Lactate and Pyruvate Activate Autophagy and Mitophagy that Protect Cells in Toxic Model of Parkinson's Disease

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ABSTRACT

Intracellular quality control regulated by autophagy process is important for maintenance of cellular homeostasis. Deregulation of autophagy and more specifically mitophagy leads to accumulation of the misfolded proteins and damaged mitochondria that in turn leads to the cell loss. Alteration of autophagy and mitophagy has shown to be involved in the number of disorders including neurodegenerative diseases. Autophagy and mitophagy could be activated by short-time acidification of the cytosol, however, most of the compounds which can induce it are toxic. Here, we tested several organic compounds which are involved in cellular metabolism on their ability to change intracellular pH and induce mitophagy/autophagy. We have found that lactate or pyruvate are able to reduce intracellular pH in non-toxic concentrations. Short-term (2 hours) and long-term (24 hours) incubation of the cells with lactate and pyruvate induced mitophagy and autophagy. Incubation of the SH-SY5Y cells or primary neurons and astrocytes with lactate or pyruvate also activated mitophagy and autophagy after MPP+ treatment that led to recovery of mitochondrial function and protection of these cells against apoptotic and necrotic death. Thus, pyruvate- or lactate-induced acidification of cytosol activates cell protective mitophagy and autophagy.

Keywords: autophagy; mitophagy; pyruvate; MPP+; Parkinson's model; intracellular pH

DECLARATIONS

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INTRODUCTION

Autophagy is intracellular process which maintains cellular homeostasis by eliminating dysfunctional cellular organelles, misfolded proteins, aged proteins or protein aggregates. Depending on the mechanism mediating the delivery of intracellular components to lysosomes, autophagy is classified as microautophagy, chaperone-mediated autophagy of protein molecules involving hsp70 and LAMP2 and macroautophagy [1]. During the process of microautophagy the substrates enter the lysosome through invagination resulting in their degradation [2]. This process involves ESCRT proteins (endosomal sorting complex required for transport) and the formation of microautophagic bodies. The Chaperone Mediated Autophagy (CMA) occurs through the recognition of a specific motif, forms a substrate/chaperone complex that fuses with the lysosome upon recognition of the CMA receptor [3,4]. The formation of a two-membrane autophagosome structure and delivery of autophagic cargo inside membrane vesicles to lysosomes for degradation characteristic of macroautophagy [5]. Autophagy is induced in response to nutrient limitation or accumulation of damaged proteins and organelles.

Specialized protective mechanism of removing impaired mitochondria through the macroautophagy/autophagy pathways was specified as mitophagy and was originally reported by Kim et al. [6]. This selective removal of mitochondria via mitophagy is an important mechanism of mitochondrial quality control in physiological and pathological conditions for maintaining the mitochondrial homeostasis. At present the most studied mitophagy pathways are ubiquitin-dependent and ubiquitin-independent [7].

Alteration of autophagy or more specifically mitophagy shown to be involved in variety of diseases, including cancer, diabetes, myopathy, and neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Huntington’s disease, neuropathy, and Parkinson’s

disease [8-11]. Two genes, which encode the kinase PINK1 and the cytosolic E3 ubiquitin ligase Parkin, involved in the clearance of damaged mitochondria, are mutated in autosomal recessive Parkinson's disease and lead to defects in mitophagy [12,13].

The processes cellular quality control through autophagy and mitophagy in particular are currently attract the attention of the researchers due to involvement in the various physiological and pathological mechanism [14]. Selective activation of autophagy and mitophagy potentially can be neuroprotective in neurodegenerative disorders because it can remove aggregated misfolded proteins and damaged mitochondria [15]. However, most commonly used method of activation of mitophagy is based on using high concentration of protonophores FCCP or CCCP, which are very toxic. Recently we have shown that activation of mitophagy and autophagy can be induced by acidification of the cytosol that can be achieved by 10 μM FCCP or ionophore nigericin [16]. Here we attempted to identify non-toxic physiological compounds which could reduce $[pH]_c$ and induce mitophagy and autophagy. We have found that millimolar concentrations of pyruvate and lactate could induce acidification of cytosol and induction of mitophagy and autophagy. Both compounds were protective in toxic (MPP+) cellular Parkinson's model.

MATERIALS AND METHODS

Materials.

Culture flasks, Petri dishes and other plastic consumables were manufactured by SPL Life Sciences; round coverslips were from Menzel-Glaser; DMEM, FBS, Neurobasal A medium, Supplement B27, sterile HBSS, trypsin solution were purchased from Gibco. 1-methyl-4-phenylpyridinium, sodium pyruvate, sodium lactate, lactic acid, L-glutamine, glucose, polyethyleneimine solution, FCCP, all inorganic salts and buffers were purchased from Sigma-Aldrich; gentamicin was obtained from PanEco, Russia; 5-(and-6)-Carboxy SNARF™-1, Rhodamine 123, Hoechst 33342, Propidium Iodide, MitoTracker Green, LysoTracker Red were obtained from Invitrogen.

Cell cultures.

SH-SY5Y. Cell culture was grown in a CO_2 incubator at 37°C and 5% CO_2 in 25 cm^2 culture flasks in DMEM medium with 10% fetal bovine serum (FBS), 15 μg/ml gentamicin, 2 mM L-glutamine and 1 mM sodium pyruvate. For the experiment cells were plated on round coverslips with a diameter of 25 mm, placed in 35 mm Petri dishes. Initial culture density was 4×10^5 cells/cm^2. Alternatively, cells were seeded onto 25 cm^2 culture flasks at the same density. Experiments were performed when cells were 70–90% confluent.

Primary rat cortical neuroglial culture. Animal studies were approved by the Animal Ethics Committee of the Institute of Cell Biophysics, Russian Academy of Sciences and were performed in compliance with the Russian Federation legislation. The primary neuroglial culture of rat cortex was obtained from newborn male pups (P1-3) of the *Sprague Dawley* rats. Pups were decapitated, the brain was removed and placed in a 60 mm Petri dish in a cold sterile HBSS solution on ice Then the meninges were removed, the tissue was placed in a microcentrifuge tube in a cold Versene solution and was cut in small pieces (1 mm). Then the medium was replaced with a 0.1% trypsin solution incubated for 15 min at 37°C. After that, trypsin was washed 3 times with DMEM medium with 10% FBS, carefully pipetting the tissue with 1000 μl tip until a homogeneous suspension was formed. Next, the cells were placed on round coverslips with a diameter of 25 mm, coated with polyethyleneimine and placed in 35 mm Petri dishes, and left for 30 min for cell attachment. After that, culture medium was replaced with 1.5 ml Neurobasal A medium contained 2% Supplement B27, 7.5 μg/ml gentamicin, 1 mM L-glutamine and cells incubated for 9–14 days at 37°C and 5% CO_2.

All microscope imaging experiments were performed at a temperature of 28°C. The working medium (HBSS) contained (in mM): 138 NaCl, 1.3 $CaCl_2$, 0.4 $MgSO_4$, 0.5 $MgCl_2$, 5.3 KCl, 0.45 KH_2PO_4, 4 $NaHCO_3$, 0.3 Na_2HPO_4, 10 glucose, 20 HEPES (pH = 7.3). To obtain a medium with different pH contents (for SNARF calibration), NaCl, $NaHCO_3$, Na_2HPO_4 were replaced with equivalent potassium salts in the medium.

Live cell imaging

Measurement of cytosolic pH. Cells were stained with 5 μM SNARF for 40 minutes. Then the cells were washed twice in HBSS working medium and intracellular pH was recorded using a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany). The fluorescence of the SNARF ratiometric probe was excited with a 543 nm Ne/He laser, signal was recorded at 550–570 nm and 650–700 nm. SNARF calibration was performed as described in [17]. Each curve is the average of three independent experiments, in each the change in fluorescence in at least 80 cells was estimated.

Mitochondrial membrane potential was measured Rhodamine 123 (Rh123) fluorescent probe. Cell cultures were loaded with 10 μM Rhodamine 123 for 15 minutes. Then the cells were washed twice in HBSS. In this protocol, fluorescent quenching (dequench) of Rhodamine 123 occurs, and an increase in fluorescence indicates mitochondrial depolarization. Measurements were carried out using a Leica DMI6000 B inverted microscope (Leica Microsystems, Germany) using a standard FITC filter cube using a 20× lens. Each curve is the average of three independent experiments, in each of which the change in fluorescence in at least 80 cells was estimated.

Measurement of NADH level. To record the NADH level, autofluorescence was measured using a Cell Observer fluorescent station (Carl Zeiss, Germany) based on an Axiovert 200M inverted microscope equipped with an illuminator with quartz optical elements and a 20× high transmittance UV lens, Hamamatsu ORCA-Flash2.8 CMOS camera. Fluorescence was excited at 340±7 nm and recorded at 460±10 nm with a frequency of 0.2 Hz. The basal level of NADH were identified in three independent experiments, in each of which were at least 50 cells.

Cell viability. To assess cell viability, the Hoechst 33342 (2 μg/ml, 10 min) and Prodium Iodide (2 μg/ml, 10 min) were used. The freely penetrating Hoechst 33342 label

DNA in all cells, and Propidium Iodide is able to penetrate only into damaged cells, indicating necrosis. The fluorescence of Hoechst 33342 and Propidium Iodide was evaluated using a Leica DMI6000 B inverted microscope (Leica Microsystems, Germany) using a 20× objective using standard filter sets for DAPI and Texas Red, respectively. For each exposure, the average culture density (cells/mm^2) and the percentage of non-viable cells (of the total counted) were calculated.

Measurement of lysosomes and mitochondria colocalization. To measure the level of mitophagy, the degree of colocalization of mitochondria and lysosomes was evaluated. Before treatment of the cell culture with 1-methyl-4-phenylpyridinium (MPP+), sodium pyruvate (SodPyr), sodium lactate (SodLact), lactic acid (Lact) mitochondria were stained with MitoTracker Green (200 nM) 40 min in the culture medium in a CO_2 incubator. After staining the cells were washed with DMEM and incubated with mitophagy inducers for 30 minutes, 2 hours, 24 hours, 48 hours in a CO_2 incubator. Lysosomes were stained with LysoTracker Red (50 nM) for 40 min in a CO_2 incubator in serum-free medium. After that, the culture medium was replaced with HBSS solution and two-channel images were obtained using a Leica TCS SP5 confocal system (Leica Microsystems, Germany) using a 63× objective. MitoTracker Green fluorescence was excited with a 488 nm line of an argon laser, and LysoTracker with 543 nm Ne/He laser. Fluorescent signal was registered at 500–550 nm and 630–700 nm respectively [16]. The values obtained for 7–15 randomly selected cells in each of three independent experiments were used.

Image analysis. The analysis of the fluorescent time-lapse images was performed using ImageJ. To process data on the level of intracellular pH, mitochondrial potential, and NADH level, the Time series analyzer plugin was used. Average values of pixel brightness in each region of interest were calculated for each frame of a series of images. Based on the results of the data, graphs and tables were built. To process the results of evaluating cell viability in the

blue (Hoechst) and red (Propidium Iodide) channels, the Cell Counter plugin was used. To quantify mitophagy, we used a specially developed macro for the ImageJ. This macro decomposes the image into green (mitochondria) and red (lysosomes) channels, then converts them to the bit format (manual threshold) and quantitatively analyzes the area occupied by mitochondria and the area occupied simultaneously by lysosomes and mitochondria. After that, the algorithm estimates the percentage of mitochondria of the cell colocalized with lysosomes.

mRNA measurement. The work was carried out on SH-SY5Y cells and primary neuroglial culture of rat cerebral cortex. The cells were treated with and MPP+, lactic acid, sodium pyruvate and sodium lactate in different concentrations. Total RNA (totRNA) was isolated from the cell culture using the MagJet kit (Invitrogen), according to the manufacturer instructions. The amount of isolated totRNA was determined spectrophotometrically at 260 nm (NanoDrop). The cDNA synthesis was carried out using MMLV RT (Eurogen). The composition of the reverse transcription reaction solution (total volume – 20 μl): 1-2 μg totRNA, 20 μM oligo(dT)primers, 10 mM dNTP, 20 mM DTT, 100 units reverse transcriptase MMLV RT in reverse transcription buffer. The reaction was carried out for 45 min at 40°C, the reaction was stopped by heating the mixture for 10 min at 70°C. The resulting cDNA preparations were used as a template for real-time PCR. The reaction mixture for PCR analysis consisted of: 3 μl of a cDNA sample, a mixture of reverse and direct primers (10 μM each), a qPCRmix-HS SYBR reaction mixture (Eurogen) containing Taq DNA polymerase. After preliminary denaturation (5 minutes at 95°C), 40 amplification cycles were carried out: denaturation at 95°C for 30 sec, annealing at a temperature specific to the primer for 20 sec, and elongation at 72°C for 30 sec. Reporter fluorescence was read at the elongation stage. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the reference gene. The expression of the studied genes was evaluated by real-time PCR using the

DTlite amplifier (NPO DNA-Technology LLC, Russia). The relative expression level was determined as $N = 2^{-\Delta\Delta Ct}$, where ΔCt is the difference between the threshold cycle values for GAPDH and the target gene (marker genes for auto/mitophagy and apoptosis) and ΔΔCt is the difference between ΔCt control cells and ΔCt treated cells. [18]. To determine the contamination of totRNA with impurities of genomic DNA, a negative control reaction was set up, where the totRNA preparation was added to the reaction mixture as a template for the PCR reaction. In these samples the fluorescent signal was absent, indicating the absence of impurities of genomic DNA.

The oligonucleotides sequence used in the work (Eurogen, Russia):

Gene (human)	**Primer**	**Sequence**	**Gene (rat)**	**Primer**	**Sequence**
Nix	For	5’-cacgtaccatcctcatcctccatccac-3’	Bnip3	For	5’-gcagcgttccagcttccgtctctat-3’
	Rev	5’-tccgcacttttcttcaaagcctcgact-3’		Rev	5’-agtagctgtgcgcttcgggtgt-3’
Beclin1	For	5’-cagatggagctaaaggagctggcact-3’	Optn	For	5'-gagaggagcggatctctgtggc-3'
	Rev	5’-gaacactgggcaggcgacc-3’		Rev	5'-ccacttcctgctccaagtcggt-3’
Lc3	For	5’-gagcgctacaagggtgagaagcag-3’	p62	For	5’-gagtcggtgtgagcagagagtgaagtc-5’
	Rev	5’-gagtaggtgggttggtgccctc-3’		Rev	5’-cccagaagcaggtgttgttccaaagttg-3’
Mfn1	For	5’-atcaactggcccatgcccttcac-3’	Lc3	For	5’-tcgtcgcgggaaccagatcgt-3’
	Rev	5’-tgtgctgtctgcgtacgtcttcca-3’		Rev	5’-accatgctgtgcccattcaccag-3’
p62	For	5’-tccctgctcttctctcgatgcaactc-3’	Parkin	For	5’-gctgggaaggagctgcagaatcac-3’
	Rev	5’-ggacgtgggctccagtttcctg-3’		Rev	5’-tggcaggggcctttgcagtag-3
Ndp52	For	5’-gcttcaggcccagctgtcaact-3’	Pink1	For	5’- cgggtctcaagcccgggag-3’
	Rev	5’-tgtaactgagcaatctgctgttctccctc-3’		Rev	5’-caacgtcgtgtgtccagtgggtc-3’
Bnip3	For	5’-ctccgacctccgctttcccac-3’	Gapdh	For	5’-ccacggcaagttcaacggcac-3’
	Rev	5’-gctactccgtccagactcatgctgt-3’		Rev	5’-gatgatgacccttttggccccacc-3’
Optn	For	5’-gccagctcctgctgtgccta-3’	Bax	For	5’-gacacctgagctgaccttggagc-3’
	Rev	5’-ggaggttcagtgcttccacttcgc-3’		Rev	5’-cgggcactttagtgcacaggg-3’
Parkin	For	5’-ccatggtttcccagtggaggtcg-3’	Bcl-2	For	5’-tggagatgaagactccgcgcccctga-3’
	Rev	5’-gagtccttcctgctgtcagtgtgc-3’		Rev	5’-cgtggcaaagcgtcccctcgcggt-3’
Pink1	For	5’-tgtgctgccctcacgcctg-3’	Bcl-xl	For	5’-ggggctatccgccaggtgc-3’
	Rev	5’-gtttccgccccgatccacgtac-3’		Rev	5’-gagcagacccagtgagtgagcaggt-3’
Gapdh	For	5’-tggtcgtattgggcgcctgg-3’	p53	For	5’-gcagagttgttagaaggcccagagg-5’
	Rev	5’-gagggggcagagatgatgacccttttt-3’		Rev	5’-gccgtcaccatcagcaacg-3’
p53	For	5’-cgacggtgacacgcttccctg-3’	Fas	For	5’-gtttggagttgaagaggagcgttcgt-3’
	Rev	5’-gaaaccgtagctgccctggtcggt-3’		Rev	5’-cattggcacactttcaggacttggg-3’
Bcl2	For	5’-ctgcatctcatgccaaggggga-3’			
	Rev	5’-cgtagcccctctgcgacagcttat-3’			
Bax	For	5’-cggcggtgatggacgggt-3’			
	Rev	5’-gtctgtgtccacggcggcaat-3’			
caspase8	For	5’-ggacagtgaagatctggcctccctc-3’			
	Rev	5’-cacccacctgtagcagaaatttgagc-3’			

