Biointerface Research in Applied Chemistry
Platinum Open Access Journal (ISSN: 2069-5837)

Article
Volume 15, Issue 1, 2025, 2
https://doi.org/10.33263/BRIAC151.002

PKA Inhibition in Alzheimer's Disease: Through the Apologia of a Straight Forward but Nontrivial Concept of Targeting Intracellular Signal Transduction to the Prologue to the Era of Novel Drugs

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Received: 20.11.2024; Accepted: 1.01.20025; Published: 11.02.2025

Abstract: A promising approach for the treatment of neurodegenerative diseases, including the treatment of Alzheimer's disease (AD), maybe "Targeted regulation of intracellular signal transduction in the regeneration-competent cells (RCCs)." The aim of the work was to study the pharmacological (including neuroregenerative) effects of the PKA inhibitor (KT 5720) in a mouse model of AD. Based on research results and analysis of previously obtained data, it was proposed for the first time to consider the intracellular signal transduction system as a deterministic chaotic system. The reasons for the development of the "butterfly effect" and "fluctuations" in its activity, as well as factors for compensation of the latter and stability of functioning (self-organization and emergence), are presented. At the same time, pronounced therapeutic effects of the PKA inhibitor were shown in aged C57BL/6 mice (characterized by a high level of pathological endogenous β-amyloid formation in the brain). The correction of disorders of their cognitive and emotional status was discovered. It was revealed that the development of these effects is based on the coordination of the activities of different types of nervous tissue progenitors, coupled with the leveling of the pathological reaction of astroglia and microglia. The morphological signs of the antisenescence effect of the pharmacological agent on some other organs and a significant increase in the life expectancy of experimental animals treated with the PKA inhibitor were also found. The findings indicate the possibility of creating on its basis not only a medicine for AD treatment but also anti-aging drugs. Controlling the regenerative potential of tissues through targeted regulation of intracellular signal transduction in the RCCs represents a promising new paradigm for a pharmacological strategy for regenerative medicine.

Keywords: regenerative medicine; pharmacology; intracellular signal transduction; targeted drugs; Alzheimer's disease; neural stem cells; cAMP; PKA.

1. Introduction

The progressive increase in the incidence of neurodegenerative diseases, including senile dementia, is a serious challenge to modern healthcare. Despite all the achievements of scientific and technological progress in the field of biomedicine, there are still no effective approaches to correcting disorders associated with these pathological conditions [1-3]. Today,

there is no talk of a complete cure for such diseases. This problem is very clearly visible from the rapid increase in the number of patients with Alzheimer's disease (AD) and the rejuvenation of this nosology [4, 5]. Existing medications are not able to significantly slow down the loss of cognitive abilities and associated social skills in patients with AD. Some researchers declare the threat of an "epidemic" of AD, primarily in socially developed countries and regions with a long life expectancy, or even a "pandemic" [6-8].

For many years, various methods of cell therapy for AD have been developed [9, 10]. In experimental and clinical studies, attempts are being made to transplant embryonic, allogeneic, and autologous postnatal progenitors, pluripotent cells obtained using genome editing technologies, etc. However, there are still no encouraging results. The reasons for this are unsolved scientific and theoretical problems. First, tumorigenicity, immunogenicity, and heterogeneity of cell lines (no matter how strictly the protocols for their production are followed) [11-14].

The explanation for the inconsistency of data from numerous studies regarding the effectiveness of cell transplantations is the recently obtained results indicating not the depletion of individual compartments of the cellular renewal systems of nervous tissue in AD but the desynchronization of their activity in conditions of a pathological continuum formed under the influence of beta-amyloid (Aβ) [15, 16]. At the same time, there is no adequate compensatory response from various neuroglial cells. Aβ aggregation in situ causes an extremely pronounced increase in the production of neurotrophins by astrocytes [16-18], which is one of the manifestations of reactive astrogliosis, the pathogenic role of which in AD is well known [19, 20]. All this is against the background of neuroinflammation initiated by microglia under the influence of endogenous toxicants, which aggravates damage to brain structures [21-23]. It is clear that no matter how many new functionally relevant progenitors enter the brain under such conditions (due to their systemic administration and/or in situ transplantation), they cannot develop normally. This also explains the ineffectiveness of attempts to stimulate neurogenesis in AD using genetically engineered neurotrophins (even when introduced in situ into brain tissue) and/or their low-molecular analogs. After all, the concentration of growth factors in nervous tissue in AD is increased due to the pathogenic activity of astrocytes described above [16, 19]. Moreover, this determines the excessive intensity of specialization of neural stem cells (NSCs), which causes aberrant development of nerve cells [24-26]. Under these conditions, there is no point in additionally introducing progenitor stimulants (neurotrophins or other growth factors) from the outside. In general, today, it is logical and fair to paraphrase Max Born's statement, which he made at the dawn of the development of quantum mechanics ("Physics, as we know it, will be over in six months" [27]) in relation to cell transplantations. Today, we can say with confidence that cell therapy, as it was seen at the beginning of the 21st century (with expectations for it), will soon be over. At the very least, large-scale clinical application of these methods in the foreseeable future should not be expected.

Widespread implementation of technologies for using the secretome of certain progenitors (extracellular vesicles, etc.) as medications in practical healthcare will also require a lot of time [28, 29]. First, it will be necessary to resolve a number of complex issues about the safety of such complex products (which do not exclude "dangerous" substances in their composition, for example, in relation to carcinogenesis) [30-32].

Therefore, from the perspective of implementation into practice, the most appropriate is the development of pharmacological approaches to treating neurodegenerative diseases, including AD [16, 30]. However, the failure of attempts to use traditional pharmaceutical

methods and therapy for these nosologies so far indicates the need to create fundamentally new drugs [3, 33]. However, this is only possible if we go beyond the current pharmacology orthodoxy, which consists of acting on mature (highly specialized) cells [34].

This approach should be considered the recently proposed new paradigm of the pharmacological strategy of regenerative medicine - "Targeted regulation of intracellular signal transduction in regeneration-competent cells (RCCs)" [16, 30, 35, 36]. Over the past five years, experiments in vitro and in vivo have shown the possibility of controlling the growth potential of various progenitors by targeting individual intracellular signaling molecules in them. At the same time, under conditions of AD modeling, the ability of some selective modifiers of the activity of signaling molecules to correct the desynchronosis of the activity of different types of nervous tissue progenitors was discovered [15, 16, 37-39]. Particularly pronounced effects have been demonstrated when targeting cAMP-dependent signaling pathways [15, 39,40]. It is known that cAMP is capable of activating PKA, CAMK, AMPK, Epac-mediated pathways, etc. [41-44]. However, in vitro experiments revealed effective coordination of the functions of NSCs and committed progenitors of nervous tissue during blockade, namely, cAMP/PKA signaling [15, 39]. In addition, it has been shown in neuroglial cell cultures that targeting PKA can significantly alleviate the dysfunction of astroglia and microglia induced by Aβ [40].

The aim of the work was to study the therapeutic effect of the PKA inhibitor KT 5720 in the AD model and its neuro regenerative properties.

