FEATURES OF CALCIUM ACTIVITY OF ASTROCYTES IN PHYSIOLOGICAL AND ACCELERATED AGING *IN VITRO*

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Abstract. In the last decade, glial cells, primarily astrocytes, have received increasing attention in studying various aspects of brain functioning. Although astrocytes are not electrically excitable cells, they are able to generate and transmit Ca^{2+} signals, which can propagate from cell to cell, forming «calcium waves». Calcium signaling allows astrocytes to interact with each other, as well as regulate neuronal function. Despite numerous studies on calcium events in astrocytes under various pathological conditions, the features of astrocytic calcium dynamics in aging are currently understudied. Our work aimed to analyze the features of calcium activity in primary astrocyte cultures during physiological and accelerated aging, as well as to assess the effect of hypoxic damage on calcium activity. For this purpose, we developed an algorithm for the accurate detection of calcium events and the calculation of indicators describing the main parameters of calcium events in a cell. It was shown that the frequency of calcium event generation in astrocytes decreases during aging while their duration and amplitude increase. Hypoxia aggravates the changes caused by cell aging.

Keywords: calcium activity, astrocytes, aging, primary astrocyte cultures, ischemia, hypoxia.

Introduction

Aging leads to morphofunctional changes and loss of both neurons and glial cells in the brain (Gudkov *et al.*, 2022). Functional changes in astrocytes are known to occur in brain regions associated with age-related neurodegenerative diseases (Diene *et al.*, 2019); however, the available data are contradictory. For example, with aging, a decrease in the number and volume of astrocyte processes contacting synapses and a consequent decrease in synaptic support has been described (Denisov *et al.*, 2021). At the same time, other researchers noted astroglial hypertrophy in old animals (Verkhratsky, 2019).

The key mechanism in the implementation of inter-astrocytic signaling is the generation of calcium events and their transmission to each other ("calcium waves"). Calcium events (waves) are spatiotemporal interconnected short-term increases in the Ca^{2+} concentration in the cytoplasm of astrocytes, which are initiated in response to various signals. Ca^{2+} can enter the cytosol through the plasma membrane from the extracellular space and intracellular Ca^{2+} stores, the endoplasmic reticulum, and mitochondria (Agulhon *et al.*, 2008). Calcium events in astrocytes trigger the release of gliotransmitters (Bazargani *et al.*, 2016), regulate K^+ uptake from the synaptic cleft, and participate in the regulation of local blood flow (Araque *et al.*, 2014). Disruption of Ca^{2+} signaling is considered by modern neurobiology as an important component of neurodegenerative diseases and aging in general (Verkhratsky *et al.*, 2017).

Astrocytes are known to become reactive with aging. GFAP expression in astrocytes has been shown to increase with age (Clarke *et al.*, 2018), which is a common feature of reactive/activated astrocytes (Liddelow *et al.*, 2017). The activation of astrocytes in the aging brain occurs according to the A1 type and has a neurotoxic character. In addition to a decrease in neurotrophic support and the synthesis of a wide range of pro-inflammatory cytokines, it has been shown that the expression of genes involved in the regulation of calcium signaling changes in old astrocytes (Clarke *et al.*, 2018). Thus, aging is associated with impaired signaling between astrocytes.

It is important to note that chronic cerebral hypoxia often develops with aging, which is associated with impaired hemodynamics and other age-related changes. Hypoxia plays a

key role in the pathogenesis of many neurological disorders, including Alzheimer's disease, Parkinson's disease, and other age-related neurodegenerative diseases (Burtscher *et al.*, 2021). From the point of view of studying the role of glial cells in various injuries, it is of interest that astrocytic networks can be morphologically and functionally rearranged as a result of injuries, pathologies, or stress factors (Charvériat *et al.*, 2017). For instance, local (non-interacting) networks of reactive astrocytes can start interacting (Oberheim *et al.*, 2008). Astrocytes are known to be more resistant to oxygen and glucose deprivation than neurons (Ouyang *et al.*, 2007). However, how the calcium activity of old astrocytes changes under hypoxic exposure and whether the response to hypoxia of old and young astrocytes differs remains unknown.

In this regard, our study aimed at analyzing the features of calcium activity in primary astrocyte cultures under physiological and accelerated aging and in modeling hypoxia. For this purpose, we developed an algorithm for the accurate detection of calcium events and assessment of their quantitative parameters.