Immunoblotting. The work was carried out using SH-SY5Y cells. Cells were grown on 25 cm^2 flasks and treated with sodium pyruvate and sodium lactate, FCCP, MPP+. The total protein was isolated from the cell culture as following. Cells were washed with cold PBS (Helicon) and then lysed with 1% Triton X100 buffer (Helicon) with the Protease/Phosphatase Inhibitor Cocktail (Invitrogen). Protein concentration was measured spectrophotometrically at 230 nm (NanoDrop). Protein separation (20 μg) was performed using denaturing electrophoresis in 10% PAAG for 1 hour. Semi-dry transfer of proteins to nitrocellulose (BioRad) was carried out for 7 minutes at a current of 1.3 A and a voltage of 25 V (Power Blotter System, ThermoScientific, USA). Membranes were blocked with a 5% blocking agent (Amersham) for 1 h at room temperature. To identify mitophagy markers (PINK1, p62, BNIP3L, LC3) and beta-actin, primary antibodies (Anti-PINK1 antibody produced in rabbit (1:1000, Abcam), Anti-LC3B antibody produced in rabbit (1:1000, Abcam), Anti-SQSTM1/p62 antibody produced in rabbit (1:1000, Abcam), Anti-BNIP3L antibody produced in rabbit (1: 1000, Abcam), Anti-ACTB antibody produced in rabbit (1:1000, Abcam)) were loaded overnight at 4°C. Membranes were washed 5 times 5 min each in PBST buffer (Sigma-Aldrich). Next membranes were incubated with secondary antibodies (Goat Anti-Rabbit IgG H&L (HRP) (1: 5000, Abcam) for 1 h at room temperature followed by 5-fold washing for 5 min each in PBST buffer. Membranes were visualized using an ECL substrate (Clarity Western ECL Substrate, BioRad) using iBright Imaging Systems (ThermoScientific, USA) and ChemiDoc MP Imaging System (BioRad, USA). The relative protein level was calculated using the ImageJ built-in gel analyzer and presented relative to beta-actin and control (untreated cells). Each bar shown in the graph is the average of at least 3 independent experiments.

Cellular model of Parkinson's disease. To study cellular toxic models of Parkinson's disease, we used SH-SY5Y neuroblastoma cells and a primary neuroglial culture of the rat

brain, which were incubated with rotenone or with MPP+ [19]. In the case of using rotenone, it was not possible to obtain quantitatively stable results. Thus, under the action of 1 μM rotenone, the number of non-viable cells after 48 hours ranged from 24 to 62% both in the culture of neuroblastoma and in the neuroglial culture of the rat brain. In contrast, the MPP+ model gave reproducible results. MPP+ was added to the cell culture medium in 0.5 ml of fresh DMEM or Neurobasal A to achieve final concentration 0.3 or 0.5 mM; 0.5 ml of culture medium was also added to control cells. Under the 48-hour exposure to 0.3 mM MPP+ the percentage of non-viable cells was 39.2±4.3%, and when exposed to 0.5 mM MPP+ it was 54.5±5.7% in neuroglial culture. In the SH-SY5Y culture the number of non-viable cells was lower (16.93±1.62%) (see Fig. 6c, b). As a result, we selected the 48-hour action of 0.5 mM of MPP+ as a toxic model of Parkinson's disease. When protective actions of organic acids were studied, we added these compounds 24 hours after the application of MPP+ and the effect was assessed 48 hours after the initial MPP+ supplementation.

Statistical analysis. Data were obtained from a minimum of three independent experiments. Statistical analysis and curve fitting were performed using Origin 2018 (OriginLab Corporation, USA) software. Data were analyzed by one way ANOVA followed by a Tukey's Multiple Comparison and significance expressed: $*\ p < 0.05$, $**\ p < 0.01$. For all graphs, error bars represent mean ± SEM (the number of experiments is indicated as N).

RESULTS

Sodium lactate, sodium pyruvate and lactic acid induce acidification of cytosol of SH-SY5Y cells.

We have tested the effect of the number of organic compounds on the $[pH]_c$: formiate, propionate, butyrate, pyruvate, succinate and lactate. We have found that 1 mM of formiate,

butyrate and propionate were toxic for cells and induced immediate de-attachment of the cells from the coverslip. Neither survival experiments nor any other microscopic measurements could be carried out under these conditions and these compounds were excluded from further studies. Sodium succinate (5–20 mM) had no significant effect of cytosolic pH (data not shown). Application of lactic acid (10 mM) induced profound and sustained acidification extracellular medium and the cytosol of SH-SY5Y cells (to pH = 6.0; N = 3 experiments, Fig. 1a). In contrast to lactic acid, addition of sodium lactate (2–50 mM) or sodium pyruvate (2–50 mM) induced fast but transient decrease in $[pH]_c$ of SH-SY5Y cells. Representative curves for 50 mM sodium lactate and sodium pyruvate are shown in Fig. 1a (average of 3 experiments). It should be noted that the amplitude of cytosolic acidification induced with 10–50 mM of sodium lactate or sodium pyruvate is comparable to the action of 10 μM FCCP (Fig. 1a). The sodium lactate- or sodium pyruvate-induced acidification is transient and cytosolic pH level recovers in 30–40 minutes. Thus, we have found organic compounds which could induce cytosolic acidification.

Sodium lactate and sodium pyruvate have no neurotoxic effect

In order to identify potential toxicity of the sodium lactate and sodium pyruvate we studied the viability of SH-SY5Y neuroblastoma cells after 24 hours incubation with these compounds in comparison to FCCP and nigericin (Fig. 1b). Considering the effects of organic compounds on the attachment of the cells to the coverslips both cell density and number of dead cells (necrosis, using Prodium Iodide and Hoechst 33342 fluorescence) was assessed. In agreement with previously published data [16] 10 μM FCCP, 1 and 3 μM nigericin significantly increased the percentage of dead cells (Fig. 1b, N = 3 and 4 independent experiments accordingly; $p < 0.05$, $p < 0.01$). Importantly, both sodium lactate and sodium pyruvate had no effect or induced only minor effect (20 mM) on cell viability (Fig. 1b).

Incubation of the SH-SY5Y cells with lactic acid dose-dependently reduced cell density on the coverslips and induced cell death (Fig. 1b).

The effect of the pH decreasing compounds on apoptosis was estimated by the level of mRNA of *p53*, *Bcl-2*, *Bax* and *caspase 8* in SH-SY5Y neuroblastoma cells. We have found that 2 hours of incubation of the cells with 5–50 mM lactic acid, sodium pyruvate or sodium lactate had no significant effect of the level of apoptotic markers (Fig. 1c, dashed line shows control value of the gene expression. N = 3–4 independent experiments; $p < 0.05$, $p < 0.01$.). In contrast, 2 hours incubation with 1 μM FCCP significantly increased the level of *Bcl-2*, *Bax* и *caspase 8* suggesting activation of apoptotic cell death (Fig. 1c). More prolonged incubation (24 hours) of the cells with 10 mM lactic acid induced gene expression of *Bax* and *caspase 8*, sodium lactate and sodium pyruvate increased the level of mRNA of *caspase 8* only in higher concentrations (20 and 50 mM; Fig. 1c). It should be noted that 24 hours incubation of SH-SY5Y cells with FCCP (1 μM) or nigericin (1 μM) had a much stronger effect on expression of proapoptotic genes *Bax* and *caspase 8* (Fig. 1c). Thus, prolonged acidification of the cytosol is toxic and can induce apoptotic and necrotic cell death. However, transient acidification of the cytosol with sodium lactate and sodium pyruvate has no effect on SH-SY5Y cells viability.

Effect of lactic acid, sodium pyruvate and sodium lactate on mitochondrial metabolism

One of the possible triggers for activation of mitophagy by FCCP is a collapse of the mitochondrial membrane potential. Pyruvate and lactate [20] could be used in energy metabolism of brain cells. Lactate itself can stimulate cell respiration also in SH-SY5Y cells [21]. Considering this we studied the effect of incubation of SH-SY5Y cells (2 or 24 hours) with lactic acid, sodium pyruvate or sodium lactate on the level of NADH autofluorescence in mitochondria (Fig. 2a). All three compounds fail to induce profound and significant changes

in NADH autofluorescence after 2 or 24 hours of incubation but in some cases, we found that the basal level of NADH tends to rise or fall (Fig. 2a).

The effect of sodium lactate and sodium pyruvate on mitochondrial membrane potential was assessed by the amplitude of Rhodamine 123 fluorescence change to the application of the uncoupler FCCP (1 μM). Short (30 min) or prolonged (24 hours) incubation of the SH-SY5Y cells with sodium lactate of sodium pyruvate (5–20 mM) had no significant effect on mitochondrial membrane potential measured by indicator Rhodamine123 (Fig. 2b). Thus, we conclude that sodium lactate and sodium pyruvate do not induce collapse of mitochondrial bioenergetics.

Sodium lactate, sodium pyruvate and lactic acid induce colocalization of mitochondria and lysosomes

We have unravelled the effect of 2- or 24-hours incubation of lactate and pyruvate on mitophagy in SH-SY5Y cells using method of colocalization of the labelled mitochondria (MitoTracker Green) and lysosomes (LysoTracker Red) (Fig. 3a, b). 24 hours incubation of the cells with lactic acid (5–20 mM), sodium lactate (5–20 mM) or sodium pyruvate (5–50 mM) induced 2–3-fold increase in colocalization of mitochondria and lysosomes that can be compared with effect of 10 μM FCCP (Fig. 3b, N = 3–4 independent experiments; $p < 0.05$, $p < 0.01$). Shorter incubation of SH-SY5Y cells with these compounds also increase percentage of mitochondria colocalized with lysosomes but the effect was expectably lower (Fig. 3b). Thus, acidification of cytosol by lactic acid, sodium lactate or sodium pyruvate induce mitophagy in SH-SY5Y cells.

Importantly, 24-hours incubation of primary neurons and astrocytes with sodium lactate of sodium pyruvate also induced 2–3-fold increase in colocalization of mitochondria with lysosomes (Fig. 3c) that confirm the effect of these compounds on the mitophagy.

Sodium lactate, sodium pyruvate and lactic acid induce expression of autophagic genes

In order to identify the specific pathways of autophagy/mitophagy which are activated by lactate and pyruvate, we estimated the level of gene expression involved in these processes (*p62, Lc3, Beclin1, Optn, Ndp52, Bnip3, Nix, Mfn1, Pink1, Parkin)* using 2- or 24-hours incubation of cells with the same range of concentrations (5–20 mM) (Fig. 4a, b, c).

2 Hours incubation of the SH-SY5Y cells with lactic acid (5 mM) induced increase in expression of *Nix* and *Parkin* genes (Fig. 4a). Increase of the concentration to 10–50 mM induced more profound effect and activated expression of most of the studied genes (*p62, Lc3, Beclin1, Ndp52, Bnip3, Nix, Mfn1)*. Sodium pyruvate at the concentration of 5 mM also induced increase in *Parkin* expression (Fig. 4b). Higher concentrations (10–50 mM) of pyruvate increased activation of the autophagic genes expression in most of the studied target excluding *Optn, Pink1, Parkin* (Fig. 4b). Application of the sodium lactate after 2 hours increased an autophagy genes expression predominantly at the 10 mM (Fig. 4c). Thus, all three compounds after 2 hours incubation able to effectively increase expression of the autophagy genes in concentrations which they induced acidification of cytosol in the cells (>10 mM).

More prolonged incubation (24 hours) of the neuroblastoma cells with lactic acid, sodium lactate and sodium pyruvate also significantly increased level of mRNA of studied genes. We have observed an increase in *p62, Lc3, Beclin1, Ndp52, Bnip3, Mfn1, Nix and Pink1* (for 5–20 mM lactic acid; Fig. 4a), *LC3, Beclin1, NDP52, Bnip3, Nix, Pink1* (for 10–50 mM sodium pyruvate, Fig. 4b), *Beclin1, Optn, Nix, Pink1* (10–50 mM sodium lactate, Fig. 4c, dashed line shows control value of the gene expression. N = 3–4 independent experiments. Comparison with control; * $p < 0.05$, ** $p < 0.01$). It should be noted that 24 hours incubation

of the cells with 1 μM FCCP or 1 μM nigericin induced increased expression of the most of the autophagic genes except *Mfn1* and *Parkin* (Fig. 4d).

The effect of 24 hours incubation lactate and pyruvate on autophagy/mitophagy was confirmed by Western blot analysis of SQSTM1/p62, LC3B, BNIP3L (Nix), PINK1 proteins. We have found that lactate and pyruvate increase expression of LC3B, BNIP3L (Nix), PINK1 compare to control. (Fig. 4e, f).

Thus, incubation of the cells with sodium lactate or lactate acid and sodium pyruvate induces autophagy and mitophagy. However, mitophagy can be induced through Pink1/Parkin-dependent and -independent way (Nix). Considering low toxicity of sodium lactate and sodium pyruvate compound to cells we have tested the effect of lactate and pyruvate on activation of autophagy and mitophagy in toxic (MPP+) model of Parkinson's disease.

Lactate and pyruvate induce mitophagy in MPP+ cell model of Parkinson's disease

Toxic cellular model of Parkinson's disease was induced by 24 hours incubation of SH-SY5Y cells or primary co-culture of neurons and astrocytes with 0.5 mM MPP+ followed by 24 hours incubation with lactate or pyruvate then mitophagy was assessed by colocalization of mitochondria (MitoTracker Green) and lysosomes (LysoTracker Red). We have found that MPP+ significantly increased percentage of mitochondria colocalized with lysosomes in SH-SY5Y cells and primary neurons and astrocytes (Fig. 5a, b, c). Importantly, 10 mM sodium lactate and 10 mM sodium pyruvate induced further increase in mitophagy in these cells (Fig. 5 a, b, c).

The effect of the lactate and pyruvate on mitophagy was confirmed by measurement of expression of the genes involved in autophagy/mitophagy using the same toxic model (0.5 mM MPP+ for 24 hours followed by 24 hours incubation with 10 mM lactate and 10 mM

pyruvate). Sodium pyruvate significantly increased expression of *Lc3, Beclin1, Bnip3, Ndp52, Parkin* in SH-SY5Y cells. Incubation of the MPP+ treated cells with 10 mM sodium lactate significantly increased *Lc3, Beclin1, Optn, Ndp52, Bnip3, Nix, Pink1* (Fig. 5d). It should be noted that 48 hours incubation of the cells with 0.5 mM MPP+ significantly increased expression of *Beclin1* and *Optn*.

Sodium lactate and sodium pyruvate increased the level of mRNA of genes *p62, Lc3, Optn* in primary neurons and astrocytes. Both lactate and pyruvate also increased mRNA of *Pink1* и *Parkin* (Fig. 5e).

The effect of lactate and pyruvate on expression of autophagic/mitophagic proteins in MPP+ cellular model of Parkinson's disease was confirmed with Western blot experiments (Fig. 4e, f). Thus, 10 mM sodium pyruvate significantly increased LC3B and PINK1 proteins while 10 mM lactate increased LC3B, BNIP3L (Nix) and PINK1 proteins (Fig. 4e, f).

