

Interrelations between nuclear-factor kappa B activation, glial response and neuronal apoptosis in gerbil hippocampus after ischemia

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Abstract. Spatial and temporal relations between transcriptional factor NFκB activation and glia reaction in gerbil hippocampus after transient cerebral ischemia has been studied. Activation of protein binding to NFκB consensus oligonucleotide was determined by electrophoretic mobility gel shift assay (EMSA) in homogenates from dorsal (DP- an equivalent of CA1 sector) and abdominal (AbP- containing CA2-4 and *gyrus dentatus*) parts of hippocampus. A significant activation of NFκB binding was observed exclusively in DP as early as 3 h after ischemia and at this time that response preceded any other morphological signs of postischemic tissue injury. This early enhancement of NFκB binding was followed by microglia activation visualized in CA1 pyramidal region at 24 h of recovery by histochemical staining with lectin from *Ricinus communis* (RCA-120). Simultaneously, only a moderate increase of immunostaining against glial fibrillary acidic protein (GFAP) was observed homogeneously in all parts of hippocampus. This uniform pattern of astrogliosis was preserved until postischemic day 3-4, when apoptotic DNA fragmentation in CA1 pyramidal neurons had been clearly documented by TUNEL staining. At this period however, continuous elevation of NFκB binding in DP corresponded with similar response manifested also in AbP of the hippocampus. These results evidence a preferential NFκB involvement in an early microglia activation in the apoptogenic CA1 sector, although its role in a later astrocytic response to ischemia could not be neglected too.

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Key words: brain ischemia, NFκB, reactive gliosis, apoptosis, microglia, astrocytes

INTRODUCTION

Animal models of cerebral ischemia provide an opportunity for studying intracellular signals leading to delayed neuronal death. Our previous work was focused mainly on neuron-specific events such as generation of an excitotoxic signal and its further propagation during reperfusion. However, it is well known that brain injury results in rapid activation and/or proliferation of glial cells. The traditional view is that this reactive gliosis constitutes merely a secondary role to neuronal destruction. More recently, however, it has become evident that glial cells are actively contributing to a cascade of events determining the final outcome after ischemic injury (Gehrmann et al. 1995, Li et al. 1995). Activated astrocytes and microglia can produce and release numerous trophic as well as cytotoxic factors influencing neuronal survival. In this respect glial cells, depending on still poorly defined circumstances, can attenuate or enhance delayed neuronal demise after ischemia (Orzyłowska et al. 1999).

Activation of microglia more often than astrocytes has been linked with neuronal destruction (Gehrmann et al. 1995, Gonzales-Scereno and Baltuch 1999). These cells are capable of releasing cytotoxic agents such as cytokines, reactive oxygen species, nitric oxide, arachidonic acid, proteolytic enzymes and so on. It should be stressed however, that no evidence has been provided yet which links activated microglia with ischemic neuronal damage *in vivo*. In contrast, reactive astrocytes are able to produce mostly (but not exclusively) neurotrophic factors promoting neuronal survival.

At a molecular level, activated glial cells show characteristic phenotypic and biochemical alternations governed by specific genes induced by ischemic stress. A number of such genes are activated by transcriptional factor NFκB including, among others, the inducible form of nitric oxide synthase (iNOS), amyloid precursor protein, trophic factors and cytokines (Baeuerle and Baltimore 1996, Baldwin 1996).

Under normoxic conditions the NFκB protein complex exists in a latent cytoplasmic form. After stimulation, NFκB is rapidly activated by the release of an inhibitory binding protein IκB and is then translocated to the cell nucleus. The trigger for NFκB activation/translocation is the phosphorylation of IκB which leads to its ubiquitination and degradation by the proteasome (Baeuerle and Baltimore 1996). It was

shown that translocation of less than 10%-20% of total NFκB content toward the nucleus is capable of inducing transcription of its target genes (Miyamoto et al. 1994).

