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13. ABSTRACT (Maximum 200 Words)

Our proposed research is focused on developing nitrone-based antioxidants as antidotes against chemical agents that induced excitatory neurotoxicity. We proposed to use kainic acid, an analog of the excitatory amino acid glutamate, to induce chronic neurological damage in adult rats. Exposure of rats to kainic acid (KA), a non-NMDA type glutamate receptor agonist, induces recurrent (delayed) convulsive seizures and hippocampal neurodegeneration reminiscent of human epilepsy. In this study, the effects of KA were studied with respect to three separate signal transduction pathways likely to regulate inflammatory and apoptotic gene expression in the hippocampus. Immunohistochemical methods and electromobility gel shift assays (EMSAs) demonstrate the concerted activation of the NFkB pathway along with the activator-1 pathway (AP-1) and the p38 mitogen-activated protein kinase pathway (p38 MAPK). Activation of these three pathways occurred simultaneously with the expression of several proapoptotic biomolecules (most notably TNF and the Fas antigen) and simultaneously with the onset of convulsive seizures but prior to the initiation of neuronal apoptosis. Co-treatment with the experimental antioxidant and anti-inflammatory compound phenyl-N-tert-butylnitrone (PBN) resulted in a diminution of NFkB, AP-1 and p38 activation, suppressed cytokine and apoptotic gene expression, inhibited neuronal apoptosis, and diminished seizure activity. These data suggest that pharmacological antagonism of multiple signal transduction pathways is achievable in the brain, and that inhibition of these processes may prevent a cascade of gene-inductive events leading to neuronal apoptosis.

14. SUBJECT TERMS
Kainic acid, phenyl-N-tert-butylnitrone, epilepsy and neurodegeneration

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Introduction

This report is a revision of final report of "DAMD17-99-1-9497" entitled "protective mechanisms of nitrone antioxidants in kainic acid induced neurodegeneration". In general, we agree with the thoughtful and insightful review provided by the reviewer. We have made substantial changes in our final report and have incorporated the comments made by reviewer into a revised report.

We wish to express our gratitude to the USAMAMC who provided the first independent grant that has helped PI established research projects that get national and international recognition. PI now has four graduate students and a postdoctoral fellow and a technician in the research group. This initial funding plays a very important roll in PI's development in scientific research career as well as management skill for research funds. The most substantial criticism of the original report was a lack of accomplishments of for the period of funding. We concur that there were publications less than PI anticipated. However, it resulted primarily from the negative findings in later part of research for testing new nitrone antioxidants that we synthesized. Nevertheless, we believed that we still managed to complete our scientific researches according to the original Statement of Work.

In response to specific comments from the reviewers, we would like to offer the following replies:

1. As reviewer suggested, we eliminated all the preliminary data with the original application and refrain from reporting data not obtained during the periods covered by this grant.
2. We only listed publications that directly or indirectly support by this grant.
3. We have integrated year 3 requested revisions into this final report.

Contractual Issues

1. We have listed the publications included as appendices with at least one of personnel supported by this grant according to reviewer's comments.
2. We have included a submitted manuscript in the appendices that addressed issue related original SOW (see appendix).
3. We have synthesized other nitrone antioxidants, namely, 2-hydroxy PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN) and 4-hydroxy PBN (4-OHPBN). These compounds have been tested both i*n vitro* and *in vivo*. Data from these experiments has placed in the appendices. Another set of data in the appendices is the effects of those compounds on hippocampal primary neuronal culture to confirm our *in vivo* findings
4. The additional findings describing the gene expression in the hippocampal tissue after kainate treatments were designed to investigate molecular mechanisms of kainate induced cell death, which fit in partially to SOW 3.

Technical Issues

1. We have included new information that reviewer asked in the appendices including a submitted manuscript, and data from in vivo experiments.

2. We agree with the reviewer's comment that there should be rigorous examination of free radical involvement in the Kainate treatment in the hippocampal cell death. However, this works were done by collaborating with other group of scientists leaded by Dr. Kim (Appendix 2). Data from these experiments can be found in the list of publications. PI has devoted significant time, effort, and resources into those projects.
3. We have reorganized data reports to be better presented and cited all the papers included in the report.

Format

1. The proposed synthesis of new and effective analogs, 2-hydroxy PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN) and 4-hydroxy PBN (4-OHPBN).was indeed carried out by Dr. Yashige Kotake. We still have a couple of grams of each compound in storage. He had failed to synthesis two other PBN analogs we proposed. However, the proposed electron paramagnetic resonance spectroscopy (EPR) was not performed in part due to PI left Oklahoma Medical Research Foundation where host the instrument that out of PI's reach.

Key Accomplishments

1. We have included six appendices which represent new information. In addition, we have presented the data from negative finding that have taken our most of last two years supported by this grant. We have tested four new above mentioned compounds in rats and in the primary neuronal cultures.
2. As reviewer suggested that we will only focus on the results obtained during the period funded by this grant and personnel who received support from this grant, including PI, were co-authors on those studies.
3. We have corrected discrepancy about time of PBN injection.
4. The data from both *in vivo* and *in vitro* for the different PBN analogs were presented in this revised report. The new compounds were synthesized as above mentioned.
5. The preliminary results from original finding were eliminated. However, we believe that it is not an unusual practice to include some of the preliminary results in the report or publication. The preliminary data is really preliminary; most times it has less number of the animals and less extensively studied. After this project was funded, we have performed additional experiment and used additional number of animals to obtain more precise and statistically sound data.

Body:

This section of the report is associated with each task outlined in the approved Statement of Work for summarizing the entire research effort.

A. *Establish kainic acid induced neuronal damage in adult rats as a model to study excitatory amino acid-induced neurodegenerative diseases. The major focus will be on the pathophysiological changes in the hippocampus. Special attention will be given to study oxidative damage induced program cell death (apoptosis), NFκB activation, and induction of p38 and AP-1 transcription factors.*

In order to establish kainic acid induced neuronal damage in adult rats as a model to study excitatory amino acid-induced neurodegenerative diseases, *in situ* TUNEL staining was performed to assess frank apoptosis. KA treatment caused DNA damage indicative of an apoptotic process within four days of subcutaneous administration (Appendix 3 Fig 7). Apoptosis was largely restricted to the CA1 and CA3 regions of the hippocampus wherein c-Fos was most strongly expressed. Administration of PBN 30 minutes after KA exposure strongly inhibited this apoptosis as indicated by diminished TUNEL staining in hippocampus from PBN treated animals (Appendix 3 Fig 7). Beginning approximately 30 minutes after KA injection, animals displayed archetypical epileptiform behavior including "wet dog" shakes, facial clonus, nodding, and forelimb clonus. Three hours after injection, KA-treated rats showed full limbic motor seizures including rearing and loss of postural control, as well as hypersalivation, circling and jumping. Rats treated with PBN 30 minutes after KA injection did not develop full limbic seizures by the 3 hour time point (Appendix 3, Table 1). Moreover, PBN rescued the KA-treated animals from mortality when evaluated at the end of the four day experiment. No behavioral, physiologic or histological alterations were observed in animals receiving PBN only.

The first immunochemical analysis of KA-treated rats was aimed at determining whether PBN could antagonize the AP-1 system *in vivo*. Immunocytochemical analysis was performed using well-characterized antibodies against the two AP-1 subunits, c-Fos and c-Jun. Within hours of KA treatment, c-Fos and c-Jun expression increased in hippocampal neurons, particularly within the CA1 and CA3 regions (Appendix 3, Fig. 2). The c-Fos and c-Jun expression was maintained throughout the 4 day experiment (not illustrated), consistent with

previously reported data (Bing et al., 1997). A single injection of PBN completely suppressed c-Jun expression in both CA regions and in the dentate gyrus (Appendix 3, Fig. 2) while c-Fos expression was suppressed by PBN only in the CA1 and CA3 regions, where most of the pathological changes were manifest. It may be significant to note that while c-Jun expression can be induced rapidly in neurons during growth factor deprivation, c-Fos expression seems to be restricted to those populations of neurons that actually commit to an apoptotic program (Estus et al. 1994).

The AP-1 pathway is but one of numerous signal transduction pathways which have been associated with cellular stress and linked to ligand-induced neurotoxicity. In particular, the p38 MAPK pathway has been repeatedly linked to neuronal apoptosis and, in some circumstances, may indirectly activate both the AP-1 and NFκB pathways (Vanden Berghe 1998; Hazzalin et al. 1997). The p38 mitogen-activated protein kinase pathway has been causally linked to neuronal apoptosis induced by growth factor withdrawal (Xia et al. 1995; Kummer et al. 1997). We therefore undertook an immunohistochemical analysis of p38 activation using an antibody specifically directed against the dual-phosphorylation motif which is present only on the active p38 kinase (Raingeaud et al., 1995). Within 4 hours of KA treatment, p38 activation was seen within the hippocampus in a pattern consistent with that of AP-1 activation (Appendix 3, Fig. 3). As in the case of AP-1, PBN suppressed p38 phospho-activation (Appendix 3, Fig. 3). The p38 system remained activated somewhat above the level of controls at the four day time point, but this chronic activation was not as dramatic as in the AP-1 case (not shown).

The NFκB transcription factor is also ubiquitously activated by physiologic stress and may potentiate excitotoxic damage in striatal neurons (Qin et al. 1998). Alternatively, NFκB seems to serve a protective role in hippocampal neurons undergoing an oxidative insult (Mattson et al. 1997) and may actually play an anti-apoptotic role in TNFα-stimulated cells (Van Antwerp et al. 1996; Wang et al. 1998). NFκB is part of a signal transduction cascade which has traditionally been thought of as distinct from the Jnk and p38 cascade modules, though correlated activation of the three pathways is often noted in cell culture experiments. We therefore sought to determine whether NFκB was activated by KA in a PBN-sensitive manner. As shown in Appendix 3, Fig. 4, NFκB-p65 immunoreactivity in the hippocampus increased dramatically within hours of KA treatment, and this effect was suppressed by PBN. The immunochemical data was corroborated by EMSA analysis which showed a dramatically-increased NFκB binding

activity in hippocampal nuclei of KA treated rats, which was partially mitigated by PBN co-treatment (Appendix 3, Fig. 4).

B. *Evaluate the nitrone antioxidant, phenyl N-tert-butyl nitrone (PBN), for its effectiveness in protection against KA induced neurodegeneration, and recurrent convulsive seizure activities. Special attention will be paid to the protective effects of PBN on apoptosis of the hippocampal neurons and on the pathophysiological changes induced by KA.*

To evaluate the nitrone antioxidant, PBN, for its effectiveness in protection against KA induced neurodegeneration and recurrent convulsive seizure activities; we injected PBN 30 minutes after KA exposure. We have found that PBN strongly inhibited this apoptosis as indicated by diminished TUNEL staining in hippocampi from PBN treated animals (Appendix 3, Fig. 7). Beginning approximately 30 minutes after KA injection, animals displayed archetypical epileptiform behavior including "wet dog" shakes, facial clonus, nodding, and forelimb clonus. Three hours after injection, KA-treated rats showed full limbic motor seizures including rearing and loss of postural control, as well as hypersalivation, circling and jumping. Rats treated with PBN 30 minutes after KA injection did not develop full limbic seizures by the 3 hour time point (Appendix 3, Table I). Moreover, PBN rescued the KA-treated animals from mortality when evaluated at the end of the four day experiment (data not shown). No behavioral, physiologic or histologic alterations were observed in animals receiving PBN only.

Hyperactivation of the JNK, NFκB and p38 signal transduction pathways could be anticipated to have numerous detrimental consequences. All three signaling pathways have been linked to transcription of inflammatory cytokines and to modulation of apoptosis (Kawasaki et al., 1997; Kummer et al., 1997; Yang et al., 1997; Qin et al., 1998). We therefore sought to determine whether cytokine and proapoptotic genes were being transcribed at a greater rate in the KA treated rats than in normal rats, and whether PBN could abrogate such an effect. Using a multiprobe ribonuclease protection assay, several inflammatory cytokines were clearly found to be transcribed following KA treatment (Appendix 3, Fig. 5). IL1α, IL1-β, IL-6 and TNF-α transcription were strongly induced by KA. Within the timeframe that cytokine transcription was enhanced, several proapoptotic genes were also induced. Most notably, the Fas antigen mRNA was strongly induced following KA and this elevation was maintained for at least four days (Appendix 3, Fig. 6). PBN treatment suppressed transcription of both inflammatory

cytokine gene products and proapoptotic gene products while having minimal effect on transcription of constitutively-expressed "housekeeping genes" including the L-32 ribosomal mRNA and glyceraldehyde phosphate dehydrogenase mRNA (Appendix 3, Figs. 5-6). PBN suppression of cytokine mRNA transcription was relatively unspecific. Interestingly, PBN displayed particular potency in suppressing Fas antigen and caspase 3 transcription, while other apoptosis-associated mRNA species analyzed by RPA were somewhat less affected by the nitrone (Appendix 3, Fig. 6).

C. *To elucidate the molecular mechanisms that underlies excitatory neurotoxin induced neurodegeneration. These will be assessed by using RNA protection assay for inflammatory cytokines and apoptosis-related genes (bcl 2, bax, caspase 1, 2, and 3), gel mobility shift assay for AP-1 and NFκB transcription factors, Northern and Western blot analyses for KA-induced mRNAs encoding Fos-related antigens, and c-Jun related transcription factors, and expression of inducible nitric oxide synthase (iNOS).*

To elucidate the molecular mechanisms that underlie excitatory neurotoxin induced neurodegeneration, we sought to determine whether cytokine and proapoptotic genes were being transcribed at a greater rate in the KA treated rats than in normal rats, and whether PBN could abrogate such an effect. Using a multiprobe ribonuclease protection assay, several inflammatory cytokines were clearly found to be transcribed following KA treatment (Appendix 3, Fig. 5, A), such as IL1α, IL1-β, IL-6 and TNF-α. Within the timeframe that cytokine transcription was enhanced, several proapoptotic genes were also induced. Most notably, the Fas antigen mRNA was strongly induced following KA and this elevation was maintained for at least four days (Fig. 6, Appendix 3). PBN treatment suppressed transcription of both inflammatory cytokine gene products and proapoptotic gene products; however, PBN treatment had a minimal effect on transcription of constitutively-expressed "housekeeping genes" including the L-32 ribosomal mRNA and glyceraldehyde phosphate dehydrogenase mRNA (Figs. 5-6, Appendix 3). PBN suppression of cytokine mRNA transcription was relatively unspecific. Interestingly, PBN displayed particular potency in suppressing Fas antigen and caspase 3 transcriptions, while other apoptosis-associated mRNA species analyzed by RPA were somewhat less affected by the nitrone (Fig. 6, Appendix 3).

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D. Test several other nitrone antioxidants for the protective action against kainic acid induced neuro-degeneration. In previous studies, we have tested several PBN derivatives for anti-inflammatory activities using a macrophage system. Based on the results of this cellular screening we selected four PBN type nitrones having a substituted phenyl group which showed the most effectiveness in neuroprotective actions. These are 2-hydroxy PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN), 4-hydroxy PBN (4-OHPBN), 2-sulfo- PBN (2-SPBN), and salicyl t-butylnitrone (SALBN).

. We have studied other nitrone antioxidants (PBN analogs) for the protective neuronal tissue against kainic acid induced damages. Those were 2-hydroxy PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN), 4-hydroxy PBN (4-OHPBN), 2-sulfo-PBN (2-SPBN). We have used the similar approaches as we did on PBN. However, to our disappointment, all of above-mentioned PBN analogs had no additional beneficial comparing with effects of PBN *in vivo* in protection of hippocampal neuronal cell death (Appendix 6, Fig 4). This may be partially due to the brain blood barrier that these reagents have to pass to have an effect on the hippocampal neurons. We have also tried to use neuron/glial mixed culture to test these reagents since they have been tested in our microglial culture system and showed anti-inflammatory effects. We performed neuron glia-mixed culture in 3 regions of rat brain, i.e., midbrain, cortex, and hippocampus (Appendix 6, Fig. 1, 2, and 3). We found that PBN can protect cultured neurons from different brain regions against KA induced neuronal damage, especially in protecting neuronal numbers and its processes in the hippocampal neuronal culture (Appendix 6, Fig. 3) However, we did not found other PBN analogs such as 2-hydroxy PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN), 4-hydroxy PBN (4-OHPBN), have additional beneficial effects.

E. Additional findings:

In our effort to elucidate the molecular mechanisms that underlies excitatory neurotoxin induced neurodegeneration, furthermore to better understanding target of PBN in central never system, we performed the suppression subtractive hybridization (SSH) and PCR-select differential screening methods (Clontech, Palo Alto, A) for comprehensive analyses of long-term, differential gene expressions in the hippocampus after KA induced degeneration. It has been reported that many genes were induced short after KA injection. However, A relatively little information is available for long-term gene expression in rat hippocampus after KA injection. In order to examine these long-term differentially expressed genes. We used the suppression subtractive hybridization (SSH) and PCR-select differential screening methods (Clontech, Palo Alto, A) for comprehensive analyses of long-term, differential gene expressions in the hippocampus. The mRNA was isolated from rat hippocampus 4 month after KA (n=3) and saline injection (n=3), cDNA was synthesized from the pooled mRNA, both forward subtracted and reverse subtracted hybridization was performed. Select-PCR was used to amplify the forward and reverse subtractive products. All of the PCR products were cloned into TA cloning vectors (Nitrogen). 432 clones were picked up (Appendix 7, Fig 1). After dot blotting and sequencing analysis 76 elevated genes and 25 suppressed genes were identified. Among them, 20 are long-term elevated genes, 11 are transient induced genes, 3 are long-term decreased genes, 8 are appear two-phase elevated, and 8 are appear to be multiple isotype genes in rat hippocampus identified by Northern blotting (Appendix 7, Fig 2, 3, and 4) after sequencing analysis and blast search 31 clones are reported in gene bank by other researchers and 23 of them have not been reported. The differential expressed genes are likely to be related to seizure activity, oxidative stress, apoptosis and some neurological disorders. These results indicated that 1). KA-induced hippocampal pathophysiological changes caused a differential gene expression that is related to neurodegenerative diseases (Appendix 7). 2). Long-term neuronal adaptation to excitatory toxicity involves a comprehensive multi-genes activation and suppression process (Appendix 7). 3). Systemic examination of all the genes that involve KA-induced neurodegeneration is possible by this methods, thus may shed a light on the molecular mechanism of neurodegenerative disease (Appendix, 4, 5, and 7).

Key Research Accomplishment:

- **Established kainic acid induced neuronal damage in adult rats as a model to study excitatory amino acid-induced neurodegenerative diseases by Terminal deoxyuridine nick-end labeling (TUNEL) for apoptotic cell death, Nissl staining and immunohistochemical assays.**
- **Demonstrated that nitrone antioxidant, PBN, inhibits KA-induced neuronal apoptosis, down regulates apoptosis-associate gene expression, and moreover, prevents seizure activity and death.**
- **Elucidated the molecular mechanisms underlying the nitrone antioxidants' protective functions against KA-induced neurodegeneration with signal transduction pathways by studying the activation of NFκB, p38, and AP-1.**
- **Tested several other PBN related antioxidants in kainic acid induced neurodegeneration.**

List of personnel receiving pay from the research effort:

Dr. Guoying Bing

Dr. Yahige Kotake

Dr. Kenneth Hensley

Dr. Lei Jin

Dr. Toyoko Arimoto

Dr. Anyang Sun

MS. Naiying Zheng

MS. Meili Zhu

Reportable Outcomes:

1. Animal Model: We have successfully used KA-induced neurodegeneration as an animal model for delayed neuronal cell death that occurred in many neurodegeneration diseases such and Alzheimer's and Parkinson's diseases.

2. A compound for treatment of excitotoxin-induced neuronal damages: We have also found that nitrone antioxidant, **PBN** can inhibits neuronal cell death in the hippocampus after KA treatment by suppressing proapoptotic signal transduction pathways.

Conclusion:

The findings of the present study extend upon previous observations concerning the broad-spectrum neuroprotective action of nitrone compounds, and provide a novel context for discussing the pathology of excitotoxicity. PBN and related nitrones have been shown to suppress striatal excitotoxic lesions induced by KA. The present data suggest that suppression of apoptosis by PBN in the KA model and possibly other models of neurodegeneration is likely due to mitigation of proinflammatory or proapoptotic gene expression under the control of the AP-1, NFκB, and p38 MAPK pathways. While the ultimate cellular target(s) for PBN action remain unclear, the present data suggest that the broad-spectrum neuroprotective action of the nitrone class of compounds might be due, in part, to antagonism of crucial oxidation-sensitive signal transduction elements linked to the initiation of apoptotic programs.

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Publications related to the researches supported by the grant:

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Appendices: Reprints, Figures, CV.

1. Floyd, R.A., Hensley, K., **Bing, G. (2000)** Evidence for enhanced neuro-inflammatory processes in neurodegenerative diseases and the action of nitrones as potential therapeutics. *J. Neural Transm*. 60:387-414.

2. Kim, H.C., **Bing, G.**, Jhoo, W.K., Ko, K.H., Kim, W.K., Suh, J.H., Kim, S.J., Kato, K., Hong, J.S. (2000) Changes of hippocampal Cu/Zn-superoxide dismutase after kainate treatment in the rat. *Brain Res*. 853:215-226.

3. Hensley, K.H., Stewart, C. A., Zheng, N. Y., Sang, S., Kotake, Y., Nguyen, X., Liu, M., Zhao., L., Jin., L., and **Bing** G. Phenyl-N-tert-butylnitrone inhibits neuronal apoptosis in the kainate acid model of epilepsy by suppressing proapoptotic signal transduction pathways. *Submitted*

4. Jin, L., Zheng, N.Y., Zhu, M., and **Bing G**. Long-term differential effects of systemic kainate treatment on neuropeptides expression. *In preparation*

5. Jin, J. Nael , N., Zheng, N.Y., Zhu, M., and **Bing. G**. Hippocampal long-term differential gene expression in the rat after systemic kainic acid injection revealed by PCR selected subtractive cloning. *In preparation.*

6. Figures

7. Jin, L., Zheng, N.Y., Zhu, M., Bing, GY. (1999) Long term, differential gene expression in the rat hippocampus after systemic kainic acid injection. Neurosci. Abstr. 25: 340.16, with poster.

8. C.V. for Guoying Bing

Note: item 4 and 5 is a incomplete manuscripts which are due to Dr. Lei Jin left lab. We are trying to complete them or rehiring him back to the lab to finished job.

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APPENDIX 1

Evidence for enhanced neuro-inflammatory processes in neurodegenerative diseases and the action of nitrones as potential therapeutics

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Summary. A brief review is presented on observations leading to the current notions regarding neuro-inflammatory processes. The greatest focus is on Alzheimer's disease (AD) since this is where the most convincing data has been obtained. A brief summary of observations on the neuroprotective action of α-phenyl-*tert*-butyl-nitrone (PBN) as well as results of research designed to understand its mechanism of action is presented. We hypothesize that the mechanism of action of PBN involves inhibition of signal transduction processes, which are involved in the upregulation of genes mediated by pro-inflammatory cytokines and H_2O_2 that cause formation of toxic gene products. Results from recent experiments on Kainic acid (KA) mediated brain damage are provided to suggest the validity of the in vivo action of PBN to inhibit neuro-inflammatory processes. The accumulating scientific facts are helping to provide concepts that may become the basis for novel therapeutic approaches to the treatment of several neurodegenerative diseases.

Introduction

Our attempts to explain the serendipitous observations made on the neuroprotective action of α-phenyl-*tert*-butyl-nitrone (PBN) several years ago provided a challenge, which lead us to postulate the occurrence of neuro-inflammatory processes in the stroked and the aging brain to help explain the results. Surprising observations made earlier by other investigators also forced them to conclude that enhanced neuro-inflammatory processes occur in the Alzheimer's Disease (AD) brain. Observations we made recently, combined with the early seminal findings and the many others made since, overwhelmingly support the notion that neuro-inflammatory processes occur in the AD brain. Results obtained in our attempts to explain the mechanistic basis of the neuroprotective action of PBN provide strong support for the notion that this compound acts, not by trapping free radicals in a mass-action

spin-trapping mode, but by effectively interfering with signal transduction mediated processes whereby stressors (such as H_2O_2 and IL1β) are prevented from upregulating genes in activated glia. This prevents the activated glia from yielding products such as nitric oxide and its reaction product peroxynitrite, which are toxic to neurons. In this report we briefly review historical observations, succinctly present data supporting these ideas, and summarize recent observations reinforcing the neuro-inflammatory notions and the activity of PBN in suppressing these processes. New findings presented relate to the action of kainic Acid (KA) induced damage to rat brain and the action of PBN in preventing this damage. Systemic administration of KA to rats was shown to mediate acute and chronic pro-inflammatory cytokine expression. Exploration of KA-mediated alteration in signal transduction pathways and the effect of PBN demonstrated immunohistochemically that p38 MAP kinase activation occurs 3 hours after toxin administration and that PBN administered after the toxin effectively suppressed p38 activation. Additionally, it was observed that KA enhanced the activation of NFκB transcription factor, as observed by the immunoreactivity of the p65 subunit epitope, in the hippocampus. PBN administration also suppressed this effect. PBN treatment shortly after KA exposure diminished the neurotoxic action of this compound assessed by death and intensity of seizures.

Alzheimer's disease; the original neuro-inflammatory observations

The first observations, which provided clues to the possibility that neuro-inflammatory processes may be occurring in the AD brain, came from the surprising results of studies by McGeer and colleagues in the late 1980's (McGeer et al., 1990; Tooyama et al., 1990; Rogers et al., 1992). Their data were the first to indicate that anti-inflammatory therapeutics taken by arthritic patients may delay the onset of AD (McGeer et al., 1990). Subsequent studies by several groups 17 in total, see their review (McGeer et al., 1996) have tended to uphold this original observation. Additionally, in early studies, McGeer and colleagues demonstrated that complement and classical markers of immune-mediated damage were expressed in affected brains (McGeer et al., 1987, 1989a, 1989b; Tooyama et al., 1990; Rogers et al., 1992) where microvessel lesions were lacking. These observations were not easily explained by the prevalent notions regarding AD development then, and in fact were dismissed or considered erroneous by many. However, since then, despite misconceptions of the notions involved (Rogers et al., 1996), increasing evidence has continued to accumulate and as the many reviews (see for example: Rogers et al., 1996; Aisen et al., 1994; Pasinetti, 1996; Eikelenboom and Veerhuis, 1996; Finch and Marchalonis, 1996; Rogers and O'Barr, 1997; Aisen, 1997; Eikelenboom et al., 1998; Floyd, 1999a) documenting the observations on the subject clearly show, the neuro-inflammatory notion is becoming more widely recognized. Cotman et al. (1996) captured the essence of the problem, "β-amyloid appears to develop properties that drive many signal

transduction processes in the classic injury cascade and also activate complement, which results in an amplified β-amyloid AD cascade". Their work shows different cell types collaborate and amplify the β-amyloid triggering events. Mediators generated by microglia (IL1, TNFα) activate astrocytes to produce other factors (IL6, etc.) that further activate nearby cells (Cotman et al., 1996). Thus β-amyloid plaques become "sparking centers" for what turns out to be "localized smoldering neuro-inflammatory processes" (Floyd, 1999a). Very recent research pertinent to the molecular events triggering the localized neuro-inflammatory processes have demonstrated that β-amyloid activation of microglia involves the interaction of CD40 receptor and the CD40 ligand (Tan et al., 1999).

Enhanced reactive oxygen species and oxidative damage are consequences of neuro-inflammatory processes

Enhanced reactive oxygen species (ROS) and the resulting oxidative damage is a characteristic feature of the AD brain (Markesbery, 1997; Smith et al., 1991; Smith et al., 1996). This is probably the result of several neuro-inflammatory events where ROS are known to be produced in excessive amounts. Activated microglia produce high levels of superoxide (Colton and Gilbert, 1987). β-amyloid activates microglia, monocytes and neutrophils to form superoxide via the NADPH oxidase pathway (Bianca et al., 1999). The amount of superoxide formed, measured as H_2O_2, was on the order of 1 nmole H_2O_2 per 3×10^5 cells in 30 minutes when stimulated with 10 μM β-amyloid peptide. β-Amyloid peptides per se also degrade to form ROS (Hensley et al., 1994), specifically H_2O_2, through transition metal ion reductive processes (Huang et al., 1999). Amyloid precursor protein per se regulates copper toxicity to neurons (White et al., 1999). H_2O_2 production by β-amyloid peptides per se or by the peptides interacting with microglia may be very important in triggering glia activation processes. We have shown that H_2O_2 itself activates cultured rat astrocytes in a manner very much like IL-1β (Robinson et al., 1999a). Clearly then H_2O_2 itself becomes a neuro-inflammatory propagating agent.

Enhanced protein oxidation associated with AD and in aging brain

Enhanced ROS formation would be expected a priori to lead to enhanced protein oxidation as well as enhanced lipid peroxidation. Significantly higher levels of protein oxidation have been noted in the AD brain versus the age-matched control brain (Smith et al., 1991). It was noted that specific brain regions had higher amounts of oxidized proteins. In general, those regions most affected by AD had higher levels of protein oxidation. It was also noted that protein oxidation increased logarithmically with age in normal, i.e. non-AD subjects. This seems to be a characteristic feature of brain aging. Increases in oxidized protein in brain with age have been noted in many

experimental models (Stadtman, 1992), including mice (Dubey et al., 1996; Forster et al., 1996), rats (Dubey et al., 1995), and gerbils (Dubey et al., 1995; Carney et al., 1991).

The increased levels of oxidized protein in brain with age could be due to a decrease in the rate of breakdown of oxidized protein by proteases. The research of Agarwal and Sohal (1994) addressed this possibility. Their results show that brain alkaline protease activity, the protease fraction shown to be responsible for the breakdown of oxidized protein, see references (Oliver et al., 1984; Mason and Rivett, 1994; Rivett, 1985; Rivett, 1989), does not decrease with age (Agarwal and Sohal, 1994). From this data they concluded decreases in alkaline protease activity could not explain the age-related increase in oxidized protein in brain. The point of this discussion is an attempt to rationalize the data obtained on the neuroprotective activity of PBN in different models and its affect on brain oxidized protein in rat and gerbil brain in relation to its proposed action of suppressing signal transduction processes. The reason why PBN suppresses the amount of oxidized protein in the aged gerbil brain (Carney et al., 1991; Floyd and Carney, 1996) may be because it suppresses the signal transduction processes leading to increased ROS generation caused by the inherent (unknown) activation processes that occur with age. In contrast to rats and gerbils it was noted, in the only study published, that the administration of PBN to older mice did not cause a significant reduction in oxidized protein in cerebral cortex (Dubey et al., 1995). A careful review of that work showed that there was a trend toward PBN-mediated reduction in oxidized protein, but it was not large enough to be significant. This may be because cerebral cortex is a brain region in mouse that does not change greatly in oxidized protein with age as other brain regions (Dubey et al., 1996; Foster et al., 1996) and possibly because the mice in the study were significantly younger (23 months) than the other studies where older mice were used. Additionally, the mice were administered PBN as bolus injections (32 mg/kg). Administration of it in drinking water, a regiment that has been shown to prolong life span in mice (Saito et al., 1998), may have been more effective.

Neuro-inflammatory processes in the aging brain

There are only a few studies in experimental animals directed toward the examination of the normal aging brain from the perspective of evaluating if neuro-inflammatory type processes occur. However, these studies do provide strong evidence to support the notion that neuro-inflammation type processes are present and do increase with age. Recent detailed studies in this area have come from Finch's lab (Rozovsky et al., 1998; Morgan et al., 1999) and from Morgan's lab (Gordon et al., 1997). The older literature was referenced by Finch and Morgan (1990). The results are consistent in showing that aging in brain is associated with an increased expression of glial fibrillary acidic protein (GFAP); and that increased GFAP expression is a marker of astrocyte activa-

tion and is a response to CNS injury. Gordon et al. (1997) showed that injury, induced by several means, including 6-hydroxy-dopamine injection or a needle stab wound, to the old brain, caused a more exaggerated astrocyte response, which persisted much longer than the same injury did in a young brain. So the old brain responded more to an injury and the response to that injury persisted for much longer. These studies reinforce the results of our work in gerbils where we noted that a stroke insult was much more serious to older animals (Carney et al., 1991; Floyd, 1990). In a careful study where microglia and astrocytes were collected from 3-, 6-, 12- and 24-month rat brains, Rozorsky et al. (1998) demonstrated that both microglia and astrocytes taken from old brains had more proliferative capacity and expressed more GFAP than those taken from young brains. TGF-β_1, which normally down-regulates inflammatory processes was less capable of suppressing proliferation of astrocytes and microglia taken from older brains when compared to younger brains (Rozovsky et al., 1998). Similarly TGF-β_1 was less capable of suppressing LPS-induced nitrate formation in the cultured microglia from older brains than the microglia from younger brains. Their data was interpreted as supporting the "hypothesis that aging promotes a proliferative microenvironment in the brain".

