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ALEXANDER S. MCNEILL
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Chapter 4

Receptor Specific Features of Excitotoxicity Induced Neurodegeneration

Sergei M. Antonov* and Dmitrii A. Sibarov
Sechenov Institute of Evolutionary Physiology and Biochemistry
RAS, Saint-Petersburg, Russia

Abstract

Excitotoxicity is a term that describes the neuronal death caused by neurotoxic effects of glutamate, which is the most abundant excitatory neurotransmitter in the vertebrate central nervous system. Glutamate is well known to be involved in cognitive functions like learning and memory, but its excessive accumulation in extracellular space can lead to neuronal damages and eventual cell death via necrosis and apoptosis. As a result excitotoxicity contributes to pathogenesis of numerous neurodegenerative diseases. Both normal function and pathological action imply an activation of the same glutamate receptors particularly of NMDA- (*N*-methyl-D-aspartate), AMPA- (α-anino-3-hydroxy 1-5-methyl-4-isoxazole-propionate) and KA- (kainate) subtypes.

Many achievements in the mechanisms of neurodegeneration were obtained using different experimental approaches on primary neuronal cultures. Double successive acridine orange and ethidium bromide staining combined with confocal microscopy offers fast, easy, sensitive and reproducible method by which necrosis and apoptosis can be recognized and quantified in a population of living neurons. Together with immunostaining they provide many research advantages and allow analysis of protein expression patterns.

The growing quantity of evidence reveals the diversity of apoptosis cascades. Whereas our data show the same profiles of excitotoxicity for NMDA and KA, we found receptor subtype specific differences in neuronal death mechanisms. For example, apoptosis caused by prolonged NMDA receptors activation develops through the caspase-indcpendent cascades via release of apoptosis inducing factor (AIF) from mitochondria

* Corresponding author: antonov@iephb.ru, dsibarov@gmail.com

and its direct action on nuclear chromatin. In contrast AMPA and KA receptors mediated apoptosis includes caspase-dependent pathway.

On the basis of our data and literature the chapter will review the contemporary state of research concerning the aspects of excitotoxicity mechanisms discussed above.

Introduction

Glutamate receptor (GluR) is the main subject of investigations, concerning excitotoxicity and neuroprotection. Functional disregulation of neuronal metabolism, resulted from overactivation of GluRs is thought to cause neuronal death and to underline a wide range of central nervous system (CNS) disorders like spinal cord and brain injuries, stroke, neurodegenerative diseases, etc. Loss of cerebral blood flow causes massive neuronal depolarization and Glu accumulation in extracellular space [Lipton, 1999]. The prolonged presence of Glu released from neurons and glial cells by non-quantum secretion [Antonov, Magazanik, 1988] and activation of ionotropic GluRs have extensive consequences for neuron functioning, starting with an increase in intracellular Ca^{2+} concentration, imbalance of transmembrane gradients of the main electrogenic ions (Na^+ and K^+), and an activation of various intracellular cascades, and ending with destruction of the plasma membrane or nuclear apparatus of neurons [Choi 1987, 1988; Jonston, 1994, Oiney, 1994; Schoepp, Sacaan, 1994; Hatanaka et al, 1996]. Massive cytoplasmic calcium ions accumulation is thought to be one of the most important activators of various cell death mechanisms.

It's important to note, that a great body of facts concerning Glu stimulated neuronal disfunction were obtained in neuronal culture models. Cell cultures provide uniform cell composition and highly controllable extracellular environment which is favorable to study fine intracellular mechanisms. However, we must take into account that this model focuses on neurons alone and ignores tissue control. Other brain tissue cell types like astrocytes and oligodendrocytes can promote or prevent excitotoxic neuronal death. Actrocytes not only regulate neuronal ion balance, but can also maintain low extracellular Glu levels due to uptake with glial GLAST and GLT-1 transporters [Rothstein et al, 1994]. Glial cells express all kinds of ionotropic GluRs and respond to Glu stimulation with neurotrophic factors secretion like BDNF (brain-derived neurotrophic factor).

Glu induced excitotoxic neuronal death involves activation of at least three types of receptors: NMDARs (selective agonist *N*-methyl-D-aspartate, NMDA), AMPARs (selective agonist α-anino-3-hydroxyl-5-methyl-4-isoxazole-propionate, AMPA) or KARs (selective agonist kainite, KA). Because KA is not strictly selective to KARs and can activate both KARs and AMPARs, below we mention these receptors together.

NMDARs are highly permeable to Ca^{2+}, as well as to Na^+ and K^+. An accumulation of free Ca^{2+} inside the cell triggers multiple predominantly Ca^{2+} -dependent mechanisms of cell death usually ending as apoptosis [Khodorov, 2004]. Apoptosis, or programmed cell death, plays an enormous role in the development and formation of organs, as well as in the functioning of rapidly renewing tissues [Abrams et al, 1993; Jonston, 1994]. However, in nervous tissue, where there is virtually no regeneration, although there are now some data on *de novo* neuron formation from glial cell precursors [Skachkov et al, 2006], apoptosis is the key factor in the pathogenesis of many nervous system disorders, along with necrosis [Jonston, 1994].

In general, AMPARs and KARs are permeable to Na^+ and K^+ , and much less permeable to Ca^{2+} than NMDARs. The impermeability of AMPARs and KARs to Ca^{2+} is determined by the presence of GluR2 subunit [Bumashev et al, 1996; Dingledine et al, 1999]. GluR2 is expressed in all brain areas and neuronal subsets [Sun et al., 2005] except motor neurons and interneurons having comparatively low GluR2 expression [Magazanik et al, 1997], which makes them selectively vulnerable to AMPAR-mediated excitotoxicity. Prolonged activation of AMPARs and KARs elevates intracellular Na an contributes to cell swelling [Choi, 1987, 1988], which increases the probability of necrotic cell death. On this basis, we expect the distinctive features of NMDARs and AMPARs/KARs to underlie the different ratio of necrotic and apoptotic neuronal death while neurodegeneration caused by selective activation of particular receptor types using NMDA or KA.

