Journal of Innovative Optical Health Sciences
Vol. 4, No. 4 (2011) 429–435

DOI: 10.1142/S1793545811001605

CHEMICAL MODULATION OF PHOTODYNAMIC INJURY OF GLIAL CELLS

M. A. KOMANDIROV, E. A. KNYAZEVA, Y. P. FEDORENKO,
M. V. RUDKOVSKII, E. V. BEREZHNAYA, V. D. KOVALEVA
and A. B. UZDENSKY*
Department of Biophysics and Biocybernetics
Institute of Neurocybernetics
Southern Federal University, 194/1 Stachky Avenue
Rostov-on-Don, 344090, Russia
**auzd@yandex.ru*

Accepted 7 May 2011

Photodynamic therapy based on photogeneration of cytotoxic singlet oxygen and following oxidative stress is currently used in neuro-oncology for destruction of brain tumors. However, along with a tumor, it damages healthy neurons and glial cells. We studied the involvement of the glutamate-related signaling pathway in photodynamic damage to normal glial cells in the crayfish stretch receptor. This model object consists of a single neuron surrounded by glial cells. It was photosensitized with alumophthalocyanine Photosens and irradiated by the diode laser (670 nm). Application of enzyme inhibitors and ion channels modulators showed that exogenous L-glutamate decreased photoinduced apoptosis of crayfish glial cells. The natural neuroglial mediator N-acetylaspartylglutamate, which releases glutamate after splitting by glutamate carboxypeptidase II, also inhibited photoinduced apoptosis. Inhibition of glutamate carboxypeptidase II, oppositely, enhanced glial apoptosis. This confirmed the antiapoptotic activity of glutamate. Glutamate agonist NMDA or inhibitor of NMDA receptors MK801 did not influence photodynamic death of glial cells, i.e., these receptors did not participate in glial apoptosis. Inhibition of metabotropic glutamate receptors mGluRI with AP-3 reduced PDT-induced apoptosis of glial cells. Thus, chemical modifiers of various signaling processes can modulate photoinduced necrosis or apoptosis of glial cells and thus modify efficiency of photodynamic therapy.

Keywords: Glia; necrosis apoptosis; glutamate.

1. Introduction

In photodynamic therapy (PDT), the energy of a photoexcited dye molecule is transferred to oxygen to transform it into the highly reactive singlet state. Intense generation of singlet oxygen triggers oxidation and peroxidation of membrane lipids and proteins, free radical production, oxidative stress and results in cell death. This effect is efficiently used in oncology for destruction of malignant cells.[1]

Treatment of brain tumors, specifically gliomas, is a difficult problem because of their resistance to chemotherapy and radiotherapy and impossibility to remove surgically a large volume of surrounding nerve tissue without impairment of brain functions.

*Corresponding author.

PDT is currently considered as a promising adjuvant method for selective destruction of brain tumors.[2−4] However, not only malignant cells but also neighboring neurons and glial cells are damaged during PDT. Therefore, photodynamic injury of normal neurons and glia should be carefully studied along with study of PDT effect on glioma cells.

The experimental data obtained in the mammalian brain studies are not easily interpreted because of high complexity of interneuronal and neuroglial interactions and difficulties in their identification. The nervous systems of invertebrates that consist of few identified neurons are much simpler. These nervous systems are better suitable for investigations of cell response mechanisms. The crayfish stretch receptor that comprises only two single neurons surrounded by glial cells is a simple but informative model object for such study.[5−8]

Intercellular interactions are of importance for responses of complex tissues to external impacts. As has been shown earlier, the crayfish stretch receptor neuron protects the surrounding glial cells from PDT-induced apoptosis.[9] This effect could be mediated by intercellular signaling molecules, primary messengers, that influence survival of neighboring cells. In the nervous system, neuromediators and neurotrophic factors are the major intercellular molecular signals. After recognition by surface receptors, they initiate cascades of secondary intracellular signaling processes, which form the cell response and determine the cell death mode, apoptosis or necrosis.[10,11] Modification of signaling pathways may either enhance cell death or protect cells.[11−14] Application of nerve growth factor NFG has been recently demonstrated to protect crayfish glial cells from PDT-induced apoptosis.[15]

