Mol Neurobiol (2015) 52:811–825
DOI 10.1007/s12035-015-9237-8

Protection of the Crayfish Mechanoreceptor Neuron and Glial Cells from Photooxidative Injury by Modulators of Diverse Signal Transduction Pathways

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Published online: 11 June 2015

Abstract Oxidative stress is the reason of diverse neuropathological processes. Photodynamic therapy (PDT), an effective inducer of oxidative stress, is used for cancer treatment, including brain tumors. We studied the role of various signaling pathways in photodynamic injury and protection of single neurons and satellite glial cells in the isolated crayfish mechanoreceptor. It was photosensitized with alumophthalocyanine Photosens in the presence of inhibitors or activators of various signaling proteins. PDT eliminated neuronal activity and killed neurons and glial cells. Inhibitory analysis showed the involvement of protein kinases Akt, glycogen synthase kinase-3β (GSK-3β), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase kinases 1 and 2 (MEK1/2), calmodulin, calmodulin-dependent kinase II (CaMKII), adenylate cyclase, and nuclear factor NF-κB in PDT-induced necrosis of neurons. Nitric oxide (NO) and glial cell-derived neurotrophic factor (GDNF) reduced neuronal necrosis. In glial cells, protein kinases Akt, calmodulin, and CaMKII; protein kinases C and G, adenylate cyclase, and p38; and nuclear transcription factor NF-κB also mediated PDT-induced necrosis. In contrast, NO and neurotrophic factors nerve growth factor (NGF) and GDNF demonstrated anti-necrotic activity. Phospholipase Cγ, protein kinase C, GSK-3β, mTOR, NF-κB, mitochondrial permeability transition pores, and NO synthase mediated PDT-induced apoptosis of glial cells, whereas protein kinase A, tyrosine phosphatases, and neurotrophic factors NGF, GDNF, and neurturin were involved in protecting glial cells from photoinduced apoptosis. Signaling pathways that control cell survival and death differed in neurons and glia. Inhibitors or activators of some signaling pathways may be used as potential protectors of neurons and glia from photooxidative stress and following death.

Keywords Neuron · Glia · Signaling · Necrosis · Apoptosis · Photodynamic

Introduction

Neuroprotection is commonly considered as a pharmacological intervention that prevents death of neuronal cells and thereby protects the nervous system from injurious impacts and treats neurodegenerative diseases [1]. The primary lesion induces simultaneously processes leading to cell death and protection that are controlled by the complex intracellular signal transduction system. Intercellular interactions in the tissues form the higher regulation level. The signaling cascades Ca^{2+}/calmodulin, phosphatidylinositol-3-kinase/protein kinase B (Akt)/glycogen synthase kinase-3β, receptor tyrosine kinase/MAP kinase, glutamate/NMDA receptors, NO/protein kinase G, and cAMP/protein kinase A are currently considered to be most important in the regulation of cell fate [2]. Neuroprotective agents may be aimed to each of these pathways. However, the current knowledge of the signal transduction system is still incomplete, many of its components are unknown, and we have not enough tools to influence them. It is unknown exactly, which signaling pathways are expressed

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in different cells and participate in their responses to various damaging impacts.

Because of intense metabolism with high oxygen consumption and limited capacity of cellular regeneration, the brain is highly susceptible to overproduction of reactive oxygen species (ROS). ROS are present in small amounts in cells as by-products of normal metabolism, but their level increases dramatically under environmental stress (e.g., ultraviolet or ionizing radiation). ROS oxidize cellular proteins and lipids, induce oxidative stress, and, finally, cause cell death. Oxidative damage of neuronal and glial cells contributes to aging and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis [3–8]. Glial cells and neurons are known to support survival of each other [8–11]. The search of efficient neuroprotective and glia-protective agents is among the central neurological problems [1, 3, 5, 8, 12].

The experiments on the mammalian brain provide the averaged data that are not easy to interpret unambiguously because of numerous interactions between neurons and between neurons and glial cells. It is difficult to identify certain neurons and glial cells that interact with them, and to registry the activity of a given neuron. In studies of the processes of neurodegeneration and neuroprotection, it is reasonable to use simpler experimental preparations exposed to stress effects in controlled conditions. The photodynamic therapy effect (PDT) is a potent and controlled inducer of oxidative stress. In PDT, the photosensitizing dye molecules generate highly toxic singlet oxygen ($^1O_2$) under light exposure in the presence of oxygen. $^1O_2$ oxidizes cellular proteins and induces peroxidation of membrane lipids that leads to cell death. PDT acts in a dose-dependent manner. It causes necrosis or apoptosis dependently on the chemical structure and concentration of the photosensitizer, as well as on light intensity. PDT efficiently and selectively destroys tumors, which accumulate a photosensitizing dye [13–15]. It is a promising adjuvant method for treatment of brain tumors [16]. However, in this case, not only malignant cells but also adjacent normal neurons and glial cells are damaged that can cause harmful side effects. Therefore, thorough experimental studies of photodynamic damage of normal neurons and glia should precede practical applications of PDT.

Here, we summarized the data on the role of diverse intercellular and intracellular signaling pathways in photodynamic injury of mechanosensory neurons and satellite glial cells in the isolated crayfish stretch receptor (CSR) that was used as a simple but informative model object. Using various inhibitors or activators of diverse signaling proteins involved in the intercellular and intracellular signaling processes, we showed the involvement of these proteins in PDT-induced death or in protection of these cells.

The Crayfish Stretch Receptor

The isolated crayfish stretch receptor is a classical neurophysiological preparation. It consists of two single mechanoreceptor neurons: slowly and rapidly adapting ones [17]. Their dendrites penetrate into the slowly and rapidly adapting receptor muscles, where they contact to the membranes of muscle fibers [18]. Receptor muscles are attached to the chitin carapace between neighboring abdominal segments. Their stretching induces transient firing responses of mechanoreceptor neurons, which are transmitted to the abdominal ganglion. The ganglion neurons generate commands for the abdominal muscles that control crayfish movements. At the stationary state, the slowly adapting mechanoreceptor neuron (MRN) fires with a constant rate proportional to the receptor muscle length, whereas the rapidly adapting neuron is silent. The frequency and number of spikes in a firing burst of this neuron correlate with the rate of receptor muscle extension [17, 19–21]. Both neurons are enwrapped by satellite glial cells [22]. Large and flattened MRN is very suitable for microscopic study (Fig. 1). The initial level of its activity is set by the experimenter. The stable firing rate provides accurate detection of small shifts induced by external impacts. Another advantage of CSR is exact identification of glial cells that contact to MRN, unlike the central nervous system where these cells are difficult to identify.

MRN has been used in classical studies of mechanisms of neuronal activity and its inhibition [19, 20], relationship between neuronal activity and metabolism [23], mechanosensory transduction [21], neuroglial interactions [22], responses of neurons and glial cells to various physical and chemical impacts [15, 23–26], and signal transduction processes in neurons and glia [15, 27–34].

CSR was isolated from the crayfish *Astacus leptodactylus* and placed in a plexiglass chamber filled with van Harreveld's saline. Spikes were derived extracellularly from the axon using a suction pipette electrode. After amplification, they were digitized and processed by a personal computer. Their frequency was continuously registered. After a 30-min control recording of neuronal activity, 10–100 nM alumophthalocyanine photosensitizer Photosens was added into the chamber. After following 30-min incubation in the dark, cells were irradiated for 30 min by a helium-neon (633 nm) or diode laser (670 nm) until irreversible firing abolition that occurred typically within 15–25 min. The methods of isolation of MRN, registration of neuronal activity, fluorescence microscopic and electron microscopic studies, and photosensitization have been described in detail earlier [22, 28–34]. Neurotrophic factors, inhibitors or activators of signaling proteins, were characterized in the papers [27–35]. In the recent research, we used also inhibitor of inducible NO synthase L-NIL (Sigma-Aldrich Co), MEK1/2 inhibitor U0126, and mammalian target of rapamycin (mTOR) inhibitor rapamycin (both from Alomone