Materials and Methods

Ethics Statement

The basic rules for keeping and caring for experimental animals were in line with the «Rules of laboratory practice with the use of laboratory animals» (Russia, 2010) and the «International guiding principles (Code of ethics) for biomedical research involving animals» (CIOMS and ICLAS, 2012). The ethical principles established by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes were also respected (Strasbourg, 2006). All experimental procedures were approved by the Bioethics Committee of Lobachevsky University.

Cultivation of primary astrocyte cultures

The object of the study was the primary dissociated cultures of astrocytes obtained from cerebral cortex of C57BL/6 mice (P1-P5). A detailed description of cultures preparation is given in Mitroshina et al., 2020. After mechanical grinding of the cerebral cortex tissues, the cells were dissociated using a trypsin-EDTA solution (ThermoFisher, USA). The nutrient medium for cultivating astrocyte cultures was DMEM culture medium (PanEco, Russia) supplemented with 10% fetal calf serum (PanEco, Russia), 0.5 mM L-glutamine, 1% sodium pyruvate (ThermoFisher, USA), and 2% B27 (ThermoFisher, USA).

The cells were cultured for 7 days, after which they were passaged. Versen-trypsin solution (3:1) (PanEco, Russia) was used to remove the cells from the substrate. Primary astrocyte culture viability was maintained under constant conditions of 35.5 °C, 5% CO_2, and a humidified atmosphere in a cell culture incubator (Binder, Germany) for more than 30 days. The cell density at planting was 4500 cells/mm^2.

In vitro model of aging

To simulate physiological aging, long-term cultivation of astrocytes was carried out for 150 days. D-galactose was used to simulate accelerated aging. Application of D-galactose at a concentration of 50 μM was performed from 7 to 21 DIV with each change of the medium. D-galactose can be oxidized to hydrogen peroxide under galactose oxidase catalysis, which leads to the formation of reactive oxygen species (ROS) (Yanar *et al.*, 2011). D-galactose can also be converted to galactitol by aldose reductase, as a result of which galactitol cannot be further metabolized; it causes increased production of free radicals, the development of a redox imbalance, and can ultimately lead to aging of the body (Azman *et al.*, 2021).

Hypoxia modeling in vitro

Acute normobaric hypoxia in the «young astrocytes» and «accelerated aging» groups was modeled at 21 DIV by replacing the normoxic culture medium with a low-oxygen medium for 30 minutes. Oxygen was displaced from the medium in a sealed chamber with an inert gas (argon). The oxygen concentration in the culture medium was reduced from 3.26 ml/l to 0.37 ml/l (Vedunova *et al.*, 2015). After 30 min incubation, the hypoxic medium was replaced

with a full growth medium. For the «old astrocytes» group, hypoxia was modeled at 150 DIV. Registration of calcium activity was performed 7 days after hypoxia modeling.

The following groups of cultures were studied:

1. «Young astrocytes» – 28 days (28 DIV) of cultivation;
2. «Hypoxia» – at 21 DIV, acute normobaric hypoxia was simulated, after which calcium activity was recorded at 28 DIV;
3. «D-galactose» - accelerated aging with D-galactose (50 μM), after which calcium activity was recorded at 28 DIV;
4. «D-galactose + hypoxia» – accelerated aging with the use of D-galactose (50 μM), modeling of hypoxia at 21 DIV, after which calcium activity was recorded at 28 DIV;
5. «Old astrocytes» (150 DIV) - duration of cultivation 150 days, registration of calcium activity at 150 DIV;
6. «Old astrocytes hypoxia» (150 DIV + hypoxia) – acute normobaric hypoxia was simulated at 143 DIV, after which calcium activity was recorded at 150 DIV.

Calcium imaging

A laser scanning microscope LSM 800 (Zeiss, Germany) with a W Plan-Apochromat 10×/0.3 objective was used for imaging studies of the functional calcium activity of nerve cells. The technique allows visualizing the functional architecture of the neural network of the culture at the cellular level. Oregon Green 488 BAPTA-1 AM (OGB-1) 0.4 μM (Invitrogen, USA) was used as a calcium sensor. LED source with a wavelength of 488 nm was used to excite fluorescence. Fluorescence emission was recorded using a light filter with a bandwidth of 500–565 nm. A time series of images were recorded to assess the dynamics of intracellular calcium concentration measurement. The resolution of the obtained image was 512x512 pixels, the size of the field of view – 693x693 μm, and the image registration frequency – 2 Hz.