Neuromediator glutamate may potentially influence survival of neurons and glial cells.[16−18] It is known to be involved in neuroglial interactions in the crayfish nervous system.[19] In fact, electric stimulation has been shown to release N-acetylaspartylglutamate (NAAG) from the crayfish giant axons. NAAG is then split in the intercellular medium by N-acetylaspartyl and L-glutamate. Following recognition of L-glutamate by metabotropic and ionotropic glutamate receptors intracellular signaling cascades in glial cells are initiated.[19]

In the present work we used specific inhibitors, ion channel agonists and blockers to study the possible involvement of NAAG, glutamate and some of its receptors in PDT-induced death of glial cells in the isolated crayfish stretch receptor used as a model object.

2. Materials and Methods

The following chemicals were used: sodium L-glutamate; its agonist N-methyl-D-aspartic acid (NMDA); antagonist of NMDA glutamate receptors (+)-MK-801 hydrogen maleate (MK801); AP-3, antagonist of metabotropic glutamate receptors; NAAG; PBDA, an inhibitor of glutamate carboxypeptidase II; fluorochromes propidium iodide and Hoechst 33342. All chemicals were supplied by the company Saflab (Moscow, Russia), the Sigma-Aldrich distributor. Photosensitizer Photosens, a mixture of sulphonated aluminum phthalocyanines, $AlPcS_n$, where mean $n = 3.1$, was a gift from NIOPIK (Moscow, Russia).

Each abdominal segment of the crayfish *Astacus leptodactylus* contains two bilateral pairs of stretch receptors. Each segment consists of two receptors, rapidly and slowly adapting, in which the sensory neurons are mounted on the appropriate receptor muscles. The rapidly adapting neuron does not fire at a constant extension of the receptor muscle, whereas the slowly adapting neuron fires regularly with a constant rate proportional to extension of the receptor muscle. The crayfish stretch receptors were isolated as described earlier.[20] Then these receptors were placed into a plexiglass chamber equipped with a device for receptor muscle extension and filled with 2 ml of van Harreveld saline (mM: NaCl, 205; KCl, 5.4; $NaHCO_3$, 0.2; $CaCl_2$, 13.5; $MgCl_2$, 5.4; pH 7.2−7.4). Neuron spikes were recorded extracellularly from axons by glass pipette suction electrodes, amplified and digitized by the analog−digital converter L-761 (L-Card, Moscow, Russia), and processed by a personal computer using the home-made software that provided continuous monitoring of firing. Experiments were carried out at 25 ± 4°C.

The experiments were performed as follows. After stretch receptor isolation and 30 min control recording of neuronal activity, Photosens (10^{-7} M) and a studied chemical agent were added into the chamber with an interval of 3−5 min. After following 30-min incubation, the cells were irradiated 30 min with the diode laser (670 nm, 0.4 W/cm^2; "Polus", Moscow, Russia). A laser beam of diameter 3 mm was introduced, so that the neuronal body and a significant part of the axon were irradiated.

The concentrations of chemical modulators were usually chosen to be approximately two times lower than the predetermined concentrations, which disturbed neuron firing for 3−4 h in the darkness. Photosens and chemical modulators were present in the chamber during and after irradiation.