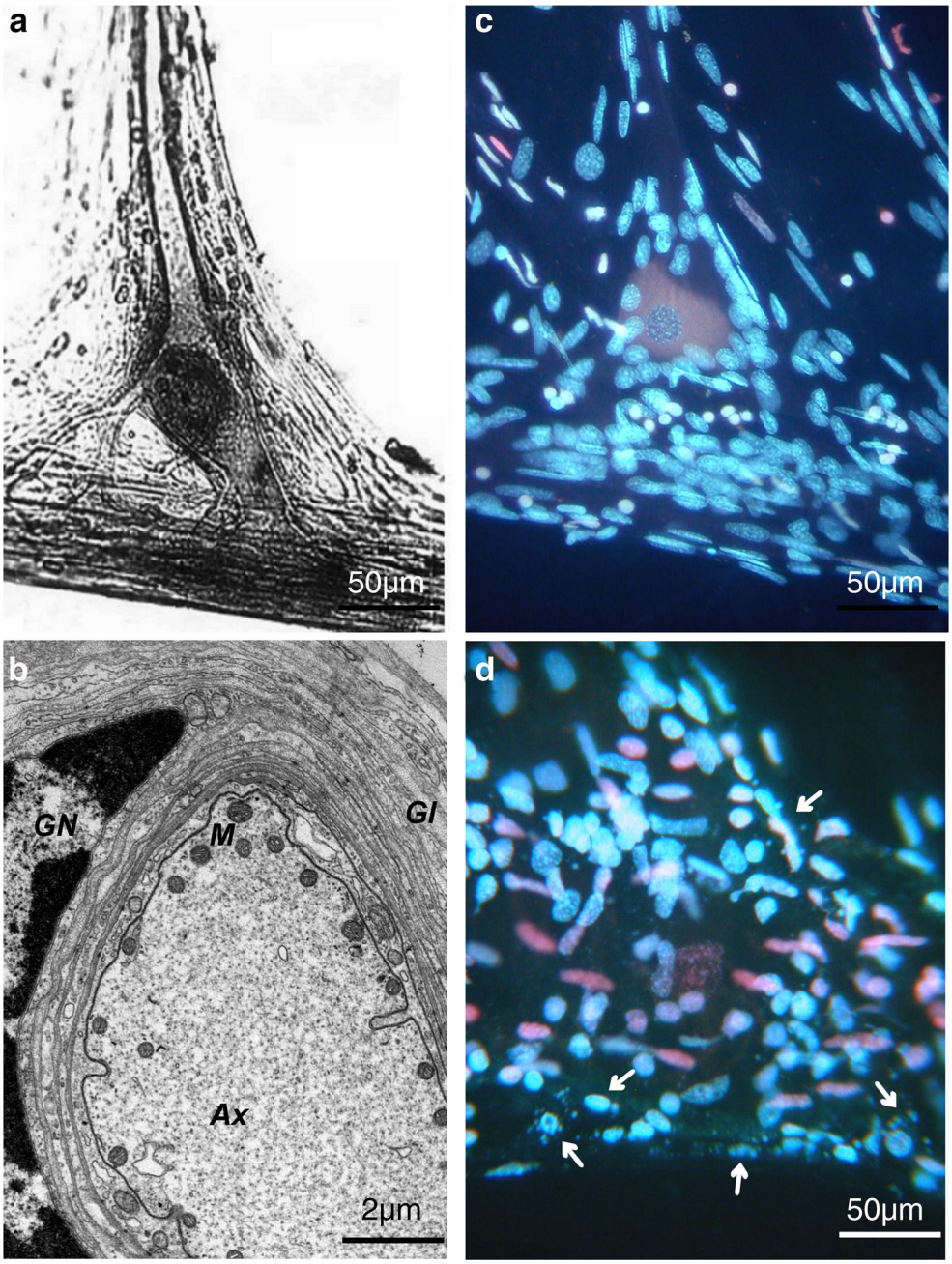


Fig. 1 The slowly adapting crayfish stretch receptor. The dendrites of the mechanoreceptor neuron penetrate to the receptor muscle and ramify between muscle fibers. The neuron body is large (50–100 μm); the axon is directed to the ventral nerve cord ganglion. **a** The bright-field image, **b** ultrastructure of the axon and satellite glial envelope, **c** fluorescence microscopic image, and **d** fluorescence microscopic image of the photosensitized preparation (100 nM Photosens). Nuclei of the neuron, glial cells, and muscle fibers stained with Hoechst 33342 have blue fluorescence. Nuclei fluorescing in red belong to necrotic cells with a compromised plasma membrane, through which propidium iodide penetrated into the cell. *Ax* axon, *Gl* glial cell, *GN* glial nucleus, *M* mitochondria. *Arrows* on **d** indicate the fragmented nuclei of apoptotic cells. *Scale bars* on **a**, **c**, and **d** indicate 50 μm, and that on **b** 2 μm

Labs, Israel). These modulators were added into the experimental chamber 25 min prior to light exposure. After following 8-h incubation in the dark, the preparations were stained with propidium iodide, which penetrates only in necrotic cells with a compromised plasma membrane and imparts red fluorescence to their nuclei. Another fluorochrome Hoechst 33342 imparts blue fluorescence to chromatin. Fragmentation of the cell nuclei is a hallmark of the final stages of apoptosis (Fig. 1c, d). The percent of propidium iodide-stained nuclei of necrotic cells were counted in the predetermined standard field around MRN soma. Fragmented apoptotic nuclei were counted around the proximal 2-mm axon fragment, where apoptosis was more significant [22, 27–34]. One-way ANOVA was used for statistical evaluations of significant differences between various experimental groups.

PDT-Induced Death of Crayfish Mechanoreceptor Neurons and Satellite Glial Cells

PDT effect on cells, in particular on MRN, strongly depended on the impact strength, i.e., on the light intensity and the photosensitizer concentration [15]. For example, intense PDT (10 μM Photosens) rapidly, within 1–3 min, increased firing

frequency from 8–10 to 40–50 Hz followed by abrupt firing abolition. Just after PDT, red fluorescence of propidium iodide was observed in almost all neuronal and glial nuclei, indicating total necrosis that had occurred during the treatment. At weaker PDT (0.1 μM Photosens), MRN necrosis was delayed by 1.7 h [30]. Therefore, neuronal necrosis under relatively weak impact may be not catastrophic but a controlled cell death mode. Fragmentation of nuclear chromatin characteristic for apoptosis was not observed in MRN under PDT or in the presence of various pro-apoptotic chemical agents. It was suggested that apoptosis is intrinsically blocked in MRN [30], like in many adult vertebrate neurons [36]. MRN is a unique neuron, vital for the animal. Unlike vertebrates, the invertebrate nervous system is much simpler, without redundancy. Each neuron plays a specific role and cannot be replaced. Accidental triggering of the cell death program can disturb the vital functions of the whole organism.

Unlike MRN, glial cells died not only from PDT-induced necrosis but also from apoptosis (Fig. 1d). These processes developed after the treatment. The level of glial necrosis that was about 10 % in the untreated CSR increased to 30 % just after PDT and to 50 % in the next 8 h. The number of apoptotic glial nuclei was negligible in the first post-treatment hours but increased significantly at 6–8 h after PDT. So, we studied PDT-induced cell death after 8 h post-treatment incubation [31].

At the ultrastructural level, membranous organelles including mitochondria, endoplasmic reticulum (ER), and dictyosomes were most photosensitive. These organelles swelled, lost the inner structure, and vacuolated. The destruction of mitochondria and loss of glycogen granules indicated suppression of bioenergetic processes and energy depletion. The elimination of rough endoplasmic reticulum and polysomes showed inhibition of protein synthesis. These changes started within the first 5 min of photodynamic impact when neuronal activity only slightly changed and increased after 30-min PDT. In the following 1 h, the ultrastructure of MRN and glial cells became obviously necrotic with swelling and vacuolation of almost all organelles [24].

Involvement of Intercellular Signaling in Neuron and Glia Killing and Protection

Neurotrophic Factors

In vertebrates neurons and glial cells exchange neurotrophic factors such as nerve growth factor (NGF), neurotrophins 3 and 4/5, brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF). These neurotrophic factors are involved in neuroglial interactions that regulate development of the nervous system and mediate resistance of neurons and glia to damaging factors [37–40]. Injured neurons stimulate surrounding glial cells to produce neurotrophins required for their survival [41–43]. After recognition by receptor tyrosine kinases, neurotrophic factors initiate intracellular signaling processes that regulate cell survival and functional activity.

Our experiments showed that mammalian NGF, GDNF, and neurturin, a member of the GDNF family, significantly reduced PDT-induced necrosis and apoptosis of crayfish glial cells. GDNF also protected crayfish neurons from PDT-induced necrosis. Other neurotrophic factors such as BDNF and CNTF as well as epidermal growth factor (EGF) were ineffective (Figs. 2a; 3a; and 4a) [29, 32].

