Research Article

Effects of mitochondrial fusion and fission regulation on mouse hippocampal primary cultures: relevance to Alzheimer's disease

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Abstract.

Background: Alzheimer's disease is a complex disease that begins long before the first well-known pathophysiological signs appear and requires, among other things, new diagnostic approaches. This is primarily due to the lack of effective treatment due to the lack of understanding of the disease mechanisms and the absence of correct biological models reflecting the cause-and-effect relationships in pathogenesis. One of the dysfunctional changes in AD is the disruption of mitochondrial fission and fusion processes.

Methods: In this study, mitochondrial fusion and fission were regulated in primary neuro-astrocytic cultures of mouse hippocampus using mitochondrial fission inhibitor, mitochondrial fusion promoter and exogenous zinc. Changes in mitochondrial and cellular morphology were assessed, as well as lipofuscin levels as an early marker of mitochondrial dysfunction. Primary neuro-astrocytic hippocampal cultures of 5xFAD mice, representing a model of hereditary AD, were used for comparison.

Results: Use of the mitochondrial fusion promoter converts the mitochondrial network to a pool of fused mitochondria and results in a drop in neuronal density by day 5 of exposure with a concomitant drop in astrocyte density by days 1 and 5 of exposure, accompanied by a drop in lipofuscin fluorescence intensity in culture. The use of mitochondrial fission inhibitor resulted in the appearance of fused mitochondria and disappearance of the pool of smallest mitochondria. This was accompanied by a decrease in neuronal density and an increase in astrocyte density with a concomitant increase in lipofuscin fluorescence intensity to the level of 5xFAD culture. Exogenous zinc induces mitochondrial fragmentation and at high concentrations leads to compensatory astrogliosis and neurodegeneration, while at low concentrations it decreases lipofuscin fluorescence intensity and affects culture morphology and changes in astrocyte immunoreactivity to GFAP.

Conclusions: The study demonstrates that changing the processes of mitochondrial dynamics affects the morphology of adult cell cultures and can lead to processes similar to those observed in 5xFAD transgenic cultures.

Keywords: mitochondria, mitochondrial fusion and fission, 5xFAD, lipofuscin, Alzheimer's disease, primary hippocampal culture

1. INTRODUCTION

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases associated with impaired cognitive function and progressive neuronal loss. Currently, researchers have no doubts that mitochondria are involved in the pathogenesis of AD; moreover, there are hypotheses that place mitochondrial dysfunction in the leading role in the onset of AD [1,2]. Decreased respiratory capacity, increased mitochondrial fragmentation, and fractures in mitochondrial cristae structure occur in the brain in Alzheimer's disease, and abnormalities in mitochondria appear before deposition of pathologic Aβ plaques [3] Consistent with the observation that impaired energy metabolism invariably precedes the clinical onset of Alzheimer's disease, mitochondrial dysfunction has been established as an early and prominent feature of the disease [4].

Cell functionality is highly dependent on the state of mitochondria, which are essential for ATP production (general support of cell metabolism) and for maintaining calcium homeostasis (regulation of neurotransmitters and communication with other cells). There are different fractions of mitochondria in different parts of the cell at the same time, performing different functions. These fractions must be in constant turnover, which maintains the normal physiological state of the cell. This form of mitochondrial dynamics is known as mitochondrial fission and fusion processes [5]. In normal conditions, mitochondrial fusion and fission occur continuously. A constant balance is maintained between mitochondria in the fusion-division process, which deviates from one side to the other in response to the energy and metabolic needs of the cell.

The search for a model of sporadic AD is incomplete, primarily due to the inability to understand and reproduce the mechanism of disease development. This leads researchers to the fact that one obvious imbalance process is reproduced on models (disturbance of balance in the systems of generation and detoxification of reactive oxygen species in rats of OXYS line [6], mice with surgically removed olfactory bulbs (olfactory bulbectomized mice) [7] or direct introduction of pathological tau protein or beta-amyloid into the culture of neurons [8,9].

In this work, we wanted to investigate whether disruption of mitochondrial dynamics in healthy cultures could lead to a process similar to the pathology manifestation in the 5xFAD mouse line. We directly affect the mitochondrial fusion-division system in primary mixed neuro-astrocyte hippocampal cultures of mice by inducing shifts

toward a prevalence of fusion, or fission. We used primary hippocampal cell culture of 5xFAD transgenic mice for comparison. Cells in the transgenic culture gradually accumulate high levels of the neurotoxic agent beta-amyloid from the beginning of their lives, causing neurodegeneration similar to AD [10]. In addition to the formation of amyloid plaques, it is characterized by marked astrogliosis and loss of neuronal and synaptic density, as well as increased levels of lipofuscin, an early marker of cellular pathology [11]. Alteration of the balance of fusion-division processes in native primary cell cultures of the hippocampus creates conditions under which an already adult neuronal culture with no pathologies encounters a forced disturbance of cellular homeostasis. In 5xFAD mice, it is amyloid overproduction that triggers a cascade of reactions affecting cellular and subcellular morphology, modeling the typical visible processes occurring in Alzheimer's disease.