2. Materials and Methods

2.1. Chemicals and drugs.

PKA inhibitor (KT 5720, Sigma-Aldrich, USA); MACS NeuroMedium; anti-PSA-NCAM MicroBeads; anti-ACSA-2 MicroBead Kit; Anti-O4 MicroBeads; Anti-CD11b (Microglia) MicroBeads (all manufactured by Miltenyi Biotec, Germany); hydroxyurea (Calbiochem, USA); dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA); primary cell culture plate (96 wells) (Corning, USA); formalin 10% buffered (HistoSafe, BioVitrum, Russia); paraffin for histological embedding (Histomix, BioVitrum, Russia); hematoxylin (H9627-250, Sigma-Aldrich, USA); eosin Y disodium salt (E6003-100G, Sigma, Sigma-Aldrich, USA).

2.2. Animals and experimental design.

During the work, we were guided by the principles of humane treatment of animals. Permission was given by the Special Ethical Committee of the Tomsk National Research Medical Center (details of protocols: GRIPhRM-2022-02/09 dated January 11, 2021; GRIPhRM-2022-02/09/a dated June 14, 2022). The experiments used 120 male C57BL/6 mice aged 16 months and 18 male C57BL/6 mice aged 2 months. The aged C57BL/6 mice are a relevant model of AD since from 8–11 months of age, genetically determined, pronounced endogenous pathological amyloid formation (Aβ plaque) occurs in their brain [17, 45].

When assessing disorders of the psychoneurological status of aged mice, their indicators were compared with those of 2-month-old male C57BL/6 mice (n=18). Mice were obtained from the Department of Experimental Biological Models of the Tomsk National Research Center.

The death rate of C57BL/6 mice before the start of the experiment (mice aged 16 months were 7.5% (9 out of 120); another 3 mice were excluded from the experiments because they were in an extremely emaciated state and practically did not move). Before the start of the

experiment, the remaining mice (n=108) were randomly divided into control and experimental groups (n=54 in each). Experimental mice were injected subcutaneously once a day for 7 days with the PKA inhibitor dissolved in 0.2 ml of 0.5% DMSO at a dose of 1 mg/kg. Control animals (including 2-month-old male C57BL/6 mice) were subcutaneously injected with 0.2 ml of 0.5% DMSO in a similar regimen.

On days 1, 3, 7, 14, and 21 after the administration of the PKA inhibitor, the behavioral and emotional profile of experimental animals was studied. On days 3, 7, and 21, the conditioned reflex activity was determined (n=18 each in the experimental and control groups). Also, 4 weeks after the start of administration of the PKA inhibitor, the body weight of the experimental animals was determined. Mice on which the effect of the PKA activity modifier on psychoneurological status was studied were subsequently used for morphological studies of internal organs and to study life expectancy.

On days 3, 7, and 14, the content and functional activity of progenitors in the subventricular zone of the brain (SVZ) were studied (n=6 each in the experimental and control groups (control aged mice). Also, at these times, the secretory function of astrocytes, oligodendrocytes, and microgliacytes (n=6 in the experimental and control groups (control aged mice) at each study period). Mice were killed by decapitation using a special guillotine.

At 23 months of life, C57BL/6 mice underwent a morphological study of the brain, heart, lungs, liver, kidneys, testes, thymus, spleen, skeletal muscles, knee joint, skin (n=6 each in the experimental and control groups (control aged mice)). For this purpose, we used mice whose neuropsychiatric status was studied. Euthanasia of animals was carried out in the CO_2 chamber. Before slaughter, the body weight of the mice was determined.

The remaining animals whose psychoneurological status was studied (n=9 in the control group (since by 23 months of life in the control group, 3 mice died on their own) and n=12 in the experimental group (since there was no spontaneous death of the animals by this time)) were kept under normal conditions to assess life expectancy.

2.3. Research methods.

2.3.1. Psychoneurological status.

2.3.1.1. Exploratory behavior.

The exploratory behavior was studied using the “open field” test [36, 37]. The following parameters were assessed in experimental animals: total, horizontal, and vertical locomotion, hole-board exploration, self-grooming, and defecation for 3 min. The asymmetry coefficient of locomotion was also determined based on the ratio of horizontal movements to total locomotor activity. The indicators of the 1^{st} min and the subsequent 2^{nd}-3^{rd} min were studied separately to assess anxiety and cognitive functions.

2.3.1.2. Conditional reflex activity.

To assess changes in the mnestic function of the brain, we used the CPAR test [36, 37]. We studied the reproduction of the acquired skill of passive avoidance by animals of pain from electric current (0.45 mA) when entering the dark compartment of the installation for the CPAR test. The test is based on assessing the suppression of the unconditioned reflex in rodents by choosing a dark chamber compartment in which, during the development of CPAR, they received electrical pain. Therefore, when checking the integrity of the conditioned reflex, they

had to be in the light compartment of the chamber, which was “uncomfortable” for rodents. The reflex was considered developed if, during testing, the animal did not move into the dark compartment of the installation for 3 minutes. The latent time of the reflex (based on the time before the animal entered the dark compartment) and the period the animals spent in the dark compartment were also determined.

2.3.1.3. Emotional status.

The semi-quantitative method was used based on the reactions of experimental animals to external influences [40]. Determined their reaction to capture in the cage, capture on a flat surface, tweezer approach, and tweezer push. The muscle tone of the mice and the level of vocalization upon contact were also assessed. The severity of each parameter was assessed on a scale from 1 to 4 points. Total emotional status was the sum of all assessed indicators.

2.3.2. Cells.

2.3.2.1. Progenitors.

Tissue was isolated from the SVZ to obtain progenitors. The NSCs and neuronal-committed progenitors (NCPs) in the cellular material were calculated by the level of colony formation in cultures of unfractionated and $CD56^+$($PSA\text{-}NCAM^+$)-expressing cells, respectively. CD56+ cells were isolated by immunosorting using PSA-NCAM MicroBead. After this, the progenitors were 5 days incubated in MACS NeuroMedium (10^5/ml) in the CO_2 incubator under standard conditions (37°C, 5% CO_2). NSCs and NCPs in the studied cellular material were assessed by colony-forming capacity (by the number of CFUs consisting of at least 100 cells). The progenitor mitotic capacity was also determined. For this purpose, we used hydroxyurea (1 μM), which blocks the mitosis S-phase. In addition, the ratio of the number of cluster-forming units (by the number of ClFU consisting of 30-80 cells) to CFU was determined by the NSCs and NCPs specialization index (specialization index) [15, 24].

2.3.2.1. Neurogliacytes.

The astrocytes, oligodendrocytes, and microgliacytes were obtained using microbeads with antibodies to ACSA-2, Anti-O4, and CD11b, respectively. The supernatants of these neuroglial cells (10^5/ml) were then obtained (2 days incubated under standard conditions). Based on the level of yield of spherical cell formations (CFU) under the influence of the resulting conditioned media, the activity stimulating neurosphere formation (NSA, the content of a complex of humoral factors stimulating neural progenitors) was determined [20, 37].

The microscopic examination of cell cultures was performed on an inverted microscope Axio Observer A1 (Zeiss, Germany).