At the ultrastructural level, GDNF has been shown to prevent PDT-induced vacuolation of the cytoplasm of MRN and glial cells caused by injury of membranes of intracellular organelles. In the presence of GDNF, mitochondria were large but did not swell; they had a moderately dense matrix and well-developed cristae that indicated efficient energetic metabolism [32]. The structures involved in protein synthesis and transport including polysomes, rough endoplasmic reticulum (ER), dictyosomes, and microtubule bundles that divide the neuronal cytoplasm by Nissl bodies were preserved. Therefore, GDNF-mediated maintaining of bioenergetic, biosynthetic, and transport processes may represent the basis of neuro- and glia protection from PDT-induced injury. Apparently, the protection effect of GDNF-mediated signaling pathways was associated with stabilization of the surface and intracellular membranes that were injured by PDT. How the binding of GDNF to its receptors on the cellular surface leads to membrane stabilization remains unknown [32].

One can suggest that NGF-like and GDNF-like signaling molecules may be involved in the neuroglial interactions in CSR, and their receptors are present on the surfaces of glial cells. Possibly, MRN was the source of neurotrophins that protect glial cells from PDT-induced necrosis and apoptosis. These results are in agreement with the previous finding that the MRN body protects glial cells from PDT-induced apoptosis: its local destruction by a focused laser beam enhanced glial apoptosis [44].

The present data are of interest from another point of view. The protective effect of exogenous mammalian NGF and GDNF on crayfish neurons and glial cells requires their binding to NGF-like or GDNF-like receptors that initiate intracellular pro-survival signaling pathways. However, neurotrophic signaling in most invertebrates including crayfish has not been established yet. This problem is of principal evolutionary significance. The emergence of neurotrophic signaling has been hypothesized to play a key role in significant increase of the number of neurons in vertebrates that led to formation of the complex brain [45, 46]. The evolution stage, in which neurotrophins emerged, is not determined yet. For example, careful genome inspection showed lack of neurotrophins and their receptors in the nervous system of *Drosophila*

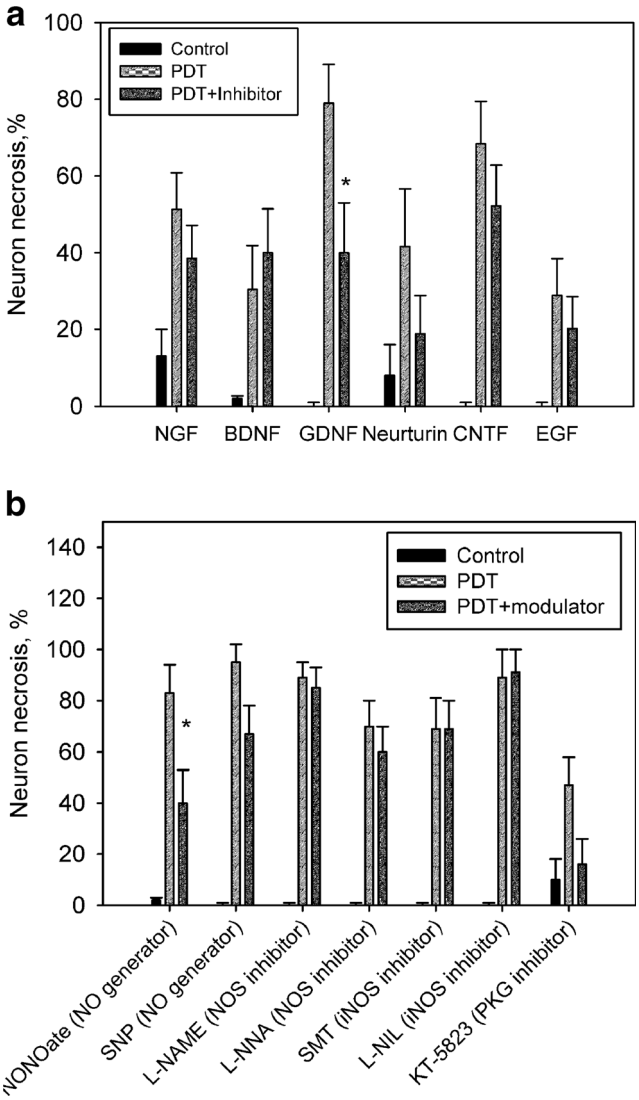


Fig. 2 The levels of necrotic neurons (%) in the photosensitized crayfish stretch receptor in the presence of **a** neurotrophic factors: NGF (100 ng/ml), BDNF (1 ng/ml), NGF (10 ng/ml), neurturin (10 ng/ml), CNTF (50 ng/ml), and EGF (100 ng/ml), or **b** modulators of NO production: NO generators NONOate (100 μM) and SNP (10 μM) or inhibitors of NO synthase: L-NAME (1 mM), L-NNA (1 mM), SMT (50 μM), and L-NIL (100 μM) or inhibitor of protein kinase G KT-5823 (10 μM). Significant difference from PDT-treated preparation in the absence of modulators, *<0.05 (one-way ANOVA)

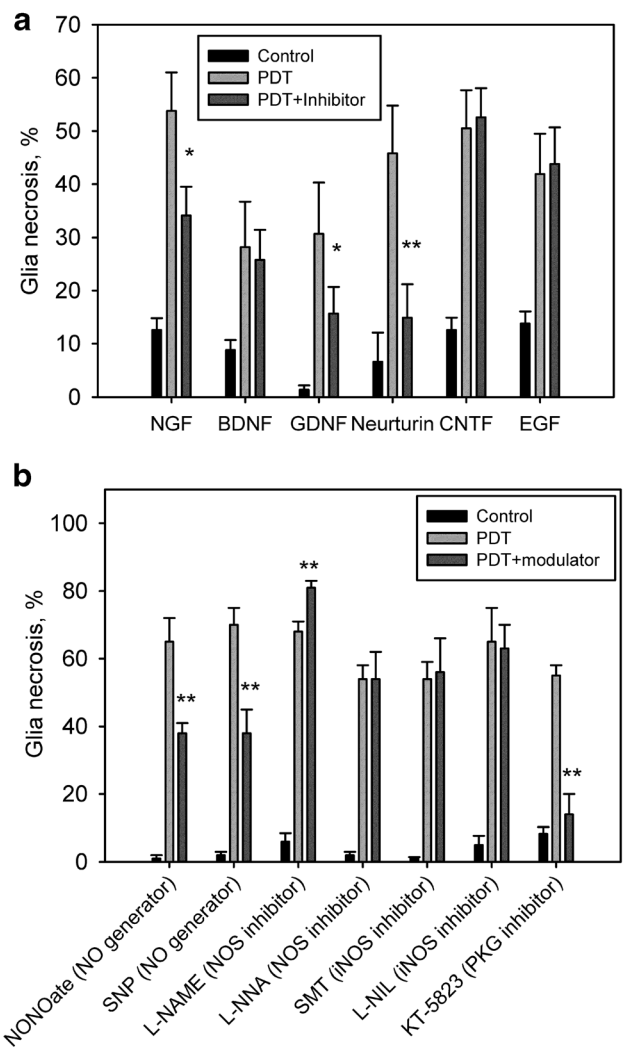


Fig. 3 The levels of necrotic glial cells (%) in the photosensitized crayfish stretch receptor in the presence of **a** neurotrophic factors: NGF (100 ng/ml), BDNF (1 ng/ml), NGF (10 ng/ml), neurturin (10 ng/ml), CNTF (50 ng/ml), and EGF (100 ng/ml), or **b** modulators of NO production: NO generators NONOate (100 μM) and SNP (10 μM) or inhibitors of NO synthase L-NAME (1 mM), L-NNA (1 mM), SMT (50 μM), and L-NIL (100 μM) or inhibitor of protein kinase G KT-5823 (10 μM). Significant difference from PDT-treated preparation in the absence of modulators, *<0.05; **<0.01 (one-way ANOVA)

melanogaster and *Caenorhabditis elegans* [47, 48]. On the other hand, the analogs of GDNF receptors have been found in some invertebrates [49]. The present data suggest the presence of NGF-like and GDNF/neurturin-like neurotrophic factors and their receptors in the crayfish nervous system [32].

NO-Mediated Pathway

Nitric oxide (NO) is an intercellular messenger, which easily moves between cells. It regulates a number of physiological and pathological processes including neurotransmission, neuroprotection, and neurodegeneration [50, 51]. However, the involvement of NO in neuroglial interactions is weakly studied.