Excess nitric oxide and peroxynitrite reaction products in AD brain

Products formed by the reaction of nitric oxide (NO) and peroxynitrite, (formed by the reaction of NO with superoxide), with cellular components were shown to be enriched in the affected regions of the AD brain (Smith et al., 1997; Hensley et al., 1998). This is also clear evidence of the involvement of neuro-inflammatory processes in the AD brain. It is known that pro-inflammatory cytokines as well as β-amyloid stimulates the production of NO in astrocytes (Akama et al., 1998). β-Amyloid enhanced NO production by astrocytes involves NFκB-mediated mechanisms (Akama et al., 1998). Enhanced NO production most likely occurs because of the induction of inducible nitric oxide synthase (iNOS) which mediates the formation of large amounts of NO. Utilizing three different antibodies to 3-nitro-tyrosine, (a product formed by the reaction of peroxynitrite with protein tyrosines), Smith et al. (1997) demonstrated significant 3-nitro-tyrosine staining in affected regions of AD brain but none in comparable age-matched control brain regions. Using novel HPLC-electrochemical detection methods to quantify the 3-nitro-tyrosine content of protein digest, we demonstrated that the content of this nitrative adduct is increased 3 to 7-fold in affected brain regions of AD subjects when compared to age-matched control brain regions (Hensley et al., 1998). In addition to 3-nitro-tyrosine, we simultaneously measured the dityrosine content of the protein digest and noted that this adduct followed in a somewhat general pattern to that observed for 3-nitro-tyrosine content (Hensley et al., 1998). Dityrosine adducts are formed by the bimolecular addition of tyrosyl free radicals.

Nitric oxide and peroxynitrite is more toxic to neurons

The clear demonstration of enhanced NO formation in affected regions of AD brain evokes a possible mechanistic basis for the mediation of neuron death or dysfunction. It has been shown that NO (and its reaction products) is more toxic to neurons than to the glia which produces it in copious quantities (Dawson et al., 1993; Dawson and Dawson, 1996). Study of the neurotoxic potency of NO and its reaction products have shown that its reaction with superoxide to form peroxynitrite is a key event in its neurotoxicity (Lipton et al., 1993). The exact molecular events involved in the neurotoxicity of nitric oxide and reaction products are not known.

Enhanced signal transduction processes near β-amyloid plaques

The involvement of neuro-inflammatory processes surrounding β-amyloid plaques is expected to cause enhanced intracellular signaling (signal transduction processes) in cells surrounding the plaques (Cotman et al., 1996). Enhanced signal transduction processes are expected because, as noted previously, β-amyloid has been shown to activate microglia via the CD40/CD40L complex (Tan et al., 1999) and to mediate formation of H_2O_2 by microglia (Colton and Gilbert, 1987) as well as to produce H_2O_2 itself (Huang et al., 1999). H_2O_2 has been shown to mediate enhanced signal transduction processes in astrocytes (Robinson et al., 1999a). Enhanced levels of IL1 and IL6 cytokines are noted near the plaques (Rogers et al., 1996; Cotman et al., 1996) and these factors are expected to mediate the enhancement of signal transduction processes. Activation of signal transduction processes involves enhanced activation (phosphorylation) of MAP kinases. Our research effort has provided a clear demonstration that enhanced signal transduction processes occur in cells surrounding the β-amyloid plaques in affected regions of AD brain (Hensley et al., 1999). We found that activated p38 was readily apparent in neurons and glia surrounding senile plaques in the AD brain. Very little if any p38 activation was found in comparable regions of age-matched control brains or in the cerebellum of AD brains. These results provided the first demonstration of p38 activation in human tissue and definitely show enhanced signal transduction processes in cells near the senile plaques in the AD brain.

P38 MAP kinase and excess nitric oxide synthase

p38 is a redox-sensitive MAP kinase (Abe et al., 1996; Huot et al., 1997). p38 activation plays a role in apoptosis and/or inflammation processes depending on the cell type. p38 is involved in apoptotic processes which are triggered in PC12 cells by deprivation of nerve growth factor (Monti et al., 1996). p38 is also involved in apoptosis in human fibroblasts (Schwenger et al., 1997). On the other hand, inhibitors of p38 prevent the biosynthesis of TNFα and IL1 in

stimulated monocytes (Ridley et al., 1997). Genes induced via the p38 kinase cascade pathway are probably very important in neurodegenerative processes. It was noted that p38 was activated in the hippocampus of gerbils 4 days after a global brain stroke was administered to these animals (Walton et al., 1998). The hippocampus is the area of the brain most susceptible to tissue injury in these animals and the brain region producing the most ROS following a global stroke (Cao et al., 1988; Carney et al., 1992). It has been shown, using inhibitors, that p38 activation is on the pathway to mediating the induction of iNOS in mouse astrocytes (Da Silva et al., 1997) and in rat glia cells (Bhat et al., 1998). Pertinent to the importance of iNOS expression and excess NO formation in stroked brain, Iadecola's group have shown that enhanced iNOS expression occurs after cerebral ischemia in rat (Iadecola et al., 1995a) and that administering catalytic inhibitors of iNOS afforded some protection from the tissue injury caused by a stroke (Iadecola et al., 1995b). PBN has been shown to prevent the induction of iNOS in a mouse septic shock model (Miyajima and Kotake, 1995).

Historical observations on neuroprotective activity of PBN in stroke

PBN has neuroprotective activities in several experimental models. We have reviewed the research in this field (see references Floyd, 1997; Hensley et al., 1996, 1997; Floyd, 1999b). The neuroprotective activity of PBN was discovered serendipitously. Utilizing the gerbil global stroke model, we attempted to make use of PBN to trap and identify free radicals during the reperfusion phase. In previous experiments, we had demonstrated using salicylate trapping that enhanced hydroxyl free radicals were formed during the reperfusion phase of stroke (Cao et al., 1988; Carney et al., 1992). PBN had been used for several years in analytical chemistry experiments to "spin-trap" and identify free radicals in chemical reactions (Janzen and Blackburn, 1969). It had also been demonstrated to be useful to trap certain free radicals in biochemical (see references Poyer et al., 1978; Poyer et al., 1980) for example) and biological systems (see references Bolli et al., 1988; Lai et al., 1979; Lai et al., 1986 for example). Our intent was to use it to see if we could elucidate the free radicals involved in experimental stroke. We found that PBN was an ineffective trap for the free radicals formed in the gerbil stroke model (Oliver et al., 1990), but discovered that it protected the gerbil from death caused by the stroke (Floyd, 1990). This observation has been replicated by other laboratories (Clough-Helfman and Phillis, 1991; Phillis and Clough-Helfman, 1990a, 1990b) and in fact PBN was shown to be neuroprotective (as assessed by brain necrosis) even if administered up to 1 hour after brain reperfusion in the gerbil model (Phillis and Clough-Helfman, 1990a). The results have since been extended to the rat middle cerebral artery occlusion (MCAO) model where PBN was shown to protect the affected brain region even if delivered up to 3 hours after the start of reperfusion (Zhao et al., 1994). A 2,5- disulfonyl PBN derivative, in development for the treatment of stroke, has also been shown to be active in the MCAO model if delivered 2 hours after the start of reperfusion

(Kuroda et al., 1999). It should be noted that Beal's group have shown considerable efficacy of PBN and its 2-sulfonated derivative (S-PBN) in several experimental models of neurodegeneration (Schulz et al., 1995). These include neuroprotective activities of S-PBN in excitotoxicity models using NMDA, KA and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid. Striatial lesions caused by MPP^+, malonate and 3-acetylpyridine were significantly inhibited by PBN as well as by S-PBN.

Chronic dosing of PBN conditioned the old brain to be less susceptible to stroke

We found that old gerbils (15–18 months old retired male breeders) were much more susceptible to a global stroke than were young (3-month-old males) gerbils (Floyd, 1990). If PBN was administered at a chronic low dose (30 mg/kg-day, twice daily) for 14 days to the old gerbils and then its administration ceased, the old treated gerbils were more resistant to a stroke, in fact nearly as resistant as were the young gerbils. This enhanced protection from stroke remained with time after ceasing PBN administration but declined to nearly 30% at 5 days (Floyd and Carney, 1996). The normal enhanced susceptibility of the old gerbils to a stroke returned by 14 days after ceasing PBN administration. There is very little chance that residual PBN remained in the dosed animals for very long after cessation of its administration, for its half-life is 132 minutes (Chen et al., 1990). Therefore, we have concluded that PBN administration mediates the alteration of the old brain such that it becomes more resistant to stroke (Floyd and Carney, 1996). In concert with this notion is the observation that chronic PBN administration lowered the normally age-enhanced oxidized protein levels in old gerbil brain back down to that noted in young gerbils (Carney et al., 1991; Floyd and Carney, 1996). Cessation of PBN administration resulted in the subsequent rise again of the oxidized protein levels in old gerbils back to the original enhanced levels (Carney et al., 1991). We also found that the enhanced behavioral errors of the older gerbils, as compared to younger gerbils, were largely reversed by the chronic 14-day PBN administration. Behavioral errors were assessed by a radial arm maze.

Neuroprotective activity of PBN is not due to its free radical trapping activity

The mechanistic basis of the neuroprotective activity of PBN has not been completely resolved. The discovery in 1969 of the mass action type reaction of PBN with free radicals made it a very useful tool to characterize free radical intermediates in analytical chemistry (Janzen and Blackburn, 1969). However, it is very clear that its neuroprotective action is not due to its ability to trap free radicals in the conventional mass action "spin-trap" mode (see our reviews Floyd, 1996; Hensley et al., 1997; Floyd, 1999b). One main reason is the fact that PBN acts to protect in stroke when delivered up to several hours

after the ischemic/reperfusion event. This means that it was not even present when the most rapid burst of free radicals occurred. The most rapid burst of free radicals in the stroked brain starts almost immediately after starting reperfusion (Cao et al., 1988; Carney et al., 1992). PBN is neuroprotective even if administered up to 3 hours after the start of reperfusion (Zhao et al., 1994). This is a very strong argument against its direct scavenging of ROS as the mechanistic basis of neuroprotective activity of PBN in the stroke model. Additionally, the fact that PBN is very active at chronic, very low levels in mediating a decrease in oxidized protein in old brain argues that its action is not merely mass action in the simple sense of the concept. Significant protein oxidation decreases have been noted in old gerbil brain after administering as little as 1 mg/kg-day PBN for 14 days (Floyd and Carney, 1996). Since PBN distributes essentially equally to all tissues within 20 minutes after its injection (Chen et al., 1990), then the maximum level of PBN that is expected to reach the brain 20 minutes after a 1 mg/kg injection is less than 1 μmolar. In chemical and biochemical experiments where the mass action type free radical trapping activity of PBN is utilized, it is normally used at 10–100 mM; and then it is assumed that it does not trap all of the free radicals present. In stroke experiments where it is administered as a bolus at 100 mg/kg 2–3 hours after reperfusion then the extracellular brain levels was shown by microdialysis to be at most 500 μM (Cheng et al., 1993). Therefore, it is not conceivable that the biological activity of PBN depends upon its classical mass action-trapping activity as noted in chemical systems. In fact, when compared to butylated hydroxytoluene (BHT) or Vitamin E its ability to shut down lipid peroxidation in rat liver microsomal systems, PBN is about 1,000-fold less active than BHT or Vitamin E (Janzen et al., 1994). Therefore, it is not even a very good antioxidant, the potency of which depends upon its ability to trap free radicals.

Behavioral deficits in brain aging/PBN effect

Arendash's group has demonstrated that aged 24-month old rats treated for 4–5 months with a combination of established antioxidants (PBN, vitamin E, and vitamin C) show improved learning and memory retention in the Morris water maze compared to aged controls (Socci et al., 1995). In a follow-up study, they injected aged 24-month old rats with PBN daily (32 mg/kg, ip) for up to 9.5 months (Sack et al., 1996). Several months into the treatment, Morris water maze testing revealed that PBN- and vehicle-treated rats had similar learning in this task. However, PBN-treated aged rats showed remarkably higher memory retention in the water maze compared to controls. In later one-way active avoidance testing, these same PBN-treated animals showed significantly greater learning than controls. These findings, in addition to an earlier study reporting PBN-induced enhancement of radial maze performance in aged gerbils (Carney et al., 1991), clearly demonstrate a cognitive-enhancing ability of PBN in aged rodents. Moreover, the PBN study (Sack et al., 1996) showed that the same group of PBN-treated animals that exhibited

cognitive enhancement also had reduced lipid peroxidation levels (as indexed by TBAR formation) in brain areas important for cognition. Results from other laboratories are consistent with several conclusions from our PBN studies. First, 14-day administration of PBN to accelerated senescence mice resulted in cortical synaptosomes showing EPR spectra indicative of less oxidative stress (Butterfield et al., 1997). Second, daily PBN injections given to accelerated senescence mice beginning in adulthood induced a 1/3 extension in average lifespan (Edamatsu et al., 1995) and PBN given in drinking water to aged mice significantly extended both average and maximal lifespan (Saito et al., 1998).

Hypothesis to explain the neuroprotective activity of PBN

We hypothesize that most, if not all, of the neuroprotective activity of PBN can be accounted for by its ability to suppress signal transduction processes, which can become exacerbated in the brain when it is suffering from any number of insults or "abnormal conditions". For the purposes of illustration, we consider three general "abnormal" conditions that a brain may experience where enhanced signal transduction processes and enhanced oxidative damage are known to occur. The three general "abnormal" conditions are: A) experiencing a large rapid insult, B) undergoing a constant, slowly accelerating-localized smoldering insult and C) experiencing a very low level constant chronic stress. The brain conditions, which generally fit these three categories, are stroke, Alzheimer's disease and an advanced aging brain, respectively. These general concepts are illustrated in Fig. 1. Clearly the conditions apply to specific brain regions for each condition. Figure 2 illustrates the production of "toxic gene products" that are formed at higher levels under each of the three

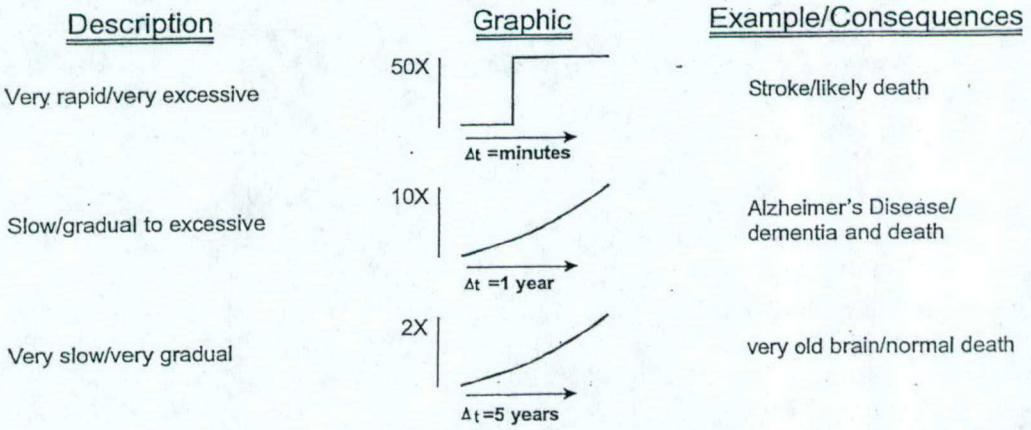

Fig. 1. Representation of brain oxidative challenge states. Particular attention should be directed to the degree of oxidative challenge (ordinate) which is very different in each of the cases and the time-frame (abscissa) which is also very different depending on each of the cases

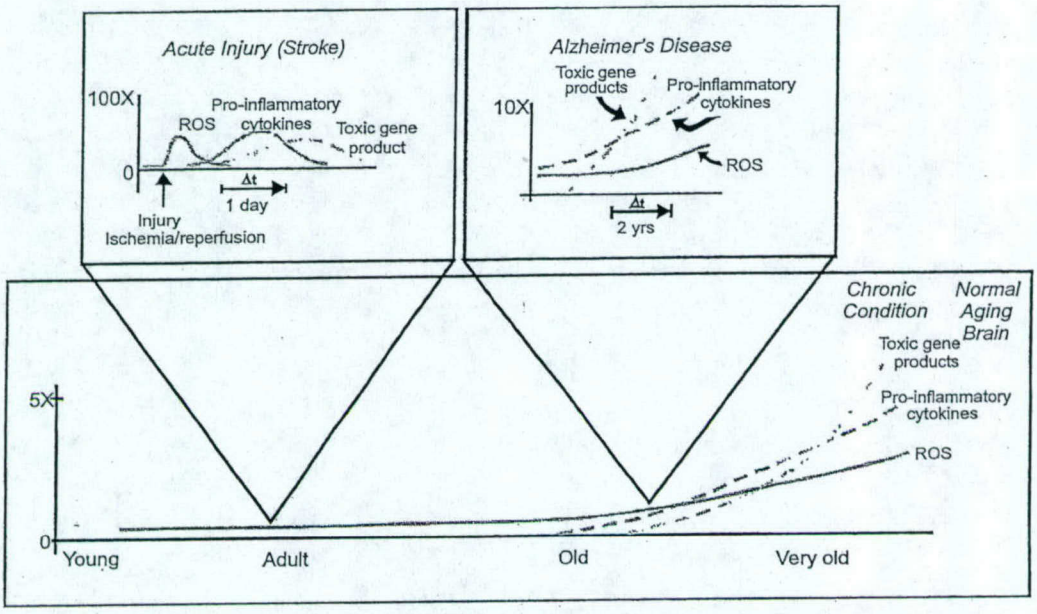

Fig. 2. Illustration of the reactive oxygen species (ROS) expected and the pro-inflammatory cytokine level and toxic gene product levels expected in a coordinated time dependent fashion. The time-frame and levels of each of the species are different in each of the conditions

conditions. The general term "toxic gene products" refers to neurotoxic compounds produced by genes that are induced or are generally upregulated by the insults or abnormal conditions that challenge the brain. We hypothesize that PBN suppresses the production of toxic gene products by suppressing the exacerbated signal transduction processes that leads to the induction of genes that form the neurotoxic products. Perusal of Fig. 2 illustrates that there is a lag time after a stroke before the gene induction processes begin and therefore, if PBN is available during this lag time, then it is expected to mediate the suppression of gene induction initiated by the stroke. In the case of the advanced aging brain, much lower levels of pro-inflammatory cytokines and other activation factors are present when compared to a stroked brain. Nevertheless the amount of cytokines present is higher than in a younger brain. The higher levels of pro-inflammatory cytokines and other factors cause the brain to experience enhanced oxidative stress over a long period of time. We postulate that this leads to enhanced protein oxidation and, for some unknown reason, the brain becomes more susceptible to a stroke. In the case of the advanced aging brain, it is then expected that chronic administration of PBN would suppress the low-grade signal transduction processes and hence lower the amount of oxidized protein. This then positions the brain to become less sensitive to a stroke. This model would then explain the results we have obtained with the stroked gerbils (Floyd, 1990) and the results Siesjo's group obtained in the rat MCAO stroke model (Zhao et al., 1994). This model would also explain the results we obtained with chronic administration of

PBN to the old gerbils (Carney et al., 1991; Floyd, 1990; Floyd and Carney, 1996). Based on this model to explain the results in the old gerbils and in the stroked brain, we think that the Alzheimer's brain suffers a condition that is represented as an intermediate somewhere between the two extremes of stroke and the advanced aging brain (see Fig. 1 and Fig. 2).

Utilizing the logic of this model we hypothesize that chronic PBN administration will suppress the enhanced signal transduction processes in the Alzheimer's brain and hence significantly lower the production of toxic gene products and decrease the amount of oxidized protein. We consider that dementia is due in part to damaged neurons caused by the production of "toxic gene products" which are made as a result of enhanced neuro-inflammatory processes that are triggered by β-amyloid plaques. Chronic PBN treatment is expected to decrease neuro-inflammatory processes and therefore, should be able to decrease dementia. It is possible that chronic PBN administration, perhaps for a relatively short period of time, may restore most of the normal functioning of the brain. If this hypothesis is valid, it is expected that PBN would have no influence on β-amyloid deposition. Therefore, the triggering stimulus would still be persistently present and hence, cessation of PBN administration would then result in the restoration of the neuro-inflammatory processes leading to enhanced protein oxidation and eventually to the redevelopment of dementia.

PBN inhibition of signal transduction processes

Our interest in signal transduction processes as the possible site of action of PBN became more intense as more and more evidence accumulated showing that ROS is involved in some fashion in signal transduction processes (see Reference Suzuki et al., 1997 for a review). There are many published reports now demonstrating that PBN suppresses signal transduction processes both in cultured cell systems as well as in animal models. The first demonstration of this fact was made evident in the stroked gerbil brain (Carney et al., 1994), where it was noted that PBN administration suppressed the induction of several genes. A more clear-cut example was then made by Miyajima and Kotake (Miyajima and Kotake, 1995) who demonstrated that PBN inhibited the induction of iNOS in the liver of a septic shock model, i.e. LPS-treated mice. They demonstrated that PBN inhibited iNOS induction but that it did not act as a catalytic inhibitor of the fully expressed and functional iNOS enzyme. Utilizing a multiprobe ribonuclease protection assay we have shown in the rat LPS-induced septic shock model that PBN suppresses a wide array of genes induced in liver (Stewart et al., 1999). Utilizing a neonatal rat model of AIDs Dementia Complex where gp120, the HIV envelope protein, is administered we demonstrated that PBN prevented the gp120-induced production of NO in the neonatal rat brain (Tabatabaie et al., 1996). Our interpretation of the results was that PBN prevented the induction of iNOS in the brain. Kotake's laboratory has recently demonstrated that PBN prevents the enhanced synthesis of NO in brain induced by a direct brain injection of LPS as an experimental model of bacterial meningitis (Endoh et al., 1999). In

cellular systems, Kotake's group has shown that PBN at higher levels inhibits LPS-mediated upregulation of iNOS and COX-2 in a macrophage cell line (Kotake et al., 1998). PBN prevented the LPS-mediated NFκB movement to the nucleus. PBN at higher concentration inhibited catalytically the expressed iNOS enzyme but did not act catalytically on the COX-2 enzyme (Kotake et al., 1998). Our group has examined the efficacy of PBN in a series of experiments involving signal transduction processes in cultured rat astrocytes. The assays have focused on p38 activation processes in the astrocytes. The results (Robinson et al., 1999a, 1999b) can be summarized as such: A) astrocytes are activated by various cytokines especially IL-1β and H_2O_2 and B) PBN as well as N-acetylcysteine (NAC) suppresses IL-1β and H_2O_2 mediated p38 activation. In this system, p38 is at first rapidly activated and then is subsequently shut down in a biphasic response. It should be noted that synthesis of cytokines is triggered in the activated cells and that PBN suppresses this.

Does PBN suppress neuro-inflammatory processes in vivo?

The previous sections provide background information which clearly implicate that PBN would be expected to suppress neuro-inflammatory processes. Prior to now, no experiment has ever been set up to directly test this notion in an in vivo model. We report here results of an experiment clearly showing that PBN does suppress signal transduction events linked to neuro-inflammatory processes in a KA — brain damage model in rats. Although the KA model is not a classical neuro-inflammatory model in the sense that AD would be, it nevertheless does provide very valuable information and surprises.

We have utilized the KA model of epilepsy, where a single systemic dose of the excitotoxin initiates a process of hippocampal neurotoxicity (Bernard and Wheal, 1995). Rats treated with KA suffer recurrent convulsive seizures and apoptotic neuron loss in the CA1 and CA3 regions of the hippocampus (Pisa et al., 1980; Schwob et al., 1980). Seizure activity is correlated with neuroanatomical changes including mossy fiber sprouting in the dentate gyrus, hippocampal sclerosis, and eventually, neuronal death (Schwob et al., 1980; Sperk et al., 1996; Cronin et al., 1992). The lesions produced by systemic KA treatment resemble those seen in hippocampi of human temporal lobe epileptics (Sommer, 1880; Schwob et al., 1980; Pisa et al., 1980; Sperk et al., 1996). KA appears to act directly on non-NMDA type ionotropic glutamate receptors (Bernard and Wheal, 1995), leading to cell death, which is predominantly apoptotic in nature (Simonian et al., 1996; Bengzon et al., 1997; Yang et al., 1997; Cheung et al., 1998). Our goal was to use KA to chronically stimulate signal transduction pathways and determine if PBN administration would suppress these events.

Materials and methods

Animals. Adult male Sprague Dawley rats (225–250 g each) were injected subcutaneously behind the neck with KA (Sigma Chemical, St. Louis MO) at a dose of 10 mg/kg, or with

vehicle alone (saline). Animals were observed for 4 hours following KA treatment and seizure activity was rated according to the scale developed by Racine et al. (1972) and modified by Mathis and Ungerer (1992). Briefly, seizure severity was scored in five stages; from Stage 1 where animals had mild myoclonus with moderate jerking movements of one or two links to State 5 where animals had status epileptic, i.e. continuous seizure activity for 30 minutes or longer with explosive jumps.

Phenyl-N-*tert*-butylnitrone was synthesized at the Oklahoma Medical Research Foundation (Oklahoma City, OK) and was injected at a dose of 150 mg/kg intraperitoneally, in saline vehicle, 90 minutes after KA treatment. The 150-mg/kg bolus of PBN is a standard dose and has repeatedly been shown effective in rodent models of ischemia-reperfusion injury and sepsis, which causes no obvious side effects such as lethargy and hypothermia that, can sometimes be seen at higher doses (Hensley et al., 1997).

Immunohistochemistry

For immunocytochemical studies, animals were anesthetized with pentobarbital and perfused with saline followed by 4% paraformaldehyde in saline. Brains were sectioned into 30 μm slices, which were incubated in 4% normal goat serum in saline for 30 min. at ambient temperature. After three washes with saline, the sections were incubated overnight at 4°C in saline plus 0.025% triton X-100, 1% goat serum, and primary antibody. Immunoreactivity was visualized by the avidin-biotin-bridged immunoperoxidase method using 3,3′-diaminobenzidine (DAB) as the chromagen (Hsu et al., 1981). The anti-phospho-p38 antibody was an affinity-purified rabbit IgG purchased from New England Biolabs (Beverly, MA), used at 1/300 dilution. Affinity purified rabbit IgG antibodies against c-Fos, c-Jun and the p65 subunit of NFκB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were used at 1/1,000, 1/1,000, and 1/300 dilution, respectively. Photomicroscopy was performed on a Zeiss Axioplan 2 spiker instrument (Carl Zeiss Inc., Thornwood, NY).

Electromobility gel-shift assays (EMSAs)

EMSAs were conducted to determine binding of activated NFκB complexes to synthetic oligonucleotide consensus sequences. The NFκB-binding oligomer was a 22-mer: 5′-GATCGAGGGGACTTTCCCTAGC-3′, purchased from Stratagene (La Jolla CA). Double-stranded oligomers were labeled with [γ-^{32}P]ATP using 10 u/reaction of T4 polynucleotide kinase (U.S. Biochemical Corp., Cleveland, OH). Hippocampi were dissected free and homogenized, and nuclear protein extracts were prepared as described (Sonnenberg et al., 1989). Binding reactions (30 μL) were performed at room temperature in reaction mixtures containing 40 μg protein, 20 mM Tris-HCL pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 50 μg/mL bovine serum albumin, 100 μg/mL sonicated salmon sperm DNA, 10% glycerol, and approximately 0.2 ng (50,000 cpm) of the specific probe. Protein-DNA complexes were separated on 5% nondenaturing polyacrylamide gels run at 150 V

in 50mM Tris/50mM boric acid/1mM EDTA. Gels were then dried and autoradiographed overnight.

Terminal deoxyuridine nick-end labeling (TUNEL)

DNA fragmentation characteristic of apoptosis was visualized by 3′end labeling with biotin-derivatized deoxynucleotides via terminal deoxynucleotidyl transferase catalysis. A commercially available TUNEL kit was used (TdT FragEL, Calbiochem, San Diego CA). Biotinylated nucleotides were detected using streptavidin-conjugated horseradish peroxidase and diaminobenzidine (Hsu et al., 1989). Tissue sections thus labeled were counterstained with methyl green as an aid to morphological evaluation.

Ribonuclease protection assays

Approximately 100mg of hippocampal tissue was homogenized in trizol isolation reagent (Life Technologies, Gaithersburg, MD) using a Dounce-type homogenizer. Total RNA in the extract was quantified by UV absorbance at 260nm. Inflammation and apoptosis-associated mRNA species were selectively visualized using a multiprobe ribonuclease protection assay (RPA). Radiolabeled probes were synthesized from DNA templates containing a T7 RNA polymerase promoter (Pharmingen, San Diego, CA). Templates were transcribed in the presence of [γ-^{32}P]ATP to yield radioactive probes of defined size for each mRNA. Probes were hybridized with total hippocampal RNA, then samples were treated with RNAse A and T1 to digest single-stranded RNA. Intact double-stranded RNA hybrids were resolved on 5% polyacrylamide/8M urea gels to produce bands detected by autoradiography.

Results

Beginning approximately 30 minutes after KA injection, animals displayed archetypical epileptiform behavior including "wet dog" shakes, facial clonus, nodding, and forelimb clonus. Three hours after injection, KA-treated rats showed full limbic motor seizures including rearing and loss of postural control, as well as hypersalivation, circling and jumping. Rats treated with PBN 90 minutes after KA injection did not develop full limbic seizures by the 3-hour time point (Table 1). Moreover, PBN rescued the KA-treated animals from mortality when evaluated at the end of the four-day experiment (Table 1). No behavioral, physiologic or histologic alterations were observed in animals receiving PBN only.

The first immunochemical analysis of KA-treated rats was aimed at determining whether PBN could antagonize the AP-1 system in vivo. Immunocytochemical analysis was performed using well-characterized antibodies

Table 1. Suppression by PBN of limbic seizures and mortality in kainic acid-treated rats. Seizure activity was ranked on a five-point scale as described in the methods

Treatment	Seizure intensity	Mortality (4 days)
Kainic acid (N = 30)	4.9 ± 0.4	12/30 (38%)
Kainic acid + PBN (N = 20)	2.3 ± 0.3*	0/20 (0%)**

*$P < 0.05$ (Student's t-test)
**$P < 0.02$ (χ^2 test)

against the two AP-1 subunits, c-Fos and c-Jun. Within hours of KA treatment, c-Fos and c-Jun expression increased in hippocampal neurons, particularly within the CA1 and CA3 regions. The c-Fos and c-Jun expression was maintained throughout the four-day experiment (not illustrated), consistent with previously reported data (Bing et al., 1997). A single injection of PBN completely suppressed c-Jun expression in both CA regions and in the dentate gyrus while c-Fos expression was suppressed by PBN only in the CA1 and CA3 regions, where most of the pathological changes were manifest (data not shown). We also have done an immunohistochemical analysis of p38 activation using an antibody specifically directed against the dual-phosphorylation motif, which is present only on the active p38 kinase (Raingeaud et al., 1995). Within 4 hours of KA treatment, p38 activation was seen within the hippocampus in a pattern consistent with that of AP-1 activation (Fig. 3). As in the case of AP-1, PBN suppressed p38 phospho-activation (Fig. 3). The p38 system remained activated somewhat above the level of controls at the four-day timepoint, but this chronic activation was not as dramatic as in the AP-1 case (data not shown).