Lactate and pyruvate protect cells against MPP+ toxicity

48 Hours incubation of SH-SY5Y cells or primary co-culture of neurons and astrocytes with MPP+ induced significant increase in percentage of Prodium Iodide positive (necrotic) cells (Fig. 6a, b, c). Application of 10 mM sodium lactate or sodium pyruvate after 24 hours of MPP+ incubation significantly reduced number of dead cells (Fig. 6b, c) while higher concentrations of lactate and pyruvate (50 mM) had no protective effect on the primary neurons and astrocytes against MPP+-induced toxicity (Fig. 6c). Interestingly, incubation of SH-SY5Y cells but not primary co-culture of neurons and astrocytes with 1 μM FCCP also induced protection against MPP+ cells death (Fig. 6b, c).

Incubation of the cells with 10 mM lactate or 10 mM pyruvate for 24 hours significantly reduced expression of the pro-apoptotic proteins induced by MPP+ (Fig. 6d, e). Thus, incubation of SH-SY5Y cells with 10 mM sodium lactate reduced the level of *caspase 8* and

Bax increased by MPP+ (Fig. 6d), lactate was also reduced the level of expression of *Bax* and *Fas* in primary neurons and astrocytes (Fig. 6e). Incubation of cells with 10 mM sodium pyruvate also significantly reduced expression of *caspase 8* and *Bax* in SH-SY5Y cells and *Bax* and *Fas* in primary co-culture of neurons and astrocytes (Fig. 6d, e). Thus, lactate or pyruvate induced mitophagy/autophagy protect cells against MPP+-induced necrosis and apoptosis.

Lactate and pyruvate recover mitochondrial membrane potential in MPP+ treated cells

Incubation of the SH-SY5Y neuroblastoma cells or primary co-culture of neurons and astrocytes for 24 hours led to almost complete mitochondrial depolarisation in primary neurons and astrocytes and significantly reduced mitochondrial membrane potential in neuroblastoma cells (Fig. 6f, g). 24 hours incubation of these cells with 10 mM lactate or 10 mM pyruvate (after first 24 hours with MPP+) significantly recover mitochondrial membrane potential in both neuroblastoma and primary neurons and astrocytes (Fig. 6f, g).

DISCUSSION

Although mechanisms of mitophagy and autophagy are well studied in detail, much less known about regulation of this processes in physiology and pathology. One of the most important finding here that two compounds, lactate and pyruvate, which are involved in cellular metabolism and shown to present endogenously in the cells induce autophagy and mitophagy via acidification of cytosol. It should be noted that changes in intracellular pH shown in cell starvation [22] and ischemia [23], and importantly these processes also shown to activate mitophagy [24]. Previously we shown that mitochondrial depolarization is not essential inductor of mitophagy [16]. Here we demonstrate that compounds, which can act as

mitochondrial substrates could prevent MPP+-induced mitochondrial depolarization and stimulate activation of mitophagy which is comparable to level of mitophagy induced by mitochondrial uncoupler 10 μM FCCP.

Importantly, acidification of cytosol with lactate or pyruvate led to complex activation of mitophagy.

Currently, two mitophagy pathways are distinguished: Pink1/Parkin-dependent and Pink1/Parkin-independent (ubiquitin-independent). In the first case mitophagy is triggered by the accumulation of the Pink1 and the attraction of Parkin to the mitochondrial membranes for further ubiquitination of proteins. In the second case, mitophagy can be triggered through receptors such as Nix, Bnip3L, Fundc1, etc., resulting in hypoxia, oxidative stress, and cell reprogramming [7]. Due to the fact that we saw in our experiments an increase in the expression of mRNA of *Pink1*, *Parkin*, *Beclin1* as well as the *Nix* and *Bnip3* when cells were treated with sodium lactate and pyruvate, including in a toxic model of Parkinson's disease, we assume that mitophagy can be triggered not only Pink1/Parkin-dependent pathway, but also through ubiquitin-independent pathways, for example, as a result of oxidative stress in Parkinson's disease. Until the end it remains unclear whether these paths can intersect and occur in parallel.

Concentration of the lactate in the body can be increased dramatically during physical exercises and these concentrations are comparable to that activated autophagy and mitophagy in our experiments [25,26]. Considering this, intensive exercises can induce autophagy and mitophagy through lactate-induced mechanism. Exercise-induced increase in plasma lactate promotes the brain takes up lactate in proportion to the arterial concentration [27]. It proves importance of the physical activity for health in general and for brain cells survival.

Pyruvate shown to be protective for various models of diseases including epilepsy and Parkinson's disease [28,29]. Although this effect is mostly can be explained by the effect of

pyruvate as additional mitochondrial complex I substrate effect of this compound on mitophagy also may have an implication. One of the pathological feature of the number of neurological disorders including Parkinson’s disease is deficiency of mitochondrial complex I [30]. Our results here are strongly indicate that pyruvate may be protective even in cells with complex I deficiency.

Lactate and pyruvate shown to be cell protective against oxidative stress through activation of Nrf2 and unfolded proteins response [31]. Mitophagy shown to be also dependent on Nrf2 [32] that can suggest possible interaction of cell acidification, Nrf2 activation and mitophagy/autophagy.

Based on the current data, we can conclude that lactate, pyruvate and possibly other agents capable of short-term cytosolic acidification, can be considered as promising pharmacological compounds for the correction of conditions such as aging, Parkinson's disease and a number of other disorders associated with mitophagy/autophagy dysfunction.

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Figure legends

Fig. 1 The effect of lactate and pyruvate on cytosolic pH and cell survival of cells. **a.** The effect of sodium lactate (SodLact), sodium pyruvate (SodPyr), lactic acid (Lact) and FCCP on intracellular pH (pH_c) in SH-SY5Y neuroblastoma cells measured using SNARF-1. Calibration was carried out in accordance with the manufacturer's instructions. The addition of 10 μM FCCP (black curve), 50 mM SodLact (blue curve), 50 mM SodPyr (pink curve), 20 mM Lact (red curve1) reduced $[pH]_c$. Each curve is the average of three independent experiments, in each the change in fluorescence in at least 80 cells was estimated. **b.** The effect of FCCP, Nigericin, SodPyr, SodLact and Lact on cell viability and on the density of SH-SY5Y neuroblastoma cells after 24 hours of exposure. Dead cells were detected by labelling with Prodium Iodide for 24 hours after addition of SodPyr, SodLact, Lact, FCCP or Nigericin. The ordinate shows the percentage of non-viable cells, the number in the column indicates the cell cultrure density (cells/mm^2). Control – untreated cells (N = 4 experiments; * $p < 0.05$, ** $p < 0.01$). **c.** The level of mRNA expression of apoptotic marker genes after 2 or 24 hours of exposure to Lact, SodPyr, SodLact, FCCP, Nigericin. The results are presented relative to the *Gapdh* reference gene and control (untreated cells). Dashed line shows control value of the gene expression. N = 3–4 independent experiments. Comparison with control, * $p < 0.05$, ** $p < 0.01$.

Fig. 2 The effect of pyruvate and lactate on mitochondrial membrane potential and mitochondrial NADH level. **a.** The effect of 2 and 24 hours incubation of SH-SY5Y neuroblastoma cells with sodium lactate (SodLact), sodium pyruvate (SodPyr), lactic acid (Lact) on mitochondrial NADH autofluorescence. Control – untreated cells. Data are presented as % of control ± standard deviation, the number in the column indicates N (number

of measured cells in 3 independent experiments). **b.** The effect of 30 min or 24 hour incubation of SH-SY5Y neuroblastoma cells with SodLact, SodPyr on Rhodamine 123 (Rh123) fluorescence. An increase in Rhodamine 123 fluorescence indicates mitochondrial depolarization induced by 1 μM FCCP. Each curve is the average of three independent experiments, in each the change in fluorescence in at least 80 cells was estimated.

Fig. 3 The effect of lactate and pyruvate on autophagy/mitophagy. **a.** Representative confocal images of SH-SY5Y neuroblastoma cells co-loaded with MitoTracker Green (green) and LysoTracker Red (red) after treatment with 20 mM sodium lactate (SodLact) for 2 hours and 24 hours. Control – untreated cells. The regions of colocalization of mitochondria and lysosomes become yellow. Scale bar – 10 μm. **b.** Percentage of mitochondria colocalized with lysosomes after 2 and 24 hours exposure of SH-SY5Y neuroblastoma cells to various concentrations of SodPyr, SodLact, Lact and FCCP. Control – untreated cells (N = 3 experiment; * $p < 0.05$, ** $p < 0.01$). **c.** Percentage of mitochondria colocalized with lysosomes after 24 hours exposure of primary cortical neuroglial culture to various doses of SodPyr, SodLact, Lact and FCCP, Nigericin. Control – untreated cells (N = 3 experiment; * $p < 0.05$, ** $p < 0.01$).

Fig. 4. The effect of lactate and pyruvate on the level of mRNA and protein expression of mitophagy/autophagy marker genes. **a.** The level of mRNA expression of genes at 2 or 24 hour exposure of SH-SY5Y neuroblastoma cells to lactic acid. The results are presented relative to the *Gapdh* reference gene and control (untreated cells). N = 3–4 independent experiments. Comparison with control; * $p < 0.05$, ** $p < 0.01$. **b.** The level of mRNA expression of genes at 2 or 24 hour exposure of SH-SY5Y cells to sodium pyruvate. The results are presented relative to the *Gapdh* reference gene and control (untreated cells). N = 3–

4 independent experiments. Comparison with control; * $p < 0.05$, ** $p < 0.01$. **c.** The level of mRNA expression of genes at 2 or 24 hour exposure of SH-SY5Y cells to sodium lactate. The results are presented relative to the *Gapdh* reference gene and control (untreated cells). N = 3–4 independent experiments. Comparison with control; * $p < 0.05$, ** $p < 0.01$. **d.** The level of mRNA expression of genes 24 hour incubation of SH-SY5Y cells to FCCP, Nigericin. The results are presented relative to the *Gapdh* reference gene and control (untreated cells). Dashed line shows control value of the gene expression. N = 3–4 independent experiments. Comparison with control; * $p < 0.05$, ** $p < 0.01$. **e.** The effect of 24 hour incubation of 10mM sodium lactate (SodLact), 10 mM sodium pyruvate (SodPyr), 1 μM FCCP, 500 μM MPP+, 10 mM sodium pyruvate against MPP+ (MPP+ SodLact) and 10mM sodium pyruvate against MPP+ (MPP+ SodPyr) on protein expression in SH-SY5Y neuroblastoma cells. Control – untreated cells. **f.** Quantitative analysis of **e**. Control – 100%; * $p < 0.05$, ** $p < 0.01$.

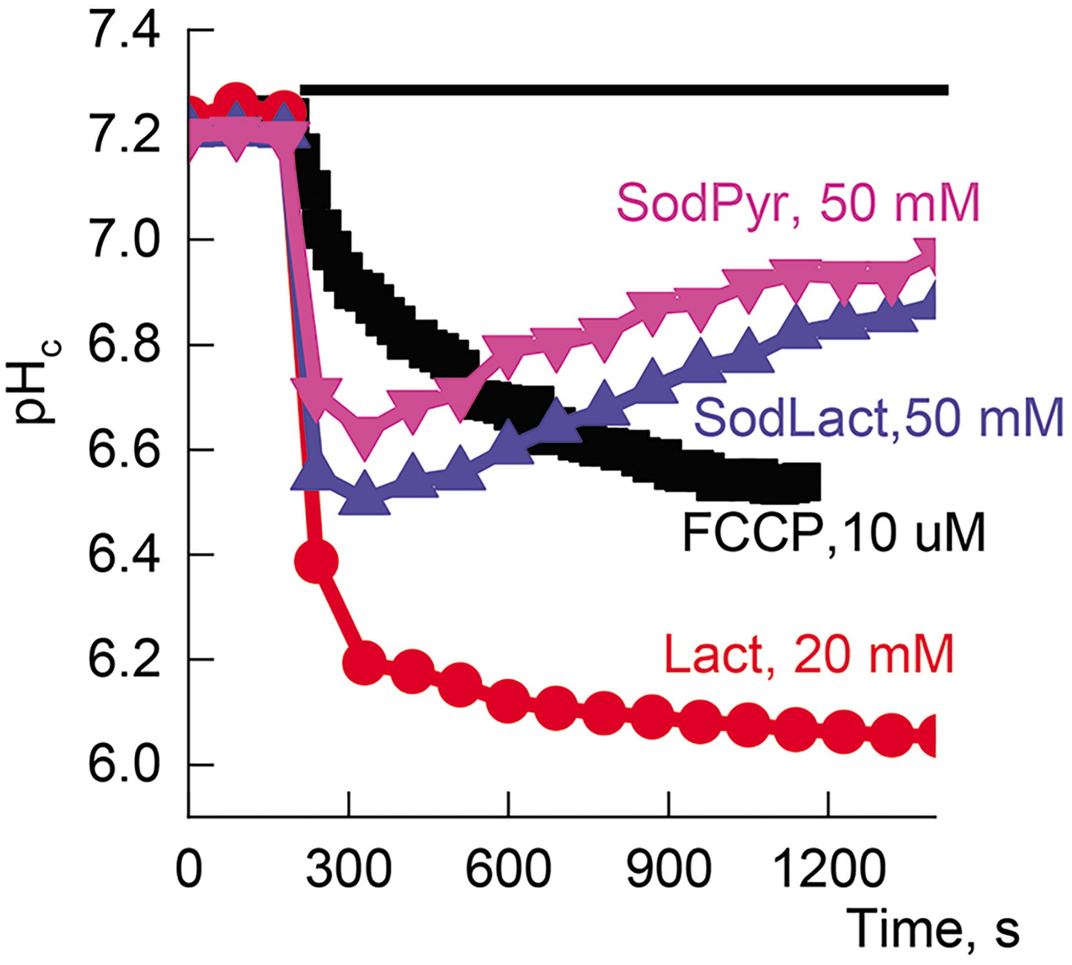
Fig. 5 The effect of sodium pyruvate and sodium lactate on autophagy/mitophagy in MPP+ treated cells. Experiment was carried out according to the following scheme: MPP+ was added to the cell culture 48 hours before measurements, 24 hours after the addition of MPP+ SodLact or SodPyr was added to some cultures. **a.** Representative confocal images of cells co-loaded with MitoTracker Green (green) and LysoTracker Red (red) in cells after MPP+ treatment (48 hours) and 24 hours SodLact or SodPyr incubation. Control – untreated cells. Scale bar – 10 μm. **b.** Percentage of mitochondria colocalized with lysosomes after 48 hours exposure of SH-SY5Y neuroblastoma cells to 500 μM MPP+ (N = 3 experiments), 10 mM SodLact against MPP+ (MPP+/SodLact, N = 3 experiments) and 10 mM SodPyr against MPP+ (MPP+/SodPyr, N = 3 experiments). Control – untreated cells; * $p < 0.05$, ** $p < 0.01$. **c.** Percentage of mitochondria colocalized with lysosomes after 48 hours exposure of primary

cortical neuroglial culture to 500 μM MPP+ (N = 3 experiments), 10 mM SodLact against MPP+ (MPP+/SodLact, N = 3 experiments) and 10 mM SodPyr against MPP+ (MPP+/SodPyr, N = 3 experiments). Control – untreated cells; * $p < 0.05$, ** $p < 0.01$. **d, e.** The effect of sodium pyruvate, sodium lactate on mRNA level in SH-SY5Y neuroblastoma cells (**d**) and primary cortical neuroglial culture (**e**). The results are presented relative to the *Gapdh* reference gene and control (untreated cells). Dashed line shows control value of the gene expression. N = 3 independent experiments; * $p < 0.05$, ** $p < 0.01$.