To understand the role of NFκB in glia activation and induction of postischemic apoptosis in a better way, we have employed a well defined model of global cerebral ischemia in gerbils (Kirino 1982) as a test system. In these animals, 5 min ligation of both carotid arteries followed by 3-4 days of reperfusion, causes apoptosis restricted solely to CA1 pyramidal neurons of the hippocampus. Thus, in our experiments, the hippocampus was divided on two parts: the dorsal part containing vulnerable CA1 neurons and the abdominal part considered as an ischemia-resistant area. In these two regions differing in their sensitivity to ischemic insult, we have measured the NFκB consensus DNA-protein binding activity during a course of postischemic recovery. The biochemical investigations were supported by assessing apoptosis by TUNEL reaction and immunocytochemistry, allowing these responses to be localized on a cellular *in situ* level. The immunochemical expression of the specific markers of activated glia: anti-GFAP antibody for astrocytes and a lectin from *Ricinus communis* RCA-120 for microglia staining, was evaluated in the course of the postischemic reaction.

METHODS

Ischemic model

The ischemic insult was performed as previously described (Domańska-Janik et al. 1999) by 5 min. bilateral ligation of the common carotid arteries under halothane/N₂O anesthesia in strictly controlled normothermic conditions. Animals subjected to ischemia were allowed to recover up to 7 days after the insult. Sham-operated animals served as controls.

Sample preparation and measurements of protein binding ability to oligonucleotide NFκB consensus sequence

Hippocampi were quickly isolated on ice in the cold-room and then divided on the dorsal (DP) and abdominal (AbP) parts. Manual "unfolding" of dorsal hippocampus, containing CA1 sector, was completed under binocular with a *fissura hippocampalis* taken as a starting orientation point. The accuracy of this dissection was standardized on cross-sectioned hippocampal slices un-

der microscope. The remained tissue (the sectors CA2 to CA4 plus *gyrus dentatus*- DG) was designed as an abdominal part (AbP).

The both pieces of tissue were manually homogenized (10 % w/v) in homogenization buffer (pH 7.9) of 10 mM Hepes-NaOH, 10 mM KCl, 0.05 % NP40 in isotonic sucrose solution with addition of 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 5 mM DTT, 10 mM β -glycerophosphate, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml antipain. The samples were centrifuged for 5 min. at 15,000 \times g and supernatants were discarded. The resulting pellets were homogenised and extracted by 10 min. on ice with 400 mM KCl in 20 mM TRIS buffer (pH 7.8) supplemented with antiprotease cocktail as in the case of homogenization buffer. After centrifugation for 10 min. at 15,000 \times g, the nuclear extract was stored at -70°C .

Evaluation of protein binding ability to an oligonucleotide probe was carried out in the extracts according to Promega Gel Shift Assay System (Promega Manual 1997). The obtained autoradiograms were scanned densitometrically and analyzed using the image analysis system Gel Scan XL (LKB, Sweden).

Protein concentrations were determined by the method of Bradford (1976). Statistic analysis consisted of the mean \pm SEM with differences between means being tested with Student's *t*-test (Pharm. Cal. System Program).

Immunohistochemistry

Animals for immunochemical analysis were perfusion-fixed with 0.9% NaCl followed by 4% paraformaldehyde in PBS. The brains were removed and allowed to fix for an additional 4 h and were then processed for routine paraffin embedding.

All procedures were performed on 10 μ m thick paraffin-embedded tissue slices processed with polyclonal anti- GFAP antibody (Sigma), anti-RelA/p65 antibody (Santa-Cruz Biotechnol.) or biotinylated RCA-120 lectin (*Ricinus Communis* Agglutinin I, Vector Lab.), followed by routine avidin-biotin-peroxidase (ABC) method. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. The specificity of the immunoreaction was tested by incubating sections without the primary antibody. Hematoxylin was used for counterstaining and hematoxylin/eosin for morphological evaluation of tissue damage after ischemia.

