

# The Use of Human Induced Pluripotent Stem Cells for Testing Neuroprotective Activity of Pharmacological Compounds

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**Abstract**—Development of therapeutic preparations involves several steps, starting with the synthesis of chemical compounds and testing them in different models for selecting the most effective and safest ones to clinical trials and introduction into medical practice. Cultured animal cells (both primary and transformed) are commonly used as models for compound screening. However, cell models display a number of disadvantages, including insufficient standardization (primary cells) and disruption of cell genotypes (transformed cells). Generation of human induced pluripotent stem cells (IPSCs) offers new possibilities for the development of high-throughput test systems for screening potential therapeutic preparations with different activity spectra. Due to the capacity to differentiate into all cell types of an adult organism, IPSCs are a unique model that allows examining the activity and potential toxicity of tested compounds during the entire differentiation process *in vitro*. In this work, we demonstrated the efficiency of IPSCs and their neuronal derivatives for selecting substances with the neuroprotective activity using two classes of compounds – melanocortin family peptides and endocannabinoids. None of the tested compounds displayed cyto- or embryotoxicity. Both melanocortin peptides and endocannabinoids exerted neuroprotective effect in the neuronal precursors and IPSC-derived neurons subjected to hydrogen peroxide. The endocannabinoid *N*-docosahexaenoyl dopamine exhibited the highest neuroprotective effect (~70%) in the differentiated cultures enriched with dopaminergic neurons; the effect of melanocortin Semax was ~40%. The possibility of using other IPSC derivatives for selecting compounds with the neuroprotective activity is discussed.

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**Keywords:** test-system, embryotoxicity, neuroprotection, induced pluripotent stem cells, oxidative stress

One of the essential problems of modern pharmacology and medicine is creation of efficient and safe drugs, which requires development of high-throughput, reproducible, and cost-effective technologies for compound screening. Currently, the average cost of a new drug development is estimated as several billion dollars, and it usual-

**Abbreviations:** alpha-MSH, alpha-melanocyte-stimulating hormone; BDNF, brain-derived neurotrophic factor; DA neuron, dopaminergic neuron; DMSO, dimethyl sulfoxide; EC, endocannabinoid; G418, geneticin; GDNF, glial cell-derived neurotrophic factor; IPSC, induced pluripotent stem cell; MC, melanocortin; N-ADA, N-arachidonoyl dopamine; N-DDA, N-docosahexaenoyl dopamine; PGP, melanocortin family peptide Pro-Gly-Pro.

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ly takes 10-15 years for a preparation to be introduced into clinical practice, starting from its synthesis and testing. The development of a new therapeutic preparation includes several steps, such as synthesis of prospective compounds, their testing in different models in order to select the most active and safest ones, clinical tests, and introduction to medical practice. Cultured animal cells (primary, transformed, and transfected) are important test models used for the screening of novel compounds. Drug testing in cell cultures is a mandatory step in the development of therapeutic preparations that precedes testing in laboratory animals. Various types of cells cultures used for screening of pharmacological compounds are shown in Fig. 1.

Mammalian cell cultures can be classified into two main types: primary and transformed. Mammalian cell

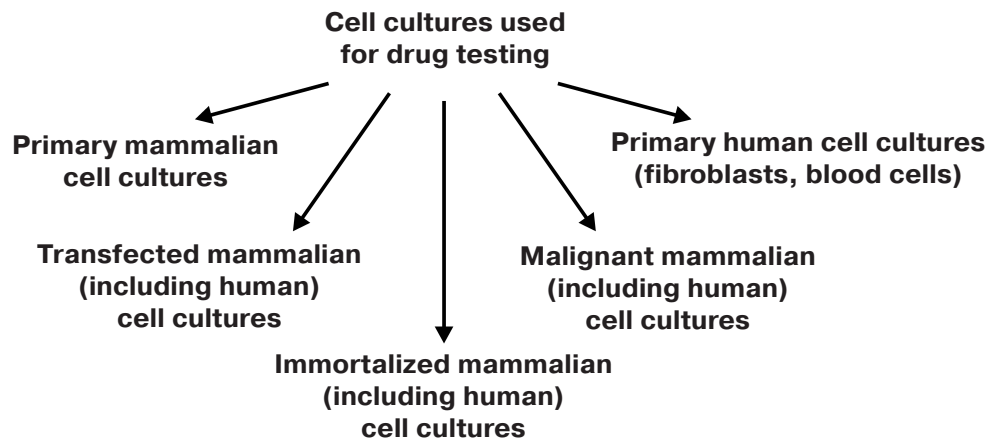


Fig. 1. Animal cell cultures used for testing pharmacological compounds.

cultures artificially transfected to contain a gene responsible for the development of one or another human disease must be also mentioned, as we have used such cultures for testing various peptide compounds for their neuroprotective activity [1, 2].