2.3.3. Morphology.

For histological examination, the brain, heart, lungs, liver, kidneys, testes, thymus, spleen, skeletal muscles, and knee joint were taken from intact mice and mice treated with the PKA inhibitor at the age of 23 months. The material was fixed in 10% buffered formalin. For bone tissue (knee joint), preliminary decalcification was performed [46]. The tissues were dehydrated in a tissue histological processing machine SNP-120 (MICROM International GmbH, USA) and embedded in paraffin using an EC 350-d paraffin embedding station

MICROM International GmbH (USA). On the rotary microtome of the HM340E series, with a Thermo Fisher Scientific (USA) section transfer system, paraffin sections with a thickness of 5 μm were prepared, which were stained with hematoxylin and eosin (ROBOT-STAINER HMS 740, MICROM International GmbH, USA). The microscopic examination was performed on an Axio Lab.A1 microscope using an Axio Cam ERc 5s video camera and AxioVision Rel.4.8 software (all devices manufactured by Zeiss, Germany).

2.3.4. Body weight, body hair, and life expectancy.

Body weight was measured in male C57BL/6 mice aged 17 and 23 months on laboratory scales ACZET CY-4102 (Citizen Scale, India). Body weight was measured in male C57BL/6 mice aged 17 and 23 months using an ACZET CY-4102 laboratory scale (Citizen Scale, India). At the same time, representative photographs of the animals' appearance were taken to assess the condition of the body hair. The mice were kept in plastic cages (no more than 4 each) with sawdust bedding; the animals had free access to drinking water ad libitum until natural death. The animal room was maintained at 22–25°C with an automatic 12-hour light-dark cycle. The timing of death of animals was recorded daily at 12-00.

2.4. 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 in tables as arithmetic means and standard error of the mean (M±SEM) in figures as arithmetic means and confidence intervals at p = 0.05. The significance level in tables and figures was $p < 0.05$ [47].

3. Results and Discussion

3.1. Psychoneurological status in aged mice.

The testing of aged C57BL/6 mice in an "open field" revealed a significant decrease in their motor activity compared to 2-month-old animals (Table 1). The total motor activity decreased due to both horizontal and vertical movements and manifestations of the hole-board exploration. At the same time, a change in the ratio of different locomotion types was observed, accompanied by an increase in the asymmetry coefficient. Moreover, the phenomenon of impoverishment of locomotor activity in mice was recorded in both the first and second periods of observation on almost all days of the study. This indicated disorders not only characteristic of increased anxiety in rodents (in such cases, these changes are observed mainly in the first period of recording the indicators) [48] and/or age-associated dystrophic changes in the musculoskeletal system (when there is a uniform decrease in various types of locomotor activity) [49], but also disorders directly exploratory behavior of mice associated with cognitive functions of the central nervous system (CNS) [35, 40].

Table 1. Locomotion in the open field test in 2-month-old male C57BL/6 mice (1); in 16-month-old male C57BL/6 mice (2); in 16-month-old male C57BL/6 mice after administration of the PKA inhibitor (3). arb. units (M±SEM).

Groups	Horizontal locomotion	Vertical locomotion	Hole-board exploration	Self-grooming	Defecation	Total locomotor activity	Asymmetry coefficient
Day 1							
First period							
1	14.50±0.88	3.25±0.39	4.25±0.72	0.42±0.11	0.33±0.19	20.75±1.41	0.71±0.02

Groups	Horizontal locomotion	Vertical locomotion	Hole-board exploration	Self-grooming	Defecation	Total locomotor activity	Asymmetry coefficient
2	10.91±1.20*	2.20±0.61	1.70±0.33*	0.20±0.10	0.00±0.00*	15.90±1.85*	0.79±0.03*
3	12.10±1.04	1.90±0.46	3.00±0.58	0.20±0.13	0.50±0.17	17.70±1.41	0.75±0.04
Second period							
1	22.67±2.19	5.17±0.96	4.92±0.78	0.67±0.26	0.92±0.29	34.33±3.24	0.61±0.02
2	13.12±2.75*	1.75±0.52*	3.11±0.47*	1.00±0.30	0.10±0.10	19.00±4.55*	0.78±0.03*
3	15.30±1.26*	2.70±0.26*	2.50±0.50*	0.60±0.16	1.00±0.25	22.10±1.43*	0.69±0.02*
Day 3							
First period							
1	12.59±1.53	1.52±0.31	3.17±0.47	0.67±0.19	0.25±0.13	18.08±1.87	0.68±0.03
2	8.44±1.03*	0.60±0.27*	1.52±0.52*	0.47±0.16	0.10±0.10	11.02±1.54*	0.80±0.04*
3	17,70±3,83#	1,30±0,33	5,10±1,35#	0,60±0,22	0,40±0,16	25,10±5,31#	0,71±0,02#
Second period							
1	23.75±1.88	3.83±0.52	8.67±1.7	0.92±0.34	1.17±0.27	38.33±3.39	0.63±0.02
2	9.24±2.15*	1.11±0.31*	1.62±0.54*	1.21±0.39	0.75±0.21	13.81±3.25*	0.71±0.03*
3	7,60±1,86*	2,50±0,60	2,50±0,72*	1,00±0,21	1,20 ± 0,20	14,80 ± 2,88*	0,48 ± 0,03*#
Day 7							
First period							
1	24.33±2.37	2.58±0.43	4.33±0.69	0.67±0.31	0.33±0.14	32.25±2.91	0.75±0.03
2	17.00±1.78*	1.74±0.56	1.43±0.37*	0.21±0.21	0.44±0.31	20.7±4.35*	0.82±0.01*
3	17,00±3,33*	2,50±0,78	4,20±0,99#	1,20±0,39#	1,10±0,23*	26,00±4,61	0,64±0,04*#
Second period							
1	22.02±2.12	4.92±0.78	6.75±1.73	1.75±0.41	0.52±0.26	35.92±2.83	0.62±0.02
2	10.95±1.56*	2.54±0.37*	2.11±0.43*	0.53±0.22*	0.38±0.21	16.34±2.45*	0.71±0.02*
3	10,90±2,51*	4,20±0,43#	4,40±0,91#	1,20±0,39	1,10±0,23#	21,80±2,32*#	0,47±0,04*#
Day 14							
First period							
1	16.58±2.62	2.00±0.44	3.75±0.98	0.67±0.33	0.08±0.08	23.08±3.59	0.71±0.03
2	12.85±2.11	1.31±0.33	2.15±0.48	0.27±0.13	0.23±0.13	16.60±1.62	0.78±0.03
3	14,10±2,06	3,00±0,80#	4,60±0,82#	0,20±0,13	0,20±0,13	22,10±2,61#	0,63±0,04#
Second period							
1	22.33±2.81	4.75±0.78	7.75±1.45	1.83±0.24	0.67±0.14	37.33±4.42	0.60±0.02
2	12.92±2.69*	2.28±0.83*	3.33±0.73*	1.27±0.47	0.61±0.22	21.24±3.88	0.71±0.02*
3	11,00±2,11*	4,30±1,12	5,60±1,26	1,20±0,44*	0,70±0,21	22,80±3,80*	0,42±0,06*#
Day 21							
First period							
1	13.83±1.66	1.33±0.33	2.0±0.69	0.42±0.19	0.25±0.13	17.83±2.18	0.67±0.02
2	5.10±1.55*	0.31±0.21*	1.42±0.6	0.00±0.00*	0.10±0.10	6.93±2.16*	0.77±0.03*
3	8,10±1,33*	1,60±0,40	2,30±0,33	0,30±0,15	0,40±0,22	12,70±1,48#	0,62±0,04#
Second period							
1	13.42±3.83	3.08±0.96	2.57±0.83	1.83±0.55	0.58±0.26	21.42±3.09	0.55±0.08
2	6.97±2.07*	2.86±1.04	1.22±0.53	0.53±0.22	0.40±0.16	11.82±3.33*	0.76±0.07*
3	10,90±0,99#	2,90±0,80	2,30±0,33	0,90±0,23	0,50±0,22	15,90±2,00	0,58±0,04#

$p < 0.05$ in compared with * group 1, # group 2.