In order to visualize dead cells, 20 μM propidium iodide and 10−20 μM Hoechst 33342 (both from Sigma-Aldrich) were added into the experimental chamber at 8 h post-irradiation. This time interval was sufficient for apoptosis development.[6,7] Then the preparations were washed with van Harreveld saline, fixed with 0.2% glutaraldehyde, repeatedly washed and mounted in glycerol. Fluorescent images were acquired using the fluorescence microscope Lumam-I3 (LOMO, Sankt-Petersburg, Russia) equipped with a digital photocamera. Propidium iodide, a membrane impermeable fluorochrome, imparts red fluorescence to the nuclei of necrotic cells with the compromised plasma membrane. Hoechst 33342 imparts blue fluorescence to the nuclear chromatin. It visualizes intact nuclei of living cells and fragmented nuclei of apoptotic cells (Fig. 1). Nucleus fragmentation is the final stage of apoptosis when the no-return point has passed.

As described earlier,[6,7] red nuclei of necrotic glial cells stained by propidium iodide were counted in the predetermined standard field around SRN soma ($100 \times 100\,\mu m^2$) so that the neuron nucleus was situated in its center. Fragmented nuclei of apoptotic glial cells were counted around the proximal 2-mm axon fragment where glial apoptosis was more pronounced than around the neuron body. Their mean number representing the level of glial apoptosis was expressed below as relative units. The Student's *t*-criterion was used for statistical evaluation of the difference between independent experimental groups. Data are presented as mean ± SEM.

3. Results

Neither laser radiation nor Photosens acting separately changed significantly neuronal activity and survival after stretch receptor isolation. However, their combined action, i.e. photodynamic effect, caused inhibition and abolition of neuronal activity, necrosis of neurons and glial cells and apoptosis of glial cells (but not neurons) as in previous experiments.[5−8] Like previously, apoptotic fragmentation of neuronal nuclei was not observed in stretch receptor neurons possibly due to intrinsic block of this program.[5−8]

Application of exogenous L-glutamate (100 μM) significantly reduced PDT-induced apoptosis but not necrosis of glial cells (Tables 1 and 2).

In order to study the possible mechanism of the antiapoptotic effect of glutamate on crayfish glial cells, we studied the role of NAAG, the natural producer of glutamate in the crayfish nervous system, and some glutamate receptors in photodynamic injury of glial cells. Exogenous NAAG (100 μM), which releases glutamate into the intercellular medium after splitting by glutamate carboxypeptidase II,[19] also significantly decreased PDT-induced apoptosis of glial cells (Table 2). This confirms the antiapoptotic effect of glutamate on crayfish glial cells. Additionally, NAAG application decreased significantly PDT-induced necrosis of glial cells (Table 1).

Inhibition of glutamate carboxypeptidase II, which splits NAAG and thus releases glutamate into the intercellular medium, with PBDA (3 μM) did not influence PDT-induced necrosis of glial cells (Table 1). However, PBDA application significantly increased the level of photoinduced apoptosis of glial cells (Table 2). This confirmed involvement of this enzyme in the antiapoptotic action of NAAG.

Application of glutamate agonist NMDA (1 mM) or MK-801 (5 μM), the blocker of ionotropic NMDA receptors, did not influence PDT-induced necrosis and apoptosis of glial cells (Tables 1 and 2). Therefore, ionotropic NMDA channels did not participate in PDT-induced death of glial cells.

AP-3 (10 or 100 μM), the antagonist of the type-I metabotropic glutamate receptors (mGluRI),[21,22] significantly reduced PDT-induced apoptosis but not necrosis of glial cells (Tables 1 and 2). This could indicate involvement of metabotropic glutamate receptors in PDT-induced apoptosis of crayfish glial cells.

4. Discussion

The present experiments have demonstrated that exogenous L-glutamate protects crayfish glial cells from PDT-induced apoptosis but not necrosis. Can this mechanism work in the natural conditions? It is known that intensely firing crayfish axons secrete NAAG into the intercellular medium, where it is split by glutamate carboxypeptidase II by N-acetylaspartyl and L-glutamate. The latter is then