Detection of fragmented DNA by TUNEL assay

Terminal transferase dUTP nick-end labeling (TUNEL) reaction was performed on paraffin sections following instructions of the reagent supplier (Boehringer, Indianapolis, USA). The reaction was visualized with BICIP/NBT as the substrate and counterstained with hematoxylin. A more detailed description of this method was provided elsewhere (Domańska-Janik et al. 1999).

RESULTS

Gel shift analysis after ischemia (Fig. 1) showed an increase of protein binding to the NF κ B DNA- consensus sequence. This response was observed up to 7 days of postischemic recovery with certain differences accord-

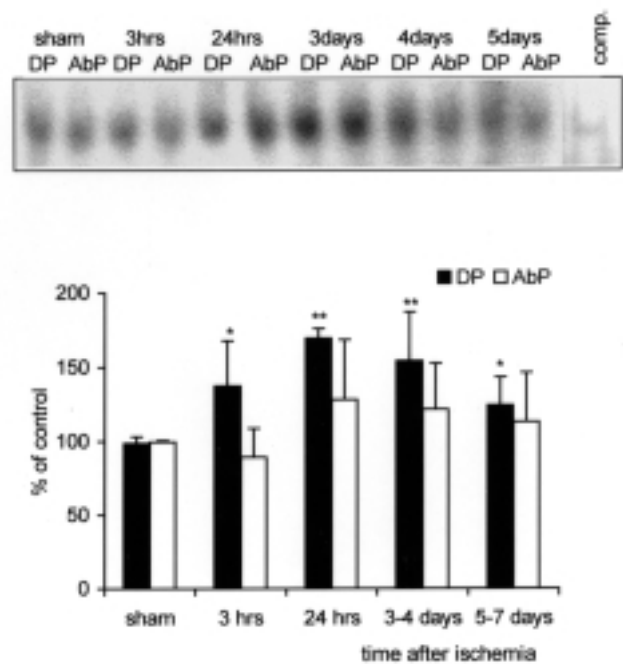


Fig. 1. Representative autoradiogram of NF κ B-consensus protein binding in dorsal part (DP) and abdominal part (AbP) of hippocampal homogenates evaluated by electrophoretic mobility shift assay (EMSA) in the course of recovery after 5 min. global, cerebral ischemia in gerbils. Competition experiments (comp.) were run in parallel with a 50-fold excess of unlabeled oligonucleotide probes. Below: Statistical analysis of the densitometric data from indicated experimental groups presented as a percent of parallel run sham-operated controls. Mean values from 6-9 independent tissue samples \pm SEM. Statistically significant differences from controls are designated by: * $P < 0.05$, ** $P < 0.001$.