Signs characteristic of senile dementia were also recorded when studying the conditioned reflex activity of aged C57BL/6 mice (Table 2). Compared to young animals, they reproduced the developed reflex of avoiding electrical pain stimulation significantly worse on all observation days. From days 7 to 21, the proportion of animals with preserved reflexes in old mice decreased much more pronounced (by 85.8%) than in young mice (by 33.3%).

Table 2. Emotional status in 2-month-old male C57BL/6 mice (1); in 16-month-old male C57BL/6 mice (2); in 16-month-old male C57BL/6 mice after the PKA inhibitor administration (3). in points (arb. units) (M±SEM)

Groups	Total reaction	Capture in the cage	Capture on a flat surface	Muscle tone	Tweezer approach	Tweezer push	Vocalization
Day 7							
1	7.64±0.41	1.86±0.14	1.57±0.14	1.79±0.19	0.36±0.17	0.71±0.22	0.93±0.20
2	4.43±0.39*	1.00±0.18*	0.86±0.23*	1.14±0.18*	0.43±0.14	0.43±0.14	0.36±0.13*
3	6.04±0.32#	1.57±0.11#	1.32±0.14	1.51±0.16	0.47±0.11	0.62±0.15	0.82±0.16#
Day 14							
1	7.71±0.47	1.57±0.14	1.93±0.16	1.71±0.22	0.39±0.13	0.86±0.23	1.00±0.18
2	5.00±0.46*	0.86±0.18*	0.71±0.19*	1.07±0.20*	0.43±0.14	0.86±0.33	0.79±0.11
3	7.28±0.32#	1.63±0.16#	1.89±0.24#	1.54±0.19	0.60±0.12	0.90±0.11	1.12±0.14#

Groups	Total reaction	Capture in the cage	Capture on a flat surface	Muscle tone	Tweezer approach	Tweezer push	Vocalization
Day 21							
1	8.00±0.46	1.36±0.13	2.14±0.23	1.86±0.23	0.50±0.14	1.00±0.21	0.79±0.15
2	4.71±0.38*	0.64±0.13*	0.43±0.14*	1.00±0.18*	0.43±0.14	0.36±0.13*	1.50±0.23*
3	7.79±0.26#	1.39±0.12#	1.54±0.19*#	1.74±0.22#	0.51±0.13	0.89±0.16	1.04±0.18

$p < 0.05$ in compared with * group 1, # group 2.

In aged mice, a significant change in emotional status was recorded (Table 3). There was a decrease in the severity of both reactions to capture and obvious hypotonia compared with similar parameters in 2-month-old animals. In addition, vocalization was altered in aged C57BL/6 mice. The initial decrease in the value of this indicator (3 days) was replaced by its increase by the end of observation (21 days). This dynamic was apparently a consequence of mental instability associated with endogenous amyloid formation in the brains of aged C57BL/6 mice [50]. It is known that the increase in the level of vocalization in such cases occurs mainly due to sounds at 22 kHz, which is a criterion for high irritability in rodents [40, 51].

Table 3. Conditioned passive avoidance response in 2-month-old male C57BL/6 mice (1); in (2); in 16-month-old male C57BL/6 mice after the PKA inhibitor administration (3) (M±SEM).

Groups	Reflex latent time (Sec)	Time spent in a dark chamber (sec)	The proportion of animals with preserved reflexes (arb. units)
Day 7			
1	156.58±18.18	13.25±10.71	0.86±0.10
2	95.71±28.75*	58.50±20.14*	0.33±0.14*
3	126.73±19.36	10.10±9.67#	0.73±0.14#
Day 14			
1	115.83±32.48	31.08±17.14	0.64±0.13
2	59.20±27.16	71.70±13.11	0.25±0.13*
3	125.17±29.21#	24.08±12.17#	0.59±0.12#
Day 21			
1	100.17±34.25	32.42±15.54	0.57±0.14
2	34.4±12.72*	63.70±21.99	0.08±0.08*
3	79.38±29.68	64.19±27.17	0.52±0.15#

$p < 0.05$ in compared with * group 1, # group 2.

The results generally confirmed the data on identifying changes in the psychoneurological status characteristic of senile dementia in aged male C57BL/6 mice [37, 40, 45]. These results indicate the adequacy of reproducing the animal model of this neurodegenerative pathology in this case and the possibility of extrapolating the results obtained to AD.

3.2. PKA inhibitor effects on neuropsychiatric status in aged mice.

The blockade of cAMP/PKA signaling significantly affected the exploratory behavior of 16-month-old C57BL/6 mice (Table 1) on all days of the study except 1 day. From the 3rd to the 21st day of observation, an increase in total locomotion was recorded, mainly due to horizontal vertical activity and hole-board exploration. It is important to note that these changes were observed to one degree or another during both observation periods (1 min and 2-3 min). In the first case, this indicates correction under the influence of the pharmacological substance of the increased irritability of aged mice [16, 29, 30], and in the second, directly their exploratory behavior [41]. Confirmation of the presence of the effect in relation to locomotion associated with cognitive functions of the CNS was the normalization of the asymmetry coefficient during all observation periods from days 3 to 21 of the experiment.

In addition, PKA targeting was accompanied by a significant improvement in the conditioned reflex activity of aged C57BL/6 mice (Table 2). This was primarily reflected in the increased proportion of animals with preserved reflex by 2.2, 2.4, and 6.5 times on the 7th, 14th and 21st days of observation, respectively.

The PKA inhibitor also corrected indicators reflecting the emotional status of aged mice (Table 3). Inactivation of this signaling molecule was accompanied by an increase in the severity of their response to capture against the background of hypotonia correction. There was also a leveling of changes in vocalization recorded in the control.

Thus, the PKA inhibitor had pronounced therapeutic effects in a mouse model of AD. The agent corrected age-related impairments in cognitive functions and emotional status of aged C57BL/6 mice with genetically determined pathological endogenous Aβ formation in the brain [17, 37, 45].