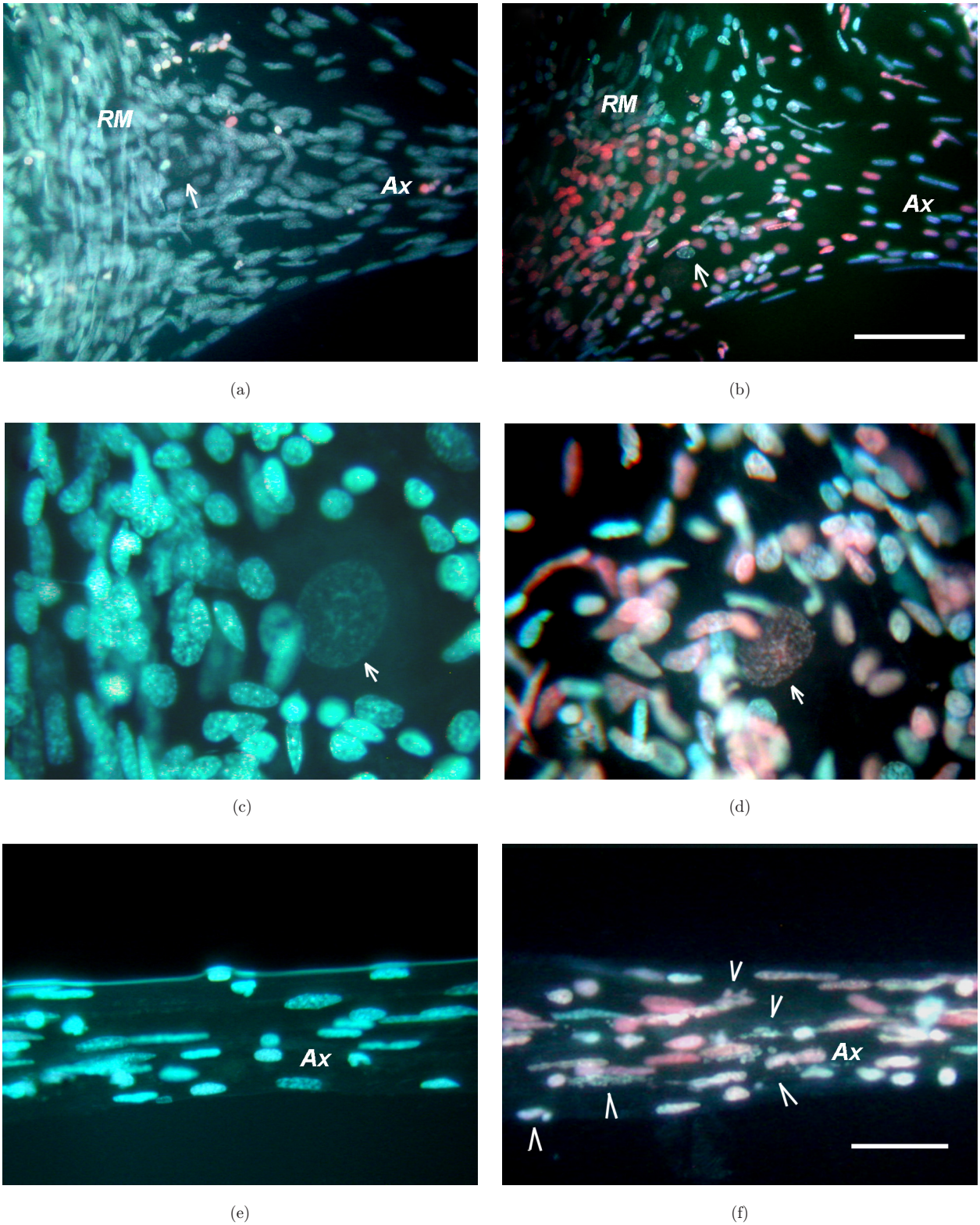

Fig. 1. The nuclear morphology of the control (a, c and e) and photosensitized (b, d and f) stretch receptor neurons and satellite glial cells. At 8 h after photodynamic treatment, the preparation was double stained with cell-impermeable propidium iodide that imparts red fluorescence to the nuclei of necrotic cells with the compromised plasma membrane and with Hoechst 33342 that imparts blue fluorescence to all cell nuclei. Arrows on (a–d) indicate the neuronal nuclei. Fragmented nuclei on (f) marked by arrowheads belong to apoptotic glial cells surrounding the proximal axon region. *Ax*, axon; *RM*, receptor muscle. The scale bar on (b) corresponds to 40 μm on (a) and (b) (objective 20×); the scale bar on (f) corresponds to 20 μm on (c–f) (objective 40×, WI).