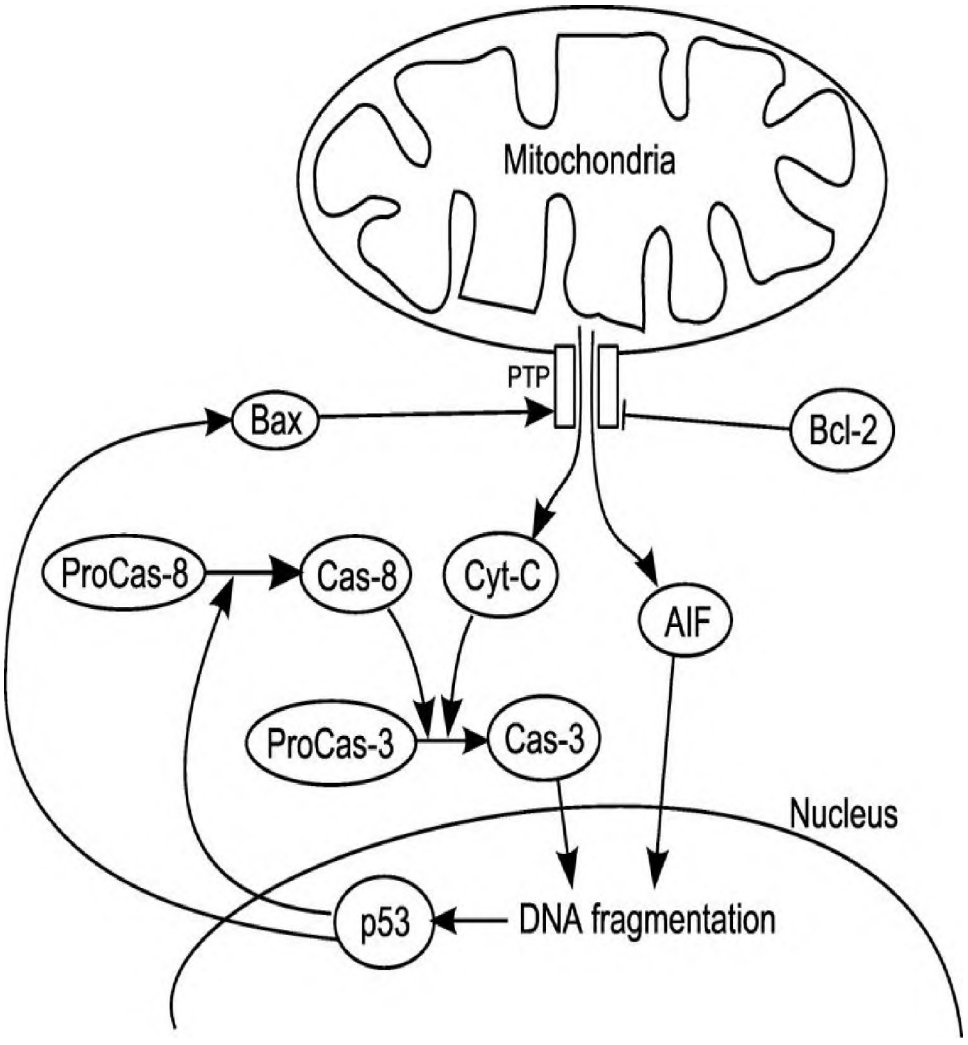

Figure 1. Simplified diagram of caspase-dependent and caspase-independent pathways of apoptosis. In response to different stress types protein P53 becomes activated and induces Bax expression and translocation to mitochondrial membrane. Bax promotes opening of mitochondrial permeability transition pores (PTP). It allows AIF and Cytochrome-C to be released to cytoplasm. AIF represents caspase-independent pathway and can directly cause DNA fragmentation. Cyt-C (caspase-dependent pathway) activates caspases phospliorilation cascade ending at Caspase-3, causing DNA fragmentation. Proapoptotic Bax protein promotes Cyt-C and AIF exit to cytoplasm. Antiapoptotic protein Bcl-2 has an opposite function

Although the cell death during apoptosis and necrosis have clear morphological and biochemical differences [Cafforio et al, 1996; Philpott et al., 1996], most methods do not allow the simultaneous identification of apoptosis and necrosis in living tissue, are very laborious and require preliminary biochemical processing, fixation, or resuspension of the study material [Gavrieli et al, 1992]. The development of a vital kit for rapid analysis of the cellular composition of neurons in tissue cultures [Mironova et al, 2007] allows the automatic quantification of cell populations to be performed for the presence of apoptosis and necrosis. It greatly facilitates the study of neurodegenerative process dynamics.

Its well known, that the development of apoptosis in various tissues can occur via two basic mechanisms (Figure 1): the caspase-dependent cascade and a cascade not involving caspases, i.e., via the direct action of apoptosis-inducing factor (AIF) on cell nuclei [Hong et al, 2004]. Inactive procaspases always exist in cytoplasm. Procaspases activation requires proteolytic splitting of proenzyme into two subunits and further cleavage of TV-terminal ends [Ermshaw et al., 1999]. Subunits are then assemble into active oligomers. Initial procaspase proteolysis can be done by various proteases, including other caspases. Caspase independent mechanism can be triggered via hyperproduction of proapoptotic proteins like Bax and Bak, which can induce apoptotic cell death even in the presence of caspase inhibitors [Xiang et al, 1996, McCarthy et al, 1997]. An appearance of caspase 8, which activates procaspases 3, 6 and 7 is enough to start apoptosis. The key enzyme in the caspase-dependent pathway is caspase 3 (Cas-3), activation of which leads to irreversible destruction of nuclear DNA. Apoptosis can be reversed before an activation of Cas-3 with Cas-8. There are several proteins promoting and preventing caspases activity at this moment [Kidd, 1998; Adams, Cory, 1998; Reed et al, 1998; Huppertz et al 1999; Gross et al, 1999]. These proteins include inhibitors of apoptosis like Al, Bcl-2, Bcl-W, Bcl-XL, Brag-1, Mcl-1 and NR13, and proapoptotic proteins Bad, Bak, Bax, Bcl-XS, Bid, Bik, Bim, Hrk, Mtd. Most of these proteins belong to Bcl-2 family of proteins which is evolutionary conservative. For example in sponges *Geodia cydomium* and *Suberites domuncula,* homologous proteins are involved in morphogenesis process [Adams, Cory, 1998].

This raises the question about the contribution of different cell death mechanisms to apoptosis evoked by selective activation of different neuronal GluR subtypes. Identification of the receptor specificities of apoptosis pathways simplifies the search of intracellular targets for new neuroprotective agents.

The goals of this work, performed on primary neuronal cultures from rat cortex were: to evaluate necrosis/apoptosis ratio during exposure to selective GluR agonists - NMDA and KA, as well as the Glu itself; to study potential neuroprotective properties of selective NMDAR, AMPAR and KAR antagonists, and to elucidate possible diversity of apoptotic cascades determining excitotoxicity triggered by excessive activation of GluR subtypes.

Methods

Experiments were performed at room temperature (20-22°C) on primary cultures of neurons from embryonic rat brain cortex. All procedures using animals were in accordance to FELASA recommendations and were approved by the local Institutional Animal Care and Use Committee. The method used for preparing cultures has been described in detail

previously [Antonov, Johnson, 1996; Mironova, Lukina, 2004]. Briefly, pregnant Wistar rats were sacrificed by CO_2 inhalation 16 days after conception. Cortices were obtained by aseptic dissection, incubated for 30-40 min at 37°C in growth medium containing 83% minimum essential medium (MEM), 25 mM Hepes, 2 mM L-glutamine, 10% D-glucose plus 0.03% trypsin, and then dispersed mechanically by trituration with a fire-polished glass Pasteur pipettes. Cells were pelleted by light centrifugation (410 x g, 5 min at 25 °C), the supernatant discarded and the cell pellet resuspended in pre-warmed Hanks solution. For better washout of reagents this procedure was repeated. A plating suspension at a density of 130,000 cells per ml was prepared in growth medium. This density was optimal for the neuronal survival, the neuronal network formation and further experimental manipulations with the cultures. The suspension of cells was plated onto 15 mm diameter glass coverslips that had been coated with poly-D-lysine in 35 mm plastic Petri dishes. Cultures were kept at 37 °C in a humidified 5% C02-containing atmosphere. The growth medium was refreshed twice a week.

