# Gene Expression in Neuronal Apoptosis — Looking for a Therapeutic Window

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## 1. Introduction

Excitotoxic neuronal loss is a phenomenon, in which an excessive neuronal stimulation through glutamate (or more broadly, excitatory amino acids) receptors results in the cell death (1). This process is believed to underlie diverse forms of neurodegeneration such as pathological consequences of brain ischemia, excessive epileptic seizures, and Alzheimer's, Parkinson's and Huntington's diseases (5,25). L-glutamate (L-glu) is the major excitatory neurotransmitter in the mammalian brain. It exerts its effects on the nervous system through interaction with specific cell surface receptors. These receptors could be classified into ionotropic: non-NMDA (kainate or AMPA), NMDA, and metabotropic receptor subtypes. Over the last years it has become evident that besides being an excitatory neurotransmitter, acts L-glu as a neuromodulator as well. In this latter role, L-glu can produce long lasting modifications in the functioning of the nervous tissue including plastic changes and neurodegeneration. It is widely expected that the understanding of molecular mechanisms of the glutamate action on the central nervous system shall greatly advance our ability to cope with, and ultimately to prevent, neurodegeneration.

Two main types of cell death — necrosis and apoptosis (or more broadly, programmed cell death) — are most often distinguished, although, they are not, apparently, homogenous entities (7,36,45). Apoptosis has been described as a genetically controlled, active process of cell death, with a highly ordered chain of events including loss of contacts among neighboring cells, cytoplasmic and nuclear shrinkage, nuclear condensation, chromatin fragmentation at internucleosomal sites, and finally degradation of cells to membrane-bound particles (apoptotic bodies) (45).

An involvement of apoptosis in neuronal excitotoxic death has recently been proposed by various authors, but nevertheless it still remains con-

troversial (for review see: 7). It has been reported that exposure of cortical neurons in culture to glutamate is associated with DNA fragmentation, and that aurintricarboxylic acid (an endonuclease inhibitor) offers a significant protection against glutamate-induced toxicity (23). However, it has also been noted that this compound may act as an antagonist of NMDA receptor (47). DNA fragmentation following the excitotoxic insult has also been observed in several other experimental paradigms involving both cultured neurons and brain cells *in situ* (13,24,30). However, it has been shown that glutamate-induced neuronal cell death in cerebellar culture does not exhibit the features of apoptosis (6,27). Obviously further studies, including, the development of new experimental models would be of great benefit to advance our knowledge on these phenomena.

Since apoptosis is supposed to be an active process, it is believed that it involves gene expression. Indeed, inhibition of RNA and/or protein synthesis have been used to prove the programmed character of excitotoxic neuronal cell death (10,38). Furthermore, it is expected that identification of genes as well as their regulatory mechanisms involved in the control of apoptosis, should be of great value for both the understanding of the process and the development of therapeutic strategies.

An ability of glutamate to influence gene expression has repeatedly been described (for review see: 18,19,20,28,39). Regulation of gene expression is brought about by transcription factors. Activation of glutamate receptors causes a dramatic increase of expression of AP-1 (activator protein-1) transcription factor (19,28) AP-1 is a dimer composed of Fos and Jun proteins. There are at least four genes encoding Fos components: *c-fos, fos B, fra-1* and *fra-2*, as well as three genes encoding Jun proteins (*c-jun, jun B, jun D*) (3,37). The complexity of AP-1, made of combinations of the Fos and Jun proteins suggests that various forms of this transcription factor may contribute to different neuronal responses. In fact, differential composition of AP-1 has recently been observed in specific neuronal responses in physiological and pathological conditions (7,21,33,43).

Possible role of AP-1, and more specificly, of the c-Fos and c-Jun proteins in active cell death has initially been supported by the results of experiments carried out on non-neuronal cells (2). In the studies on the central nervous system using *c-fos-lacZ* transgenic mice expressing β-galactosidase under control of *c-fos* regulatory elements, Smeyne et al. (42) observed that its β-galactosidase expression was associated with apoptotic cell death of neurons following kainate injection. It has also been documented that kainate treatment evokes not only an increase of expression of AP-1 in the brain, but is responsible for changes in the composition of this transcription factor as well (21). Dragunow et al. (9) have reported a prolonged increase in immunoreactivity for c-Jun protein in a model of *status epilepticus* coupled to neurodegeneration. Recently, Estus et al. (11) and Ham et al. (16) have demonstrated that c-Jun is induced in sympathetic neurons undergoing programmed cell death after NGF withdrawal. Moreover, blocking of AP-1 activity with either microinjection of specific antibodies (11) or introduction into the

cells of dominant negative mutant of c-Jun (16) prevented neuronal death, whereas overexpression of c-Jun promoted the neuronal death (16,46).