2. MATERIALS AND METHODS

2.1 Animal Models

The work was performed on animals that are a model of Alzheimer's disease: transgenic mice of 5XFAD (TG6799) line and healthy littermates. Cells in the transgenic culture gradually accumulate high levels of the neurotoxic agent beta-amyloid, which causes neurodegeneration in AD, from the beginning of their life. Mice of the 5xFAD line were obtained on a congenic SJL/C57B16 background to minimize concerns related to allelic segregation and high variability in the original hybrid background. 5xFAD transgenic mice overexpress the following five FAD mutations: the APP(695) transgene contains the Swedish (K670N, M671L), Florid (I716V), and London (V7171) mutations, and the PSEN1 transgene contains the M146L and L286V mutations. Expression of both transgenes is regulated by murine Thy1.promoter elements to drive their overexpression specifically in neurons. The transgenes are inserted at a single locus Chr3:6297836, where they have no effect on any known genes. Animals were kept in a specialized vivarium with free access to water and standardized feed at 22 - 24℃ and natural light. The laboratory animals were treated in accordance with the European Convention for the Protection of Vertebrates used for experimental and other purposes (Strasbourg, 1986) and the principles of the Helsinki Declaration (2000). All animal procedures performed with mices were approved by the Commission on Biosafety and Bioethics (Institute of Cell Biophysics – Pushchino Scientific Center for Biological Research of the Russian

Academy of Sciences) in accordance with Directive 2010/63/EU of the European Parliament.

First, to form groups of animals for breeding, mice were genotyped by classical PCR using DNA isolated from ear biopsy specimens. The presence of a Tg cassette of 377 bp in length was detected using primers 5'-AGG ACT GAC CAC TCG ACC AG-3' and 5'-CGG GGG TCT AGT TCT GCA T-3', followed by electrophoretic visualization.

2.1 Primary culture of brain hippocampus.

After genotyping and formation of groups of healthy and 5xFAD animals for breeding, mice (age 0-1 day) were genotyped to further obtain primary hippocampal cell culture. For this purpose, the hippocampus was mechanically crushed and treated with Trypsin-EDTA solution (Gibco, USA). The cell suspension obtained by enzymatic and mechanical dissociation was then added to the wells of a 12-well plate coated with Poly-D-Lysine support substrate (Gibco, USA) or to slides, cells were added to 2 or 1 ml of Neurobasal Medium (Gibco, USA) containing 2% B-27 Supplement (Gibco, USA) and 1% Penicillin-Streptomycin-Glutamine (Gibco, USA), respectively. Half of the medium was replaced with new medium every five days. Cells were cultured until the required time in a CO2 incubator at 37℃ and 5% CO2. Thus, we obtained both control transgenic (Tg) culture and nontransgenic (nTg) culture from hippocampi of littermate animals. Cultures 14 days old were used in the experiments. At this term transgenic cultures already well reproduce such characteristic features of AD as: decrease in neuronal density on the background of increase in astrocyte density, decrease in the number of synaptic contacts, as well as a significant increase in the concentration of beta-amyloid.

2.3 Regulation of mitochondrial fusion and fission processes.

Two substances with similar functions but different pathways were selected for mitochondrial fusion shifts. The first substance, Mitochondrial Division Inhibitor 1 (Sigma-Aldrich 475856) (mdivi-1), is a cell-permeable quinazolinone compound that reversibly inhibits dynamin-like proteins (Drp1) (dynamin-related GTPases) responsible for mitochondrial fission. The second substance: Mitochondrial Fusion Promoter M1 (Sigma-Aldrich SML0629) (MFP), is a cell-permeable hydrazone that enhances mitochondrial fusion and does not affect the morphology of the endoplasmic network and lysosomes. Preparation of solutions of substances and application into wells was performed according to the manufacturer's recommendations. In our

experiments, we used short (1 day) and long (5 days) observation after Mdivi-1 and MFP administration to evaluate the response and dynamics of changes in the structure of the primary hippocampal culture over time.

Forcing mitochondrial fission seemed to be a much more difficult task. Currently, the commercial market cannot provide compounds capable of directly affecting mitochondrial fission processes. In our experiments we used the introduction of zinc chloride solution of high (1μM) and low concentration (0.1μM) into the cultures. "Overloading" the culture cells with zinc causes too much cellular and mitochondrial stress, which directly affects both mitochondrial and culture morphology, so this experiment was performed within a single day only. The concentration we chose allowed for effects on mitochondrial morphology without causing immediate cytotoxicity, which is primarily reflected in the integrity of the neurite structure. This experiment reflects short (1 day) and long (5 days) follow-up.

2.4 Visualization of mitochondria.

To visualize mitochondria in cells, we used transduction/transfection of cell cultures with the CellLight BacMam 2.0 Red system. This ready-to-use construct is transduced/transfected into cells using BacMam 2.0 technology, where it expresses a fluorescent protein fused to the E1-alpha-pyruvate dehydrogenase leader sequence. A suitable volume of reagent is injected into a cell culture of desired density (reagent calculation is based on the number of cells in the culture according to the manufacturer's recommendations) and gently mixed. The cells are returned to the culture incubator overnight, and after approximately 16 hours, the culture is ready for experiments.