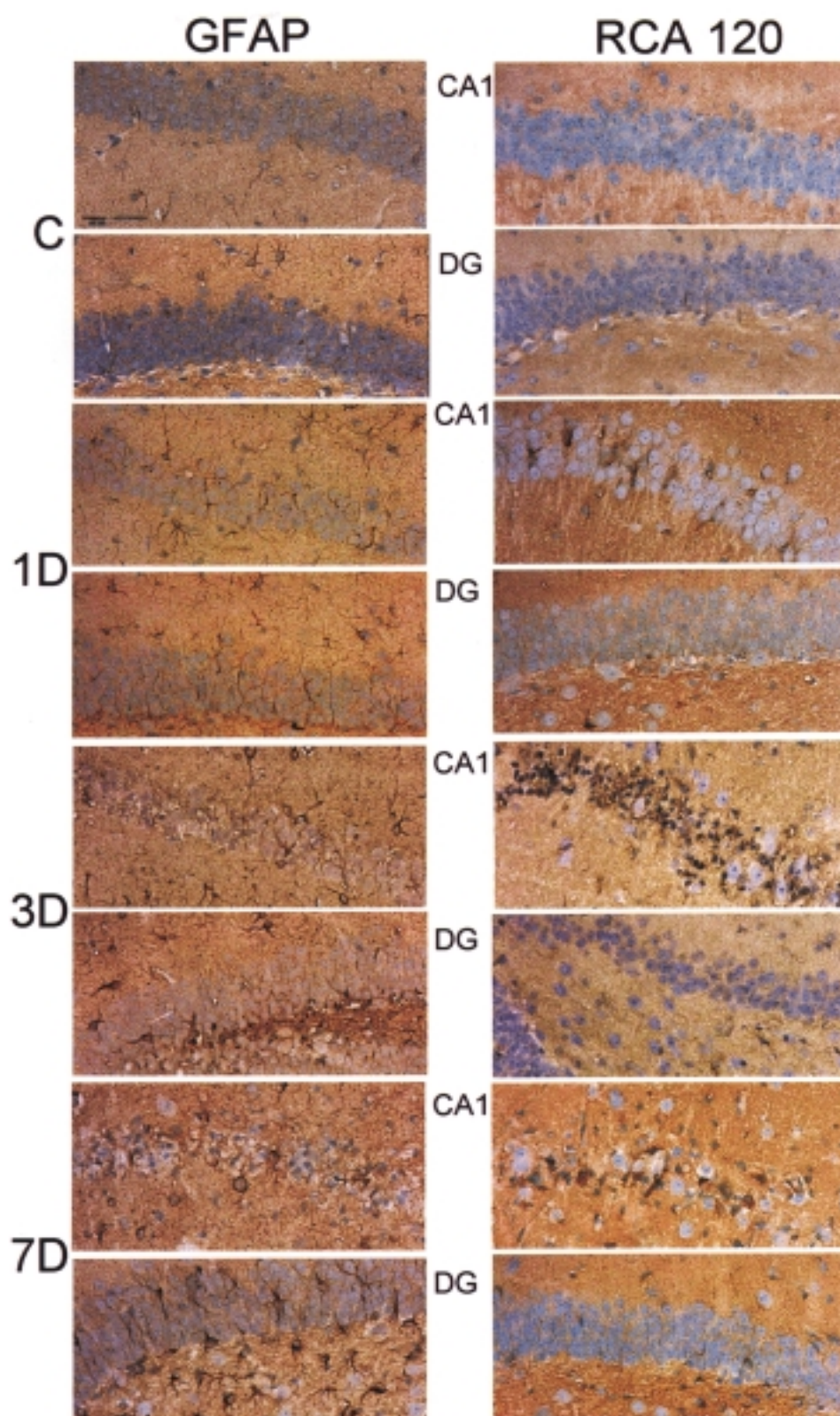


Fig. 2. Postischemic changes of GFAP and RCA-120 immunoreactivity in CA1 pyramidal layer (CA1) or granular layer of dentate gyrus (DG) in gerbil hippocampus. C, controls, D, days after ischemia. Sections were counterstained with hematoxylin and shown at 40 x magnifications (scale bar equal to 50 μ m is inserted to upper left microphotograph).

ing to hippocampal region studied. Generally, the reaction occurred earlier and was more accentuated in the dorsal (DP), apoptogenic region of hippocampus compared with its abdominal (AbP), ischemia-resistant area. However, in this later part too, a permanent tendency toward NF κ B activation, although statistically not significant, was clearly seen in all groups of animals examined at time longer than 24 hour after ischemia.

To evaluate extend and distribution of GFAP and RCA-120 immunoreactivities as markers of astroglia and microglia activation (Fig. 2) during the course of postischemic degeneration, we have compared them with the appearance of apoptosis being detected at 3 to 4 days of recovery by the terminal transferase nick-end labeling (Fig. 3).

In control animals only a low number of each type of immunostained cells were detected in hippocampus. They were localized mostly around vessels of the hippocampal fissure (not shown) and evidently apart from CA1- pyramidal or DG- granular cell body layers (Fig. 2).

In postischemic hippocampus, GFAP immunoreactivity was maintained at near the control level during the first 24 h. After this time, the immunoreaction increased successively in the glial cells located in different hippocampal regions without any noticeable spatial preferences to CA1 sector (Fig. 2). Then suc-

cessively, GFAP immunoreaction precipitated mostly in CA1 (*stratum radiatum, oriens* and cell body layer) with the highest response being seen at 7 days after the insult.