However, animals and animal-derived cell cultures are often non-adequate models for screening therapeutic preparations because of species-specific responses. It is known that some drugs successfully tested in such models later proved to be unsuitable or even toxic in humans [3, 4].

Reprogramming of somatic cells and production of induced pluripotent stem cells (IPSCs) from mammals, including humans, has opened new prospects in transplantation and studies of molecular and cellular mechanisms of human diseases *in vitro* [5, 6]. This technology has created new opportunities for the development of models for severe human pathologies, including neurodegenerative disorders, and facilitated development of test systems for the high-throughput *in vitro* screening of components of therapeutic preparations tailored to individual patients [7-9].

The most important characteristic of IPSCs is their capacity to differentiate into all cell types of an adult organism. Various derivatives have been generated from human IPSCs, including retinal pigment epithelium, motoneurons, cortical neurons, GABAergic, dopaminergic, and sensor neurons, Purkinje cells, oligodendrocytes, endothelial cells, cardiomyocytes, hematopoietic cells, osteogenic cells, neutrophils, etc. [5, 6]. The majority of these cell were derived from patients with genetic pathologies and, therefore, displayed altered genotypes typical for particular diseases. Such cells have been used as models for both molecular genetic investigation of disease pathogenesis and screening of potential therapeutic preparations [6].

As a model system, human IPSCs present several advantages as a model system for investigating the functions and pathologies of the nervous system, as well as for

searching efficient treatments for neurological disorders because of the following:

- 1) impossibility to obtain neurons from adult human brain (with exception of surgical removal of malignant tumors);
- 2) inability of differentiated human neurons to grow in cell culture;
- 3) possibility of production of unlimited amounts of IPSCs and their derivatives;
- 4) possibility of obtaining IPSCs from a particular donor, as well as from patients with neurodegenerative diseases;
- 5) possibility of targeted differentiation of IPSCs to neuronal or glial lineage *in vitro* with the generation of particular type of neurons.

In our laboratory, we have used human IPSCs to develop a test system for the high-throughput screening of various pharmacological compounds and evaluation of their cytotoxicity, embryotoxicity, and neuroprotective potential [10, 11].

The objective of this study was to examine the efficiency of the developed and patented test system in the screening of compounds with the neuroprotective activity using two groups of chemical compounds, such as peptides of the melanocortin family [alpha-melanocyte-stimulating hormone (alpha-MSH), Semax (Met-Glu-His-Phe-Pro-Gly-Pro), and PGP (Pro-Gly-Pro)] and endocannabinoids [*N*-docosahexaenoyl dopamine (*N*-DDA) and *N*-arachidonoyl dopamine (*N*-ADA)].

## MATERIALS AND METHODS

**Cell culturing and synthesis of compounds.** Human IPSCs and their derivatives produced in [12] were used in this work. Culturing and evaluation of IPSC proliferative activity were carried out as described previously [13]. Melanocortins (MCs) were synthesized at the Division of

Chemistry of Physiologically Active Compounds, Institute of Molecular Genetics, Russian Academy of Sciences; endocannabinoids (ECs) were synthesized in the Laboratory of Oxylipins, Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences.

**Evaluation of compound cytotoxicity in iPSCs.** iPSCs were seeded at 40,000 cells per well in 24-well plates and cultured in 1 ml of mTeSR1 medium (STEMCELL Technologies, Canada) in a CO<sub>2</sub> incubator (Sanyo, Japan) at 37°C and 5% CO<sub>2</sub>. Neuronal precursors produced from the iPSCs as described previously [14] were seeded at 20,000 cells per well in a 96-well plate and cultured in the neuronal medium containing DMEM/F12 (Gibco, USA), 2% serum replacement (Gibco), 1% B27 supplement (50×; Gibco), 2 mM glutamine (ICN Biomedicals, USA), 1 mM non-essential amino acids (PanEko, Russia), 50 units/ml penicillin-streptomycin (PanEko), 10 μM SB431542 (Stemgent, USA), 80 ng/ml Noggin (Stemgent) in 5% CO<sub>2</sub> at 37°C and. The plates were coated with Matrigel (Corning, USA) prior to the experiments. Next day, the medium in the wells was replaced with the medium containing investigated compounds at the final concentration of 0.001, 0.1, and 10 μM and antibiotic geneticin (G418; Gibco) at the concentrations of 50, 100, and 200 μg/ml. After 48 h, the medium was replaced with the fresh medium containing the corresponding compound. On day 3 of incubation, the proliferative activity of the cells was examined with the MTT assay. For this purpose, an aliquot (400 μl) of freshly prepared 0.3 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well and incubated for 3 h in a CO<sub>2</sub>-incubator. Next, the MTT solution was replaced with DMSO (400 μl per well) followed by incubation for 2 h at room temperature on a shaker (Eppendorf, Germany). The optical density in the wells was recorded at 600 nm with an Infinite M200 Pro plate reader (Tecan, Switzerland).