Mitochondrial morphology was examined on a Leica DM IL LED microscope (Leica, Germany) using a x100 oil-immersion objective. We found that fixation of nerve cells disrupts mitochondrial morphology and triggers an immediate cascade of mitoptosis reactions with displacement of mitochondrial material together with fluorescent agent from the cell, which is especially noticeable when working with astrocytes. Therefore, all data on mitochondrial morphology were obtained on live, unfixed cell cultures.

2.5 Detection of intracellular lipofuscin

We also used spectrofluorimetric detection of lipofuscin, an autofluorescent aging pigment, as a marker of changes in cellular metabolism. For this purpose, culture plates with primary hippocampal cultures were washed three times from the medium with PBS and poured into a solution of 5% sodium dodecyl sulfate in water for 5

minutes with simultaneous stirring on a shaker equipped with a refrigerant, which allowed to completely destroy cell cultures and transfer them into solution. The resulting suspensions were frozen and further used for experiments. Sodium dodecyl sulfate, disintegrates protein complexes, destroying cell membranes, but does not destroy lipofuscin, due to its N-substituted imines - Schiff bases. The addition of detergent in our experiments, in addition to chemical dissociation, reduced light scattering and eliminated the effect of Tyndall-Rayleigh hypochromism, directly affecting the registration of spectral characteristics.

Protein concentration in the obtained suspensions was determined and equilibrated by the Lowry method and UV-express method by the optical density of tryptophan protein in ultraviolet at 286 nm [12]. UV spectroscopy methods are based on the ability of solutions to absorb light at around 280 nm due to the presence of the amino acids' tryptophan, tyrosine and phenylalanine in proteins. The detection results are directly proportional to the content of these amino acids in proteins. To obtain reliable and accurate results, the optical density values of the solutions must meet the requirements of linearity over the range of protein concentrations to be determined.

Lipofuscin formation was detected by a characteristic fluorescence band at about 450 nm under excitation at a wavelength of 360 nm. Lipofuscin content in homogenates was determined on a Perkin Elmer MPF-44B spectrofluorimeter (USA) in multiway mirror microcuvettes for measuring fluorescence of weakly absorbing solutions. The cuvettes provide multiple enhancement of fluorescence intensity due to the increased optical path length of the excitation light and the added light collection of the emission. Excitation light entering through a narrow window in the frontal mirror wall of the cuvette passes through the solution and onto the mirror opposite side, is deflected and undergoes two or three reflections within the cuvette. The radiation is collected at right angles. Additional light collection of radiation is provided by a mirror side wall that directs the fluorescence into the registration channel. When it is used, light losses and artifact polarization are minimal compared to a conventional cuvette located near concave mirrors.

2.6 Immunocytochemistry

For immunocytochemical staining for neuronal and astrocyte markers, cell cultures were fixed for 10 minutes with 4% paraformaldehyde. Membrane permeability was then increased using 0.2% Triton X-100 solution and nonspecific binding to antigens was blocked for one hour at room temperature in PBST (PBS+0.1% Tween 20) + 1%

BSA solution using 10% normalized donkey serum (Abcam, ab7475) and 5% normalized goat serum (Invitrogen, 31872). Cultures were left overnight at 4℃ for incubation with primary antibodies. After incubation with primary antibodies, binding was performed with appropriate secondary antibodies conjugated to fluorescent tags for 2 hours at room temperature. After each treatment, cells were washed 3 times with PBS solution (pH 7.4) for 5 minutes each. The following primary and secondary antibodies were used to stain for neurons - Anti-MAP2 antibody (ab32454, 1:200) and corresponding Alexa594nm (ab150080, 1:400); astrocytes - Anti-GFAP antibody (ab4674, 1:800); Alexa488nm (ab150169, 1:1000).