The NFκB transcription factor is also ubiquitously activated by physiologic stress and may potentiate excitotoxic damage in striatal neurons (Qin et al., 1998). Alternatively, NFκB seems to serve a protective role in hippocampal neurons undergoing an oxidative insult (Mattson et al., 1997) and may actually play an antiapoptotic role in TNFα-stimulated cells (Van Antwerp et al., 1996; Wang et al., 1998). NFκB is part of a signal transduction cascade, which has traditionally been thought of as distinct from the Jnk and p38 cascade modules, though correlated activation of the three pathways is often noted in cell culture experiments. Several lines of evidence now suggest that p38 and other MAPK enzymes may hyperactivate NFκB (reviewed in Schulze-Osthoff et al., 1997), while inhibition of p38 can suppress transactivational potential of NFκB (Vanden Berghe et al., 1998). We therefore sought to determine whether NFκB was activated by KA in a PBN-sensitive manner. NFκB activation can be indexed several ways. Immunologically, NFκB activation can be inferred from increased immunoreactivity of an epitope on the p65 subunit, which is exposed upon NFκB recruitment (Rice and Ernst, 1993). As shown in Fig. 4, NFκB-p65 immunoreactivity in the hippocampus increased dramatically within hours of KA treatment, and this effect was suppressed by PBN. The immunochemical data was corroborated

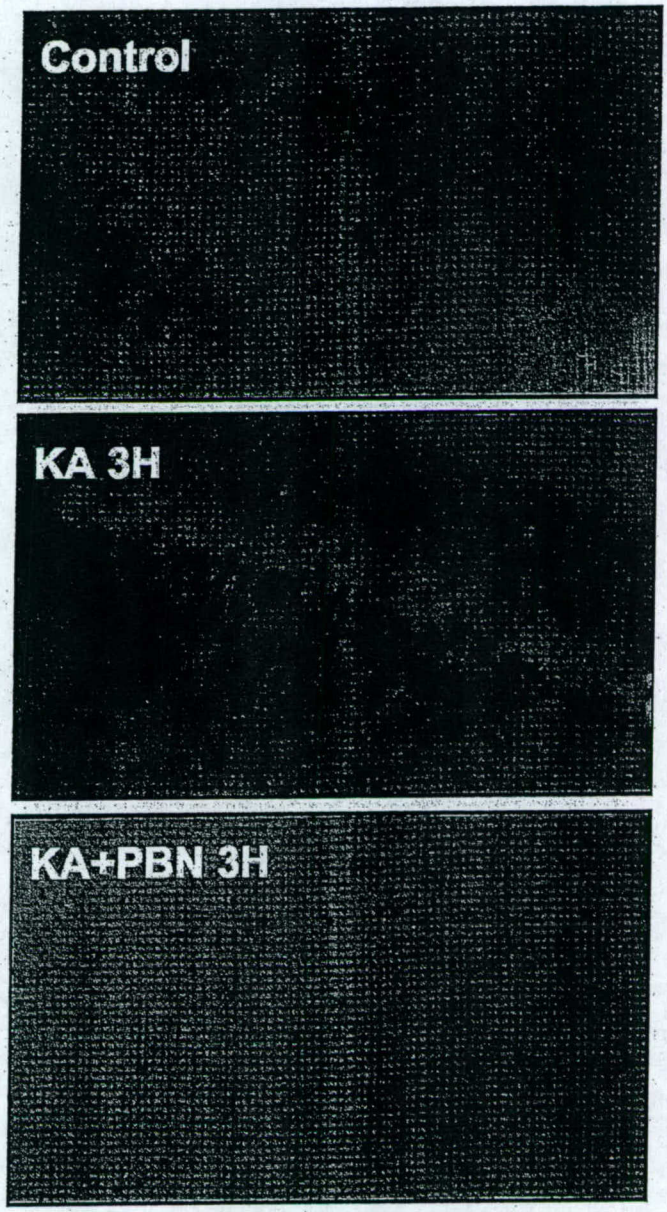

Fig. 3. Kainic acid increases p38-MAPK activation in the hippocampus as indicated by increased phosphorylation of the p38-MAPK activation domain. The CA1 subregion is depicted. Immunohistochemistry was performed using an antibody directed against the phosphorylation domain of the active p38 MAPK enzyme ($pThr^{180}$-Gly^{181}-$pTyr^{182}$)

by EMSA analysis, which showed a dramatically increased NFκB binding activity in hippocampal nuclei of KA, treated rats, which was partially mitigated by PBN cotreatment (Fig. 4).

Hyperactivation of the Jnk, NFκB and p38 signal transduction pathways could be anticipated to have numerous detrimental consequences. All three signaling pathways have been linked to transcription of inflammatory

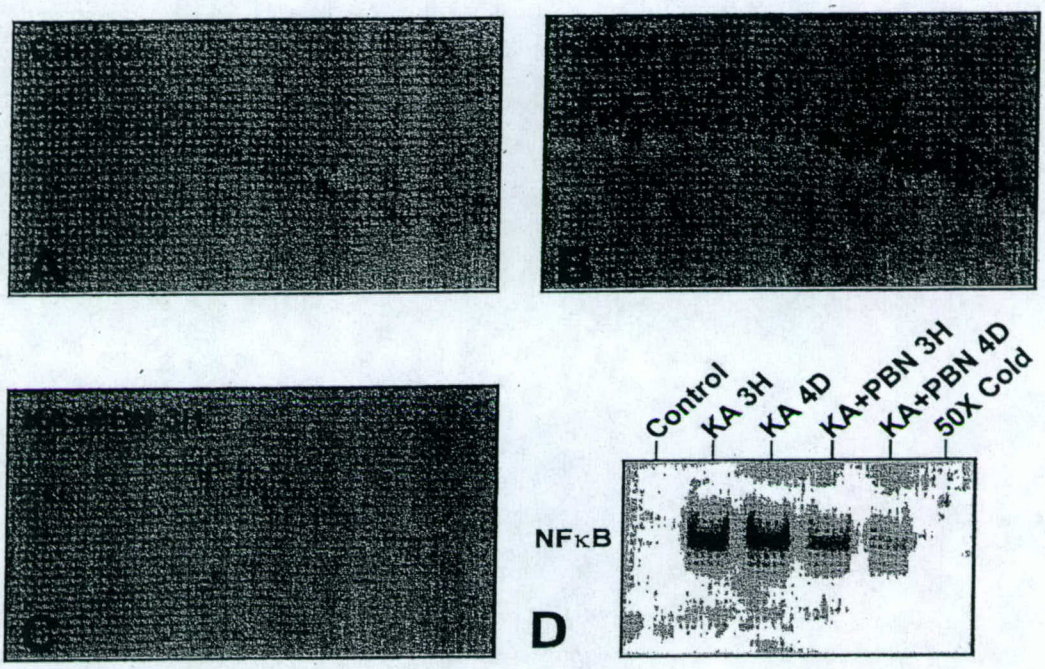

Fig. 4A–D. Kainic acid increases NFκB activation in the hippocampus. **A**, **B**, and **C** illustrate exposure of the p65 subunit of the NFκB complex following KA treatment (arrows). **D** Electromobility gel shift assay demonstrating increased NFκB binding activity in nuclear extracts induced by KA treatment and suppressed by cotreatment with PBN. Specificity of binding was evidenced by competition for the NFκB complex by an unlabeled (cold) oligonucleotide probe (rightmost lane)

cytokines and to modulation of apoptosis (Kawasaki et al., 1997; Kummer et al., 1997; Yang et al., 1997; Qin et al., 1998). We therefore sought to determine whether cytokine and proapoptotic genes were being transcribed at a greater rate in the KA treated rats than in normal rats, and whether PBN could abrogate such an effect. Using a multiprobe ribonuclease protection assay, several inflammatory cytokines were clearly found to be transcribed following KA treatment (Fig. 5). IL1α, IL1-β, IL-6 and TNF-α transcription were strongly induced by KA. Within the timeframe that cytokine transcription was enhanced, several proapoptotic genes were also induced. Most notably, the Fas antigen mRNA was strongly induced following KA and this elevation was maintained for at least four days (Fig. 6). PBN treatment suppressed transcription of both inflammatory cytokine gene products and proapoptotic gene products while having minimal effect on transcription of constitutively-expressed "housekeeping genes" including the L-32 ribosomal mRNA and glyceraldehyde phosphate dehydrogenase mRNA (Figs. 5, 6). PBN suppression of cytokine mRNA transcription was relatively unspecific. Interestingly, PBN displayed particular potency in suppressing Fas antigen and caspase 3 transcription, while other apoptosis-associated mRNA species analyzed by RPA were somewhat less affected by the nitrone (Fig. 6).

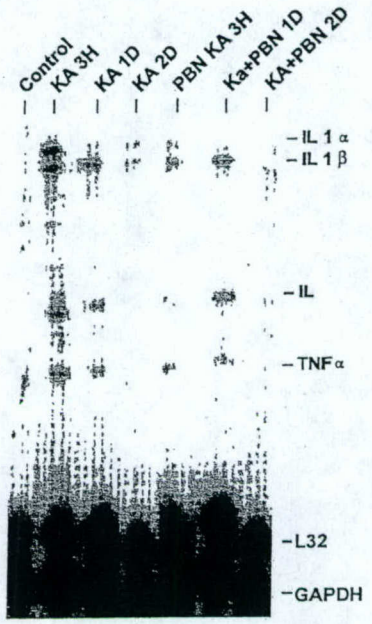

Fig. 5. Kainic acid stimulates the transcription of proinflammatory cytokines in the hippocampus as determined by multiprobe ribonuclease protection assay (RPA)

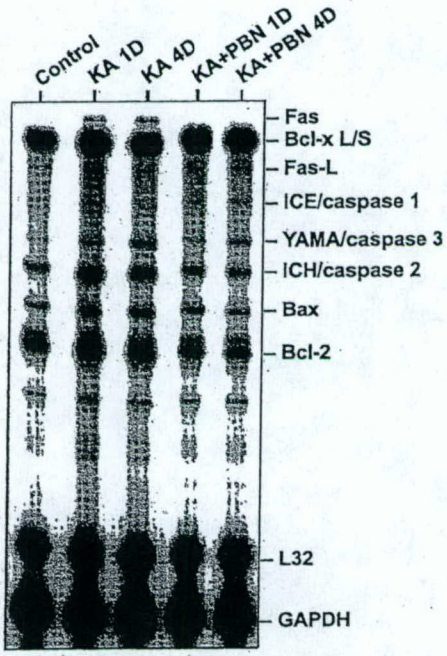

Fig. 6. Kainic acid stimulates transcription of proapoptotic genes in the hippocampus as determined by multiprobe ribonuclease protection assay (RPA)

As a final indication of KA-induced hippocampal damage, in situ TUNEL staining was performed to assess frank apoptosis. KA treatment caused DNA damage indicative of an apoptotic process within four days of subcutaneous administration (data not shown). Apoptosis was largely restricted to the CA1 and CA3 regions of the hippocampus wherein c-Fos was most strongly expressed. Administration of PBN 30 minutes after KA exposure strongly inhibited this apoptosis as indicated by diminished TUNEL staining in hippocampi from PBN treated animals. TUNEL staining for apoptotic nuclei therefore corroborates the pattern of KA-induced and PBN-sensitive immediate early gene expression, and the pattern of proapoptotic gene induction illustrated in Fig. 6.

Discussion

The results of the KA induced brain damage experiment highlights several important points. These include: A) the clear demonstration of the neuroprotective activity of PBN in the KA-induced epilepsy model and B) the potent activity of PBN in suppressing signal transduction processes in the three MAP kinase pathways (AP-1, NFκB and p38) in an in vivo model where excitoxicity and apoptosis have already been implicated. This suggests an inhibition of these three pathways by the experimental compound phenyl-*tert*-butylnitrone was associated with diminished cytokine elaboration, prevention of neuronal apoptosis, reduced seizure activity, and reduced mortality. While the AP-1, NFkB, and p38 pathways are known to respond positively to oxidants and negatively to antioxidants in cell culture (Suzuki et al., 1994; Guyton et al., 1996; Robinson et al., 1999a), the data in this present study are the first to demonstrate the sensitivity of these three pathways to PBN (sometimes classed as an antioxidant compound) within the context of an established in vivo model of hippocampal neurodegeneration.

The findings of the present study extend upon previous observations concerning the broad-spectrum neuroprotective action of nitrone compounds, and provide a novel context for discussing the pathology of excitotoxicity. PBN and related nitrones have been shown to suppress striatal excitotoxic lesions induced by NMDA, KA, and AMPA, though not by virtue of any obvious direct interaction with glutamate receptors (Shultz et al., 1995). Similarly, PBN and a sulfated analog inhibit striatal lesions caused by mitochondrial inhibitors such as malonate and the 1-methyl-4-phenylpyridinium (MPP+; Shultz et al., 1995). Nitrones suppress apoptosis and oxidative stress in cultured Down's syndrome neurons (Busciglio and Yankner, 1995), and similarly inhibit chemically induced thymocyte apoptosis in vitro (Slater et al., 1995), though the influence of nitrones on apoptosis in vivo has not been well studied. Unfortunately, the pharmacologic effects of nitrones in most previous investigations were not correlated with biomarkers of oxidative stress, inflammation or apoptosis. The present data suggest that suppression of apoptosis by PBN in the KA model and possibly other models of neurodegeneration is likely due to mitigation of proinflammatory or

proapoptotic gene expression under the control of the AP-1, NFκB, and p38 MAPK pathways. While the ultimate cellular target(s) for PBN action remain unclear, the present data suggest that the broad-spectrum neuroprotective action of the nitrone class of compounds (Hensley et al., 1997) might be due, in part, to antagonism of crucial oxidation-sensitive signal transduction elements linked to the initiation of apoptotic programs.

PBN neuroprotection and future novel therapeutics

The data clearly show that administration of PBN at least 90 minutes after the administration of KA affords significant protection. It is not known the time to give PBN in reference to KA for achieving maximum efficiency. However, in preliminary experiments, we noted a lack of protection and in fact, perhaps an enhancement of KA toxicity if PBN was given 30 minutes prior to giving the toxin. It is possible in this case that PBN perhaps inhibits metabolic processes whereby KA is rendered inactive, although this has not been studied. The fact that PBN was effective after the KA administration, again as in the case of stroke, indicates that an insult to the brain sets off processes which require some time to reach their full destructive potential. Much evidence in the case of stroke, and now as we have presently demonstrated in the KA model, suggests that signal transduction processes lending to gene induction is a requisite to begin the events leading to brain injury. Agents, such as PBN, which interfere or suppress these processes occurring during the lag phase, may be good candidates for therapeutics of several neurodegenerative diseases.

In the case of Alzheimer's disease, we consider the β-amyloid plaques are localized constant trigger centers. Therefore, to suppress this constant stress it requires the constant administration of an agent that would suppress the localized neuro-inflammatory processes. We envision that treatment with the novel therapeutic, based on the notions outlined here, although it probably would not reverse the β-amyloid deposition, it would however ideally suppress the brain damage caused by the neuro-inflammatory processes triggered by the senile plaques. We consider it likely that the dementia associated with AD is the indication that would benefit the most from the novel therapeutics that may be developed based on these concepts. These ideas have yet to be thoroughly tested but do offer a new approach and possibly an inordinate potential for the treatment of several neurodegenerative diseases.

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APPENDIX 2

BRAIN RESEARCH

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Research report

Changes of hippocampal Cu/Zn-superoxide dismutase after kainate treatment in the rat

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Abstract

In order to evaluate the putative role of Cu,Zn-superoxide dismutase (SOD-1) in the antioxidant defense mechanism during the neurodegenerative process, we examined the level of mRNA, the specific activity and immunocytochemical distribution for SOD-1 in the rat hippocampus after systemic injection of kainic acid (KA). Hippocampal SOD-1 mRNA levels were significantly increased by the seizure intensity 3 and 7 days after KA. These enhanced mRNA levels for SOD-1 were consistent with the increased specific activities for SOD-1, suggesting that the superoxide radical generated in neurotoxic lesion, induced SOD-1 mRNA. The CA1 and CA3 neurons lost their SOD-1-like immunoreactivity, whereas SOD-1-positive glia-like cells mainly proliferated throughout the CA1 sector and had an intense immunoreactivity at 3 and 7 days after KA. This immunocytochemical distribution for SOD-1-positive non-neuronal elements was similar to that for glial fibrillary acidic protein (GFAP)-positive cells. Each immunoreactivity for SOD-1-positive non-neuronal cell or GFAP in the layers of CA1 and CA3 disappeared 3 and 7 days after a maximal stage 5 seizure. On the other hand, activated microglial cells as selectively marked with the lectin occurred in the areas affected by KA-induced lesion. Double-labeling immunocytochemical analysis demonstrated the co-localization of SOD-1-positive glia-like cells and reactive astrocytes as labeled by GFAP or S-100 protein immunoreactivity. This finding suggested that the mobilization of astroglial cells for the synthesis of SOD-1 protein is a response to the KA insult designed to decrease the neurotoxicity induced by oxygen-derived free radicals. Therefore, these alterations might reflect the regulatory role of SOD-1 against oxygen-derived free radical-induced neuronal degeneration after systemic KA administration.

Keywords: Kainic acid; Neurodegeneration; Hippocampus; Superoxide; Cu,Zn-superoxide dismutase; Free radical; Astrocyte; Microglia

1. Introduction

The Cu,Zn-superoxide dismutase (SOD-1; EC 1.15.1.1), the most affected antioxidant enzyme during neurodegeneration [45], catalyzes the dismutation of superoxide into hydrogen peroxide which is then converted to water by glutathione peroxidase (EC 1.11.1.9) and/or catalase (EC 1.11.1.6). This enzyme is known to be involved in the pathophysiology of Down's syndrome [16,38] and mutations in its gene have been found to be responsible for autosomal dominant inherited forms of amyotrophic lateral sclerosis [51]. Since hydrogen peroxide can decompose to form the very reactive hydroxyl radical, it is possible that overexpression/high activity of SOD-1 could increase basal oxidative stress. An increase in basal oxidative stress in neurons could theoretically lead to an increased vulnerability to a neurotoxic insult.

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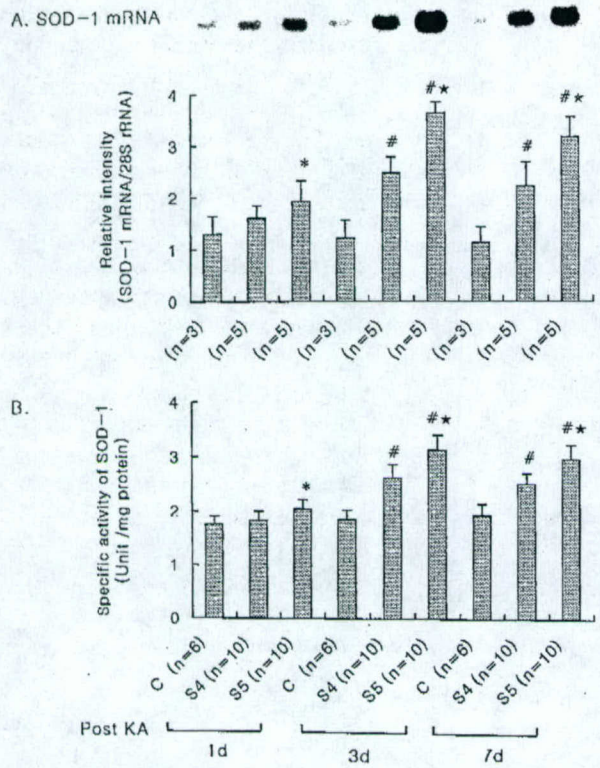


Fig. 1. Level of mRNA (A) and specific activity (B) for SOD-1 in rat hippocampus. (A) Northern blots and the ratio of SOD-1 mRNA/28S rRNA of the detected bands from autoradiograms. (B) Changes of SOD-1 activity in rat hippocampus after systemic KA injection. The level of mRNA for SOD-1 on each time point was clearly correlated with the specific activity for SOD-1. Each value is the mean ± S.E.M. n = animal number in each group. C = control in each time point. S4 = a stage 4 seizure. S5 = a stage 5 seizure. $^{*}P < 0.05$ vs. the corresponding control; $^{\#}P < 0.01$ vs. the corresponding control; $^{*}P < 0.05$ vs. corresponding S4 (one-way ANOVA followed by Dunnett's post-hoc test).

The role of oxygen-derived free radicals in the pathogenesis of neurodegeneration has been well recognized [6,45]. Kainic acid (KA) has been used to generate animal models for both limbic seizures and several neurodegenerative disorders [26,55]. Increasing evidence suggest that KA promotes the generation of oxyradicals, not only in vivo [9,10,25,30,37,58], but also in vitro [5,12,21,53]. Alterations in the levels of cellular antioxidant systems have been proposed to explain conditions that lead to free radical generation; seizure [9,10,25,30,37,58], ischemia [32,35] and neurodegeneration [6,45].

Dynkens et al. [21] have shown that SOD possesses a beneficial effect on kainate/ischemia mediated neurotoxicity. However, the role of endogenous SOD in the pathogenesis of an oxidative stress-related neurotoxic injury in the brain has not been clearly elucidated yet. Neurodegenerative processes produced by excessive cellular SOD-1 activity have been proposed in different experimental paradigms [11,25,40]. To explore the oxygen-derived free radical hypothesis in KA-induced neuronal degeneration, we examined the level of mRNA, specific activity, and the immunological localization for SOD-1 in the rat hippocampus.

2. Materials and methods

2.1. Treatment of animals and preparation of samples

All rats were treated in strict accordance with the NIH Guide for the Humane Care and Use of Laboratory Animals. Male Sprague–Dawley rats (Charles River Laboratories, Seoul, Korea) weighing about 250 g were maintained on a 12:12 h light:dark cycle and fed ad libitum. They were adapted for 2 weeks to the above conditions before KA (10 mg/kg, i.p.) administration. Control rats received the same volume of saline. Under the automated video tracking system (Noldus Information Technology, Wageningen, Netherlands), seizure activity was rated during a 5-h period following the KA challenge according to the

Table 1
Alterations of SOD-1-like immunoreactivity after kainic acid (KA) administration in the rat hippocampus

Time periods		Neurons				Non-neuronal cells			
After KA		CA1	CA3	CA4	DG	CA1		CA3	
						SO	SR	SO	SR
Saline		2.9 ± 0.4	2.1 ± 0.4	1.2 ± 0.6	2.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1 day	S4	0.9 ± 0.4*	0.6 ± 0.5*	0.4 ± 0.6*	2.7 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	S5	0.3 ± 0.5**	0.4 ± 0.4*	0.2 ± 0.3*	2.7 ± 0.2	0.2 ± 0.3	0.2 ± 0.2	0.1 ± 0.2	0.0 ± 0.0
3 days	S4	0.1 ± 0.3**	0.5 ± 0.6*	0.1 ± 0.2**	2.6 ± 0.3	0.4 ± 0.2*	0.5 ± 0.3*	0.1 ± 0.1	0.1 ± 0.1
	S5	0.1 ± 0.2**	0.2 ± 0.1**	0.0 ± 0.0**	2.7 ± 0.2	0.2 ± 0.2	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
7 days	S4	0.1 ± 0.0**	0.2 ± 0.1**	0.1 ± 0.0**	2.7 ± 0.4	1.3 ± 0.3**	1.6 ± 0.0**	0.2 ± 0.1	0.2 ± 0.2
	S5	0.0 ± 0.0**	0.1 ± 0.1**	0.0 ± 0.0**	2.6 ± 0.3	0.05 ± 0.1	0.05 ± 0.1	0.0 ± 0.0	0.0 ± 0.0

Each value for intensity of SOD-1-like immunoreactivity was graded as an absolute value using image analysis systems with polaroid digital microscopic camera (Optimas version 6.2). Each value is the mean ± S.E.M. of *five animals*. S4 = a stage 4 seizure. S5 = a stage 5 seizure. DG = dentate gyrus. SO = striatum oriens. SR = stratum radiatum. Negligible induction of SOD-1-positive non-neuronal cells was noted in the stratum pyramidale or dentate hilus area after KA treatment. $^{*}P < 0.05$ vs. saline. ** $P < 0.01$ vs. saline (Williams–Wilcoxon multiple rank sum test).

scale devised by Racine [50]: Stage 1, facial clonus; Stage 2, nodding; Stage 3, forelimb clonus; Stage 4, forelimb clonus with rearing; Stage 5, rearing, jumping and falling. Animals were scored after having three consecutive seizures at each stage. From 'stage 4' to 'stage 5', animals were employed for Northern blot analysis and assay of SOD-1 activity. In order to understand reactive glia-specific responses related to seizure intensity, animals exhibiting either 'stage 4' or 'stage 5' were used for immunocytochemistry. Rats were sacrificed after KA on the following

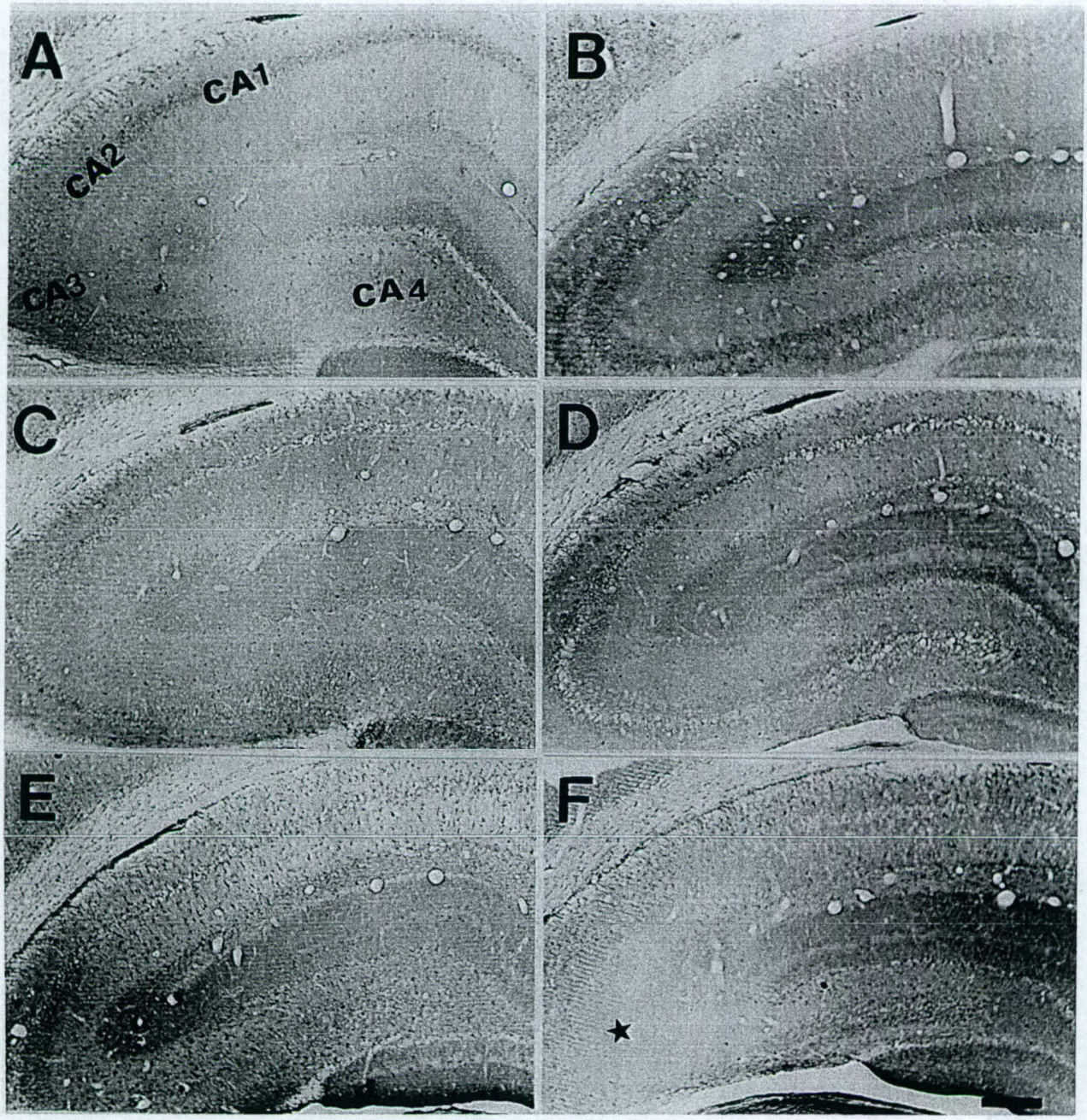
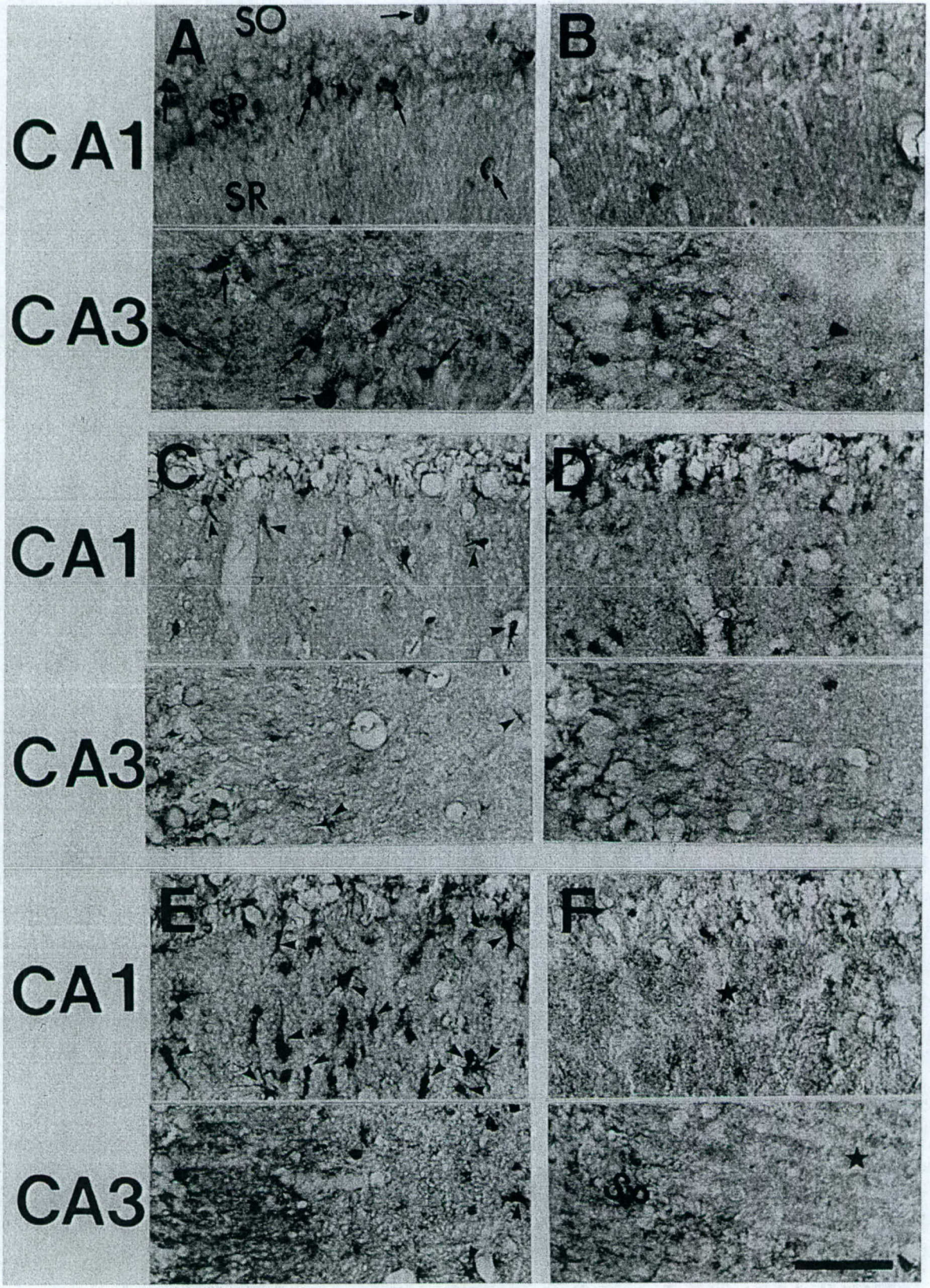


Fig. 2. Photomicrographs of immunostained sections with SOD-1 after systemic administration of KA. Hippocampal section from normal animals showed an intense SOD-1-like immunoreactivity in the pyramidal neurons and dentate gyrus (A). The SOD-1-like immunoreactivity was apparently reduced in the pyramidal neurons 1 day after a stage 4 seizure (B). The SOD-1-positive neurons disappeared in the CA1 and CA3 regions 3 days after KA injection (C,D). These neuronal losses were more pronounced in the animal showing a stage 5 seizure (D). Seven days later, SOD-1-immunoreactive non-neuronal elements were mainly increased in the CA1 field of animal exhibiting a stage 4 seizure (E), but these elements were barely induced in the CA1 after a stage 5 seizure (F). Furthermore, SOD-1-positive glia-like cells were completely absent from the CA3 area and mossy fiber layers 7 days after a maximal seizure. Star represents the lesioned area devoid of SOD-1-positive glia-like cells (F). Scale bar = 250 μm.

schedule: 1, 3 and 7 days. The rats were anesthetized at each time-point with pentobarbital (50 mg/kg) and were perfused transcardially with ice-cold 0.9% NaCl (80 ml/100 g body weight) to remove the free radical-



scavenging sources and the free radical-generating sources in the brain [8]. Brains were rapidly removed, hippocampi were dissected and then stored at −70°C.