The time course analysis of RCA 120 labeling being characteristic for activated microglia, showed that this reaction was expressed after ischemia comparatively earlier than GFAP and TUNEL. Noticeable activation of lectin binding appeared in CA1 cell body layer at 24 h after the insult (Fig. 2) and preceded any other morphological signs of neuronal injury. Then it increased further at day 3 with clear preferences to the CA1 cell body layer. In comparison, at the same time point, numerous pyramidal neurons in CA1 became labeled by *in situ* terminal transferase reaction (Fig. 3), whereas GFAP expressing activated astrocytes could be still found throughout the whole hippocampus without any obvious preferences to CA1 (Fig. 2). The additional immunolabeling with RelA/p65 antibody revealed temporal appearance of numerous stained cells and their nuclei localized beyond the limits of pyramidal cell body layer in postischemic CA1 sector (Fig. 3). Moreover, at the later time point (7 day), when apoptotic neurons almost disappeared in the CA1 layer, RCA-120 as well as GFAP immunoreactions were still well expressed by the scar-forming and infiltrating glial cells.

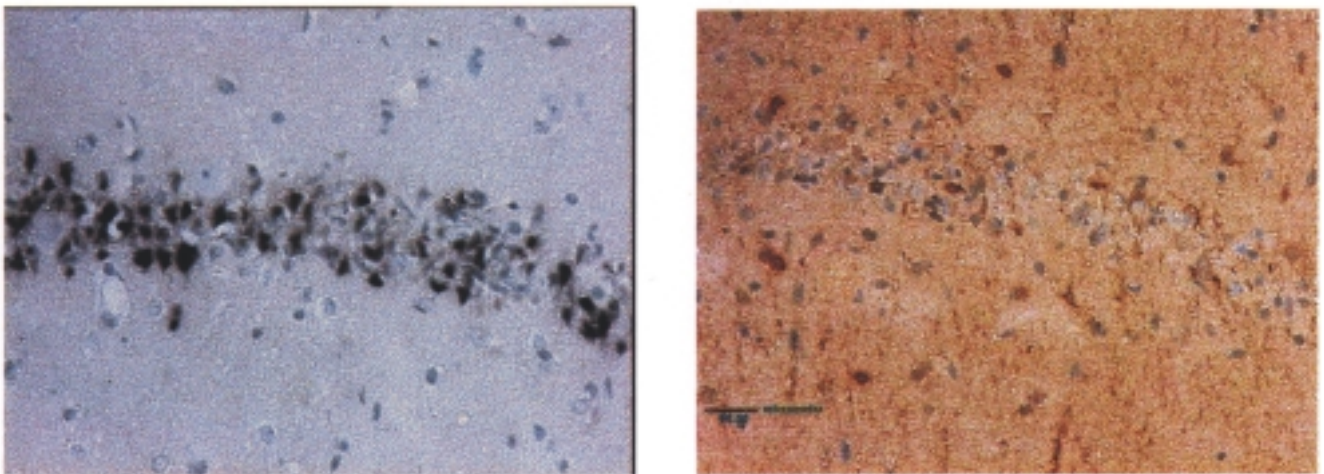


Fig. 3. TUNEL staining (left) appearing at a third day of reperfusion is restrained to the pyknotic, pyramidal neurons of CA1 sector. In contrast, RelA/p65 immunoreactivity (right) is expressed mainly in the cells localized apart from CA1 neuronal cell body layer. Sections counterstained with hematoxylin are shown at 40x magnification (scale bar equal to 50 μ m is inserted to right microphotograph).

DISCUSSION

In order to get a more clear understanding of the brain response to ischemia, neurons cannot be studied in isolation but must be put into the context of surrounding glia. Such an approach raises the question of intracellular signals that couple short-term external triggers with long-lasting changes within activated glia. We have shown that the expression of NF κ B, one of the transcription factors strongly linked with control of neurogenic inflammation (Gonzalez-Scerano and Baltuch 1999), increased early and significantly in the ischemia-injured hippocampus. Enhanced protein binding to the NF κ B consensus DNA sequence measured by gel shift analysis was observed in CA1 as early as 3 h after the insult (Fig. 1). This early increase is probably due to rapid translocation of NF κ B binding protein from the cytoplasm to the nuclei of activated cells after release from I κ B subunit inhibition (Baeuerle and Baltimore 1996), where it exerts its specific effect on target genes (Baldwin 1996) affecting glial cells phenotype.