2.7 Statistical Analysis

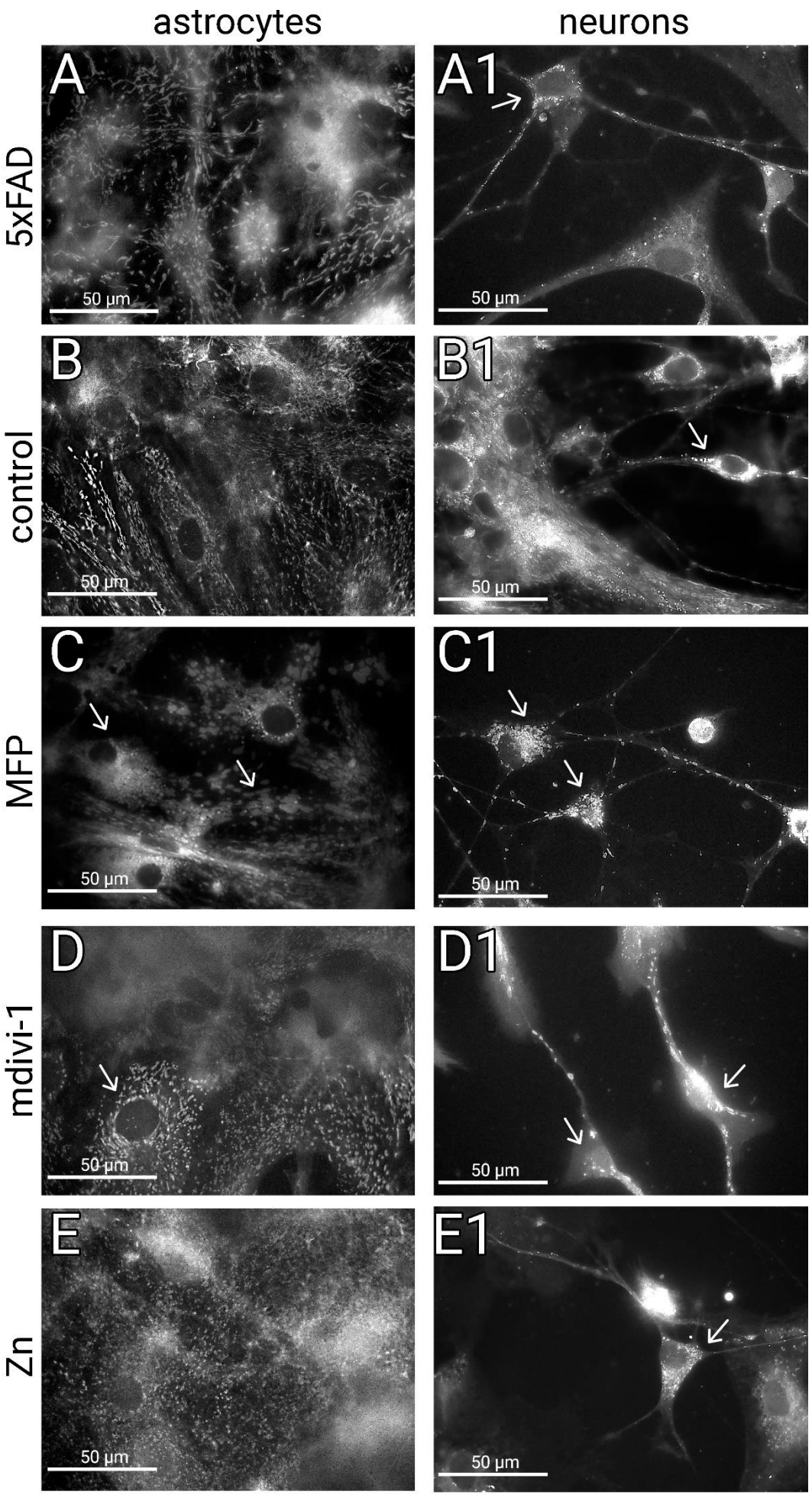
Statistical analysis of the results was performed using Sigma Plot 12.5 software. Data are presented as mean and standard error of mean for visual convenience. The hypothesis of normal distribution was tested using the Shapiro-Wilk test. One-factor ANOVA analysis of variance with a posteriori comparison of groups using Dunn's and Bonferroni methods were used to compare differences between groups and to determine statistical significance.

3. RESULTS

3.1 Forced activation of mitochondrial fusion leads to slow degradation of the culture.

Utilization of the mitochondrial fusion promoter M1 (MFP) in astrocytes converts the mitochondrial network into a pool of fused and hyperfused mitochondria (**Figure 1 C**). A similar pattern is observed in neurons, in which fused mitochondria become like elongated sticks or become large fused mitochondria (**Figure 1, C1**). **Figure 2, B-B1** is a tendency for neuronal density to fall by day 1 of exposure, and it falls significantly relative to the control native culture level by day 5 (47.4±1 at native level, 36.7±1.4 at day 5, $p < 0.05$, one-way ANOVA followed by Dunn's post-tests). There was a decrease in astrocytic density by day 1 (52,3±1 in control, 48,6±1.6 at day 1, $p < 0.05$, one-way ANOVA followed by Dunn's post-tests), and a further progressive decrease in density by day 5 (37.9±1,5 at day 5, $p < 0.05$, one-way ANOVA followed by Dunn's post-tests). We also observe an interesting effect of decreasing lipofuscin fluorescence by day 1 (345±2 in native levels, 277±2 at day 1, $p<0.001$ one-way ANOVA followed by Dunn's post-tests) and day 5 (131±1,5, $p<0.001$ one-way ANOVA followed by Dunn's post-tests) of fusion activator

246 exposure at **Figure 3, B.** Overall, prolonged exposure to MFP resembles a depletion
247 of internal culture resources.


astrocytes
neurons
5xFAD
control
MFP
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A
A1
B
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D
D1
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E1
50 µm