Fig. 6 The effect of sodium pyruvate and sodium lactate on MPP+-induced cell toxicity. The experiment was carried out according to the scheme as in Figure 5: MPP+ was added to the cell culture 48 hours before measurements, 24 hours after the addition of MPP+, sodium lactate or sodium pyruvate was added. **a.** Representative images of SH-SY5Y neuroblastoma cells stained with Hoechst 33342 (blue) and Propidium Iodide (Red) in control conditions, after 48 hours action of 500 μM MPP+ and when 10 mM sodium lactate (SodLact) was added according the experimental protocol. Scale bar – 100 μm. **b, c.** Percentage of viable SH-SY5Y neuroblastoma cells (**b**) and cells in primary cortical neuroglial culture (**c**) in the control (untreated cells) and after 48 hours of 500 μM MPP+, 10 mM SodLact against MPP+ (MPP+/SodLact) or 10 mM SodPyr against (MPP+/SodPyr). N = 3 experiments; * $p < 0.05$, ** $p < 0.01$. **d, e.** The level of mRNA expression after 48 hours exposure to 500 μM MPP+, when exposed to 10 mM SodLact together with MPP+ (MPP+/SodLact) and 10 mM SodPyr together with MPP+ (MPP+/SodPyr) in SH-SY5Y neuroblastoma cells (**d**), as well as under the action of SodLact or SodPyr, MPP+ and in primary cortical neuroglial culture (**e**). The results are presented relative to the *Gapdh* reference gene and control (untreated cells). Dashed line shows control value of the gene expression. N = 3 experiments; * $p < 0.05$, ** $p < 0.01$. **f, g.** The effect of sodium lactate and sodium pyruvate on FCCP-induced change in

Rhodamine 123 fluorescence in SH-SY5Y neuroblastoma cells (**f**) and in primary cortical neuroglial culture (**g**). The changes in Rhodamine 123 fluorescence response to 1 μM FCCP in control (untreated cells; black curve, N = 3 experiments); after 24 hours of exposure to MPP+ (green curve); after 48 hours of exposure to MPP+ (red curve), with the addition 10 mM sodium lactate for 24 hours after the application of MPP+. (MPP+/SodLact, blue curve) or 10 mM sodium pyruvate (MPP+/SodPyr, pink curve). Each curve is the average of three independent experiments, in each the change in fluorescence in at least 80 cells was estimated.

a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pH$_c$
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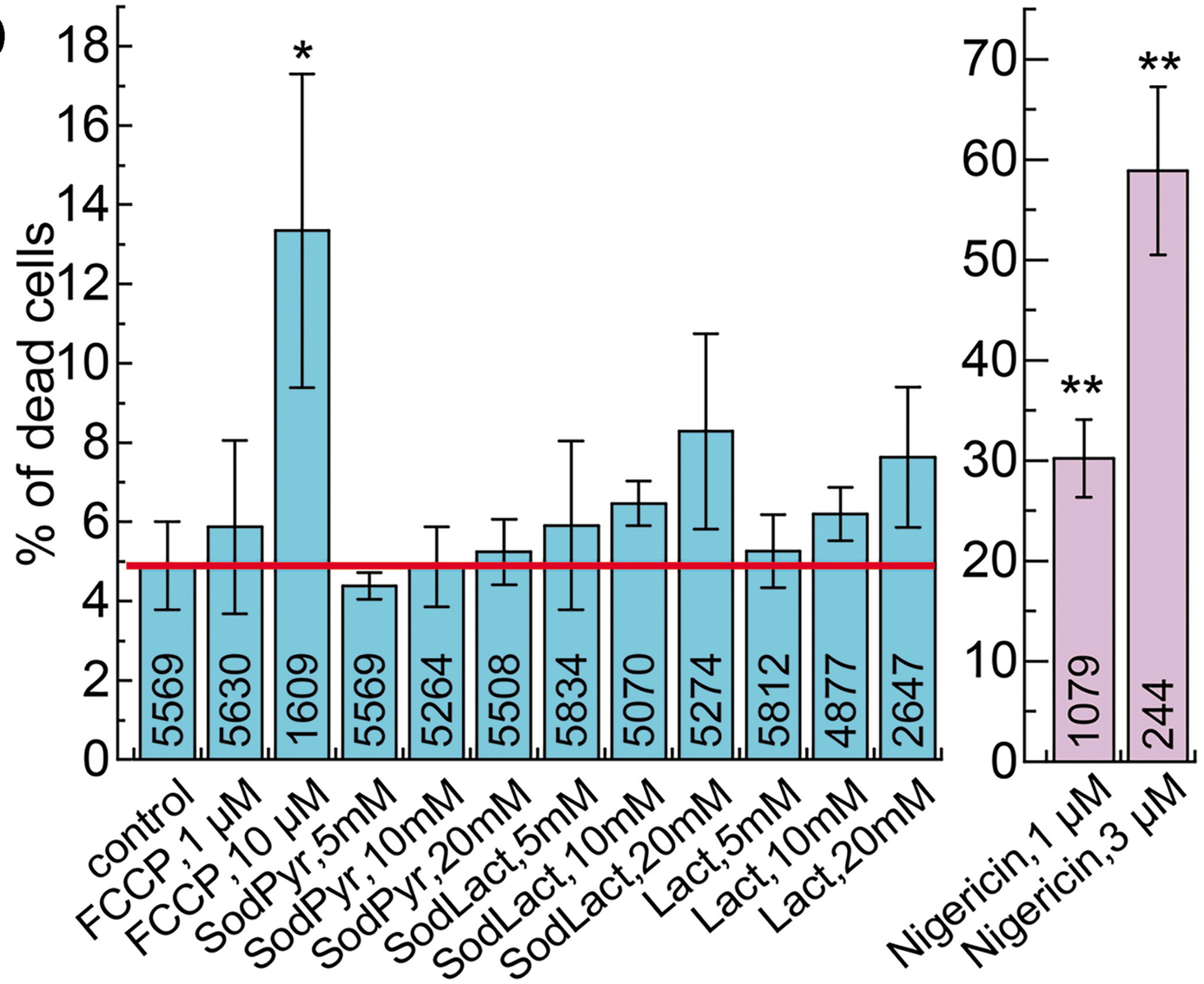
600
900
1200
Time, s
SodPyr, 50 mM
SodLact,50 mM
FCCP,10 uM
Lact, 20 mM

b


% of dead cells
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10
12
14
16
18
70
60
50
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20
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0
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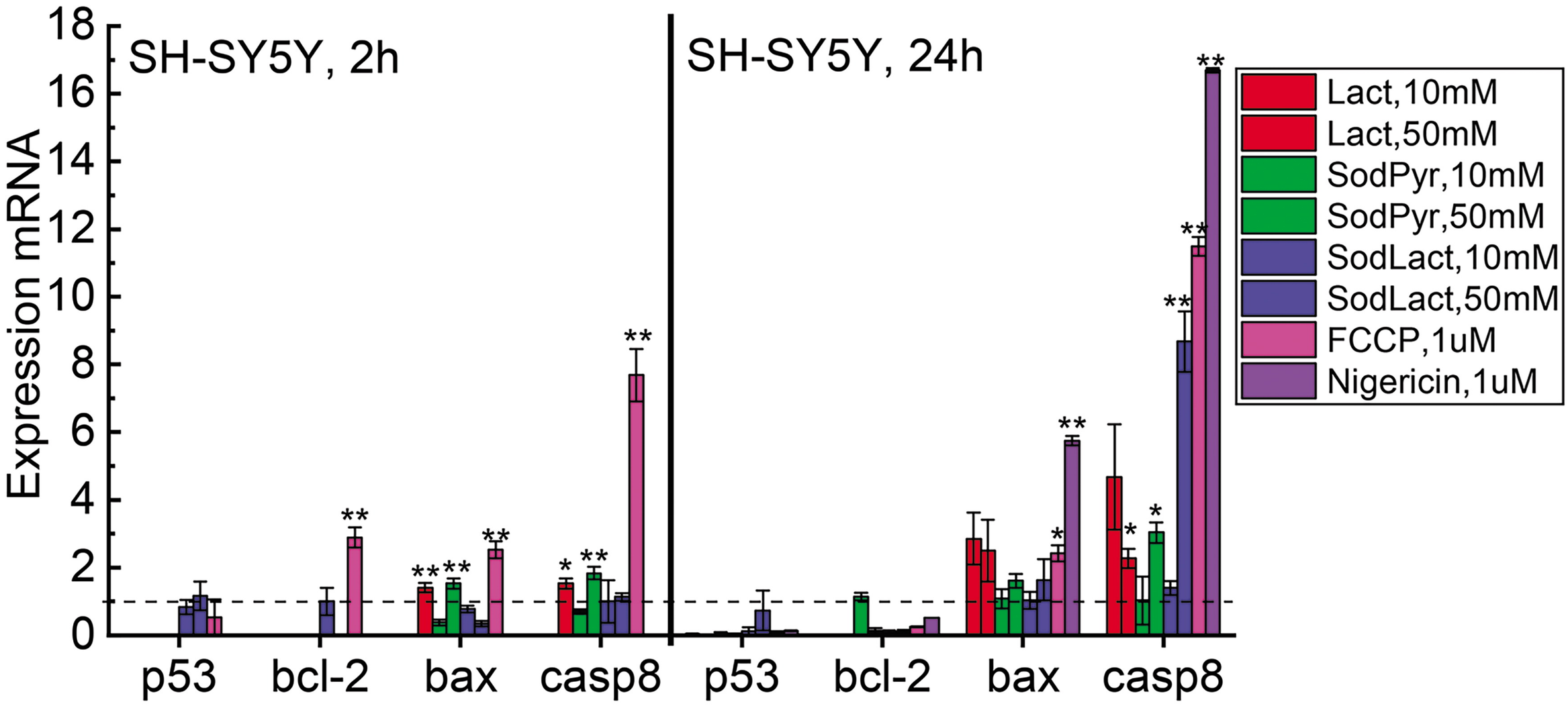
5630
1609
5569
5264
5508
5834
5070
5274
5812
4877
2647
1079
244
control
FCCP,1 μM
FCCP,10 μM
SodPyr,5mM
SodPyr,10mM
SodPyr,20mM
SodLact,5mM
SodLact,10mM
SodLact,20mM
Lact,5mM
Lact,10mM
Lact,20mM
Nigericin,1 μM
Nigericin,3 μM

c


Expression mRNA
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4
6
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14
16
18
SH-SY5Y, 2h
SH-SY5Y, 24h
p53
bcl-2
bax
casp8
p53
bcl-2
bax
casp8
Lact,10mM
Lact,50mM
SodPyr,10mM
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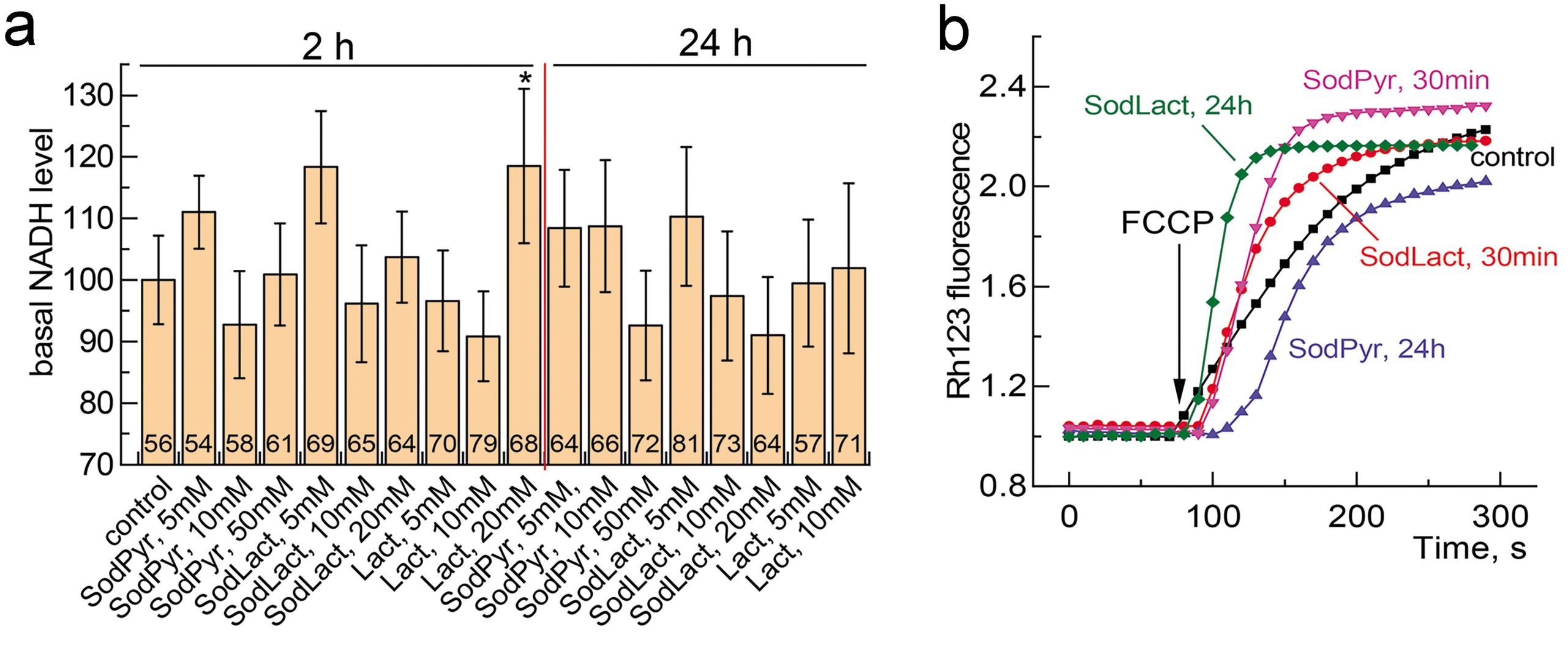
FCCP,1uM
Nigericin,1uM