2.2. Northern blot analysis of SOD-1 mRNA

Total RNA was isolated from pooled hippocampi from three rats as described by Chomczynski and Sacchi [15]. Isolated total RNA (10 μg/lane) was denatured with 6.7% formaldehyde and 50% formamide, electrophoresed on a 1% agarose/formaldehyde gel and transblotted directly onto a nylon membrane (Hybond N, Amersham). Blotted RNAs were fixed by baking. Blots were probed with a synthetic 48-mer oligonucleotide, indicating a sequence complementary to bases 372–419 of the rat Cu/Zn-SOD mRNA [19]. Probe was radiolabeled using a random prime-labeling kit (Amersham) [43]. Blots were prehybridized for 4 h at 37°C in hybridization buffer containing 4 × Denhardt's solution, 50% formaldehyde, 0.5% sodium dodecyl sulfate (SDS), 5% dextran sulfate, 100 μg/ml yeast tRNA and 500 μg/ml sonicated salmon sperm DNA. Hybridizations were performed at 37°C for 18 h in the same buffer containing 10^6 dpm/ml of a ^{32}P-labeled probe. Following hybridization, membranes were washed in 4 × SSC containing 0.1% SDS for 30 min at 50°C, 30 min at room temperature and were then exposed to X-ray film (Hyperfilm MP, Amersham) with two intensifying screens for 7–10 days at 70°C. The optical densities of signals on the autoradiograms were measured using a computerized imaging analysis system. The ratio of the Cu/Zn-SOD mRNA to the constitutively expressed 28S rRNA was calculated for each sample [43].

2.3. SOD-1 biochemical assay

Hippocampal homogenates used to measure SOD-1 activity were centrifuged at 25,000 × *g* for 15 min at 4°C and supernatant dialyzed in 50 mM phosphate buffered saline (PBS; pH 7.8) containing 1 mM EDTA. The protein content of the tissue supernatants was determined by the Coomasie® protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard. SOD-1 activity was measured by the method of Crapo et al. [17]. One unit of SOD-1 is defined as the quantity required to inhibit the rate of cytochrome C reduction by 50%. The specific activity of SOD-1 was calculated by substracting the Mn-SOD activity from the total activity.

2.4. Immunocytochemistry

Animals were perfused transcardially with 50 ml of PBS (pH 7.4) followed by 120 ml of 4% paraformaldehyde in PBS. Brains were then removed, stored in 4% paraformaldehyde overnight, and cut on a horizontal sliding microtome 40 μm transverse free-floating sections. Prior to incubation with the primary antibodies, sections were preincubated with 0.3% hydrogen peroxide in PBS for 30 min (to block endogenous peroxidase activity), then in PBS containing 0.4% Triton X-100 for 20 min and 1% normal serum for 20 min. After a 48-h incubation with the primary antibody at 4°C, sections were incubated with the secondary biotinylated antisera (1:1000 dilution; Vector, Burlingame, CA) for 1 h, washed, and immersed in avidin–biotin–peroxidase complex (ABC Elite kit, Vector) for 1 h. Sections were always washed three times with PBS between each incubation step. 3,3′-Diaminobenzidine (DAB) was used as the chromogen. The whole process for preparation of the purified SOD-1 [33] and SOD-1 antibody has been described elsewhere [4,33,35]. The SOD-1 antibody was used at 1:1000 as the optimal dilution. The specificity of the SOD-1 antibody has been proven by immunoblotting [33].

In order to characterize SOD-1-immunoreactive non-neuronal cells after KA-induced neuronal loss, the immunodistribution (regional proliferation) pattern of these cells was compared with that of glial fibrillary acidic protein (GFAP)-positive astrocytes or lectin-positive microglial cells. In addition, double-labeled immunocytochemical analysis was performed. Microglial cells were identified by lectin histochemistry using biotinylated α-D-galactosyl-specific isolectin B_4 conjugated with horseradish peroxidase derived from Griffonia simplicifolia seeds (GSA I-B_4-HRP, Sigma, St. Louis, MO), which is considered as a reliable method to stain selectively rat microglial cells in a pathologically altered brain [57].

For double-labeling immunocytochemistry, sections were first stained with the GFAP or S-100 protein antibody (using DAB-nickel as the developer, bluish purple staining), and then with the SOD-1 antibody (DAB used as the chromogen, brownish staining). The GFAP mouse

Fig. 3. Magnified photomicrographs of immunostained sections with SOD-1 in the CA1 and CA3 regions. In the normal hippocampus, the CA1 and CA3 pyramidal neurons show an intense SOD-1-like immunoreactivity. The SOD-1-like immunoreactivity was more strongly induced in the interneurons (arrows) (A). One day after a KA-induced stage 4 seizure, the CA1 and CA3 pyramidal neurons lost their SOD-1-like immunoreactivity without significant neuronal losses (B). The SOD-1-immunoreactive neurons disappeared due to neuronal death 3 days after KA (C,D). After a stage 4 seizure, the SOD-1-positive glia-like cells (arrowheads) mainly appeared in the SR of the CA1 area (C), but these glia-like cells were not clearly induced in the animal exhibiting a stage 5 seizure (D). Seven days later, the proliferation of SOD-1-positive glia-like cells (arrowheads) was mainly increased in the SR of the CA1 sector after stage 4 seizures (E); however, because pyramidal neurons and glia-like cells were damaged, this proliferation was not seen in the SR of the animal exhibiting a maximal seizure (stage 5). Stars represent the lesioned area devoid of SOD-1-positive glia-like cells in the SR (F). Note the SP; SOD-1-positive glia-like cell barely induced in the SP. SO = stratum oriens. SP = stratum pyramidale. SR = stratum radiatum. Scale bar = 125 μm.

monoclonal antibody was purchased from Boeringer Mannheim (Indianapolis, IN). The S-100 protein rabbit polyclonal antibody was purchased from Zymed Laboratories (San Francisco, CA). The antibodies against GFAP, S-100 protein, and GSA I-B_4-HRP were diluted 4000, 500 and 500 times, respectively.

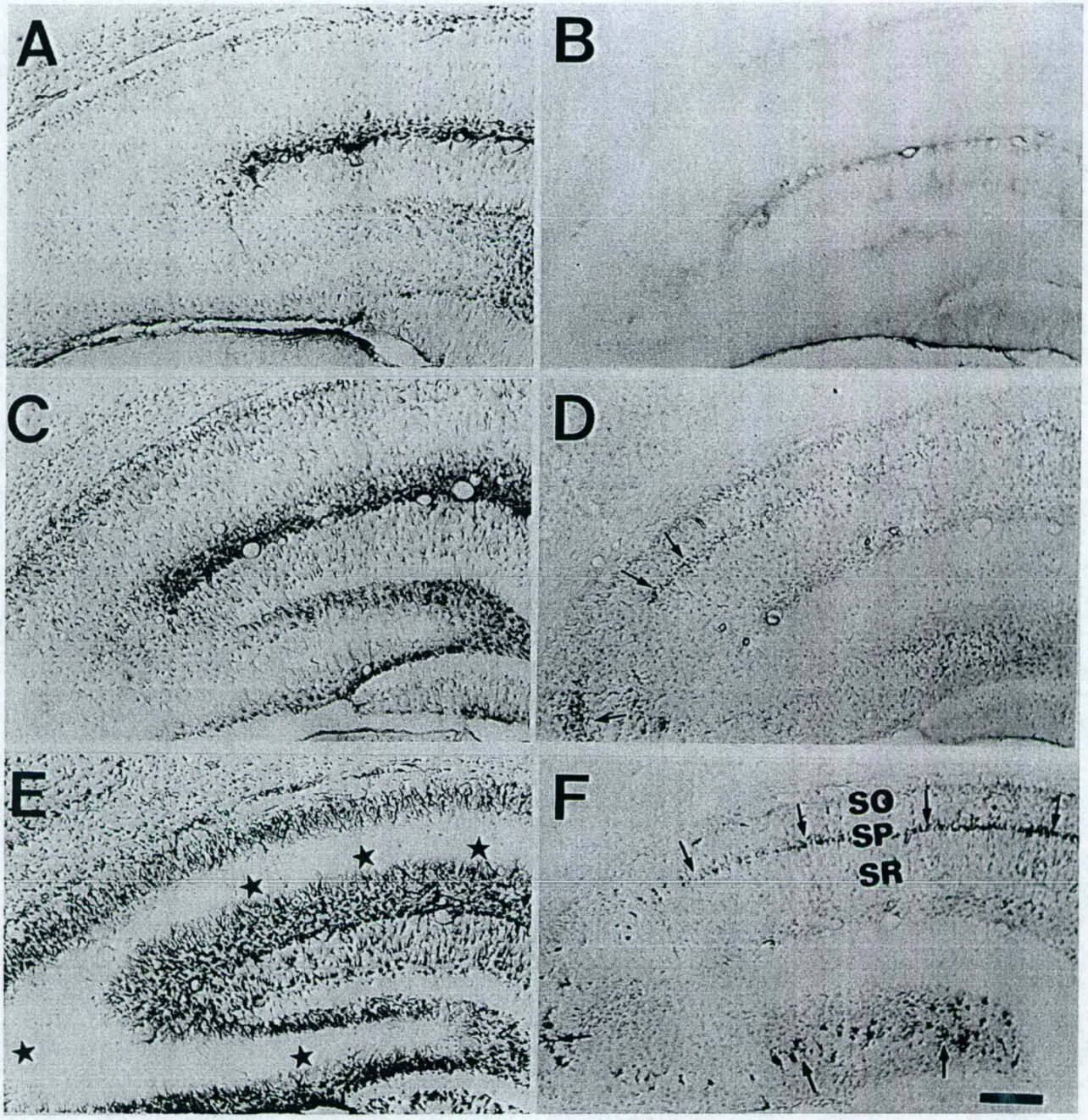


Fig. 4. Photomicrographs of immunostained sections with GFAP or GSA I-B_4-HRP (lectin) after systemic administration of KA. GFAP-like immunoreactive astrocytes showed a homogenous distribution, and were found in all regions of the hippocampal formation in the normal animal (A). Seven days after a stage 4 seizure, GFAP-like immunoreactivity were diffusely stained in the CA1 and CA2 regions, while GFAP-positive astrocytes in the CA3 and dentate hilus were apparently decreased 7 days after a stage 4 seizure (C). Moreover, GFAP-immunoreactive astrocytes completely disappeared (the area is represented by stars) in the SR of the CA1, CA3 and in the dentate hilus in the animal exhibiting a stage 5 seizure (E). GFAP-immunoreactive astrocytes were rarely observed in the SP. Lectin-positive microglial cells were rarely observed in the normal hippocampus (B). By 7 days, reactive microglial cells proliferated mainly SR of CA3 region, and increased in the SP (arrows) of the CA1 and CA3 regions and in the dentate hilus after a stage 4 seizure (D). Strong proliferation of reactive microglial cells apparently observed in the SP (arrows) of the CA1 and CA3 fields, and in the dentate hilus (arrows) in the animal exhibiting a stage 5 seizure (F). SO = stratum oriens. SP = stratum pyramidale. SR = stratum radiatum. Scale bar = 250 μm.

2.5. Statistics

Statistical analysis was performed by either the one-way ANOVA followed by Dunnett's post-hoc test or the Williams–Wilcoxon multiple rank sum test. A significant level of less than 0.05 was accepted for comparisons.

3. Results

3.1. The changes of hippocampal SOD-1 mRNA level after KA administration

Northern blot analysis using the SOD-1 specific probe showed hybridization to a single band with a molecular

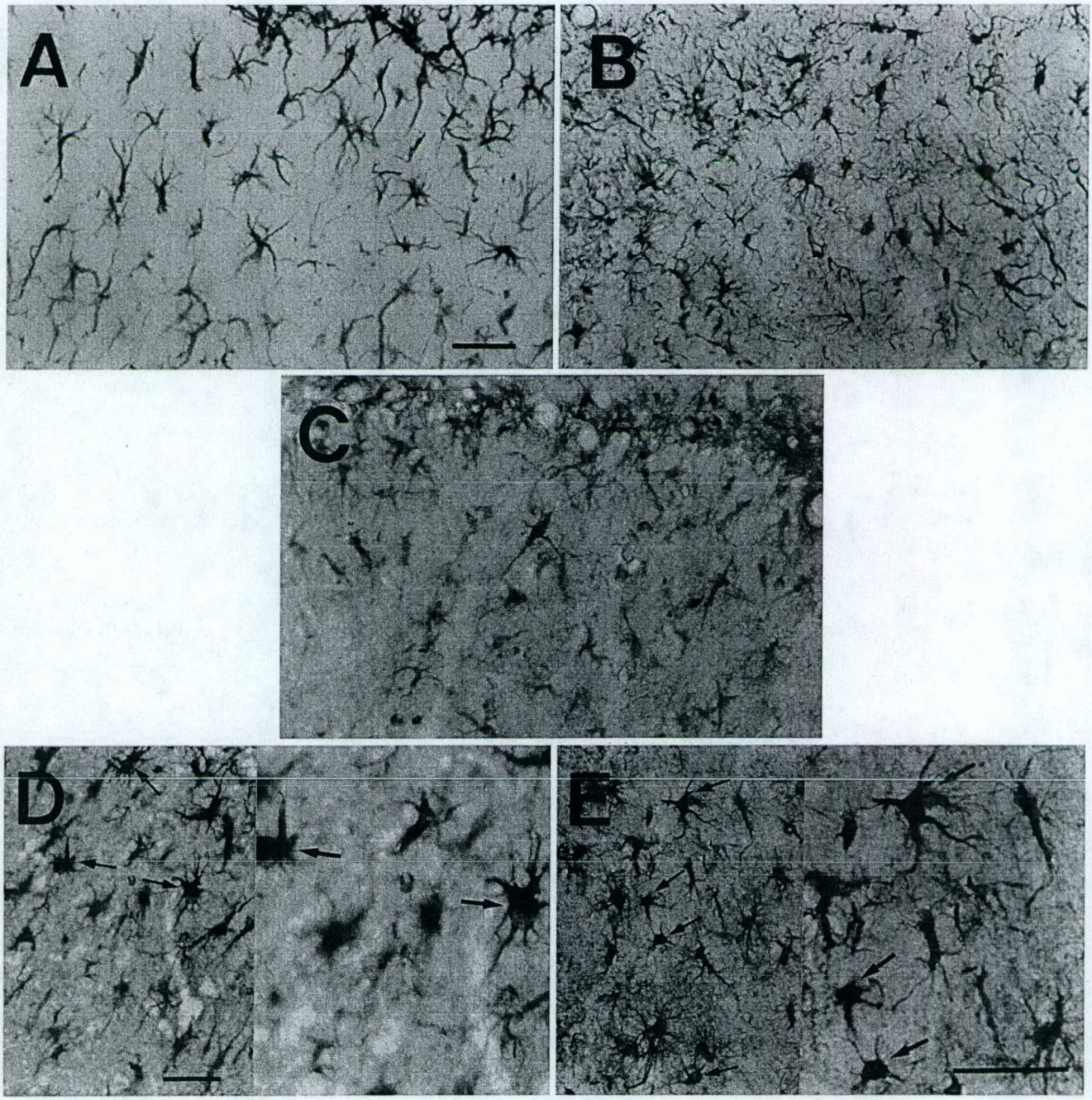


Fig. 5. Double-labeling immunocytochemistry for the characterization of SOD-1-immunoreactive cells. The photomicrographs of double-labeled cells were focused on the CA1 area 7 days after a stage 4 seizure. For double-labeling, either GFAP-positive astrocytes (A) or S-100 protein-positive astrocytes (B) were marked with a bluish purple color, and then SOD-1-containing cells were visualized with a brown color (C). The examples of double-labeled cells are indicated by arrows: "GFAP and SOD-1" (D), and "S-100 protein and SOD-1" (E). Because it is difficult to prove co-localized astrocytes in an area of intense proliferation, the double-labeling immunocytochemistry was done in an area with a moderate rate of proliferation. Each scale bar = 125 μm.

weight of about 0.65 kb. Stage 4 seizures caused a slight increase of the signal; more pronounced increase was observed in rats exhibiting stage 5 seizures ($P < 0.05$ vs. con) 1 day after KA. The signal intensity was significantly increased by seizure intensity (stage 4 or 5 seizures vs. con; $P < 0.01$, stage 5 seizures vs. stage 4 seizures; $P < 0.05$) 3 days after KA treatment. The signal still remained significantly elevated (stage 4 or 5 seizures vs. con; $P < 0.01$, stage 5 seizures vs. stage 4 seizures; $P < 0.05$) 7 days after KA administration (Fig. 1A; Table 1).

3.2. The changes of specific activity of hippocampal SOD-1 after KA administration

One day after KA injection, no significant change in the specific activity of hippocampal SOD-1 was observed in the animals expressing a stage 4 seizure, while this activity was increased ($P < 0.05$ vs. con) after stage 5 seizures. The specific activity of SOD-1 was significantly elevated (stage 4 or 5 seizures vs. con; $P < 0.01$, stage 5 seizures vs. stage 4 seizures; $P < 0.05$) by the seizure severity 3 days following KA administration. This activity of SOD-1 still remained significantly increased (stage 4 or 5 seizures vs. con; $P < 0.01$, stage 5 seizures vs. stage 4 seizures; $P < 0.05$) 7 days after KA administration (Fig. 1B).

3.3. SOD-1-positive cells after KA administration in the rat hippocampus

SOD-1-like immunoreactivity showed a high intensity in the pyramidal cells and granule cells of dentate gyrus in the normal hippocampus(Figs. 2A and 3A). By contrast, SOD-1-like immunoreactivity in the CA1 and CA3 pyramidal cells was apparently reduced without significant neuronal losses 1 day after stage 4 seizures (Figs. 2B and 3B). Because of the significant neuronal cell loss in the CA1 and CA3 regions 3 and 7 days after KA treatment, most SOD-1-immunopositive neurons in the CA1 and CA3 sectors disappeared in the animals exhibiting stage 4 (Fig. 2C and E, Fig. 3C and E) or 5 seizures (Fig. 2D and F, Fig. 3D and F); while the appearance of SOD-1-positive non-neuronal populations was noted in the stratum radiatum (SR) of CA1 3 days after stage 4 seizures (Figs. 2C and 3C). This proliferation of non-neuronal elements was not pronounced in the SR of CA3 (Figs. 2C and 3C). Negligible induction of SOD-1-positive non-neuronal cells was observed 3 days after stage 5 seizures (Figs. 2D and 3D). By 7 days, animals expressing a seizure stage 4 had activated SOD-1-positive glia-like cells in the SR of CA1 (Figs. 2E and 3E), while others showing a seizure stage 5 had a marked loss of SOD-1-positive glia-like cells in the SR of CA1 (Figs. 2 and 3F).

3.4. GFAP-positive astrocytes have a SOD-1-positive non-neuronal cell-like immunodistribution after KA administration in the rat hippocampus

GFAP-positive astrocytes in normal hippocampus showed a homogenous distribution in the whole hippocampus. However, GFAP-positive astrocytes were rarely observed in the pyramidal cell layer (stratum pyramidale) (Fig. 4A). At 7 days after KA, GFAP-like immunoreactivity was significantly increased in the animal exhibiting a seizure stage 4. However, in the CA3 area, this GFAP-like immunoreactivity was apparently decreased (Fig. 4C). In addition, a significant loss of GFAP-positive elements adjacent to the degenerating pyramidal cells was also noted 7 days after stage 5 seizures (Fig. 4E).

3.5. Activated microglia as labeled by lectin occurs in areas of neuronal cell loss after KA administration in the rat hippocampus

Microglial cells were rarely found with GSA I-B_4-HRP staining in the normal hippocampus (Fig. 4B). The microglial reaction in CA3 increased to a maximum at day 7 after stage 4 seizures (Fig. 4D). The typical changes were dense accumulations of microglial cells in the stratum pyramidale of the CA1 and CA3 regions, and in the dentate hilus 7 days after stage 5 seizures. In addition, an increased microglial reaction was also noted in the SR of CA1 and CA3 region (Fig. 4F).

3.6. SOD-1-positive cells have an astrocyte-like morphology, and co-localize with GFAP- or S 100 protein-positive reactive astrocytes

Double-labeling immunocytochemical experiments confirmed the astrocytic localization of the SOD-1-positive cells (Fig. 5). The astrocytic processes had a bluish purple color, indicative of GFAP-immunoreactivity (Fig. 5A) or S-100 protein-immunoreactivity (Fig. 5B). The cell body of the SOD-1-positive cells in the CA1 field was stained brown in the animal showing a stage 4 seizure (Fig. 5C). This SOD-1 staining was found in reactive astrocytes that stained positively for GFAP (Fig. 5D) or S-100 protein (Fig. 5E). Activated microglia were not observed earlier than 2days after KA treatment (data are not shown). Although the sites of activated microglia overlapped with those of SOD-1-induction, the morphology and immunodistribution of both populations was dissimilar.

4. Discussion

KA-induced seizures resulted in a significant increase in the specific activity of SOD in the *hippocampus* of the adult rat, indicating that KA-induced seizure activity could contribute to the increased formation of oxygen-derived free radicals [5,9,10,25,30,37,53,58]. The present finding is partially in line with the results of Bruce and Baudry [9]. In this article, we focused on the regulatory role of endogenous SOD-1 in the neurodegeneration induced by KA administration. Our results clearly showed that *endoge-*

nous induction of SOD-1 mRNA level depends on the seizure intensity after KA tretament. Although the precise mechanism for the induction in SOD-1 mRNA is not known, the increased level of hippocampal SOD-1 mRNA might reflect a prolonged oxidative stress to specific neuronal elements [11]. An increase in SOD-1 mRNA in this study was correlated with the change in the specific activity of SOD-1, suggesting that these events are needed to scavenge superoxide radicals during the neuronal degeneration [43]. In addition, increased SOD activity has been described in the brains of kindled rats [39] and in the cerebral cortex following chemically induced seizures in rats [54], indicating that the formation of superoxide in the brains could be increased following convulsive behaviors [60]. Because of the role of SOD-1 is the dismutation of superoxide to form hydrogen peroxide which, in turn, is reduced to water by peroxidases, it has been proposed that increased SOD-1 activity in the neurodegenerative model could result in an enhanced formation of hydrogen peroxide and, as a consequence, in an increased lipid peroxidation and/or oxidative damage [11]. The levels of glutathione peroxidase and catalase in neuronal tissue appear to be relatively low for the prevention from peroxide-induced lesions. Furthermore, the neuronal cell membrane contains high levels of polyunsaturated fatty acids [24]. Thus, brain cells may be extremely susceptible to peroxidative damage. We also agree with the speculation that the early rise in lipid and protein peroxidation products and enzyme activity corresponds directly to KA-induced pathology, while prolonged elevation in enzyme activity may be due to subsequent glial activity [9,27,30,37,61].

The present results showed that normal hippocampus contains an intense SOD-1-like immunoreactivity, which is considered to be very useful scavenger of superoxide anions in the brain. The localization of SOD-1 was mainly in the CA1 pyramidal neurons and granule cells of the dentate gyrus and relatively less in other regions. This finding is identical to previous observations [20,35]. The SOD-1-like immunoreactivity obviously changed in the hippocampus after KA insult; SOD-1-like immunoreactivity was reduced in the CA1, CA3 and CA4 neurons by 1 day and never recovered. This reduction may be caused by the large amount of superoxide radicals generated during the early stage of neurotoxicity induced by KA, and may support the hypothesis that generated free radicals induce a vicious cycle leading to delayed neuronal damage.

SOD-1-like immunoreactivity was not induced in the pyramidal layer of the hippocampus, because of the delayed neuronal death after maximal seizures. However, SOD-1-like immunoreactivity in the dentate gyrus remained the constant throughout the entire period, since KA-induced insult failed to induce neuronal death in this region. This indicates that KA-induced seizures increased hippocampal SOD-1 mRNA without the production of SOD-1 protein in the pyramidal neuron. Thus, it is suggested that the prolonged induction of hippocampal SOD-1 mRNA after KA reflect a persistent stimulus to specific neurons, and that the prolonged reduction in the synthesis of endogenous SOD-1 protein is important in the neurons vulnerable to KA-elicited insults. However, the precise mechanisms for this phenomenon still remain to be clarified.

Our immunocytochemical analysis clearly demonstrated a decreased level of SOD-1 protein in pyramidal neuron before histological signs of neuronal death (1 day post-KA). Whereas the SOD-1-like immunoreactivity was induced in the proliferated glia-like cells by 3 and 7 days after KA-induced stage 4 seizures, suggesting that the protein expression for SOD-1 differs among the types of cells that receive the KA insult, and this glial induction of SOD-1 protein reflects an adaptive process against attack from superoxide. The morphology of SOD-1-positive cells is extremely similar to that of the S-100 protein or GFAP-labeled reactive astrocytes, and the location and time course of the appearance of SOD-1 immunoreactive cells closely resembles those characteristics of reactive gliosis. Double-labeled immunostaining for SOD-1 and S-100 protein or GFAP showed a co-localization of SOD-1-labeled cells and reactive astrocytes, suggesting that SOD-1 was specifically induced in this particular type of glial cell in response to neuronal injury. Since the superoxide radical has been postulated to be an initiator of the oxyradical chain reaction [24,45], the demand for SOD-1 should be greatly increased after KA toxicity to scavenge superoxide. Thus, SOD-1-positive reactive astrocytes may act as an important defensive mechanism against free radical-mediated cellular damage [61]. However, the significance of the induction of SOD-1 protein in the reactive astroglial cells following the KA insult still remains to be clarified.

A striking finding in the animals showing maximal seizure behavior (stage 5) was a significant loss of SOD-1-positive reactive astrocytes or GFAP-labeled reactive astrocytes from the SR of the CA1 and CA3 pyramidal cell layer 3 and 7 days after KA administration. This phenomenon could be due to: (1) a consequence of massive neuronal death, which might induce the release of numerous substances, including glutamate and potassium, which cause astrocytic damage [61], or (2) a profuse leakage of the blood-brain barrier and the massive microglial reaction [2]. This finding corresponded with the results produced by intraventricular injection of KA [27]; intracerebral injection of α-aminoadipate (α AA), an astrogliotoxin, resulted in selective ablation of astrocytes, and in rapid neurodegenerative changes, as manifested by a loss of structural integrity at the ultrastructural level. The neuroprotective role of astrocytes appears most evident following reactive gliosis. For example, reactive gliosis is also characterized by upregulation of astrocytic antioxidants in an animal model of epilepsy and in human degenerative disease [61]. Thus, the α AA might reduce astrocytic antixoidant such as astroglial glutathione peroxidase protein levels [61]. This reaction could facilitate excitotoxic neuronal death.

The α AA could be binding to an astrocyte-specific glutamate-like receptor; excess of α AA could then initiate a sequence of excitotoxic events, similar to the one hypothesized to cause neuronal degeneration [13]. Correspondingly, an intrastriatal injection of α AA caused a significant reduction in the level of glutamine synthetase 6-h post-injection. In addition, neuronal necrosis was observed following systemic administration of L-α AA [46]. A profound loss of GFAP-positive astrocytes was observed within a large area around the site of injection, while lectin-positive reactive microglial cells proliferated at the site of astrocytic degeneration [28].

Recently, the histochemical localization of scavenging activity of superoxide anions in the rat brain was visualized by tissue-blotting technique; the finding demonstrates that the localization of the scavenging activity of this oxyradical is identical to the immunodistribution of SOD-1, indicating that SOD-1-free zone in the brain is extremely susceptible to toxicity caused by superoxide [44]. The consistent result observed in this study that the reactive microglial cells as labeled by lectin, proliferated in the SOD-1-positive astrocyte-free zone induced by the maximal seizure, indicating that SOD-1-immunoreactive astrocyte-free zone is more susceptible to superoxide-mediated damage. Activated microglia can become phagocytic, and release oxygen-derived free radicals in order to remove necrotic tissue debris [14,59]. Correspondingly, this astrocyte-free zone was replaced by severe necrotic edematous change. Therefore, we raise the possibility that activated microglial cells induced by KA potentiate oxidative stress, especially in the astrocyte-free zone. Activated microglia has been also well known from numerous studies to be a marker of neural injury [18,27,48,57,59]. Thus, it is possible that reactive astroglial cells without surrounding activated microglia are, at least, less severely injured to hippocampal tissue than reactive astroglial cells which are surrounded by activated microglia, suggesting that seizure intensity is consistently correlated with neuronal damage.

In the hippocampus, the highest density of KA receptors is in the CA3 region [23], the area most severely damaged after applications of KA [23,41,42,49]. On the other hand, after systemic KA administration (8–12 mg/kg, i.p. or s.c.; optimal systemic dose to induce severe seizure [55]) in the rat, some KA lesions did not affect the entire CA3 on both sides, but did extensively include CA1 [36,48,52,56] in spite of a comparatively low density of KA receptors [23], suggesting that some additional unknown KA-responsive mechanism exist. KA receptor stimulation leads to depolarization of CA3 pyramidal cells, which secondarily may leads to endogenous glutamate release in the CA1 region, *N*-methyl-D-aspartate (NMDA) sensitive glutamate binding sites are predominantly bound in CA1 [3,22,31]. Most NMDA receptor antagonists have been shown to be protective against brain damage associated with seizures, especially in the CA1 field of the hippocampus [55]. In addition, the selective neuronal protection in the CA1 field by NMDA antagonists may be related to the major distribution of NMDA receptors in the CA1 field [29,31,53]. The formation of free radicals increased following activation of NMDA and KA receptors [5,7,25,34,53,58]. Thus, it is possible that a facilitative action of NMDA toward KA receptors may be involved in oxyradical generation following KA-induced seizure.

The proliferation of SOD-1-positive astroglial cells in the CA1 field may be a compensatory induction to scavenge oxyradical formation facilitated by KA. In contrast, the basal level of SOD-1-like immunoreactivity in the CA3 is lower than that in the CA1 [35]. After systemic KA administration, the proliferation of SOD-1-immunoreactive astroglial cells in CA3 is also less than that in CA1. Thus, we cannot rule out the possibility that the CA3 area may be susceptible to oxidative stress derived from KA receptor activation (although the basal level of Mn-SOD-like immunoreactivity in CA3 is relatively higher than that in CA1 [1,35], the proliferation of SOD-2-positive astroglia-like cells in the SR of the CA3 is also less than that in the CA1 [data not shown]). However, this selective seizure-related damage induced by KA may involve various mechanisms [27,47,48,55]. These complex etiologic factors are still being debated.