Table 1. Effect of glutamate and modulators of glutamate receptors on the level of necrosis of crayfish glial cells after photodynamic treatment.

		Necrosis level, %	
Protein	Modulator, concentration	PDT	PDT + modulator
Ionotropic and metabotropic glutamate receptors	L-glutamate, 100 μM	$43 \pm 5(9)$	$43 \pm 6(9)$
Ionotropic and metabotropic glutamate receptors	NAAG, 100 μM	$46 \pm 6(11)$	$28 \pm 5(10)$*
Glutamate carboxypeptidase II	PBDA, 3 μM	$52 \pm 7(7)$	$37 \pm 7(9)$
Ionotropic glutamate receptor	NMDA, 1 mM	$46 \pm 9(6)$	$36 \pm 5(10)$
Ionotropic NMDA receptor	MK-801, 5 μM	$45 \pm 4(14)$	$54 \pm 7(13)$
Metabotropic glutamate receptor	AP-3, 10 μM	$37 \pm 7(7)$	$33 \pm 9(9)$
	AP-3, 100 μM	$37 \pm 5(10)$	$28 \pm 8(8)$

Percent of red nuclei of necrotic glial cells stained by propidium iodide was calculated in the predetermined standard field ($100 \times 100\,\mu m^2$) around the neuron body. The data are expressed as mean ± SEM. The number of the experiments is given in brackets. *$p < 0.05$.

Table 2. Effect of glutamate and modulators of glutamate receptors on the level of apoptosis of crayfish glial cells after photodynamic treatment.

		Number of apoptotic glial nuclei	
Protein	Modulator, concentration	PDT	PDT + modulator
Ionotropic and metabotropic glutamate receptors	L-glutamate, 100 μM	$11 \pm 1(10)$	$6 \pm 1(9)$**
Ionotropic and metabotropic glutamate receptors	NAAG, 100 μM	$10 \pm 1(11)$	$6 \pm 1(11)$*
Glutamate carboxypeptidase II	PBDA, 3 μM	$11 \pm 4(6)$	$31 \pm 8(7)$*
Ionotropic glutamate receptor	NMDA, 1 mM	$4 \pm 1(8)$	$3 \pm 1(11)$
Ionotropic NMDA receptor	MK-801, 5 μM	$13 \pm 2(14)$	$9 \pm 2(13)$
Metabotropic glutamate receptor	AP-3, 10 μM	$10 \pm 1(7)$	$5 \pm 1(12)$***
	AP-3, 100 μM	$6 \pm 1(10)$	$4 \pm 1(8)$*

The number of fragmented nuclei of apoptotic glial cells was counted around the proximal 2 mm axon fragment. The data are expressed as mean ± SEM. The number of the experiments is given in brackets. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

recognized by ionotropic and metabotropic glutamate receptors on the surface of glial cells.[19] Application of exogenous NAAG in the present experiments prevented PDT-induced apoptosis of glial cells similar to the action of glutamate. Oppositely, glial apoptosis was augmented when glutamate carboxypeptidase II, which splits NAAG, was inhibited by PBDA. This confirmed involvement of NAAG and glutamate carboxypeptidase II in glutamate-mediated antiapoptotic processes in the photosensitized crayfish stretch receptor.