The time course of the GFAP and RCA-120 expression, in comparison with NF κ B activation/translocation, failed to show any spectacular specificity of this factor toward microglia or astrocyte activation. However, we have noticed a significantly earlier and higher NF κ B activation in DP than in AbP of the hippocampus. This initial NF κ B reaction parallels more closely the pattern of microglia activation which, similarly to previous reports (Clemens et al. 1997), has been evidenced in CA1 as early as 24 h after ischemia (Fig. 2). In contrast, appearing comparatively later ischemia-activated-GFAP-expressing astrocytes were dispersed rather uniformly throughout the whole hippocampus. Thus, the NF κ B activation/translocation, simultaneously assessed by EMSA in both, DP as well as AbP regions, would be, at this time, more closely associated with the astrocytic response. Nevertheless, activated microglia could be still an additional contributor, responsible for the differences in NF κ B binding, noticed between DP and AbP subfields in all examined time points (Fig. 1). This is in agreement with earlier studies showing NF κ B/p65 positive cells double labeled with GFAP in the penumbra surrounding a cerebral infarct (Terai et al. 1996). In other pathologies however, for example in experimental autoimmune encephalomyelitis, NF κ B has been implicated as a main signaling molecule also in microglia activation (Kaltschmidt et al. 1994). The immunohistochemical data presented here with NF κ B-specific anti-RelA/p65

antibody, exclude rather a third possibility, that CA1 pyramidal neurons themselves can express NF κ B after ischemia. This have been implicated by reports, mostly from cell culture studies (between them also our unpublished observations on N2a neuroblastoma), demonstrating transient but significant activation of NF κ B after a variety of adverse stimulation signals linked either with apoptosis (Kolesnik 1994) or cellular endogenous protection (Mattson 1998). Certainly, in our experiments we can not differentiate between glial cell type expressing NF κ B in ischemia-injured CA1 hippocampus, nevertheless, their histological localization seems to exclude the involvement of dying, TUNEL-stained neurons.

The role of activated glia in ischemic pathology is still under debate. Numerous reports suggest that microglia especially can actively participate in the induction of delayed neuronal death (Banati et al. 1993, Lees 1993). In contrast, more recently demonstrated dissociation between microglia activation and neuronal degeneration in tumor-necrosis-factor-receptor and colony-stimulating-factor-1 knockout mice, questions again the role of microglia as a key player in ischemic encephalopathies (Bruce et al. 1996, Fedoroff et al. 1997). Furthermore, there are also some suggestive evidences about a possible beneficial role of factors secreted by activated microglia for neuronal survival (Mattson and Furukawa 1996, Li et al. 1999). As in the case of microglia, it seems that also astrocytes and their products certainly assist and possibly modify the apoptotic reaction in postischemic CA1 neurons. However, in accord with our data, astrocytosis does not seem to be causally associated with the induction phase of delayed neuronal death after ischemia, but rather with a more generalized stress response of post-injured tissue as well as with late formation of the glial scar in the vulnerable CA1 sector.

ACKNOWLEDGEMENT

The study was supported by the State Institute for Scientific Research grant # 6.P04A.010.14 and PHARE SCI-TECH II project PL 9611/03.01.

REFERENCES

- Baeuerle P.A., Baltimore D. (1996) NF kappa B: ten years after. *Cell* 87: 13-20.
- Baldwin A.S. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insight. *Annu. Rev. Immunol.* 14: 649-681.