Figure 1. *Alteration of mitochondrial morphology under the action of different fusion and fission modulators. Mitochondria staining was performed by transfection/transduction with the CellLight BacMam 2.0 system. Mitochondrial morphology was examined on a Leica DM IL LED microscope using a x100 oil-immersion objective. A - mitochondrial network with a tendency to mitochondrial fusion in 5xFAD astrocytes. A1 - mitochondria with a tendency to reduce their size in the neuron of cultures of 5xFAD mice (arrow indicates the body of the neuron). B - Mitochondrial network in healthy astrocytes, has more elongated mitochondria. B1 - Neurons in healthy cultures, you can see mitochordria of different sizes (arrow indicates the body of the neuron). C - Mitochondria under the action of MFP in astrocytes fuse into grouped round (fused) or hyperfused mitochondria. Bright examples are indicated by the arrow. C1 - Mitochondria in neurons become larger under the action of MFP (arrow indicates neuron bodies). D - Mitochondria in astrocytes under the action of mdivi-1 lose the shape of the mitochondrial network and the number of smallest mitochondria also decreases. D1 - Mitochondria in neurons under the influence of mdivi-1 become larger, the smallest mitochondria disappear (arrow indicates neuron bodies). E - Mitochondria in astrocytes under the action of zinc completely lose the shape of the network. E1 - Mitochondria in neurons under the action of zinc become small (arrow indicates neuron bodies).*

3.2 Inhibition of mitochondrial fission makes the culture similar to that of 5xFAD.

Morphologically, the use of the mitochondrial fission inhibitor mdivi-1 resulted in larger mitochondria being found in neuron bodies and neurites. At the same time, in astrocytes there was fragmentation of the mitochondrial network and instead fused mitochondria were found in greater numbers. We also observed a decrease in the pool of smallest mitochondria, which almost completely disappeared in astrocytes and remained only in neurons (**Figure 1, D-D1**). After one day of mitochondrial fission inhibition, neuronal density decreased relative to the control group (47,4±1 at native level, 35±2 at day 1, $p<0.05$, one-way ANOVA followed by Dunn's post-tests) and did not decrease further by day 5. There was an increase in astrocytic density by day 1, and by day 5 (52,3±1.2 at native level, 58,2±1,7 at day 1, 68,1±1,8 at day 5, $p<0.05$, one-way ANOVA followed by Dunn's post-tests). In addition, there was a significant increase in lipofuscin fluorescence correlating with the time of mdivi-1 exposure

(Figure 3, C). Moreover, by day 5 of mitochondrial fission inhibition, the intensity of lipofuscin fluorescence increased such that it was not significantly different from lipofuscin fluorescence in transgenic culture (571±3 in Tg culture, 571±2 at day 5 $p<0.001$ one-way ANOVA followed by Bonferroni post-tests). Thus, inhibition of mitochondrial fission in healthy cultures leads to a cascade of events reminiscent of those in transgenic culture **(Figure 2, C**).