3.3. PKA inhibitor effects on rccs functioning in aged mice.

3.3.1. Progenitors.

The pharmacological inactivation of PKA in aged mice was accompanied by an "explosive" increase in the number of NSCs in the SVZ: 267.9%, 245.5%, and 163.2% of similar indicators in untreated mice on days 3, 7, and 14 after administration of the agent, respectively (Figure 1A). On the part of the NCPs, a slightly different dynamic was observed. The number of these types of progenitors increased to a maximum on the 14th day (reaching 204.7% of the control) (Figure 2A). Changes in the content of RCCs in the cambial zone of the brain were a consequence of the adjustment of their mitotic activity (Figures 1B, 2B). At the same time, the proliferation of NSCs was the highest in the initial periods of observation (days 3 and 7) and in NSCs – on the 14th day. In addition, PKA inhibition had different effects on the process of progenitor specialization (Figures 1B, 2C). The differentiation of NSCs was significantly inhibited (up to 50.0%, 39.3%, and 38.8% on days 3, 7, and 14, respectively), while the specialization of NCPs, on the contrary, accelerated (up to 220.2%, 222.7% and 271.3% on days 3, 7 and 14, respectively) compared with similar parameters in aged C57BL/6 mice that were not administered the pharmacological agent.

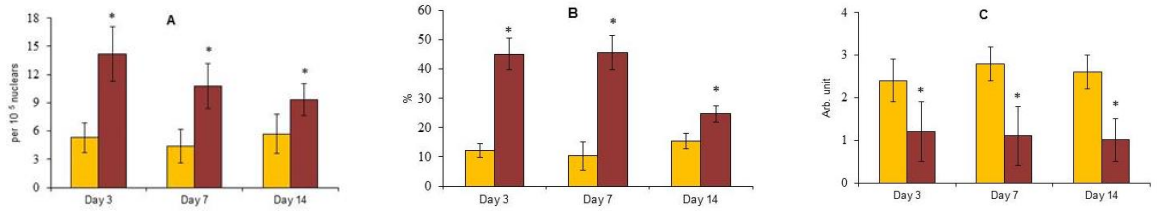

Figure 1. (A) NSC amount; **(B)** NSC proliferative activity; **(C)** NSC differentiation index. Here and in Figures 2-3: in aged C57BL/6 mice (yellow columns); aged male C57BL/6 mice treated with the PKA inhibitor (red columns); * - significance level at $p < 0.05$.

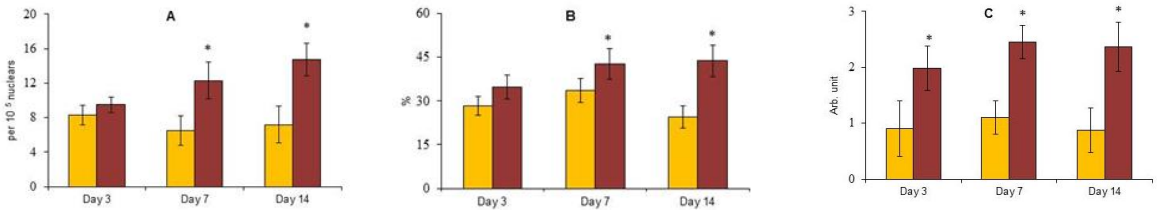

Figure 2. (A) NCP amount; **(B)** NCP proliferative activity; **(C)** NCP differentiation index.

The identified phenomena (in exact accordance with the previously discovered effect of the PKA inhibitor on the functioning of NSCs and NPCs in vitro [15]) reflected pathological changes in the activity of progenitors caused by Aβ. It is known that this neurotoxin blocks the ability of NSCs to undergo mitosis, stimulating their extremely rapid aberrant development [24-26]. While NPCs are under its influence, on the contrary, they actively proliferate against the background of a maturation block [16, 37].

Thus, the PKA inhibitor effectively corrected the disruption of the coupling of the activities of different types of progenitors. Moreover, it uniquely synchronized their proliferation and specialization (differentiation/maturation). As a result, this led to an increase in the intensity/efficiency of neurogenesis in nervous tissue affected by pathological Aβ formation [45], which, in turn, was the reason for the normalization of behavior and cognitive status of experimental animals described in Section 3.2.

3.3.2. Neurogliacytes.

The blockade of the PKA-dependent cAMP-mediated pathway had a pronounced but unequal effect on the functioning of different neurogliocytes. Astrocytes (ACSA-2+ cells), in response to exposure to a pharmacological agent, significantly reduced the production of neurotrophic growth factors. The NSA of their supernatants was 67.1% and 68.0% of the control values on days 3 and 7 after the start of administration of the PKA inhibitor, respectively (Figure 3A).

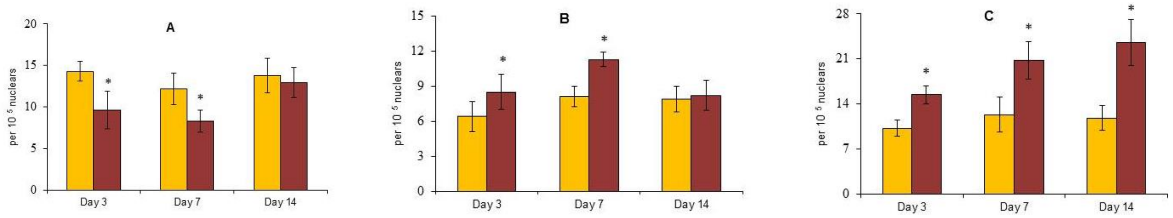

Figure 3. NSA of supernatants from **(A)** astrocytes; **(B)** oligodendrocytes; **(C)** microgliocytes.

The changes in the neurotrophin-producing function of oligodendrocytes (O4+ cells) were fundamentally different. In this case, on the contrary, stimulation of the secretion of growth factors (activating NSCs) was observed during the same periods of the study (up to 132.8% and 139.5% on days 3 and 7, respectively) (Figure 3B). On the 14th day, the function of the macroglial cells studied remained at the control values level.

In contrast, when studying microglia, the most pronounced shifts in the parameter were noted towards the end of the observation period (Figure 3B). The NSA of their conditioned media increased to 151.0%, 168.3%, and 199.2% of those values in the control on days 3, 7, and 14, respectively. However, when analyzing these data, it is necessary to take into account that the yield of NSA in the test system under the influence of supernatants depends both on the concentration of NSC growth stimulators (neurotrophins) and blockers of this process, which primarily include pro-inflammatory cytokines [35, 53].

Thus, PKA inhibition caused different vectoriality and dynamics of reactions of individual types of neuroglial cells. Moreover, its character in relation to each type of neurogliocytes indicated compensation for disturbances in their functioning in AD (at a minimum, correction of manifestations of astrogliosis [19, 20], and also, probably, neuroinflammation [17, 21, 37]).

3.4. PKA inhibitor effects on the appearance and weight of aged mice.

By the end of the experiments to study the effect of the pharmacological agent on the indicators described in Sections 3.2.-3.3., significant differences in the appearance of mice of control and experimental age were noted. Before the start of the experiments, all 16-month-old C57BL/6 mice had severe diffuse and focal alopecia. By 21 days after the start of administration of the PKA inhibitor in control animals, its signs naturally persisted. There was significant baldness of the entire skin with multiple foci (up to 5 mm each) of complete absence of hair (Figure 4A). The preserved wool was characterized by a dull appearance and brittleness.