Statistical analysis

Statistical analysis was performed using Statistica (v.6.1) and GraphPad Prism (v.9.0). The distribution of quantitative values was checked for normality using the Shapiro-Wilk test (Kim, 2011). Mann-Whitney-Wilcoxon test was used to compare two unrelated groups. The difference between the groups was considered significant if the p-value was less than 0.05. To counteract the problem of multiple comparisons, p-values were adjusted using the Holm-Bonferroni method. At least three independent biological repeats were used for all experiments. Differences between groups were considered significant if the corresponding p-value was less than 0.05 ($p < 0.05$).

Results

Development of an algorithm for the detection of calcium events

At the first stage of the work, we developed an algorithm for the accurate detection of calcium events in cells. To detect calcium activity peaks, data on the brightness of each pixel in the cell were obtained using the previously implemented Astrolab program (Mitroshina *et al.*, 2020); the data were used for further analysis of calcium events. A Python algorithm was developed to construct a baseline calcium concentration below which there is an activity related to potential biological noise. The baseline corresponds to the inactive state of the cell (no change in intensity - no information signal). We chose the MCA205 cell line (murine fibrosarcoma) as a negative control for assessing the level of biological noise. The algorithm we developed finds a baseline based on a moving average, one of the most popular algorithms for smoothing fluctuations in time series. The principle of processing is to calculate the average value of neighboring w data for each value of the argument x_i. The number w is called the moving average window: the larger it is, the more data are involved in the calculation of the average, respectively, the smoother the curve is obtained.

The following functions were created:

1. building_baseline is a function, as an input of which the user sets the values of the glow intensity of the pixels of the cell and the window, the number of values of the initial function for calculating the moving average (smoothing interval). The wider the smoothing interval is, the

smoother the graph of the moving average is obtained.

The moving average is calculated iteratively as finding the arithmetic mean of the elements in the window, which becomes the value of the center of the window.

$$\overline{x} = \frac{x_1 + x_2 + x_3 + \ldots + x_w}{w}, \qquad (1)$$

where $\overline{x}$ is the sample mean; $x_1, \ldots, x_w$ are values in the interval specified by the window; w is the value of the window.

Next, the values of the moving average under the pixel glow intensity line are subtracted from the corresponding values of the pixel glow intensity line.

The standard deviation S is calculated using the following formula:

$$S = \frac{1}{n-1}\sqrt{\sum_{i=1}^{n} c_i^2}, \qquad (2)$$

where S is the standard deviation; n is the sample size; c is the difference between the pixel glow intensity and the moving average, where all values less than 0 are taken as 0.

The iterations are repeated until the S value is less than the previously calculated threshold, which was taken to be ±3σ of the pixel glow intensity in the time series of images of MCA205 cells.

2. plotting_base_line is a function for plotting the dependence of the pixel glow intensity for one cell on time in seconds and the respected baseline (Fig. 1).

3. event_borders is a function for finding the beginning and end of the peaks of astrocyte calcium activity. The beginning of a peak is found as a point preceded by a value below the baseline and followed by a value above the baseline. The end of a peak is found as a point preceded by a value above the baseline and followed by a value below the baseline. Only those peaks that lie above the +3σ baseline are taken as event peaks.

4. absolute_value is a function for calculating the event amplitude value. The amplitude of events is the difference between the value of the glow intensity at the maximum point between the beginning and end of the event and the value of the point on the baseline lying below it (Supplementary 1).

$$A = \int [i] - base[i], \qquad (3)$$

where A is the amplitude of events; int[i] is the glow intensity value at the maximum point between the beginning and end of the event; base[i] is the value of the point on the baseline that lies under the value of int[i].

To improve the accuracy of calcium event detection, manual labeling of events was carried out, as well as visualization of image stacks of astrocyte calcium activity in MATLAB using the implay function (Attaway, 2016). A smoothed video of a three-dimensional matrix 512 x 512 x 1200 was analyzed (Fig. 2); the inspect pixel values function allowed us to see the changes in the glow intensity of each pixel, thereby separating calcium events (significant changes in the glow intensity of pixels within the cell) from the usual calcium activity of an astrocyte.

1030 activity peaks of biological noise and peaks that are not related to events in intact cell cultures and 775 peaks of calcium events in intact cell cultures were labeled.