**Effect of MC peptides on the formation of embryoid bodies from iPSCs.** In order to produce embryoid bodies, iPSC colonies were detached from the support using dispase (Gibco). Next, the colonies were transferred to a 24-well ultra-low attachment with (Costar, USA) at 160,000 cells per well in 1 ml of mTeSR1 growth medium for non-differentiated cells containing 50 μg/ml G418; MC peptides were added to the wells at the concentrations of 0.001, 0.1, and 10 μM, and the cells were incubated for 24 h at 37°C in 5% CO<sub>2</sub>. Next, half of the medium was replaced with the medium for the embryoid body growth containing DMEM/F12 (Gibco), 20% fetal bovine serum (Hyclone, Denmark), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1 mM non-essential amino acids, and 50 units/ml penicillin-streptomycin. After 24 h, the medium was completely replaced with the embryoid body growth medium containing G418 and MC peptides in the corresponding concentrations. Embryoid bodies were

counted under the microscope 24–48 h after cell seeding. For this purpose, the entire cell suspension was transferred to a round-bottom 96-well plate (100 μl per well) to provide the maximal accuracy in determining the number of embryoid bodies in each case.

**Generation of differentiated cultures enriched with dopaminergic neurons.** Neuronal precursors were seeded into Petri dishes treated with Matrigel at 200,000 cells per cm<sup>2</sup>. Next day, the medium was replaced with the type I differentiation medium containing DMEM/F12, 2% serum replacement, 1% B27 supplement (50×), 2 mM glutamine, 1 mM non-essential amino acids, 50 units/ml penicillin-streptomycin, 100 ng/ml recombinant Sonic hedgehog protein (PeproTech, USA), 20 ng/ml FGF8 (PeproTech), and 10 μM purmorphamine (Stemgent). The cells were cultured for 10 days; the medium was changed every other day. Next, the cells were transferred to the type II differentiation medium containing DMEM/F12, 2% serum replacement, 1% B27 supplement (50×), 2 mM glutamine, 1% amino acid mixture, 50 units/ml penicillin-streptomycin, 20 ng/ml brain-derived neurotrophic factor (BDNF; PeproTech), 20 ng/ml glial cell-derived neurotrophic factor (GDNF; PeproTech), 200 μM ascorbic acid (PanEko), and 4 μM forskolin (Stemgent) followed by incubation for 14–21 days at 37°C in 5% CO<sub>2</sub>, (the medium was replaced every 48 h).

**Assessment of neuroprotective effect of MC peptides and ECs on the neuronal precursors and dopaminergic neurons under oxidative stress.** Neuronal precursors were seeded into a Matrigel-coated 96-well plate at 20,000–30,000 cells per well and incubated for 24 h. Dopaminergic neurons were produced by culturing under conditions described in the above section. Following generation of mature neurons, the medium was replaced with the test medium (DMEM/F12, 2 mM L-glutamine, 50 units/ml of penicillin-streptomycin) containing MC peptides (Semax, alpha-MSH, PGP; 50 μl per well) at the final concentration of 1 nM or ECs (*N*-DDA, *N*-ADA) at the final concentrations of 2.5 or 5.0 μM. An equal volume of vehicle (PBS for MC peptides and DMSO for ECs) was used as a control. The cells were incubated for 40 min; then, 50 μl of the medium containing 200 μM H<sub>2</sub>O<sub>2</sub> was added per well, and the cells were incubated for another 3 h. Next, the medium was replaced with the test medium, and the number of viable neuronal precursors was determined on the next day using the MTT assay.

**Immunofluorescence assay.** Dopaminergic (DA) neurons were fixed with 4% paraformaldehyde and stained with polyclonal rabbit anti-tyrosine hydroxylase antibodies (1 : 1000; Abcam, USA) and monoclonal mouse antibodies anti-βIII-tubulin antibodies (1 : 2000; Abcam, USA) with the following detection using goat polyclonal antibodies against mouse and rabbit immunoglobulins conjugated with Alexa 488 and Alexa 546 dyes, respectively (1 : 2000; Thermo Fischer, USA) as described previously [12]. Stained cells were counted

under an AXIO Imager Z1 fluorescence microscope (Zeiss, Germany). The intensity of fluorescence signal in the plate wells was evaluated with an Infinite M200 Pro plate reader (Tecan). The images were analyzed for the number of nuclei and cells using the ImageJ-win64 program (USA), plugin ITCN.