a
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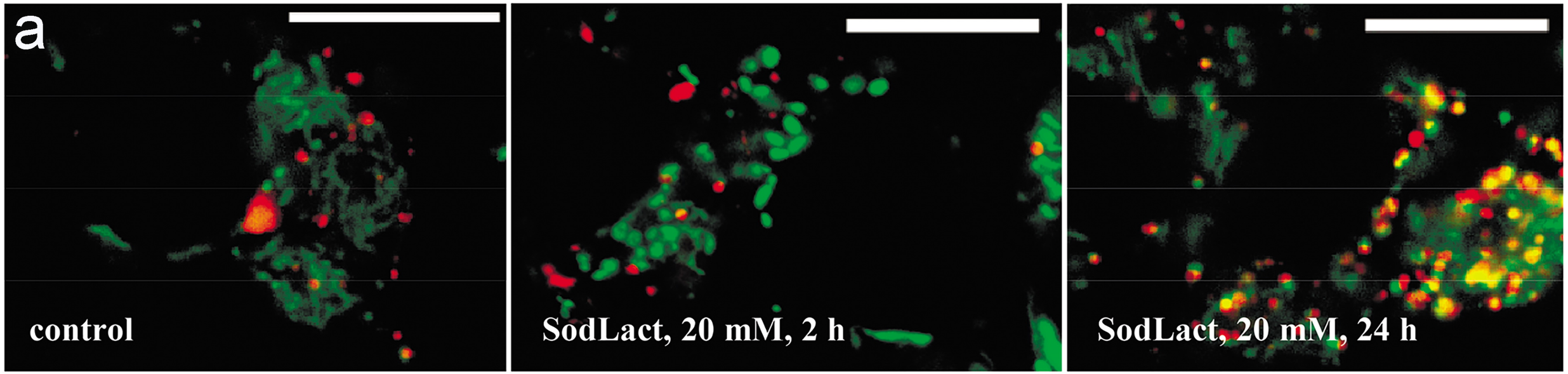
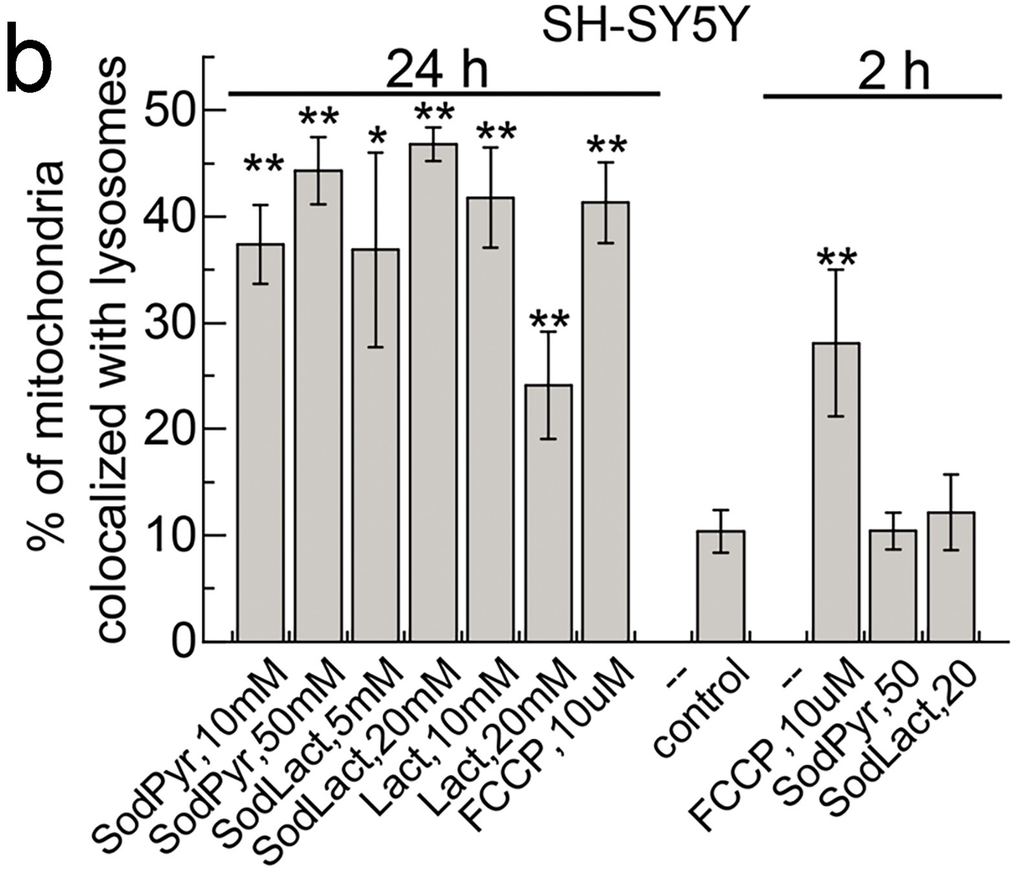
basal NADH level
130
120
110
100
90
80
70
*
56
54
58
61
69
65
64
70
79
68
64
66
72
81
73
64
57
71
control
SodPyr, 5mM
SodPyr, 10mM
SodPyr, 50mM
SodLact, 5mM
SodLact, 10mM
SodLact, 20mM
Lact, 5mM
Lact, 10mM
Lact, 20mM
SodPyr, 5mM,
SodPyr, 10mM
SodPyr, 50mM
SodLact, 5mM
SodLact, 10mM
SodLact, 20mM
Lact, 5mM
Lact, 10mM
b
Rh123 fluorescence
2.4
2.0
1.6
1.2
0.8
0
100
200
300
Time, s
SodLact, 24h
SodPyr, 30min
control
FCCP
SodLact, 30min
SodPyr, 24h

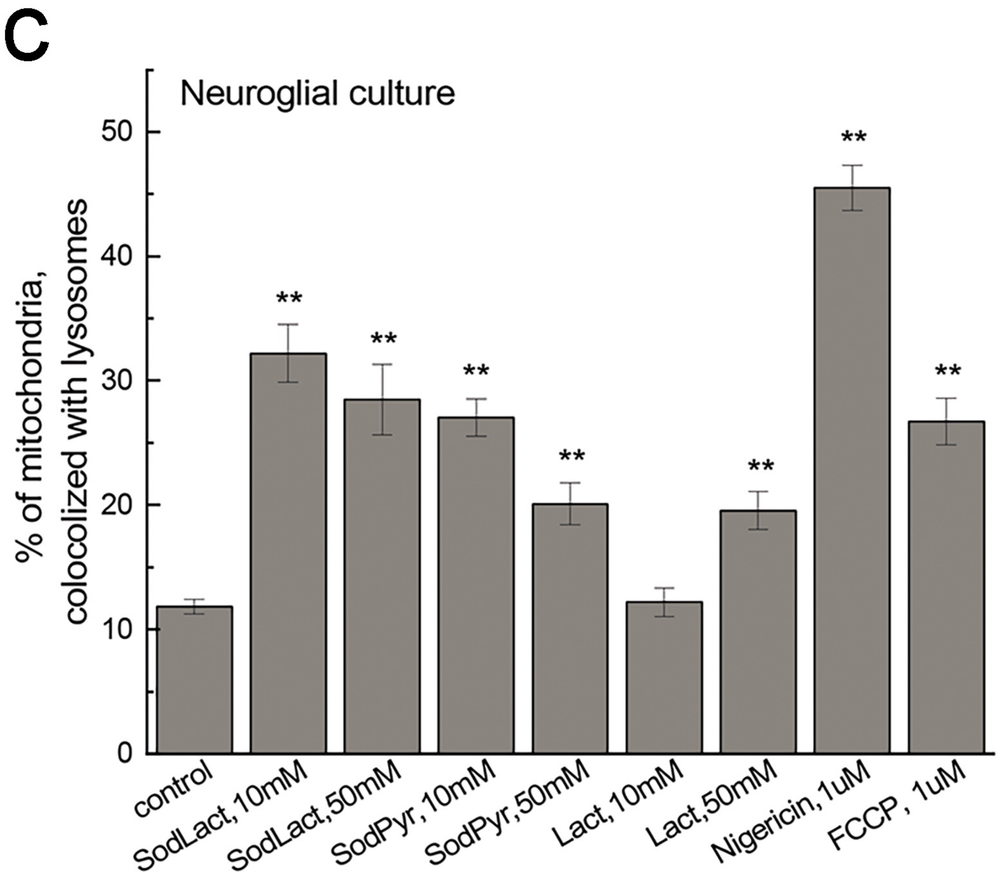
a


control
SodLact, 20 mM, 2 h
SodLact, 20 mM, 24 h

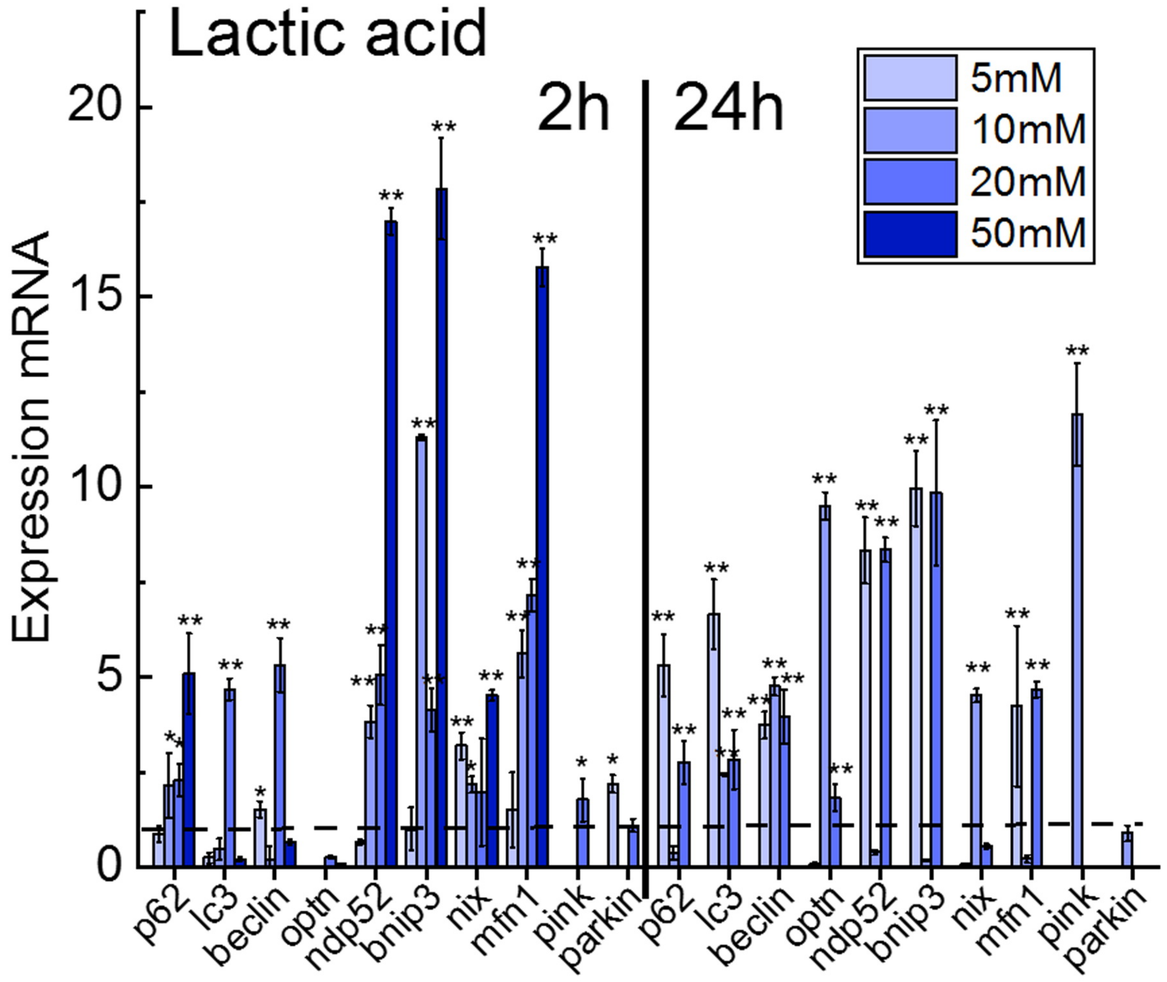
b


SH-SY5Y
24 h
2 h
% of mitochondria colocalized with lysosomes
0
10
20
30
40
50
SodPyr,10mM
SodPyr,50mM
SodLact,5mM
SodLact,20mM
Lact,10mM
Lact,20mM
FCCP,10uM
--
control
--
FCCP,10uM
SodPyr,50
SodLact,20

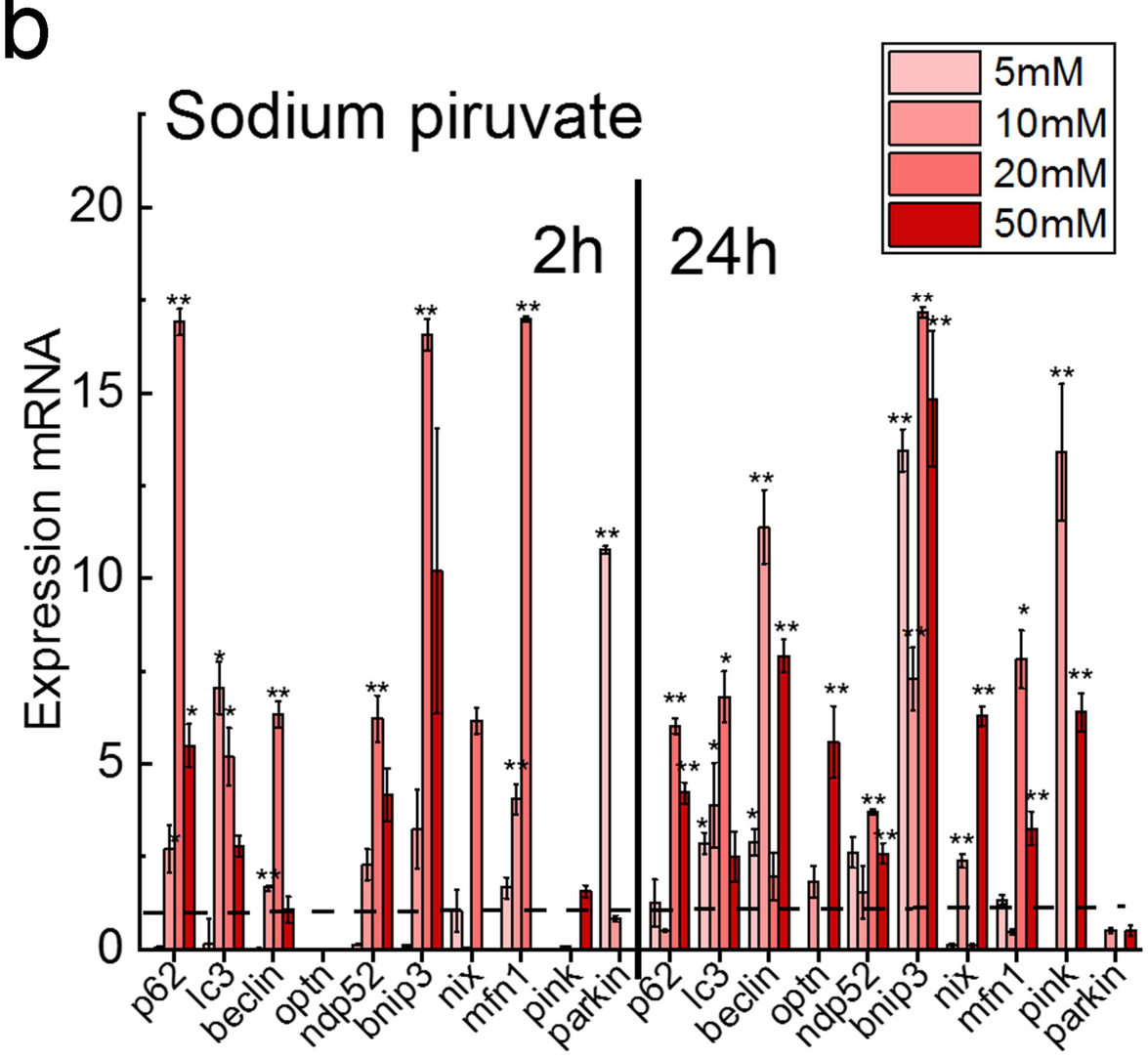
c


Neuroglial culture
% of mitochondria, colocolized with lysosomes
0
10
20
30
40
50
control
SodLact,10mM
SodLact,50mM
SodPyr,10mM
SodPyr,50mM
Lact,10mM
Lact,50mM
Nigericin,1uM
FCCP, 1uM

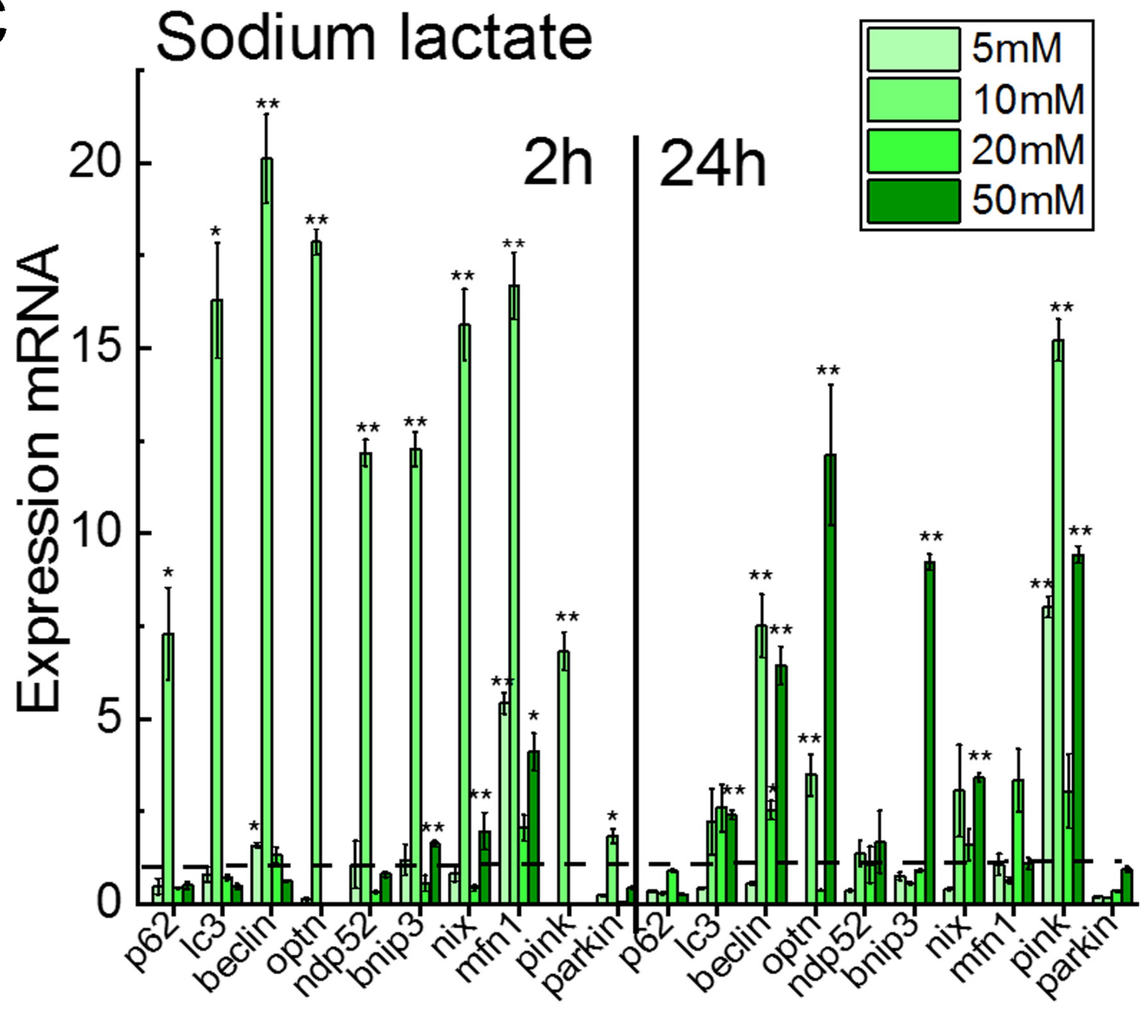
a


Lactic acid
2h
24h
5mM
10mM
20mM
50mM
Expression mRNA
0
5
10
15
20
p62
lc3
beclin
optn
ndp52
bnip3
nix
mfn1
pink
parkin

