INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

Université d'Ottawa · University of Ottawa

IMPLICATION OF ANTI-APOPTOTIC GENES IN NEURONAL DEATH FOLLOWING FOCAL CEREBRAL ISCHEMIA IN RATS

By

Zhigao Huang, M.D.

Department of Anatomy and Neurobiology

Submitted in partial fulfilment of

the requirements for the degree of

Doctor of Philosophy

Faculty of Medicine

School of Graduate Studies

University of Ottawa

Ottawa, Canada

September 2001

© Zhigao Huang 2001

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-72813-7

Canada

UNIVERSITY OF OTTAWA

SCHOOL OF GRADUATE STUDIES

FACULTY OF MEDICINE

CERTIFICATE OF EXAMINATION

Chief Advisors	Examining Board
_____	_____
Advisory Committee	_____
_____	_____
_____	

Thesis by

Zhigao Huang

Entitled

Implication of anti-apoptotic genes in neuronal death following cerebral ischemia in rats

is accepted in partial fulfilment of the requirements of the degree of

Doctor of Philosophy

Date _____ Chair of Examining Board _____

DEDICATION

To my wife Yinan, who supported my studies,

To my grandfather, my parents, who have taught me

to be a responsible, hard working man, and

To my beloved daughter Solana, who inspired me,

and

To all my mentors, who have taught me.

ACKNOWLEDGEMENTS

I would first like to thank my supervisor, Dr. George S. Robertson, for his support throughout the project and completion of the thesis.

Special thanks go to my committee chairman, Dr. William Staines, for his continuous support, guidance, and friendship, which has helped me through some of the most difficult times, and for the encouragement he has so generously given me in the course of the completion of my thesis.

I would like to thank Drs. Dan C. McIntyre and Alastair Buchan for giving me the opportunity to conduct scientific research in University of Ottawa and Carlton University.

I would also like to thank Dr. J. P. MacManus, who helped me through the entire research project and encouraged me to complete the program at University of Ottawa; for that, I will never forget.

I would also like to thank Dr. Bin Hu for sitting on my advisory committee.

I am grateful to Drs. Mary-Ellen Kelly, S.T. Hou, C. Tompson, Y. Tu and D. G. Xu for their expertise and scientific inputs to my studies.

My appreciation also extends to Mrs. M. St-Jean and Y. X. Zhu for their technical assistance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Ac-DEVD-CHO Ac-Asp-Glu-Val-Asp-al

Ac-YVAD-cmk; Ac-Tyr-Val-Ala-Asp-cmk

AD; afterdischarge

AMPA; α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid

AP-1; activator protein-1

Apaf-1; apoptotic protease activating factor-1

ATP; adenosine triphosphate

ATPase; adenosine triphosphatase

Bad; Bcl-2 –associated death promoter homology

Bax; Bcl-2-associated partner

Bcl; B cell leukemia

BIR; baculoviral inhibitor of apoptosis protein repeat domains

BIRPs; BIR-containing proteins

bp; base pair

CAD; caspase-3 activated DNaseI

CalM; calmodulin

cAMP; cyclic adenosine monophosphate

CBF; cerebral blood flow

CCA; common carotid artery

Cdk; cyclin-dependent kinase

cDNA; complementary deoxyribonucleic acid

C. elegans; Caenorhabditis elegans

Ced; c. elegans death gene

c-fos; cellular FBJ (Finkel, Biskis and Jinkins) murine osteosarcoma virus oncogenes

cGMP; guanosine 3',5'-cyclic monophosphate

c-jun; cellular *ju-nana* oncogene

CNS; central nervous system

CpIAP; Cydia pomonella granulosis virus IAP

Cpm; counts per minute

CrmA; cytokine response modifier

CSD; cortical spreading depression

Cyto c; cytochrome C

DAB; diaminobenzidin

DAG; diacylglycerol

dATP; deoxyadenosine triphosphate

DNA; deoxyribonucleic acid

dNTP; deoxynucleotide triphosphate

Dpm; disintegrations per unit time

DTT; dithiotreitol

EAA; excitatory amino acid

EDTA; ethylene-diamine tetraacetic acid

ER; endoplasmic reticulum

FADD; Fas activated death domain

FasL; Fas ligand

FOS; FBJ murine osteosarcoma virus oncogene protein

GABA; gamma-amminobutyric acid

Gadd45; growth arrest and DNA damage inducible gene45

GluR; glutamate receptor

GSH; glutathione

HCl; hydrogen cloride

HO2.; perhydroxyl radical

H2O2; hydrogen peroxide

IAP; inhibitor of apoptosis protein

ICE; interleukin converting enzyme

Ich; ice and ced-3 homologue

IEG; immediate early gene

IEGs; immediate early genes

IP3; inositol-1,4,5-triphosphate

ISSH; *in situ* hybridization histochemistry

JUN; *ju-nana* oncogene protein

KCl; potassium cloride

kD; kilodalton

LTP; long-term potentiation

MCA; middle cerebral artery

MCA-o; middle cerebral artery occlusion

MCID; microcomputer-based image display system

MD; transmembrane domain

Mdm2; murine double minute 2

MGlu; metabotropic glutamate

MRI; magnetic resonance image

MgCl; magnesium cloride

MORT1; mediator of receptor-induced toxicity

mRNA; messenger ribonucleic acid

NaCl; sodium cloride

NAIP; neuronal apoptosis inhibitory protein

Nedd; neuronal embryonic development and differentiation

NeuN; Neuronal Nuclei

NGF; nerve growth factor

NMDA; N-methyl-D-aspartate

NO; nitric oxide

NO2; nitrogen dioxide

NOS; nitric oxide synthase

OD; optical density

O2.-; superoxide

.OH; hydroxyl

ONOO-; peroxynitrite

OpIAP, orgyia pseudotsugata nuclear polyhedrosis virus IAP

ORFs, open reading frames

PARP; poly ADP-ribose polymerase

PBS; phosphate buffered saline

PCD; programmed cell death

PCNA; proliferating cell nuclear antigen

PCR; polymer chain reaction

PIP_2; phosphatidylinositol-4,5-bisphosphate

PKC; protein kinase C

PT; permeability transition

Rb; retinoblastoma tumor suppressor protein

rCBF; regional cerebral blood flow

RNA; ribonucleic acid

RNase; ribonuclease

ROS; reactive oxygen species

RP; reperfusion

RT-PCR; reverse transcript polymer chain reaction

SE; status epilepticus

SHR; spontaneously hypertensive rat

SMA; spinal muscular atrophy

SND; selective neuronal death

SOD; superoxide dismutase

SSC; standard saline citrate

TBS; Tris-HCl-buffered saline

TNF; tumor necrosis factor

TRAF; tumor necrosis factor receptor-associated factor

TUNEL; terminal deoxytransferase-mediated dUTP-nick end labeling

VOCC; voltage operated calcium channel

XIAP; X-linked inhibitor-of-apoptosis protein

ZVAD-fmk; Z-Val-Ala-Asp-fluoromethylketone

ABSTRACT

Accumulating biochemical and morphological evidence suggests that apoptosis contributes to neuronal cell death following cerebral ischemia. Recent research which has examined changes in expression of proapoptotic proteins has further strengthened the important role of apoptosis in ischemic cell death. In this thesis I first addressed the role of apoptosis in ischemic death by examining p53, which is itself a complex multifunctional tumor suppresser gene, following transient focal ischemia. Of particular note were the alterations of the anti-apoptotic gene, *naip*, that were observed under stress conditions. The anatomical distribution of *naip* expression and neuronal survival following middle cerebral artery occlusion (MCA-o) were closely examined.

In experiment I, SHR rats were subjected to 90 minute MCA-o followed by 22.5 hr reperfusion (RP) and compared with sham operated controls. Animals were then sacrificed at 1, 4, 8, 12 and 24 hr of RP (n=3-4). Fresh frozen or fixed brain tissue sectioned for ISHH and immunohistochemical detection of p53 expression. Fresh frozen tissue was also dissected from the ischemic penumbra at each time point for mRNA extraction and RT-PCR to confirm p53 mRNA expression. In experiment II, sections obtained from fresh frozen or fixed brain tissue of long-Evans hooded rats (n=3-4) that had been subjected to hippocampal kindling were used for ISHH or immunohistochemistry for *naip* expression. *naip* expression was examined 3h, 1, 7 or 21d after the last episode of kindling. Neuronal protection against cerebral ischemia by hippocampal kindling was assessed in experiment III. 21 days after the last kindling treatment, kindled (n=8) and operated control animals (n=6) were subjected to 90 min of transient MCA-o followed by 22.5 hrs. Infarct volume was histologically assessed. Hippocampal kindled animals were also used to study the time course of *naip*

expression in the frontoparietal cortex, which is mainly supplied by MCA. Kindled animals were sacrificed at 3h, 1, 7 or 21d, and fresh frozen, fixed sections or frozen tissues were dissected from the frontoparietal cortex (n=3). These tissues were used for ISHH, immunohistochemistry or Western blotting to assess naip expression.

Both p53 mRNA and protein were elevated in the ischemic penumbra in experiment I. The induction of p53 peaked within 8-12 hr then returned to basal levels within 24 hr after RP. The short duration of p53 induction in ischemic penumbra may suggest that p53 activate downstream genes responsible for growth arrest, DNA repair or/and apoptosis. Experiment II demonstrated a significant elevation in *naip* mRNA and proteins in piriform cortex and hippocampus, where neuronal populations known to be protected by kindling. The duration of the elevation lasted up to three weeks. In contrast, *naip* mRNA and protein remained at baseline levels in regions that are not protected, such as endopiriform cortex and medial thalamus. We also demonstrated that hippocampal kindling attenuated cortical infarct induced by MCA-o to 57.7± 4.6mm^3 as compared to 156.5 ± 12.6mm^3 in controls. This neuroprotection was associated with a two to three fold elevation of naip expression in the corresponding areas by kindling treatment.

In summary, the studies presented in this thesis demonstrated that *naip* induction is positively correlated with neuroprotection in many aspects. More importantly, the data show that hippocampal kindling modulates the injury produced by noxious treatment. The close temporal and spatial relationship between *naip* induction and the neuroprotective effects of hippocampal kindling suggest that induction of this antiapoptotic gene might mediate the neuroprotection and that treatments capable of increasing in *naip* expression might be a potential treatment for stroke.

CHAPTER 1

INTRODUCTION AND HISTORICAL REVIEW

The brain is so critically dependent on the continuous supply of oxygen and glucose that even brief periods of hypoxia can cause serious disruptions of neuronal homeostasis leading to cell death. Many hypotheses have been proposed in order to explain the physiological and biochemical basis for ischemic brain damage (Chan, 1996; Choi, 1996, 1992; Dawson, 1992; Olanow, 1993; Pulsinelli, 1992; Siesjo, 1992). The pathophysiology of the ischemic brain may involve the cumulative effects of multiple factors, such as increased intracellular free calcium, lactate levels, free radical formation and elevated synthesis of nitric oxide (Choi, 1996; Pusinelli, 1992; Siesjo, 1992). Neuronal injury resulting from cerebral ischemia has long been regarded to be necrotic in nature, however, recent studies suggest that programmed cell death (PCD) or apoptosis may contribute to the loss of neurons following cerebral ischemia (Charriayt-Marlangue et al., 1995; Choi, 1996; Choop and Li, 1996; Du et al., 1996; Linnik et al., 1993; MacManus et al., 1993; 1994). Since PSD is mediated by alterations in gene expression, in the present study I have focused on the alteration in the expression of pro and anti-apoptotic genes, p53 and *naip* respectively. The purpose of this study is to confirm that apoptosis is indeed involved in the process of cell death following cerebral ischemia, and that specific changes in the levels of apoptotic genes are correlated with the susceptibility to neuronal cell death following cerebral ischemia.