Finding the threshold for the best detection of calcium events

For further analysis, a threshold was determined to separate biological noise from calcium events in terms of amplitude. The threshold finding was implemented using ROC analysis based on plotting the receiver operating characteristic curve (ROC curve). ROC curve is used to analyze the behavior of classifiers at various thresholds. It allows us to consider all

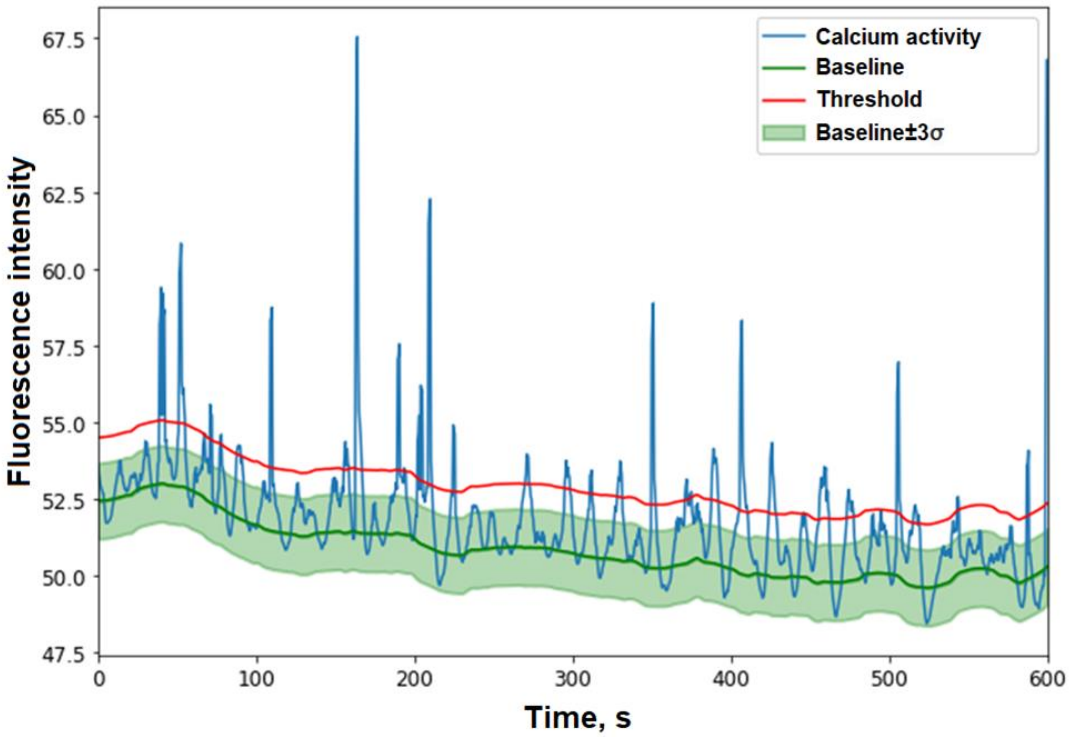

Fig. 1. Graph of the dependence of the pixel glow intensity for one cell on time, where the blue line is the pixel glow intensity, the green line is the baseline, and the red line is the threshold found using manual labeling

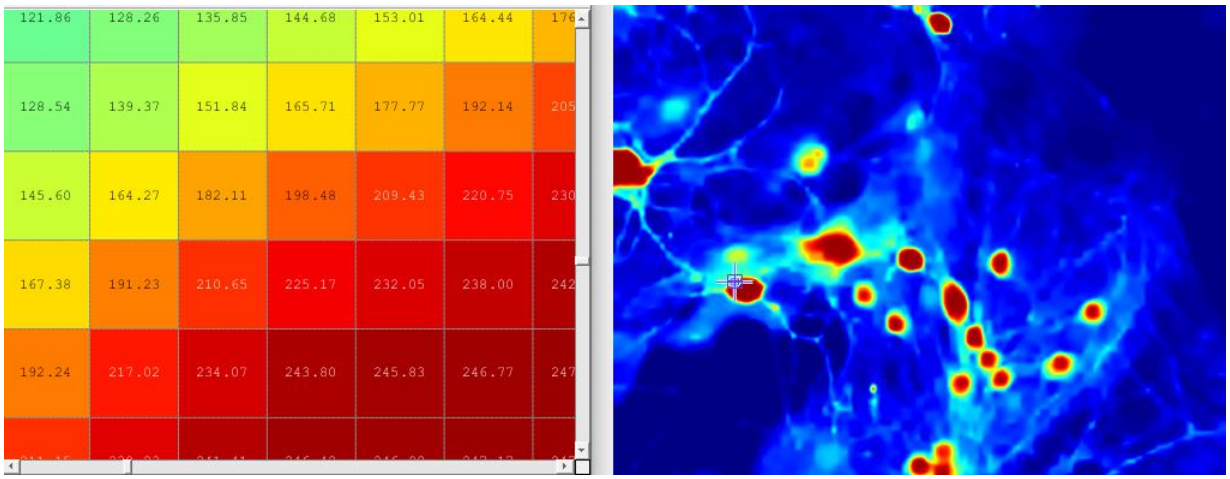

Fig. 2. Visualization and analysis of astrocyte calcium events and biological noise using the MATLAB

thresholds for a given classifier (Wixted *et al.*, 2017) and shows the proportion of false positive rates (FPR) versus true positive rates (TPR). An algorithm to calculate these metrics was written in the Python programming language; it was used to find the optimal threshold values to separate biological noise from calcium events in terms of event amplitude (Supplementary 1).

