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Regulation of Morphological and Functional Properties of Astrocytes by Hydrogen Peroxide

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Abstract—Effects of hydrogen peroxide on morphological characteristics, proliferation index, and menadione-dependent lucigenin-enhanced chemiluminescence of C6 glioma cells were studied. It was established that H_2O_2 at 5×10^{-7}–1×10^{-8} M concentrations acted as a regulator of morphological and functional properties of astrocytes, inducing their reactivation, which is manifested as cell body hypertrophy and an increase of proliferative activity and menadione-induced production of superoxide anion radicals ($O_2^{\bullet -}$). Cytodestructive action of hydrogen peroxide at a concentration higher than 1×10^{-6} M on C6 glioma cells shows itself as a decrease of their proliferation index and the ability to generate $O_2^{\bullet -}$ under the effect of menadione. Use of lipopolysaccharide B as a functional stimulator has shown that H_2O_2 modifies signaling pathways leading to an increase of mitotic activity of C6 glioma cells and decreases the yield of lucigenin-dependent chemiluminescence of astrocytes under the action of menadione to the level of control values.

Key words: C6 glioma cells, chemiluminescence, hydrogen peroxide.

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INTRODUCTION

Hydrogen peroxide, depending on its concentration, produces dual effects on the functional state of various types of cells. H_2O_2 in cells at millimolar concentrations in vitro induces ruptures of DNA helixes, lipid peroxidation, a decrease of intensity of glycolysis, and morphological changes of plasma membranes to produce cell death by the mechanisms of apoptosis or necrosis [1, 2]. At low concentrations, hydrogen peroxide is a secondary messenger, which takes part in the processes of signal transduction; initiates protein phosphorylation [3]; activates transcription factors, specifically NF-kB [4], MAP-kinases [5, 6], protein kinases [7], etc.; and thereby affects the functional properties of cells [8].

One source of H_2O_2 in the central nervous system is microglial cells which are activated by both cytokines and β-amyloid peptide as well as by neurons during the appearance of a deficit of L-arginine or tetrahydrobiopterine [9]. The high permeability of the membrane to H_2O_2 and the relatively long lifetime lead to the fact that hydrogen peroxide affects not only H_2O_2-producing cells, but also neighboring cells, specifically, astrocytes. It is known that astrocytes in the brain perform several important functions: they regulate extracellular concentration of ions, metabolites, and neurotransmitters; maintain neuronal and synaptic functions; and, together with cerebral endothelial cells, form the blood-brain barrier (BBB) [10, 11], as well as participating in initiation and regulation of immune and inflammatory responses to damage and pathological states of the brain [12]. Therefore, study of effect of H_2O_2 on properties of astrocytes is vital.

In our earlier work using methods of chemiluminescence (ChL), no formation of active oxygen forms was observed after stimulation of the metabolism of C6 cells by latex particles, arachidonic acid, liposaccharide (LPS), and phytoagglutinin [13]. However, on addition of menadione, astrocytes generated superoxide anion radicals [14]. Therefore, changes in the intensity of menadione-dependent lucigenin-mediated ChL reflect the functional state of these cells. Thus, we have established that the ability of C6 cells to produce $O_2^{\bullet -}$ correlates positively with changes of their mitotic activity [14, 15]. Based on this, we used the parameters of the

menadione-dependent ChL of C6 cells in the presence of lucigenin for evaluation of functional cell activity.

In the present work, we studied the effect of H_2O_2 on proliferative activity, menadione-dependent $O_2^{\cdot-}$ production, morphological characteristics, and vitality of transplanted cells of rat glioma C6 cells.

MATERIALS AND METHODS

The transplanted strain of rat glioma C6 cells was obtained from the Collection of Cultures at the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg).

Used in the work were Eagle medium MEM with the addition of HEPES (Sigma, USA); Earl balanced salt solution (mole/l): 0.12 NaCl, 5.4×10^{-3} KCl, 0.9×10^{-3} $NaHPO_4 \cdot 2H_2O$, 0.8×10^{-3} $MgSO_4 \cdot 7H_2O$, 5.6×10^{-3} glucose, 1.8×10^{-3} $CaCl_2$, 26.2×10^{-3} $NaHCO_3$ (Analiz X, Republic of Belarus); cattle fetal serum (Research Institute of Epidemiology and Microbiology, Republic of Belarus Ministry of Health); gentamicin (Belmedpreparaty, Republic of Belarus); lucigenin (10,10-dimethyl-biacrydinium) (Fluka, Switzerland); H_2O_2; and Triton X-100 (Dialek, Republic of Belarus), as well as LPS, hematoxylin, eosin, menadione (2-methyl-1,4-naphthoquinone), dimethylsulfoxide, and propidium iodide (Sigma, USA).