1.1 Definition of Cerebral Ischemia

Ischemic cerebrovascular disease (stroke) results from arterial narrowing, thrombosis or from arterial occlusion by embolism. These pathological processes impair the blood supply to the brain resulting in cerebral ischemia and subsequent neuronal cell damage. Clinical features may differ from patient to patient, but typical symptoms include hemiplegia, hemianesthesia and hemianopsia. Aphasia is also present if the dominant hemisphere is affected.

1.2 Epidemiology

Cerebrovascular disease, the third leading cause of death after heart disease and cancer in developed countries, has an overall prevalence of 800 per 100,000. Five percent of the population over 65 years old are affected by stroke in North America. It is estimated that about one third of stroke victims die and another one third are disabled from the devastating disease (Wolf and D'Agostino, 1998). The loss of these patients from the work force and the extended hospitalization they require during recovery make the economic impact of the disease one of the most devastating in medicine.

1.3 Pathogenesis

The pathogenic basis of stroke can be divided into those caused by atherosclerotic occlusion and stenosis, emboli of cardiac origin, vasculopathies (inflammation of the blood vessels), hypertensive lacunar infarction (infarction less than one centimetre in diameter, usually caused by hypertension) and cerebral artery

thromboses due to coagulation abnormalities, polycythemia (abnormal elevation of the red cells) or thrombocytosis (abnormal elevation of the platelets).

Atherosclerosis is the major cause of arterial disease (Wolf and D'Agostino, 1998) and it accounts for most ischemic stokes due to its effect on the major extracranial arteries. The most common site for atherosclerosis is in the region of the carotid sinus just beyond the origin of the internal carotid artery. The vertebral artery is more commonly affected at its origin and at the C1-2 level but disease can occur intracranially, frequently between the origin of the posterior inferior cerebellar artery and its junction with the basilar artery. The development of an atherosclerotic plaque may not only lead to potential occlusion of the artery but also become the site of ulceration and the cause of luminal platelet thrombus.

Despite the decline in the incidence and sequelae of rheumatic heart disease, cardiac causes of stroke are being recognized with increasing frequency. The present estimated figure for the incidence of cerebral infarction resulted from heart diseases is between 20% to 35% (Wolf and D'Agostino, 1998). Most cerebral lesions of cardiac origin arise from emboli. The thrombi are produced in the chambers or on the valves. Because the most direct laminar flow from the internal carotid artery is to the middle cerebral artery (MCA), the MCA territory is most commonly affected, with a predilection for the main trunk or the parietal and angular branches. Emboli seldom lodge in the anterior cerebral artery.

1.4 Experimental Animal Models for Cerebral Ischemia

Experimental animal models have been used to study the mechanisms of neuronal cell death following cerebral ischemia and to establish potential therapies.

i. Focal Cerebral Ischemia

The most common site for cerebral ischemia is within the middle cerebral artery (MCA) distribution territories. Focal ischemia can be achieved by blocking the blood flow in the MCA. Models of permanent and transient MCA occlusion have been developed (Brint et al., 1988; Buchan et al., 1992). MCA occlusion is done in conjunction with laser Doppler flowmetry in order to determine to what severity and for what duration the brain tissue has become ischemic. Regional cerebral blood flows are maintained at approximately 10% of baseline throughout the ischemic period.

ii. Transient Forebrain Ischemia

Following a cardiac arrest or during open-heart surgery employing cardiopulmonary bypass methods, periods of hypotension may result in global ischemia. Four-vessel occlusion models or models involving two vessel occlusion coupled with hypotension are two commonly employed methods for simulating clinical global ischemia. These models produce severe forebrain ischemia with blood flow to the hippocampus, cortex and striatum being reduced to 2-10 mls/100gm/min (Pusinelli et al., 1982b).

1.5 Regional Cerebral Blood Flow (rCBF) Dynamics and Cerebral Ischemia

Cerebral ischemia resulting from single or multiple vascular occlusion causes rCBF reduction. The severity of ischemia, depth and duration of rCBF reduction, determines the degree of neuronal damage. CBF in the forebrain is severely disrupted during global ischemia (Pusinelli et al., 1982b). The animals may only tolerate an ischemic insult for a few minutes before they die. In the focal ischemic model there is a moderately severe reduction rCBF to 10-17 ml/100gm/min (Astrup et al., 981, Heiss, 1983), which leads to infarction if this state of hypoperfusion is maintained for about 1 to 2 hours (Buchan et al., 1992). On the edge of the "core" region of severely reduced flow there is a so-called "penumbral" zone (Hakim, 1998; Heiss, 1983) where a moderate reduction in blood flow 20-40 ml/100gm/min is associated with either a partial or no reduction in energy charge.

1.6 Energy Failure, Homeostasis and Metabolic Changes following Cerebral Ischemia

Traditionally, the main cause of ischemia-induced cell death was attributed to energy failure (Duffy et al., 1972). It is reasoned that the life of a cell is terminated by serious disruption of homeostatic mechanisms. The mechanisms that maintain cellular homeostasis (ion pumps, macromolecular transport, transcription and translation) are energy-dependent, so that impairment of cellular respiration due to ischemia results in a disruption of homeostasis, causing death of the cell (Levy and Duffy, 1977; Pusinelli

et al., 1982a). The evolutional time for ischemic injury can be minutes to hours and is usually associated with the formation of brain edema. This type of cell injury has generally been referred to as "death of energy failure".

In contrast, post-ischemic neuronal death occurs gradually in the presence of normal cerebral blood flow (Levy et al., 1975), normal energy metabolism, and normal homeostasis as far as Na+, K+, and water are concerned (Pulsinelli and Duffy, 1983; Arai et al., 1986). In fact, the recovery of the energy state of the brain after transient brain ischemia is very rapid (Onodera et al., 1986). In this event, neuronal cell death occurs through a slowly progressive process over a few hours to days following a short episode of ischemia. The presence of "delayed neuronal death" a few days after ischemia has been demonstrated in the hippocampus and striatum following transient forebrain ischemia (Pusinelli et al., 1982a) and in the cortical areas after transient focal cerebral ischemia (Du et al., 1996; Li et al., 1995).

1.7 Acidosis and Ischemic Neuronal Death

Many mechanisms contribute to neuronal cell death following cerebral ischemia, among which the concept of acidic damage is perhaps the earliest. The brain, with its high metabolic rate, is very susceptible to anaerobic acidosis. Lack of oxygen supply to the brain easily triggers the Pasteur effect, stimulating glycolysis to keep pace with energy demand (Siesjo, 1988; Siesjo, 1990). Ischemia thereby couples enhanced lactate production with ATP hydrolysis, accelerating proton production. Accordingly, oxygen deprivation in rodents enhances cerebral glucose uptake and utilization (Torbati et al., 1986; Izumiyama et al., 1987) and evokes a pronounced rise

in tissue lactate (Pusinelli and Duffy, 1983). As ATP consumption outstrips production, and as $[H^+]i$ exceeds the buffering capacity within ischemic neurons, protons are exported via active extrusion mechanisms to the interstitial space where they accumulate (Kraig et al., 1985). However, the rapid loss of Na^+, K^+-ATPase pump activity during periods of energy depletion compromises the Na^+ gradient, impairing both H^+ and HCO_3^- exchange systems. As a result, pH_i in both neuronal and glial cells drops during ischemia (Kraig and Chesler, 1990) and is apparently linear with respect to lactate concentration.

The acidotic hypothesis of ischemic injury has been strengthened by studies showing that H^+ and lactate accumulation are closely correlated with the extent of ischemic brain damage (Siesjo, 1988). More direct evidence has come from examining the damaging potential of low pH solutions *in vitro* or following their direct injection into the living brain (Kraig et al., 1987; Goldman et al., 1989). Although precisely how acidosis damages nervous tissue remains unclear, several mechanisms have been proposed. One of these theories suggests that the build-up of intracellular acid concentrations promote the iron-catalyzed formation of free radicals (Rhencrona et al., 1989), which mediate lipid peroxidation and disrupt membranes (see below). Low tissue pH may also cause a nonselective denaturation of proteins and nucleic acids (Kalimo, et al., 1981) and trigger cell swelling via stimulation of the Na^+/H^+ and Cl^-/HCO_3^- exchangers (Kimelberg et al., 1990), potentially causing cellular edema and osmolysis. Acidosis enhances membrane depolarization, which in turn activates voltage sensitive calcium channels and facilitates increased intracellular Ca^{2+} concentrations. When coupled with energy failure, intracellular acidification may also

facilitate Ca^{2+} release from intracellular stores and impair cytosolic Ca^{2+} buffering (Abercrombie and Hart, 1986). In addition, acidosis may hinder postischemic metabolic recovery by inhibiting mitochondrial energy metabolism (Shields et al., 1980; Hillered et al., 1985) and by impairing blood flow via vascular edema.

1.8 Exitotoxicity following Cerebral Ischemia

Severe forebrain ischemia of short duration induces selective neuronal death (SND) (Kirino, 1982; Pusinelli et al., 1982a). Transient global cerebral ischemia results in energy failure (Pusinelli and Duffy, 1983); however, the observed homogeneous restoration of blood flow results in the ubiquitous recovery of energy charge, electrophysiological and membrane function for all cells, at least initially (Pusinelli et al., 1982b). After a delay, the death of certain neuronal population occurs, such as hippocampal CA1 pyramidal cells, neocortical neurons in layer 3, 5 and 6, medium sized striatal neurons and cerebellar Purkinje cells, leaving other neurons and glial cells untouched (Kirino, 1982; Pusinelli et al., 1982a). Similarly, selective cortical neuronal loss is observed following transient focal cerebral ischemia (Du et al., 1996; Choop and Li, 1996). Why are specific populations of neurons exquisitely sensitive to transient ischemia while other populations of neurons are relatively resistant to this treatment? What accounts for the delayed and gradual temporal sequence of this death? While the release of unchecked excitatory activity has recently been blamed for the selective sensitivity of brain neurons to ischemia, the final common cellular pathway is speculated to be a slow but lethal intracellular calcium ion accumulation (Choi, 1988; Siesjo and Bengtsson, 1989).