Based on the amplitude of glow events, the threshold p = 2.06 was found by plotting the ROC curve (Fig. 3).

With the threshold found, the share of negative samples that were incorrectly classified as positive (FPR) = 0.06, and the share of positive results that were correctly identified (TPR) = = 0.89. The area under the curve (AUC) = 0.97. Since AUC > 0.05 and the resulting value is

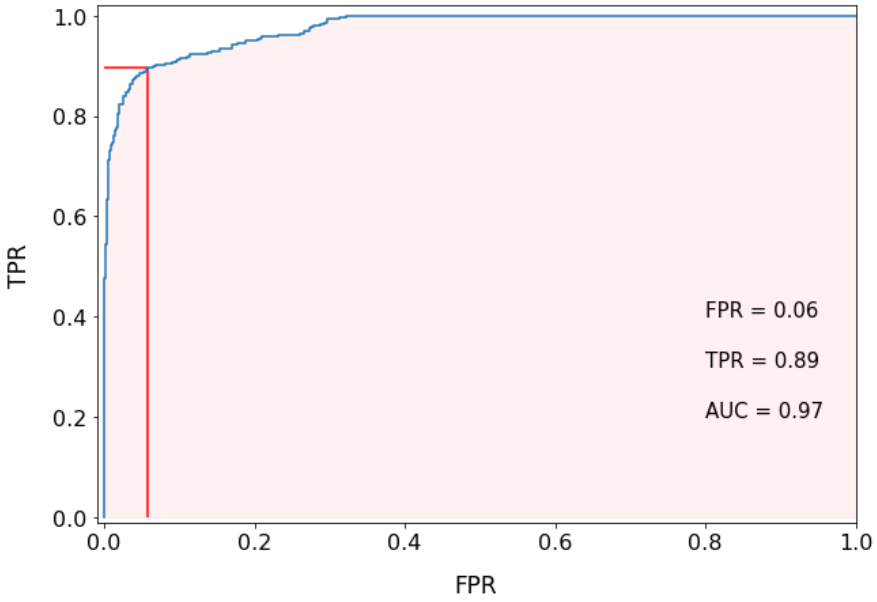

Fig. 3. ROC curve for absolute glow intensity values, where TPR is the sensitivity, FPR is the share of negative samples that were incorrectly classified as positive, AUC is the area under the curve

close to 1, we can conclude that the accuracy of the classifier is high, and it will detect more true positives and true negatives than false negatives and false positives. The accuracy of the developed algorithm was 93%.

Developing an algorithm for detecting calcium events and the boundaries of calcium events

An algorithm to search for calcium events and their boundaries was written in the Python programming language, using the previously found threshold p = 2.06 in terms of the absolute glow intensity value. The beginning of a peak is found as a point preceded by a value below the baseline and followed by a value above the baseline. For each peak, the event amplitude (3) is calculated, and the algorithm leaves only peaks whose event amplitude is higher than the found threshold.

- plotting_borders is a function for plotting the dependence of the intensity of the glow of pixels for one cell on time in seconds with the boundaries of calcium events depicted on it, the baseline, ±3σ-standard deviation from the baseline, as well as the maximum point of events (Fig. 4).

We implemented a graphical interface of the program in the Python programming language using the libraries tkinter, pandas, numpy, matplotlib, statistics (Alan, 2018). The user selects a CSV file with pixel glow intensity values of a cell as an input; the output is a graph of the dependence of pixel glow intensity per cell on time (Fig. 4), and the following parameters are calculated:

1) The number of events in the cell;

2) Average duration of an event in a cell (in seconds);

2) Average frequency of events per minute in a cell;

3) All durations of events in the cell (in seconds);

4) All values of the amplitude of events in the cell.