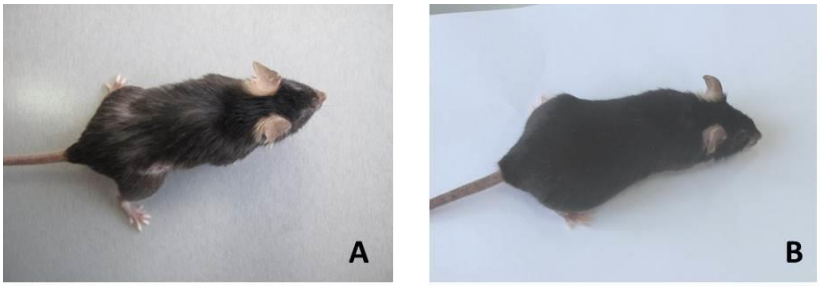

Figure 4. Photo of aged C57BL/6 mice 21 days after the start of PKA inhibitor administration: **(A)** mouse from the control group; **(B)** mouse from the experimental group.

Unlike control animals, all aged C57BL/6 mice in the experimental group (who received the PKA inhibitor) experienced complete hair restoration. At the same time, the fur was characterized by a bright shine, similar to that of young mice. In some cases, there were signs of loss of melatonin pigment (gray hair) in individual hairs (Figure 4B). At the same time, even these depigmented skin derivatives looked shiny and elastic to the touch.

There was also a significant difference in the weight of the control (22.03±0.37 g) and experimental (24.87±0.26 g) animals despite being kept under the same conditions.

Taking this into account, it was decided to continue observing the animals (n=18 each in the control and experimental groups) and conduct a histological examination of the internal organs and skin after 6 months (at the age of 23 months). In addition, from this moment on, a study was carried out on the life expectancy of control and experimental mice (additional approval from the Ethical Committee of the Tomsk National Research Medical Center (details of protocol: GRIPhRM-2022-02/09/a dated June 14, 2022).

At the age of 23 months. the appearance and weight of the animals also differed significantly. In the control group, the external manifestations of diffuse and focal alopecia worsened. In the experimental group, the hairline remained almost the same. Signs of loss of pigment in individual hair hairs only increased slightly (an increase in the number of gray hairs was observed). The weight of the animals in the control group was 21.26±0.52 g, and in the experimental group, 24.49±0.73 g. In addition, in the control group, by this time, 3 mice had already died.

3.5. Morphological studies of aged mice.

3.5.1. Macroscopy.

At autopsy, a significant increase in liver size was found in control mice. In this case, the presence of round-shaped nodes with a smooth surface, ranging in size from 2 to 5 mm,

was noted on the organ's surface. In the section, the nodes were dense and varied in color. The macroscopic examination did not reveal any other noteworthy features.

The brain, heart, lungs, liver, kidneys, testes, thymus, and spleen were isolated and weighed in both groups. Analysis of the data obtained did not reveal differences in organ weight between groups, with the exception of liver weight. In control aged C57BL/6 mice, it was significantly greater than in aged mice administered the PKA inhibitor (4.26 ± 0.06 g and 2.15 ± 0.05 g, respectively).

3.5.2. Histology.

For histological examination, the organs are specified in Section 3.5.1. were taken, as well as skeletal muscle (quadriceps femoris), knee joint, and skin. When examining histological preparations, the following changes were found in all aged C57BL/6 mice. In the cerebral cortex in both groups, both swelling and shrinkage of nerve cells were observed, and the phenomena of neuronophagy were also observed (Figures 5 A1-A2). In the lungs, there were clear signs of focal pneumofibrosis (thickening and infiltration of the interalveolar septa). In both groups of old mice, pronounced age-related involution of the thymus was observed. At the same time, small lobules were preserved in the adipose tissue. In the spleen of all animals, lymphoid follicles were reduced in size. Skeletal muscle fibers were swollen, crimped, and dislocated (Figures 5B1-B2). The pulpy nerve fiber is edematous, the axial cylinders are fragmented in places, and the membranes are vacuolated. In the myocardium, hypertrophy of individual cardiomyocytes was noted, and the muscle fibers, as in skeletal muscles, were swollen and disintegrated (Figures 5C1-C2). When studying the knee joints in the femur and tibia, dissolution of the hyaline cartilage of the epiphyseal growth plate was recorded. The hyaline cartilage of the articular surfaces in the area of the knee joint was thinned and, in some places, completely absent. In the convoluted seminiferous tubules of the testes of mice of both groups, pronounced atrophy of the spermatogenic epithelium was observed.

It is important to note that in animals treated with the PKA inhibitor, the described pathological changes in the skeletal and cardiac muscles were significantly less pronounced (Figures 5B2, 5C2).

However, the greatest difference between the groups occurred in the histological picture of the skin and liver (Figures 5D1-2, 5E1-2). In the skin of control mice, thinning of the epidermis and collagen fibers in the dermis, hyperkeratosis, and an almost complete absence of hair follicles and sebaceous glands were observed compared to animals treated with the PKA inhibitor. In these mice, all layers of the epidermis were preserved; a large number of sebaceous glands and hair follicles were observed in the dermis (Figure 5D2). In the liver of control mice, signs of fatty small- and large-droplet degeneration of hepatocytes were observed. There was also a massive focus on necrosis of the liver tissue, which had different periods of occurrence. Around the foci, the formation of a connective tissue capsule of varying degrees of maturity was noted, but the organization of foci of necrosis was poorly expressed. In addition, the accumulation of macrophages between hepatocytes was noticed (Figure 5E1). Animals in the experimental group showed other changes. Signs of protein (hydropic) dystrophy predominated (Figure 5E1). At the same time, foci of necrosis were found, just as in the control, but of significantly smaller sizes.