i. Excitatory Amino Acids

There are abundant levels of glutamate and aspartate in the CNS that participate in multiple metabolic functions under normal physiological conditions. Glutamate is the predominant excitatory amino acid (EAA) in the mammalian CNS. The neurotransmitter pool of glutamate is stored in synaptic vesicles and, upon neuronal depolarization, is released into the synaptic cleft in a Ca^{2+}-dependent fashion (Shepherd, 1994). Glutamate is cleared from the synaptic cleft by high affinity, Na^{+}-dependent uptake carriers located in both neurons and glia. Glutamate acts on several distinct families of receptors, each of which has multiple subtypes with distinct pharmacological and physiologic properties. Under some conditions, glutamate and related compounds act as excitotoxins that might participate in the events leading to neuronal damage in a variety of acute and chronic neurological disorders (Choi, 1988). Membrane depolarization caused by ischemia and energy failure results in a decrease of cellular glutamate uptake and increased glutamate release (Drejer et al., 1985); when the net increase of extracellular glutamate concentration exceeds the physiological limit (10 mM), this neurotransmitter becomes toxic to neurons.

ii. Glutamate Receptors

Glutamate activates several classes of receptors, each of them has a distinct pharmacology and physiology, and they are named after their selective artificial ligands. Therefore, these different type receptors have been classified as a-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), N-methyl-D-aspartate

(NMDA), and kainate receptors (Johnson et al, 1987; Hollman et al, 1989: Agrawal and Evans, 1986). All of these receptors are ligand-gated ion channels (ionotropic receptors). In contrast, metabotropic glutamate receptors are linked to G-proteins which couple with enzymes that produce changes in cyclic nucleotides or phosphoinositol metabolism.

ii-a. NMDA Receptor

NMDA receptors are activated by glutamate. In addition to a binding site for glutamate, there is also a binding site for glycine. Both the glutamate and glycine sites must be occupied for receptor activation to occur. Thus, glutamate and glycine are referred to as "co-agonists" of the NMDA receptor (Johnson et al., 1987; Kleckner et al., 1988). NMDA receptor activation is also modulated by polyamines, such as spermine and spermidine. One of the most important and defining features of the NMDA receptor is the voltage-dependent blockade of the receptor ion channel by Mg^{2+} (Nowak L et al., 1984). At the resting membrane potential, the ambient extracellular concentration of Mg^{2+} blocks the NMDA receptor ion channel and prevents the current flow, even when the glutamate and glycine sites are occupied. However, because $Mg2^{+}$ blockade is voltage dependent, the degree of Mg^{2+} block is reduced as a neuron becomes depolarised. Thus, with graded depolarization, the amount of Ca^{2+} current flowing inward through the NMDA receptor ion channel increases.

Unlike the AMPA receptor, the NMDA subtype of glutamate receptor ion channel is highly permeable to calcium, as well as sodium and potassium ions. This Ca^{2+} influx has been implicated in a range of physiological phenomena including

synaptic integration (Watkins, 1994) and long-term potentiation (LTP) (Shepherd, 1994). Thus, NMDA receptor activation is involved in the fine control of co-ordinated movement, behavioural patterns, and learning and memory. Under pathological conditions, sustained activation of the NMDA receptor resulting in prolonged neuronal depolarisation has been implicated in a variety of neurological disorders, such as epilepsy, stroke, amyotrophic lateral sclerosis and Huntington's disease. In fact, NMDA receptors have become increasingly prominent as potential therapeutic targets for these disorders. (Croucher et al.,1982; Simon et al., 1984).

ii-b AMPA Receptor

Activation of AMPA receptors by glutamate appears to mediate most of the fast excitatory neurotransmission in the CNS. The AMPA receptor is linked to a channel providing a nonselective conductance mechanism for the monovalent cations Na^+ and K^+. The binding of glutamate or AMPA to the receptor is associated with influx of Na^+ from the extracellular space to the intracellular compartment coupling with the efflux of K^+.

It has been reported that certain isoforms of the AMPA receptors are highly permeable to Ca^{2+} (Gilbertson et al., 1991; Hollmann et al., 1991). This is a result of the subunit composition of individual AMPA receptors. Like other ligand-gated ion channels, the AMPA receptors are thought to be multimeric heteromers composed of several distinct subunits. GluR1 through GluR4 (also known as GluRA through GluRD) are AMPA receptor subunits that can assemble in various combinations to form functional receptors (Hollman et al., 1989; Keinanen et al., 1990).

The permeability of AMPA receptors to Ca^{2+} is determined by receptor subunit composition. GluR1 and GluR3 can form homomeric or heteromeric receptor channels that are permeable to Ca^{2+}. However, inclusion of a GluR2 subunit in the receptor assembly prevents Ca^{2+} permeability (Hollman et al., 1991). Site directed mutagenesis has revealed that the transmembrane domain in GluR2 (AMPA subtype) carries a positively charged arginine residue at amino acid position 586 (Verdoorn et al., 1991), which prevents Ca^{2+} permeability in GluR2. Alterations in GluR2 expression therefore regulate the AMPA receptor-dependent divalent cation permeability of a cell (Burnashev et al., 1992). This determinant is not encoded by the gene but appears to be introduced by RNA editing with the CGG codon for arginine converted to a CAG codon for glutamine, a condition that could be caused by excessive glutamate activated Ca^{2+} influx under ischemic conditions (Sommer et al., 1991). In post-ischemic CA1, there is a change in the mRNA ratio of GluR1 + GluR3/GluR2 resulting in a relative post-ischemic loss of mRNA for GluR2, which might serve as a mechanism for increased Ca^{2+} permeability in CA1 neurons of the hippocampus (Pellegrini-Giampietro et al., 1994). This change in the ratio does not occur in CA3, which is resistant to ischemia. Influx of Ca^{2+}through AMPA receptor channels activated by exogenous application of glutamate analogous has been demonstrated (Zeilhofer et al., 1993). This influx is reported to cause Ca^{2+} -mediated cell death in cultured neurons (Brorson et al., 1994).

ii-c Kainate Receptor

Ligand binding experiments and biophysical studies of recombinant receptors indicate that the GluR5, 6, 7 and the KA1, KA2 subunits assemble into receptors that are reminiscent of the kainate receptors (Agrawal and Evans, 1986; Bettler, et al., 1990; Huettner, 1990; Egebjerg et al., 1991; Herb et al., 1992; Sakimura et al., 1992; Sommer et al., 1992). The kainate receptor subunit GluR5 and GluR6 form homomeric receptors and can also form heteromeric receptors in pairwise combination with the KA1 or KA2 subunits (Partin et al., 1993). The agonist profile for GluR7, as determined in radioligand binding studies, is very similar to those of the GluR5, 6 subunits (Bettler et al., 1992). Similar to the GluR2 subunit in the AMPA receptor, RNA editing of the Q/R site, in which the editing mechanism involves a transition from arginine to glutamine, has also been observed for the kainate receptor subunits GluR5, 6, but not for GluR7. In contrast to the very high degree of GluR2 editing, significant amounts of both edited and unedited GluR5, 6 transcript variants can be found in the adult brain. In addition to the Q/R site, two extra sites of editing have been identified in the putative transmembrane domain1 (MD1) of GluR6 transcripts, converting the codon for isoleucine to valine and the codon for tyrosine to cysteine (Kohler et al., 1993). Significantly, recombinant GluR6(R) and GluR6(Q) receptors are differentially permeable to Ca^{2+}, most pronounced in instances where the MD1 sites are edited (Egebierg and Heinemann, 1993). Unlike AMPA receptors, GluR6 (R) and GluR6 (Q) are both permeable to Ca^{2+} with GluR6 (R) having a higher Ca^{2+} permeability (Egebierg and Heinemann, 1993).

Like the NMDA and AMPA receptors, sustained activation of the kainate receptors also leads to excessive Ca^{2+} influx resulting in neuronal damage following cerebral ischemia. Injection of kainate in rodents produces limbic seizures causing neuronal injury in select brain areas as well.

ii-d Metabotropic Glutamate Receptor

The role of metabotropic glutamate (mGlu) receptors in excitotoxic injury has been difficult to define because of the lack of selective pharmacological agents. The mGlu-receptor family consists of transmembrane receptors coupled to G-proteins. The family is comprised of eight subtypes, which are subdivided into three groups on the basis of sequence similarity and transduction pathways. Group I includes mGlu1 and mGlu5 receptors, which are coupled to polyphosphoinositide turnover (Nakanishi, 1992; Brabet et al., 1995; Knopfel et al., 1995). Binding of this subtype of the receptors by glutamate activates the coupling G-protein and initiates inositol phosphate metabolism. The activated G-protein stimulates phospholipase C, which then hydrolyses PIP2 resulting in the formation of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Nicoletti et al., 1996). IP3 acts as a second messenger, which triggers the release of Ca^{2+} from intracellular stores, such as endoplasmic reticular (ER). DAG in turn activates protein kinase C (PKC). In addition to the release of intracellular Ca^{2+}, metabotropic glutamate receptor activation has several actions likely to promote excitotoxic injury due to the activation of PKC, including: (a) increasing NMDA and AMPA receptor-mediated membrane current; (b) promoting slow onset

potentiation or long term potentiation at excitatory synapses; (c) reducing GABAergic inhibition; and (d) reducing inhibitory K^+ currents (Shapher, 1994).

In contrast to the Group I subtype, which includes mGlu1 and mGlu5, Group II comprises mGlu2 and mGlu3, and Group III consists of mGlu4, mGlu6, mGlu7 and mGlu8. The later two groups are negatively coupled to adenylate cyclase activity (Nakanishi, 1992; Pin and Duvoisin, 1995), as well as to voltage operated calcium channels (VOCC) (Chavis et al., 1994; Ikeda et al., 1995). A presynaptic localization has been shown for some of the members of group II or III mGlu receptors, including mGlu2, mGlu4, and mGlu7 (Pin and Duvoisin, 1995). Pharmacological activation of all these receptor subtypes reduces glutamate release, where mGlu2 receptor antagonists amplify the depolarization-induced release of glutamate (Glaum and Miller, 1995). Thus, these receptor subtypes might function as inhibitory autoreceptors, which mediate long-term depression and reduce Ca^{2+} influx through VOCCs (Nicoletti et al., 1996). Hence, it is generally thought that activation of Group I mGlu receptors increase neuronal excitation and excitability, whereas activation of Group II or Group III mGlu receptors reduces synaptic excitation.

1.9 Intracellular Calcium and Ischemic Injury

Calcium is a prominent second messenger in neurons, where it plays pivotal roles in many fundamental physiological processes, including regulation of neurite outgrowth and synaptogenesis, neurotransmitter release, and synaptic plasticity (Neurobiology, 1994). However, excessive and sustained increase in intracellular calcium can kill neurons (Choi, 1995; Kristian and Siesjo, 1998). It has been suggested

that excessive intracellular calcium play a very important role in the process of neuronal cell death following cerebral ischemia. Depolarization due to energy failure allows entry of Ca^{2+} through voltage operated calcium channels (L and T types). Excessive glutamate release resulting from depolarization activates NMDA, AMPA, kainate and metabotropic receptors leading to a increase in Ca^{2+} influx as well as Ca^{2+} mobilization from intracellular stores (see discussion in Sec. 1.8, iii-d).