Cells were used for experiments after culturing 7 days *in vitro* (DIV). Directly before the experiments, coverslips with neuronal cultures were placed in the bathing salt solution. Since extracellular Mg^{2+} , which blocks NMDAR channels [Antonov, Johnson, 1999], has neuroprotective effects on "young" neurons [Antonov et al, 2006], Mg^{2+} -free bathing salt solution was used of the following composition: 140 mM NaCl, 2.8 mM KC1, 1.0 mM $CaCl_2$, 10 mM Hepes; pH was adjusted to 7.2 - 7.4 with NaOH. To initiate excitotoxic insults 3 mM Glu, 30 (μM NMDA and 30 μM KA were added to the bathing salt solution. In the case of NMDA in all experiments 30 μM glycine was coapplied, as a coagonist of NMDAR [Johnson, Ascher, 1987]. When effects of antagonists and modulators were studied the compounds were coapplied with the GluR agonists. Measurements of the proportion of dead cells among whole cell population were performed after 120 and 240 min treatment with the compounds.

Cell viability was determined utilizing the vital fluorescence assay. Confocal images captured after 120 and 240 min of incubation with compounds were subjected for automatic cell counting after staining of all nuclei with acridine orange and dead cell nuclei with ethidium bromide. First, cells were treated with 0.001% acridine orange for 30 s. After complete washout of contaminating acridine orange cells were exposed to 0.002% ethidium bromide for 30 s. This procedure was applied directly before every measurement. As a result in confocal microscopy experiments the nuclei of live neurons, labeled with acridine orange, looked green and the nuclei of injured neurons, labeled with ethidium bromide, looked red (Figure 2, A) [Pulliam et al, 1998; Mironova et al, 2007]. In the absence of correlated pixels (Figure 2) the cell viability was estimated by the ratio of green pixels (the number) to the total number of lightened pixels (red plus green, Figure 2, B). If some population of neurons exhibited apoptotic transformations their nuclei looked yellow-orange, revealing the colocalization of fluorescence in green and red spectral regions (Figure 3). In this case the fractions of live, apoptotic and necrotic cells were calculated on the basis of correlation plot (Figure 3) as the ratio of green, yellow-orange and red pixels to the total number of lightened pixels (the sum of green, yellow-orange and red), correspondently. To improve the validity of cell viability measurements, 3 non-overlapping images from a single coverslip were pooled to calculate the mean value for this particular coverslip, which represented a single experimental measurement.

Fluorescence images were captured using a Leica (Leica Microsystems, Heidelberg, Germany) TCS SL scanning laser confocal microscope (upright) equipped with argon laser of 50 mW (excitation wavelengths 458, 476, 488 and 514 nm, approximately 10 mW each). Cultures were viewed with a 40x water objective (HCX APO L 40x/0.80, Leica Microsystems, Heidelberg, Germany). To resolve fine details an additional electronic zoon with a factor of 1.5 -3.5 was used. Since ethidium bromide has a second peak of excitation, which is consistent with the excitation of acridine orange, both acridine orange and ethidium bromide could be visualized using the same laser line. For two-channel imaging of acridine orange and ethidium bromide, neuronal cultures were excited with 488 nm laser line, which could be varied between 0.1 - 10 mW by means of a neutral density filter. The emitted fluorescence was acquired at 500 - 560 nm (green region of spectrum, for acridine orange) and > 600 nm (red region of spectrum, for ethidium bromide) and collected simultaneously using separate photo multiplier tubes. Microscope settings were adjusted so that imaging conditions for both channels were kept equal and constant. To improve signal-to-noise ratio 6 scans (512 x 512 pixel array) were averaged at each optical section. The confocal images from both channels were merged using standard Leica software and program ImageJ (http://rsb.info.nih.gov). In order to quantify colocalized and non-colocalized fluorescence the correlation plot, which sorts values of given pixels in the first image as the x-coordinate and values of corresponding pixels in the second image as the y-coordinate, was generated for each of measurements. On the resulting image non-correlated pixels looked green and red and were attributed to live and necrotic neurons, correspondently. Correlated pixels looked yellow-orange and were attributed to nuclei of apoptotic neurons.

Apoptotic proteins were identified immunochemically using mouse monoclonal antibodies to proapoptotic proteins P53 (dilution 1:400), AIF (dilution 1:1000), Cas-3 (dilution 1:100) and Bax (dilution 1:200). Monoclonal antibodies were visualized using secondary antibodies conjugated with the fluorochromes FITC, Phr, and Cy3, diluted 1:150. Images were recorded using a Leica TCS SL confocal scanning microscope. Fluorochromes were excited by light at a wavelength of 488 nm (for FITC), 488 nm (for Phr), and 514 nm (for Cy3). Emission was recorded in the green spectral region (emission peak at 525 nm) for FITC, in the red spectral region (emission peak at 620 nm) for Phr, and in the yellow spectral region (emission peak at 570 nm) for Cy3. All antibodies were obtained from Sigma.

Before fixation, neurons on coverslips were treated with neurotoxic factors (Glu, NMDA or KA) separately or in combination with antagonists for 240 min. Cells were then fixed with 4% paraformaldehyde solution in PBS (phosphate-buffered saline) for 30 min. After fixation, cells were washed out twice with PBS (15 min x 2). Before treatment with BSA (bovine serum albumin, 2%), cells were incubated with Triton X-100 (0.2%) for 15 min, washed out with PBS, and exposed to monoclonal antibodies for 12 h at 4°C. After washing out to remove primary antibodies, fluorochrome-conjugated secondary antibodies were added. Reactions with secondary antibodies lasted 40 min at a room temperature of 23 °C. Before recording of data, antibody-bound preparations were fixed on slides with Moviol glue to prevent fading of fluorochromes.

All drugs were kept frozen in stock solutions at concentrations of 1 - 100 mM and were diluted to the required concentrations before use. All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Data were analyzed statistically using Excel, Origin 6.1, and SigmaPlot 8. All histograms and Table 1 show means ± standard errors of mean (s.e.m.). Student's two-tailed ***t***-test and ANOVA were used for statistical comparisons.

Results

Double sequential staining with acridine orange and ethidium bromide allowed cells dying by apoptosis or necrosis and living cells to be discriminated [Mironova et al, 2007]. In course of exposing of neuronal cultures to 3 mM Glu for 120 min green nuclei of live neurons and red nuclei of injured neurons were observed (Figure 2, A). The intensity correlation plot (Figure 2, B) reveals non-overlapping areas of red and green pixels. An absence of colocalization between green and red fluorescence points on necrotic type as a mechanism of neuronal death under these particular conditions. This data suggests that during 120 min excitotoxic insult cells die by necrosis. To induce apoptosis a longer treatment by Glu or its agonists is required.