PDT rapidly, within the first minute, stimulates NO production in diverse cell lines [52, 53]. However, the reports on the role of NO on PDT-induced cell death are controversial. Some authors have demonstrated the protective effect of NO in photosensitized cells [54, 55], whereas others stated the involvement of NO in PDT-induced apoptosis [53, 56].

In order to study the role of NO in PDT-induced injury of MRN and satellite glial cells, we used different modulators of NO production including inhibitors of various NO synthase (NOS) isoforms, NO generators, and scavengers (Fig. 2b; 3b;

and 4b). In the presence of NO generators diethylamine NONOate or sodium nitroprusside (SNP), the level of PDT-induced necrosis of MRN and glial cells significantly decreased (Figs. 2b and 3b). This suggests the involvement of NO in the protection of MRN and glial cells from photoinduced necrosis. At the same time, PDT-induced apoptosis of glial cells significantly increased in the presence of NONOate (Fig. 4b). Therefore, NO has mediated apoptosis of glial cells. In the presence of nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of both neuronal and inducible NO synthase isoforms (nNOS and iNOS, respectively), the level of PDT-induced necrosis of glial cells but not neurons increased (Fig. 3b), which suggests the involvement of NOS in the anti-necrotic processes in glial cells. Non-specific NOS inhibitors, L-NAME and nitro-L-arginine (L-NNA), as well as *S*-methylisothiourea (SMT) and L-*N*-iminoethyl-lysine (L-NIL), selective inhibitors of the inducible NOS isoform (iNOS), reduced PDT-induced apoptosis of glial cells (Fig. 4b). This also indicates the pro-apoptotic activity of NO, which was possibly generated by iNOS. On the contrary, iNOS was not involved in PDT-induced necrosis of neurons and glia. Therefore, the anti-necrotic activity of NO in glial cells was associated rather with activation of nNOS than iNOS. Effects of NO could be mediated by protein kinase G (PKG), which is activated by cyclic GMP that is produced by guanylate cyclase in a NO-dependent manner. In fact, KT-5823, an inhibitor of protein kinase G, also reduced PDT-induced apoptosis of glial cells (Fig. 4b). Protein kinase G possibly participated also in necrosis of glial cells and MRN, because its inhibition decreased the level of PDT-induced necrosis of these cells (Figs. 2b and 3b).

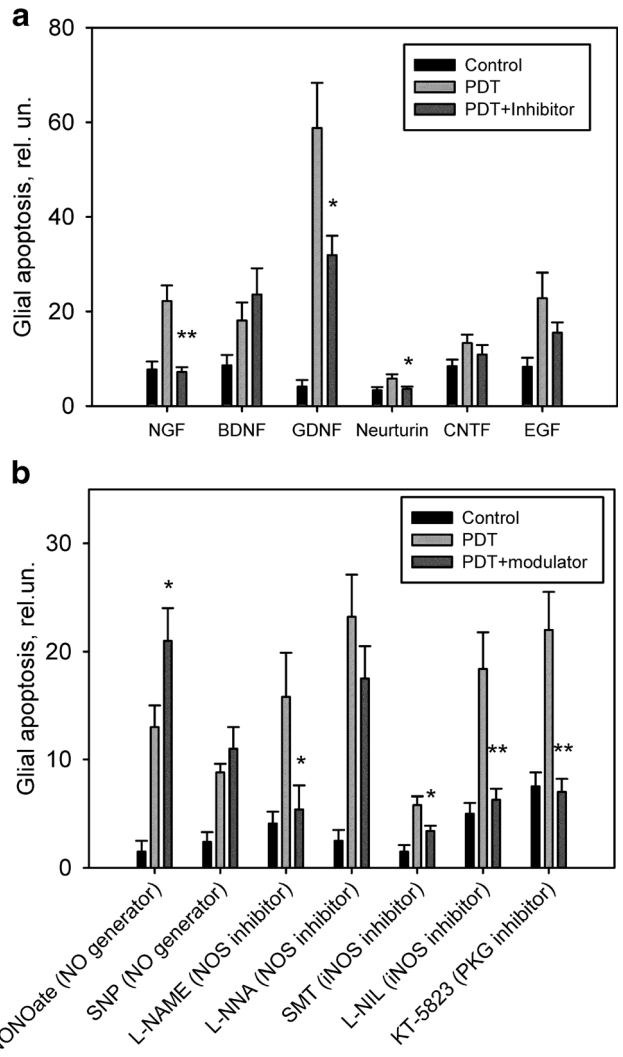
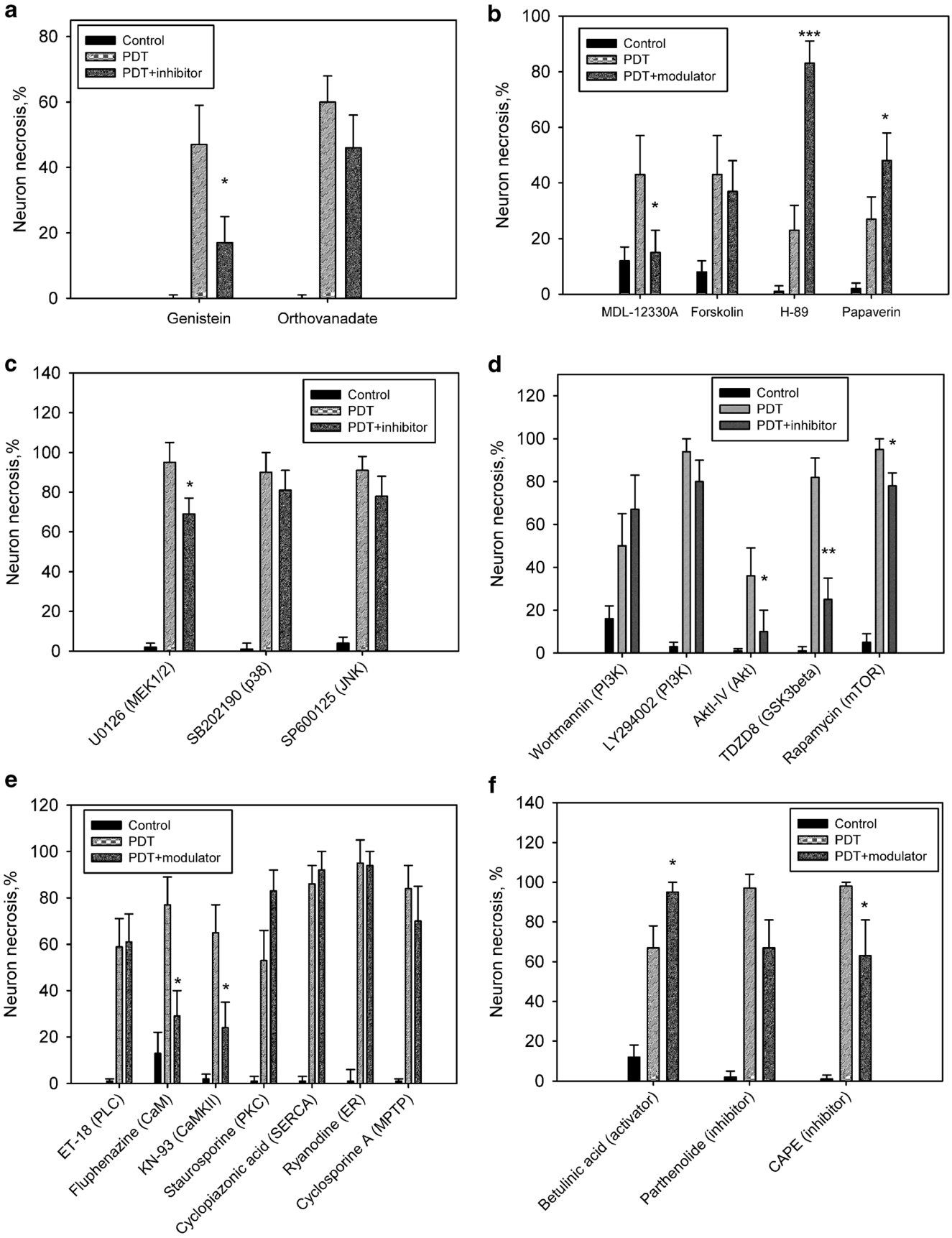


Fig. 4 The levels of apoptotic glial cells (rel. un.) in the photosensitized crayfish stretch receptor in the presence of **a** neurotrophic factors: NGF (100 ng/ml), BDNF (1 ng/ml), NGF (10 ng/ml), neurturin (10 ng/ml), CNTF (50 ng/ml), and EGF (100 ng/ml), or **b** modulators of NO production: NO generators NONOate (100 μM) and SNP (10 μM) or inhibitors of NO synthase L-NAME (1 mM), L-NNA (1 mM), SMT (50 μM), and L-NIL (100 μM) or inhibitor of protein kinase G KT-5823 (10 μM). Significant difference from PDT-treated preparation in the absence of modulators, *<0.05; **<0.01 (one-way ANOVA)

NO synthase needs NADPH as cofactor, which is produced by NADPH diaphorase. The expression of NADPH diaphorase serves as a marker of NO synthase in cells and tissues. Its activity displays NO production [57, 58]. Our histochemical study has shown that in the crayfish stretch receptor, NADPH diaphorase is located mainly in the MRN perikaryon but not in the glial envelope. Just after PDT, its activity increased significantly by 80 % but decreased in 4 h post-treatment 2.7 times relatively to control preparations (Kovaleva and Uzdensky, unpublished data).