Analysis of calcium activity parameters in primary astrocyte cultures during physiological and accelerated aging

Using the algorithms we developed, we studied the features of astrocyte cultures in modeling aging and hypoxic damage. Within 10 minutes of recording calcium activity in young astrocytes, from 1 to 13 calcium events were recorded (Fig. 5). The frequency of event

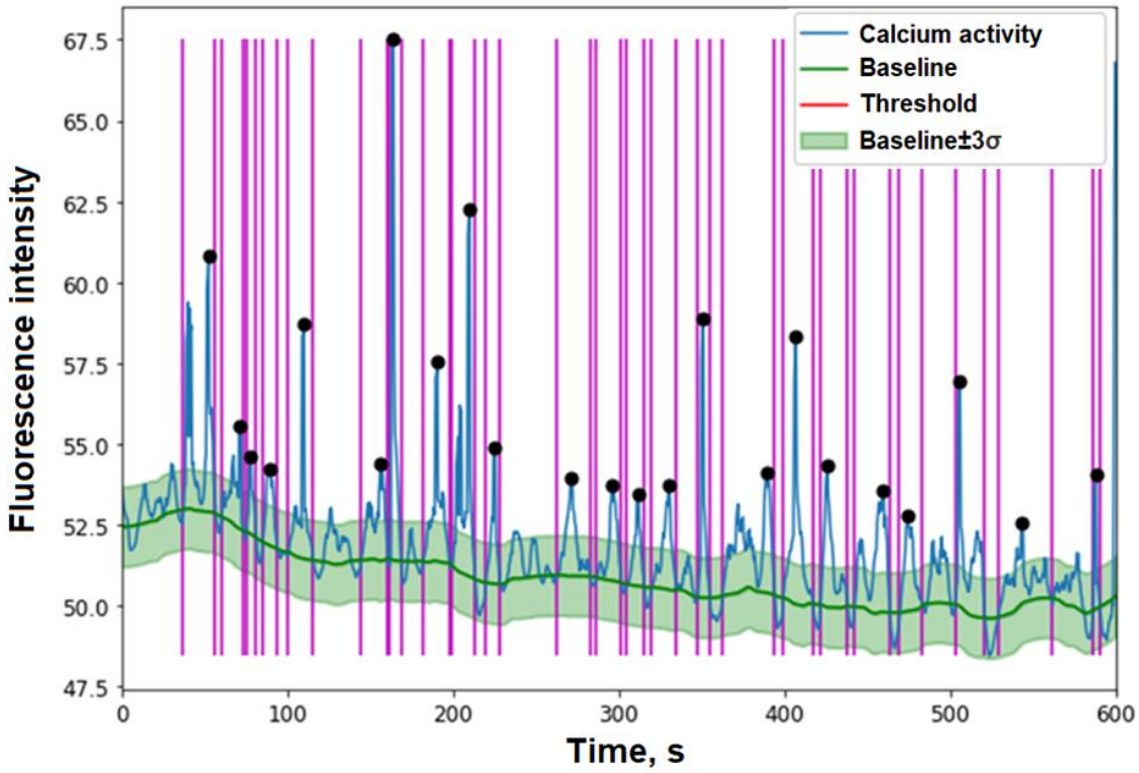

Fig. 4. Graph of the dependence of pixel glow intensity on time for one cell, where the blue line is the pixel glow intensity, the green line is the baseline, and the purple lines are the boundaries of astrocyte calcium events

generation was 0.21 [0.08; 0.44] events/min. Hypoxic exposure caused a significant decrease in the frequency of calcium events in the posthypoxic period (0.12 [0.06; 0.21] events/min). In the «D-galactose», «D-galactose + hypoxia» groups, there was also a significant decrease in the frequency of calcium events relative to young astrocytes. At the same time, there were no statistically significant differences between the «Intact» and «150 DIV» groups in terms of the frequency of occurrence of calcium oscillations. In the posthypoxic period, the frequency of occurrence of events in old astrocytes significantly decreased (Fig. 5A).

A significant increase in the duration of calcium events was shown both in cultures with the simulation of accelerated and physiological aging and in young astrocytes exposed to a hypoxia. Interestingly, the duration of calcium events in old astrocytes did not change after the hypoxic episode (Fig. 5B).

Characterization of the amplitude of calcium events in primary astrocyte cultures showed a statistically significant decrease in the amplitude of calcium events for all groups relative to the «Intact» group. There was a statistically significant decrease in the amplitude of calcium events in the «D-galactose» group compared to the «Intact», «DIV 150», and «Hypoxia» groups, which indicates pronounced metabolic disorders of astrocytes during accelerated aging. In the «D-galactose + hypoxia» group, the amplitude of calcium events was significantly lower compared to the «Hypoxia» and «150 DIV + hypoxia» groups, indicating that accelerated aging increases the sensitivity of astrocytes to hypoxic damage (Fig. 5C).