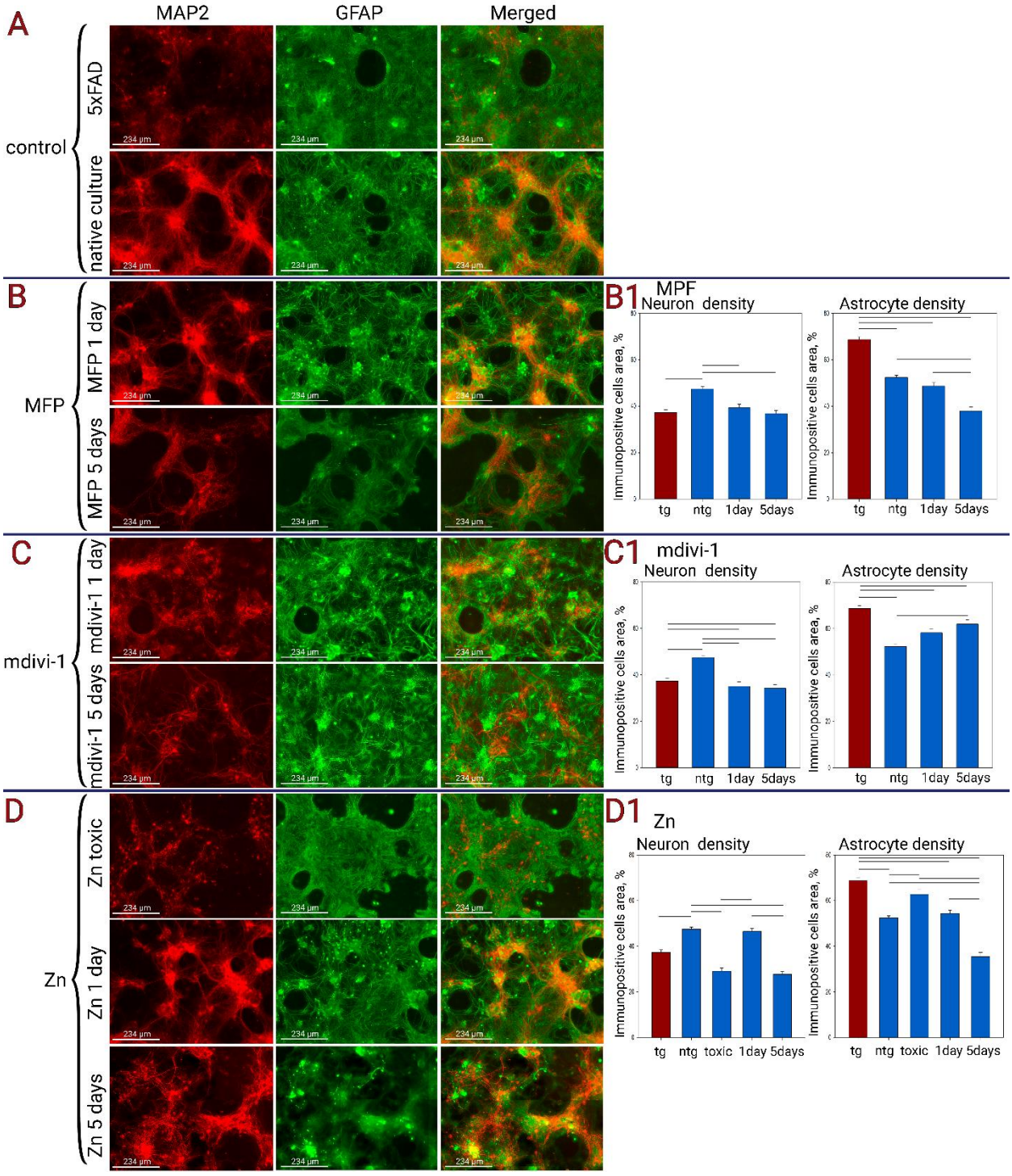

Figure 2. *Immunopositivity to an astrocyte marker (GFAP in green) and a neuronal marker (MAP2 in red) in primary neuronal cultures under the influence of fusion and fission activators. A - Control transgenic 5xFAD culture that served as a reference for modeling pathogenesis and control healthy culture. B - Effect of MFP on culture morphology after 1 and 5 days of administration. B1 - Neuronal and astrocytic densities (in %) under the effect of MFP. C - Effect of mdivi-1 on culture morphology after 1 and 5 days of administration. C1 - Neuronal and astrocytic densities (in %) under the effect of mdivi-1. D1 - Effect of Zn on culture morphology after 1 and 5 days of administration. D1 - Neuronal and astrocytic densities (in %) under the effect of Zn.*

3.3 Exogenous zinc induces mitochondrial fission, alters cell culture morphology and induces GFAP aggregation.

We used exogenous zinc chloride solution to modulate mitochondrial fission. In this case, zinc overload at high, rather uncomfortable concentrations for nerve cells, induces cellular and mitochondrial stress affecting mitochondrial morphology, which is consistent with the results of [13]. Primary culture neurons are the first to suffer from zinc overload, at the same time in culture it was observed compensatory astrogliosis (**Figure 2, D).** Treatment of cultures with excessive zinc resulted in neurite destruction and a drop in neuronal density relative to control cultures (47,4±1 in native, 29±1,5 under toxic influence, $p < 0.001$, one-way ANOVA followed by Dunn's post-tests), while there was a strong compensatory astrocyte overgrowth (52,38±1 in native, 62,7±2,2 under toxic influence, $p<0.001$, one-way ANOVA followed by Dunn's post-tests) and an increase in lipofuscin fluorescence intensity (**Figure 3, D**) (344,9±2,7 in native, 440±1,2 under toxic influence, $p<0.05$ one-way ANOVA followed by Dunn's post-tests). Using lower concentrations of zinc chloride solution allowed us to achieve the same morphologically detectable mitochondrial fission (**Figure 1, E-E1**), in which neurites do not receive such a high zinc overload (**Figure 2, D).** Low zinc concentrations caused no significant change in neuronal and astrocytic density by day 1, but reduced lipofuscin fluorescence intensity (265±1 at day 1, $p<0.05$ one-way ANOVA followed by Dunn's post-tests). However, on day 5 (**Figure 2, D1)** of the experiment, a fall in neuronal density was already again observed (47.4±1 at native, 27,7±1,3 at day 5, $p<0.001$, one-way ANOVA followed by Dunn's post-tests) with a concomitant drop in astrocyte density (52,3±1 at native,

35,4±1,8 at day 5, $p < 0.001$, one-way ANOVA followed by Dunn's post-tests), and the cluster morphology of the culture was impaired, neurons became less likely to assemble into clustered structures, and began to show altered immunoreactivity to glial fibrillary acidic protein, and pathologic aggregation of GFAP increased, however, lipofuscin fluorescence intensity was still reduced (190±3 at day 5, $p<0.05$ one-way ANOVA followed by Dunn's post-tests).