In conclusion, the increases of SOD-1 mRNA and SOD-1 enzyme activity after KA injection were increased by the seizure activities, which provide early biochemical markers for identifying hippocampal neuronal cells exposed to oxidative stress. Our results also indicate that an increase of SOD-1 mRNA after KA administration occurs in response to a consumption of intrinsic SOD-1 molecules following a prolonged oxidative stress to specific neuronal components, and that this altered regulatory mechanism of SOD-1 synthesis contributes to the neurodegeneration induced by KA. Consistently, an increase of the specific activity of SOD-1 also indicates superoxide generation in the neurotoxic lesion induced by KA. The mobilization of SOD-1-positive astroglial cells may also be a response to neurodegeneration to minimize superoxide attack. In contrast, the loss of SOD-1-immunostaining exhibited tissue areas where activated microglia occurred in the areas undergoing neuronal death. Thus, it is suggested that if the content of endogenous SOD-1 proteins were sufficient to scavenge the oxygen-derived free radicals following seizure behavior, the cells would be protected against KA-induced injury. In view of the selective vulnerability of hippocampal cell populations after convulsive behavior, the role of astroglial cells in this area deserves further attention.

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Phenyl-N-*tert*-butylnitrone Inhibits Neuronal Apoptosis in the Kainic Acid Model of Epilepsy by Suppressing Proapoptotic Signal Transduction Pathways

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ABSTRACT

Exposure of rats to kainic acid (KA), a non-NMDA type glutamate receptor agonist, induces recurrent (delayed) convulsive seizures and hippocampal neurodegeneration reminiscent of human epilepsy. In this study, the effects of KA were studied with respect to three separate signal transduction pathways likely to regulate inflammatory and apoptotic gene expression in the hippocampus. Immunohistochemical methods and electromobility gel shift assays (EMSAs) demonstrate the concerted activation of the NFκB pathway along with the activator-1 pathway (AP-1) and the p38 mitogen-activated protein kinase pathway (p38 MAPK). Activation of these three pathways occurred simultaneously with the expression of several proapoptotic biomolecules (most notably TNFα and the Fas antigen) and simultaneously with the onset of convulsive seizures but prior to the initiation of neuronal apoptosis. Cotreatment with the experimental antioxidant and antiinflammatory compound phenyl-N-*tert*-butylnitrone (PBN) resulted in a diminution of NFκB, AP-1 and p38 activation, suppressed cytokine and apoptotic gene expression, inhibited neuronal apoptosis, and diminished seizure activity. These data suggest that pharmacological antagonism of multiple signal transduction pathways is achievable in the brain, and that inhibition of these processes may prevent a cascade of gene-inductive events leading to neuronal apoptosis.

Keywords: Kainic acid, inflammation, apoptosis, nitrone, kinase.

The ability to commit apoptosis, or programmed self-destruction, is inherent to most somatic cells and doubtlessly serves a vital function during periods of tissue development or remodeling, or as a defense against neoplastic transformation. Apoptosis must be tightly regulated in order to avoid capricious destruction of healthy tissue. Nonetheless, apoptosis occurs in numerous pathological states, under conditions where deliberate cell death confers no obvious adaptive benefit. In these latter circumstances, an arrest of apoptosis by agents which antagonize the appropriate signal transduction pathways may result in a net benefit to the damaged tissue, and the organism.

With the goal of identifying new strategies for inhibiting neuronal apoptosis, we have begun to explore the molecular basis for apoptosis in a commonly used animal model of epilepsy. In the kainic acid (KA) model of epilepsy, a single systemic dose of the excitotoxin kainic acid initiates a process of hippocampal neurotoxicity (Bernard and Wheal 1995). Rats treated with kainic acid suffer recurrent convulsive seizures and apoptotic neuron loss in the CA1 and CA3 regions of the hippocampus (Pisa et al., 1980; Schwob et al., 1980; Ben Ari et al., 1985; Tauk et al., 1985). Seizure activity is correlated with neuroanatomical changes including mossy fiber sprouting in the dentate gyrus, hippocampal sclerosis, and eventually, neuronal death (Schwob et al., 1980; Sauk et al., 1985; Sperk et al., 1985; Cronin et al., 1992). The lesions produced by systemic kainic acid treatment resemble those seen in hippocampi of human epileptics (Sommer et al., 1880; Schwob et al., 1980; Pisa et al., 1980; Ben Ari et al., 1985; Sperk et al., 1985). Kainic acid appears to act directly on non-NMDA type ionotropic glutamate receptors (Bernard and Wheal 1995), leading to cell death which is predominantly apoptotic in nature (Simonian et al., 1996; Bengzon et al., 1997; Yang et al., 1997; Cheung et al., 1998).

We hypothesized that KA chronically stimulates signal transduction pathways linked to apoptotic gene induction within sensitive populations of hippocampal neurons. Associations between immediate early gene expression and neuronal apoptosis have been previously noted in the KA model (Goodenough et al. 1997),

while less attention has been focused on the association between activated signal transduction pathways and KA-induced apoptosis. We now present evidence that several interrelated signal transduction pathways are activated in the hippocampus between 3 hours and 4 days following systemic exposure to KA. In particular, immunochemical studies and electromobility gel shift assays (EMSAs) demonstrate activation by KA of the NFκB (nuclear factor kappa B) system, the AP-1 (activator protein 1) system, and the p38 mitogen activated protein kinase (p38 MAPK) pathway (Fig. 1). The AP-1 activation may be particularly relevant to KA-induced neuronal apoptosis, since mice lacking the neuronal Jnk-3 kinase (an upstream regulator of AP-1 activation; Fig. 1) are highly resistant to KA-induced seizure activity and hippocampal apoptosis (Yang et al. 1998). Within the timeframe that these signal transduction systems became hyperactivated in the KA-treated rat, numerous proinflammatory and proapoptotic genes were transcribed in a concerted fashion. Frank apoptosis was observed in the time period after activation of the several signal transduction pathways and following expression of inflammatory gene products.

Most intriguingly, treatment of the KA-exposed animals with the compound phenyl-N-*tert*-butylnitrone (PBN, a brain-accessible antioxidant with potent anti-inflammatory and anti-excitotoxic action; Fig. 1) (Hensley 1997) inhibits KA-induced neuronal apoptosis, down-regulates apoptosis-associated gene expression, and prevents seizure activity and death. PBN has an established history as a neuroprotective agent in stroke and age-related neurological impairment (reviewed in Hensley 1997), while a sulfonated analog of the nitrone reportedly inhibits excitotoxicity induced by direct injection of KA into the striatum (Schulz et al. 1995). The neuroprotective mechanism(s) of nitrone action remains unclear. Recently, we have found that PBN inhibits activation of the p38 MAPK and NFκB pathways in cell culture (Robinson et al. 1999a; Kotake et al. 1998). This action is apparently manifest by an inhibition of cytokine-mediated H_2O_2 generation and protection of oxidant-sensitive protein phosphatase activity in the face of a cytokine challenge (Robinson et al. 1999a, 1999b). The data in this present paper are the first to demonstrate PBN inhibition of the p38 MAPK, NFκB

and AP-1 systems *in vivo* during a neurotoxic insult. Intriguingly, PBN suppressed AP-1 activation most strongly in the CA3 and CA1 subfields of the hippocampus, where most of the KA-induced apoptosis occurred, while the dentate gyrus was relatively unaffected by PBN. The results of the current study further define the molecular basis for KA-induced seizure activity and further corroborate the action of nitrone-based pharmacological agents as antagonists of oxidation-sensitive protein kinase cascades and their downstream genetic targets.

MATERIALS AND METHODS

Animals. Adult male Sprague Dawley rats (225-250 g each) were injected subcutaneously behind the neck with KA (Sigma Chemical, St. Louis MO) at a dose of 10 mg/kg, or with vehicle alone (saline). Animals were observed for 4 hours following KA treatment and seizure activity was rated according to the scale developed by Racine (1972) and modified by Mathis and Ungerer (1992). Seizure severity was scored in five stages as follows:

Stage 1. Mild myoclonus with moderate jerking movements of one or two limbs.

Stage 2. Whole body clonus with dramatic movements involving all limbs; loss of righting reflex.

Stage 3. Clonic-tonic seizures consisting of three successive components: a) wild running characterized by episodes of jumping; b) a clonic phase resembling a very brief whole body clonus; c) a tonic phase characterized by extreme rigidity of the whole body and arching of the neck. This phase is sometimes followed by respiratory arrest.

Stage 4. Status epilepticus defined by continuous seizure activity lasting 30 minutes or longer.

Stage 5. Stage four plus vigorous explosive jumps.

Phenyl-N-*tert*-butylnitrone was synthesized at the Oklahoma Medical Research Foundation (Oklahoma City, OK) and was injected at a dose of 150 mg/kg intraperitoneally, in saline vehicle, 90 minutes after KA treatment. The 150 mg/kg bolus of PBN is a standard dose has repeatedly been shown effective in rodent

models of ischemia-reperfusion injury and sepsis, which causes no obvious side effects such as lethargy and hypothermia that can sometimes be seen at higher doses (Hensley et al. 1997). Radiotracer studies indicate that peripherally administered KA permeates the rat brain to near-maximal concentrations within 60 minutes after injection (Berger et al. 1986). Similar pharmacokinetics of KA penetration have been reported in the mouse with no strain differences being reported (Ferraro et al. 1995). Therefore, the 90 minute time lapse between KA and PBN injections in the rat largely negates the possibility that PBN might interfere with KA transition across the blood-brain barrier, or that PBN might stimulate the expression of detoxification enzymes which would alter the pharmacokinetics of systemically-administered KA.

Immunohistochemistry. For immunocytochemical studies, animals were anesthetized with pentobarbital and perfused with saline followed by 4 % paraformaldehyde in saline. Brains were sectioned into 30 μm slices, which were incubated in 4 % normal goat serum in saline for 30 min. at ambient temperature. After three washes with saline, the sections were incubated overnight at 4°C in saline plus 0.025 % triton X-100, 1 % goat serum, and primary antibody. Immunoreactivity was visualized by the avidin-biotin-bridged immunoperoxidase method using 3,3'-diaminobenzidine (DAB) as the chromagen (Hsu et al., 1981). The anti-phospho-p38 antibody was an affinity purified rabbit IgG purchased from New England Biolabs (Beverly, MA), used at 1/300 dilution. Affinity purified rabbit IgG antibodies against c-Fos, c-Jun and the p65 subunit of NFκB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were used at 1/1000, 1/1000, and 1/300 dilution, respectively. Photomicroscopy was performed on a Zeiss Axioplan 2 instrument (Carl Zeiss Inc., Thornwood, NY).

Electromobility gel-shift assays (EMSAs). EMSAs were conducted to determine binding of activated NFκB complexes to synthetic oligonucleotide consensus sequences. The NFκB-binding oligomer was a 22-mer:

5'-GATCGAGGGGACTTTCCCTAGC-3', purchased from Stratagene (La Jolla CA). Double-stranded oligomers were labeled with [γ-^{32}P]ATP using 10 u/reaction of T4 polynucleotide kinase (U.S. Biochemical Corp., Cleveland, OH). Hippocampi were dissected free and homogenized, and nuclear protein extracts were prepared as described (Sonnenberg et al., 1989). Binding reactions (30 μL) were performed at room temperature in reaction mixtures containing 40 μg protein, 20 mM Tris-HCL pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 50 μg/mL bovine serum albumin, 100 μg/mL sonicated salmon sperm DNA, 10 % glycerol, and approximately 0.2 ng (50,000 cpm) of the specific probe. Protein-DNA complexes were separated on 5 % nondenaturing polyacrylamide gels run at 150 V in 50 mM Tris/ 50 mM boric acid / 1 mM EDTA. Gels were then dried and autoradiographed overnight.

Terminal deoxyuridine nick-end labeling (TUNEL). DNA fragmentation characteristic of apoptosis was visualized by 3'end labeling with biotin-derivatized deoxynucleotides via terminal deoxynucleotidyl transferase catalysis. A commercially available TUNEL kit was used (TdT FragEL, Calbiochem, San Diego CA). Biotinylated nucleotides were detected using streptavidin-conjugated horseradish peroxidase and diaminobenzidine (Hsu et al., 1989). Tissue sections thus labeled were counterstained with methyl green as an aid to morphological evaluation.

Ribonuclease protection assays. Approximately 100 mg of hippocampal tissue was homogenized in trizol isolation reagent (Life Technologies, Gaithersburg, MD) using a Dounce-type homogenizer. Total RNA in the extract was quantified by UV absorbance at 260 nm. Inflammation and apoptosis-associated mRNA species were selectively visualized using a multiprobe ribonuclease protection assay (RPA). Radiolabeled probes were synthesized from DNA templates containing a T7 RNA polymerase promoter (Pharmingen, San Diego, CA). Templates were transcribed in the presence of [γ-^{32}P]ATP to yield radioactive probes of defined

size for each mRNA. Probes were hybridized with total hippocampal RNA, then samples were treated with RNAse A and T1 to digest single-stranded RNA. Intact double-stranded RNA hybrids were resolved on 5 % polyacrylamide / 8 M urea gels to produce bands detected by autoradiography.

RESULTS

Beginning approximately 30 minutes after KA injection, animals displayed archetypical epileptiform behavior including "wet dog" shakes, facial clonus, nodding, and forelimb clonus. Three hours after injection, KA-treated rats showed full limbic motor seizures including rearing and loss of postural control, as well as hypersalivation, circling and jumping. Rats treated with PBN 90 minutes after KA injection did not develop full limbic seizures by the 3 hour time point (Table I). Moreover, PBN rescued the KA-treated animals from mortality when evaluated at the end of the four day experiment (Table I). No behavioral, physiologic or histologic alterations were observed in animals receiving PBN only.

KA treatment causes alteration in glutaminergic neurotransmission, which is intimately linked to recruitment of certain transactivating factors such as the AP-1 complex (Cheung et al., 1998). The AP-1 complex is a heterodimer composed of members of the Fos and Jun families of immediate-early gene products. AP-1 transactivation is greatly increased by phosphorylation on specific c-Jun residues (Ser-63 and Ser-73) via the c-Jun amino terminal kinase (Jnk; see Fig. 1). AP-1 transactivation leads to expression of other immediate-early gene products, including further c-Fos and c-Jun expression (Griffiths et al. 1998). Interestingly, mice lacking the Jnk-3 gene product (a brain-specific Jnk isoform) are resistant to kainic acid-induced seizures and neuronal apoptosis (Yang et al., 1997). The first immunochemical analysis of KA-treated rats was therefore aimed at determining whether PBN could antagonize the AP-1 system *in vivo*. Immunocytochemical analysis was performed using well-characterized antibodies against the two AP-1 subunits, c-Fos and c-Jun. Within hours of KA treatment, c-Fos and c-Jun expression increased in hippocampal neurons, particularly within the

CA1 and CA3 regions (Fig. 2). The c-Fos and c-Jun expression was maintained throughout the four day experiment (not illustrated), consistent with previously reported data (Bing et al., 1997). A single injection of PBN completely suppressed c-Jun expression in both CA regions and in the dentate gyrus (Fig. 2) while c-Fos expression was suppressed by PBN only in the CA1 and CA3 regions, where most of the pathological changes were manifest (Fig. 2 and discussed further below). It may be significant to note that while c-Jun expression can be induced rapidly in neurons during growth factor deprivation, c-Fos expression seems to be restricted to those populations of neurons that actually commit to an apoptotic program (Estus et al. 1994).

The AP-1 pathway is but one of numerous signal transduction pathways which have been associated with cellular stress and linked to ligand-induced neurotoxicity. In particular, the p38 MAPK pathway has been repeatedly linked to neuronal apoptosis and, in some circumstances, may indirectly activate both the AP-1 and NFkB pathways (Schulze-Osthoff et al. 1997; Vanden Berghe 1998; Hazzalin et al. 1997). The p38 mitogen-activated protein kinase pathway has been causally linked to neuronal apoptosis induced by growth factor withdrawal (Xia et al. 1995; Kummer et al. 1997). Moreover, pharmacological antagonism of p38 protects cultured neurons against glutamate excitotoxicity (Kawasaki et al. 1997) and we have shown PBN to antagonize cytokine- and hydrogen peroxide-induced p38 activation in cell culture (Robinson et al., 1999a). We therefore undertook an immunohistochemical analysis of p38 activation using an antibody specifically directed against the dual-phosphorylation motif which is present only on the active p38 kinase (Raingeaud et al., 1995). Within 4 hours of KA treatment, p38 activation was seen within the hippocampus in a pattern consistent with that of AP-1 activation (Fig. 3). As in the case of AP-1, PBN suppressed p38 phospho-activation (Fig. 3). The p38 system remained activated somewhat above the level of controls at the four day timepoint, but this chronic activation was not as dramatic as in the AP-1 case (not shown).

The NFκB transcription factor is also ubiquitously activated by physiologic stress and may potentiate excitotoxic damage in striatal neurons (Qin et al. 1998). Alternatively, NFκB seems to serve a protective role in

hippocampal neurons undergoing an oxidative insult (Mattson et al. 1997) and may actually play an antiapoptotic role in TNFα-stimulated cells (Van Antwerp et al. 1996; Wang et al. 1998). NFκB is part of a signal transduction cascade which has traditionally been thought of as distinct from the Jnk and p38 cascade modules, though correlated activation of the three pathways is often noted in cell culture experiments. Several lines of evidence now suggest that p38 and other MAPK enzymes may hyperactivate NFκB (reviewed in Schulze-Osthoff et al. 1997), while inhibition of p38 can suppress transactivational potential of NFκB (Vanden Berghe et al. 1998). We therefore sought to determine whether NFκB was activated by KA in a PBN-sensitive manner. NFκB activation can be indexed several ways. Immunologically, NFκB activation can be inferred from increased immunoreactivity of an epitope on the p65 subunit which is exposed upon NFκB recruitment (Rice and Ernst, 1993). As shown in Fig. 4, NFκB-p65 immunoreactivity in the hippocampus increased dramatically within hours of KA treatment, and this effect was suppressed by PBN. The immunochemical data was corroborated by EMSA analysis which showed a dramatically-increased NFκB binding activity in hippocampal nuclei of KA treated rats, which was partially mitigated by PBN cotreatment (Fig. 4).

Hyperactivation of the Jnk, NFκB and p38 signal transduction pathways could be anticipated to have numerous detrimental consequences. All three signaling pathways have been linked to transcription of inflammatory cytokines and to modulation of apoptosis (Kawasaki et al., 1997; Kummer et al., 1997; Yang et al., 1997; Qin et al., 1998). We therefore sought to determine whether cytokine and proapoptotic genes were being transcribed at a greater rate in the KA treated rats than in normal rats, and whether PBN could abrogate such an effect. Using a multiprobe ribonuclease protection assay, several inflammatory cytokines were clearly found to be transcribed following KA treatment (Fig. 5). IL1α, IL1-β, IL-6 and TNF-α transcription were strongly induced by KA. Within the timeframe that cytokine transcription was enhanced, several proapoptotic genes were also induced. Most notably, the Fas antigen mRNA was strongly induced following KA and this elevation was maintained for at least four days (Fig. 6). PBN treatment suppressed transcription of both

inflammatory cytokine gene products and proapoptotic gene products while having minimal effect on transcription of constitutively-expressed "housekeeping genes" including the L-32 ribosomal mRNA and glyceraldehyde phosphate dehydrogenase mRNA (Figs. 5-6). PBN suppression of cytokine mRNA transcription was relatively unspecific. Interestingly, PBN displayed particular potency in suppressing Fas antigen and caspase 3 transcription, while other apoptosis-associated mRNA species analyzed by RPA were somewhat less affected by the nitrone (Fig. 6).

As a final indication of KA-induced hippocampal damage, *in situ* TUNEL staining was performed to assess frank apoptosis. KA treatment caused DNA damage indicative of an apoptotic process within four days of subcutaneous administration (Fig. 7). Apoptosis was largely restricted to the CA1 and CA3 regions of the hippocampus wherein c-Fos was most strongly expressed. Administration of PBN 30 minutes after KA exposure strongly inhibited this apoptosis as indicated by diminished TUNEL staining in hippocampi from PBN treated animals (Fig. 7). TUNEL staining for apoptotic nuclei therefore corroborates the pattern of of KA-induced and PBN-sensitive immediate early gene expression depicted in Fig.2, and the pattern of proapoptotic gene induction illustrated in Fig. 6.

DISCUSSION

Kainic acid is a well-studied neurotoxin which elicits an animal model of temporal lobe epilepsy and delayed neuronal death in the CA1 and CA3 hippocampal regions. Kainic acid kills neurons in culture by an apoptotic pathway involving binding to non-NMDA type glutamate receptors and consequent expression of immediate early genes including *c-Jun* (Cheung et al. 1998). In the present study, we extend upon these cell culture experiments by documenting increased c-Fos and c-Jun expression (i.e., activation of the AP-1 signaling pathway) within the rat hippocampus following systemic administration of KA. Furthermore, we document the activation of two distinct signal transduction pathways, the NF*k*B pathway and the p38 MAPK pathway,

following the KA challenge. Activation of these three distinct signal transduction pathways correlated temporally with the transcription of both proinflammatory cytokines and proapoptotic mRNA species. Inhibition of these three pathways by the experimental compound phenyl-*tert*-butylnitrone was associated with diminished cytokine elaboration, prevention of neuronal apoptosis, reduced seizure activity, and reduced mortality. While the AP-1, NF*k*B, and p38 pathways are known to respond positively to oxidants and negatively to antioxidants in cell culture (Suzuki et al. 1994; Guyton et al. 1996; Robinson et al. 1999a), the data in this present study are the first to demonstrate the sensitivity of these three pathways to an antioxidant compound within the context of an established *in vivo* model of hippocampal neurodegeneration.

The findings of the present study extend upon previous observations concerning the broad-spectrum neuroprotective action of nitrone compounds, and provide a novel context for discussing the pathology of excitotoxicity. PBN and related nitrones have been shown to suppress striatal excitotoxic lesions induced by NMDA, KA, and AMPA, though not by virtue of any obvious direct interaction with glutamate receptors (Shultz et al., 1995). Similarly, PBN and a sulfated analog inhibit striatal lesions caused by mitochondrial inhibitors such as malonate and the 1-methyl-4-phenylpyridinium (MPP+; Shultz et al. 1995). Nitrones suppress apoptosis and oxidative stress in cultured Down's syndrome neurons (Busciglio and Yankner 1995), and similarly inhibit chemically-induced thymocyte apoptosis *in vitro* (Slater et al. 1995), though the influence of nitrones on apoptosis *in vivo* has not been well-studied. Unfortunately, the pharmacologic effects of nitrones in most previous investigations were not correlated with biomarkers of oxidative stress, inflammation or apoptosis. The present data suggest that suppression of apoptosis by PBN in the KA model and possibly other models of neurodegeneration is likely due to mitigation of proinflammatory or proapoptotic gene expression under the control of the AP-1, NFkB, and p38 MAPK pathways. While the ultimate cellular target(s) for PBN action remain unclear, the present data suggest that the broad-spectrum neuroprotective action of the nitrone

class of compounds (Hensley et al. 1997) might be due, in part, to antagonism of crucial oxidation-sensitive signal transduction elements linked to the initiation of apoptotic programs.

It may be impossible to separate the anti-inflammatory, anti-apoptotic and antioxidant effects of PBN or other, similar pharmacophores. For instance, stimulation of primary astrocytes or fibroblasts with the inflammatory cytokine IL1β elicits cellular H_2O_2 production (Meier et al., 1989, Robinson et al. 1999a) which is partially inhibited by PBN (Robinson et al. 1999a). Moreover, addition of exogenous H_2O_2 causes *de novo* cytokine expression in these cells (personal observations). These and similar findings have led to the postulate that H_2O_2 is an intracellular messenger involved with inflammatory signal transduction (Suzuki et al., 1994; Robinson et al. 1999a). Agents such as PBN which uncouple ligand-receptor binding from intracellular oxidant production might therefore inhibit inflammation and apoptosis as well as diminish cellular oxidative stress. Consistent with such a notion and shown in the present study, PBN inhibits cytokine and apoptosis-associated gene expression *in vivo* following KA challenge. Thus, some of the antioxidant effects of PBN reported in previous studies might reflect a secondary consequence of the gene suppressive and anti-inflammatory action of this compound.

In the KA model of hippocampal neurodegeneration, pharmacological inhibition of pathways leading to apoptosis is correlated with a positive physiologic outcome (survival and diminished seizure activity). Thus, aversion of apoptosis is beneficial in this particular model. While the present study does not address the relative importance of the several signal transduction pathways that are inhibited by PBN, the results suggest that certain drugs may antagonize multiple stress-related signal transduction pathways. Pharmacological strategies designed to specifically inhibit individual signaling modules, such as the NFκB, AP-1 or p38 modules, might therefore be unnecessary and might be more difficult to execute *in vivo* than alternative strategies designed to suppress multiple signaling processes simultaneously. Evaluation of these issues must await future investigations aimed at identifying the central control systems which regulate proinflammatory and proapoptotic

signaling in the hippocampus, particularly with respect to localizing specific oxidation-sensitive elements that may be targeted by nitrone-type neuroprotective agents.

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TABLE I. Suppression by PBN of limbic seizures and mortality in kainic acid-treated rats. Seizure activity was ranked on a five-point scale as described in the methods.

Treatment	Seizure Intensity	Mortality (4 days)
Kainic acid (N = 30)	4.9 ± 0.4	12 / 30 (38 %)
Kainic acid + PBN (N = 20)	2.3 ± 0.3 *	0 / 20 (0 %) **

* $P < 0.05$ (Student's t-test)
** $P < 0.02$ (χ^2 test)

Figure Legends

Figure 1. A. Illustration of the p38-MAPK, AP-1 and NFκB signaling pathways that are activated by KA *in vivo*. Solid arrows indicate directionality of the kinase cascades; dashed arrows indicate putative interactions which have been suggested but not yet confirmed. B. Structure of phenyl-N-*tert*-butylnitrone (PBN).

Figure 2. Kainic acid increases the expression of AP-1 transcription factor components in the hippocampus as evidenced by increases in c-Fos and c-Jun immunoreactivity. Cotreatment with PBN suppresses c-Jun expression globally, however, c-Fos expression is suppressed only in the CA1 and CA3 regions while c-Fos expression in the dentate gyrus was largely unaffected by PBN.

Figure 3. Kainic acid increases p38-MAPK activation in the hippocampus as indicated by increased phosphorylation of the p38-MAPK activation domain. The CA1 subregion is depicted. Immunohistochemistry was performed using an antibody directed against the phosphorylation domain of the active p38 MAPK enzyme ($pThr^{180}$-Gly^{181}-$pTyr^{182}$).

Figure 4. Kainic acid increases NFκB activation in the hippocampus. A,B, and C illustrate exposure of the p65 subunit of the NFκB complex following KA treatment (arrows). D: Electromobility gel shift assay demonstrating increased NFκB binding activity in nuclear extracts induced by KA treatment and suppressed by cotreatment with PBN. Specificity of binding was evidenced by competition for the NFκB complex by an unlabeled (cold) oligonucleotide probe (rightmost lane).

Figure 5. Kainic acid stimulates the transcription of proinflammatory cytokines in the hippocampus as determined by multiprobe ribonuclease protection assay (RPA).

Figure 6. Kainic acid stimulates transcription of proapoptotic genes in the hippocampus as determined by multiprobe ribonuclease protection assay (RPA).

Figure 7. Kainic acid induces apoptosis in the hippocampus as indicated by TUNEL staining. Brown staining (arrows) indicates DNA fragmentation. Tissue was counterstained with methyl green. Boxed areas indicate magnification of CA1 (insets) and CA3 subregions (rightmost panels).