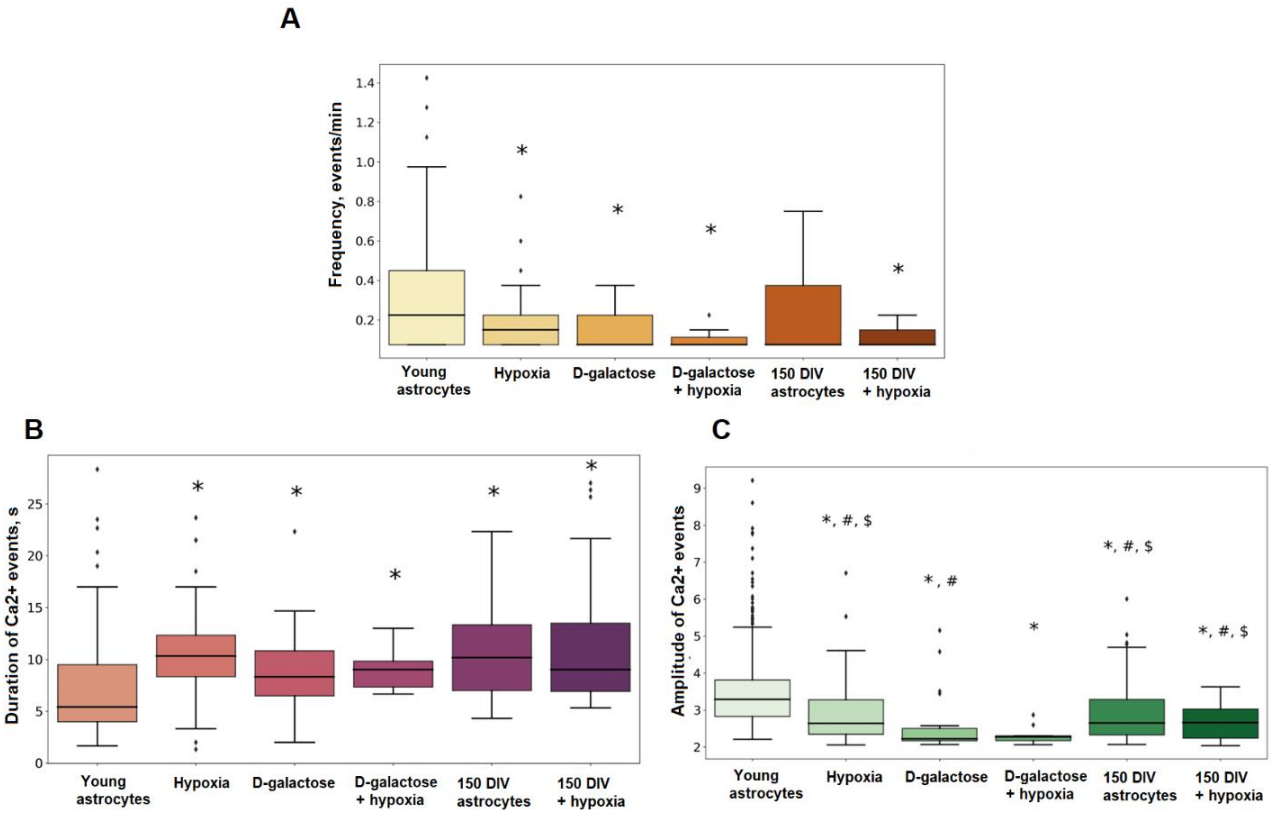

Fig. 5. The main parameters of astrocyte calcium activity in the experimental simulation of physiological and accelerated aging. A – frequency of event generation in a cell (number/min), B – duration of events (s), C – amplitude of calcium events, relative units. * – differences are significant relative to the «Young astrocytes» group, # – differences are significant relative to the «D-galactose + hypoxia» group, $ – differences are significant relative to the «D-galactose» group (p-value ≤ 0.05)

Discussion

Aging is a complex and multifactorial process. It is known that aging changes a number of physiological processes in which astrocytes are involved, including their interactions through gap contacts, the release of neurotrophic factors and water-ion metabolic balance, their ability to regulate synaptic transmission, etc. In the CNS, oxidative stress and neuroinflammation are two major pathological features associated with aging. Increased levels of reactive oxygen species and chronic inflammation in aging can contribute to the development and progression of many neurodegenerative diseases, including Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD), etc.