**Statistical analysis.** Results are shown as mean values  $\pm$  standard deviation of the mean for 3-4 independent experiments. In each experiment, 3-4 replicates per point were carried out. The significance of differences between the experimental groups was estimated using dispersion analysis (one-way ANOVA); significance level was set at  $p < 0.05$ .

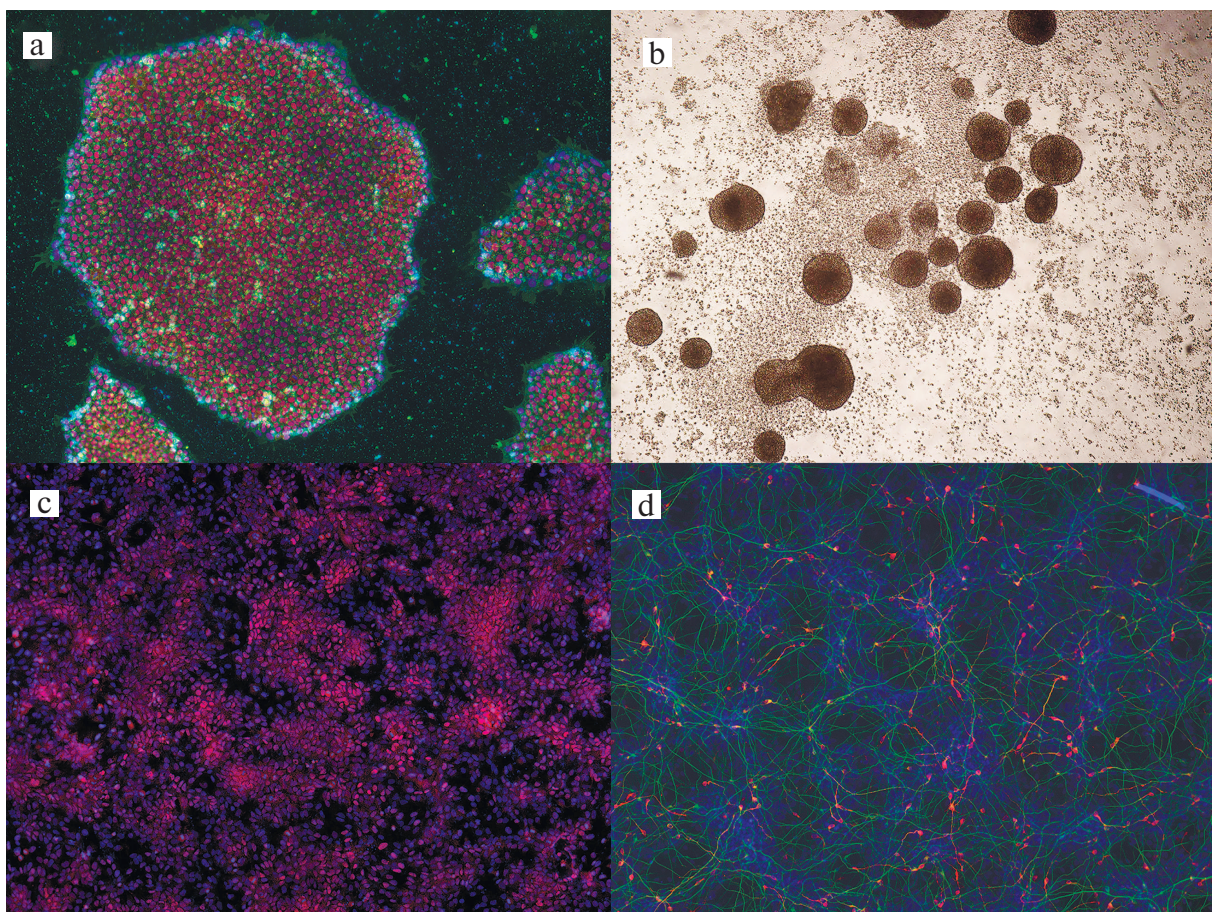
## RESULTS

As mentioned above, we have previously developed a test system based on human iPSCs for the high-throughput screening of pharmacological compounds for cytotoxicity, embryotoxicity, and neuroprotective potential

[10, 11]. Non-differentiated iPSCs, embryonic bodies, neuronal precursors, and cells cultures differentiated into neuronal lineage and enriched with DA neurons were used for verifying the developed test system (see Fig. 2 for the microphotographs of the used cell cultures).