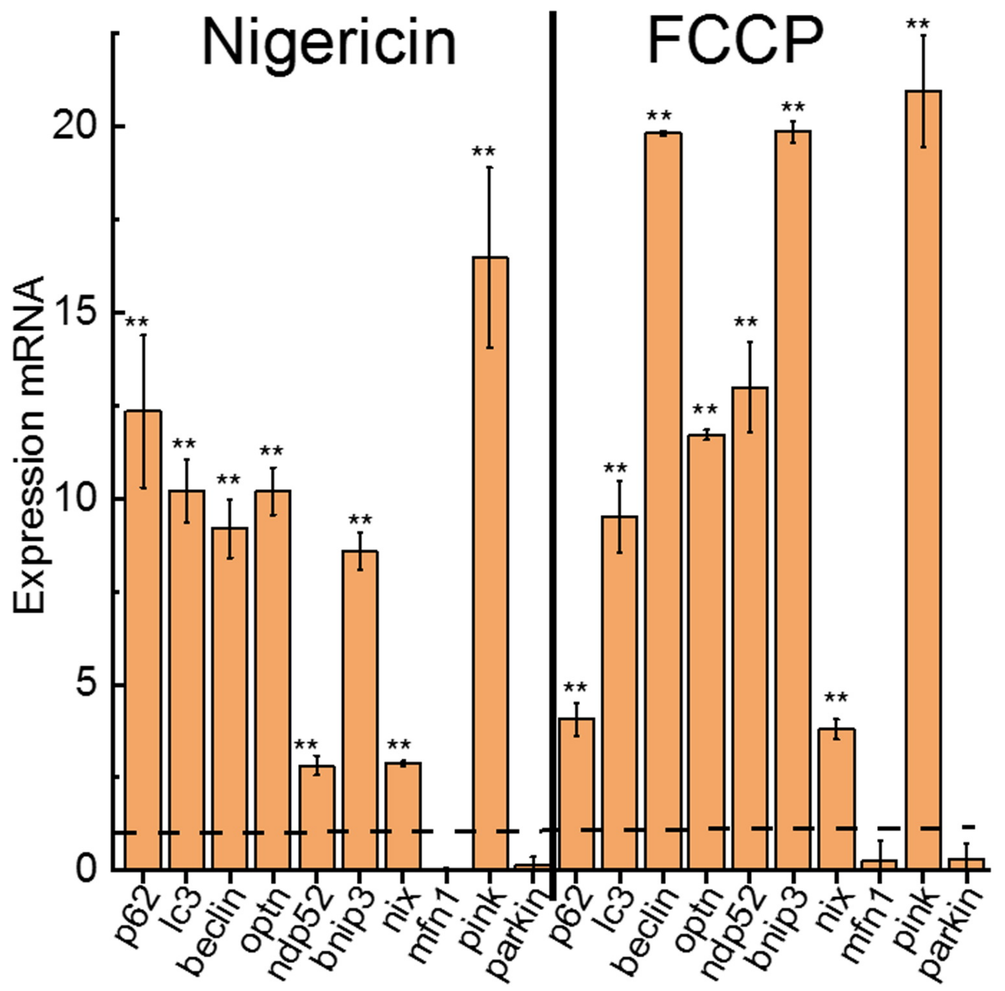
b


Sodium piruvate
2h
24h
5mM
10mM
20mM
50mM
Expression mRNA
0
5
10
15
20
p62
lc3
beclin
optn
ndp52
bnip3
nix
mfn1
pink
parkin

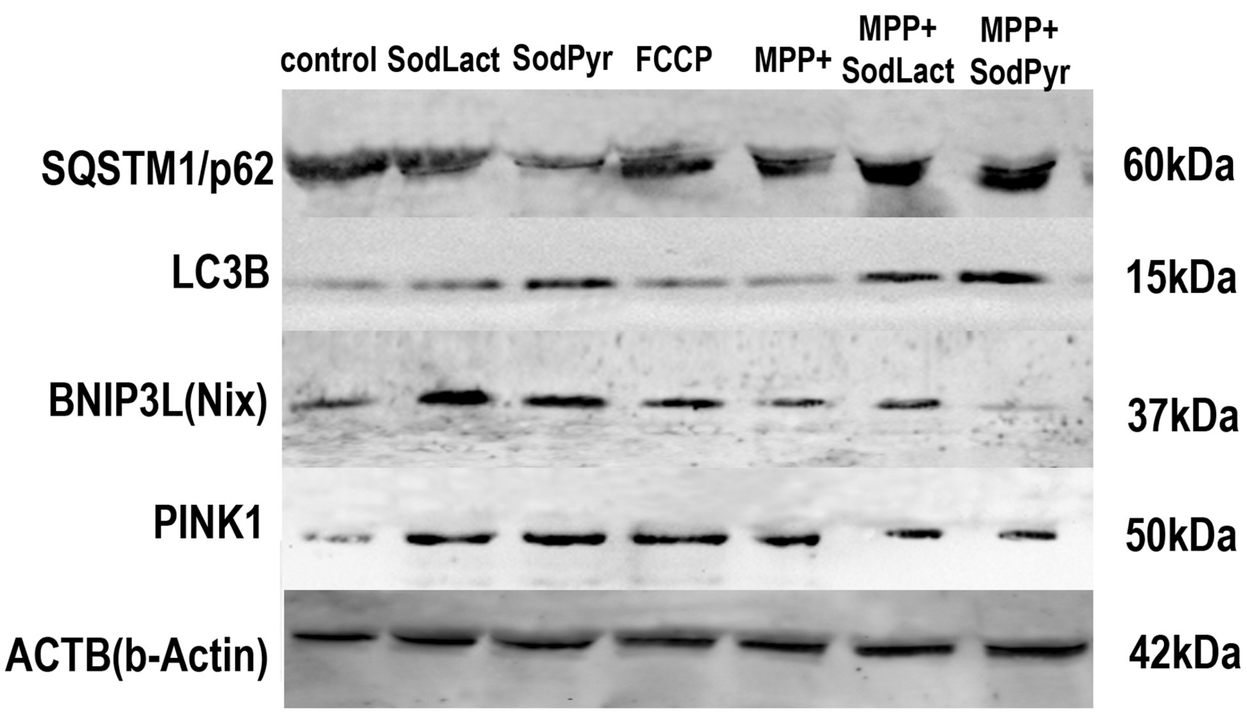
c


Sodium lactate
2h
24h
5mM
10mM
20mM
50mM
Expression mRNA
0
5
10
15
20
p62
lc3
beclin
optn
ndp52
bnip3
nix
mfn1
pink
parkin

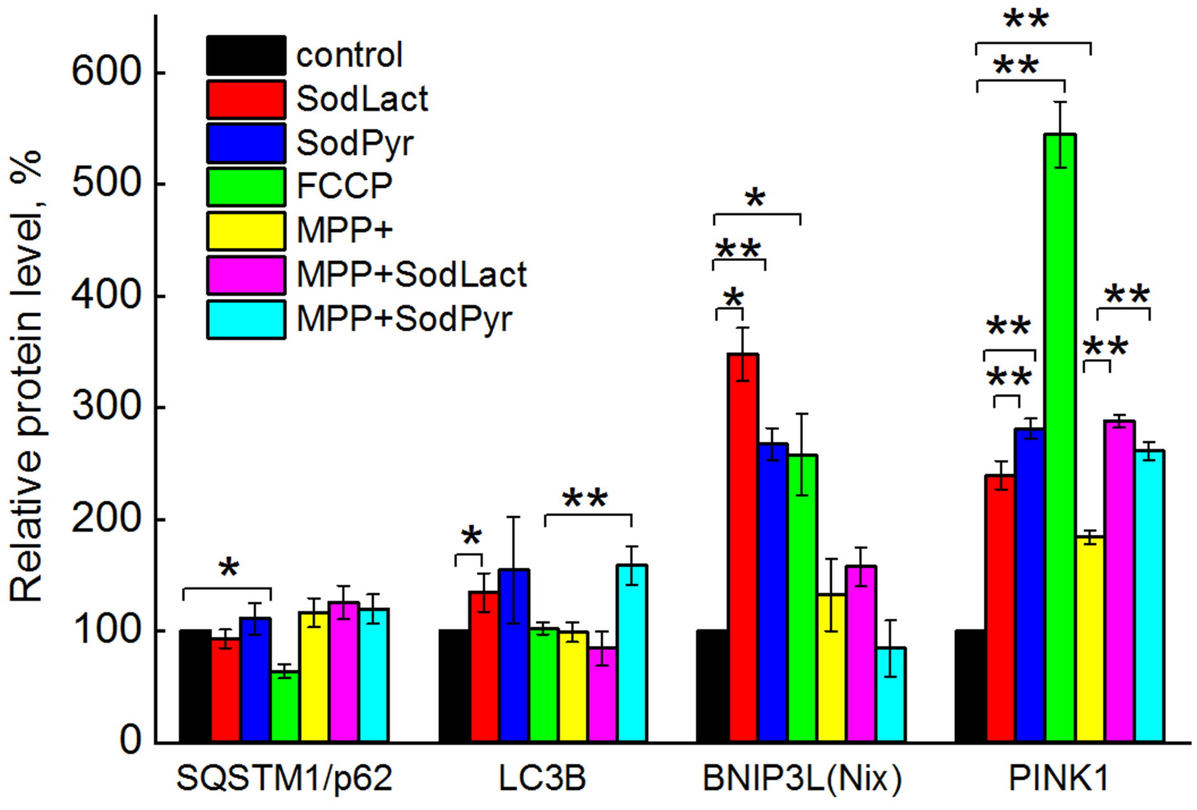
d


Nigericin
FCCP
Expression mRNA
0
5
10
15
20
p62
lc3
beclin
optn
ndp52
bnip3
nix
mfn1
pink
parkin

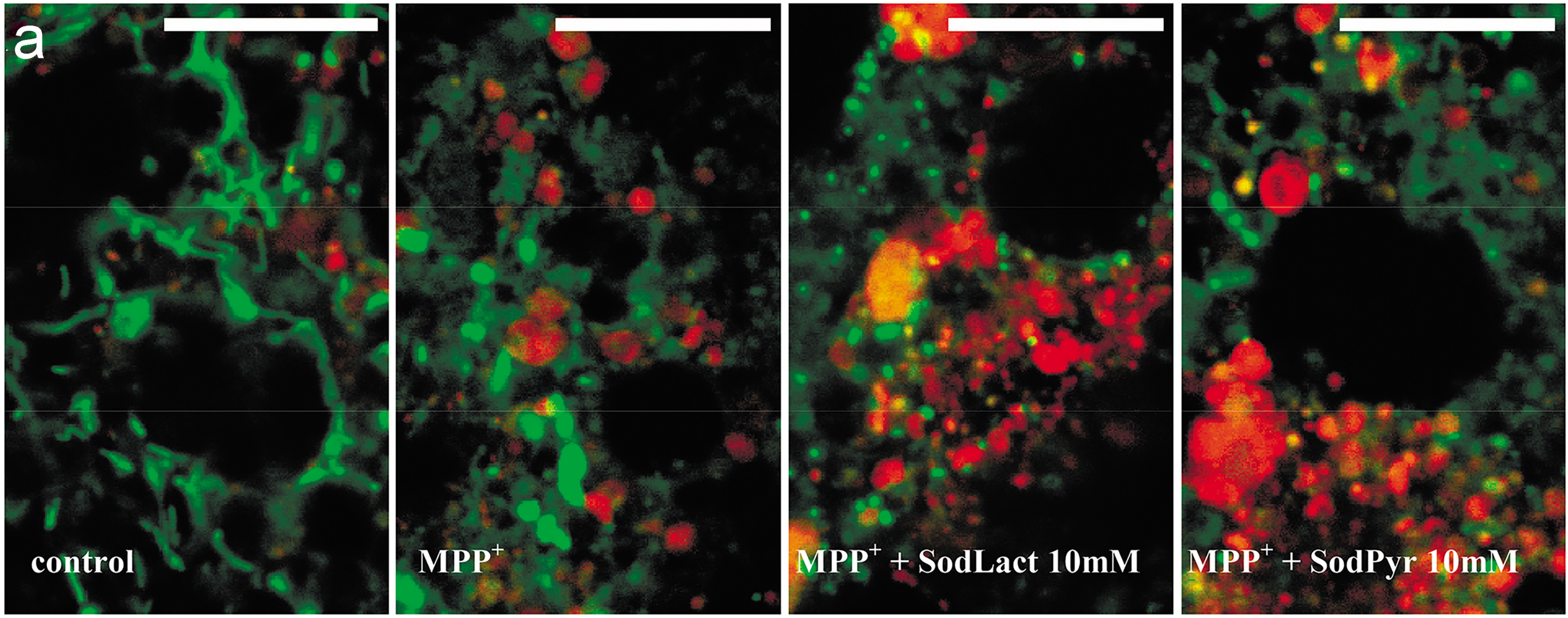
e


control
SodLact
SodPyr
FCCP
MPP+
MPP+ SodLact
MPP+ SodPyr
SQSTM1/p62
LC3B
BNIP3L(Nix)
PINK1
ACTB(b-Actin)
60kDa
15kDa
37kDa
50kDa
42kDa