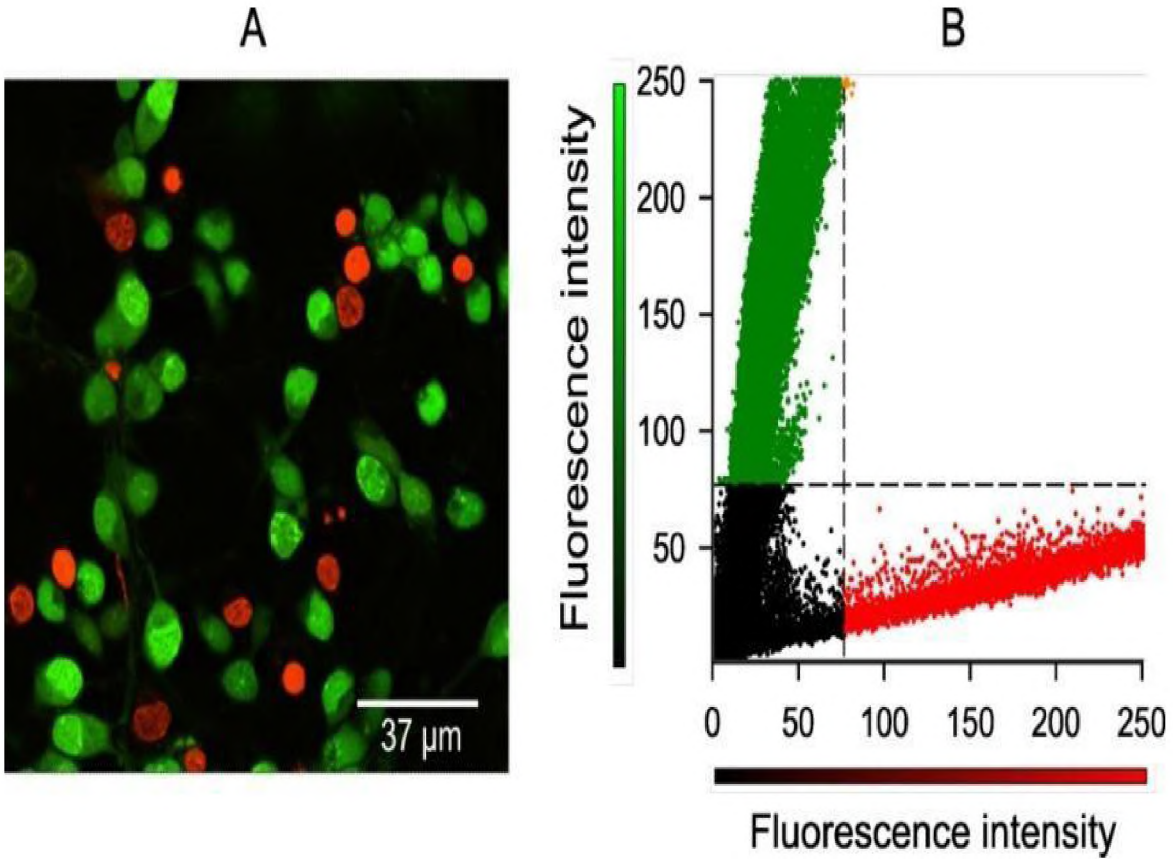

Figure 2. Algorithm of recognition and automatic quantification of live and dead neurons using the sequential acridine orange and ethidium bromide confocal microscopy assay. (A) Neurons after exposure of neuronal culture to 3 mM Glu during 120 min. Dead neurons are labeled with ethidium bromide and have red nuclei. Live neurons are labeled with acridine orange and have green nuclei. (B) The correlation plot for the image A. The absence of correlation between images obtained in green and red spectral regions demonstrates the lack of apoptotic nuclei. Dashed lines indicate thresholds, chosen empirically, that separates visible fluorescence from dark pixels

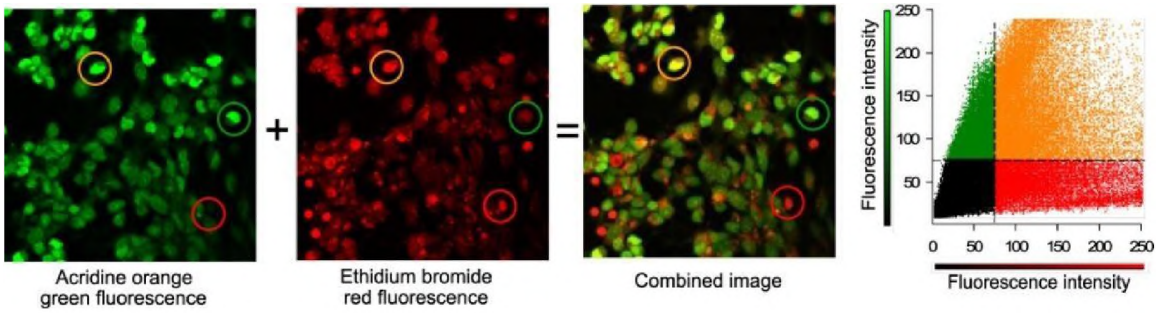

Figure 3. Distribution and automatic counting of living and dead cortical neurons in cultures treated with 3 mM glutamate for 240 min. Vital sequential staining with acridine orange and ethidium bromide using confocal microscopy is shown

Three groups of nuclei can be distinguished: live neurons (example marked with green circle are visible only in green spectral region, left panel); neurons dying by necrosis (marked with red circle are visible only in red spectral region, intermediate panel), and apoptotic neurons (orange circle mark can be seen in both parts of the spectrum. The right image represents the result of merging red and green spectral regions). Frame size is 139 x 139 μιη. The correlation plot shows correlated pixels or colocalization of fluorescence between the images recorded in the green and red spectral region. Correlating pixels are orange. The dotted lines show the empirically selected limits discriminating lighting-up pixels from non-lighting-up pixels. The proportion of live cells (37% for this experiment) was evaluated by the ratio of the number of green pixels to the total number of pixels lighting up.

Figure 3 shows images captured in the green and red spectral regions after exposure to 3 mM Glu for 240 min. The combined image shows the most typical situation, in which neurons can be discriminated into three classes on the basis of their nuclei. The nuclei of live neurons are visible only in the green part of the spectrum. They are detected by staining with and fluorescence of acridine orange. Yellow-orange nuclei on combined image (Figure 3) are seen in both the green and red areas and are detected because of the shift of acridine orange fluorescence towards the red spectral region as a result of nucleus acidification while apoptosis. [Mpoke, Wolfe, 1997]. Red nuclei are seen only in the red part of the spectrum, and are the nuclei of neurons with plasma membrane destruction, i.e., cells which have died by necrosis and which are detected by the fluorescence of ethidium bromide.