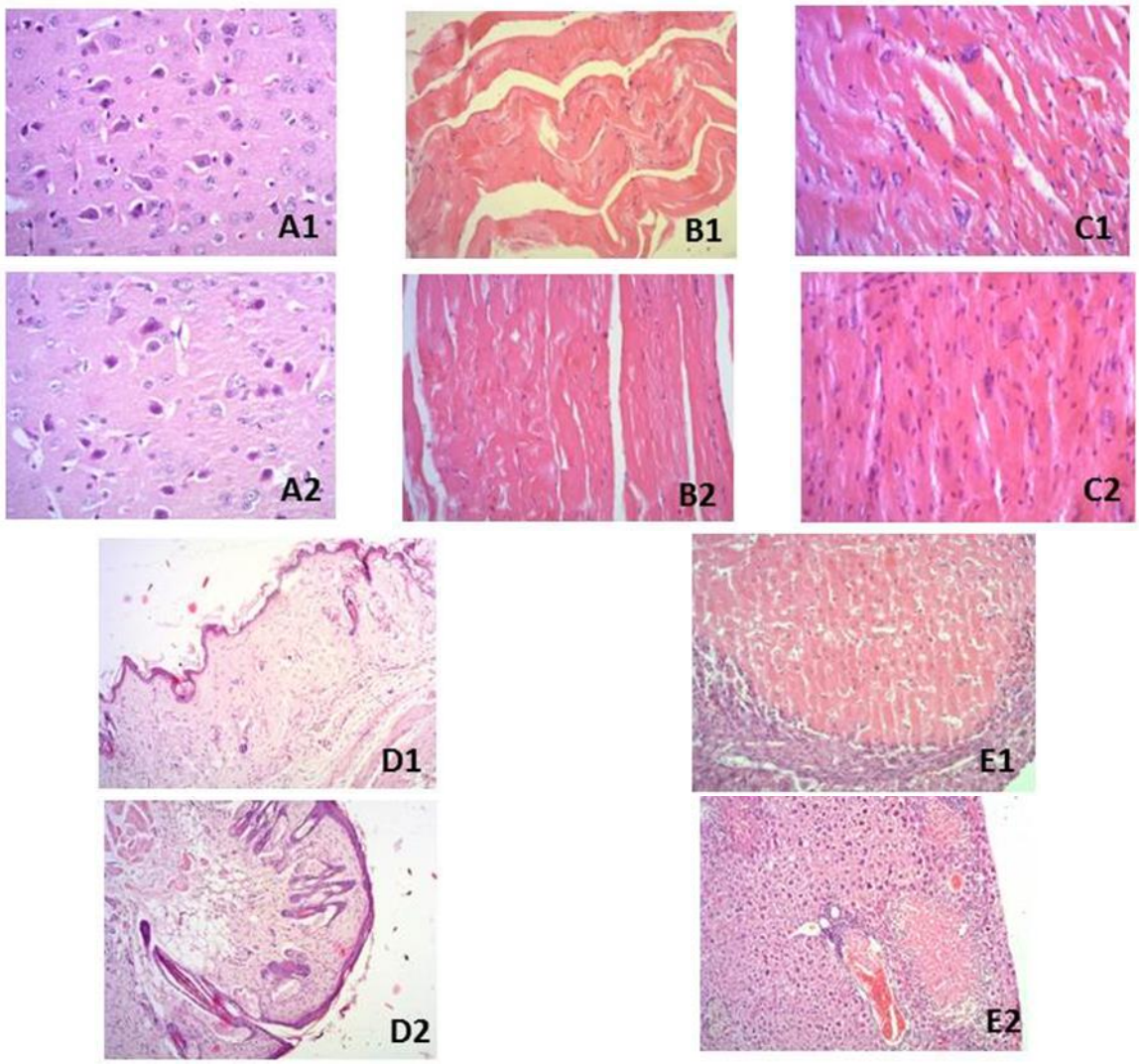

Figure 5. Histology of the **(A)** cerebral cortex; **(B)** skeletal muscle; **(C)** myocardium; **(D)** skin; **(E)** liver in the control aged C57BL/6 mouse **(1)** and the aged C57BL/6 mouse treated with the PKA inhibitor **(2)**. Hematoxylin and eosin staining; A, B, C - magnification ×600; D, E - magnification ×150.

Thus, in general, aged C57BL/6 mice showed characteristic senile histological changes in internal organs [45, 54, 55]. However, undoubtedly, the effect of a single course of the PKA inhibitor administration (7 days) on the morphology of vital organs such as the liver, heart, and skeletal muscles is interesting, despite the fact that the formation of morphological features is always a sign of very pronounced and quite long-term changes in the functions of cells and tissues [56, 57]. However, of particular interest, of course, is the severity and persistence of correction under the influence of the PKA blocker of senile dystrophic signs of the skin [58, 59].

3.6. PKA inhibitor effect on life expectancy.

The average life expectancy in the control group was 737.92±25.9 days. The spread of this parameter ranged from 589 to 845 days. In the experimental group, the mouse with the shortest lifespan lived 729 days, and the animal had a maximum lifespan of 1086 days (almost 3 years). The average life expectancy in animals that received a PKA inhibitor for only 7 days at 16 months was 870.08 ± 29.60 days, statistically significantly longer than in the control by 17.9%.

Based on the results obtained in general, it follows that PKA blockade was accompanied by the development of pronounced therapeutic effects in a mouse model of AD. At the same time, correction of cognitive and emotional status disorders in aged C57BL/6 mice occurred against the background of coordination of the activity of different types of nervous tissue progenitors (NSCs and NCPs) associated with leveling the pathological reaction of astroglia and microglia (Figure 6). The identified changes in the functioning of RCCs were accompanied by an increase in the efficiency of neurogenesis under conditions of its impairment during genetically determined Aβ formation in the brain of old mice [17, 45]. It is important that neuroregeneration not only leads to an increase in the potential of neuroplasticity but also to its implementation in the CNS, which is disorganized by the pathological process [4, 16].

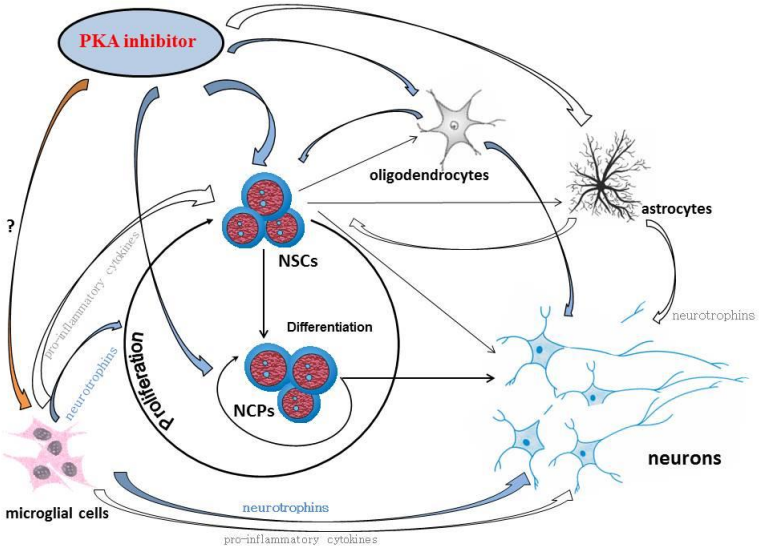

Figure 6. Coordinate the activities of the RCCs during the PKA blockade. Solid lines – stimulating influence; dotted lines – inhibitory influence; wide blue arrows—increased neurotrophic influence by neurogliocytes; wide white arrows - decreased production of relevant factors.

In addition, the effects of the PKA inhibitor were not limited to the effect on nervous tissue. Clear signs of antisenescence effects of the pharmacological agent on other organs and tissues of the body were also recorded [59, 60]. Ultimately, this led to a noticeable increase in the life expectancy of experimental animals. However, it is not possible to unambiguously determine the reason for the development of these effects based on the data obtained alone. It could be realized due to the PKA blockade in both target tissues (skin, heart, skeletal muscles, and liver (primarily in resident progenitors [30])) and as a result of the normalization of the activity of the CNS and restoration/optimization of nervous regulation of organs and systems [61, 62]. The answer to this question, of course, can only be answered by additional research. Moreover, the fact that no significant morphological changes in the brain cortex of mice treated with the PKA inhibitor were found using the methods used does not indicate their absence. They manifested themselves at the ultrastructural level [63].