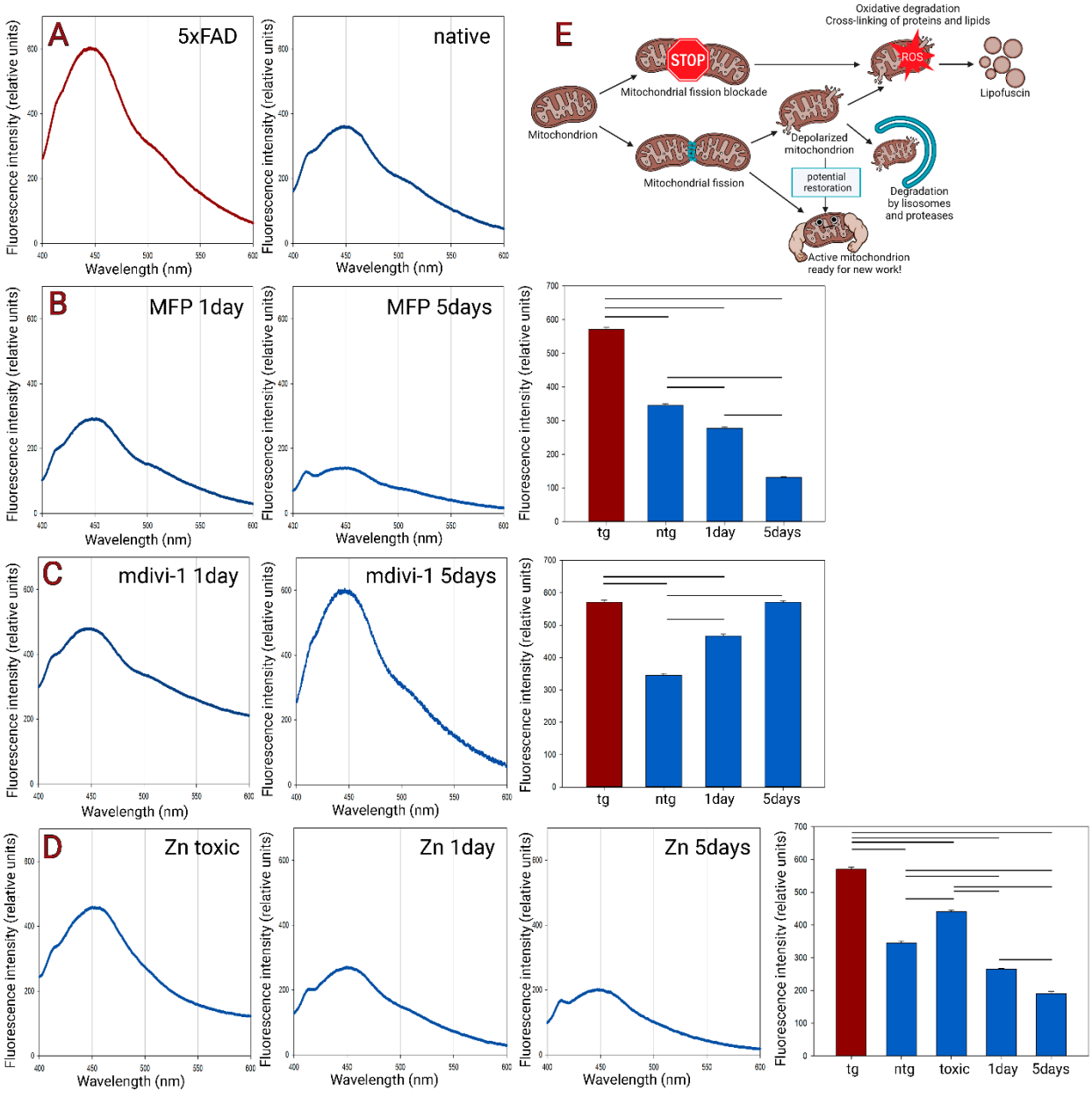

Figure 3. *Emission spectra of lipofuscin fluorescence (360/450) and fluorescence intensity at the maximum point. A - Fluorescence emission spectra in 5xFAD hippocampal cultures and healthy cultures. B - Effect of MFP on lipofuscin fluorescence intensity. C - Effect of mdivi-1 on lipofuscin fluorescence intensity. D -*

Effect of Zn on the intensity of lipofuscin fluorescence. E - Suggested mechanism of lipofuscin formation from mitochondria.

4. DISCUSSION

Normal mitochondrial fusion and fission dynamics is the basis for maintaining a healthy mitochondrial pool also through mitophagy, the disruption of which leads to the degradation of mitochondria into lipofuscin granules [14].

First, we would like to talk about lipofuscin. It is a substance consisting of oxidized lipids, covalently cross-linked proteins, oligosaccharides and metals [15]. The composition of lipofuscin is largely dependent on the intracellular site of action of reactive oxygen species on proteins and lipids. Lipofuscin accumulation is directly proportional to pathologies of various genesis and also increases under conditions of oxidative stress and reactive oxygen species produced by damaged mitochondria **(Figure 2, E)**. Although, lipofuscin is thought to be a marker of senescent cells, recent work shows its close association with pathologic tau protein and Aβ in AD [16,17]. Moreover, a new study, shows that α-structured protein lipofuscin is a toxic component of β-structured amyloid plaques [18]. Since lipofuscin has the property of autofluorescence, it can be used as a marker of disorders even before the first morphologic manifestations. That said, one of the most frequent descriptions of lipofuscin sounds like "intracellular trash". This term denotes one of the interesting properties of lipofuscin - extreme resistance to cellular proteolysis, which is explained by "cross-linking" of aldehyde and amino groups of the peptide to form stable polymeric structures.