Table 1. Comparison of the proportions of necrotic, apoptotic, and living neurons by activation of different types of glutamate receptors for 240 min. Values marked with * are significantly different from the control ($p < 0.05$, Student's two-tailed t test)

Treatment	Necrosis, %	Apoptosis, %	Living cells, %	Number of experiments
Control	3± 1	15 + 5	82 + 6	4
30 μM NMDA	15+1*	45 + 9*	40+10*	5
30 μM NMDA + 50 μM AP5	3 + 1	16 + 4	81 + 5	4
30 μM kainite	16 + 2*	52 + 5*	32 + 6*	5
30 μM kainate + 30 μM CNQX	1 + 0.5	16 + 4	83 + 4	4
3 mM Glu	35 + 8*	31 + 3	34 + 5*	4
3 mM Glu + 30 μM CNQX	19 + 6*	16+13	65 + 8	4
3 mM Glu + 50 μM AP5	49+13*	17 + 3	34 + 6*	4

The proportion of neurons in each of three physiological states can be determined as ratios of the numbers of correlating pixels (lighting up in both parts of the spectrum) and non-correlating pixels (lighting up in only one part of the spectrum) to the total number of pixels lighting up. In the experiment shown in Figure 3, 3 mM Glu induced the apoptotic processes in 39% of neurons; 24% died via necrosis and 37% of neurons remained live. Average data are shown in Table 1. Under the control conditions (incubation in the bathing salt solution for 240 min) only 3% of necrotic and 15% of apoptotic neurons were found. Significantly increased number of necrotic and apoptotic cells at the cost of decreased number of live neurons illustrates the strong neurotoxic action of Glu. Obviously, neurodegeneration triggered by excitotoxic effects of Glu has two components. Neuronal death by necrosis occurred rather quickly and was accompanied by delayed activation of apoptotic process.

The first step in necrotic cell death is usually cell swelling. Multiple cell swellings or varicosities along dendrites were observed after treatment of neurons with 30 μM NMDA. The dynamics of varicosities swelling can be seen on Figure 4. The process of cell swelling in response to NMDA application is possibly related to activity of volume-sensitive chloride channels (VSOR Cl^-) [Inoue and Okada, 2007]. VSOR Cl^- channels play a great role in cell volume regulation, but cause neuronal body and processes swelling after prolonged NMDA excitotoxic insult, which leads to necrotic cell death. Despite the data about the mechanisms of NMDA induced necrosis [Inoue and Okada, 2007], information about necrotic processes caused by KA is scanty.

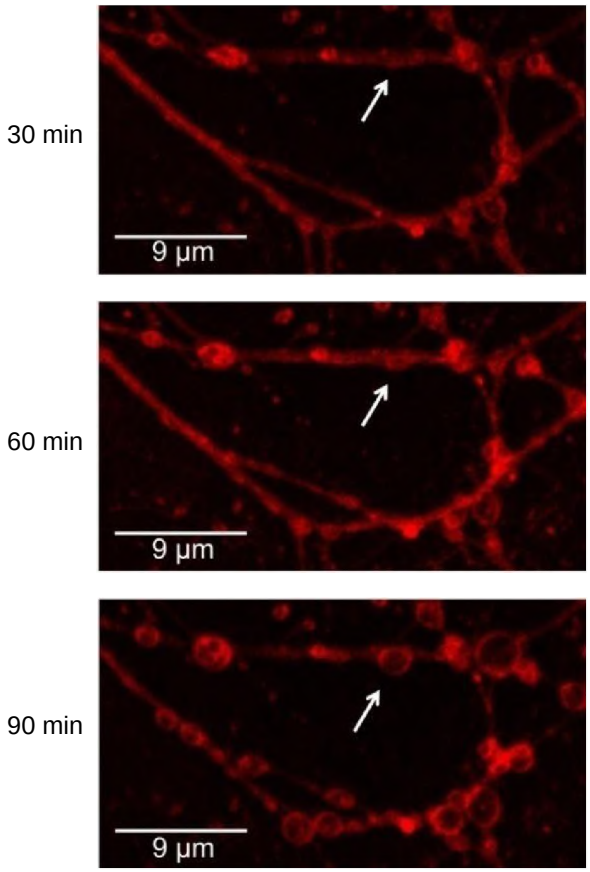

Figure 4. Confocal microscopy images, demonstrating the dynamics of varicosities formation in neuronal cell culture continuously exposed to 30 μM NMDA. Vital staining with ANEPPS. Arrows indicate the place of single varicosity growth

Selective agonists of different Glu receptor subtypes can be utilizes to assess the particular receptor type that determines the major contribution to neurodegeneration induced by natural neurotransmitters. In order to evaluate the functional role of NMDARs and AMPAs/KARs in Glu induced excitotoxic stress, we used the approach described above. We have analyzed the effects of 3 mM Glu in combination with 50 μM AP5, a selective competitive antagonist of NMDARs, and 30 μM CNQX, a selective antagonist of AMPARs/KARs [Dingledine et al, 1999]. CNQX was found to provide highly significant protection of neurons against the toxic action of 3 mM Glu: the viability of neurons in the presence of CNQX increased, on average, from 34% to 65% (Figure 5). On the contrary, AP5 did not have any neuroprotective action suggesting that NMDARs are not involved in the transmission of 3 mM Glu excitotoxicity. This observation clearly demonstrates that an activation of AMPARs/KARs provide a dominant contribution to the neurotoxic effect of 3 mM Glu, which is consistent with data obtained using trypan blue as an indicator of necrosis [Mironova et al, 2006].

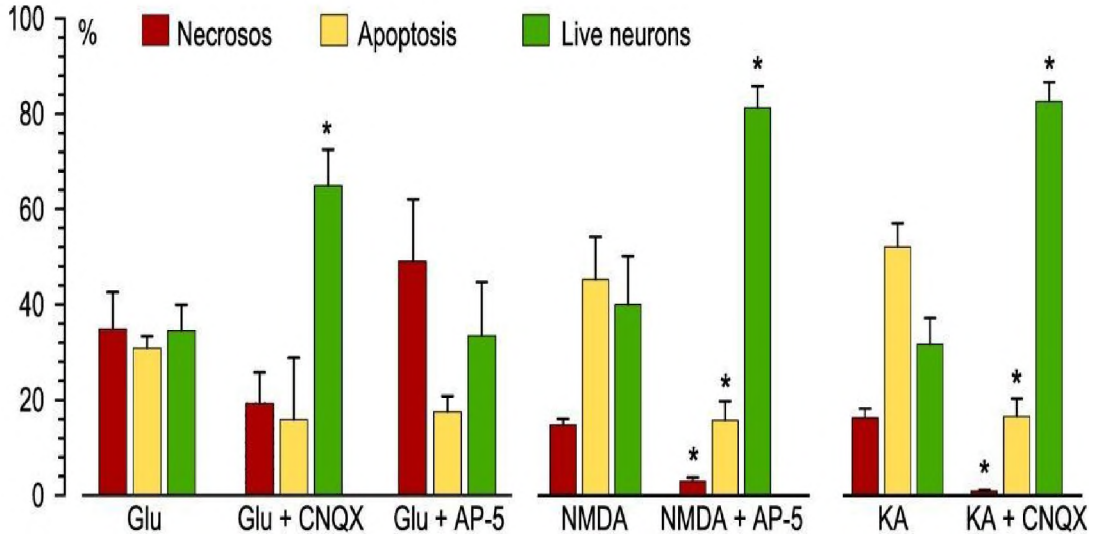

Figure 5. Effects of 240-min exposure of glutamate receptor agonists and antagonists on ratio of live, necrotic and apoptotic neurons in whole cellular population. (Glu) 3mM glutamate; (NMDA) 30 μM NMDA; (KA) 30 μM KA; (CNQX) 30 μM CNQX, (AP-5) 50 μM AP-5. * The data significantly differ from corresponding values, obtained in the absence of antagonists ($p < 0.05$, Student's two-tailed t test).