The transplanted cells of the rat glioma C6 were cultivated at 37°C in flat flasks and on coverslips placed in flasks containing Eagle medium MEM with HEPES and with addition of 10% cattle fetal serum and 1×10^{-4} g/ml gentamicin. The implanted cell dose amounted to 1.5×10^5 cells/ml [16]. After 48 h of cultivation (in the logarithmic growth phase), the growth medium was replaced by the maintaining medium (the Eagle medium without serum). Then H_2O_2 at final concentrations from 1×10^{-3} to 1×10^{-9} mole/l and LPS at a final concentration of 0.1×10^{-6} g/ml were introduced into the flasks for 24 h. The volume of the added solution did not exceed 10% of the total sample volume. The cell preparations obtained were fixed in Dubosque–Brasil–Bouin's fluid, stained with hematoxylin-eosin, and examined under a light microscope, using a 40× objective and a 10× eyepiece.

The effect of H_2O_2 on cell morphological characteristics and proliferative activity was studied by light microscopy. Proliferative activity was determined from accumulation of cells in the monolayer formed in the stationary growth phase and was expressed as the proliferation index: the ratio of the number of cells in the flask to the number of implanted cells.

Vitality of the cells was determined by the fluorescent method in an LSF 1211A spectrofluorimeter (Solar, Republic of Belarus) with the use of propidium iodide [17], whose molecules can only penetrate into cells through damaged cytoplasmic membranes and then insert themselves between DNA and RNA nucleotides. After cultivation of C6 sample with H_2O_2 for 24 h, the cell monolayer was twice washed out with the balanced Earl salt solution, then added with propidium iodide at a concentration of 4×10^{-6} mol/l and mixed, and 5 min later intensity of fluorescence F_{d1} (λ_{exc} = 530 nm, λ_{reg} = 645 nm) was measured. After that, the cells were destroyed with Triton X-100 and the intensity of fluorescence F_{t1} was measured. Vitality of the cells was determined by the formula

$$\frac{(F_{t1} - F_{t2}) - (F_{d1} - F_{d2})}{(F_{t1} - F_{t2})} \times 100\%,$$

where F_{d2} and F_{t2} are intensity of fluorescence of propidium iodide and Triton X-100, respectively, in the balanced Earl solution.

Menadione-dependent $O_2^{\cdot-}$ formation in the C6 cell suspension was recorded by the chemiluminescent method on a BChL-1 biochemiluminometer at 37°C and pH 7.4 with use of 1.25×10^{-5} mole/l lucigenin as a specific emitter of $O_2^{\cdot-}$ ChL [18]. The C6 cells were cultivated with H_2O_2 for 24 h as described above. Prior to the ChL measurement, the cells attached to the flask surface were picked up mechanically using a scraper. The obtained suspension was washed out with the balanced Earl salt solution and the cell concentration was brought up to 1×10^6 cells/ml. Menadione was used at a concentration of 1.6×10^{-5} mole/l.

In a mathematical treatment of results, the mean value was determined for a group of measurements (more than three). The obtained data were presented as the means and their standard deviations.

RESULTS

Upon cultivation of the transplanted cells of the rat glioma C6 for 24 h with H_2O_2, changes of morphological characteristics were revealed, whose degree of expression depended on the preparation dose. H_2O_2 at concentrations of 1×10^{-3}–1×10^{-6} mole/l caused destruction of the cell C6 monolayer (10–100%). Figure 1b demonstrates a decrease of density of the cell C6 monolayer as compared with intact culture (Fig. 1a) under the effect of H_2O_2 at a concentration of 1×10^{-5} mole/l. Cultivation of astrocytes with H_2O at concentrations of 5×10^{-7}–1×10^{-8} mole/l did not destroy the monolayer, and the cells preserved their vitality; in a portion of them, hypertrophy of cell bodies was observed (Fig. 1c). Action of H_2O_2 at concentrations of 1×10^{-8}–1×10^{-9} mole/l did not affect morphological characteristics of the C6 cells.