## 2. Kainate-Evoked Neuronal Death

In our studies, we analyzed the effects of kainate (KA), a potent excitatory and neurotoxic agent, on gene transcription including expression of genes encoding components of AP-1 transcription factor (21,22,17). We also investigated functional aspect of AP-1, i.e., its DNA binding activity in nuclear protein extracts collected from rat brain at different times after systemic injection of kainate. Two phases of elevated AP-1 DNA binding activity were observed in the hippocampus and entorhinal cortex, correlated with the period of seizures (2 and 6 hours following KA injection) and neuronal damage (72 h), which was found to be of apoptotic character (13,21,34,38). During the first phase, AP-1 consisted predominantly of Fos B, c-Fos and Jun B, while during the other phase, Jun D comprised major AP-1 component in place of Jun B. Lower AP-1 levels were seen at 24 h after the onset of seizure. Unlike for AP-1, no effect of KA on the levels of two other transcription factors, namely CREB (cAMP responsive element binding proteins) and OCT (octamer element DNA binding activity) was observed (21). Northern blot analysis of mRNA levels and immunocytochemical investigation of the proteins' expression confirmed these obervations (21; Filipkowski, unpublished results). These findings imply that two different, although possibly intimately linked, kainate effects, i.e., neuronal excitation and neuronal cell loss, may involve the same transcription factor, namely AP-1, but of a different protein composition.

We also identified various potential target genes for two waves of KA-evoked expression of transcription factors. During the first phase, initiated early after the kainate administration, genes encoding secretogranin II, clathrin heavy chain and heat shock cognate (Hsc) 70 (29) were activated as a secondary (dependent on previous protein synthesis) event (22). Neuronal cell death-associated expression of cathepsin D and reactive astroglia expression of glial acidic fibrillary protein could be observed only at the later times, being apparently dependent on the second wave of activation of the transcription factors (17).

## 3. Neuronal Loss in the Retrosplenial Cortex Provoked by Application of NMDA Receptor Antagonist

Another model system we employed to investigate neuronal cell death was treatment of female rats with high (>1 mg/kg) doses of MK-801. This compound blocks an open channel of NMDA receptor in the brain, in a non-competitive manner. Since $Ca^{2+}$ influx through NMDA receptor is be-

lieved to provide major trigger for neuronal cell death, it has repeatedly been proposed that antagonists of this receptor should provide main pharmacological tool to prevent neuronal damage. However, their potential clinical application is complicated on at least two counts. First, NMDA receptor is indispensable for neuronal plasticity, including learning and memory phenomena, and utmost care is required to develop strategies which would not be detrimental to brain cognitive functions. Secondly, it has been found that high doses of NMDA receptor antagonists, such as MK-801, may evoke cell death mainly in layer III of the retrosplenial cortex in the rat (32,31,14).

In our studies (Hetman et al., submitted) we confirmed that MK-801 provoked the appearance of degenerating neurons in the rat retrosplenial cortex 3 days after the administration of the antagonist. We, and others, have also found that this treatment induces c-Fos expression (Rydz et al., unpublished observations). However, the c-Fos expression was much more widespread than the neurodegeneration, as it involved not only the retrosplenial cortex but the neocortex as well. Thus, it was not spatially limited to the area of dying cells. On the contrary, we found that cathepsin D immunoreactivity was mostly confined to the dying neurons, similarly as it was observed in the hippocampus and the limbic cortex in kainate-treated rats.

This result did not confirm a role of AP-1 in neuronal death. However, it did not exclude such possibility, either. On the other hand, our study has implicated cathepsin D as protease, whose increased levels conspicuously increase in two different model systems of neurodegeneration. So far, no evidence has been provided for apoptotic character of neuronal death after MK-801 administration. Obviously, low number of cells involved in the process makes such studies particularly challenging.

## 4. Apoptosis in Dentate Gyrus Neurons Cultured *in vitro*

To further analyze the correlation between c-Fos protein expression and neuronal apoptosis, we have employed *in vitro* cultures of hippocampal dentate gyrus (12). Previous studies have shown clear differences in the vulnerability to glutamate-induced neurotoxicity in cultures obtained from different hippocampal regions (26). In particular, the rank order of sensitivity to glutamate between regions was: DG<CA2<CA3<CA1. This pattern of selective vulnerability in cell cultures seems to well correspond to the pattern of selective cell loss seen *in situ* in acute and chronic neurodegenerative disorders (1,40,35,44,25,4,41).

Unexpectedly, in our culture conditions, based on growing cells collected from dentate gyri of 5-day old pups, and culturing the cells in chemically defined medium, we observed an extensive neuronal loss after treating the cultures with 0.5 mM L-glu. We therefore decided to use this system first to investigate whether the neuronal degeneration involved apoptosis, and then to ask for the correlation between cell death and expression of two AP-1 components, namely, c-Fos and c-Jun proteins.

Two major morphological cell types could be distinguished after 6 days in culture: large and flat cells, containing large pale nuclei and abundant cytoplasm, and smaller and round cells, with extended processes which were often long or branched, and usually grew on top of the monolayer of flat cells. Immunocytochemical staining revealed that the former were astrocytes, which were GFAP (selective, cytoskeletal markers of astroglia)-positive, while most of the latter were neurons, which stained for MAP2 (selective for neurons).

