed neuronal precursors when exposed to Aβ [5, 10, 11, 27]. The inhibition of the progression of the NSC cell cycle with an increase in the mitotic activity of NCP was revealed. Moreover, the differentiation rate of multipotent progenitors increased against the background of a decrease in that of committed precursors. That is, the desynchronization of the proliferative activity of NSC and NCP developed against the background of dissociation of the processes of proliferation and differentiation of both types of progenitors. At the same time, the discovered extreme acceleration of NSC differentiation/maturation is probably one of the key factors in the aberrant pathway of nerve cell development under conditions of a pathogenic biochemical continuum in the nervous tissue in AD [15, 22, 23]. Such disturbances will necessarily reduce the efficiency of neurogenesis [28, 29]. Simultaneously, the contribution of individual types of neuroglia to the development of the detected discoordination of the functions of NSC and NCP remains unclear [14, 30, 31]. The increase in the secretion of neurotrophins by astrocytes and microglial cells under the influence of βA may be excessive (including the reason for the high intensity of NSC specialization). In this case, their reaction is decompensatory and is involved in the dysregulation of the cell renewal system [30, 32]. Moreover, it should be taken into account that under the influence of phosphorylated tau proteins, disorders of the cholinergic system, and other problems of tissue homeostasis in AD [33, 34], these disarrangements in the functioning of progenitors in situ will be multiplied and aggravated. Based on this, it follows that it is necessary to develop pharmacological approaches to synchronize the activity of the RCC to stimulate the restoration of the structure and functions of the central nervous system in AD [3, 5, 10].

At the same time, it was found that cAMP-mediated signal transduction (including via PKA) under neurotoxic exposure to an even greater extent, inhibits the NSC proliferation and stimulates the process of their specialization caused by Aβ. However, only PKA-dependent signaling is involved in forming a new pattern of NCP functioning. The identified patterns of changes in intracellular signaling in the event of impaired progenitors functioning under the influence of Aβ represent one of the mechanisms of disadaptation of the system of cell renewal of the nervous tissue in AD [17, 18, 35].

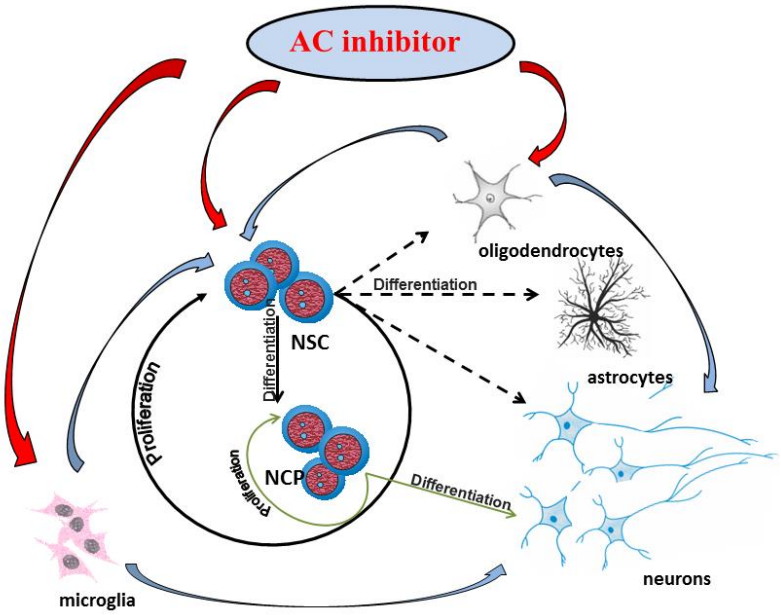

Figure 4. Synchronization of RCC activity under the influence of the AC inhibitor under conditions of AβIN.

Furthermore, the experimental results demonstrate the unique prospect of coordinating the functioning of different types of progenitors by targeting cAMP-dependent pathways [10, 12]. The possibility of stimulating the progression of the NSC cell cycle and pairing this process with their differentiation using selective inhibitors of AC and PKA was revealed (Figure 4, 5). However, concerning NCP, the PKA inhibitor was the most acceptable corrector.

Here and in Figure 5: 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.

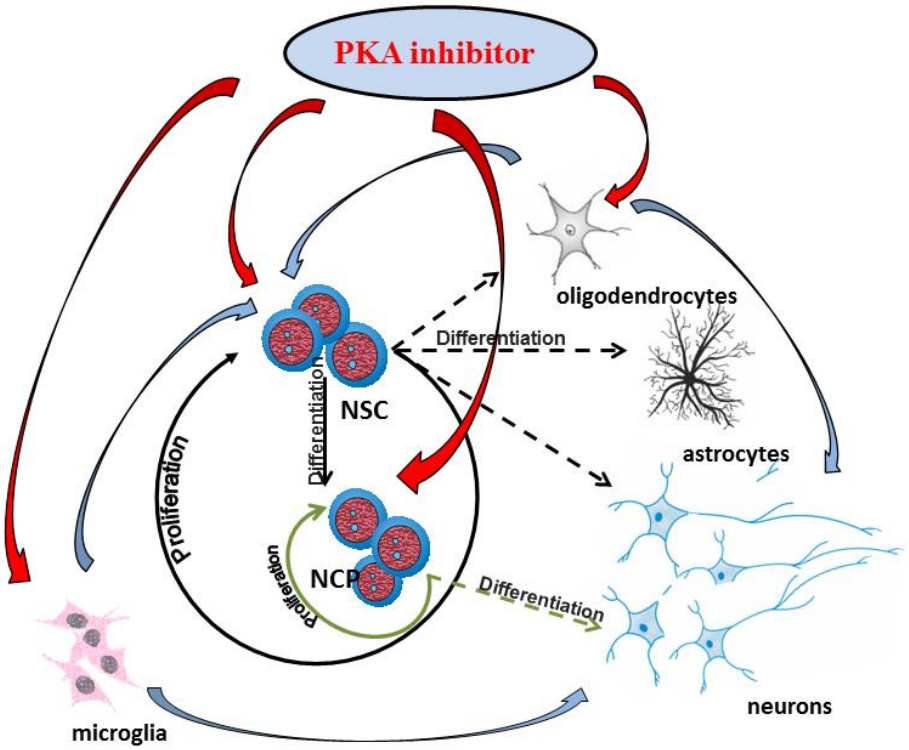

Figure 5. Synchronization of RCC activity under the influence of the PKA inhibitor under conditions of AβIN.

Also, such targeted regulation of intracellular signal transduction, based on the study's data, is not able to negatively affect the implementation of the secretory function of neuroglial cells. Inactivation of AC and PKA somewhat reduced the severity of the neurotrophin production ability of oligodendrocytes (which, as noted earlier, could be excessive) and, on the contrary, significantly increased the NSA of supernatants from microglial cells (probably due to a decrease in the production of pathogenic pro-inflammatory cytokines [1, 25, 26]).

4. Conclusions

The results obtained indicate the prospect of developing new methods of pharmacotherapy for AD with the use of AC and PKA inhibitors. However, based on these experiments, PKA is a more promising target for potential fundamentally novel drugs with neuroregenerative activity for treating AD.

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Conflicts of Interest

The authors state that there is no conflict of interest here.

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