Cytotoxicity and embryotoxicity of the tested compounds and proliferative activity of treated cells were evaluated with the MTT assay as the simplest technique for a high-throughput screening. The neuroprotective potential of the investigated compounds in differentiated cell cultures was determined by immunofluorescence staining from the expression of specific markers ( $\beta$ III-tubulin for neurons and tyrosine hydroxylase for DA neurons). The peptide Semax, which is a basic component of therapeutic preparation with the established neuroprotective properties, was used as a positive control [15, 16]; the antibiotic gentamicin (G418) exhibiting the cytotoxic effect on eukaryotic cells was used as a negative control.

**Effect of MC peptides on the iPSC proliferative activity and ability to form embryoid bodies.** The effect of 0.001-10  $\mu$ M MC peptides on the survival and prolifera-



**Fig. 2.** IPSC cultures at different stages of differentiation: a) non-differentiated iPSCs stained for SSEA-4 (green) and Oct3/4 (red); b) embryonic bodies without staining; c) neuronal precursors stained for Sox1 (red); d) differentiated neurons stained for  $\beta$ III-tubulin (green) and tyrosine hydroxylase (red);  $\times 100$ . Cell nuclei were stained with DAPI (blue).

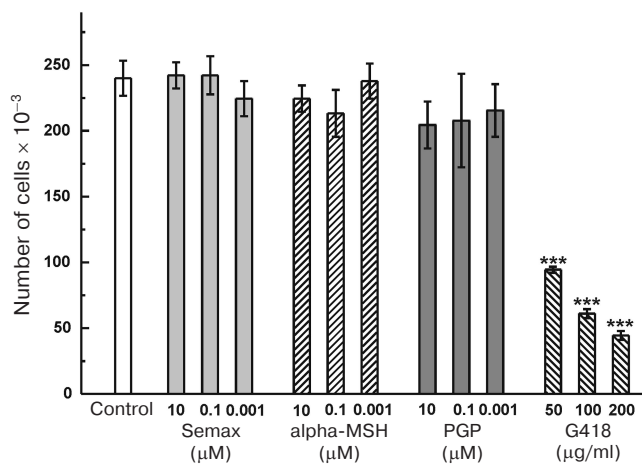


Fig. 3. Effect of MC peptides and G418 on the proliferative activity of IPSCs;  $n = 3$ ; \*\*\*  $p \leq 0.005$ .

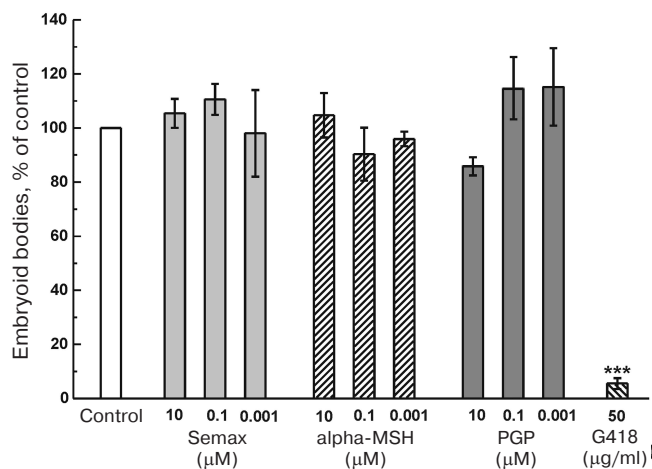


Fig. 4. Effect of MC peptides and G418 on the ability of IPSCs to form embryoid bodies;  $n = 4$ ; \*\*\*  $p \leq 0.005$ .

tion of non-differentiated IPSCs treated with G418 (50, 100, and 200  $\mu\text{g/ml}$ ) (Fig. 3). At all the tested concentrations, G418 caused pronounced cell death. Semax, alpha-MSH, and PGP did not affect proliferation of IPSCs in a wide concentration range and did not display the cytotoxic activity (Fig. 3).

Next, we studied the effect of the tested compounds on the embryoid body formation, which models initial stages of embryonic development. For this purpose, IPSCs were detached from the support and placed in ultra-low attachment plates to eliminate cell attachment to the support. Under these conditions, IPSCs enter initial stages of differentiation and form embryoid bodies. The number of formed embryoid bodies was evaluated after 72 h of cell incubation in the presence of tested MC peptides by direct counting under a microscope (Fig. 4).

We found that the MC peptides did not produce any significant effect on the number of embryoid bodies. At 50  $\mu\text{g/ml}$ , G418 significantly decreased the number of formed embryoid bodies (Fig. 4), while 100 and 200  $\mu\text{g/ml}$  G418 completely inhibited embryoid body formation (data not shown).