Therefore, PDT first activated NOS in the neuronal body, and then, 4 h later, NOS activity decreased below the initial level. NO synthesized by nNOS in the MRN body during a 30-min photodynamic impact diffused to glial cells and contributed to their protection from PDT-induced necrosis. As suggested above, neuronal iNOS was involved in apoptosis

Fig. 5 The levels of necrotic neurons (%) in the photosensitized crayfish stretch receptor in the presence of **a** inhibitor of tyrosine kinase genistein (50 μM) or inhibitor of tyrosine phosphatase sodium orthovanadate (500 μM); **b** inhibitor of adenylate cyclase MDL-12330A (50 nM), activator of adenylate cyclase forskolin (10 μM), inhibitor of protein kinase A H-89 (1 μM), or inhibitor of phosphodiesterase papaverine (1 μM); **c** inhibitors of MEK1/2 U0126 (2 μM), MAP kinase p38 SB202190 (5 μM), or MAP kinase JNK SP600125 (10 μM); **d** inhibitors of PI3K wortmannin (120 nM) and LY294002 (10 μM), Akt inhibitor IV (1 μM), inhibitor of GSK3β TDZD-8 (2 μM), or inhibitor of mTOR rapamycin (50 μM); **e** inhibitor of phospholipase Cγ ET-18 (20 μM), inhibitor of calmodulin fluphenazine (1 μM), inhibitor of calmodulin kinase II KN-93 (2 μM), inhibitor of protein kinase C staurosporine (1 nM), inhibitor of Ca^{2+}-ATPase cyclopiazonic acid (30 μM), blocker of ryanodine-sensitive channels ryanodine (10 μM), or blocker of mitochondrial permeability transition pores cyclosporine A (5 μM); and **f** NF-κB activator betulinic acid (5 μM) or NF-κB inhibitors parthenolide (20 μM) and CAPE (30 μM). Significant difference from PDT-treated preparation in the absence of modulators, *<0.05; **<0.01 (one-way ANOVA)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Forskolin
H-89
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SP600125 (JNK)
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LY294002 (PI3K)
AktI-IV (Akt)
TDZD8 (GSK3beta)
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KN-93 (CaMKII)
Staurosporine (PKC)
Cyclopiazonic acid (SERCA)
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Neuron necrosis, %
Betulinic acid (activator)
Parthenolide (inhibitor)
CAPE (inhibitor)

of glial cells that was developed later, 6–8 h after PDT. Since the activity of neuronal NADPH diaphorase and, consequently, NO production decreased 4 h after PDT, the pro-apoptotic effect of iNOS could initiate the pro-apoptotic processes during or shortly after PDT.

It should be noted that NO could not participate in the MRN-mediated protection of glial cells from PDT-induced apoptosis as reported in [44]. This role was possibly played by NGF-like and/or GDNF-like neurotrophic factors.

Involvement of Intracellular Signaling in Neuron and Glia Killing and Protection

Binding of growth factors to receptor tyrosine kinases initiates a range of intracellular signaling pathways such as (1) phospholipase Cγ and following mobilization of intracellular Ca^{2+}; (2) MAP kinase (MAPK) pathways that include ERK, JNK, and p38 MAP kinases; and (3) phosphatidylinositol-3-kinase (PI3K) and protein kinase B/Akt. They regulate cell survival, functional activity, proliferation, differentiation, and apoptosis [2].

Tyrosine Kinases and Phosphatases

Inhibition of tyrosine kinases by genistein decreased the number of necrotic neurons in the photosensitized CSR (Fig. 5a). Orthovanadate, an inhibitor of tyrosine phosphatases, which dephosphorylates tyrosine residues and thereby ceases the neurotrophic signaling, reduced necrosis of glial cells but not neurons (Fig. 6a). Therefore, tyrosine phosphorylation is involved in necrosis of neurons, but protects glial cells from PDT-induced necrosis [31]. This creates the opportunity to protect neurons or glial cells from photoinduced necrosis (but not apoptosis) selectively by genistein or orthovanadate, respectively. No significant effects of genistein or orthovanadate on PDT-induced glial apoptosis were observed (Fig. 7a). At first glance, this does not confirm the involvement of tyrosine kinase receptors in the anti-apoptotic activity of neurotrophic factors such as NGF or GDNF [29, 32]. Nevertheless, genistein, if applied in combination with NGF, almost totally eliminated the anti-apoptotic effect of NGF on photosensitized glial cells. This evidences the involvement of receptor tyrosine kinases in its anti-apoptotic activity [29].