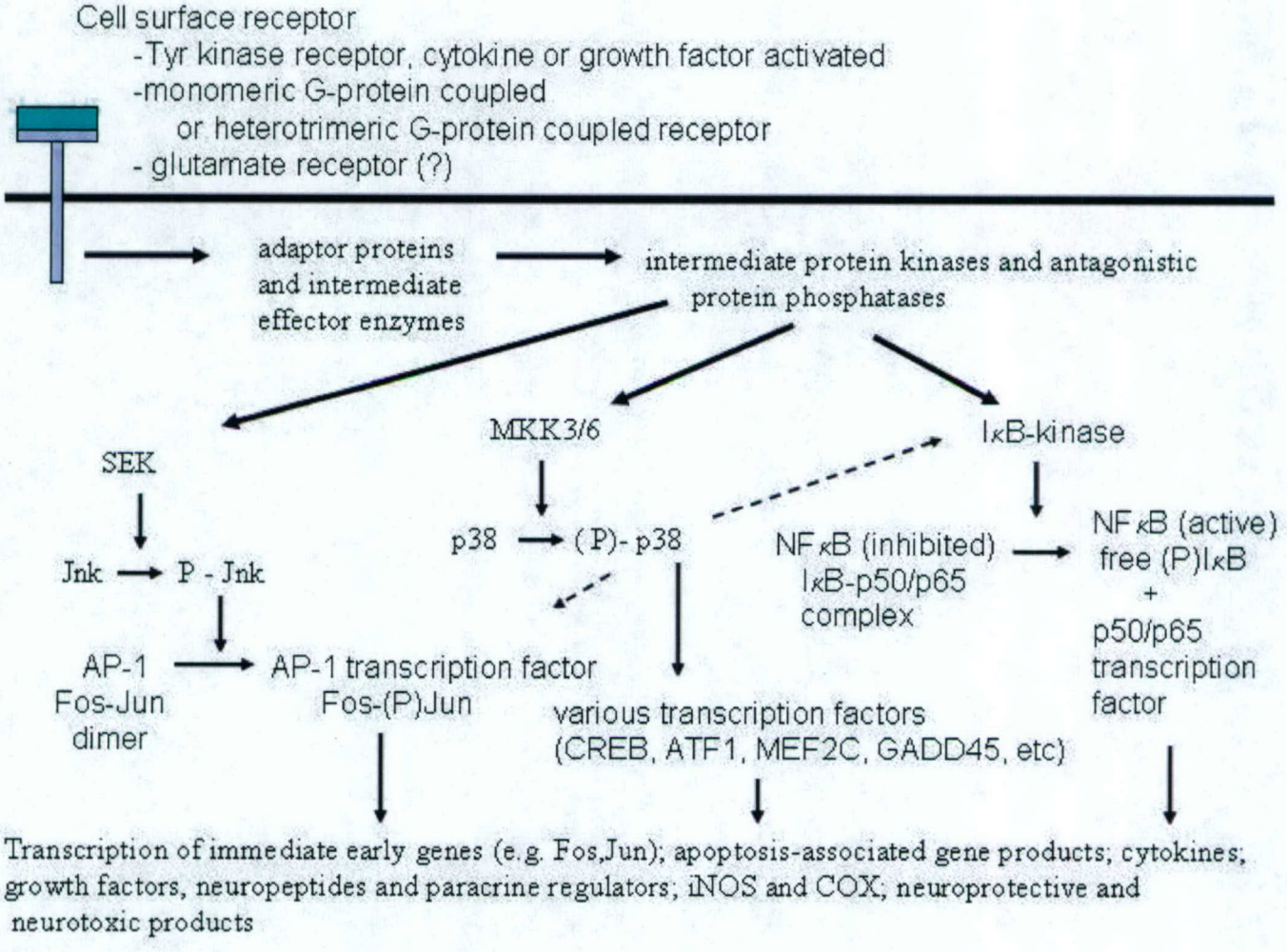
Cell surface receptor
-Tyr kinase receptor, cytokine or growth factor activated
-monomeric G-protein coupled
or heterotrimeric G-protein coupled receptor
- glutamate receptor (?)
adaptor proteins
and intermediate
effector enzymes
intermediate protein kinases and antagonistic
protein phosphatases
SEK
MKK3/6
IκB-kinase
Jnk
P - Jnk
p38
(P)- p38
NFκB (inhibited)
IκB-p50/p65
complex
NFκB (active)
free (P)IκB
+
p50/p65
transcription
factor
AP-1
Fos-Jun
dimer
AP-1 transcription factor
Fos-(P)Jun
various transcription factors
(CREB, ATF1, MEF2C, GADD45, etc)
Transcription of immediate early genes (e.g. Fos,Jun); apoptosis-associated gene products; cytokines;
growth factors, neuropeptides and paracrine regulators; iNOS and COX; neuroprotective and
neurotoxic products

Fig. 1

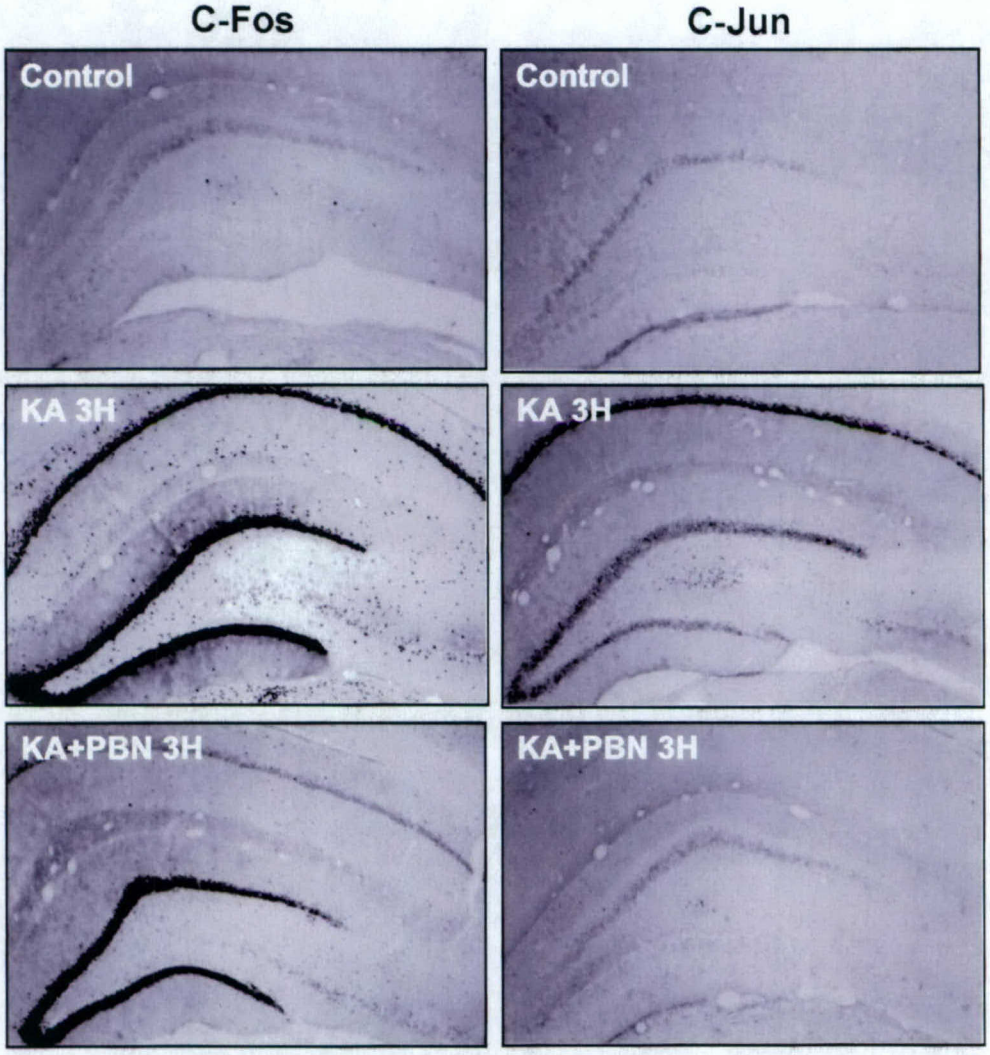

Fig. 2

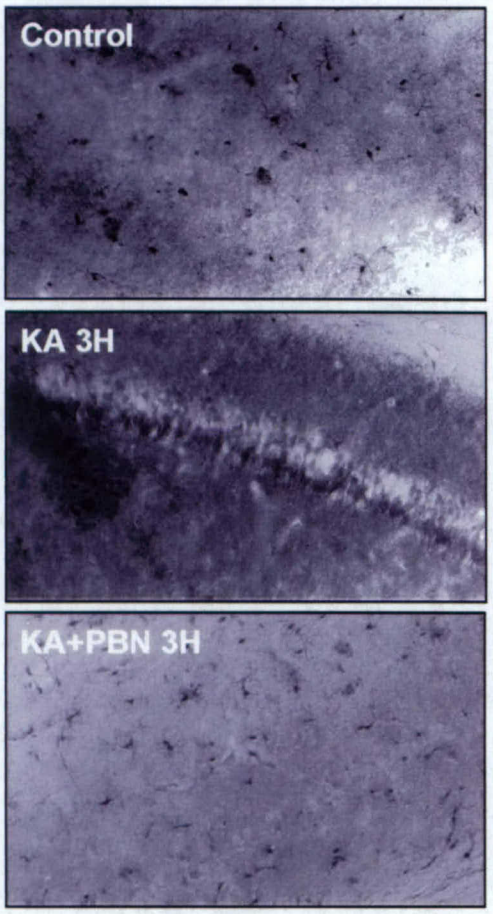

Fig. 3

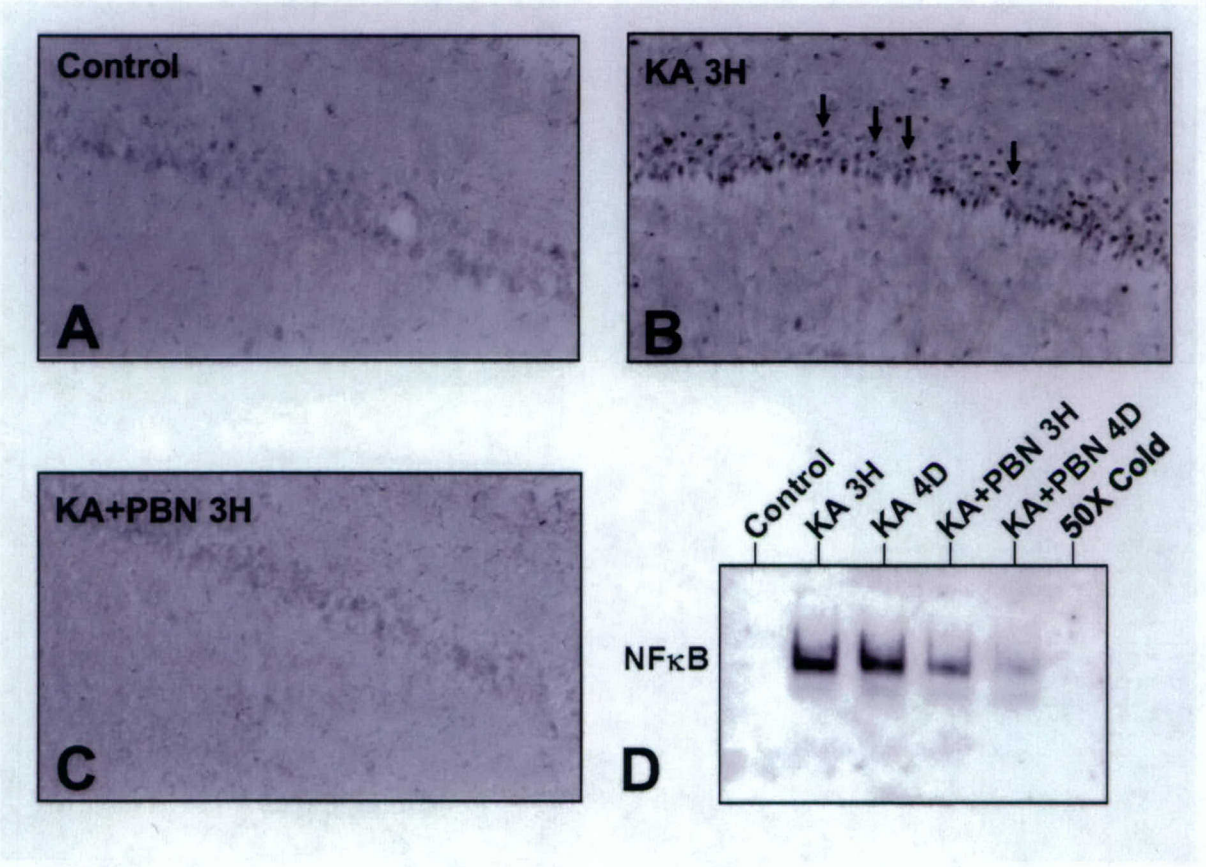
Control
A
KA 3H
B
KA+PBN 3H
C
Control
KA 3H
KA 4D
KA+PBN 3H
KA+PBN 4D
50X Cold
NFκB
D

Fig. 4

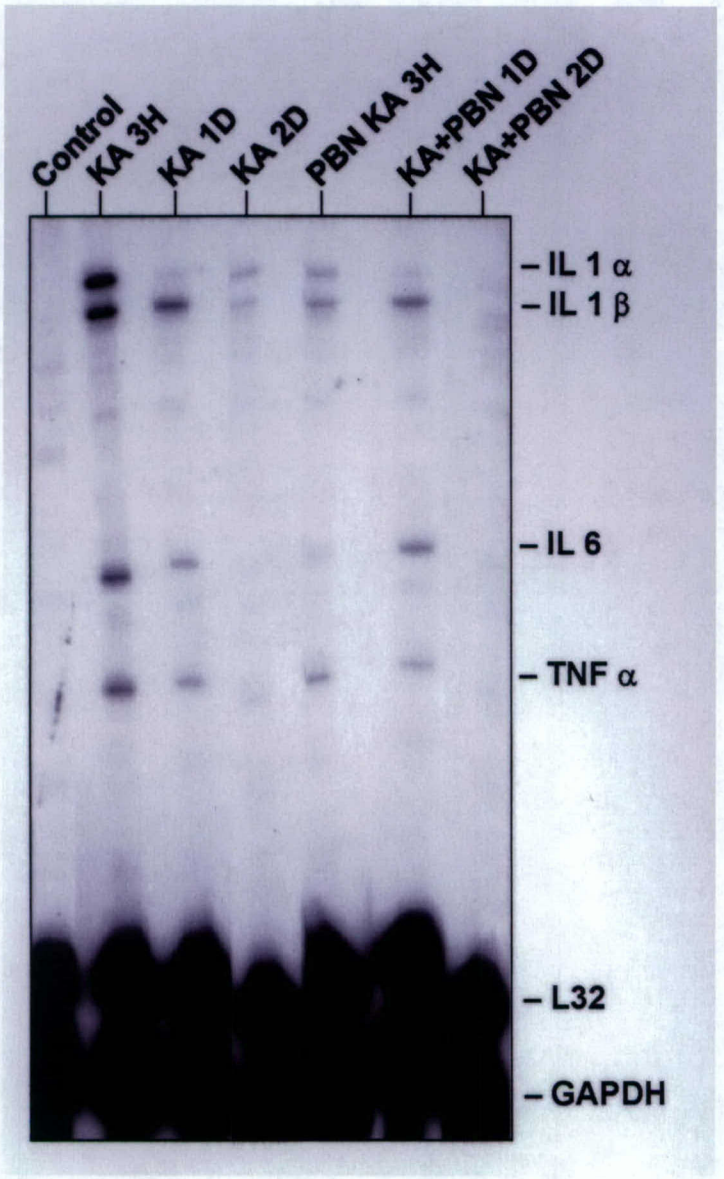
Control
KA 3H
KA 1D
KA 2D
PBN KA 3H
KA+PBN 1D
KA+PBN 2D
– IL 1 α
– IL 1 β
– IL 6
– TNF α
– L32
– GAPDH

Fig. 5

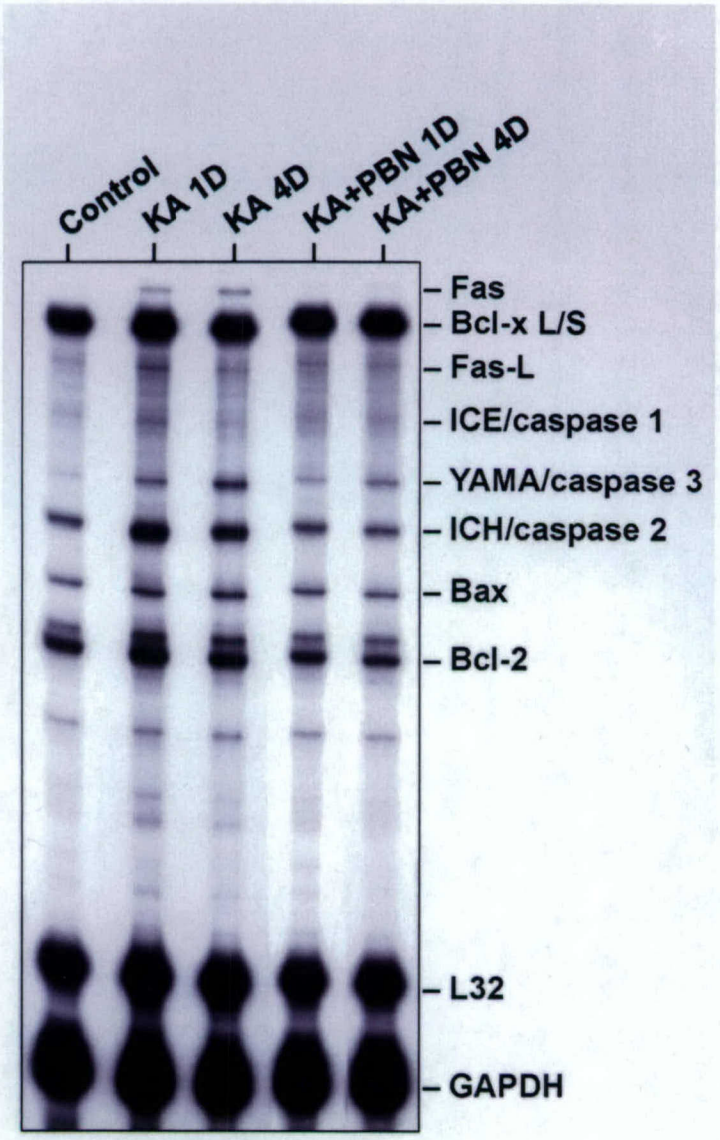
Control
KA 1D
KA 4D
KA+PBN 1D
KA+PBN 4D
– Fas
– Bcl-x L/S
– Fas-L
– ICE/caspase 1
– YAMA/caspase 3
– ICH/caspase 2
– Bax
– Bcl-2
– L32
– GAPDH

Fig. 6

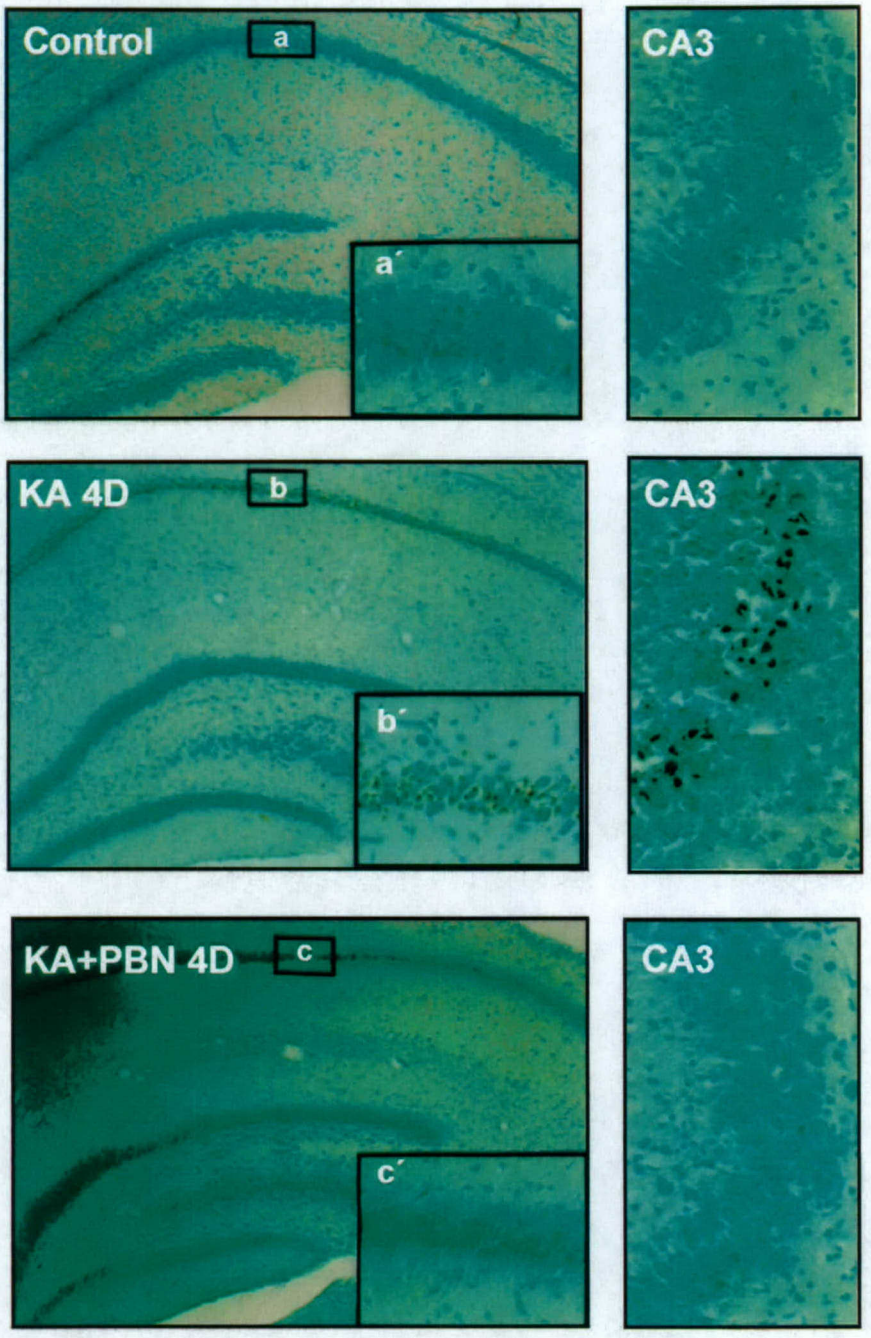

Fig. 7

LONG TERM, DIFFERENTIAL EFFECTS OF SYSTEMIC KA TREATMENT ON NEUROPEPTIDES EXPRESSION.

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Abstract

Although the acute effects of kainic acid (KA) on the expression of various neuropeptides, such as enkephalin (ENK), dynorphin (DYN) and neuropeptide Y (NPY), have been well characterized, little is known about the long-term, differential effects of KA on these neuropeptides. We have found that both mRNA and protein for enkephalin were persistently elevated for at least 1 year after single systemic injection of KA . In order to investigate the differential and long-term effects of KA on other neuropeptides in the hippocampus, we examined the prodynorphin (PDYN), proneuropeptide Y (PNPY), proenkephalin (PENK), prosomatostatin (PSOM) and procholecystokinin (PCCK) mRNA mRNA levels in the rat hippocampus at progressive time points following! KA administration. Using Northern Blot triple labeled with cDNA probes for all 3 neuropeptides, we have found that mRNA for both ENK and NPY exhibits bi-phasic increases. The first peak of the increase occurs at 6 hours and 1 day respectively, but the levels of mRNA then return to control level at 3 days to 2 weeks. However, the levels of mRNA for PENK and PNPY were elevated again after 3 weeks and persist for at least 7 months. In contrast, the mRNA levels for PDYN only showed the first peak of increase at about 6 hours and then were down regulated at 3 days to 2 weeks. Immunocytochemical staining for NYP and ENK revealed a marked increase of immunostaining in the inner molecular layer of granule cells suggesting mossy fiber sprouting. Our results indicate that long-term, differential expression of the neuropeptides after KA injection may underlie the molecular mechanisms for spontaneously convulsive seizure activity of the KA-treated rats.

Introduction

Epilepsy and seizures affect 2.3 million Americans of all ages. 10% of the American population will experience a seizure in their lifetimes. According to most recent estimates, approximately 181,000 new cases of seizures and epilepsy occur each year (Begley CE, Annegers JF, et al 1998). It has been being ¡¡that the long-term, recurrently, spontaneously convulsive seizures occurs in theses patients. A single systemic injection of a convulsive dose of kainic acid(KA), a chemical analog of the excitatory amino acid glutamate, result in both short-term and long-term convulsive seizures in the rat. The KA-induced epileptic seizures in the rat have been widely used as a model for human epilepsy(! Sutula T, Cavazos J, et al 1992; Tauck DL and Nadler 1985; Sloviter RS. 1992). Many studies indicated that some neuroprptides in the brain may play a critical role in the regulation of seizure activity(Erickson JC, Clegg KE, et al 1996; Vezzani A, Monhemius R et al 1996 ;¡.)Although the short-term effects of kainic acid (KA) on the expression of various neuropeptides, such as enkephalin (ENK), dynorphin (DYN) and neuropeptide Y (NPY), have been well characterized, little is known about the long-term, differential effects of KA on these neuropeptides. In our previous study we have found that both

mRNA and protein for enkephalin were persistently elevated for at least 1 year after single systemic injection of KA . In order to investigate the differential an! d long-term effects of KA on other neuropeptides in the hippocampus, we examined the prodynorphin (PDYN), proneuropeptide Y (PNPY), prosomatostatin (PSOM) and procholecystokinin (PCCK) mRNA levels in rat hippocampus at progressive time points following KA administration. Our results suggested that¡¡.

MATERIALS AND METHODS

Animals and Treatments

Adult male Fischer 344 (225-250 g body weight) was used throughout the study. Control animals were injected s.c with physiological saline. Experimental animals were injected with kainic acid (Sigma, St. Louis, MO, and 7.25 mg/kg, S.C.). The animals were rated according to the scale devised by Racine (Racine, 1972) for the initial 4 hours following the KA injection. Only animals with full limbic seizures (forelimb clones with rearing, stage 4) were chosen for further studies.Animals were perfused with 4% paraformaldehyde in phosphate-buffed saline (PBS) at different time points.

RT-PCR and Gene Cloning

Plasmid DNAs for Dynorphin, CCK, Somatostatin, and G_3PDH were obtained by RT-PCR (GIBCO BRL Superscript Preamplificaiton System) and TA Cloning (Invitrogen, Carlsbad, CA). Total RNA was isolated from the rat hippocampus using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to procedures recommended by the manufacturer. 1.0 ug RNA was reverse transcribed into cDNA in a 20 ul reaction using olig-dT as primer. cDNA template was used in a 50 ul PCR reaction containing 5 ul 10X PCR Buffer, 0.5 ul 50 mM dNTPs, 1 uM of each primer, and 1 U Taq to amplify the desired genes. The sense primer to amplify Dynorphin was 5¡¯-GGGGCTTTTGGTCTTTTCTCAC-3¡¯ and the anti! sense primer was 5¡¯-ATAGAGCGGTTGGGCTGATGTC-3¡¯. The PCR conditions for Dynorphin consisted of 35 cycles at 94° C for 2 min., 94° C for 1 min., 63.6° C for 30 sec., 72° C for 2 min., 72° C hold for 10 min., and 4° C hold. The sense primer for amplifying CCK was 5¡¯-TATGAAGTGCGGCGTGTGTCTG-3¡¯ and the antisense primer was 5¡¯-AAACATTAGAGGCGAGGGGTCGTG-3¡¯. The PCR conditions for CCK consisted of 42 cycles at 94° C for 2 min., 94° C for 1 min., 65° C for 30 sec., 72° C for 2 min., 72° C hold for 10 min., and 4° C hold. The sense primer for Somatostatin was 5¡¯-ATGCTGTCCTGCCGTCTCCAGT-3¡¯ and the antisense primer was 5¡¯-ACAGGATGTGAATGTCTTCCAG-3¡¯. The PCR conditions for Somatostatin consisted of 35 cycles at 94° C for 2 min., 94° C for 1 min., 60.5° C for 30 sec., and 70° C for 1.5 min. The sense primer for G_3PDH was 5¡¯-ACCACAGTCCATGCCATCAC-3¡¯ and the antisense primer was 5¡¯-TCCACCACCCTGTTGCTGTA-3¡¯. The PCR conditions for G_3PDH consisted of 28 cycles at 94° C for 1 min., 94° C for 30 sec., 60° C for 30 sec., and 68° C for 2 min. Amplified PCR products were ligated into the pCR11-TOPO vector in a reaction containing 0.5-2 ul PCR product and 1 ul pCR11-TOPO vector to a total volume of 5 ul and transformed into One Shot cells to generate plasmid DNA. DNA isolation was carried out according to Plasmid Midi-Kit protocal! described by the manufacturer (QIAGEN, Santa Clarita, CA). Isolated plasmids were used to synthesize probes for Northern blot analysis and *In situ* hybridization. Probes for Northern blot analysis were synthesized according to DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim). 1 ug template DNA in a 16 ul reaction was denatured by boiling for 10 min. and incubated in 4 ul of DIG-High Prime at 37° C. Reaction was stopped by 2 ul of 0.2 M EDTA (pH 8.0). *In situ* hybridization probes were made according to ¡¡..

Northern Blot Analysis

Total RNA Iso total RNA was isolated from the rat hippocampus using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to procedures recommended by the manufacturer. Briefly, brain tissues were homogenized in 1 mL of TRI Reagent. 0.2 mL of chloroform was added to the homogenized mixture and mixed vigorously. The solution was stored at room temperature for 15 min., and the aqueous phase was collected. A 1:1 volume of isopropanol was added to the aqueous phase, mixed, and stored at ¨C20° C overnight. RNA pellet isolated after centrifugation (13,200 rpm) was washed wi! th 75% Ethanol, dissolved in DEPC-treated water, and quantified.2.25 ug RNA sample in sample buffer (5% 20X MOPS, 16% formaldehyde, and 79% formamide) was denatured by heating at 65° C for 8 min. and placed immediately on ice for 3 min. RNA sample was electrophoresed on 1.0% agarose/formaldehyde gel containing 5% MOPS, 3% formaldehyde (37%), and 1.5 ul Ethidium bromide and transferred overnight by blotting to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL) with 10X SSC (3.0 M NaCl and 0.3 M Sodium Citrate). Filters were baked at 80°C under vacuum for 2 hrs. Membranes were pre-hybridized in high sodium dodecyl sulfate (SDS) buffer for 1 hr. and hybridized overnight with dig-labeled cDNA probes for Dyn, NPY, Enk, CCK, Somatostatin, and G_3PDH. in hybridization buffer (40 mM NaPO! $_4$, 1 mM EDTA, 50% formamide, 2% SDS and 10 mg/mL salmon sperm DNA) at 55° C. After hybridization, the membranes were washed 30 min. with a solution of 2x SSC and 0.1% SDS at room temperature followed by a solution of 0.1xSSC and 0.1 % SDS at 60° C. The membranes were blocked in 4x block solution (10% blocking reagent from Boehringer Mannheim in 1x Maleic acid buffer) for 1 hr. at room temperature and incubated in antibody solution (10% block solution, 1:10,000 Anti-Digoxigenin-Ap) for 30 min. After washing in washing buffer (1x Maleic Acid buffer, 0.3% Tween 20, pH 7.5) for 30 min., the membranes were incubated it in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 5 min. The membranes were coated with chemiluminescent substrate CSPD (Boehringer Mannheim) in an envelope for 5 min. and stored at 37°! C for 15 min. The membranes were then placed against the Amersham Hyperfilm (SIGMA), and the film was processed.

***In Situ* Hybridization**

Precedure was carried out at described in the ????? cDNA probe was linearized at two different sites in 25 ul reactions containing 1ug/ul cDNA template, 10x buffer D (company???) and the appropriate restriction enzyme. Following incubation at 37° C for 2 hrs., reaction was heated to 95° C for 2 min. and placed on ice for 2 min. 50 ul of TE Buffer (1 M Tris-HCl, and 0.5 M EDTA, pH 8.0) was added to the reaction and extracted with phenol/chloroform? The reaction was centrifuged (13,000xg) and the supernatant was collected. 10 ul of 3 ! M Sodium Acetate and 300 ul of chilled Ethanol (100%) was added to the supernatant and left to precipitate at ¨C70° C. Pellet was isolated by centrifugation (13,000xg) for 30 min. at 4° C and dissolved in DEPC-treated water. To label the isolated RNA, 2 ul of RNA polymerase was added to an 18 ul reaction mixture containing 1 ug purified template, 2 ul NTP labeling mixture (10x), 2 ul transcription buffer (10x), and 1 ul Rnase inhibitor, and incubated at 37° C for 2 hrs. 2 ul of DNase I was added to the reaction and incubated for 15 min. at 37° C. To stop the reaction, 2 ul of 0.2 M EDTA was added. The solution was incubated at ¨C70° C for 1 hr. following addition of 4 M LiCl and prechilled Ethanol, and centrifuged (12000xg) for 30 min. at 4°! C to isolate the pellet. Pellet was centrifuged (12000xg) at 4° C in 70% ethanol, and dissoved in appropriate volume of DEPC-water. Estimate the yield of labeled probes by direct blotting procedure described in ???? Store labeled probes at ¨C80° C

Tissue sections were washed in DEPC-treated PBS (pH 7.4), DEPC-treated PBS containing 0.3% Triton X-100, and DEPC-treated PBS, respectively. Sections were permealized with TE

buffer (100 mM Tris-HCl, 50mM EDTA, pH 8.0 containing RNase-free Proteinase K) for 30 min. at 37° C. Sections were treated with DEPC-treated PBS containing 4% paraformaldehyde followed by DEPC-treated PBS. Sections were incubated on a rocking platform in 0.1 M triethanolamine (TEA) buffer (pH 8.0) containing 0.25% acetic anhydride, followed by incubation with prehybridization buffer (4x SSC containing 50% deionized formamide) at 37° C. Sections were incubated overnight at 42° C in hybridization buffer c! ontaining DIG-labeled RNA probe. Sections were wahsed in 2xSSC and 1xSSC at 37° C. The sections were incubated in NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) containing 20 ug/mL RNase A for 30 min. at 37° C. The sections were washed in 0.1xSSC at 37° C and in buffer 1(100 mM Tris-HCl, pH 7.5, 150 mM NaCl). After blocking fro 30 min. in a solution of 0.1% Triton X-100 and 2 % normal serum, sections were incubated with buffer 1 containing 0.1% Triton X-100, 1% normal serum, and suitable dilution of anti-DIG-alkaline phasphatase. Sections were washed in buffer 1 and buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and incubated in color reaction in a dark chamber for 2-24 hrs. Color reaction was stopped by incubating in buffer 3 (10 mM Tris-HCl, pH 8.1, 1 mM EDTA).

Immunohistochemistry

Brain tissues were sectioned at 30 μm on a sliding microtome. All steps were carried out at room temperature on free-floating tissue sections. Alternate sections of the rat hippocampus were stained with primary antibodies against Dynorphin (1:1000), Enkephalin (1:1000), and CCK (1:1000) diluted in solution of KPBS, 0.4% Triton X-100, 1% normal serum, and 0.25% BSA. Biotinylated anti-rabbit IgG secondary antiserum (Vector Lab! oratory, Burlingame, CA) was diluted (1:600) in washing buffer (KPBS, 0.02% Tx,, 0.25% BSA). The avidin-biotin immunoperoxidase method with 3,3-diaminobenzidine tetrahydrochloride as the chromagen was used to visualize immunoreactive cells (ABC Kits, Vector Laboratory).

Sections were incubated in 4% normal serum in PBS for 30 min. at room temperature to block non-specific immunostaining. After 3 washes in KPBS, the sections were incubated in solutions of primary antiserum overnight at 4° C. After extensive washes in washing buffer, the sections were incubated in solution of secondary antiserum for 60 min. Sections were rinsed in KPBS and incubated for 60 min. in avidin-biotin complex. Sections were then washed in KPBS followed by Tris-Imid Buffer (0.2 M Imidazole, pH 9.2, 1.0 M Trizma, pH 7.2). Color reaction was carried out in a solution of 0.05% diaminobenzidine tetrachloride and 0.01% hydrogen peroxide in Tris-Imid Buffer.!