We see that the accumulation of lipofuscin in our experiments occurs in two cases. The first is the toxic effect of high concentrations of exogenous zinc, which is quite explained by the activation of peroxidation and, in general, quite a common pathway of lipofuscin increase. The second is inhibition of mitochondrial fission. Thus, by day 5 of fission inhibition, the fluorescence intensity of lipofuscin in the experimental groups is compared to control 5xFAD transgenic animals. The issue [19] states that the normal mitochondrial fission cycle involves the formation of two daughter mitochondria, one of which has a higher membrane potential and goes on to the fusion-fission cycle, and the other, with a more depolarized membrane, remains separated until membrane potential is restored or until elimination by autophagy. And we can assume that the increase in lipofuscin in this case is a process of accumulation

of intracellular debris associated with insufficient mitochondrial division and clearance. And therefore, activation of fission by non-toxic concentrations of zinc, although leading to deleterious effects in culture, decreased the level of lipofuscin. At the same time, the activation of mitochondrial fusion, although leading to the degradation of the culture, did not lead to the formation of lipofuscin. On the contrary, it decreased over time. This can be taken to mean that by acting on the fusion promoter, we are not blocking fission, and mitochondria still have the ability to maintain fission dynamics. Also, given the close relationship between lipofuscin and peroxidation, we can assume that when mitochondria fuse, cells are not subjected to oxidative stress.

It has been reported that 5xFAD transgenic mice and a reproducible neuro-astrocytic culture from them, which we considered as a control, already have a shift in mitochondrial balance toward excess fission [20]. Also, loss of the central mitochondrial fission protein dynamin-related mitochondrial fission protein 1 (Drp1) increases the toxicity of mutant APP in vivo [21]. In the 5xFAD mouse model, all mutations target the hyperproduction of human Aβ1-42 [22], it is the overproduction of toxic agents that is the cause of dysfunction in this model. We assume that the increased mitochondrial fission in this model is secondary and is related to the fact that the cells are trying to get rid of toxic amyloid in this way. In our experiments, inhibition of mitochondrial fission resulted in a cascade of reactions characteristic of 5xFAD culture. In addition to a pathologic increase in lipofuscin, we observed a decrease in neuronal density and compensatory overgrowth of astroglia, which within this model can be considered as a manifestation of astrogliosis. That is, cellular toxin accumulation and mitochondrial fission mediate each other.

At the same time activation of cell division did not lead to such consequences. But we can assume that zinc is a toxic agent in general and its mechanism of operation is a reflection of how the introduction of an external toxic agent (similar to Aβ1-42 expression in 5xFAD) leads to an attempt of mitochondria to clean themselves and the cell from contaminants.

In doing so, the prolonged action of the fusion promoter resembles the depletion of internal resources in a healthy culture. This process of slow degeneration is broadly similar to what one pathway of cell culture degeneration looks like. It loses clustering and shrinks (although in general there is another pathway of culture degradation, abrupt neurosphere clustering, where thin neuro-astrocytic strands stretch between

densely packed neurospheres, linking them to other neurospheres, this is characteristic of primary cultures with high initial stem cell content or their increased level of activity). Mitochondria fusion is thought to result in enhanced ATP energy supply to cells because they have more cristae, increased levels of dimerization and ATP synthase activity and support ATP synthesis [23,24]. However, excess ATP leads to neuronal dysfunction and death [25] and is a prerequisite for the realization of the cellular apoptosis cascade [26]. In this case, the mitochondria resulting from fusion may be too large for mitochondrial transport. We find it difficult to say what the mechanism of culture degeneration is in this case. However, the absence of oxidative stress and the drop in lipofuscin levels shows that this process is more likely a controlled extinction of activity rather than an abrupt pathology. It can be stated unequivocally that only harmonious functioning of this system can ensure normal physiological functions of mitochondria, and any shift to one side of the dynamic process leads to disorders.

5. CONCLUSIONS

Forced alteration of mitochondrial fusion and fission leads to morphologic changes in primary neuro-astrocytic cultures of mouse hippocampus. Activation of mitochondrial fusion leads to slow depletion of the culture in the absence of visible degenerative processes and oxidative stress. Inhibition of mitochondrial fission leads to a cascade of events reminiscent of those occurring in a culture of 5xFAD animals (a genetic model of AD) - astrogliosis, decreased neuronal density, and increased lipofuscin levels. Exogenous zinc induces mitochondrial fission and provokes compensatory astrogliosis at toxic concentrations, and at lower concentrations leads to a slow decrease in culture clustering, changes in astrocyte immunoreactivity to GFAP and its aggregation, whereas lipofuscin fluorescence was still decreased. We suggest that mitochondrial fission in models with toxic mitochondrial damage is a compensatory process in which mitochondria strive to clear the cell of toxic agents, but which may lead to a cascade of reactions that provoke further development of pathologies.

6. DECLARATION

Acknowledgments

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Authors' contributions

The authors contributed equally to the preparation of the study.

Availability of data and materials

The results of the work can be found at the google link. Or write to the corresponding author.

https://docs.google.com/spreadsheets/d/1qnVotSWUwW1XUEAVkRCA3HBqfkvMVetg/edit?usp=sharing&ouid=105509595749562341769&rtpof=true&sd=true

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The laboratory animals were treated in accordance with the European Convention for the Protection of Vertebrates used for experimental and other purposes (Strasbourg, 1986) and the principles of the Helsinki Declaration (2000). All animal procedures performed with mices were approved by the Commission on Biosafety and Bioethics (Institute of Cell Biophysics – Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences) in accordance with Directive 2010/63/EU of the European Parliament.

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