However, we can already say that the results of these experiments, as well as the data obtained earlier during the development of the "Strategy for targeted regulation of intracellular signal transmission in RCCs" [16, 30, 35-40], not only revealed a number of new effects of PKA inhibition but also significantly expanded ideas about the possibilities of implementing this concept of pharmacotherapy. The identified spectrum of properties of the activity modifier of just one signaling molecule forces us to look at the intracellular signaling system in many

ways differently (including outside the framework of classical determinism [64] of changes in dissipative systems [65-67]).

In most cases, the peculiarities of signal transduction in various tissue-specific progenitors do not allow us to extrapolate the nature of the participation of individual signaling molecules in the regulation of the functions of some progenitors to other types of cells. In addition, the previously discovered high specificity of the role of individual secondary messengers in various RCCs under the influence of the same stimulating and/or pathogenic factors [15-18, 30, 35-40] often does not allow us to predict its inversion and/or modification of the degree of participation in the regulation of functions when the living conditions of cells change. The same phosphorylation of PKA can be accompanied by both an increase in the proliferative activity of committed neuronal precursors (for example, under conditions of Aβ exposure in vitro) and, on the contrary, its suppression (e.g., under conditions of optimal life activity in vitro) [15, 39]. Similar phenomena have also been revealed in relation to many other signaling molecules in different types of progenitors under extreme influences (such as ERK1/2, p38, JNK, STAT3 in ethanol-induced neurodegeneration [35, 36, 68], etc.). At the same time, the tissue specificity of the expression of different types and subtypes of different signaling molecules [16, 30, 69-71] is unlikely to be the main (and certainly not the only) factor in this kind of features, the participation of certain signaling molecules in the regulation of their functions. Perhaps compartmentalization (subcellular localization) plays a certain role in this. That is the involvement in signaling in each case of identical molecules but located in different intracellular compartments [72, 73]. However, the range of circumstances that determine the nature of the participation of individual components of signal transduction is, in fact, so wide [30] that it is not possible in principle to give a definitive list of them.

The intracellular signal transduction system is unknowable. It is this way even without taking into account Heisenberg's uncertainty principle [74] (although quantum entanglement, tunneling, and other phenomena of quantum mechanics are known in biological objects [75, 76], as well as the presence of quantum wave nature in polypeptides [77]) and Gödel's incompleteness theorem (valid for any system) [78]. In essence, this is a deterministic chaotic system [79], the nonlinearity and cascade nature of which determine the "butterfly effect" known from synergetics and chaos theory [80]. At the same time, the "sensitive dependence on initial conditions" [80, 81] (on the living conditions of the cell - numerous factors of the external and internal environment, even unknown) makes the change in the role of signaling molecules in the regulation of cell functions in some cases random/stochastic for the observer. Often, this role can seem completely out of character, such as the occurrence of the anti-proliferative effect of JNK phosphorylation in relation to hematopoietic precursors when exposed to an alkylating cytostatic agent [30]. After all, it is known that most often, the activation of JNK signaling is accompanied, on the contrary, by the progression of the cell cycle [17, 20]. That is, if we use the terminology of chaos theory, then "fluctuations" arise (i.e., random deviations from the seemingly canonical role of individual molecules) [81]. However, in most cases, they compensate for each other and are not functionally manifested. This is ensured due to the highest self-organization and emergence [82] of the signal transduction system, guaranteeing its stability, i.e., "order out of chaos" [83]. Therefore, it is possible to create only very simplified unified models (schematic designs) of individual signaling and not of intracellular signaling as a whole.

Therefore, within the framework of the development of applied biomedicine, the strategy of targeted regulation of intracellular signal transduction in RCCs should certainly be

considered a nontrivial but promising pharmacological concept (Figure 7). After all, the implementation of its potential in practice will be based on using traditional approaches to create drugs, namely, identifying specific patterns in absolutely specific conditions [16, 30, 34].

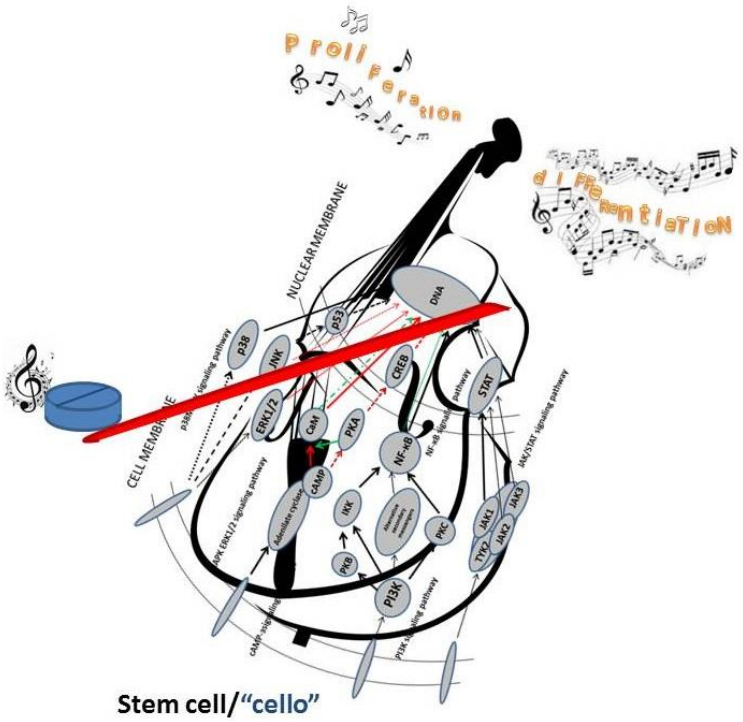

Figure 7. Scheme of the nontrivial pharmacological concept of targeting intracellular signal transduction in RCCs/stem cells (artistic depiction of changes in cell functioning under the influence of a drug, the cell is represented in the image of a violoncello (cello)).

4. Conclusions

The identified therapeutic properties of the PKA inhibitor (including its effects on aging and life expectancy) indicate the possibility of creating on its basis not only a medicine for the treatment of AD but also fundamentally new geroprotectors and anti-senescent drugs [59, 84]. Controlling the regenerative properties of tissues by implementing a strategy of targeted regulation of intracellular signal transduction in RCCs is a phenomenon that deserves attention, both from the standpoint of fundamental pharmacology and the potential for its practical application. This concept represents a promising new paradigm for a pharmacological strategy for regenerative medicine.

Funding

The study was carried out within the framework of the state assignment of the Ministry of Science and Higher Education of Russia on the topic FGWM-2022-0018 with the support of the Grant Council of the President of the Russian Federation (grants No. MD-893.2013.7, No. MD-3096.2015.7), the Russian Foundation for Basic Research (grant No. 18-015-00013), and the Russian Science Foundation (grant No. 22-25-00069).

Acknowledgments

We thank the directorate of the Tomsk National Research Medical Center for providing the necessary conditions for work.

Conflicts of Interest

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

Authorship Contributions

Concept, design, data analysis, and interpretation, writing: G.N.Z.; Data Collection: L.A.M., T.Yu.P, A.V.C., T.I.F., L.A.S.

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