- Banati R.B., Gehrmann J., Schubert P., Kretzberg G.W. (1993) Cytotoxicity of microglia. *Glia* 7: 111- 118.
- Bradford M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Acta Biochem.* 72: 248-254.
- Bruce A.J., Boling W., Kindy M.S., Peschon J., Kraemer P.J., Carpenter M.K., Holtzman F.W., Mattson M.P. (1996) Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptor. *Nat. Met.* 7: 788- 794.
- Clemens J.A., Stephenson D.T., Dixon E.P., Smalstig E.B., Mincy R.E., Rash K.S., Little S.P. (1997) Global ischemia activates nuclear factor kappa-B prior to evidence of DNA fragmentation. *Mol. Brain Res.* 48: 187-196.
- Domańska-Janik K., Bong P., Bronisz-Kowalczyk A., Zając H., Zabłocka B. (1999) AP1 transcriptional factor activation and its relation to apoptosis of hippocampal CA1 pyramidal neurons after transient ischemia in gerbils. *J. Neurosci. Res.* 57: 840-846.
- Fedoroff S., Berezovskaya O., Maysinger D. (1997) Role of colony stimulating factor-1 in brain damage caused by ischemia. *Neurosci. Biobehav. Rev.* 21: 187-191.
- Gehrmann J., Banati R.B., Wiessner C., Hossmann K-A., Kretzberg G.W. (1995) Reactive microglia in cerebral ischemia: an early mediator of tissue damage? *Neuropathol. Appl. Neurobiol.* 21: 277-289.
- Gonzalez-Scarano F., Baltuch G. (1999) Microglia as mediators of inflammatory and degenerative diseases. *Ann. Rev. Neurosci.* 22: 219-240.
- Kaltschmidt C., Kaltschmidt B., Lannes-Vieira J., Kretzberg G.W., Wekerle H., Baeuerle P.A., Gehrmann J. (1994) Transcription factor NF kappa B is activated in microglia during experimental autoimmune encephalomyelitis. *J. Neuroimmunology* 55: 99-106.
- Kirino T. (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res.* 239: 57-69.
- Kolesnick R. (1994) Signal transduction through the sphingomyelin pathway. *Mol. Chem. Neuropathol.* 21: 287- 297.
- Lees G.J. (1993) The possible contribution of microglia and macrophages to delayed neuronal death after ischemia. *J. Neurol. Sci.* 114: 110- 122.
- Li Y., Chopp M., Zhang Z.Z., Zhang R.L. (1995) Expression of glial fibrillary acidic protein in areas of focal cerebral ischemia accompanies neuronal expression of 72 kDa heatshock protein. *J. Neurol. Sci.* 128: 134-142.
- Li Y-B., Kaur C., Ling E-A. (1999) Neuronal degeneration and microglial reaction in the fetal and postnatal rat brain after transient maternal hypoxia. *Neurosci. Res.* 32: 137-148.
- Mattson M.P. (1998) Free radicals, calcium, and the synaptic plasticity - cell death continuum: emerging roles of the transcription factor NFκB. *Int. Rev. Neurobiol.* 42: 103-168.
- Mattson M.P., Furukawa K. (1996) Programmed cell life: anti- apoptotic signalling and therapeutic strategies for neurodegenerative disorders. *Restorative Neurol. Neurosci.* 9: 191-205.
- Miyamoto S., Chio P.J., Verma I.M. (1994) Enhanced I-kappa B alpha degradation is responsible for constitutive NF kappa B activity in mature murine B-cell lines. *Mol. Cell. Biol.* 14: 3276-3282.
- Orzyłowska O., Oderfeld-Nowak B., Zaremba M., Januszewski S., Mossakowski M. (1999) Prolonged and concomitant induction of astrocyte immunoreactivity of interleukin-1beta and interleukin-6 in the rat hippocampus after transient global ischemia. *Neurosci. Lett.* 263: 72-76.
- Terai K., Matsuo A., McGeer E.G., McGeer P.L. (1996) Enhancement of immunoreactivity for NFκB in human cerebral infarction. *Brain Res.* 739: 343-349.

Received 9 May 2000, accepted 5 October 2000