Under normal physiological conditions, an initial Ca2+ influx from the extracellular space is rapidly followed by an intracellular release of Ca2+ from mitochondria and ER compartments. Energy failure or very low oxygen tensions (reduced ATP concentrations) decreases mitochondrial calcium uptake, leading to a rise in cytosolic calcium levels following ischemia. Acidosis following cerebral ischemia may also inhibit intracellular calcium buffer system and replace H^+ from the buffer proteins, leading to the further accumulation of intracellular free calcium (Seisjo, 1988). The excessive accumulation of calcium via NMDA receptor activation binds calcium-dependent calmodulin, which activates nitric oxide synthase and leads to the formation of nitric oxide (NO) (Bredt et al., 1990; Bredt and Snyder, 1992). NO can diffuse to the adjacent cells and activate soluble guanyl cyclase leading to increases in the second messenger cGMP (Trump and Berezasky, 1992). cGMP is thought to act in part by activating the ryanodine receptor causing stored calcium to be released from the ER (Berridge, 1993).

Intracellular calcium overload can induce a sequence of events: (1) Activation of phospholipase A2 (Farber and Young 1981) that catalyzes the hydrolysis of membrane phospholipid and leads to the production of free radicals. (2) Activation of

proteases, such as calpain I, which degrades several major neuronal structural proteins, including tubulin, microtubule-associated proteins, neurofilament polypeptides and spectrin (Siman and Noszek, 1988). (3) Activation of endonuclease, it has been shown that calcium mediated endonuclease activation is involved in cytotoxicity (Jones et al., 1989; McConkey et al., 1988; McConkey et al., 1989; Trump et al, 1992) (Figure 1).

1.10 Free Radicals and Ischemic Injury

Free radicals can be generated from ischemic-reperfusion related events. Several oxidizing radical species, including superoxide ($O_2^{\cdot-}$), perhydroxyl ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$), are formed following the initial reduction of oxygen. Superoxide dismutase (SOD) is an endogenous antioxidant, which catalyzes the conversion of toxic superoxide into hydrogen peroxide under physiological conditions. Hydrogen peroxide is then converted into water products by glutathione reductase (Olanow, 1993,). It is generally believed that energy failure can result in the compromised function of antioxidant enzymes such as SOD and glutathione reductase leads to the accumulation of injurious superoxide and hydrogen peroxide (Chan, 1996; 1994).

There are also other pathways in the process of free radical generation following cerebral ischemia. Cerebral ischemia cause intracellular calcium overload (see above discussion) that activates phospholipase A2 and leads to the liberation of arachidonic acid (Lazarenwiez et al., 1988).

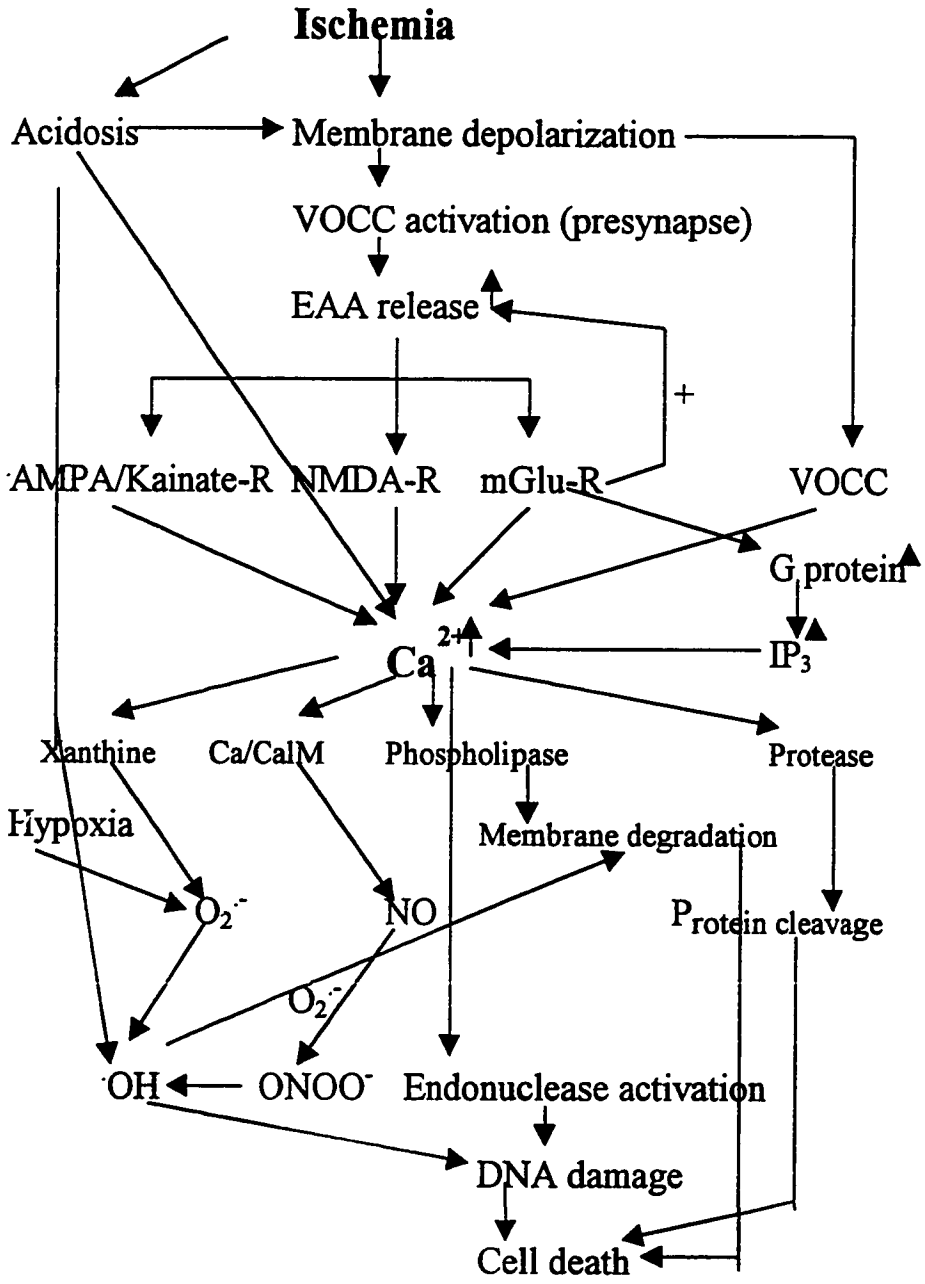


Figure 1. Mechanisms of ischemic neuronal death pathway.

The oxidative metabolism of arachidonic acid leads to the production of oxygen free radicals (Chan and Fishman, 1978). Superoxide radicals are produced by the enzymatic action of xanthine oxidase, which is generated by calcium-activated protease cleavage of xanthine dehydrogenase (Dykens et al., 1987) (see above for details regulating calcium overload). Superoxide radicals have a longer half-life and can form hydroxyl radicals through a Haber-Weiss reaction (Figure 2). This reaction proceeds rapidly in the presence of the trace metal iron, which is released from the iron binding protein transferrin or ferritin during ischemia or acidosis.

Another pathway for the formation of hydroxyl radicals is through the reaction of superoxide and nitric oxide (Dawson et al., 1992). Activation of the NMDA receptor leads to a calcium influx; calcium then binds to calcium/calmodulin. Calcium/calmodulin is an essential activator of nitric oxide synthase (NOS). NOS converts arginine to citrulline resulting in the generation of NO (Bredt and Snyder, 1990). Nitric oxide and superoxide react together and form peroxynitrite ($ONOO^-$) (Beckman et al., 1990). Peroxynitrite degrades instantly to hydroxyl ($^{\cdot}OH$) and nitrogen dioxide ($NO_2^{\cdot}$) (Figure 2).

Frec radicals are compounds with an unpaired electron in their outer electron shell. They are highly reactive and capable of inducing long polymerization reactions in biological systems. These radicals, especially hydroxyl radicals, are known to initiate lipid peroxidation and to cause protein oxidation (Schmidley, 1990) and DNA damage (Liu et al., 1996), which if uncontrolled, are rapidly lethal to cells.

$$O_2 + e^- \Longrightarrow O_2^{\cdot -} \quad (1)$$

$$O_2^{\cdot -} + H^+ \Longrightarrow HO_2^{\cdot} \quad (2)$$

$$O_2^{\cdot -} + O_2^{\cdot -} \Longrightarrow H_2O_2 \quad (3)$$

$$H_2O_2 \Longrightarrow H_2O + O_2 \quad (4)$$

$$O_2^{\cdot -} + H_2O_2 \Longrightarrow OH + OH^- + O_2 \quad (5)$$

$$O_2^{\cdot -} + NO^{\cdot} \Longrightarrow ONOO^- \quad (6)$$

$$ONOO^- + H^+ \Longrightarrow ONOOH \quad (7)$$

$$ONOOH \Longrightarrow OH + NO_2^{\cdot} \quad (8)$$

Figure 2. Simplified equations for free radical generation.

1.11 Mitochondria and Ischemic Neuronal Injury

A variety of key events in apoptosis focus on mitochondria, including the generation of ROS, loss of mitochondrial transmembrane potential, changes in electron transport, altered cellular oxidation-reduction, calcium accumulation, release of caspase activators, and participation of pro- and antiapoptotic bcl-2 family proteins.

i Mitochondrial Alteration and Mitochondrial Permeability Transition Pore

Measurements of mitochondrial transmembrane potential (White and Reynolds 1996; Bruce-Keller et al., 1999), and membrane permeability transition (PT) and release of cytochrome *c* (Hortelano et al., 1997) have documented mitochondrial alteration known to occur in cells undergoing apoptosis in several of the cell culture models. In many apoptosis scenarios, the mitochondrial inner transmembrane potential collapses (Petit et al., 1996), indicating the opening of a large conductance channel known as the mitochondrial permeability transition pore (Herrmann et al., 2000; Crompton, 1999). The PT pore consists of inner membrane proteins, such as the adenine nucleotide translocator (ANT), and outer membrane proteins, such as porin (voltage-dependent anion channel) and cyclophilin D, which operate in concert, presumably at inner and outer membrane contact sites, and create a channel through which molecules < 1.5 kD pass (Petit et al., 1996; Bernardi et al., 1994). Opening of this nonselective channel in the inner membrane allows for an equilibration of ions

within the matrix and intermembrane space of mitochondria, thus dissipating the H+ gradient across the inner membrane and uncoupling the respiratory chain and leading to cessation of ATP production. Perhaps more importantly, PT pore opening results in a volume dysregulation of mitochondria due to the hyperosmolality of the matrix, which causes the matrix space to expand. This matrix volume expansion can eventually cause outer membrane rupture.

Mitochondrial PT pore opening is regulated by voltage, electron flow, oxidative stress, and by the two of the pore constituents, cyclophilin D and ANT (Vander Heiden et al., 1999; Fontaine et al., 1998, Crompton, 1999). Interestingly, ROS, such as NO and peroxynitrate, predispose to a mitochondria PT and could also act as triggers of a pore opening in partially calcium-loaded mitochondria (Richter et al., 1994; Schweizer and Richter, 1994; Packer and Murphy, 1995).

Mitochondria are normally impermeable to H+ and other ions, and only allow passage of ions for which specific transport system exists. However, the literature on mitochondrial function *in vitro* contains many reports that exposure of mitochondria to calcium causes them to swell and release intramitochondrial components into the medium (Gunter and Pfeiffer, 1990; Gunter et al., 1994). It is now realized that this sequence of events reflects the assembly of mitochondrial PT pore. This pore allows the release of Ca2+ and Mg2+ as well as various compounds including cytochrome *c*.