The ability to induce neurodegeneration by activation of AMPARs/KARs was supported by experiments using KA - a selective agonist of these receptors. After 240 min exposure to 30 μM KA 16% necrotic neurons and 52% of neurons dying by apoptosis ware observed (see Table 1, Figure 5). KA produced a statistically significant reduction in the proportion of live cells and increased the proportions of necrotic and apoptotic cells in comparison to the control (see Table 1). When KA was coapplied with CNQX, the neurotoxic effect was significantly weakened; the proportion of apoptotic and necrotic cells decreased, on average, to 16% and 1% respectively (Figure 5), and did not significantly differ from the control values (see Table 1).

The role of NMDARs in induction of neurodegenerative processes in neuron cultures was evaluated by studying the neurotoxic action of 30 μM NMDA. All experiments with NMDA were performed in the presence of a saturating concentration of glycine (30 μM) which is a coagonist of NMDARs [Johnson, Ascher, 1987; Dingledine et al, 1990]. The data are presented on figure 5. Activation of NMDARs for 240 min induced neurodegeneration mostly via apoptosis (45%) rather than necrosis (15%). The data obtained in the presence of NMDA with respect of all necrosis, apoptosis and live neurons were significantly different from those of the control (see Table 1). NMDA induced neurodegeneration was completely prevented by an addition of AP5 (Figure 5). The ratio of apoptotic, necrotic, and live cells obtained after application of 30 μM NMDA combined with 50 μM AP5 did not significantly differ from the control (see Table 1). This demonstrates the neuroprotective properties of NMDAR antagonists.

At this stage of our study we employed a simple procedure using vital fluorescence stains acridin orange and ethidium bromide. Recently it has been demonstrated the double sequential acridine orange and ethidium bromide staining combined with the confocal microscopy offers an express, fast, easy, sensitive and reproducible method by which necrosis, apoptosis and live neurons can be recognized and automatically quantified in a population of living cells [Mironova et al., 2007]. The utility of this assay has a broad experimental potential ranging from the analysis of dynamics and mechanisms of cell death to the pharmacological studies on a variety of live preparations.

The data suggests that either NMDARs or AMPARs/KARs can mediate excitotoxicity. Excessive and selective activation of these Glu receptor types results in the development of neurodegeneration via both mechanisms, necrosis and apoptosis, although apoptosis is predominant. Overall, the neurotoxic action of KA was more pronounced than of NMDA, as the total number of necrotic and apoptotic neurons was greater when excitotoxic insults were induced by KA. Selective antagonists produced neuroprotective effects, preventing the neuronal death by necrosis as well as the induction of apoptotic processes. Long-lasting presence in the media of the natural neurotransmitter, Glu, which at concentrations used in our experiments, presumably, open all NMDARs, AMPARs and KARs, led to substantially more remarkable neurodegeneration in comparison with the selective agonists of particular receptor types (Figure 5). In case of Glu the necrotic component was noticeably larger, as the quantity of necrotic neurons was significantly greater when Glu was applied as a neurotoxic agent, than those obtained after NMDA or KA exposure ($p<0.05$ for both comparisons, Student's two-tailed ***t*** test). In consistence with previous studies [Mironova et al, 2006; Mironova et al, 2007] our experiments favor the conclusion that onset of necrosis requires much less prolonged Glu receptor agonists exposure then of apoptosis. As could be seen from Figure 2, 120 min agonist exposures caused rundown of live neuron because of necrosis. At this period of time no visible features of apoptosis were found. The development of apoptosis required a longer agonist treatment so, as a large portion of neurons exhibited apoptotic features after 240 min agonist exposures. In conclusion, Glu and selective agonists of Glu receptors are triggering neurodegeneration, which mechanistic pattern changes in time: at the beginning of excitotoxic insults neurons predominantly die via necrosis, to start apoptosis a longer period of time is required.

The vital fluorescent assay applied to neuron cultures provides an estimate of integral pattern of apoptosis and can not provide any information about the particular intracellular cascades involved in this pathology process. The particular apoptotic pathways that

participate the neurodegeneration evoked by different Glu receptor agonists (3 mM Glu, 30 μM NMDA and 30 μM KA) were analyzed in immunocytochemical experiments. We studied an expression patterns of two apoptotic proteins P53 and Bax and the key proteins of two basic apoptotic cascades AIF (apoptosis inducing factor) and Cas-3 (caspase 3). Neuron cultures were incubated in the bathing salt solution supplemented with one of the agonists for 240 min. Cells then were fixed and the proteins of interest were visualized using immune reactions with monoclonal antibodies.

Each of the agonists induced an increase of the number of cells that were immunopositive to proapoptotic protein P53 (Figure 6, A). After exposure of neuronal cultures to any agonist the number of neurons expressing P53 was significantly greater than those under the control conditions (Figure 6, A), though the proportion of P53-positive cells exhibited no significant difference between agonists ($p>0.05$, ANOVA). P53 is known to be involved in apoptosis regardless of its particular cascades. This is a protein which is involved in the reparation when DNA damages are present, and P53 expression increases as the cell progresses along the apoptosis [Bates, Vousden, 1999; Miller et al, 2000]. In the case of our experiments P53 expression level in neuron cultures increased during excitotoxic stress caused by any agonist and could be taken as an integral measure of the apoptosis intensity. This is supported by an agreement in assessments of apoptosis either by P53 immune-positive cell count in fixed tissues and the vital fluorescence assay. For example, the proportion of apoptotic neurons among whole cellular population in course of treatment with 30 μM NMDA (240 min) were 45 ± 9% ($n = 5$) and 40 ± 10% ($n = 4$) obtained using the vital fluorescence assay and the reaction for P53, respectively, and did not differ significantly ($p > 0.76$, Student's two-tailed ***t*** test).

The same experimental protocol as for P53 has been used to study another proapoptotic protein Bax, which demonstarated similar to P53 expression profile. Numerous Bax positive cells were found after treatment of neurons with 30 μM NMDA, 30 μM KA and 3 mM Glu (Figure 6, B). Glu was the most effective inductor of Bax expression, since in it's presence nearly 90% of neurons were Bax-positive. Selective activation of NMDARs or KARs was less effective, causing substantial increase of Bax-positive neurons up to 50% (Figure 6, B). It is well evaluated, that an elevation of Bax expression observed during apoptosis is independent of involved apoptotic cascade (caspase-dependent or caspase-independent) [Saikumar et al, 1998]. Bax translocation to mitochondrial membrane is critical for these processes. With this respect an estimation of P53 and Bax expression can serve as two reliable marks of apoptosis onset. These data suggest that any type of Glu receptors can mediate neurotoxic insult, that promotes Bax expression.