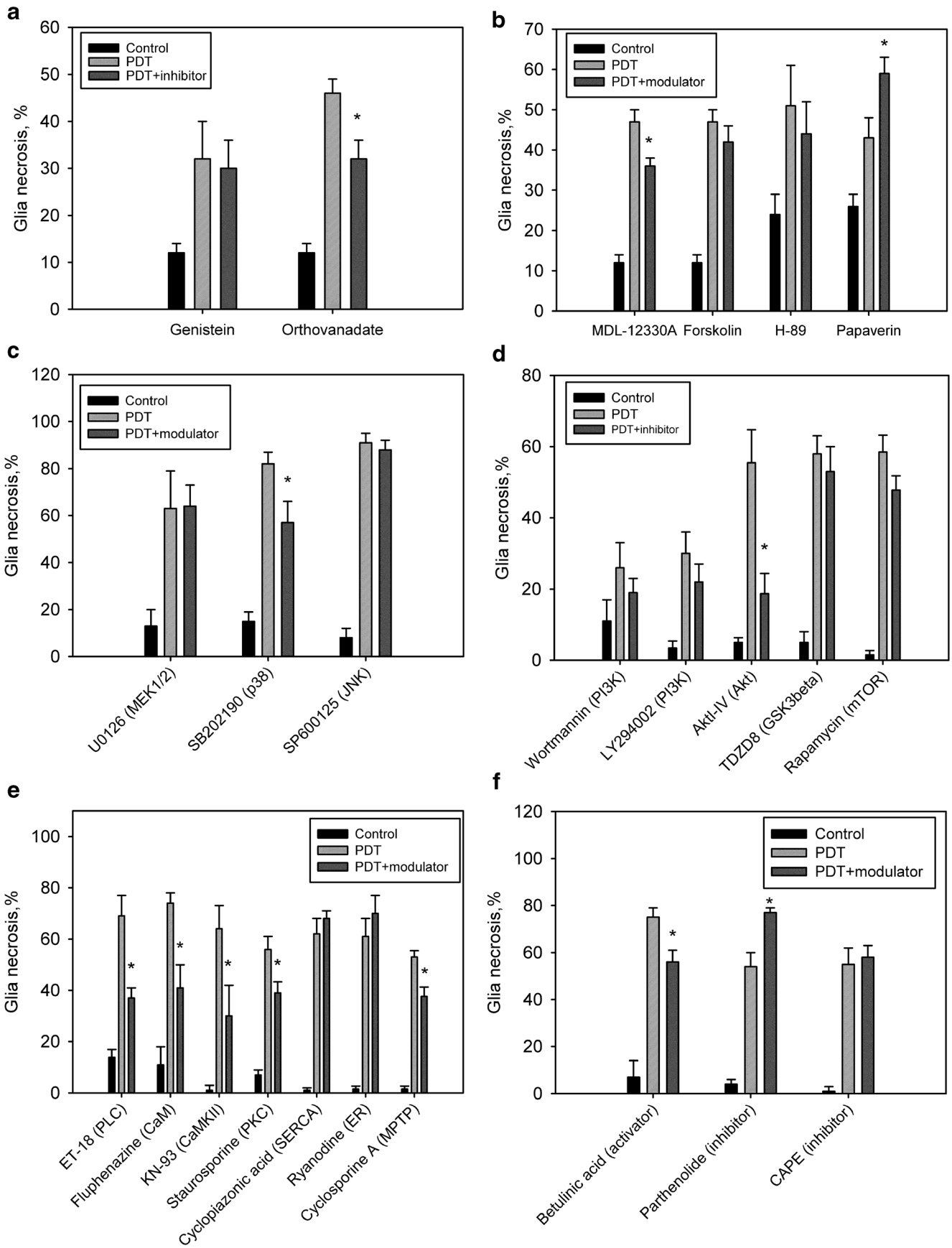
MAP Kinase Pathway

U0126, an inhibitor of mitogen-activated protein kinase kinases 1 and 2 (MEK1/2), which activates extracellular signal-regulated kinases (ERK1/2), did not influence PDT-induced necrosis and apoptosis of glial cells (Figs. 6c and 7c) but reduced necrosis of MRN (Fig. 5c) (Uzdensky et al., unpublished data). Therefore, the cascade MEK/ERK was involved in necrosis of crayfish neurons but not glial cells. It did not also regulate apoptosis of glial cells. Similarly, using SB202190, an inhibitor of another mitogen-activated protein kinase p38, we showed the involvement of this enzyme in PDT-induced necrosis of photosensitized glial cells (Fig. 6c). SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), did not influence PDT-induced death of MRN and glial cells. However, it eliminated the anti-apoptotic effect of NGF on photosensitized glial cells that confirmed its involvement in the anti-apoptotic activity of NGF [29].

PI3K/Akt Pathway

Receptor tyrosine kinases also activate the PI3K/Akt cascade that regulates cell survival and death under injurious impacts. Its protective, anti-apoptotic activity is usually associated with Akt-mediated inhibition of pro-apoptotic glycogen synthase kinase-3β (GSK-3β). However, in our experiments, PI3K inhibitors wortmannin and LY294002 did not influence PDT-induced death of MRN and glial cells (Figs. 5d, 6d, and 7d). Therefore, PI3K did not participate in PDT-induced cell death. The inhibition of Akt with Akt inhibitor IV (AktI) did not influence PDT-induced apoptosis of glial cells (Fig. 7d), but protected MRN and glial cells from PDT-induced necrosis (Figs. 5d and 6d). Therefore, Akt mediated photoinduced necrosis but not apoptosis of neurons and glial cells. TDZD-8, an inhibitor of GSK-3β, reduced significantly PDT-induced necrosis of MRN and apoptosis but not necrosis of glial cells (Figs. 5d, 6d, and 7d). This indicates involvement of GSK-3β in photoinduced necrosis of neurons and in apoptosis of glial cells. Thus, the PI3K/Akt/GSK-3β pathway as a whole did not protect crayfish neurons and glial cells. Akt and GSK-3β modulated PDT-induced death of MRN and glial cells in an uncoordinated manner, not as an integrated signaling pathway but separately. We have suggested that the pro-necrotic

Fig. 6 The levels of necrotic glial cells (%) in the photosensitized crayfish stretch receptor in the presence of **a** inhibitor of tyrosine kinase genistein (50 μM) or inhibitor of tyrosine phosphatase sodium orthovanadate (500 μM); **b** inhibitor of adenylate cyclase MDL-12330A (50 nM), activator of adenylate cyclase forskolin (10 μM), inhibitor of protein kinase A H-89 (1 μM), or inhibitor of phosphodiesterase papaverine (1 μM); **c** inhibitors of MEK1/2 U0126 (2 μM), MAP kinase p38 SB202190 (5 μM), or MAP kinase JNK SP600125 (10 μM); **d** inhibitors of PI3K wortmannin (120 nM) and LY294002 (10 μM), Akt inhibitor IV (1 μM), inhibitor of GSK3β TDZD-8 (2 μM), or inhibitor of mTOR rapamycin (50 μM); **e** inhibitor of phospholipase Cγ ET-18 (20 μM), inhibitor of calmodulin fluphenazine (1 μM), inhibitor of calmodulin kinase II KN-93 (2 μM), inhibitor of protein kinase C staurosporine (1 nM), inhibitor of Ca^{2+}-ATPase cyclopiazonic acid (30 μM), blocker of ryanodine-sensitive channels ryanodine (10 μM), or blocker of mitochondrial permeability transition pores cyclosporine A (5 μM); and **f** NF-κB activator betulinic acid (5 μM) or NF-κB inhibitors parthenolide (20 μM) and CAPE (30 μM). Significant difference from PDT-treated preparation in the absence of modulators, *<0.05; **<0.01 (one-way ANOVA)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Glia necrosis, %
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Orthovanadate
b
Glia necrosis,%
Control
PDT
PDT+modulator
MDL-12330A
Forskolin
H-89
Papaverin
c
Glia necrosis,%
Control
PDT
PDT+modulator
U0126 (MEK1/2)
SB202190 (p38)
SP600125 (JNK)
d
Glia necrosis,%
Control
PDT
PDT+inhibitor
Wortmannin (PI3K)
LY294002 (PI3K)
Akt-IV (Akt)
TDZD8 (GSK3beta)
Rapamycin (mTOR)
e
Glia necrosis,%
Control
PDT
PDT+modulator
ET-18 (PLC)
Fluphenazine (CaM)
KN-93 (CaMKII)
Staurosporine (PKC)
Cyclopiazonic acid (SERCA)
Ryanodine (ER)
Cyclosporine A (MPTP)
f
Glia necrosis,%
Control
PDT
PDT+modulator
Betulinic acid (activator)
Parthenolide (inhibitor)
CAPE (inhibitor)

activity of Akt could be associated with its ability to inhibit anti-oxidant enzymes and stimulate generation of reactive oxygen species [27].

The pro-survival protein mTOR is known to form two protein complexes: mTORC1, which, being activated by Akt, inhibits autophagy and stimulates protein synthesis, and mTORC2 that activates Akt. The Akt/mTOR pathway is a mediator between signaling processes, metabolism, and ultrastructural changes. The inhibition of mTOR by rapamycin in our experiments partially protected neurons from PDT-induced necrosis (Fig. 5d) like Akt inhibitor IV did, and suppressed apoptosis of glial cells (Fig. 7d) (Uzdensky et al. [32], unpublished data). Hence, mTOR was involved in necrosis of neurons and in apoptosis of glial cells. Possibly, it regulated necrosis in the same signaling cascade with Akt.

Ca^{2+}-Dependent Processes

Ca^{2+} is a key regulator of numerous processes in the cell. Photodynamic impact can increase the level of cytosolic Ca^{2+} in various ways: directly through ruptures in the plasma membrane or in the membranes of mitochondria and endoplasmic reticulum (ER) that store calcium, or indirectly through signaling pathways that control calcium homeostasis. We studied the role of different Ca^{2+} mobilization mechanisms in PDT-induced necrosis and apoptosis of MRN and glial cells [33].

Binding of neurotrophic factors to receptor tyrosine kinases is known to activate phosphatidylinositol-dependent phospholipase Cγ (PI-PLC). Following liberation from the plasma membrane, inositol-3-phosphate (IP_3) stimulates Ca^{2+} release from the ER into the cytosol. In our experiments, the inhibition of PI-PLC with ET-18 significantly reduced necrosis and apoptosis of glial cells (Figs. 6e and 7e) but not neurons (Fig. 5e) [59]. This is in agreement with the well-known role of cytosolic Ca^{2+} in initiation of both necrosis and apoptosis [60]. Another mechanism of Ca^{2+} release trough ryanodine channels in ER was ineffective. Inhibition of Ca^{2+}-ATPase in ER by cyclopiazonic acid also did not influence PDT-induced death of MRN and glial cells (Figs. 5e; 6e; and 7e). Cyclosporin A, an inhibitor of mitochondrial permeability transition pores (MPT), decreased PDT-induced necrosis and apoptosis of glial cells (Figs. 6e and 7e), which indicated possible involvement of Ca^{2+} released from MPT in death of glial cells [59].