Analyses of the role of mitochondrial alteration in neuronal death following cerebral ischemia provide further support for a major contribution of apoptosis to the pathophysiology of stroke. Studies have demonstrated that cyclosporin A, a inhibitor of the mitochondrial PT pore, protected cultured neurons against apoptosis induced by

ischemia-relevant insults (Keller et al., 1998), and protected hippocampal neurons against hypoglycemic injury in vivo (Friberg et al., 1998).

ii Mitochondria and ROS

Mitochondria are the major intrinsic source of oxyradicals, with superoxide anion radical O2.- being constantly generated during the process of electron transport in mitochondria. Superoxide dismutases located in the mitochondria (also called SOD2) and cytoplasm (also called SOD1) covert O2.- to H2O2. H2O2 is not a free radical, but is a major source for generation of hydroxyl radical, which is formed in the fenton reaction, catalyzed by Fe2+. Other pathways for oxyradical production in neurons include interaction of nitric oxide and superoxide to form peroxidenitrite, and superoxide production via activity of various oxygenases (e.g., cyclo-oxygenases).

As mentioned previously, ischemia with reperfusion is associated with production of ROS. Evidence now exists that a substantial part of this production emanates from mitochondria (Dykens, 1994; Piantadosi and Zhang, 1996). This notion is supported by results obtained in vitro on primary neuronal cultures. The concept is based on the hypothesis that excitotoxic cell death is, at least in part, mediated by a coupling between glutamate-induced production of ROS and EAA-induced cell damage (Pelligrino-Giampietro et al., 1992). Subsequent results showed that exposure of cells to glutamate gave rise to production of ROS and that a substantial part of this production occurred in the mitochondrial fraction (Dugan et al., 1995; Reynolds and Hastings, 1995).

Mitochondria–derived ROS appear to play a particularly important role in triggering changes that lead to neuronal apoptosis, because overexpression of Mn-SOD can prevent apoptosis induced by an array of insults, including ischemia (Keller et al, 1998). Infarct size following MCA-o reperfusion was significantly decreased in transgenic mice overexpressing mitochondrial superoxide dismutases (Mn-SOD), indicating that mitochondrial superoxide production contributes to ischemic neuronal death (Keller et al., 1998). The latter study further showed that cultured neurons overexpressing Mn-SOD are resistant to apoptosis induced by insults relevant to stroke. The antiapoptotic property of Bcl-2 is associated with its ability to suppress accumulation of ROS (Hockenbery et al., 1993), and Bcl-2 may act locally in membranes (particularly mitochondrial and plasma membranes) to suppress lipid peroxidation (Bruce-Keller et al., 1997). Overexpression of Bcl-2 in cultured neural cells results in resistance of the cells to oxidative stress-induced apoptosis (Kruman et al., 1997).

iii Mitochondria and Calcium

One of the major determinants of intracellular calcium movements is the mitochondrion. It is now widely accepted that the balance between influx and efflux of Ca2+ across the inner membrane regulates mitochondrial dehydrogenases, which are rate-limiting for citric acid cycle metabolism (Denton and McCormack, 1990). When cell activity leads to a substantial rise in intracellular Ca2+, the mitochondria may accumulate large amounts of calcium (Nicholls, 1985). This is because the uniporter, carrying calcium into the mitochondria along the electrochemical gradient, has a much

higher total activity than the export pathways, which encompass 2Na+-Ca2+ exchange. In other words, if the net influx of Ca2+ exceeds the capacity of the extrusion pathway through 2Na+/Ca2+ exchange, intramitochondrial Ca2+ concentration increases, and Ca2+ will be sequestered within the mitochondria. When the concentration exceeds the "set point" for calcium sequestration in the mitochondria, this accumulation will result in mitochondrial dysfunction. Subsequently, intramitochondrial calcium accumulation was observed in various ischemic conditions (Dux et al., 1987; Silver and Erecinska, 1992; Zaidan and Sims 1994), and the calcium accumulation occurs before cell death becomes manifest (Zaidan and Sims 1994).

Cytochrome *c* release from the mitochondria is mediated by Ca2+, which stimulates opening of the PT pore in the inner membrane of mitochondria (Green et al., 1998, Crompton, 1990), resulting in osmotic swelling of the matrix, secondary rupture of the outer membrane, and release of proteins, such as cytochrome *c*, stored within the intermitochondrial space (Bernardi et al., 1994, Green and Reed, 1998).

iv Mitochondria and Caspase

Intermitochondrial calcium overload and ROS production lead to cytochrome *c* release. An alternative pathway, however, may involve changes in the permeability of the outer membrane of mitochondria, without the alteration in inner membrane function that produce mitochondrial swelling. In this regard, Bax induces release of cytochrome *c* (Jurgensmeier et al., 1998), presumably as a result of its ability to form pores in the membrane or to interact with other channel proteins in mitochondrial membranes (Antonsson et al., 1997; Matsuyama et al., 1998).

Caspase activation is considered to be the *sine que non* of apoptosis. Both a receptor-mediated and a mitochondrial pathway for caspase activation have been described. In the receptor-mediated pathway, Fas ligand or tumor necrosis factor *a* binds to its receptor, causing receptor aggregation and recruitment of death adapter molecules on the cytoplasmic side of the membrane. Caspase 8 or 10 is activated via this pathway and activated caspase 8 or 10 can then cleave procaspase 3, which serves as an efficient executioner to cleave multiple targets within the cell, including DNases (Muzio et al., 1998). Caspase 8 also targets the mitochondria through the proteolytic activation of Bid (Liu et al., 1997).

A second pathway of caspase activation is activated in response to a variety of cellular stresses, including DNA damage, protein kinase inhibition, and loss of survival signaling. In fact, cytochrome *c* release was observed on cerebral ischemia. Fujimura et al., (1998) demonstrated that cytochrome *c* redistributes from the mitochondrial fraction to the cytosolic fraction in cortical tissue following transient focal ischemia in rats. In this pathway, termed the mitochondrial pathway, a proapoptotic member of the bcl-2 family, such as Bax or Bid, associated with the mitochondria and directs the dissociation and eventually release of cytochrome *c* to the cytosol. Release of cytochrome *c* from the mitochondria is a key step leading to PCD (Green and Reed, 1998; Yang et al., 1997; Li et al., 1997). Although apoptosis can occur in the absence of cytochrome *c* release, cytochrome *c* directly activates downstream effectors when injected into the cytosol in the absence of upstream signals (Crompton, 1990). Following release of cytochrome *c*, a complex is formed with another key factor, Apaf-1 (Peter and Krammer 1998). Apaf-1 binds cytochrome *c*, dATP or ATP, and forms a

large multimeric complex (700kD), termed the apoptosome, which also includes molecules of caspases 9 and 3 (Nijhawan et al., 1997). Caspase 9 is activated when bound to Apaf-1, where it processes caspase 3 to its active form (Cain et al., 2000).

1.12 Neuronal Cell Death in Ischemia: Necrosis vs Apoptosis or Programmed Cell Death

Cell death occurs by different mechanisms throughout the life of a metazoan organism. Apoptosis is a distinct type of cell death that occurs normally during development and continually in many tissues in the adult organisms. Necrosis is the classic, pathological form of cell death due to noxious injury or trauma. It often affects groups of cells and promotes an inflammatory response due to the release of intracellular contents. Necrosis is characterized by dilation of the ER and swelling of mitochondria, followed by loss of ion transport, general cell swelling, breakdown of the plasma membrane and lysis. Flocculation of the chromatin in the nucleus and non-specific degradation of DNA occur presumably because of release of cytoplasmic contents including lysosomal enzymes. Release of cellular contents into the extracellular fluid also kills neighbouring cells due to production of an inflammatory response.

Apoptosis derives from the Greek word referring to falling off, similar to leaves falling from a tree. Apoptosis is a markedly different form of cell death than necrosis. In brief, apoptosis is characterized by an orderly dismantling of the cell that prevents an inflammatory response. It is manifested by cell shrinkage (Kerr et al., 1972). Early

reports even referred to this process as shrinkage necrosis. In apoptotic cells, lysosomes remain intact, and cellular fragmentation rather that swelling occurs. Marked condensation and nucleosomal fragmentation of the chromatin is a key feature of apoptotic cells. The cytoplasm also condenses and the cell morphology is rapidly altered. Cellular blebbing and protuberances of the cell surface occur, resulting in the production of many membrane-bound cell fragments of varying sizes with well-preserved cellular organelles. The formation of apoptotic bodies of fragmented cells is the first stage of apoptosis, which is followed by phagocytosis of the fragments by surrounding parenchymal cells or macrophages. The entire process of apoptosis can occur within a few hours to days. Apoptotic cells disappear following phagocytosis and degradation. (Table 1).

Table 1 Comparison between apoptosis and necrosis

Characteristic	Apoptosis	Necrosis
Morphology	condensation of cell apoptotic bodies	lysis of cell
Membrane integrity	persists until late	early failure
Mitochondria	morphology unaffected	swelling
Chromatin	condensation	pyknosis
Occult phase	minutes to hours	none
Protein synthesis	death sometimes blocked by antibiotics	not affected by antibiotics
Cytoplasmic biochemical change	c-myc, c-fos	no protein synthesis
Nuclear biochemical changes	laddering (internucleosome cleavage)	diffuse degradation
First manifestation	activation of endonuclease	swelling

i. Apoptosis vs. Programmed Cell Death

Apoptosis has been defined as " an active process of gene-directed cellular self-destruction that ... serves a biologically meaningful, homeostatic function" (Kerr and Harmon, 1991). This concept of a "suicide program" in which the cell actively participates in its own demise, was originally reserved for specialized cells and unique circumstances. For example, lymphocyte selection in the thymus leads to apoptosis of T cells bearing receptors that are autoreactive (Golstein et al, 1991; Duvall and Wylli 1986). Similarly, developmental structures such as the tadpole's tail, which must be dispensed with at the appropriate time in morphogenesis, also succumb to an apoptotic death (Lockshin 1981). However, the increasing number of circumstances under which apoptosis is now observed suggests that many, if not all, cells possess an innate program for cell death.

The gradual acceptance that cell death through the active process of apoptosis is widespread in biological systems has several important consequences (Wyllie et al, 1980; Williams et al, 1992). First, it has been suggested that cell populations may be regulated by modulation of the rate of cell death in a manner which parallels more familiar controls by modulation of cell proliferation and cell differentiation (Williams 1991). Such a role in physiological control would be difficult to envisage for the essentially degenerative process of necrosis. Control involving regulation of apoptosis, however, has been demonstrated in a growing number of circumstances and that it is likely to be mediated by specific effects on the proteins involved in the pathway or pathways of apoptotic cell death.

It has become apparent that apoptosis, like the cell cycle, is not a simple metabolic cascade but rather a complex network of pathways that influence one another in a manner that appears to be either pro-apoptotic or anti-apoptotic. As in the development of cell cycle control models, the molecular basis of apoptosis control has emerged as a distinct area of investigation. Similar to the cell cycle, the output of this dynamic and complex system of molecular interactions seems to be an orderly cascade of events marked most reliably by the appearance of ultrastructural changes in the nucleus.