Unlike P53 and Bax, AIF and Cas-3 are key molecules mediating caspase-independent (AIF) or caspase-dependent (Cas-3) apoptotic cascades. AIF or Cas-3 immunostaining allows recognition of particular cascades involved in excitotoxicity. Under the control conditions AIF protein was present in the cytoplasm of most neurons and only a few (on average 6%) contained AIF in the nucleus (Figure 6, C). Treatment with 30 μM NMDA for 240 min produced a statistically significant increase in the proportion of cells containing AIF in the nucleus ($p < 0.001$, Figure 6, C). The effects of 30 μM KA and 3 mM Glu on AIF expression did not differ, but were significantly weaker than 30 μM NMDA induced increase of AIF expression.

An elevation of Cas-3 expression depended on Glu mimetic used (Figure 6, D). The histogram in Figure 6, D summarizes the results obtained. The quantity of neurons exhibiting

high nuclear content of Cas-3 during 30 μM NMDA exposure did not differ from the control level. The number of cells containing nuclear Cas-3 in the presence of 3 mM Glu and 30 μM KA were significantly greater than in the control (Figure 6, D).

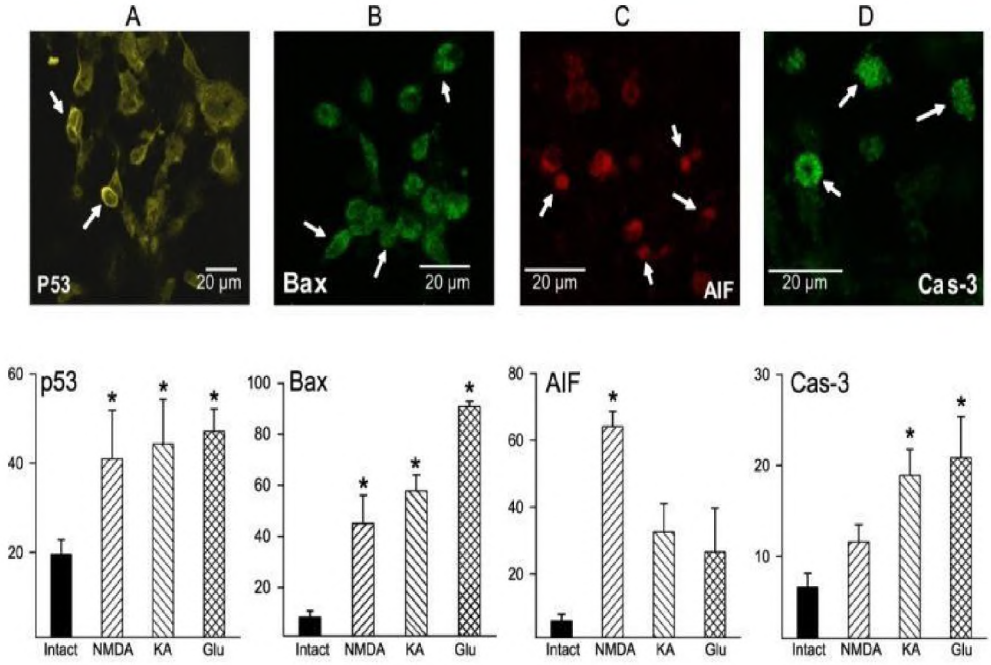

Figure 6. Apoptotic proteins P53, Bax, AIF and Cas-3 expression in response to glutamate, NMDA and KA for 240 min exposure. (A) immunostaining for P53 after exposure to 30 μM KA; (B) immunostaining for Bax after exposure to 30 μM NMDA; (C) immunostaining for AIF after exposure to 30 μM; (D) immunostaining for Cas-3 after exposure to 30 μM kainate. Immunoreactive neurons are indicated by arrows. Histograms presented below the images show percentage of neurons immunopositive for P53, Bax, AIF and Cas-3. (Intact) incubation in the bathing salt solution; (NMDA) treatment with 30 μM NMDA; (KA) treatment with 30 μM kainate; (Glu) treatment with 3 mM glutamate. * The data differ significantly from the data obtained under the control condition ($p < 0.05$, Student's two-tailed t-test)

In fact, 30 μM NMDA was successful to increase the quantity of AIF-positive cells, but did not affect the expression of Cas-3. In opposite, 3 mM Glu and 30 μM KA, whose effects are realized by AMPARs and KARs, increased the number of Cas-3-positive cells, but did not affect AIF nuclear expression (Figure 6). In two sets of our experiments performed to evaluate the apoptosis (immunostaining for P53 and Bax in fixed culture and the vital fluorescence assay) regardless the methods NMDA and KA induced similar apoptosis intensity. Observed difference of expression pattern for Cas-3 and AIF in the case of NMDA and KA allows us to suspect that NMDARs-mediated apoptosis predominantly involves AIF-dependent cascades, while AMPARs and KARs operate via the Cas-3-dependent apoptosis pathway.

Conclusion

It is generally accepted that research to investigate the nature of processes and the extent of changes in cells and cellular structures under different physiological states, including pathogenesis, provides complex understanding of morphological and functional processes

occurring in development, normal functioning, regeneration and neurodegeneration of CNS. A variety of stains [Noraberg, 1999; Patterson, 1979] and biochemical methods [Bergmeyer, Bernt, 1974; Koh, Choi, 1978; Uliasz, Hewett, 2000] have recently become available whose combination allows an assessment of the whole cellular cycle from the cell appearance till the death. Each of these approaches has its own advantages and disadvantages.

Analysis of neurodegeneration processes in our studies was addressed by the recognition of neuron death using sequential staining with acridine orange and ethidium bromide. The characteristics, suitability for experiments on living tissues, effectiveness, and reliability of this method of identifying necrosis and apoptosis have recently been described in detail [Mironova et al, 2007]. The method is based on the ability of acridine orange to penetrate membranes and stain the nuclei of living cells; ethidium bromide can only detect cells with a disintegrated plasma membrane, staining their nuclei [Pulliam, 1998]. In addition, acridine orange allows apoptosis to be detected because of the difference in staining of apoptotic and living nuclei which has previously been used to identify apoptosis in cell populations of *Drosophila* embryos [Abrams et al, 1993; White et al, 1994], in *Tetrahymena,* and chick chondrocytes [Mpoke, Wolfe, 1997]. Many studies demonstrated that apoptosis is accompanied by acidification of cells [Gottlieb et al, 1995; Li, Eastman, 1995], that appears as a result of nuclear disintegration and is associated with decreases in pH due to fusion of nuclei with lysosomes [Mpoke, Wolfe, 1997]. The difference in staining of living and apoptotic nuclei results from a displacement of acridine orange emission to the red spectral region while acidification is occurring during apoptosis [Zelenin, 1966].