However, to date, little is known about the physiology of senescent astrocytes. It has been shown that their general electrophysiological properties (membrane potential at rest, K^+ membrane permeability, etc.) do not change with age (Lalo *et al.*, 2011). The preservation of the main types of neurotransmitter receptors on the astrocytic membrane has been noted (Lalo *et al.*, 2011; Gómez-Gonzalo *et al.*, 2017). However, the level of functional expression of the main receptors on astrocytes changes with age: the density of ionotropic glutamate and purinergic receptors in mice increased from 1 to 3–6 months of age, and then rapidly decreased with aging (Lalo *et al.*, 2011). Aging has been found to reduce the density of astroglial aquaporin 4 and therefore impair the clearance of proteins, including amyloid proteins, which may contribute to the development of dementia (Kress *et al.*, 2014).

The state of calcium signaling in senescent astrocytes is of great interest. However, most studies are aimed at evaluating calcium signal-

ing in various age-associated pathologies, primarily Alzheimer's disease (Mustaly-Kalimi *et al.*, 2018; Verkhratsky *et al.*, 2019.). There are very few studies on calcium signaling in healthy but senescent astrocytes. Therefore, the data we obtained on the features of calcium dynamics in astrocytes in modeling physiological and accelerated aging are of great interest.

We developed an original algorithm for calcium event detection based on building a baseline, as well as finding a threshold for separating calcium events from biological noise using ROC analysis. This made it possible to significantly improve the accuracy of event detection compared to existing algorithms.

Analysis of the data obtained showed that during physiological aging in primary astrocyte cultures (long-term cultivation for 150 days), events of lower amplitude and increased duration compared to young astrocytes were observed. With chronic administration of D-galactose («accelerated» aging) in primary astrocyte cultures, significantly lower frequency, prolonged calcium events of reduced amplitude compared to intact cultures were observed. These changes were more pronounced than in physiological aging.

We also revealed differences in the response of young and old astrocytes to hypoxic damage and lower amplitude of events in comparison with the «150 DIV» and «Hypoxia» groups. Hypoxic exposure leads to a significant decrease in the frequency and amplitude as well as an increase in the duration of calcium events compared to intact young astrocytes. At the same time, hypoxia did not cause a significant change in activity in the «old» cultures. However, hypoxia causes further aggravation of the changes caused by D-galactose: in the group «D-galactose + hypoxia» the most low-amplitude (in comparison with all groups), long-term, low-frequency calcium events were revealed.

The data we obtained are in agreement with the study by Palygin et al., which showed that the amplitude of neurotransmitter-induced Ca^{2+} signals decreased in mice with age (Palygin *et al.* 2010). This decrease in Ca^{2+} signals, in turn, led to an age-related decrease in astroglial ATP secretion and aberrant astroglial regulation of metaplasticity (Lalo *et al.*, 2014, 2018).

Summing up, we revealed functional changes in calcium metabolism in aging astrocytes. In the future, we will focus on studying inter-astrocytic signaling and evaluating network parameters of calcium activity.

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Supplementary 1

Calcium activity threshold calculation

```
import pandas as pd
import glob
import numpy as np
iint = []
bbase = []
for py in glob.glob("C:/Users/Figurova/Documents/Разметка данных/порог/*.csv"):
  b = py.split("\\")
  a = (b[-1])
  data = pd.read_csv(a, sep = "; ", engine='python', header = None)
  data["int"] = data[0]
  a = data["int"]
  d = []
  for i in range(0, len(a)):
    c = a[i].replace(',', '.')
    d.append(c)
  data["int"] = [float(x) for x in d]
  intensity = list(data["int"])
  window = 65
  intensity_copy, base_line, base_lines = intensity.copy(), intensity.copy(), []
  f = 0
  for k in range(int(window / 2)):
    intensity_copy.insert(k, intensity[0])
  for n in range(int(window / 2)):
    intensity_copy.append(intensity[-1])
  L = int(window/2)
  base_line = []
  for i in range(L, len(intensity_copy)-L):
    if (i + L + 1) >= int(window / 2):
      a = intensity_copy[i-L:i+L+1]
      c = sum(intensity_copy[i-L:i+L+1])/ window
      base_line.append(c)
  for i in intensity:
    iint.append(i)
  for j in base_line:
    bbase.append(j)
difference = np.array(bbase) - np.array(iint)
difference_mod = [0 if x < 0 else x for x in difference]
s = ((1 / (len(iint) - 1)) * sum([i**2 for i in difference_mod]))**0.5
sd = difference.std()
print(s)
print(3 * sd)
```