Observation of glutamate-treated cultures under phase contrast microscope revealed morphological changes associated with nerve cells' death. Generally, in cultures treated with 0.5 mM L-glu we noted somal rounding, thinning and fragmentation of dendrites. In some cases, cell body became enlarged, the nucleus vacuolated, and eventually the cell body detached from the substrate. On the other hand, 0.1 mM L-glu caused the loss of dendrites and somal rounding a in significantly smaller number of cells with morphology of dentate granule neurons. Immunostaining with antibodies directed against GFAP or MAP2 revealed that cells with altered morphology such as dendrite fragmentation and/or regression, somal shrinkage and rounding were MAP2 positive, thus indeed the cell death affected neurons.

The specific DNA stain Hoechst 33258 was used to assess changes in DNA and nuclear structure following exposure to glutamate. It was found that nuclei in control neurons were large and exhibited diffuse staining of chromatin. On the other hand, a significant number of nuclei in neurons treated with 0.5 mM L-glu demonstrated a variety of abnormal morphologies, including highly condensed and fragmented chromatin. When the concentration of glutamate was decreased to 0.1 mM, these morphological changes appeared only occasionally.

To investigate whether these apoptotic-like morphological changes were accompanied by DNA fragmentation, thought to be another marker for programmed cell death, we used the method of TdT-mediated dUTP-digoxigenin 3' end labeling (TUNEL) (15). Cultures treated with 0.5 mM L-glu showed the most extensive DNA fragmentation, whereas lower concentration of glutamate evoked only moderate effect. On the other hand, in control cultures we observed only a few DNA fragmentation-positive cells.

Finally, we employed immunocytochemistry to investigate c-Fos and c-Jun levels in the control and glutamate-treated cells. We found that in L-glu-treated cultures the level of c-Fos protein was significantly increased in comparison to control cultures. However, the pattern of this immunoreactivity was almost identical regardless of the concentration of glutamate. Moreover, there was much higher number of neurons expressing c-Fos, than the number of dying cells identified with an aid of the aforementioned approaches. c-Jun expression was found only in dying cells.

To conlude from the studies based on excitotoxicity in the dentate gyrus cultures, we may suggest that c-Fos expression directly result from activation of glutamate receptor, whereas c-Jun expression is correlated with neuronal cell death.

## 5. Conclusions

There is a growing body of evidence that neurodegenerative processes may involve programmed cell death phenomena. In our studies, we could support this view by employing three different model system. The differences between them are apparently profound, however, although all of them involve glutamate receptors, it is difficult to understand how agonists such as kainate and glutamate may share the mechanisms of action with antagonist, MK-801. There is no proof for DNA fragmentation — believed to be diagnostic for apoptosis — after MK-801 administration. Nevertheless, neuronal death occurring in all of the systems shares common features. In particular, the process is always delayed to the primary insult, suggesting that it requires an activation of a still poorly defined chain of events. In all cases, we have also observed c-Fos expression. Although not restricted in dose-response, time, and spatial pattern of expression to the regions of cell death, it always extensively occurred in those areas. This may at least imply that gene expression phenomena are correlated with neuronal damage, further suggesting an active form of the process. Finally, in two *in situ* models employed, activation of major lysosomal aspartic protease, cathepsin D, on both mRNA and protein levels was observed. This could suggest that common executor mechanisms for various modes of neuronal death may exist. Altogether, these data, as well as a number of other studies (see: 7, for review) offer a hope that there is a therapeutic window for the treatment of neurodegenerative diseases, both in respect to time between the insult and cell death, and through possible common mechanisms to be targeted by future therapies. One can even speculate that such therapies might target transcription factors (e.g., AP-1 of a specific composition) and/or executor hydrolytic enzymes (e.g., cathepsin D).

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Summary

Excitotoxicity — cell loss occurring after an excessive neuronal stimulation with excitatory amino acids — has been suggested to underlie major neurodegenerative disorders. Recent studies imply that this phenomenon may have an apoptotic character, i.e., it may be an active process. In our studies, reviewed herein, we confirmed and extended this view by demonstrating a gene expression component in the processes of neuronal cell loss in three different experimental models: i. kainate administration, resulting in severe damage of the limbic system; ii. high-dose MK-801 treatment, evoking selective neuronal loss in the retrosplenial cortex; iii. glutamate stimulation of dentate gyrus neurons cultured *in vitro*. In conclusion, we suggest that these data, as well as the results of a number of other studies offer a hope that there is a therapeutic window for the treatment of neurodegenerative diseases, both in respect to time between the insult and cell death, and through possible common mechanisms to be targeted by future therapies. One can even speculate that such therapies might aim at transcription factors, e.g., AP-1 of a specific composition and/or executor hydrolytic enzymes, e.g., cathepsin D.

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