To estimate quantitatively the action of H_2O_2 on the cell C6 morphological characteristics in culture (after cultivation for 24 h), the cell viability was determined using propidium iodide. As seen in Fig. 2, with

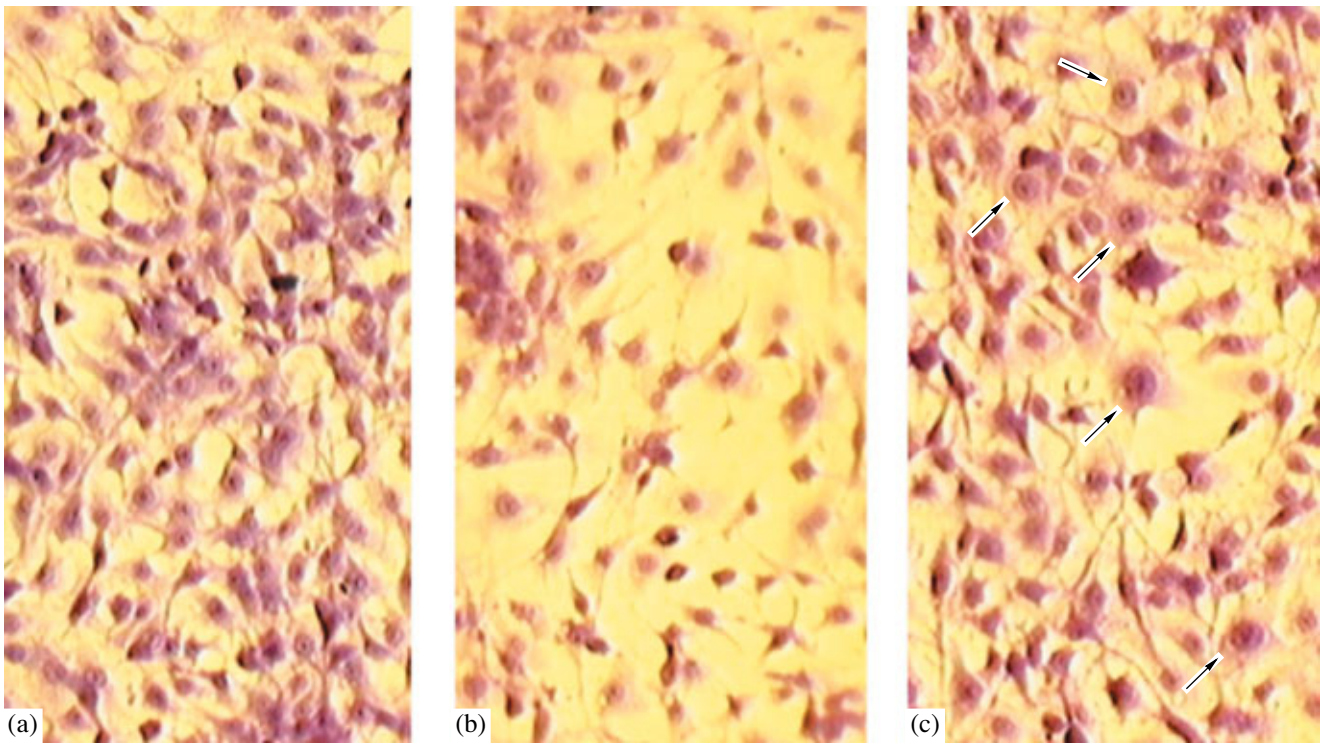

Fig. 1. Morphological changes of transplanted cells of rat glioma C6 under the action of hydrogen peroxide for 24 h.
(a) Monolayer formed of C6 cells, (b) cytotoxic action of H_2O_2 at a concentration of 1×10^{-5} mole/l, (c) reactivation of astrocytes during cultivation with 5×10^{-7} mole/l H_2O_2 (reactivated cells are indicated by arrows). Staining with hematoxylin-eosin. Objective 40×, eyepiece 10×.