Results

Changes in the mRNA Levels of DYN, ENK, and NPY

Bing et al has previously reported significant biphasic increases in PENK mRNAs in the rat hippocampus after kainate injection. They report short-term increases in PENK mRNA beginning at 6 hr after treatment, which return to control levels by 7 days. This is followed by a long-term increase at 2 weeks, which persists at higher levels than control for at least one year (Bing, et al 1997). Our Northern blot results reveal comparable biphasic increases in PENK mRNA levels after kainate treatment. We observed a significant increase in PENK mRNA starting at 3 hr and persisting up to one day after treatment (Figure A). The maximum increase in PENK mRNA level was 20 fold of the control and occurred at 6 hr after treatment. PENK mRNA levels returned to control level at 3 days, followed by a 10- fold increase, which persisted up to 4 months after treatment, the longest time-point studied. In contrast to the biphasic long-term change in PENK levels after kainate treatment, PDYN mRNA levels showed a short-term

increase at 3 hr, persisting up to 1 day after treatment, with a maximum of 15- fold at 6 hr. Although short-term changes in the mRNA levels of NPY after kainate acid treatment has been investigated, we discovered a biphasic increase in NPY mRNA levels in the hippocmapus after kainate treatment. Our results indicate a 6-fold increase in NPY mRNA levels 1 day after KA treatment. The NPY mRNA levels return to control at 3 days followed by a long-term increase starting at 4 weeks and persisting up to 4 months, the longest time-point studied. The maximum increase in mRNA levels observed for NPY was 10-fold of control at 4 months after treatment. mRNA levels of DYN, ENK, and NPY for 4 months control animals were similar to that of control animals at lower time-points.

Changes in the mRNA Levels of ENK and CCK

We also compared the mRNA levels of ENK and CCK in the rat hippocmapus after kainate treatment. PENK mRNA levels were reported under the previous subheading. We discovered significantly lower biphasic increases in CCK mRNA levels after kainate treatment compared to the PENK mRNA levels (Figure B). The CCK mRNA levels increased 3-fold at 3 hr after treatment and gradually declined to control levels at 1 day. However, we observed a long-term increase in CCK mRNA levels at 4 weeks, which increased to a maximum of 5-fold increase at 6 weeks. The increase in CCK mRNA levels remained consistently higher than control up to the final time-point of 4 months after treatment. The long-! term increase in CCK mRNA level after kainate treatment has not been investigated previously.

Changes in the mRNA Levels of DYN and Soma

The mRNA levels of DYN and Soma were compared after kainate treatment in the rat hippocampus. PDYN mRNA levels were as reported under the first subheading. However, the time-point after kainate treatment was extended to 12 months. No changes in PDYN mRNA levels was observed after 4 months. In contrast to PDYN, Soma mRNA levels revealed a biphasic change after kainate treatment. The level of Soma increased 1-fold 6 hr and returned to control level 1 day after treatment. However, the level of Soma mRNA was significantly lower than the PDYN mRNA level at that time point, and the increase does not persist as long as the PDYN mRNA level at 1 day.&n! bsp; The Soma mRNA level gradually increases beginning at 5 months and reaches a maximum of 4-fold increase at 12 months, the longest time-point studied. This is the first reported investigation of the long-term induction of Soma mRNA after kainate treatment in the rat hippocampus.

Changes in Levels of Neuropeptide mRNA in the Rat Hippocampus after Kainate Treatment Revealed by *in Situ* Hybridization. We investigated the localization and induction of mRNA levels of various neuropeptides in the rat hippocampus after kainate treatment using *in Situ* Hybridization. We used tissue sections from various time-points after treatment including control, 1 day, 1 week, and 2 months.

PENK mRNA

Our results indicate a significant increase in the PENK mRNA from the control 1 day after kainate treatment (Figure E and F). PENK mRNA was comparable to control 1 week after treatment followed by another induction 2 months after treatment (Figure G and H). The increase in PENK mRNA was primarily observed in the granule cell layer of dentate gyrus.

PDYN mRNA

We observed an induction in the PDYN mRNA from the control 1 day after kainate treatment (Figure A and B). PDYN mRNA was mainly induced in ???? cells in the ??? region of the hippocampus. PDYN mRNA was comparable to control 1 week and 2 months after kainate treatment (Figure C and D). ??????????????????

Expression of Neuropeptide Immunoreactivity in the Rat Hippocampus after Kainate Treatment. We employed immunohistochemistry to determine whether the levels of various neuropeptides correlated with their mRNA levels in the rat hippocampus at various time-points after kainate treatment.

ENK Immunoreactivity

We observed a significant increase in ENK immunoreactivity from the control 1 day after kainate treatment (Figure E and F). ENK immunoreactivity gradually decreased at 1 week and returned to control 2 months after treatment (Figure G and H). Increase in ENK-immunoreactivity was prominent in the mossy fibers of hippocampus. ?????????

DYN Immunoreactivity

Our results indicate a significant increase in DYN immunoreactivity from the control 1 day after kainate treatment in the ???? cells of the ???? region of the hippocampus (Figure A and B). DYN immunoreactivity returns to control 1 week and 2 months after kainate treatment (Figure C and D)

NPY Immunoreactivity

NPY-immunoreactivity significantly increased from the control 1 day and 1 week after kainate treatment (Figures I-K). NPY-immunostaining was mainly observed in the ???? cells of the ??? region in the hippocampus. NPY expression decreased 2 months after kainate treatment (Figure L).

Discussion

Dyn , Enk and NPY gene expressions in the rat hippocampus after KA injection

Northern blotting analyses have been used previously to identify the gene expressions of Dyn, Enk and NPY in rat hippocampus after KA treatment( ). In our studies, Northern Blot triple labeled with cDNA probes for all 3 neuropeptides were used to compare the their long-term different expression patterns onto the same nylon membrane on the same hippocampus tissue at the progressive time points following KA administration.

Fig 1 showed that PDYN mRNAs were induced by KA , peaked around 6 hours after KA treatment, and returned to control levels by 1 week and stayed for four months. In the same way PENKlevels were increased about 12-fold at 6 h after KA injection. These increases returned by

7 days to control levels, but 2 weeks later PENK mRNA rose again and persisted at this higher level for four months. A biphasic increases in PENK mRNAs were found.

In our previous studies ENK mRNA levels were elevated by KA in the rat hippocampus for up to 1 year and a biphasic increases in PENK mRNAs were found (Bing et al.,1997).

Using Northern Blot triple labeled with cDNA probes for all 3 neuropeptides, we have found that mRNA for both ENK and NPY exhibits bi-phasic increases. The first peak of the increase occurs at 6 hours and 1 day

CCK long-term expression in the rat hippocampus after KA injection

SOMA long-term expression in the rat hippocampus after KA injection

Changes in Neuropeptide mRNA levels in the Rat Hippocampus after Kainate Injection Revealed by Northern Blot Analysis. We compared changes in the mRNA levels of various neuropeptides in the hippocampus after Kainic Acid treatment using Northern blot analysis.

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HIPPOCAMPUS LONG TERM, DIFFERENTIAL GENE EXPRESSION IN THE RAT AFTER SYSTEMIC KAINIC ACID INJECTION REVEALED BY PCR-SELECTED SUBSTRACTIVE CLONING

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A single systemic injection of convulsive dose of KA can cause a selective degeneration in hippocampus and result in long-term spontaneously recurrent seizures in rats. It has been reported that many genes were induced short after KA injection. However, A relatively little information is available for long-term gene expression in rat hippocampus after KA injection. In order to examine these long-term differentially expressed genes. We used the suppression subtractive hybridization (SSH) and PCR-select differential screening methods (Clontech, Palo Alto, A) for comprehensive analyses of long-term, differential gene expressions in the hippocampus. The mRNA was isolated from rat hippocampus 4 month after KA (n=3) and saline injection (n=3), cDNA was synthesized from the pooled mRNA, both forward subtracted and reverse subtracted hybridization was ! performed. Select-PCR was used to amplify the forward and reverse subtractive products. All of the PCR products were cloned into TA cloning vectors (Nitrogen). 432 clones were picked up. After dot blotting and sequencing analysis 76 elevated genes and 25 suppressed genes were identified. Among them, 20 are long-term elevated genes, 11 are transient induced genes, 3 are long-term decreased genes, 8 are appear two-phase elevated, and 8 are appear to be multiple isotype genes in rat hippocampus identified by Northern blotting. after sequencing analysis and blast search 31 clones are reported in gene bank by other researchers and 23 of them have not been reported. The differential expressed genes are likely to be related to seizure activity, oxidative stress, apoptosis and some neurological disorders. These results indicated that 1). KA-induced hip! pocampal pathophysiological changes caused a differential gene expression that is related to neurodegenerative diseases. 2). Long-term neuronal adaptation to excitatory toxicity involves a comprehensive multi-genes activation and suppression process. 3). Systemic examination of all the genes that involve KA-induced neurodegeneration is possible by this methods, thus may shed a light on the molecular mechanism of neurodegenerative disease.

INTRODUCTION

Epilepsy , recurrently, spontaneously convulsive seizures, is affecting an estimated 2.5 million people in the United States and 40 million worldwide. A recent study by the Epilepsy Foundation estimated that 10% of the American population will experience a seizure in their lifetimes,181,000 new cases of seizures and epilepsy occur each year, and the annual financial cost of this disorder is $12.5 billion in the United States alone.

Despite recent advances in treatment, many people with epilepsy still suffer from uncontrolled seizures or from the side effects of treatment. The science of predicting which drugs are most likely to be successful in treating patients based on expression of different genes. For this reason it will be ¡..step for this disorder to investigate how many genes, which kind of genes, are involved in seizure activity

A single systemic injection of a convulsive dose of kainic acid(KA), a chemical analog of the excitatory amino acid glutamate, result in both short-term and long-term convulsive seizures in the rat. The KA-induced epileptic seizures in the rat have been widely used as a model for human epilepsy(1-3). Short-term expressed genes induced by KA in rat hippocampus have been

reported (4). Our previous studies indicated that some long-term differential genes in the rat hippocampus after KA injection may play a critical role in the regulation of seizure activity, such as enkephalin (ENK)(5) and neuropeptide Y (NPY)(6).

There are several different techniques for cloning of differential expressed genes in two populations. 1. Differential display (7) 2. Representational differential display (8)3. Enzymatic degradation subtraction (9) 4. Linker capture subtraction (10) and 5. Subtraction Suppression Hybridization (SSH) and PCR-Selected differential Screening (11). We think the later, PCR-Selected substractive cloning, is a more powerful technique for enrichment of rarely differential expressed genes! . We used PCR-selected subtractive cloning to reveal rat hippocampus long-term, differential gene expression 4 months after KA injection. Our results suggested that¡¡

MATERIALS AND METHODS

Animals and Treatments

Adult male Fischer 344 (225-250 g body weight) was used throughout the study. Control animals were injected with physiological saline. Experimental animals were injected with kainic acid (Sigma, St. Louis, MO, and 10 mg/kg, S.C.). The animals were rated according to the scale devised by Racine (12) for the initial 4 hours following the KA injection. Only animals with full limbic seizures (forelimb clones with rearing, stage 4) were chosen for further studies.

Suppression subtractive hybridization (SSH)

Tester and driver preparation. Tester and driver ds cDNA were synthesized from 2 ug of two different poly (A) RNA after mRNA were isolated by mRNA purification kit (Pharmacia Biotech) from hippocampus 4 month after kainic acid (n = 3) and saline (n = 3). For forward subtraction KA-induced samples were used as tester and control samples were used as driver. For reverse subtraction control samples were used as tester and KA-induced samples were used as driver. First- and second ¨Cstrand cDNA synthesis and blunt-ending of DNA ends by T4 DNA polymerase were carried out according to the CLONTECH¡¯ protocol. The resulting cDNA pellet was dissolved in 10 ul of dionized water and digested by Rsa I in a 50-ul reaction m! ixture containing 15 units of enzyme at 37 0C for 1.5 hr. Digested tester cDNA (1 ul) was diluted in 5 ul of water. The diluted tester coda (2 up) was then ligated to 2 ul of adapter 1 and adapter 2R (10 um) in separate ligation reactions in a total volume of 10 ul at 16 0C overnight, using 0.5 units of T4 DNA ligase, 1 ul of 0.2 M EDTA was added and the samples were heated at 70 0C for 5 min to inactivate the ligase and stored at ¨C 20 0C.Forward and reverse subtraction were performed using the PCR-Selected cDNA Subtraction Kit (CLONTECH) according to the manufacturer¡¯s protocol. The first and second hybridization were performed on a Perkin-Elmer 9600 thermalcycler.

PCR Amplification and T/A cloning

Two PCR amplifications were performed for each subtraction. The primary PCR was conducted in 25 ul. It contained 1 ul of diluted, subtracted cDNA, 1ul of PCR primer 1 (10 um) and 23 ul of master mixture prepared using the Advantage cDNA PCR kit (CLONTECH). PCR was performed with following parameters: 75 oC for 5 min; 94oC 25 sec; 27 cycles at (94 oC for 10 sec; 66oC 30 sec; 72 oC for 1.5 min). The amplified products were diluted 10-fold in dionized water. The second PCR was conducted in 25 ul. It contained 1 ul of diluted primary PCR product, 1 ul of Nested PCR primer 1 (10 um), 1 ul of Nested PCR primer 2R (10 um). PCR conditions were as follows: 10-12 cycles at (94 oC 10 sec; 68 oC 30! sec; 72 oC 1.5

min).Products from the secondary PCR were inserted into pCR II using a T/A cloning kit (Nitrogen) according to the manufacturer¡¯s protocol. All of positive clones were picked up.

Dot blot analysis

Plastid DNA minipreparation of 432 clones were performed by QIAprep Spin Miniprep Kit (QIAGEN) and diluted into 100 ng/ul. 1 ul of plasmid DNA (100 ng) was dropped on nylon membranes for screening with forward subtracted probe and reverse subtracted probe. Random primer labeling of subtracted probe and reverse subtracted probe and hybridizations for dot blot analysis were performed using PCR-Select Differential Screening Kit (CLONTECH).

Northern blot analysis

Probes preparation. A non-radioactive Northern blot analysis was used to detect every subtracted product after dot blot analysis. All of coda probes were prepared by PCR with DIG dNTP (Boehringer Mannheim, Indianapolis, IN). The PCR was conducted in 25 ul. It contained 1 ul of diluted subtracted cDNA (100 ng/ul), 1 ul of nested PCR primer 1 (10 um) and 1 ul of nested PCR primer 2R(10 um), and 22 ul of PCR master mixture prepared using the PCR kit (Boehringer plasmid Mannheim, Indianapolis, IN). PCR was performed with the following parameters: 95 0C 2 min; 32 cycles at (94 0C for 30 sec; 68.5 0C for 30 sec; 72 0C 1 min 30 sec); and final extension at 72 0C for 7 min. The amplified products were purified by G50 (¡.).

Total RNA was isolated from immediately frozen hippocampal tissues (control, 6 hr, 1 d, 1 w, 2 w and 3 m after KA injection) according to the Tri Reagent protocol (Molecular Research Center, Cincinnati, OH) and electrophoresed through 1.2-% agars/formaldehyde gels (1 X MOPS and 6% formaldehyde). After transfer onto nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) with 10 x SSC, the filters were baked at 80 0 C under vacuum for 1 hr. The membranes were hybridized with the PCR-DIG labeling probes in high SDS concentration hybridization buffer (7% SDS, 50% formamide, 5 x SSC, 2% Blocking Reagent, 50 mM sodium-phosphate, pH 7.0 and 0.1% N-lauroylsarcosine) for 14 h at 50 0C and washed in solution containing 2 x SSC and 0.1% SDS at room temperature and followed by another two washes with 0.1 x SSC and 0.1% SDS fo! r 15 min at 68 0 C. The membranes were incubated for 30 min in antibody solution (75 mU/mL anti-DIG-AP conjugate, 1% blocking reagent in 0.1 M maleic acid) after 1 h blocking (2% blocking reagent, 0.1 M maleic acid and 0.15 M NaCI) at room temperature. The membranes, then, were washed twice for 15 min in washing buffer (0.1 M maleic acid buffer plus 0.3% Tween 20) and equilibrated for 5 min in detection buffer (0.1 M Tris-HCI, 0.1 M NaCI, pH 9.5.). The membranes were incubated for 5 min at room temperature in a development folder with CSPD solution (Boehringer Mannheim) for chime-luminescent reaction and then incubated at 37 0C for 15 min. Filters were exposed to X-ray film.

Sequence analysis

DNA sequencing was performed by automated means at¡¡¡ Nucleic acid homology searches were performed using the BLAST program through e-mail servers at the National Center for Biotechnology Information (National Institutes of Health, Bethesda).

Result

432 clones were picked up after suppression subtractive hybridization (SSH) and PCR- select differential screening.76 elevated clones and 25 suppressed clones were identified after dot blotting and sequencing analysis 46 clones are long-term elevated genes and 8 of those are

suppressed genes in rat hippocampus identified by Northern blotting. 31 clones are reported in gene bank by other researchers and 23 of them have not been reported after sequencing analysis and blast search

Differential Expression of mRNAs after KA injection

1. Long-term increased genes

2. Transient induced genes

3.Long-Term decreased genes

4. **Two-Phase elevated genes**

5. **Multiple isotype genes**

Figure 1. Dot blots hybridized with subtracted and reverse subtracted cDNA probes, which were l. A. Dot blots hybridized with subtracted cDNA probes, which were made from tester (synthesized from rat hippocampal mRNA 4 month after KA injection) and driver (synthesized from rat hippocampal mRNA 4 month after physiological saline) B. Dot blots hybridized with reverse subtracted cDNA probes, which were made from tester cDNA (synthesized from rat hippocampal mRNA 4 month after physiological saline) and driver cDNA (synthesized from rat hippocampal mRNA 4 month after KA injection).

Figure 2. Non-radioactive Northern blot analysis with cloned cDNA after dot blot. These cDNAs were cloned into T/A vector. The probes were prepared by PCR with DIG dNTP, nested PCR primer 1 and nested PCR primer 2R. The labled inserts were hybridized with different RNA samples (2 ug of total RNA isolated from rat hippocampus). Control. after saline injection. 6 h. 6 hour after KA injection. . 1 d. 1 day after KA injection. . 1 w. 1 week after KA injection. . 2 w. 2 weeks after KA injection. . 3 m. 3 month after KA injectio! n.

Discussion

Neuronal Activity-Assiociated genes :

K1-44 (NPY) and K2-22 (ENK)

Inflammation-Associated Proteins:

K2-17 (Cathepsin D) (13) and K3-244 (cox-2?)

Neurodegeneration-Associated Protein:

K2-33 (Lowe Oculocerebrorenal Syndrome)(14), rare transcripts and K1-17 (B-Amyloid Binding Protein)(15)

Xenobiotic Biotransformation-Associated Proteins:

K1-9 (GST)(16) and K3-144(Selenoprotein P)(17-19)

Phosphatase and Kinase:

K3-205 (MKP-5)(20) and K3-184 (AKAP 220)(21)

Unknown transcripts:

K3-163-K2-33-Dyn; K3-173, 241-K1-9; K3-241-K3-205¡

Implication on Disease prevention and treatment

Conclusion

1. KA-induced hippocampus pathophysiologicl changes caused a differential gene expression which has at least 5 different spatial and tempal patterns after KA treatment.

2. The sequence analysis and gene bank homology search revealed that the differential expressed genes after KA injection may not only related to seizure activity but also related to the long-term processes of neurodegeneration which may resemble the neurodegenerative diseases.

3. Long-term neuronal adaptation to excitatory toxicity involves a comprehensive multigenes activation and suppression process.

4. Systemic examination of all the genes that involve KA- induced neurodegeneration is possible by this methods, thus may shed a light on the molecular mechanism of neurodegenerative disease.

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13.

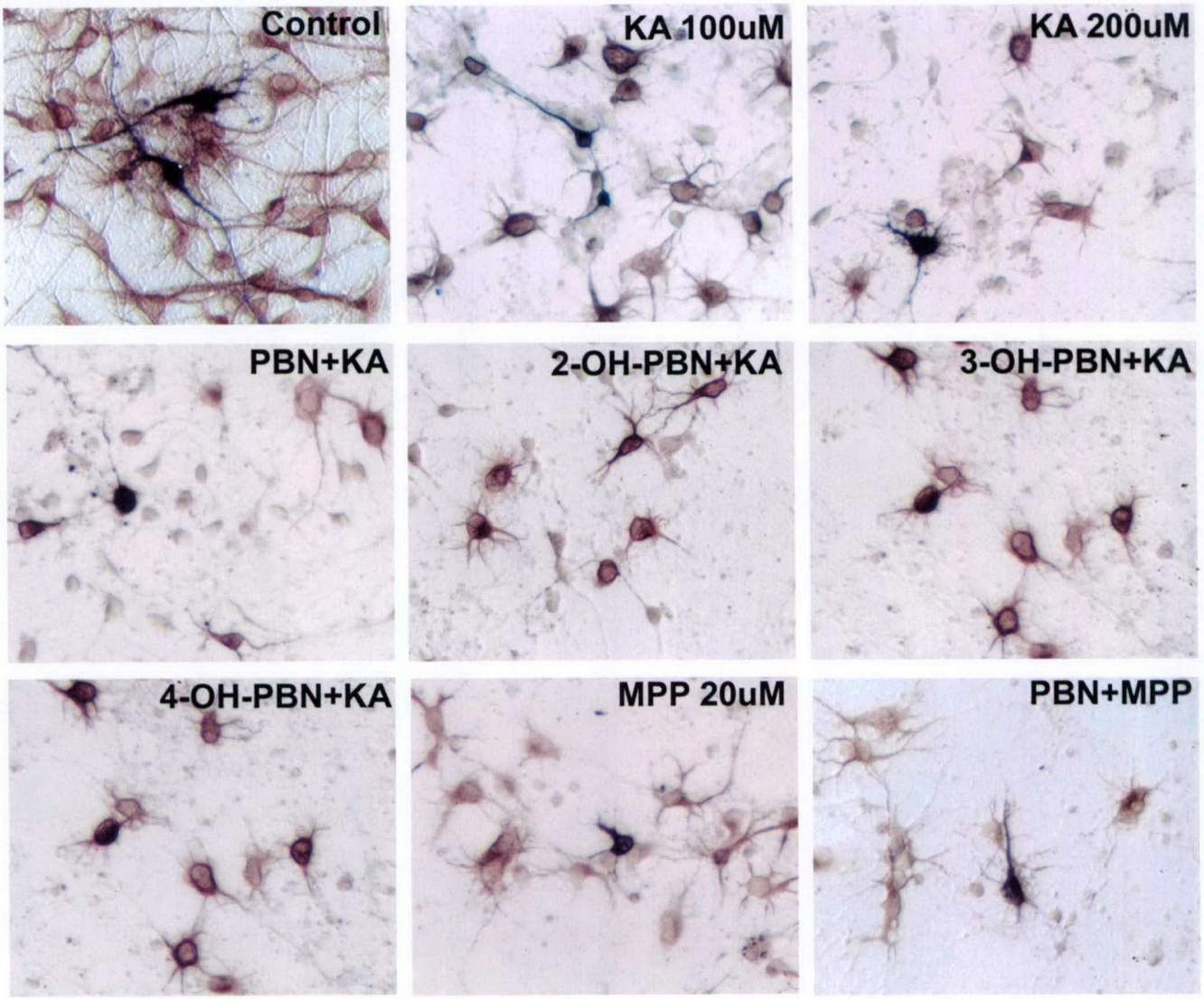


Fig 1. Double labeling immunocytochemical study showed effects of PBN and its analogs (PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN), 4-hydroxy PBN (4-OHPBN) on KA or MPTP -induced neurotoxicity in midbrain neuron-glial mixed cultures. Six days after placement of the cells in a 24 vials culture plate, the cultures were treated with KA or MPP+ (20 micro M) and PBN or its analogs(100 miro M). Three days after treatment, cultures were washed with PBS and fixed with 4% paraformaldehydefor 30 min.Immunocytochemistryfor MAP 2 (brown color) and TH (black color) was performed to show total neuronal number and dopaminergic neurons.

Note: PBN and its analogs fail to protect TH-positive neuron loss but neuronal processes were protected by PBN and its analogs but there was no significant effects between PBN and its derivatives.

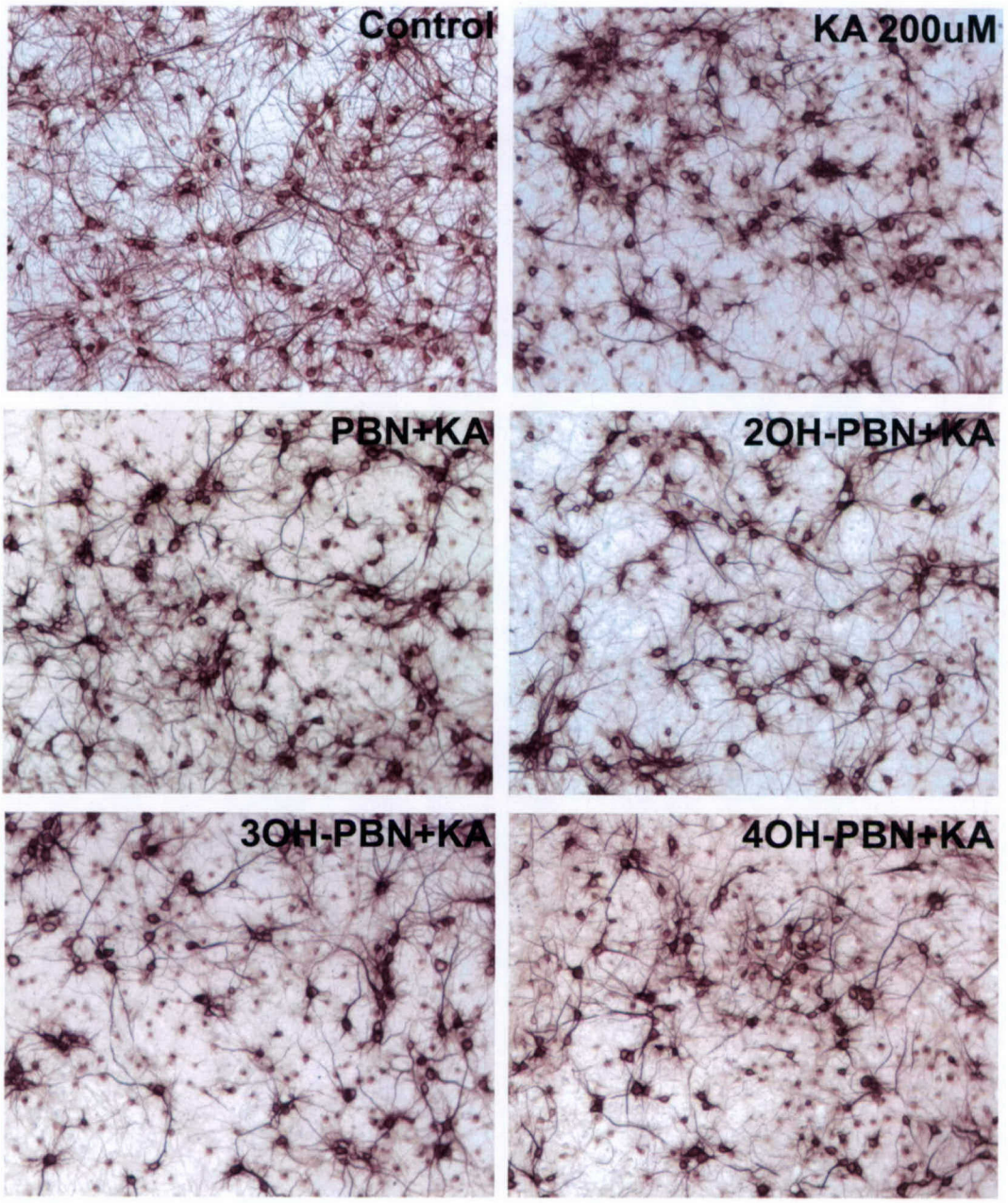


Fig 2. Effects of PBN and its analogs (PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN), 4-hydroxy PBN (4-OHPBN) on Kainic acid-induced neurotoxicity in corticall neuron-glial mixed cultures. Six days after placement of the cells in a 24 vials culture plate, the cultures were treated with of Kainate acid (400 micro M) and PBN or its analogs(100 miro M). Three days after treatment, cultures were washed with PBS and fixed with 4% paraformaldehyde for 30 min.Immunocytochemistry for MAP 2 was performed to show the neurons.
Note: neuronal processes were specially protected by PBN and its analogs but there was no significant effects on cell numbers between PBN and its derivatives.

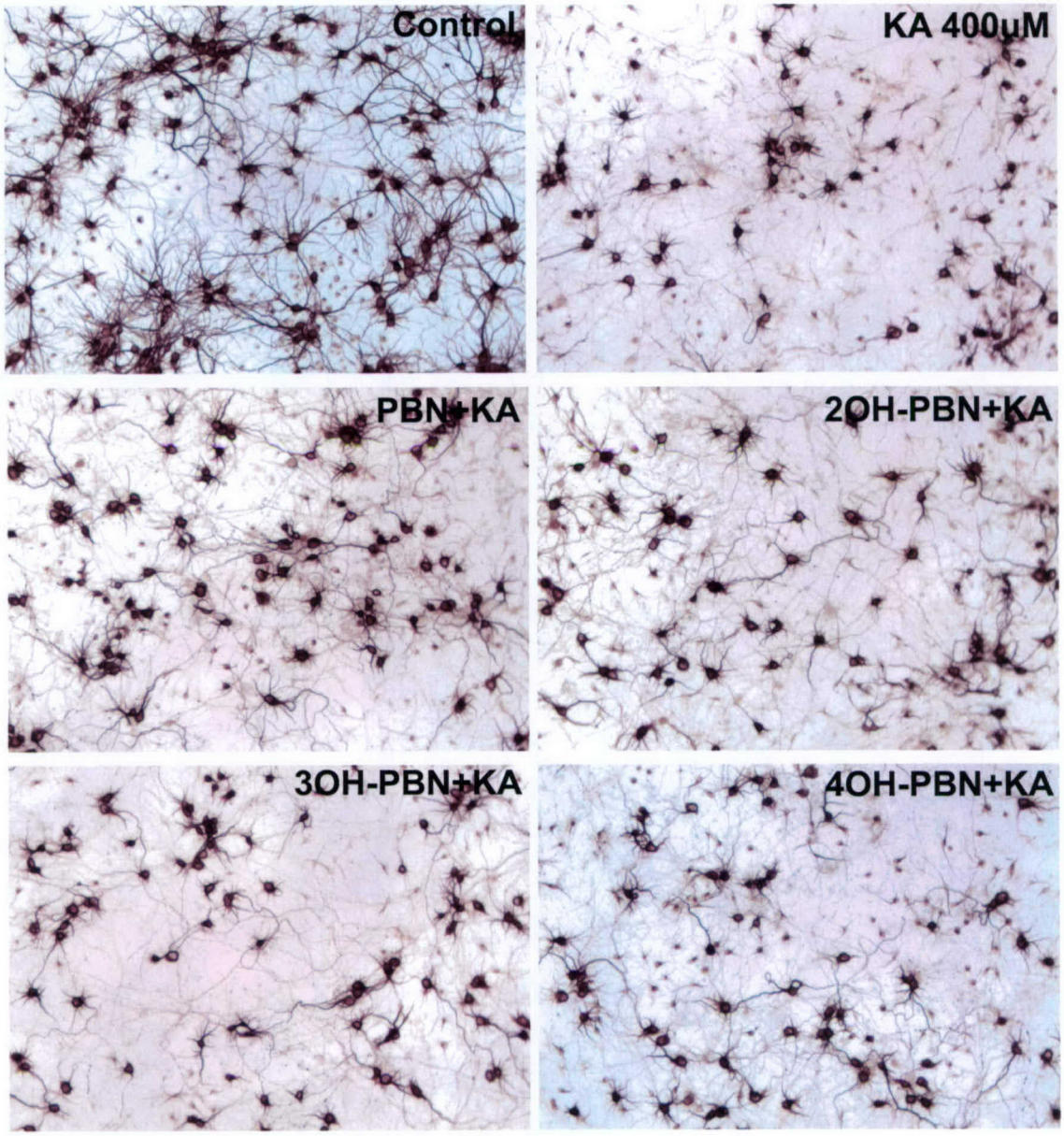


Fig 3. Effects of PBN and its analogs (PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN), 4-hydroxy PBN (4-OHPBN) on Kainic acid-induced neurotoxicity in hippocapal neuron-glial mixed cultures. Six days after placement of the cells in a 24 vials culture plate, the cultures were treated with of Kainate acid (400 micro M) and PBN or its analogs(100 miro M). Three days after treatment, cultures were washed with PBS and fixed with 4% paraformaldehyde for 30 min.Immunocytochemistry for MAP 2 was performed to show the neurons.
Note: both nuronal numbers and neuronal processes were protected by PBN and its analogs Compare with KA treated along but there was no significant effects between PBN and its derivatives.