Cytosolic Ca^{2+} activates protein kinase C (PKC), calmodulin, and calmodulin-dependent protein kinase II (CaMKII) that regulate enzyme activity, metabolism, cytoskeleton remodeling, ionic transport, survival, and proliferation [35, 61]. We showed that inhibition of PKC by staurosporine decreased PDT-induced necrosis of glial cells (Fig. 7e). Inhibition of calmodulin or CaMKII by fluphenazine or KN-93, respectively, decreased PDT-induced necrosis of MRN and glial cells (Figs. 5e and 6e) but not glial apoptosis (Fig. 7e). This indicates the involvement of these enzymes in photoinduced necrosis of neurons and glia [33].

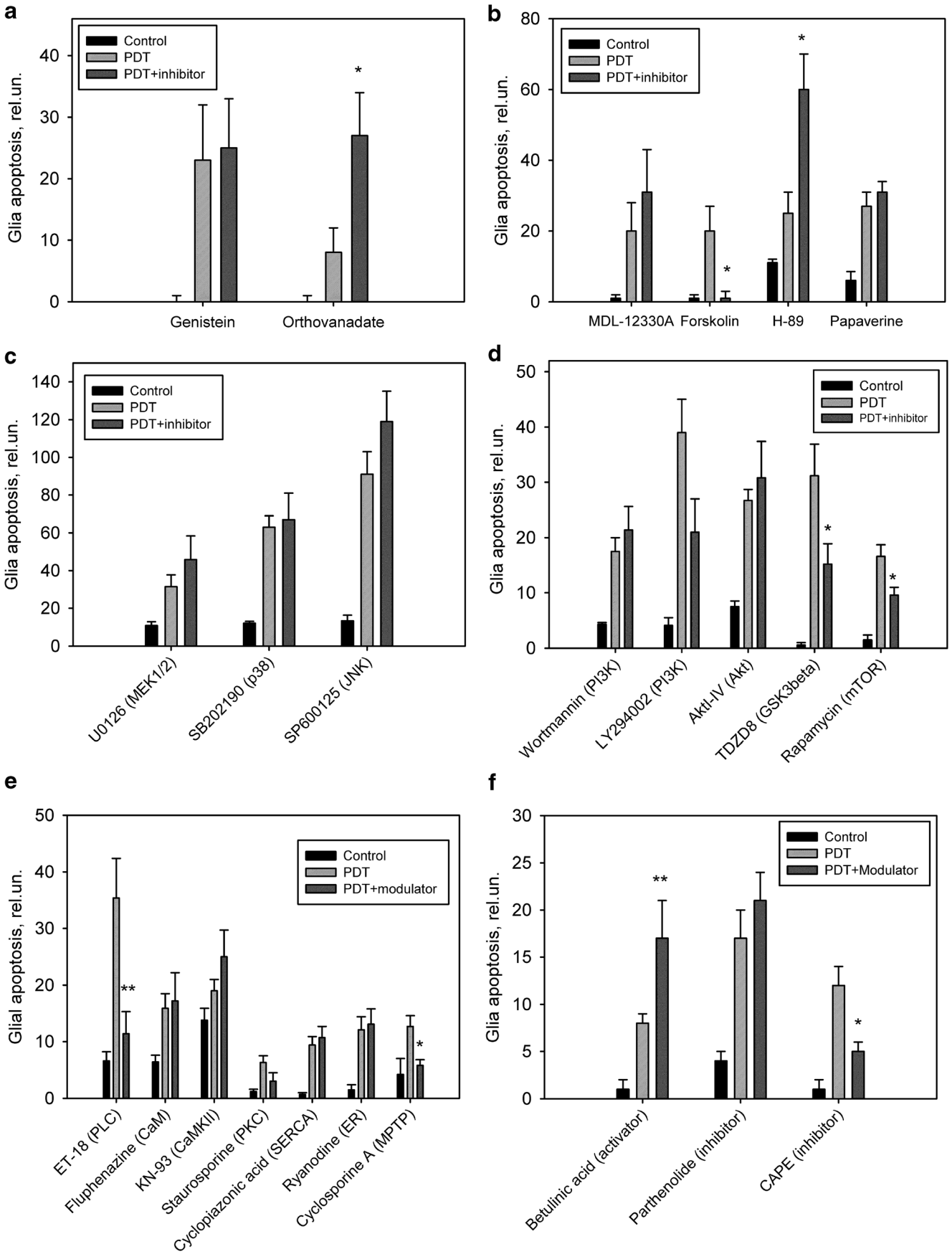
cAMP/Protein Kinase A Pathway

The adenylate cyclase/cAMP/protein kinase A pathway is stimulated by receptors coupled with G proteins but not receptor tyrosine kinases. Nevertheless, cAMP-related enzymes played a significant role in PDT-induced necrosis of MRN and glial cells. Actually, in the presence of MDL-12330A, the inhibitor of adenylate cyclase that produces cAMP, necrosis of photosensitized neurons and glial cells significantly decreased (Figs. 5b and 6b). Papaverine, the inhibitor of phosphodiesterase that splits cAMP and stops its signaling activity, increased necrosis of neurons and glial cells (Figs. 5b and 6b). Activation of adenylate cyclase by forskolin suppressed PDT-induced apoptosis of glial cells (Fig. 7b), which indicated the anti-apoptotic activity of cAMP in glial cells. This finding correlates with the pro-apoptotic action of H-89, an inhibitor of protein kinase A (PKA) (Figs. 5b, 6b, and 7b). Therefore, cAMP-activated protein kinase A mediated protection of photosensitized glial cells [33].

NF-κB

Activation of gene expression by diverse signaling pathways is mediated by transcription factor NF-κB [62]. NF-κB is known to control PDT-induced cell death [63]. In our experiments, NF-κB mediated PDT-induced necrosis of neurons and apoptosis of glial cells. On the other hand, it protected glial cells from photodynamic necrosis. In fact, activation of NF-κB by betulinic acid increased the levels of PDT-induced necrosis of MRN (Fig. 5f) and apoptosis of glial cells (Fig. 7f). It also exerted anti-necrotic action on glial cells (Fig. 6f). In

Fig. 7 The levels of apoptotic glial cells (rel. un.) in the photosensitized crayfish stretch receptor in the presence of **a** inhibitor of tyrosine kinase genistein (50 μM) or inhibitor of tyrosine phosphatase sodium orthovanadate (500 μM); **b** inhibitor of adenylate cyclase MDL-12330A (50 nM), activator of adenylate cyclase forskolin (10 μM), inhibitor of protein kinase A H-89 (1 μM), or inhibitor of phosphodiesterase papaverine (1 μM); **c** inhibitors of MEK1/2 U0126 (2 μM), MAP kinase p38 SB202190 (5 μM), or MAP kinase JNK SP600125 (10 μM); **d** inhibitors of PI3K wortmannin (120 nM) and LY294002 (10 μM), Akt inhibitor IV (1 μM), inhibitor of GSK3β TDZD-8 (2 μM), or inhibitor of mTOR rapamycin (50 μM); **e** inhibitor of phospholipase Cγ ET-18 (20 μM), inhibitor of calmodulin fluphenazine (1 μM), inhibitor of calmodulin kinase II KN-93 (2 μM), inhibitor of protein kinase C staurosporine (1 nM), inhibitor of Ca^{2+}-ATPase cyclopiazonic acid (30 μM), blocker of ryanodine-sensitive channels ryanodine (10 μM), or blocker of mitochondrial permeability transition pores cyclosporine A (5 μM); and **f** NF-κB activator betulinic acid (5 μM) or NF-κB inhibitors parthenolide (20 μM) and CAPE (30 μM). Significant difference from PDT-treated preparation in the absence of modulators, *<0.05; **<0.01 (one-way ANOVA)


a
Control
PDT
PDT+inhibitor
Glia apoptosis, rel.un.
Genistein
Orthovanadate
b
MDL-12330A
Forskolin
H-89
Papaverine
c
U0126 (MEK1/2)
SB202190 (p38)
SP600125 (JNK)
d
Wortmannin (PI3K)
LY294002 (PI3K)
AktI-IV (Akt)
TDZD8 (GSK3beta)
Rapamycin (mTOR)
e
PDT+modulator
Glial apoptosis, rel.un.
ET-18 (PLC)
Fluphenazine (CaM)
KN-93 (CaMKII)
Staurosporine (PKC)
Cyclopiazonic acid (SERCA)
Ryanodine (ER)
Cyclosporine A (MPTP)
f
Betulinic acid (activator)
Parthenolide (inhibitor)
CAPE (inhibitor)

line with these data, inhibition of NF-κB by caffeic acid phenethyl ester (CAPE) reduced necrosis of photosensitized neurons (Fig. 5f) and apoptosis of glial cells (Fig. 7f). Another NF-κB inhibitor parthenolide increased necrosis of glial cells (Fig. 6f) [34].