How exogenous glutamate reduces glial apoptosis? The observed antiapoptotic effect of glutamate differed from the known excitotoxic action of glutamate on various neuronal cells.[16–18] Glutamate receptors are classified by (i) ionotropic receptors that open ionic channels permeable for Ca^{2+}, Na^+ and K^+ in response to binding of agonists (NMDA, AMPA or kainate), and (ii) metabotropic receptors associated with G-proteins that initiate intracellular Ca^{2+}- and cAMP-related signaling cascades upon glutamate binding. Penetration of Ca^{2+} through ionotropic NMDA receptors leads to mitochondria damage and necrosis or apoptosis depending on the level of mitochondrial depolarization, production of free radicals and release of proapoptotic factors.[21] However, in the present experiments neither NMDA nor MK801, which is the antagonist of NMDA receptors, influenced PDT-induced glial apoptosis. Therefore, NMDA receptors were not involved in PDT-induced apoptosis of glial cells. Application of kainate did not significantly change the firing of the crayfish stretch receptor neurons (data not shown), which indicated the possible absence of kainate receptors on this cell.

One can, therefore, suggest possible involvement of metabotropic glutamate receptors in glutamate-mediated protection of glial cells from PDT-induced

apoptosis. The application of AP-3, inhibitor of the group I metabotropic glutamate receptors (mGluR1), inhibited PDT-induced apoptosis of glial cells. This indicated involvement of mGluR1 in PDT-induced glial apoptosis but not in the anti-apoptotic glutamate activity. This effect was possibly related to mGluR1-mediated activation of phospholipase C and following Ca^{2+} release from endoplasmic reticulum into the cytosol.[22] However, the observed antiapoptotic effect of glutamate in photosensitized glial cells was possibly related to its binding to other receptors. For example, Matias *et al.*[22] showed that activation of mGluR2 subtype of metabotropic glutamate receptors protected hippocampal neurons from NO-induced apoptosis. Possible antiapoptotic activity of mGluR2 in photosensitized glial cells is to be checked in further experiments.

The present data as well as the results of the previous experiments showed that necrotic and apoptotic cell death is controlled by diverse signaling pathways and may be modulated by chemical modulators of various signaling proteins. These pathways differ in glial and neuronal cells.[5–9,14,15] One can suggest that signaling pathway controlling cell death in normal glial cells and malignant glioma cells also differ. This may represent the basis for selective injury of brain tumors and simultaneous protection of surrounding healthy neurons and glial cells with the help of pharmacological modulators of signaling pathways or their combinations.

Acknowledgments

The work was supported by the grants of the Russian Foundation for Basic Research No. 05-04048440 and 08-04-01322 and the grant No. 2.1.1/6185 of the Ministry of Education and Science of Russian Federation.