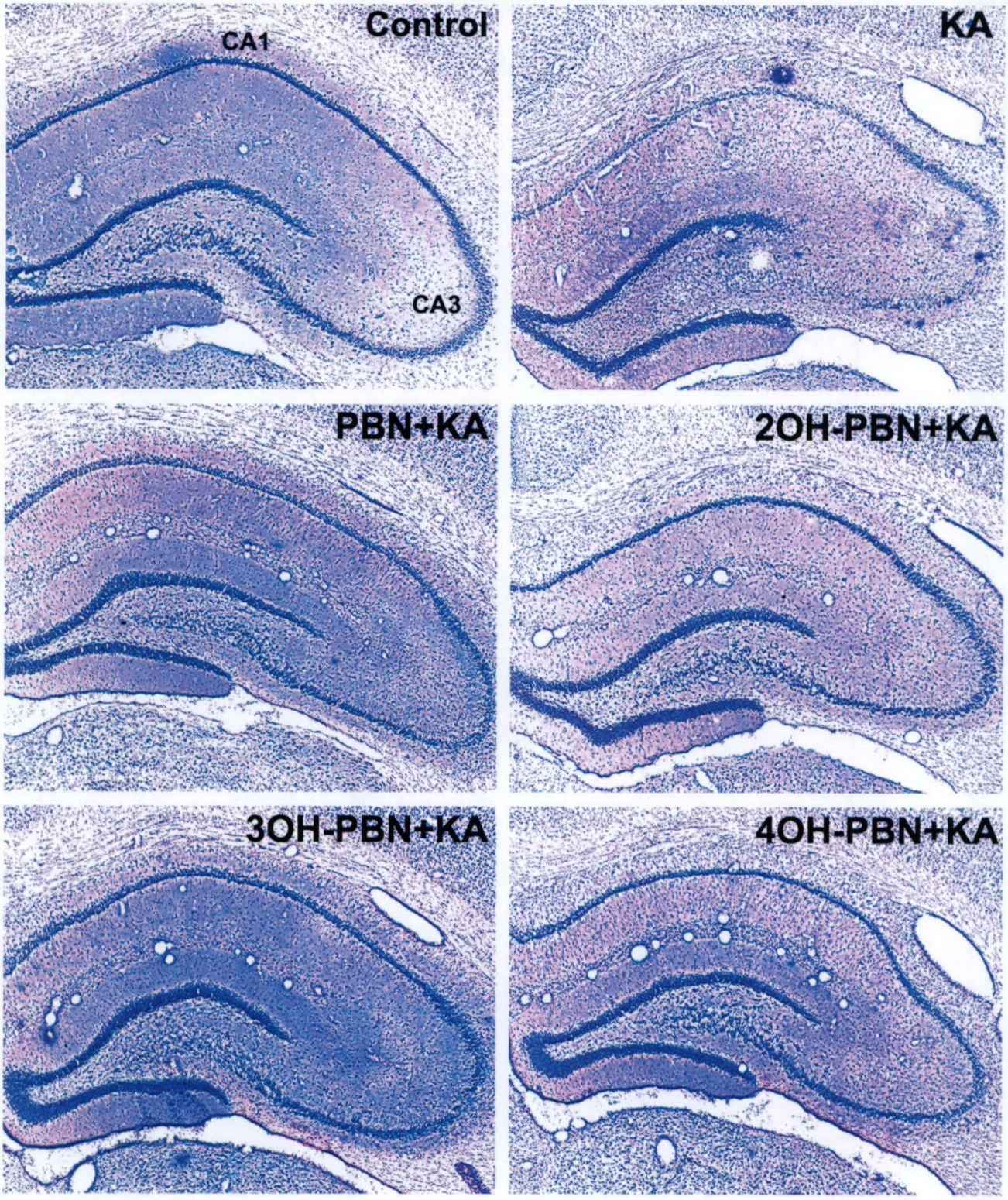


Fig. 4. PBN and its analogs protect hippocampal neurons from KA-induced cell death. Rats were injected with PBN and its analogs (PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN), 4-hydroxy PBN (4-OHPBN) (150 mg/kg, i.p.) 90 minutes after KA (10 mg/kg., i.p.) injection. One week after the injection, animals were processed for histological analysis. Representative photomicrographs showed that there is marked neuronal loss in hippocampus, especially in the CA1 and CA3 areas after KA treatment but treatment with KA and its analogs significantly protected that cell loss. However, PBN analogs did not show additional beneficial effects comparing with KA if not less effective than PBN.

Long-Term Differential Gene Expression in the Rat Hippocampus After Systemic Kainic Acid Injection Revealed by PCR-Selected Substractive Cloning

L. Jin, N. Y. Zheng, M. Zhu, R. Nael and G. Y. Bing

Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104

340.16

ABSTRACT

A single systemic injection of convulsive dose of KA can cause a selective degeneration in hippocampus and result in long-term spontaneously recurrent seizures in rats. It has been reported that many genes were induced short after KA injection. However, A relatively little information is available for long-term gene expression in rat hippocampus after KA injection. In order to examine these long-term differentially expressed genes. We used the suppression subtractive hybridization (SSH) and PCR-select differential screening methods (Clontech, Palo Alto, A) for comprehensive analysis of long-term, differential gene expressions in the hippocampus. The mRNA was isolated from rat hippocampus 4 month after KA (n=3) and saline injection (n=3), cDNA was synthesized from the pooled mRNA, both forward subtracted and reverse subtracted hybridization was performed. Select-PCR was used to amplify the forward and reverse subtractive products. All of the PCR products were cloned into TA cloning vectors (Nitrogen). 432 clones were picked up. After dot blotting and sequencing analysis 76 elevated genes and 25 suppressed genes were identified. Among them, 20 are long-term elevated genes, 11 are transient induced genes, 3 are long-term decreased genes, 8 are appear two-phase elevated, and 8 are appear to be multiple isotype genes in rat hippocampus identified by Northern blotting. The differential expressed genes are likely to be related to seizure activity, oxidative stress, apoptosis and some neurological disorders. At least 15 transcripts are unknown. These results indicated that 1). KA-induced hippocampal pathophysiological changes caused a differential gene expression that is related to neurodegenerative diseases. 2). Long-term neuronal adaptation to excitatory toxicity involves a comprehensive multi-genes activation and suppression process. 3). Systemic examination of all the genes that involve KA-induced neurodegeneration is possible by this methods, thus may shed a light on the molecular mechanism of neurodegenerative disease. This work was supported by the OCAST (HR98-004).

METHODS

Animals and treatment

Adult male Fischer 344 (225–250 g body weight) was used throughout the study. Control animals were injected with physiological saline. Experimental animals were injected with kainic acid (Sigma, St. Louis, MO, and 7.25 mg/kg, S.C.). The animals were rated according to the scale devised by Racine (Racine, 1972) for the initial 4 hours following the KA injection. Only animals with full limbic seizures (forelimb clones with rearing, stage 4) were chosen for further studies.

Suppression subtractive hybridization (SSH)

Tester and driver preparation tester and driver ds cDNA were synthesized from 2 μg of two different poly (A) RNA from hippocampus 4 month after kainic acid (n = 3) and saline (n = 3). KA-induced samples were used as a tester and control samples were used as a driver for forward subtraction. For reverse subtraction, control samples were used as tester and KA-induced samples were used as driver. First- and second -strand cDNA synthesis and blunt-ending of DNA creation were carried out according to the manufacturer's protocol. In brief, the resulting cDNA pellet was dissolved in 10 μl of deionized water and digested by Rsa I in a 50-μl reaction mixture containing 15 units of enzyme at 37°C for 1.5 hr. Digested tester cDNA (1 μl) was diluted in 5 μl of water. The diluted tester cDNA (2 μl) was then ligated to 2 μl of adapter 1 and adapter 2R (10 μM) in separate ligation reactions in a total volume of 10 μl at 16°C overnight, using 0.5 units of T4 DNA ligase, 1 μl of 0.2 M EDTA was added and the samples were heated at 70°C for 5 min to inactivate the ligase and stored at –20°C. Forward and reverse subtractive hybridization were performed using the PCR-Selected cDNA Subtraction Kit (Clontech) according to the manufacturer's protocol. The first and second hybridization were performed on a Perkin-Elmer 9600 thermalcycler.

PCR amplification and T/A cloning

The primary PCR was conducted in 25 μl. It contained 1 μl of diluted, subtracted cDNA, 1μl of PCR primer 1 (10 μM) and 23 μl of master mixture prepared using the Advantage cDNA PCR kit (CLONTECH). PCR was performed with following parameters: 75° C for 5 min, 94° C, 25 sec, 27 cycles at (94°C for 10 sec, 66°C, 30 sec; 72°C for 1.5 min). The amplified products were diluted 10-fold in deionized water. The second PCR was conducted in 25 μl. It contained 1 μl of diluted primary PCR product, 1 μl of Nested PCR primer 1 (10 μM), 1 μl of Nested PCR primer 2R (10 μM). PCR conditions were as follows: 12 cycles at 94°C for 10 sec, 68.5°C, 30 sec, 72°C for 1.5 min.

Products from the secondary PCR were inserted into pCR II using a T/A cloning kit (Invitrogen) according to the manufacturer's protocol. All of positive clones were picked up.

Dot blot analysis

Plasmid DNA mini-preparation of 432 clones were performed by QIAprep Spin Miniprep Kit (QIAGEN) and diluted into 100 ng/μl. 1 μl of plasmid DNA (100 ng) was dropped on nylon membranes for screening with forward and reverse subtracted probe. Random primer labeling of subtracted probe and reverse subtracted probe and hybridizations for dot blot analysis were performed using PCR-Select Differential Screening Kit (CLONTECH).

Northern blot analysis

Probes preparation A non-radioactive Northern blot analysis was used to analyze every positive clones after dot blot analysis. All of cDNA probes were prepared by PCR with DIG-labeling dNTP mixture (Boehringer Mannheim, Indianapolis, IN). The PCR was conducted in 25 μl. It contained 1 μl of diluted plasmid DNA (100 ng/μl), 1 μl of nested PCR primer 1 (10 μM) and 1 μl of nested PCR primer 2R (10 μM), and 22 μl of PCR master mixture prepared using the PCR kit (Boehringer Mannheim). PCR was performed with the following parameters: 95°C for 2 min, 94°C 30 sec, 68.5°C, 30 sec, 72°C for 1.5 min for 32 cycles and final extension at 72°C for 7 min. The amplified products were purified by G50 column (Amersham Pharmacia, Inc).

Total RNA was isolated from immediately frozen hippocampal tissues (control, 6 hr, 1 d, 1 w, 2 w and 3 m after KA injection) according to the Tri Reagent protocol (Molecular Research Center, Cincinnati, OH) and electrophoresed through 1.2% agare-formaldehyde gels (1 x MOPS and 6% formaldehyde). After transfer onto nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) with 10 x SSC, the filters were baked at 80°C under vacuum for 1 hr. The membranes were hybridized with the DIG labeled probes in high SDS hybridization buffer (7% SDS, 50% formamide, 5 x SSC, 2% Blocking Reagent, 50 mM sodium-phosphate, pH 7.0, and 0.1% N-lauroylsarcosine) overnight at 50°C and washed in solution containing 2 x SSC and 0.1% SDS at room temperature and followed by another two washes with 0.1 x SSC and 0.1% SDS for 15 min at 68°C. The membranes were incubated for 30 min in antibody solution (75 mU/ml. anti-DIG-AP conjugate, 1% blocking reagent in 0.1 M maleic acid) after 1 h blocking (2% blocking reagent, 0.1 M maleic acid and 0.15 M NaCl) at room temperature. The membranes, then, were washed twice for 15 min in washing buffer (0.1 M maleic acid buffer plus 0.3% Tween 20) and equilibrated for 5 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). The membranes were incubated for 5 min at room temperature in a development folder with CSPD solution (Boehringer Mannheim) for chime-luminescent reaction and then incubated at 37°C for 15 min. Filters were exposed to x-ray film.

Sequence analysis

DNA sequencing was performed by OMRF sequence facility. Nucleic acid homology searches were performed using the BLAST program through National Center for Biotechnology Information (National Institutes of Health, Bethesda).

Differential, Long-term Expressed Genes Induced by Kainic Acid Screening with cDNA Probes

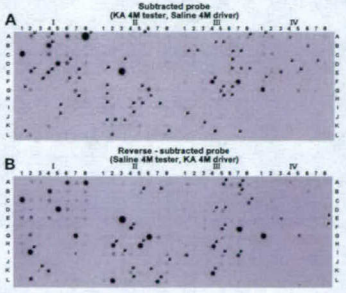
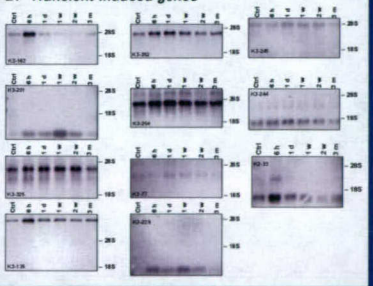


Figure 1. Dot blots hybridized with subtracted and reverse subtracted cDNA probes. A. Dot blots hybridized with cDNA probes made from forward-subtracted cDNA (KA 4 m as tester, and saline 4 m as driver). Arrows indicate positive clones compare from the control sample. B. Dot blots hybridized with reverse-subtracted cDNA probes (saline 4 m as tester, KA 4 m as driver). Arrows indicate positive clones compare from the KA treated hippocampal samples.

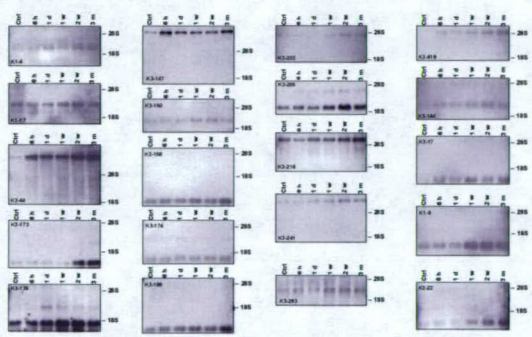
B. Transient-induced genes



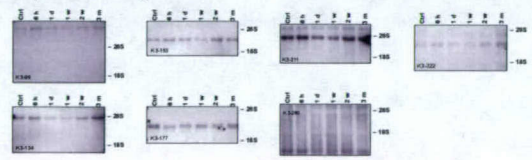
C. Long-term decreased genes

Differential Expression of mRNAs after KA Injection

A. Long-term increased genes



D. Two-phase elevated genes



E. Multiple isotype genes

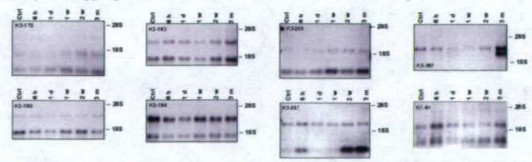


Figure 2. Non-radioactive Northern blot analysis with positive cloned cDNA probes selected from dot blot analysis. The probes were prepared by PCR with DIG-labeled dNTP using nested PCR primer 1 and nested PCR primer 2R as primer pair. The labeled inserts were hybridized with RNA samples from the rat hippocampus after KA injection (2 μg of total RNA isolated from rat hippocampus). The lanes indicate: con after saline injection; 6 h. 6 hour after KA injection; 1 d. 1 day after KA injection; 1 w. 1 week after KA injection; 2 w. 2 weeks after KA injection; 3 m. 3 month after KA injection.

Long-Term Induced Genes and Their Possible Function

Neuronal activity-associated proteins		
Clones	Products	Gene Bank Acc. #
K1-44	Neuro Peptide Y	
K1-24	Neuron activity-regulated protein	S82649

Inflammation-associated proteins		
Clones	Products	Gene Bank Acc. #
K1-4	IFN-B2	
K1-54	Infection response serine protease	AF000675
K3-22	Plasminogen	AF029692
K2-17	Aspartic Protease	X54467
K3-244	Cox-2	S67722

Apoptosis-associated proteins		
Clones	Products	Gene Bank Acc. #
K2-10	Synuclein 1	AF007758
K1-61	Proapoptotic Protein	AF033165

Ubiquitin-Associated Protein		
Clones	Products	Gene Bank Acc. #
K1-12	Ubiquitin-protein Ligase	AF018071
K2-28	Polyubiquitin Ligase	D16554

Xenobiotic Biotransformation-Associated Proteins		
Clones	Products	Gene Bank Acc. #
K1-93	Carbonic anhydrase II	X58294
K1-9	GST Yc subunit	X78845
K3-144	Selenoprotein P	D25221

Neurodegeneration-Associated Protein		
Clones	Products	Gene Bank Acc. #
K1-17	B-amyloid binding protein	M88162
K2-33	Lowe oculocerebrorenal syndrome mRNA	U57627

Oncogene and Transcription Factors		
Clones	Products	Gene Bank Acc. #
K2-15	Fra	M27266
K3-77	Lac	U58203

Metabolic-Associated proteins		
Clones	Products	Gene Bank Acc. #
K3-153	Pyruvate dehydrogenase E1 alpha form 1	AF120584
K3-170	CDK 10B	Y17328.1
K3-217	Matrilin-2 precursor	U65262
K3-174	Ma-crystalin	AF039391

DNA binding proteins		
Clones	Products	Gene Bank Acc. #
K3-201	Rat enhancer factor 1 subunit A	M57299
K3-218	Mouse type II DNA topoisomerase	D38046

Phosphatase and Kinase		
Clones	Products	Gene Bank Acc. #
K3-205	Dual specificity phosphatase MKP-5	AF046907

Others		
Clones	Products	Gene Bank Acc. #
K3-150	Myelin protein zero-like protein MPZL1	AF092425
K3-139	Rat mitochondrial genome	X14850
K3-200	Human C3f	U72515
K3-181	Matrilin-2 precursor	U65262
16 Clones	Unknown K1-44, K1-99, K3-147, K3-163, K3-164, K3-173, K3-177, K3-198, K3-203 K3-229, K3-241, K3-256, K3-322, K3-352, K3-367, K3-419	

Note: All 92 cDNA were partially sequenced from both ends and subjected to BLAST II searches for sequence homologies. The clones shown shared homologies with the genes indicated the numbers designation the clones represent their identification numbers during screening.

CONCLUSION

1. KA-induced hippocampus pathophysiological changes caused a differential gene expression that has at least 5 different spatial and tempal patterns after KA treatment.
2. The sequence analysis and gene bank homology search revealed that the differential expressed genes after KA injection may not only be related to seizure activity but also be related to the long-term processes of neurodegeneration that may resemble the neurodegenerative diseases.
3. Long-term neuronal adaptation to excitatory toxicity involves a comprehensive multigenes activation and suppression process.
4. Systemic examination of all the genes that involve KA-induced neurodegeneration is possible by this method, thus may shed a light on the molecular mechanism of neurodegenerative disease.

APPENDIX 7

CURRICULUM VITAE

Guoying Bing, M.D., Ph.D.

Associate Professor, Department of Anatomy & Neurobiology
University of Kentucky, School of Medicine

PERSONAL DATA

310 Davis Mills Building
Department of Anatomy & Neurobiology
University of Kentucky School of Medicine
Lexington, KY 40536-0298
E-mail: gbing@uky.edu
Phone: (859) 323-9708
Fax: (859) 257-3625

543 Lake Tower Dr. Unite 133
Lexington, KY 40502
(859) 335-0116

EDUCATION

Doctorate of Philosophy, Anatomy and Neurobiology October, 1988
Advisor: Dr. Don Gash
University of Rochester, Rochester, NY

Doctor of Medicine (equivalent) September, 1977
Jilin Medical College, Jilin, China

PROFESSIONAL EXPERIENCE AND ACADEMIC APPOINTMENTS

2000-present **Associate Professor**, Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Lexington, KY

1991-present **Visiting Professor**, Beijing Institute of Neuroscience, Beijing, China

1997-2000 **Adjunct Professor**, Department of Cell biology, University of Oklahoma, Health Science Center.

1997-2000 **Assistant Member,** Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation

1993-1997 **Senior Fellow,** National Institute of Environmental Health Science

1991-1993 **Assistant Professor,** New York University Medical Center, Department of Psychiatry

1989-1991 **Postdoctoral Fellow, NYU Medical Center, Department of Psychiatry**

RESEARCH INTERESTS

Major research interests focus on the molecular and cellular mechanisms underlying the neurodegenerative diseases. Currently, there are three research projects are actively carried on in the laboratory: 1) the role of neuro-inflammatory processes in the etiology and pathophysiology of Parkinson's disease. 2). Long-term neuronal adaptation to excitatory neurotoxicity---Molecular cloning long-term, differential expressed genes in the hippocampus after KA-induced epileptic seizures; and 3). The role of xenobiotic metabolite enzymes in the central nervous system---The effects of environmental and endogenous toxins on neurodegeneration.

HONORS & AWARDS

1981-1982	Excellent in Teaching Award, Jilin Medical College
1982-1983	Fellowship for Chinese Graduate Student Study in USA, Chinese Government
1983-1984	Fellowship from Educational Commission for Foreign Medical Graduate, USA
1984-1985	Teaching Assistantship, University of Rochester
1985-1986	Research Fellowship, Society of Physiology

PATENT AWARDED

1. *Methods of using alpha 2 agonist for the treatment of neurodegenerative diseases.*
   Inventor: **Guoying Bing,** and Eric Stone. 1993.
   USA Patent Number: **5,252,816**

2. *A method for preventing and treating the degeneration of neurons.*
   Inventor, **Guoying Bing**, Naiying Zheng, Lei Jin, and Xin Lu, 1999
   USA Patent Number: **60,114,214.**

PATENT PENDING:

A method for preventing and treating the neurodegenerative diseases.
Inventor, **Guoying Bing** and Jordan Tang, 2000

GENE BANK SUBMISSION

1. *Molecular cloning of a new gene for Fos-related antigen (FRA) in the kainic acid treated hippocampus.*

Submitted by: **Guoying Bing,** Qiping Qi, Zhihuei Feng and Jau-Shyong Hong.

Accession Number: **U34932**

2. *Rat striatum genomic DNA of c-fos intron 3 and flanking cDNA sequence.*

Submitted by:, Zhihuei Feng, Kong L, Qiping Qi, No, S. Tiao, N., and **Guoying Bing**

Accession Number: **341647**

RESEARCH GRANTS

(*Principal Investigator unless otherwise noted*)

Active Supports

Guoying Bing, (P.I.) **12/1/99 - 11/ 31/04**
NIH R01 Grant-NS39345
Project Title: Microglia Activation Induces Parkinsonism in rats

The major goal of this grant is to develop a new animal model that may be used in the development of novel therapeutic treatment for Parkinson's disease and other neurodegenerative diseases.

Total Amount: **$830,000**

Guoying Bing, (P.I.) **9/17/01 - 9/16/06**
NIH/NIMH F 30 Grant-MH65055
Project Title: Dynorphin in Age-related Impairment of Learning and Memory

The major goal of this grant is to investigate the mechanisms underlying age-dependent changes in neuronal or synaptic function and the potential role of dynorphin in mediating these changes.

Total Amount: **$117,263**

Guoying Bing, (P.I.) **7/1/03 – 6/30/08**
NIH R01 Grant- NS044157
Project Title: COX-2 Deficient Mice are Resistant to MPTP Neurotoxicity

The goals of this study are to elucidate the changes in inflammatory processing affected by COX-2 deficiency, to explore the etiology and molecular mechanisms underlying Parkinsonian symptoms in the experimental MPTP model, and to develop novel therapeutic treatments for PD and other neurodegenerative diseases.

Total Amount: **$1,425,000**

Guoying Bing, (P.I.) **11/1/03**
UK Microarray Pilot Program
Project Title: Microarray Detection of Patterns of Aging-Associated Genes Affected by

Endogenous Dynorphin

The goal of this study is to investigate the mechanisms underlying age-dependent changes in neuronal or synaptic function and the potential role of dynorphin in mediating these changes; we propose to examine the effects of aging on memory in knockout mice lacking the coding exons for the precursor prodynorphin.

Total Amount: **$5,000**

Past Supports

Guoying Bing, (P.I.) **10/1/99 –9/30/03**
US Army Medical Research Grant
Project Title: Protective Mechanisms of Nitrone Antioxidants in Kainic Acid Induced Neurodegeneration
Total Amount: **$540,000**

Guoying Bing, (P.I.) **7/1/98 –6/30/01**
Principal Investigator for OCAST
Project Title: KA-induced gene expression in the hippocampus.
Total Amount: $150,000

Guoying Bing, (P.I.) **5/1/02**
UK Microarray Pilot Program
Project Title: Differential Gene Expression in Hippocampus of Dynorphin Knockout mice.
Total Amount: **$5,000**

Pending:

Guoying Bing, (P.I.) **4/1/04—3/31/06**
Michael J. Fox Foundation
Project Title: COX-2 regulation of neuroinflammation in Parkinson's disease
Total Amount: **$200,000**

Guoying Bing, (P.I.) **6/1/04—5/31/06**
Alzheimer Health Assistance Foundation
Project Title: **Role of xenobiotic metabolism in Alzheimer's disease**
Total Amount: **$300,000**

TEACHING EXPERIENCE

University of Kentucky College of Medicine, Lexington, KY

2003 **ANA 534;** Human Gross Anatomy; *Lecturer & Lab Instructor*

2002 **ANA 534;** Human Gross Anatomy; *Lecturer & Lab Instructor*
2001 **ANA 534;** Human Gross Anatomy; *Lecturer & Lab Instructor*

Oklahoma University, Oklahoma City, OK

1999 **Neuroscience Methods,** *Lecturer*

University of Rochester, Rochester NY

1987 **ANA 531;** System Neuroscience; *Lab Instructor*

Jilin Medical College, Jilin, China

1981 **Human Gross Anatomy**; *Lecturer & Lab Instructor*

GRADUATE STUDENTS

Thesis Advisor

Current MD., Ph.D. or Ph.D. candidates

Xuan Nguyen,	MD. Ph.D. candidate	1999-
Rattanavijit Vijitruth	Ph.D. Candidate	2001-
Bin Xing	Ph.D. Candidate	2003-

Supervisor/Advisor

Undergraduate or graduate students

Raha Neal,	MD., Ph.D. Student 1999-2000
Candice Turner,	Bio 395undergraduate student 2001
Monica	Bio 395undergraduate student 2001

Current Postdoctoral Fellows

Mei Liu,	**MD,** 2001-
Deanna McCullers,	**Ph.D.** 2002-

Past Postdoctoral Fellows	Current Position
Yi Zhang, M.D. 1991-1993	Editorial assistant, Society of Physiology
Lei Jin, Ph.D. 1995-1999	Professor, Peking Union Medical University
Lingling Zhao, M.D., 1999-2001	Professor, Hunan Medical University
Toyoko Arimoto, Ph.D., 1999-2002	Staff Fellow, NIH
Anyang Sun, Ph. D., 1999-2002	Research Associate, Harvard Medical School

Professional trainees

Hyoung-Chun Kim, Ph.D., 1994-1996	Professor, Kangwon National University, Korea
Qiping Qi, Ph.D., 1995-1996	Director, Institute of National Academy of Preventive Medicine, China
Xianxi Liu, M.D., 1997	Professor, Shandong Medical University
Yahui Qi, M.D., 1998	Associate Professor, Capital University of Medical Science, Beijing

INVITED LECTURES

1. "Cografts of Adrenal Medullary cells with Neurotrophic producing Cells" Veterans Administration Hospital, Bedford, MA 01730, 1987.

2. "Transplantation of Adrenal Medullary, Carotid Body Glomus Cells with C6 Glioma Cells into the rat brain" Department of Anatomy, Boston University School of Medicine Boston, MA 02118, 1987.

3. "Neurotransplantation: Present and Future" Capital Institute of Medicine, Beijing, China, 1988.

4. 4, "Animal models used in neurotransplantation" New York University, Medical Center, New York, NY 10016, 1991

5. "Locus coeruleus lesions potentiate neurotoxic effects of MPTP in dopaminergic neurons of the substantia nigra" NIEHS/NIH, Research Triangle Park, NC 27709, 1993.

6. "Long-term genomic effects of administration of kainic acid in the rat brain" Centaur Pharmaceutical Inc., Sunnyvale, CA 94086, 1995.

7. "The regulation of the opioid peptide by seizure activities ----Role of long-term AP-1 transcription factors". Oklahoma Medical Science Foundation, City, OK 73104, December, 1996.

8. "The regulation of the opioid peptide by seizure activities ----Role of long-term AP-1 transcription factors". University of Oklahoma, Oklahoma Center for Neuroscience, Oklahoma City, OK 73104, January, 1997.

9. Capital University of Medical Science, Beijing, China. March, 1997.

10. "Microglia mediated neuronal death----A new animal model for Parkinson's disease" Kangwon National University, Korea. April, 1997.

11. "Long-term gene induction in the hippocampus by excitatory amino acid----A PCR-selected subtractive cloning methods" Shanghai Medical University, Shanghai, China. September, 1998.

12. "Current trends in research for neurodegenerative diseases" Shandong Medical University, Shandong, China. September,1998

13. "Microglia mediated neuronal death----A new animal model for Parkinson's disease" National Institute of Radiation Research, Ciba, Japan. June, 1999.

14. "Microglia mediated neuronal death----A new animal model for Parkinson's disease" Yamagata University, School of Medicine, Yamagata, Japan, June, 1999

15. "Recent development of Molecular biological techniques in Neuroscience Research". Capital University of Medical Science, Beijing, China. March, July, 1999.

16. "Microglia mediated neuronal death----A new animal model for Parkinson's disease". University of Missouri-Kansas City, School of Pharmacy, Kansas City, MS, August, 1999.

17. "Direct Visualization of Neurofibrillary Pathology in Alzheimer's Disease" Kangwon National University, Korea. June, 2001.

18. "Gene therapy in neurological disease". Capital University of Medical Science, Beijing, China. June, 2001.

19. "Microglia-activation Induced Parkinsonism" Capital University of Medical Science, Beijing, China. June, 2002

20. "A new animal model for Parkinson's disease: microglial activation" 4th Ilsong international Symposium on Aging and Neurodegenerative Diseases in Seoul, Korea. December 2002

21. "Inflammation induced neurodegeneration" Xiangya Medical University, Hunan, China. September, 2003

22. "A single intrapallidal LPS injection induces Parkinsonism in rats: A new animal model for Parkinson's disease" International symposium on Parkinson's disease, Beijing, China, September 2004.

23. "Inflammation and Parkinson's disease" College of Pharmacy, Kangwon National University, Korea. September, 2004.

24. "Animal Model of Parkinson's disease" Capital University of Medical Science, Beijing, China. June, 2004

COMMITTEE & SERVICE

2000-present University of Kentucky Medical Research Advisory Committee
2000-Present Graduate Faculty Committee, University of Kentucky, Medical Center
1997-2000 Graduate Faculty Committee, University of Oklahoma Health Sciences Center
1997-2000 Fleming Scholar Select Committee, Oklahoma Medical Research Foundation

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REVIEW ACTIVITY:

1997-present Ad Hoc reviewer, Alzheimer's Association.

Brain Research; Brain Research Protocol

Neuroscience; Neuroscience Letter

Neurodegeneration J. Neuroscience

Free Radical Biology and Medicine

PUBLICATIONS:

1. Gash, D.M., Notter, M.F.D., **Bing, G.**, Kordower, J.F. **(1986)** Neural implants into primates: Studies employing differentiated neuroblastoma cells. *Cell and Tissue Transplantation into the Adult Brain* pp. 37.

2. Hansen, J.T., **Bing, G.**, Notter, M.F.D., Gash, D.M. **(1987)** Ultrastructure of striatal implants of adult adrenal chromaffin cells in unilateral 6-OHDA lesioned rats. *Anat. Rec.* 218:56A.

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