**Evaluation of neuroprotective effect of MC peptides and ECs on human IPSCs at different stages of neuronal differentiation.** We investigated the ability of MC peptides (Semax, alpha-MSH, and PGP) and ECs (*N*-DDA and *N*-ADA) to exhibit the neuroprotective effect under conditions of oxidative stress induced by hydrogen peroxide. For this purpose, neuronal precursors were seeded into 96-well plates coated with Matrigel. The medium was replaced after 24 h with simultaneous addition of the investigated compounds. Next, the cells were incubated for 1 h, followed by addition of hydrogen peroxide at the indicated concentrations. After 24 h, the number of viable cells was estimated using the MTT assay (Fig. 5). We found that both MC peptides and ECs produced the neuroprotective effect in the generated IPSC-derived neuronal precursors. In particular, the number of survived cells in the presence of Semax, alpha-MSH, and PGP was approximately 15, 20, and 10% higher, respectively, than in the control. The number of survived cells in the presence of *N*-ADA and *N*-DDA was by 36 and 47% higher, respectively, than in the control.

To obtain cultures enriched with DA neurons, neuronal precursors were seeded into 96-well plates coated with Matrigel and cultured as described in the "Materials

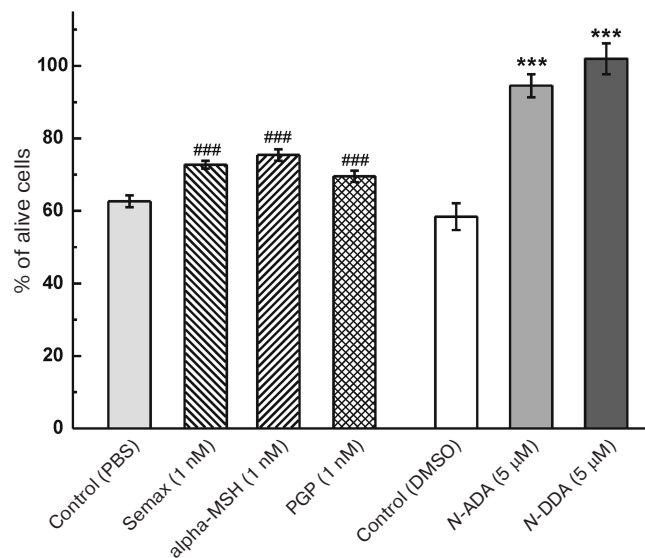


Fig. 5. Effect of MC peptides and ECs on the survival of neuronal precursors under oxidative stress induced by 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . The number of alive cells in the presence of investigated compounds without  $\text{H}_2\text{O}_2$  addition was taken as 100%;  $n = 4$ ; \*\*\*  $p \leq 0.001$  in comparison with control (PBS); ###  $p \leq 0.001$  in comparison with control (DMSO).

Effect of MC peptides (alpha-MSH and Semax) and ECs (*N*-ADA and *N*-DDA) on the survival of differentiated neurons under oxidative stress

Differentiated neurons	Number of survived neurons					
	MC			EC		
	control, PBS	Semax, 1 nM	alpha-MSH, 1 nM	control, DMSO	<i>N</i> -ADA, 5 μM	<i>N</i> -DDA, 5 μM
Tub <sup>+</sup> cells	1108 ± 84	1620 ± 276	1109 ± 59	2608 ± 280	3300 ± 105**	2434 ± 595
TH <sup>+</sup> cells	132 ± 10	189 ± 28**	185 ± 19***	181 ± 21	236 ± 43**	323 ± 14***

Note: Tub<sup>+</sup>, cells stained with antibodies against βIII-tubulin (total neuronal population); TH<sup>+</sup>, cells stained with antibodies against tyrosine hydroxylase (DA neurons); *n* = 4; \*\* *p* ≤ 0.01; \*\*\* *p* ≤ 0.005.

and Methods” section. After 15 days of culturing, the medium was replaced with the new one containing the studied compounds (1.0 nM MC peptides, 2.5 or 5.0 μM of ECs). The cells were incubated for 1 h followed by addition of H<sub>2</sub>O<sub>2</sub> to the final concentration of 200 μM. After 24 h, the cells were fixed and stained immunofluorescently using antibodies against βIII-tubulin (total neuronal population) and tyrosine hydroxylase (DA neurons). The neuroprotective effect of the analyzed MC peptides varied (table). Thus, Semax increased the number of survived cells by 40% vs. control in both total (Tub<sup>+</sup>) and DA (TH<sup>+</sup>) neurons, while alpha-MSG demonstrated the same protective activity only for the DA neurons (TH<sup>+</sup>) (table).