Signaling and Potential Protectors of Neurons and Glial Cells from Photooxidative Damage

The search of glial and neuron protectors should be based on a thorough understanding of the molecular targets of PDT, mechanisms of PDT-induced cell death, and involvement of diverse signaling pathways in the regulation of cell resistance.

The results of our studies have demonstrated that diverse intercellular and intracellular signaling pathways control PDT-induced necrosis or apoptosis of isolated neurons and satellite glial cells [15, 27–33, 59]. These data are summarized in the Table 1.

It is of interest that not only apoptosis but also necrosis caused by a moderate impact is controlled by various signaling enzymes. Therefore, necrosis that has been traditionally considered as a catastrophic and passive cell death mode may be regulated by the signal transduction system, and chemical modulators of signaling proteins can modulate it. The data about controlled necrosis have been accumulated over the past several years [64–68]. Moreover, the specific program for implementation of necrosis has been recently discovered. The programmed necrosis was called as necroptosis [69]. The problem of control of neuronal death is highly significant for neuropathology. In stroke, for example, necrosis is the main cell death mechanism [70]. Its pharmacological suppression can limit brain damage.

The primary PDT targets in the cell are known to be the plasma membrane and the membranes of intracellular organelles where the photosensitizer is accumulated [13–15]. Upon light exposure, these membranes are injured. This deteriorates ion homeostasis and induces swelling and dysfunction of cellular organelles. The shifts in neuronal activity and swelling of some mitochondria, ER, and Golgi cisterns have been observed in our experiments within the first minutes of photodynamic impact. Longer light exposure completely abolishes neuronal activity and strongly disturbs the ultrastructure of intracellular organelles [15, 24, 30].

Ca^{2+} influx though the plasma membrane and its release into the cytosol from Ca^{2+}-storing organelles (mitochondria, ER, dictyosomes) are the critical events in PDT-induced cell injury [15, 71, 72]. Actually, PDT rapidly, within the first minute, increases the cytosolic Ca^{2+} level [73]. At the ultrastructural level, primary alterations in photosensitized neurons and glial cells were observed in mitochondria, ER, and Golgi apparatus [24], which, being damaged, liberate calcium ions. Cytosolic Ca^{2+} activates many signaling and proteolytic enzymes [2] and induces necrosis and/or apoptosis [60, 74]. In our experiments, pharmacological agents preventing the liberation of stored Ca^{2+} (ET-18 and cyclosporine A) reduced apoptosis of glial cells (Fig. 7e) [33, 59]. Ca^{2+}-dependent enzymes such as calmodulin, CaMKII, and protein kinase C were involved in PDT-induced necrosis of MRN and glial cells (Figs. 5e and 6e) [33]. So, Ca^{2+} could be a primary intracellular initiator of signaling processes that determine the fate of photosensitized cells.

The present results have demonstrated that different intracellular signaling pathways such as stress-induced MAP kinases p38 or JNK; adenylate cyclase/cAMP/PKA; Akt/mTOR; and Ca^{2+}-dependent, GSK-3β-dependent, and NF-κB-dependent cascades were involved in the regulation of survival or PDT-induced death of crayfish neurons and glia. Some of them could be primarily initiated by the intracellular signals like Ca^{2+} or ROS, whereas others were triggered by extracellular signals such as neurotrophic factors or NO. This broad spectrum of inhibitors or activators of various signaling proteins involved in regulation of cell survival and death provides the set of potential pro- and anti-necrotic or apoptotic agents for selective protection or enhancement of damage of neurons or glial cells subjected to photoinduced oxidative stress.

The difference in signaling mechanisms that control survival and death of neurons and glia may be associated with different sets of signaling proteins and different organization of signaling networks in these cells. It would be very desirable to find pharmacological tools that increase selectively the photosensitivity of glioma tissue and protect simultaneously surrounding normal neurons and glial cells. Of course, the biochemistry of mammalian cells differs in some details from that of invertebrates, but this difference may be not very significant and some of these results may be valid for the mammalian nervous system.

Intercellular neuroglial signaling based on neurotrophic factors NGF, neurturin, and GDNF but not NO-mediated signaling has protected crayfish glial cells from photoinduced necrosis and apoptosis. However, the mechanisms of these effects remained unclear. Since inhibition of tyrosine kinases by genistein did not influence PDT-induced necrosis and apoptosis of glial cells, receptor tyrosine kinases did not participate in the glia-protective effects of these neurotrophic factors. We also did not observe significant involvement of PI3K and MEK1/2 that are also activated by receptor tyrosine kinases upon binding of neurotrophic factors in neurotrophin-mediated protection of glial cells. The PLC/Ca^{2+}-mediated pathway that can be also activated by receptor tyrosine kinases mediated necrosis and apoptosis of glial cells but not their protection. The protective effect of GDNF was possibly associated with other mechanisms associated with protection of intracellular organelles and normalization of metabolism. The details of these processes have to be disclosed in the future.

Table 1 Modulation of PDT-induced death of the crayfish neurons and glial cells by neurotrophic factors and inhibitors or activators of signaling proteins

Cell death modality	Signaling proteins	Protection	Enhancement	References
Necrosis of neurons	Neurotrophic factor	GDNF		[32]
	Tyrosine kinase	Genistein		[31]
	MEK1/2	U016		Uzdensky et al. (unpublished data)
	Protein kinase B/Akt	Akt inhibitor IV		[27]
	GSK3β	TDZD-8		[27]
	mTOR	Rapamycin		Uzdensky et al. (unpublished data)
	Calmodulin	Fluphenazine		[33]
	Calmodulin kinase II	KN-93		[33]
	NF-κB inhibitor	CAPE		[34]
	NF-κB activator		Betulinic acid	[34]
	Adenylate cyclase inhibitor	MDL-12330A		[31]
	Protein kinase A		H-89	[33]
	Phosphodiesterase		Papaverine	[33]
	NO generator	NONOate		[28]
Necrosis of glial cells	Neurotrophic factors	NGF GDNF Neurturin		[29, 32]
	Tyrosine phosphatase	Orthovanadate		[31]
	MAP kinase p38	SB202190		[29]
	Protein kinase B/Akt	Akt inhibitor IV		[27]
	Calmodulin	Fluphenazine		[33]
	Calmodulin kinase II	KN-93		[33]
	Protein kinase C	Staurosporine		[33]
	Mitochondrial permeability transition pores	Cyclosporine A		[59]
	NF-κB activator	Betulinic acid		[34]
	NF-κB inhibitor		Parthenolide	[34]
	Adenylate cyclase inhibitor	MDL-12330A		[31]
	Phosphodiesterase		Papaverine	[33]
	NO generator	NONOate		[28]
	NO generator	SNP		[28]
	Protein kinase G	KT-5823		[28]
Apoptosis of glial cells	Neurotrophic factors	NGF GDNF Neurturin		[29, 32]
	Tyrosine phosphatase		Orthovanadate	[31]
	GSK3β	TDZD-8		[27]
	mTOR	Rapamycin		Uzdensky et al. (unpublished data)
	Phospholipase Cγ	ET-18		[59]
	Mitochondrial permeability transition pores	Cyclosporine A		[59]
	NF-κB inhibitor	CAPE		[34]
	NF-κB activator		Betulinic acid	[34]
	Adenylate cyclase activator	Forskolin		[31]
	Protein kinase A		H-89	[33]
	NO generator		NONOate	[28]
	NOS	L-NAME		[28]
	iNOS	SMT		[28]
	iNOS	L-NIL		Kovaleva et al. (unpublished data)
	Protein kinase G	KT-5823		[28]

Thus, the results obtained on the simple model object, isolated crayfish stretch receptor neurons and satellite glial cells, revealed a variety of inhibitors or activators of diverse intracellular signaling proteins as well as intercellular signaling molecules, NO and neurotrophic factors (NGF, GDNF, and neurturin), that can protect glial cells and/or neurons from PDT-induced oxidative injury. Of course, these results cannot be translated directly to the mammalian nervous system because the biochemistry of mammalian cells can differ in some details from that of invertebrates. Nevertheless, this difference may be not very high and some of these results may be valid for the mammalian nervous system.

Acknowledgments The work was supported by grants of the Russian Scientific Foundation (14-15-00068) and Russian Foundation for Basic Research (05-04-48440; 08-04-01322; 11-04-01476; 14-04-00741).

Conflict of Interest The authors declare that they have no competing interests.

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