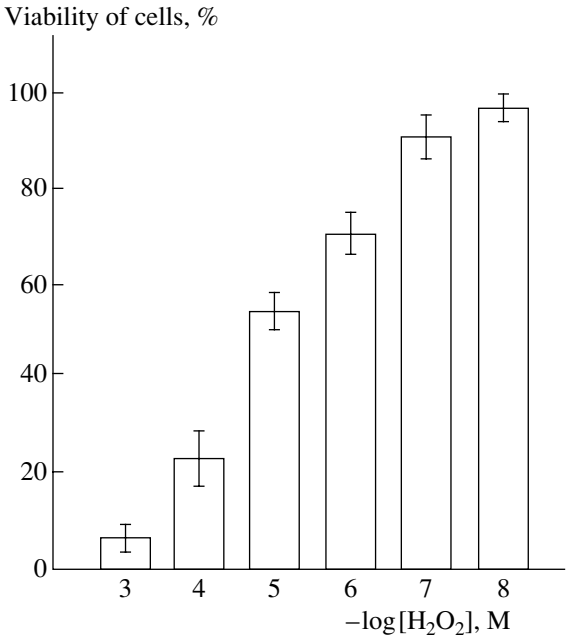

Fig. 2. Viability of transplanted cells of the rat glioma C6 after action of H_2O_2 in the concentration range from 1×10^{-3} to 1×10^{-8} mole/l for 24 h. The viability of cells cultivated in medium free of H_2O_2 for 24 h is taken as 100%.

decrease of the H_2O_2 concentration from 1×10^{-3} to 1×10^{-8} mole/l, the number of viable cells increased to the level recorded in control samples.

It is reported in the literature that active oxygen forms at micromolar concentrations can stimulate the proliferative activity of cells [19]. We determined the proliferation index (PI) of the transplanted cells of the rat glioma C6 after the H_2O_2 action for 24 h. As seen in Fig. 3, the decrease in the H_2O_2 concentration in the cultivation medium from 5×10^{-7} to 5×10^{-8} mole/l led to a considerable (up to 1.6-fold) increase of the cell proliferative activity. With further elevation of the H_2O_2 concentration, PI decreases and reaches the level of control values already at a H_2O_2 concentration equal to 1×10^{-6} mole/l.

Figure 4 shows the dependence of the integral intensity of the lucigenin-mediated ChL on the H_2O_2 concentration on addition of metadione to the C6 cell suspension. It is seen that cultivation of the cells with H_2O_2 at concentrations of 1×10^{-7}–1×10^{-8} mole/l for 24 h leads to an increase of the ChL intensity. Maximal values of the integral ChL intensity were found at H_2O_2 concentration of 1×10^{-7} mole/l. A further rise in ChL concentration was accompanied by a decrease in the release of $O_2^{\bullet -}$ until the level of control values was

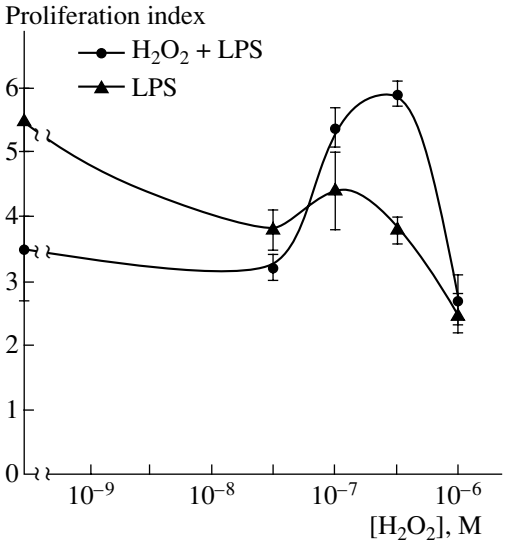

Fig. 3. Effect of H_2O_2 on proliferation index of transplanted cells of the rat glioma C6 in the absence or in the presence of 0.1×10^{-6} g/ml LPS. Duration of cultivation with LPS and/or with H_2O_2 is 24 h.