Although Kerr and his colleagues originally used the term "apoptosis" to refer to a specific morphologic type of programmed cell death (PCD), the term is now used by many investigators as a synonym for PCD because apoptosis is probably the most common morphologic type. Despite this common use of the term, it is important to be aware that apoptosis is a descriptive term used to define a specific type of cell death exhibiting a distinct set of morphological features. PCD, on the other hand, is a functional term, used to describe cell death that is a gene-mediated process. Apoptosis refers to only a single very specific morphologic pattern (Kerr et al., 1972). The failure to demonstrate apoptotic morphology in a pathologic condition does not definitively exclude a possible role for PCD. It remains formally possible that an alternate morphology of PCD may be present.

ii. Is Ischemic Neuronal Cell Death Apoptotic?

Apoptotic neuronal death has been described during the development of the nervous system (Oppenheim, 1991). Withdrawal of growth factors, such as nerve growth factor (NGF), from developing neurons, such as those of the superior cervical ganglion, readily induces apoptosis (Martin, 1988). Although the role of apoptosis in the adult nervous system is less well understood, it clearly occurs under some conditions. For example, following adrenalectomy and steroid replacement, a sudden withdrawal of glucocorticoid results in widespread apoptosis of hippocampal dentate granule cells (Sloviter et al, 1993).

Neuronal cell death following cerebral ischemia has long been regarded as necrosis due to the acute cellular swelling resulted from the rapid entry of Na^+/H_2O. Recently, compelling evidence indicating that excitotoxicity and intracellular calcium overload results in delayed neuronal cell death following cerebral ischemia has been reported (Choi, 1988; Siesjo, 1989). These reports have characterized the delayed neuronal cell death as distinct from conventional necrosis based on biochemical, morphological and molecular criteria. Endonuclease digestion producing cleavage of double stranded DNA as revealed by laddering of DNA fragments characteristic of apoptosis has been reported following transient cerebral ischemia in animal models (MacManus et al, 1993; MacManus et al., 1994; Heron et al., 1993; Tominaga et al, 1993). Evidence of ordered DNA laddering in the hippocampus, striatum in global ischemia and cortex following transient focal ischemia have also been note, with a time course that is similar to the delayed evolution of this injury (MacManus et al, 1993; Heron et al., 1993; Du et al., 1996). An endonuclease inhibitor, aurintricarboxylic

acid, protects against neuronal cell death following cerebral ischemia, supporting a role for apoptotic-like cell death that is different from necrosis (Rosenbaum et al., 1994).

Activation of a genetic program is required for PCD. Thus PCD can be inhibited by either RNA or protein synthesis inhibitors (Oppenheim, 1991). These same inhibitors have been reported to protect against delayed neuronal cell death following cerebral ischemia. Inhibition of protein synthesis with anisomycin (Shigeno et al., 1990) and cyclohexamide (Goto et al., 1990) can achieve a reduction of delayed or selective neuronal death. Studies show that the vast majority of the apoptotic-like neurons are located along the inner boundary of the ischemic core of the lesion in the case of focal ischemia (Chopp and Li, 1996). The localization of apoptotic-like cells at the inner boundary of the ischemic lesion suggests that the apoptotic process contributes to the expansion of the ischemic lesion. In fact, the reduction in cortical infarct by the use of continuous cyclohexamide infusion demonstrated that the expansion of the ischemic lesion could be reduced by protein synthesis inhibitors (Linnik, et al., 1993). This further argues for a role of gene activation and protein synthesis in neuronal degeneration following cerebral ischemia.

The criteria of morphological alterations characteristic of delayed neuronal death showing apoptosis following cerebral ischemia have yet to be established. Some reports maintain that the conventional morphologic features of the so-called shrinking death of apoptosis such as chromatin condensation and segmentation of the nuclei have not clearly been demonstrated in cerebral ischemia (Desphande, et al., 1992; Colbourne et al, 1999). Others point out that characteristics of apoptotic death such as the appearance of dense chromatin masses and apoptotic bodies have been detected by

electron microscopy in the nuclei of degenerating CA1 neurons after transient global ischemia (Nitatori et al., 1995). Therefore, it is essential to establish the molecular profiles of delayed cell death so that this morphological controversy can be resolved using molecular criteria. Investigation of the involvement of apoptotic genes in cerebral ischemia may therefore provide further evidence as to whether this cells death is actually apoptotic in nature.

1. 13 A Role for Anti-apoptotic Genes in the Ischemic Neuronal Injury following Transient Cerebral Ischemia

Despite the overall suppression in protein synthesis, cerebral ischemia rapidly increases neuronal expression for certain genes, such as immediate-early genes (IEGs) (Estus et al., 1994; Dragunow et al., 1994; Kiessling et al., 1993; Jorgensen et al., 1989; An et al., 1993), and p53 (Li et al., 1994). IEGs encode transcriptional regulating factors known to participate in gene signalling pathways that mediate PCD (Colotta et al., 1992; Smeyne et al., 1993). The tumor suppressor gene p53 has been implicated in growth arrest, DNA repair and PCD resulting from DNA damage (Lowe et al., 1993). Wild type-p53 can act as a transcription factor that regulates the expression of p53-dependent genes. It is possible that p53 function is mediated by downstream effector genes among which are Gadd45 (growth arrest and DNA damage inducible gene) and Mdm2 (murine double minute), although the respective roles of the products of these two genes in the regulation of growth control, apoptosis, amplification, and response to DNA damage remain to be determined (Figure 3).

KEY EVENTS IN APOPTOSIS

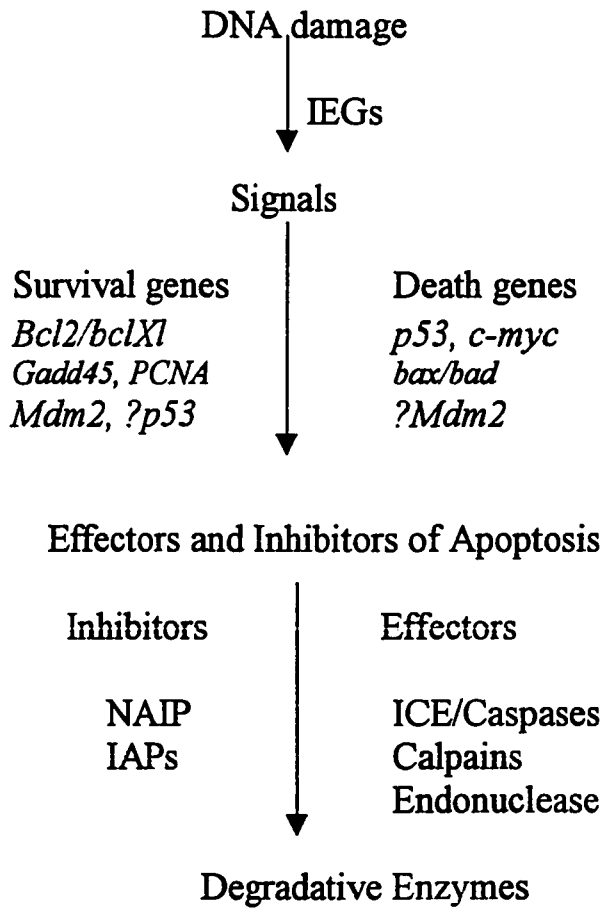


Figure 3. Genetic alteration and the cell death cascade (adopted from McManus and Linnik, 1997).

Gadd45 has been shown to interact with proliferating cell nuclear antigen (PCNA), which is a subunit of the polymerase involved in DNA replication and repair (Smith et al., 1994), while Mdm2 is classically considered to be an inhibitor of p53, that forms an auto-regulatory loop (Momand et al., 1997; Wu et al., 1993). p53-induced apoptosis has been shown to be prevented by Mdm2 (Chen et al., 1996). Therefore, the ability of gadd45 and mdm2 to inhibit p53 suggests that those two genes mediate damaged DNA repair in cerebral ischemic conditions. In fact, an increased Gadd45 expression occurs following p53 overexpression (Chin et al., 1997) in tumor cell lines ML-1 (Zhan et al., 1997). Expression of Mdm2 is also increased in response to DNA damage in certain cell lines (Price and Park, 1994; Kondo et al., 1995). However, the involvement of gadd45 and mdm2 in neuronal cell death following cerebral ischemia is still unclear. Recent studies have demonstrated elevated gene expression of p53 downstream genes, mdm2 and gadd45 following cerebral ischemia (Hou et al., 1997; Tu et al., 1998), suggesting that p53 may mediate DNA repair following ischemic neuronal injury.

The proto-oncogene bcl-2 plays a negative role in the process of apoptosis. In fact, bcl-2 inhibits apoptosis in proliferating cell lines (McDonnell et al., 1989; Mah et al., 1993) and well-differentiated neurons (Allsopp et al., 1993; Garcia et al., 1992; Kane et al., 1993) induced by various insults. It has also been proved that bcl-2 protects against neuronal cell death following cerebral ischemia. Transgenic mice that overexpress the anti-apoptotic protein Bcl-2 are more resistant to ischemic brain damage (Martinou et al., 1994). Similarly, hippocampal and striatal damage induced

by focal ischemia can be attenuated by overexpression of Bcl-2 using a herpes simplex viral vector (Lawrence et al., 1996).

1.14 Does NAIP Pose a Protective Effect to Neurons Injured by Cerebral Ischemia?

Previous exposure to a mild stressor can also alter the susceptibility of neurons to more severe insults. A typical example of this phenomenon is the observation that animals which have undergone hippocampal kindling display considerably less brain injury than controls following kainic acid-induced status epilepticus (SE) (Kelly and McIntyre, 1994). Cerebral cortical spreading depression produced by application of a KCl solution to the cortical surface reduces cortical infarction in a focal model of transient cerebral ischemia (Yanamoto et al., 1998). In a process called ischemic preconditioning, a brief, sublethal ischemic insult protects tissue from a subsequent, more severe injury. Animals exposed to a 2-min preconditioning period of global ischemia show a protection of CA1 neurons of the hippocampus against subsequent 10-min periods of forebrain ischemia (Perez-Pinzon et al., 1997). Similarly, brief middle cerebral artery occlusion, one to five min preconditioning, given 30 min before MCA occlusion confers significant protection as assessed by infarct volume (Stagliano et al., 1999).

Unlike the brief seizures triggered during kindling, kainic acid induces a period of prolonged seizure activity that can last between six to twelve hours. This prolonged period of continuous seizure activity results in extensive neuronal damage in several brain regions including the piriform cortex, hippocampus, midline thalamus and

substantial nigra (Kelly and McIntyre, 1994). The severity of the SE-induced pathology observed in control rats highlights the powerful nature of kindling induced neuroprotection. Despite experiencing SE that is as severe as that observed in naïve rats as determined by measurements of brain electrical seizure activity, hippocampal kindled rats show little evidence of cell loss in the piriform cortex or hippocampus (Kelly and McIntyre, 1994), while damage similar to controls is evident in the midline thalamus and endopiriform nuclei. The selective nature of this neuroprotection provides a powerful paradigm with which to investigate the molecular basis of this phenomenon. The protective capacity of kindled seizures illustrates the remarkable capacity of the brain to protect itself against a metabolic stressor that is normally lethal.