We also studied the effect of 2.5 and 5.0 μM ECs (*N*-DDA and *N*-ADA) on the survival of differentiated neurons under the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. No protective effect of 2.5 μM ECs was observed (Fig. 6), while at the concentration of 5.0 μM, both ECs demonstrated reliable neuroprotective properties (table). The number of survived cells in the presence of *N*-ADA in both cell populations was ~30% higher than in the control; *N*-DDA demonstrated the highest neuroprotective effect among the all investigated compounds (~70%) (table).

## DISCUSSION

Because of the normal karyotype and capacity to differentiate into all types of cells of an adult organism, iPSCs represent a unique model for fundamental, pharmacological, and toxicological studies [7, 9, 17]. By transforming consequently through the differentiation stages from immature precursors to terminally differentiated cells, iPSCs allow to evaluate various aspects of the actions of pharmacological compounds. Testing compounds in human iPSCs has made it possible to replace similar experiments in animal cells. In recent years, the use of iPSCs and their differentiated derivatives as test systems for drug screening has received wide recognition [6, 9, 17]. In particular, hepatocytes and cardiomyocytes have been used to search for compounds for the treatment

of hypercholesterolemia and some cardiovascular diseases [18, 19]. Much attention has been paid to the use of iPSCs differentiated into neuronal lineage for selecting compounds with the neuroprotective activity [20-23].

Our research group has developed and patented an iPSC-based test system for screening of various compounds for their cytotoxicity, embryotoxicity, and neuroprotective activity [10, 11]. This test system allows to evaluate not only the cytotoxicity of the investigated compound, but also its embryotoxicity and teratogenic properties, since formation of embryoid bodies mimics the pre-implantation stage of human embryo. Further differentiation of embryoid bodies *in vitro* for 3-5 days is similar to the embryo development from the blastocyst (3.5 days) to the gastrula (7.5 days) [24], which makes it possible to relate embryonic development *in vitro* to embryogenesis *in vivo*. Here, we demonstrated that MC peptides do not affect the proliferative activity of iPSCs and their ability to form embryoid bodies, i.e., lack cytotoxicity and

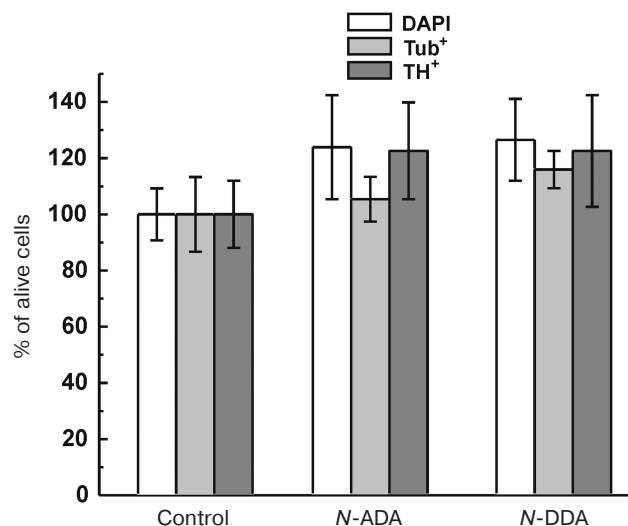


Fig. 6. Effect of 2.5 μM ECs (*N*-ADA and *N*-DDA) on the survival of neurons under oxidative stress. The number of alive cells in the presence of ECs without H<sub>2</sub>O<sub>2</sub> addition was taken as 100%; *n* = 3.

embryotoxicity. At the same, the antibiotic geneticin caused the death of non-differentiated iPSCs and blocked their ability to form embryoid bodies, thus displaying pronounced cytotoxic properties. The capacity of iPSCs to differentiate into neurons allows identification of compounds with neurotoxic and neuroprotective activities. We analyzed the effect of two groups of compounds (MC peptides and ECs) at different stages of neuronal differentiation. Both MC peptides and ECs are considered as potential therapeutic preparations with neuroprotective properties [15, 16, 25, 26]. However, the vast majority of studies demonstrating their beneficial effect have been conducted in either human tumor cells or animal-derived cells [25-27]. Thus, *N*-ADA and *N*-DDA were found to exhibit neuroprotective properties in the primary neuronal cultures derived from the rat brain [25, 26]. Similar effects were observed for the MC peptide Semax [15, 16].

The data of these studies should be extrapolated to “normal” human cells with great care. Here, we investigated for the first time the effect of MC peptides and ECs on the neuronal population of human cells at different stages of differentiation. All the tested compounds demonstrated neuroprotective effect in cells at the neuronal precursor stage subjected to the hydrogen peroxide-induced oxidative stress. Furthermore, the neuroprotective efficiency was different for different groups of compounds. The number of cells that survived oxidative stress in the presence of ECs was ~40% higher than in the control; while the MC peptides increased cell survival by 10-20% only. The differentiated cultures enriched with DA neurons were protected by the investigated compounds to a varying degree. While ECs at the concentration of 2.5  $\mu$ M did not display neuroprotection properties, at higher con-

centration (5.0  $\mu$ M) *N*-DDA demonstrated the highest neuroprotection with regards to DA neurons (~70%). The neuroprotective effect 5.0  $\mu$ M of *N*-ADA was only ~30%. The MC peptides Semax and alpha-MSH also exhibited the neuroprotective effect in these cultures (~40%) (table).