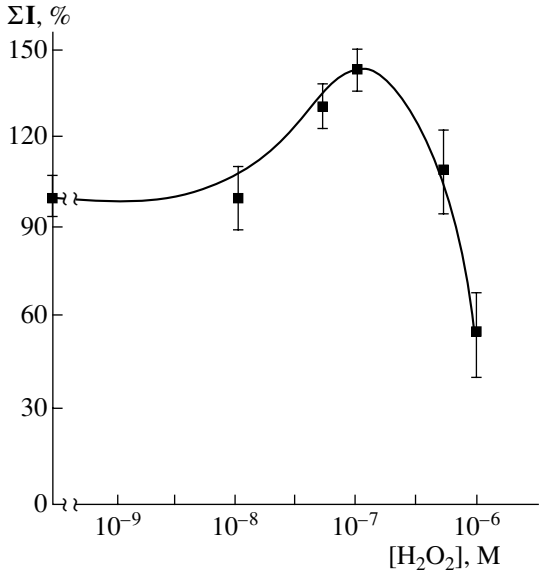

Fig. 4. Dependence of integral intensity of lucigenin-mediated ChL (ΣI) upon the action of menadione on a suspension of transplanted cells of the rat glioma C6 on H_2O_2 concentration. Concentration of menadione is 1.6×10^{-5} mole/l, of lucigenin 1.25×10^{-5} mole/l. Duration of cultivation with H_2O_2 is 24 h.

reached, while upon an increase of the H_2O_2 concentration to 1×10^{-6} mole/l the $O_2^{\cdot -}$ release decreased by a factor of 2 compared with the control.

LPS is an efficient stimulator of immunocompetent cells, including astrocytes [20]. As demonstrated in Fig. 3, upon cultivation of cells of the rat glioma C6 with LPS at a concentration of 0.1×10^{-6} g/ml for 24 h, proliferation index increased 1.5 times as compared with the control. It is seen that at the combined action of LPS and H_2O_2 at a concentration of 1×10^{-6} mole/l, PI decreases by a factor of 2 and reaches the value recorded on addition of H_2O_2 in the absence of LPS. At the same time, a decrease of the H_2O_2 to 1×10^{-8} mole/l on the background of the LPS action leads to restoration of PI until the level found in control samples with LPS is reached.

Functional changes in the C6 cells after the combined action of H_2O_2 and LPS agree with results of studying the menadione-dependent ChL. Figure 5 presents the results of the measurement of integral intensity (ΣI) of lucigenin-mediated ChL of suspension of the C6 cells obtained after coincubation with 0.1×10^{-6} g/ml LPS and H_2O_2 at various concentrations for 24 h. The combined action of LPS and H_2O_2 at concentrations of 10^{-7}–10^{-8} mole/l produces a decrease in the yield of lucigenin-mediated ChL astrocytes upon menadione action down to the control level, which indicates a decrease of activity of the menadione-dependent $O_2^{\cdot -}$-generating systems of astrocytes.

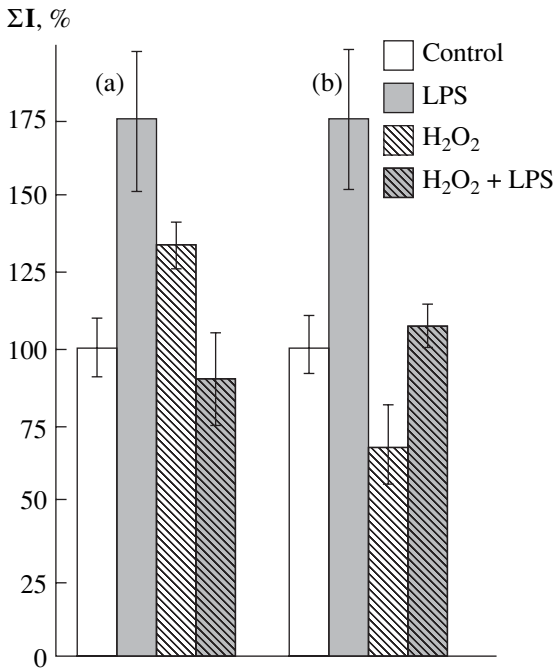

Fig. 5. Integral intensity of lucigenin-mediated ChL (ΣI) upon the action of menadione on a suspension of transplanted cells of the rat glioma C6 after their cultivation with LPS and H_2O_2 separately or in combination for 24 h. The H_2O_2 concentration is 1×10^{-7} (a) or 1×10^{-8} mole/l (b). The concentration of menadione is 1.6×10^{-5} mole/l; of lucigenin, 1.25×10^{-5} mole/l; of LPS, 0.1×10^{-6} g/ml.