There is little known about the endogenous mechanism(s) responsible for the ability of kindling to produce a state of increased resistance. Given that kainic-acid induced neuronal damage is believed to involve both necrotic and apoptotic processes, there are a number of potential cellular and molecular changes that could influence cell viability during periods of prolonged seizure activity as well. Although initial investigations into the mechanisms associated with SE-induced neuronal damage focused on those related to necrosis, more recent studies have observed morphological and biochemical changes following SE, that are indicative of 'programmed cell death', or apoptosis.

Kainic acid-induced SE produces selective neuronal injury. Using silver degeneration techniques, neuronal cell loss is evident in the piriform cortex and certain subregions of the hippocampus within a few hours following the onset of SE (Kelly and McIntyre, 1994). Combined use of genomic DNA gel electrophoresis, light and

electron microscopy and *in situ* labeling of DNA-breaks as assessed by TUNEL provides strong evidence of an apoptotic component to the neuronal death observed with kainic acid (Simonian et al., 1996; Pollard et al., 1994; Filipkowski et al., 1994).

Alterations in neuronal gene expression have been postulated to be one of the underlined mechanisms causing the apoptotic neuronal death induced by SE. The levels of several proteins, known to influence the likelihood of apoptotic cell death, fluctuate dramatically following the onset of SE (Sakhi et al., 1994; 1996). In a recent report it was shown that bcl-2, a protein believed to prevent the induction of cell death, decreased by 45% following SE, whereas bax, a related protein believed to promote apoptotic cell death, was increased substantially in vulnerable regions of the mouse forebrain (Zhang et al., 1998).

Based on the premise that kainic acid-induced neuronal damage may be apoptotic in nature, we became interested in whether the amelioration of damage observed in previously kindled rats is related to kindling-induced alterations in the levels of genes known to inhibit apoptosis. Of interest was the potential involvement of the recently identified gene known to encode neuronal apoptotic inhibitory protein (*naip*). Isolated as a candidate gene for spinal muscular atrophy (SMA), *naip* itself has shown to be potentially important in preventing cell death induced by a variety of apoptotic triggers (Liston et al., 1996). Basal levels of Naip-like immunoreactivity (Naip-Li) are found throughout the rat CNS (Xu et al., 1997a), yet following transient forebrain ischemia there is a rapid induction of *naip* expression in neurons known to be resistant to ischemic injury. This rapid induction of *naip* is not observed in regions known to degenerate. Recent observations by Xu and colleagues (Xu et al., 1997b)

have been consistent with the view that increasing Naip levels may protect neurons against neuronal apoptosis induced by transient cerebral ischemia. Injection with the bacterial alkaloid K252a, a treatment leading to a transient elevation of Naip-Li in the rat hippocampus, significantly reduced the depletion of CA1 neurons produced by global ischemia by about 50%. Similarly, elevations in Naip induced by intracerebral injection of an adenovirus vector capable of overexpressing *naip in vivo,* conferred a similar resistance to hippocampal neurons during ischemia.

There are many similarities between ischemia and SE induced neuronal cell damage. Thus, I hypothesize that Naip induction may contribute to the neuroprotection to kainic acid induced-neurodegeneration posed by kindling. Hippocampal kindling may decrease the susceptibility of neurons to cerebral ischemia as a result of Naip induction. Therefore, in the present study, I propose to examine the effects of kindling on the expression of both *naip* mRNA and NAIP protein. Furthermore, I will study the neuroprotective effects by hippocampal kindling on transient focal ischemic model, and a temporal and spatial correlationship between kindling induced in Naip overexpression and resistance to ischemic injury will be determined.

CHAPTER 2

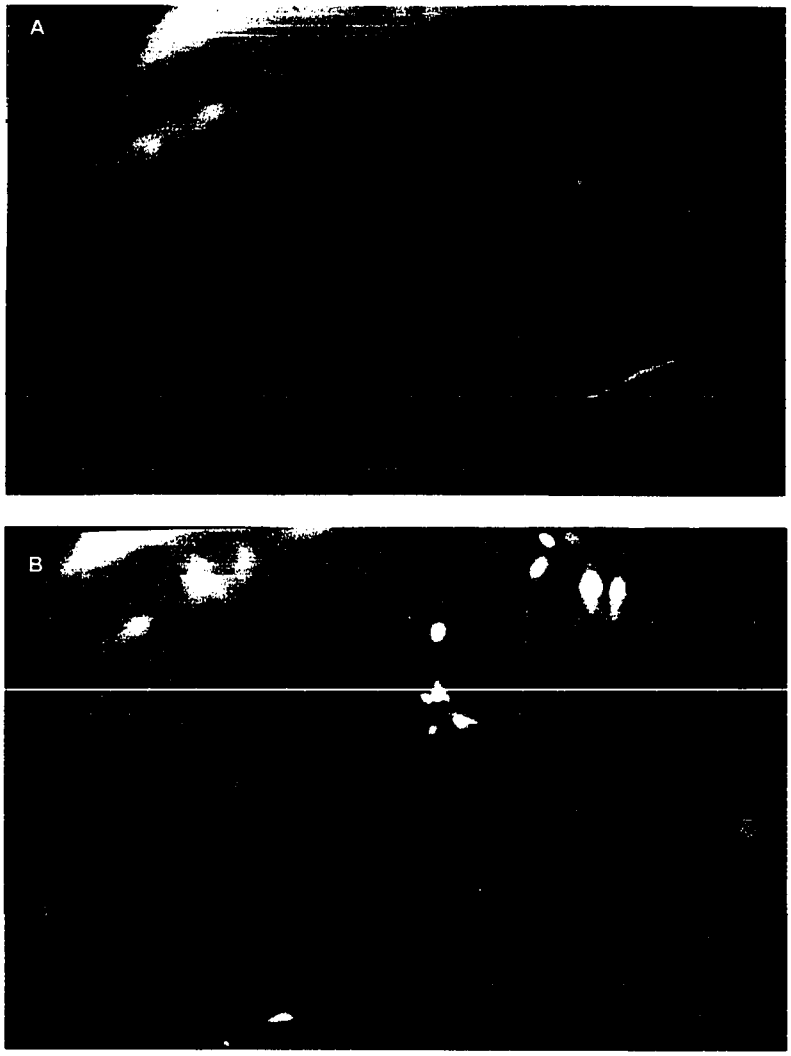
METHODS AND MATERIALS

2.1 Focal Middle Cerebral Artery Occlusion Model

Transient focal ischemia was performed according to Buchan et al (1992). Adult male spontaneously hypertensive rats (SHR) (225-250 g) were kept on a 12 hr light-dark cycle with free access to food and water except for the night prior to the procedure. Animals were anesthetized with a halothane/nitrogen/oxygen mixture (3%/ 70%/27%) during induction, with the halothane reduced to 1-2% for maintenance.

The ventral surface of the neck region was shaved and the exposed skin surface cleansed with Betadine solution. A 1.5cm incision was made in the ventral surface of the neck. The right common carotid artery (CCA) was permanently occluded with #2-0 silk and the wound was closed. The area behind the right eye was shaved and cleansed with Betadine solution and a 1-cm incision was made behind the right eye in front of the external auditory canal to expose the skull. Using a dissecting microscope, the right middle cerebral artery (MCA) was exposed through a 2-3 mm burr hole in the skull 2-3 mm rostral to the fusion of the zygomatic arch with the squamous part of the temporal bone. Drilling was done under a continuous drip of saline to prevent any heat injury to the underlying cortex. The dura was then retracted and animals underwent a temporary occlusion of the middle cerebral artery for 90 minutes by using a 1-mm (Codman clip, Johnson and Johnson) micro-aneurysm clip that was applied to the vessel (Figure 4). Following MCA-o the wound was closed with surgical clips.

Figure 4



Ninety minutes after the initial occlusion of the MCA, the rats were re-anesthetized and the clip was removed under microscopic observation. Upon recovery, animals were returned to their home cages for the remainder of the experiment. Sham-operated control groups underwent the same surgical procedures except that the middle cerebral artery was not clamped.

Figure 4. Photomicrograph illustrating the middle cerebral artery in a rat before (A) and after (B) occlusion.

2.2 Regional Cerebral Blood Flow Monitor

Body temperature was maintained throughout surgery at 37.5°C using a rectal thermistor probe connected by feedback to a heating lamp. While still under general anesthesia, regional cerebral blood flow was measured in the core of the ischemic region using a laser Doppler flow meter. A 1-2 mm diameter burr hole was drilled 3 mm dorsal to the site of middle cerebral artery occlusion (MCA-o) and 3 mm caudal to the bregma. The dura at the site was left intact. The flow meter probe was then advanced with a Narishige micromanipulator under microscopic guidance to a site free of large pial vessels, and positioned to rest on the dura without indenting the cortex. Cerebral blood flow was measured prior to CCA/MCA occlusion (to calculate baseline flow) and then again just after CCA/MCA occlusion. Relative flows could also be measured before and just after reperfusion. Blood flows were recorded as the mean of the maximum and minimum recorded flows over a period of 5 min. and expressed as a percentage of pre-ischemic baseline values.

2.3 Histology

The subjects were decapitated twenty-four hours after the MCA occlusion under the same anaesthetic condition, and their brain was removed and frozen in dry ice cooled isopentane (-65°C). Coronal brain sections, 20 micrometers thick cut at a 1 in 25 intervals, were stained with cresyl violet. The infarct areas (shown in figures 5 and 17) along the brain's entire anterior-posterior axis were measured by a microcomputer-based image display system (MCID, Imaging Research Inc., St. Catherines, Ontario) using the method described by Swanson et al (1990). Infarct

volume was calculated as the integral of the infarct areas for the cortex and expressed as mm^3.

2.4 Hippocampal Kindling

Male Long-Evans hooded rats, weighing 250-300 g at the time of surgery, were housed individually with food and water available at all times. After one week of daily handling, animals were anaesthetised with sodium pentobarbital (60mg/kg, i.p.) and implanted bilaterally with bipolar stimulating/recording electrodes in the dorsal hippocampus (Kelly and McIntyre, 1994). The stereotaxic coordinates were: 2.5mm posterior to bregma, 3.7mm lateral to midline and 3.0 mm below the skull. The electrodes were cut to length, surgically implanted, secured to the skull with six jeweller's screws and a covering of dental acrylic.