Hence, in this work, we confirmed the efficiency of the developed test system in the evaluation of cytotoxicity, embryotoxicity, and neuroprotective potential of the tested compounds. The question on the molecular mechanisms of this neuroprotective effect remains open. It is possible that it is mediated through specific receptors located on the iPSC surface. The presence of receptors for MC peptides and ECs has been established by us earlier [13, 28]. It was demonstrated that the neuroprotective effect of ECs is associated with the presence of respective type 1 and 2 receptors on the cells [29].

Apparently, testing therapeutic preparations on specialized cells at certain stages of differentiation is very important. However, a potential negative effect on the population of surrounding cells should be taken into account. The number of glial cells that surround neuron in the human brain is approximately the same as the number of neurons themselves. So far, the studies on the involvement of glial cells in the development of neurodegenerative disorders have been very scarce; although, it has been found that astrocytes, for example, play an important role in the development of some neurodegenerative diseases [30, 31]. That is why we plan in the future to generate glial cultures from iPSCs in order to develop similar test system for screening potential drugs with the neuroprotective activity. Earlier, we have used similar test system based on the primary cultures of rat brain neurons and glial cells [27]. It seems especially important to use iPSCs obtained from the patients with genetic forms of neurodegenerative diseases, because this will give the possibility not only to monitor effects on specialized neurons undergoing degeneration in the brain, but also to evaluate the contribution of glial cells to this process [31, 32].

In Fig. 7, we summarized the possible uses of iPSCs and their derivatives in the selection of compounds with the neuroprotective activity.

The use of three-dimensional neuronal-glial cultures (organoids) that was first described in 2013, is considered a very promising approach for development of more complex models for investigating human nervous system pathologies and screening potential therapeutic preparations with neuroprotective activity [33]. Currently, such cultures are widely used as models for studying brain pathologies [34, 35]. Despite the attractiveness of this *in vitro* method, there are certain limitations that prevent considering formed organoids as an adequate model of complex three-dimensional structures formed from the cells in the brain. This is related mostly to different sets of trophic and differentiation factors present in an

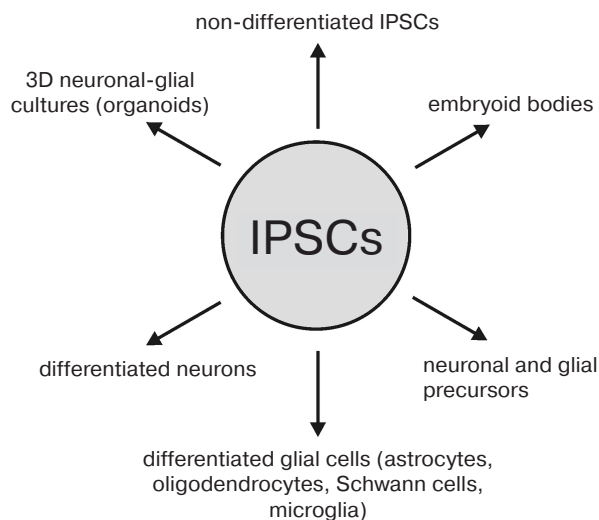


Fig. 7. iPSCs and their neuronal and glial derivatives used for screening compounds with the neuroprotective activity.

organism *in vivo* and used by researchers *in vitro*. Nevertheless, numerous studies are underway pursuing production of more standardized organoids [36].

It must be also emphasized that the developed test system can be used for high-throughput screening of chemical compounds for their neuroprotective activity. The use of 96- and 384-well plates and highly sensitive fluorescence or cytochemical detection methods, as well as modern analyzers, offer promise of simultaneous screening of thousand and more compounds per day. At present, such approaches are known and used in a number of foreign laboratories [37-42]. In the case of differentiated neuronal cultures, the problem that still needs to be addressed is a long period of time required for the terminal differentiation of these cells *in vitro* (more than 3-4 weeks).

In conclusion, our experiments provided evidence that the use of iPSCs and their neuronal derivatives at different stages of differentiation is a promising approach for screening neuroprotective compounds, which allowed us to identify a number of compounds with neuroprotective activity.

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**Ethical approval.** This article does not contain any studies with human participants or animals performed by any of the authors.

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