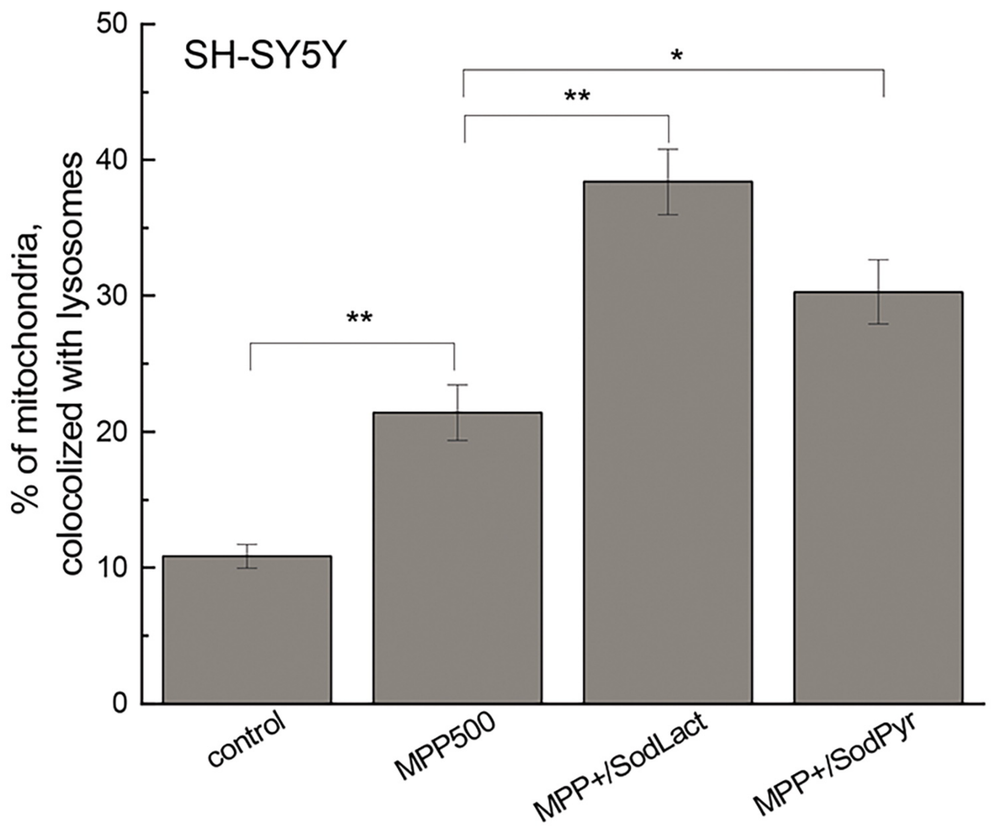
f


control
SodLact
SodPyr
FCCP
MPP+
MPP+SodLact
MPP+SodPyr
Relative protein level, %
0
100
200
300
400
500
600
SQSTM1/p62
LC3B
BNIP3L(Nix)
PINK1

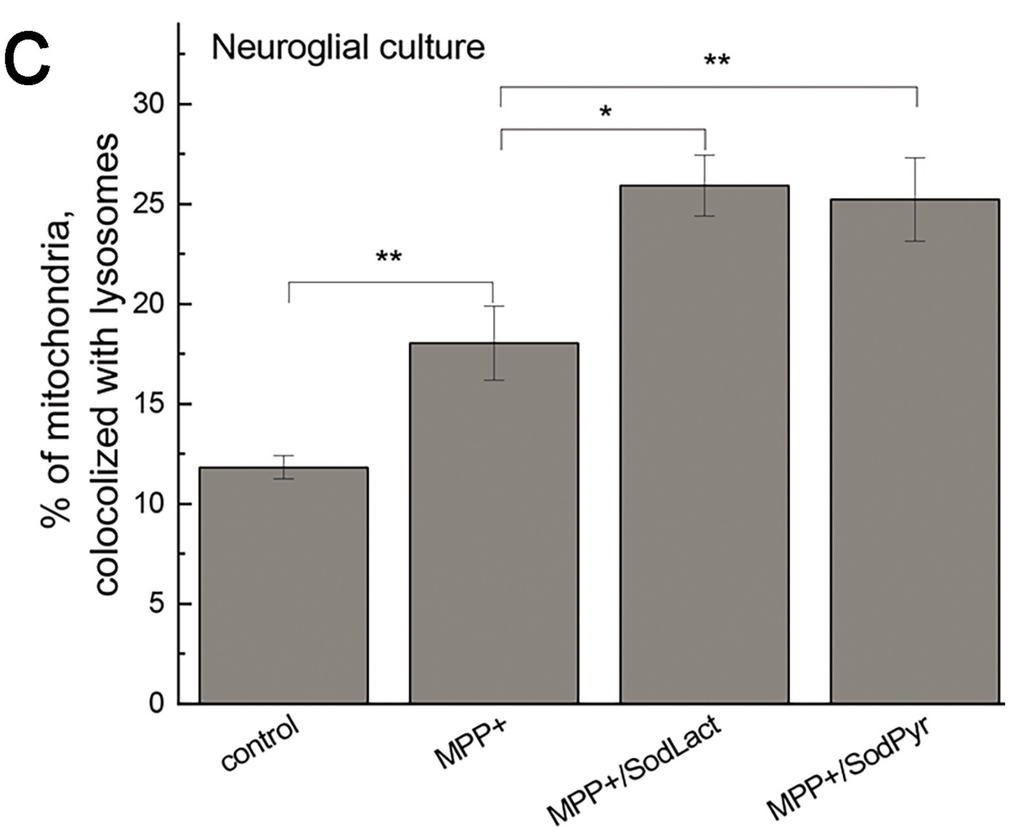
a


control
MPP+
MPP+ + SodLact 10mM
MPP+ + SodPyr 10mM

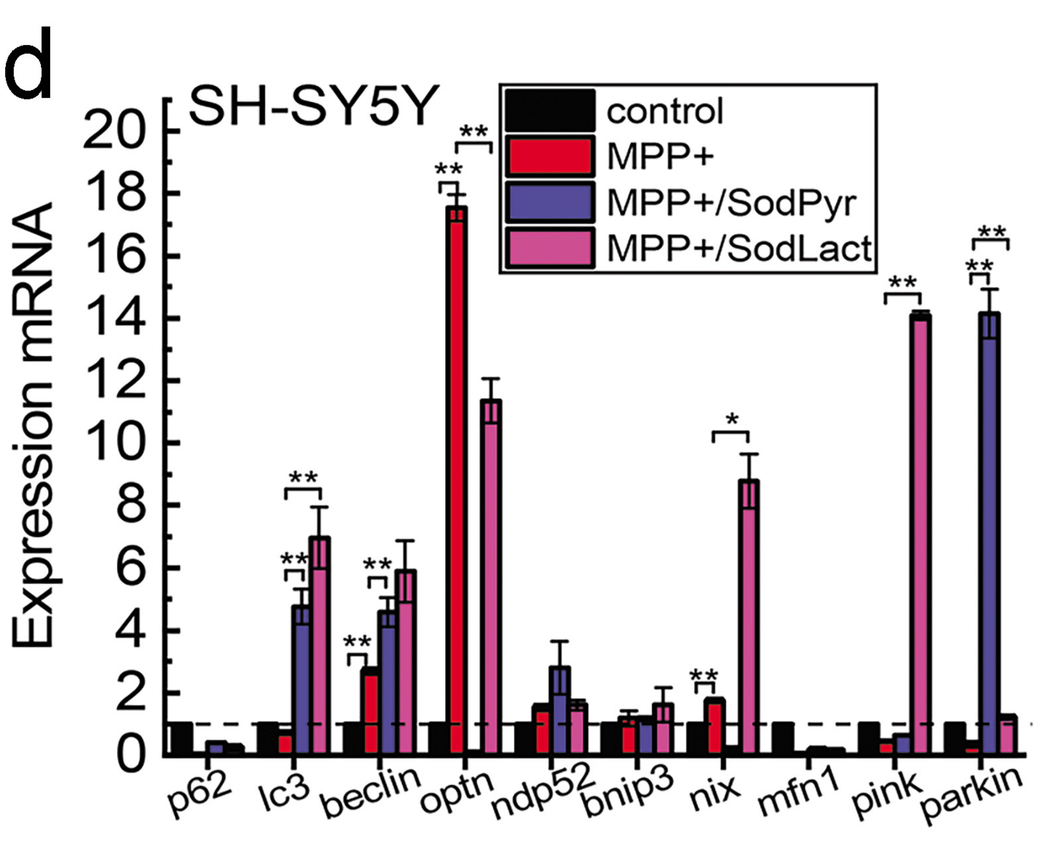
b


SH-SY5Y
% of mitochondria, colocolized with lysosomes
0
10
20
30
40
50
**
**
*
control
MPP500
MPP+/SodLact
MPP+/SodPyr

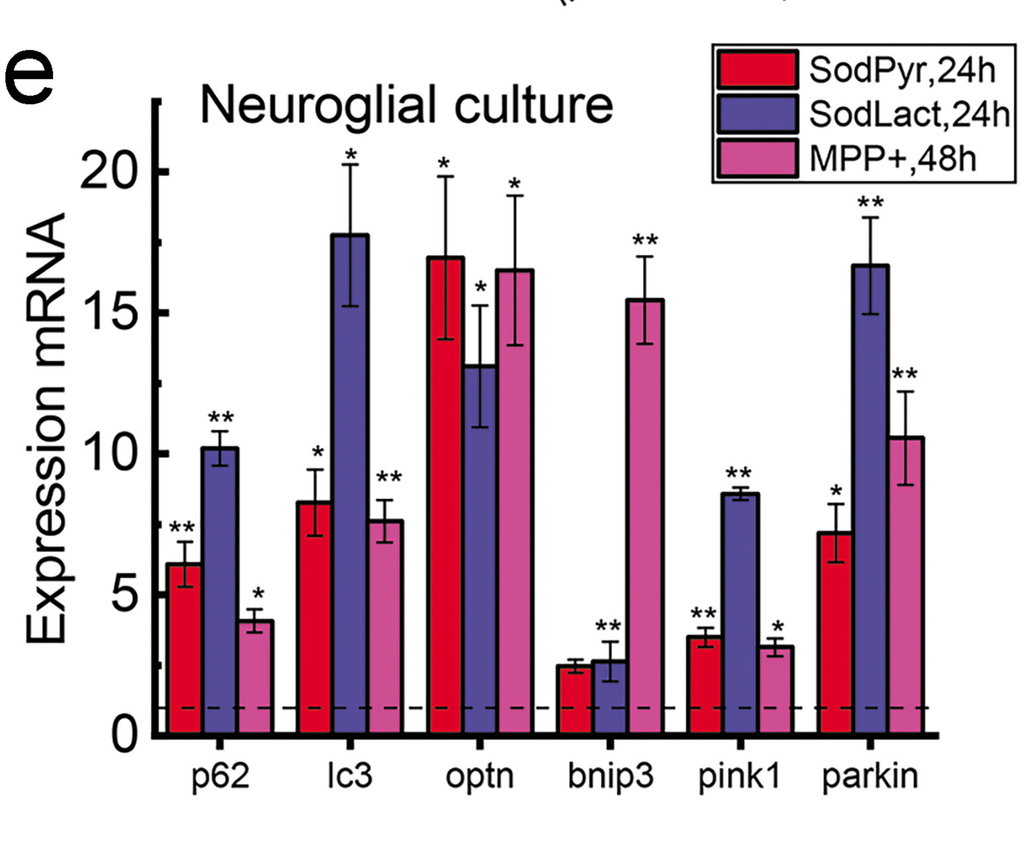
c


Neuroglial culture
% of mitochondria, colocolized with lysosomes
0
5
10
15
20
25
30
**
*
**
control
MPP+
MPP+/SodLact
MPP+/SodPyr

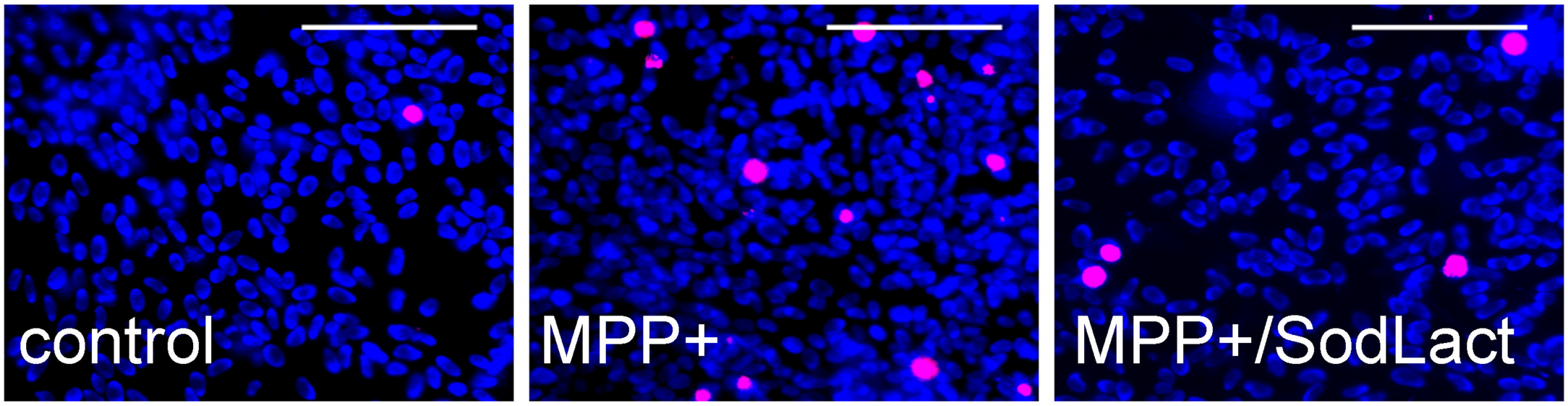
d


SH-SY5Y
Expression mRNA
0
2
4
6
8
10
12
14
16
18
20
control
MPP+
MPP+/SodPyr
MPP+/SodLact
p62
lc3
beclin
optn
ndp52
bnip3
nix
mfn1
pink
parkin

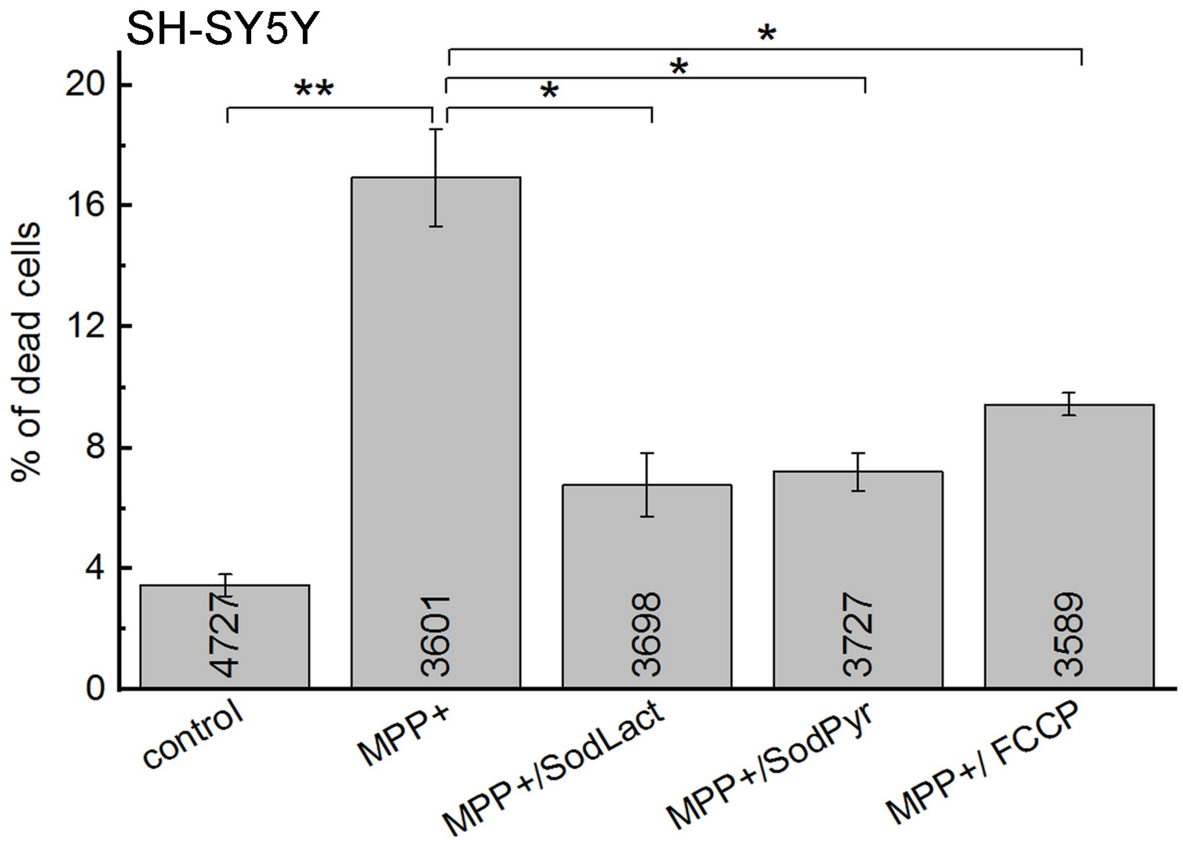
e


Neuroglial culture
Expression mRNA
0
5
10
15
20
SodPyr,24h
SodLact,24h
MPP+,48h
p62
lc3
optn
bnip3
pink1
parkin