Experiments have demonstrated that prolonged exposure of neurons to the GluR agonists induces neurodegeneration, with substantial contributions being made by both apoptosis and necrosis. Unlike Glu, the neurotoxic actions of NMDA and KA mainly induce apoptosis rather than necrosis. This is not surprising because NMDA and KA are specific agonists of a particular receptor type, whereas Glu can simultaneously activate any Glu receptors [Gibb, Colquhoun, 1992]. Furthermore, the large contribution of necrosis to neurodegeneration evoked by 3 mM Glu can also be explained by the fact that it is a natural neurotransmitter and, therefore, has broad physiological effects which are not restricted by the activation of ionotropic GluRs only. In the continuous presence in extracellular media, Glu may interfere and interrupt the functioning of neuronal and glial Glu transporters, presynaptic structures by the interaction with metabotropic Glu receptors as well as induce an impairment of the transmembrane ionic gradients at the least for Na^+, K^+ and Ca^{2+} [Antonov, Magazanik, 1988; Antonov, 2001]. The Glu concentration producing the neurotoxic effects in our experiments on primary cortical neuron cultures of 7 DIV was rather high. This concentration was chosen since a lower one (1 mM) had no particular effect throughout 240 min experiment [Mironova et al, 2006]. This would appear to be associated with the receptor expression level, as the effective (in terms of excitotoxicity) Glu concentrations for neurons of 14 DIV were almost 100-orders of magnitude lower than for 7 DIV [Mironova et al, 2007]. These lead us to the conclusion that the endogenous agonist at high concentrations is more effective in the induction of necrosis than its synthetic analogs. The opposite situation was observed in relation to the promotion of apoptosis.

Overall our study on rat cerebral cortical neurons of 7 DIV revealed two components of neurotoxic Glu action. One component was associated with the induction of necrotic cell death, while the other was associated with triggering of apoptotic mechanisms. Both components were clearly recognized for selective and natural agonists of Glu receptors rising

up the possibility of separate characterization of their pharmacological and neurochemical properties.

Neurodegeneration induced by NMDA and KA was completely abolished (to the control level) by the competitive NMDAR antagonist - AP5 and the competitive AMPAR and KAR antagonist - CNQX, respectively. This observation suggests that selective activation of any receptor type is sufficient to trigger apoptosis that is consistent with previously published data [Xiao et al, 2001; Wise-Faberowski et al, 2006]. This also demonstrates the potential of selective antagonists of Glu receptors revealing different mechanisms of action, including non-competitive inhibitors [Antonov, Johnson, 1996; Antonov et al, 1998], as neuroprotective agents that are able to prevent neuronal injuries and the development of apoptotic processes.

The neurotoxic action of Glu was slightly reduced by CNQX, as a significant increase in neuronal viability was found in the presence of CNQX, although no significant decrease in necrosis and apoptosis was observed. At the same time, the effects of 3 mM Glu were completely resistant to AP5, a specific antagonist of NMDARs. These suggest that the neurotoxic effects on neurons of 7 DIV induced by 3 mM Glu are presumably mediated by an activation of non-NMDA receptors [Mironova et al, 2006].

We identified cells expressing proapoptotic peptides P53, Bax, AIF and Cas-3 using immunocytochemical methods. P53 is known to be a universal protein which is indirectly involved in all apoptotic mechanisms. In our experiments, P53 expression is the same when selective agonists or Glu were used, which is consistent with other data [Miller et al, 2000]. Under similar conditions, the number of neurons expressing P53 coincided well with the apoptosis estimates obtained with the vital fluorescence assay. Immunostaining for P53 is often utilized as universal marker for apoptosis, since an expression of this protein is proportional to the degree of cell genome damages [Bates, Vousden, 1999]. In the case of moderate genome damages, the cell division stops, DNA reparation occurs, and the cell survives. In conditions of extreme genome fragmentation, when DNA can no longer be repaired, receptor- and Cyt-C - dependent apoptotic cascades activate caspases. The first data concerning the involvement of caspases in the neuronal death were obtained from studies of their inhibitor - P53 - in cultures of substantia nigra neurons [Rabizadeh et al, 1993]. In these cells, P53 blocked caspases, which resulted in inhibition of apoptosis induced by hypoglycemia, Ca" excess and deprivation of neurotrophic factors. An activation of caspases is evidently one of the possible mechanisms of neuronal death in neurodegenerative conditions.

P53 induces expression of proapoptotic Bax protein. In normal conditions small amounts of Bax exist in the cytoplasm. During apoptosis Bax can be translocated to mitochondrial membrane where it interacts with proteins of mithochondrial permeability transition pores (PTP), forcing them to open. Opened PTP are permeable to AIF and Cytochrome-C. An active involvement of Bax into Glu induced neuronal death has been demonstrated in experiments with Bax-knockout cortical neurons cultures [Dargusch et al., 2001]. Antibodies for Bax are now widely used in investigations of neurodegeneration [Zhang, Bhavnani, 2005; Raghupathi et al., 2003]. We also have some preliminary data that increased expression of Bax during apoptosis is accompanied with suppressed production of antiapoptotic Bcl-2 protein. The balance between pro- and antiapoptotic Bcl-2 family proteins it thought to be the key parameter in switching neuronal programmed cell death.

An activation of ionotropic receptors (usually NMDARs) results in increased influx of Ca^{2+} into cells, triggering proteolysis and degradation of cellular structures [Hatanaka et al,

1996; Jonston, 1994]. This process is also accompanied by increased lipid peroxidation and subsequent development of oxidative stress [Tapia, 1992; Waters, 1995; Boldyrev, 2001]. Although an activation of Glu receptors is evidently accompanied with neuronal oxidative stress [Waters, 1995], there are nevertheless specific cascades triggering apoptosis whose involvement depends on activation of a given receptor type. This statement is supported by our experimental data on AIF and Cas-3 expression. The induction of apoptosis via the activation of NMDARs was not accompanied with increased Cas-3 expression, though overall assessment of apoptosis using P53 and the vital fluorescence assay gave high values. On the contrary the significant increase in the number of neurons expressing AIF was observed. Induction of apoptosis by KA and Glu led to the significant increase in Cas-3 expression as compared to the control, though AIF production was substantially lower than in the case of NMDA application.

It can be suggested that hyperactivation of AMPARs and KARs, which have low Ca^{2+} conductance, is associated with impairments to energy metabolism and complex alterations of mitochondrial and sarcoplasmic reticulum functions [Khodorov, 2004]. Complex disregulation of mitochondrial membrane permeability is associated with the release of several apoptogenic factors: Cyt-C and procaspases 2, 3, and 9. Cyt-C catalyzes Cas-3 activation which determined caspase-dependent pathway of apoptosis. The increase in Cas-3 in the experiments with 3 mM Glu and 30 μM KA provide evidence that apoptosis was triggered via the caspase pathway.

Another mechanism regulating apoptotic death occurs when NMDARs are activated. This mechanism involves the release of AIF, which is translocated from the mitochondrial membrane to the nucleus, inducing DNA degradation via the caspase-independent pathway [Yu et al., 2002]. It is supported by observed increase of nuclear AIF presence after 30 μM NMDA excitotoxic insults. The results described in this chapter are indirectly supported by data that NMDARs mediated apoptosis can not be blocked with caspase inhibitors, but is prevented in the case of Bcl-2 hyperexpression [Wang et al., 2004].

Thus, we can conclude that apoptosis induced by the NMDARs activation develops through the caspase-3 - independent pathway that involves direct AIF accumulation in nuclei. The AMPARs/KARs mediated apoptosis includes a caspase-3 - dependent mechanism. An existence of the receptor specific apoptosis cascades during excitotoxicity reveals new directions in selective therapy of neurodegenerative states.

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