References

1. S. B. Brown, E. A. Brown, I. Walker, "The present and future role of photodynamic therapy in cancer treatment," *Lancet Oncol.* **5**, 497–508 (2004).
2. M. S. Eljamel, "Brain PDD and PDT unlocking the mystery of malignant gliomas," *Photodiagn. Photodyn. Ther.* **1**, 303–310 (2004).
3. S. S. Stylli, A. H. Kaye, "Photodynamic therapy of cerebral glioma — A review. Part I. A biological basis," *J. Clin. Neurosci.* **13**, 615–625 (2006).
4. H. Kostron, "Photodynamic diagnosis and therapy and the brain," in *Photodynamic Therapy. Methods and Protocols. Meth. Mol. Biol.*, C. J. Gomer, Ed., Vol. 635, pp. 261–280, Springer-Verlag, Science + Business media (2010).
5. A. Uzdensky, D. Bragin, M. Kolosov, O. Yu. Dergacheva, G. M. Fedorenko, A. A. Zhavoronkova, "Photodynamic inactivation of isolated crayfish mechanoreceptor neuron," *Photochem. Photobiol.* **76**, 431–437 (2002).
6. A. Uzdensky, M. Kolosov, D. Bragin, O. Dergacheva, O. Vanzha, L. Oparina, "Involvement of adenylate cyclase and tyrosine kinase signaling pathways in response of crayfish stretch receptor neuron and satellite glia cell to photodynamic treatment," *Glia* **49**, 339–348 (2005).
7. A. Uzdensky, A. Lobanov, M. Bibov, Y. Petin, "Involvement of Ca^{2+}- and cyclic adenosine monophosphate-mediated signaling pathways in photodynamic injury of isolated crayfish neuron and satellite glial cells," *J. Neurosci. Res.* **85**, 860–870 (2007).
8. A. B. Uzdensky, M. S. Kolosov, A. V. Lobanov, "Neuron and gliocyte death induced by photodynamic treatment: Signal processes and neuroglial interactions," *Neurosci. Behav. Physiol.* **38**, 727–735 (2008).
9. M. Kolosov, A. Uzdensky, "Crayfish mechanoreceptor neuron prevents photoinduced apoptosis of satellite glial cells," *Brain Res. Bull.* **69**, 495–500 (2006).
10. B. Fadeel, S. Orrenius, "Apoptosis: A basic biological phenomenon with wide-ranging implications in human disease," *J. Intern. Medicine* **258**, 479–517 (2005).
11. A. B. Uzdensky, "Controlled necrosis," *Biochemistry* (*Moscow*). *Suppl. A: Membr. Cell Biol.* **4**, 3–12 (2010).
12. R. D. Almeida, B. J. Manadas, A. P. Carvalho, C. B. Duarte, "Intracellular signaling mechanisms in photodynamic therapy," *Biochim. Biophys. Acta* **1704**, 59–86 (2004).
13. E. Buytaert, M. Dewaele, P. Agostinis, "Molecular effectors of multiple cell death pathways initiated by photodynamic therapy," *Biochim. Biophys. Acta* **1776**, 86–107 (2007).
14. A. B. Uzdensky, "Signal transduction and photodynamic therapy," *Curr. Sign. Transd. Ther.* **3**, 55–74 (2008).
15. A. V. Lobanov, A. B. Uzdensky, "Protection of crayfish glial cells but not neurons from photodynamic injury by nerve growth factor," *J. Mol. Neurosci.* **39**, 308–319 (2009).
16. D. T. Monagham, R. J. Bridges, C. W. Cotman, "The excitatory amino acid receptors: Their classes, pharmacology, and distinct properties in the function

of the central nervous system," *Annu. Rev. Pharmacol. Toxicol.* **29**, 365−402 (1989).
17. S. Nakanishi, Y. Nakajima, M. Masu, Y. Ueda, K. Nakahara, D. Watanabe, S. Yamaguchi, S. Kawabata, M. Okada, "Glutamate receptors: Brain function and signal transduction," *Brain Res. Rev.* **26**, 230−235 (1998).
18. C. Matute, M. Domercq, M.-V. Sanchez-Gomez, "Glutamate-mediated glial injury: Mechanisms and clinical importance," *Glia* **53**, 212−224 (2006).
19. A. K. Urazaev, R. M. Grossfeld, P. L. Fletcher, H. Speno, B. S. Gafurov, J. G. Buttram, E. M. Lieberman, "Synthesis and release of N-acetylaspartylglutamate (NAAG) by crayfish nerve fibers: Implications for axon-glia signaling," *Neuroscience* **106**, 237−247 (2001).
20. E. Florey, E. Florey, "Microanatomy of the abdominal stretch receptors of the crayfish (*Astacusfluviatili L.*)," *J. Gen. Physiol.* **39**, 69−85 (1955).
21. K. Maiese, I. Ahmad, M. TenBroeke, J. Gallant, "Metabotropic glutamate receptor subtypes independently modulate neuronal intracellular calcium," *J. Neurosci. Res.* **15**, 472−485 (1999).
22. C. Matias, P. Foley, M. E. Quinta-Ferreira, "L-AP3 blocks rises in intracellular calcium associated with hippocampal CA1 LTP," *Neuroreport* **7**, 751−755 (2002).