DISCUSSION

Upon pathological changes of the brain (e.g., traumas or neurodegenerative diseases) and posttraumatic regeneration, migration of astrocytes is known to be directed into the area of damage and reactivation of these cells [21, 22]. This is accompanied by hypertrophy of cells and their nuclei (astrogliosis), proliferation (astrocytosis), elongation and branching of processes, and an increase of synthesis of glial acid fibrillary protein, vimentin, and nectin—the structural units of the astrocytic cytoskeleton. Reactivation is also characterized by an enhancement of astrocytic metabolic activity that consists in the production of various trophic and growth factors, cytokines, and nitrogen monoxide, and the expression of cytosol antioxidant proteins (glutathione-S-transferase-1, ceruloplasmin, etc.) [23, 24].

Results of the present study indicate that hydrogen peroxide at concentrations from 5×10^{-7} to 5×10^{-8} mole/l leads to reactivation of transplanted cells of the rat glioma C6. Thus, in our system, under the effect of H_2O_2 at these concentrations, hypertrophy of cell bodies and an elevation of the proliferation index, as well as an increase of the intensity of the menadione-dependent lucigenin-mediated ChL of C6 cells, are found. Based on data obtained earlier [14], it can be concluded that H_2O_2 at these concentrations stimulates functional activity of C6 cells. However, with the increase in H_2O_2 concentration to 1×10^{-6} mole/l, destruction and loosening of the cell monolayer and a slight decrease of PI and ΣI of the menadione-dependent lucigenin-mediated ChL of C6 cells are observed, which indicates a cytodestructive effect of the preparation.

Data in the literature indicate constant exchange of substances and information between astrocytes and other brain cells—both neurons and glial cells [11]. In this exchange, one astrocyte can simultaneously contact multiple cells of various types by performing the function of a messenger in signal transduction in the brain and participating in processes of neurogenesis, synaptogenesis, vasculogenesis, angiogenesis, remyelinization, etc. [24]. Hence, the destructive effect of H_2O_2 will lead to the changes of intercellular interactions.

It is also shown in the literature that H_2O_2, as a second messenger, interacts with some participants of signal transduction cascades in cells of various types, including astrocytes, by modulating activation of transcription factors and gene expression [25]. To find out which role is played by H_2O_2 in the functioning of stimulated astrocytes, we studied the effect of this intermediate on the functional state of LPS-stimulated C6 cells. It is known that under the effect of this endotoxin, intracellular receptor-mediated signal cascades are activated and cells produce various cytotoxins, adhesion proteins, and nitrogen oxide (II) [26, 27].

Our results for the estimation of PI after the combined action of H_2O_2 and LPS indicate a modifying H_2O_2 effect on signal pathways leading to an enhancement of the mitotic activity of astrocytes. This agrees with data obtained in studying proliferative activity of lymphocytes [28]; the authors of this study have shown H_2O_2 to produce a dose-dependent inhibition of proliferation of B and T lymphocytes stimulated by LPS and concanavalin A, respectively, these effects being associated with the inhibitory effect of H_2O_2 on protein kinase C that is activated by LPS.

Thus, our results allow the conclusion that H_2O_2 in a range of concentrations between 5×10^{-7}–1×10^{-8} mole/l acts as a regulator of the morphological and functional properties of cells of the rat transplanted glioma C6 by induction of astrocyte reactivation that is manifested as hypertrophy of cellular bodies and an increase of proliferative activity and of menadione-induced $O_2^{\bullet -}$ production. In the C6 cell culture, the cytodestructive action of H_2O_2 at a concentration above 1×10^{-6} mole/l is manifested as a decrease of the cell PI and of the cell's ability to generate $O_2^{\bullet -}$ under the influence of menadione.

Our data on the effects of H_2O_2 on proliferative activity and the lucigenin-dependent ChL of astrocytes (stimulated and non-stimulated by LPS) suggest that the signal transduction cascades which are affected by H_2O_2 at micromolar concentrations, and by LPS as well, depend on the "crossing" MAP kinases found in signal cascades, all of which results in a change in the functional response of astrocytes.

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