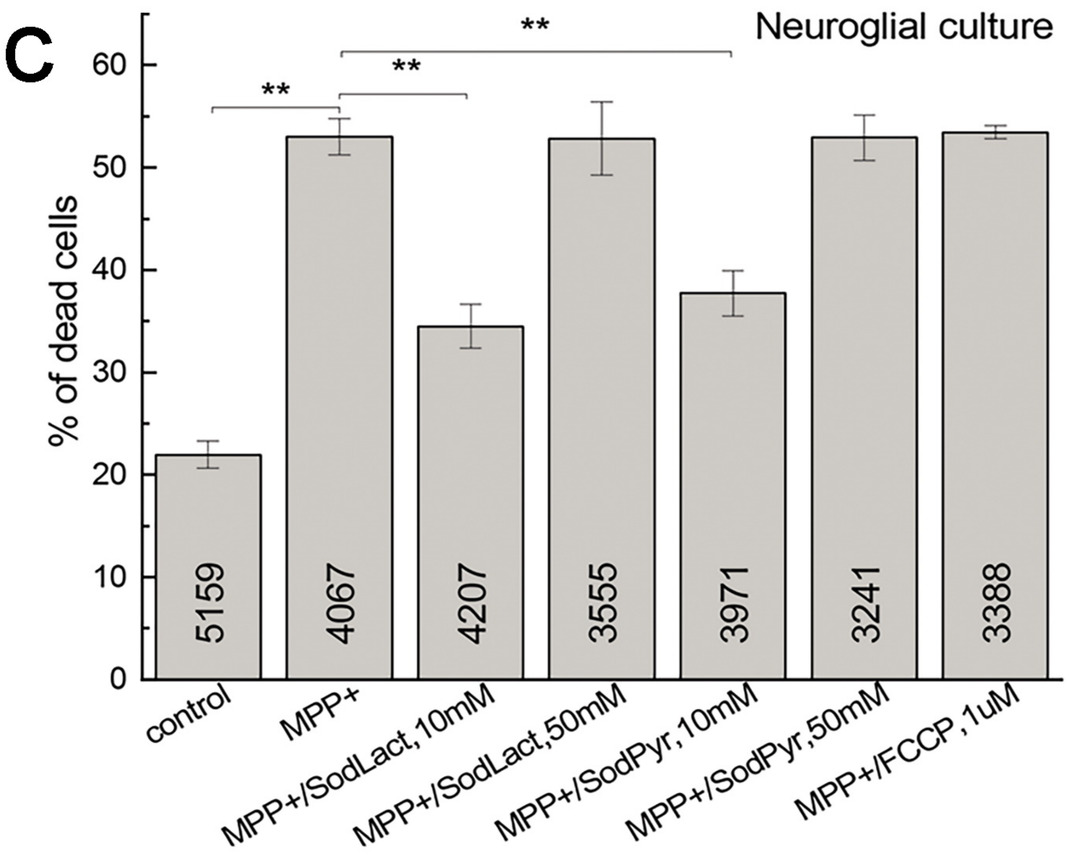
a


control
MPP+
MPP+/SodLact

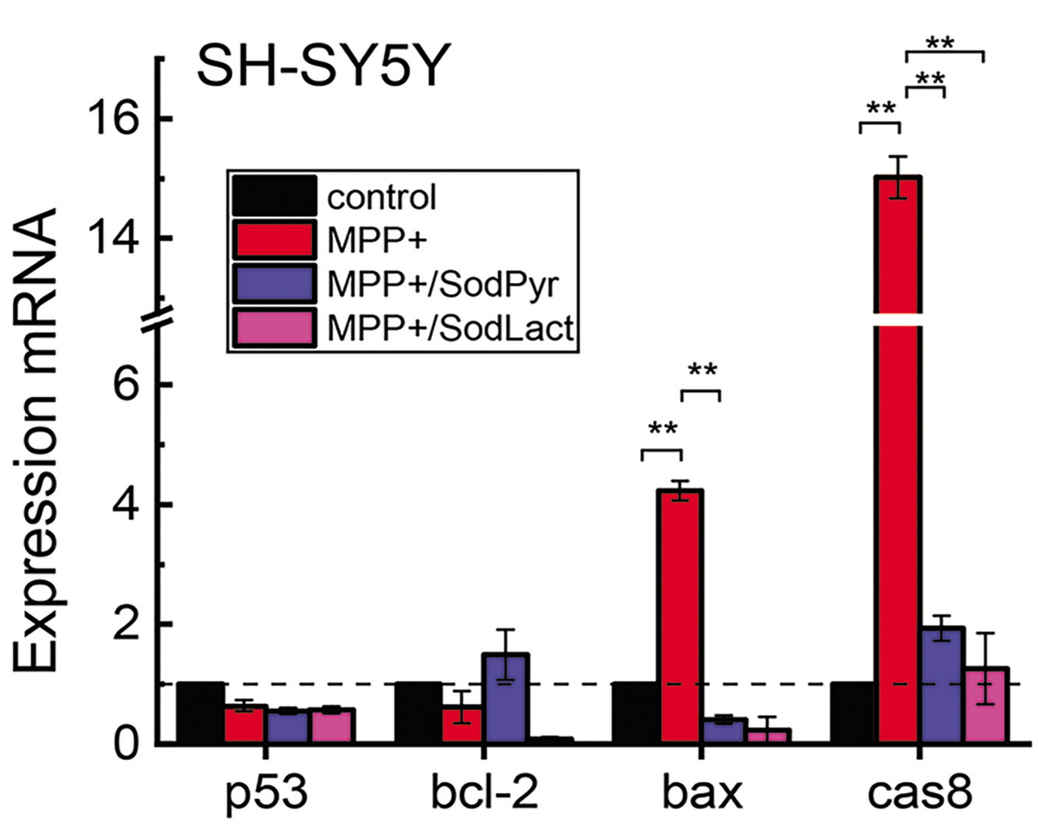
b


SH-SY5Y
% of dead cells
0
4
8
12
16
20
**
*
*
*
4727
3601
3698
3727
3589
control
MPP+
MPP+/SodLact
MPP+/SodPyr
MPP+/ FCCP

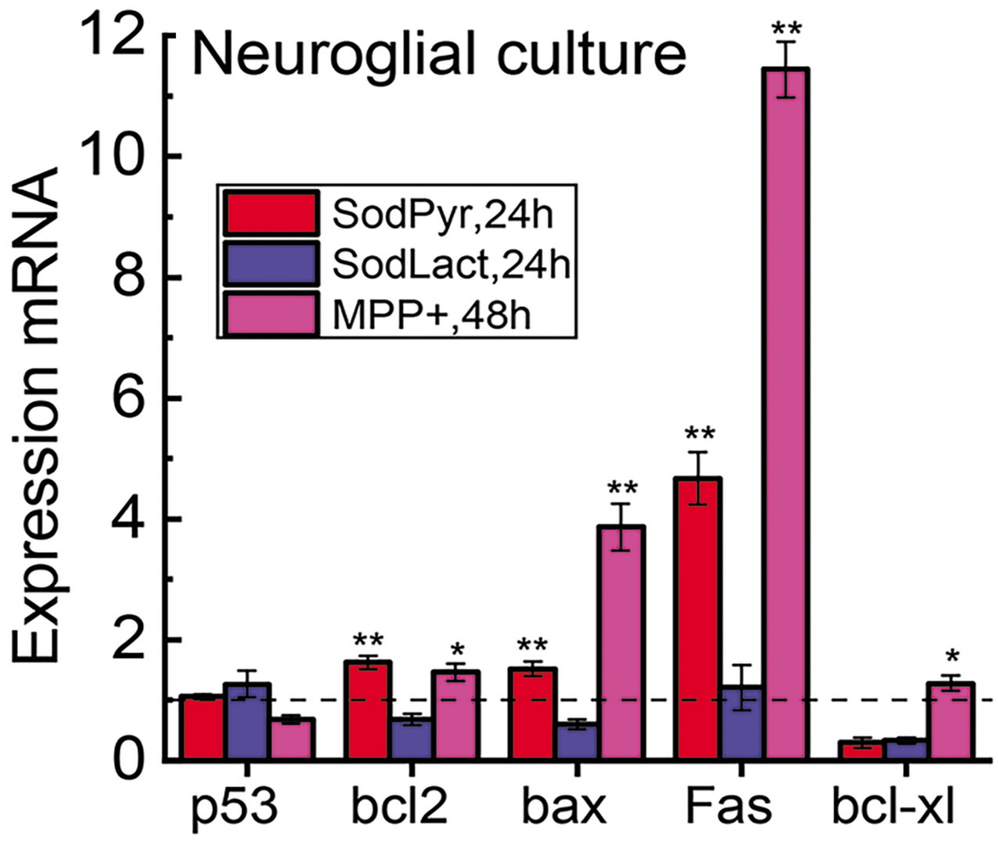
c


Neuroglial culture
% of dead cells
0
10
20
30
40
50
60
**
**
**
5159
4067
4207
3555
3971
3241
3388
control
MPP+
MPP+/SodLact,10mM
MPP+/SodLact,50mM
MPP+/SodPyr,10mM
MPP+/SodPyr,50mM
MPP+/FCCP ,1uM

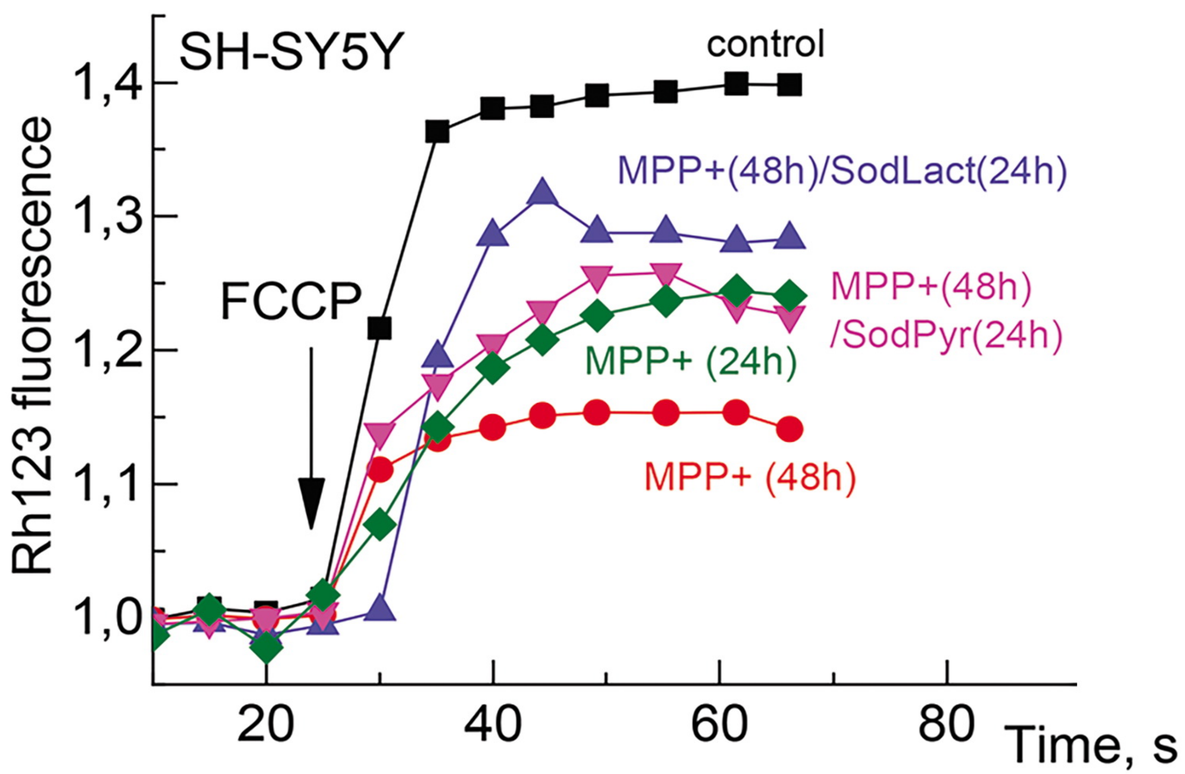
d


SH-SY5Y
Expression mRNA
0
2
4
6
14
16
control
MPP+
MPP+/SodPyr
MPP+/SodLact
p53
bcl-2
bax
cas8

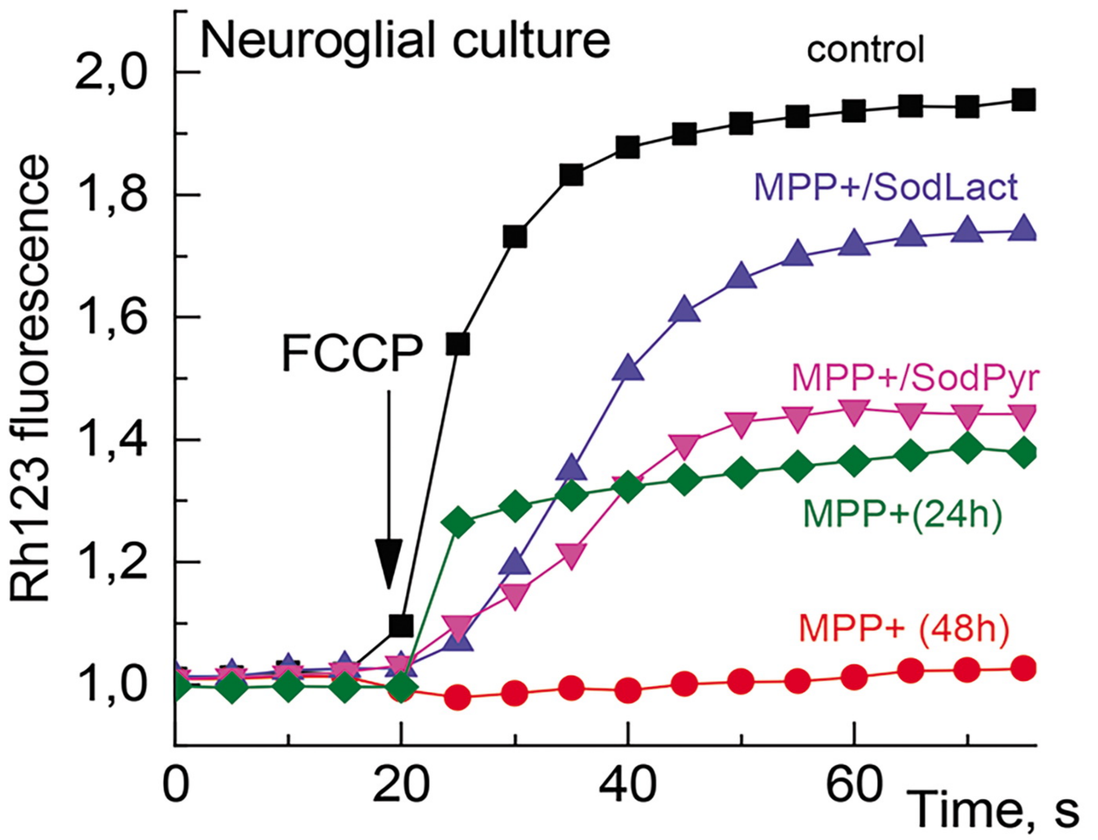
e


Neuroglial culture
Expression mRNA
0
2
4
6
8
10
12
SodPyr,24h
SodLact,24h
MPP+,48h
p53
bcl2
bax
Fas
bcl-xl

f


SH-SY5Y
Rh123 fluorescence
1,0
1,1
1,2
1,3
1,4
FCCP
control
MPP+(48h)/SodLact(24h)
MPP+(48h)
/SodPyr(24h)
MPP+ (24h)
MPP+ (48h)
20
40
60
80
Time, s

g


Neuroglial culture
Rh123 fluorescence
1,0
1,2
1,4
1,6
1,8
2,0
FCCP
control
MPP+/SodLact
MPP+/SodPyr
MPP+(24h)
MPP+ (48h)
0
20
40
60
Time, s