Afterdischarge (AD) thresholds were determined one week after the surgery at the electrode sites in the group to be kindled. The AD threshold, defined as the minimum stimulus intensity required to provoke an AD, was determined by challenging with a 2 s, 60 Hz sin wave stimulus of progressively increasing intensity (15, 25, 35, 50, 75, 100, 150, 200, 250, 300 and 350 μA peak to peak) until an AD was triggered. Dorsal hippocampal kindling commenced 24 hrs later. Kindling

Figure 5

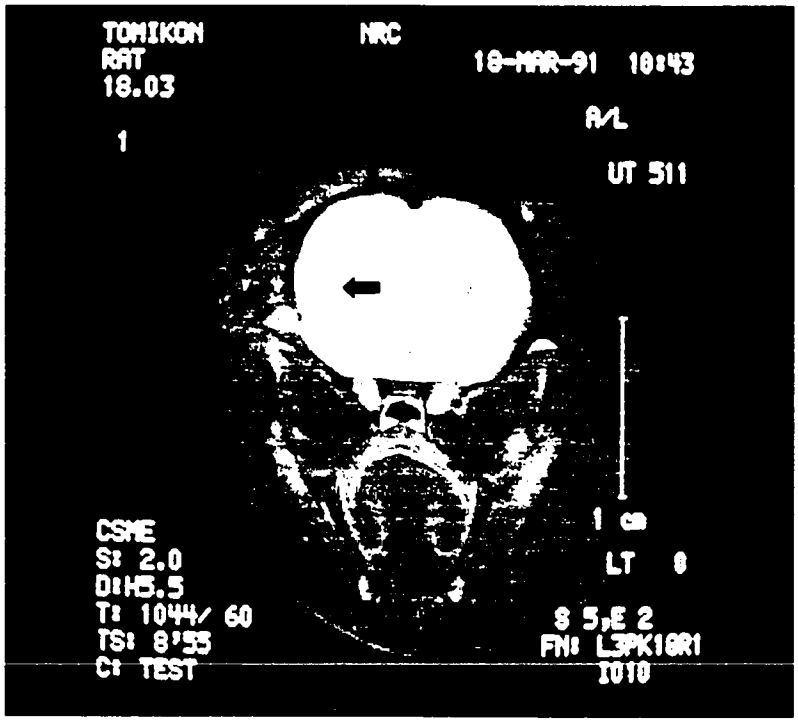

TOMIKON
RAT
18.03
1
NRC
18-MAR-91 10:43
A/L
UT 511
1 cm
LT 0
CSME
S: 2.0
D:H5.5
T: 1044/ 60
TS: 8'55
C: TEST
S 5,E 2
FN: L3PK16R1
I010

Figure 5. Photomicrograph showing a rat brain coronal section by MRI. Notice cortical edema of the right middle cerebral territories after 90min occlusion followed by 22.5 hrs reperfusion (aquired from previous study in same MCA-o model with MRI).

entailed stimulating the dorsal hippocampus at AD threshold with a 2 s, 60 Hz sin wave once daily. The kindling procedure continued until six Stage 5 generalized convulsions were recorded (Racine et al., 1972).

2.5 Oligodeoxynucleotide Probes and In Situ Hybridization Histochemistry

Expression of p53 mRNA was detected by *in situ* hybridization histochemistry (ISHH) (Robertson et al., 1992). Briefly, p53 cDNA antisense probe (Soussi et al., 1988) complementary to bases 13449-13484 of rat p53 (5'-CAGAGACCCAGCAACTACCAACCCATTCCCCAATC-3') was used for the ISHH studies. The probe was 3' end-tailed with ^{35}S-labelled dATP using terminal deoxynucleotidyl transferase to a specific activity of 6 x 10^9 d.p.m./μg. ISHH was performed on fresh frozen brain sections. Twelve-μm-thick sections were cut with cryostat from the prefrontal cortex to lateral septal nucleus. The sections were first immersed into 4% paraformaldehyde for 10 min at 21°C, rinsed 5 min twice in PBS for 5 min and then allowed to dry. Next, the sections were incubated for 1.5 hrs at 42°C in hybridization buffer containing the following: 4 X standard saline citrate buffer (SSC); 0.6 M NaCl; 0.06 M sodium citrate (pH 7.0); 50% deionized formamide; 1 X Denhardt's (0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin); 10% dextran sulphate; 1% N-lauroylsarcosine; 1 mg/kg salmon sperm DNA; 0.2 M dithiothreitol. After this step was completed, the sections were hybridized overnight at 42°C with ^{35}S-labelled probe contained in hybridization buffer (1 x 10^6 d.p.m./100 μl/slide). After

hybridization, the sections are washed at 21°C (20 min) and 1 X SSC (4 x 20 min) at 35 °C, then at 55°C with 1 X SSC (20 min). Finally, the sections were washed again in double-distilled water at 30°C (1 min) and 21°C (5 min twice) and allowed to dry at room temperature. Slides were then dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled water) at 42°C, dried and exposed for 2-3 weeks at 4°C. The slides were developed with Kodak D-19 for 4 min at 18°C, rinsed in water, fixed in Kodak fixer and washed in water. The sections were counterstained with cresyl violet (0.01%, Sigma), dehydrated through a graded series of alcohol and two changes of xylene, and coverslipped for microscopic observation.

2.6 Riboprobe and In Situ Hybridization Histochemistry

The template for a rat *naip* riboprobe was generated by RT-PCR from rat hippocampal mRNA using primers directed against nucleotide sequences conserved in mouse and human *naip* (Roy et al., 1995). A 223 bp sequence was ligated into the EcoR I site of the plasmid pcDNA3 (Invitrogen, Inc.). The automated sequencer starts sequencing at either the T7 polymerase site (antisense) (ACAAAATGTTCTCCCGCT ACATGAAGAAATCCCGTGTAGCTTTGAATATACTGGTTAATTTCCTCTGTGG ATTTCTTACTTTGAAGAAATTCACATTTGGGGAACCACTTGGCATGCTCCTT CCAGGGATCATCTCCTTCTTCCCAGTTTCCCAAGCATCCACCACAGGAGAA ACACTGCACAGTGTCCCTTTTACCTGTAAAGACAAAGCCAGCTGCAAGCC) or the Sp6 site (sense) so that the actual probe sequence doesn't start until it comes to the EcoRI site and it ends at the next EcoRI site, even though the sequencer keeps

going. Both sense and antisense ^{33}P-labeled riboprobes were generated from linearized plasmid templates with the MAXIscript *In Vitro* Transcription Kit (Ambion, Inc.) using the Sp6 (sense) and T_7 (antisense) sites. Specific activities ranged from 0.6 - 1.2 x 10^8 cpm/µl. Tissue sections were immersed in 4% paraformaldehyde for 10 min at 21° C, rinsed 5 min twice in PBS and then dried. Sections were then incubated in hybridization buffer (50% Formamide, 600mM NaCl, 1 X Denhardt's- 0.2% polyvinylpyrrolidone and 0.2% bovine serum albumin, 10mM Tris, 1mM EDTA, 10mM Dtt, 10% Dextran Sulfate and 0.2 µg/µL tRNA) containing either a sense or an antisense *naip* riboprobe (1 x 10^6 cpm/100 µl/slide) overnight at 56°C. Sections were treated with ribonuclease A (10 µg/ml) at 35°C for 30 minutes and washed in 2X SSC for 30 minutes at 21°C. After drying, the slides were dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled water) at 42°C, dried and exposed for 2-3 weeks at 4°C. The slides were developed with Kodak D-19 for 4 min at 18°C, rinsed in water, fixed in Kodak fixer and washed in water. The sections were then counterstained with cresyl violet (0.01%, Sigma), dehydrated through a graded series of alcohol and two changes of xylene, and coverslipped for microscopic observation.

2.7 RNA Extraction, Reverse Transcription and Amplification

Total RNA was extracted from fresh frozen tissues dissected from the ischemic cortex and the corresponding cortical region of sham-operated animals using the QIAGENE protocol. Samples (30 µg) were homogenized in 350 µl of denaturing solution with a Polytron set at maximum speed (15 sec twice). RNA pellets were

washed three times in 70% ethanol, air-dried and redissolved in 25 µl of DEPC-treated water. From this RNA stock solution, a 1:10 dilution was made and 2 µl (1 µg) of this dilution was added to the reverse transcription mixture. This mixture consisted of 5mM $MgCl_2$, 1 X PCR buffer II (50mM KCl and 10mM Tris-HCl, pH 8.3), 1mM of dNTPs, 20 units of RNase inhibitor, 50 units of MMLV reverse transcriptase and 2.5mM oligodT (GeneAmp RNA PCR kit, Perkin Elmer Cetus, Norwalk CT) prepared as a master mix. Reverse transcription was performed at 42° C for 45 min and then heated to 95°C for 5 min to terminate the reaction. For amplification, PCR primers were designed on the basis of the rat p53 sequence (Soussi et al., 1988). The 5' (GTGCAGTTGTGGGTCACCTCC) and 3' (TCCTCTGTCCGACGGTCTCTC) primers generated a 432bp product. PCR was performed as described by Jasmin et al (1993). Briefly, 5µL of the reverse transcription mixture was added to the PCR reaction mixture (0.625 units of AmpliTaqDNA polymerase, 0.125 µg of each of the 5' and 3' primers, $MgCl_2$ in PCR buffer II at a final concentration of 2mM), respectively. Each cycle of amplification consisted of denaturation at 94°C for 1 min, primer annealing at 60°C for 3 min and extension at 72°C for 3 min. Thirty-four cycles were sufficient to generate a strong p53 band on ethidium bromide-stained gels. Control reactions consisting of reaction mixtures without templates were run in parallel both for RT and PCR. Ten µl aliquots of each PCR reaction mixture were electrophoresed in 1.5% agarose gels in Tris-borate EDTA buffer containing ethidium bromide.

2.8 Antisera and Immunohistochemistry for p53

Antisera from two different commercial suppliers were used to detect p53-like immunoreactivity. Mouse monoclonal antibodies Ab-5 (Oncogene science) reacted preferentially with wild type p53. Ab-3 (Oncogene Science) bound specifically to common mutant p53. A mouse monoclonal antibody PAb 240 (Santa Cruz) detected amino acid residues 212 - 217 of the common mutant p53, while mouse monoclonal antibody PAb-246 (Santa Cruz) recognized wild type p53 protein.

Immunohistochemistry was performed according to Robertson et al (1992). Sham and the MCA-o animals were perfused with saline followed by phosphate buffer (pH 7.2) containing 4% paraformaldehyde. Coronal sections (30μm) were cut through the cortex using a vibratome. Sections were washed in 0.01 M phosphate buffeted saline (PBS) containing 0.3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by microwave treatment (two times, 10 sec, 750 W) in 0.01M PBS buffer, sections were then washed three times in PBS and incubated in PBS containing 0.3% Triton X-100, 0.02% azide and either Ab-3 or AB-5 (PAb-240 or PAb-246) primary antisera (1:1000) for 48 hours at 4°C. The sections were then washed 3 times with PBS and incubated with purified biotin-labeled donkey anti-mouse (Jackson laboratories; 1:200) for 12 hours. The sections were washed 3 times with PBS and incubated for 3 hours with PBS containing 0.3% Triton X-100 and streptavidin-horseradish peroxidase (Amersham; 1:200). After 3 washes in PBS, the sections were rinsed in 0.1 M acetate buffer (pH 6.0). The reaction was visualized using a glucose oxidase-DAB-nickel method described previously. Washing in acetate buffer terminated the reaction, and the sections were mounted on

chromalum-coated slides. After drying, the sections were dehydrated through a graded series of alcohols, and two changes of xylene, and coverslipped for microscopic observation.

2.9 Antibodies and Immunohistochemistry for Naip

The antibody against Naip was raised in rabbits against Exons 7-11 of *naip* (150 amino acid fragment) and affinity-purified. Immunohistochemical detection of Naip (diluted 1:1000) was performed on free-floating cryostat sections (12 μm thick) using a previously described method (Robertson et al., 1992). Sham and the kindled animals were perfused with saline followed by phosphate buffer (pH 7.2) containing 4% paraformaldehyde. Tissues were then immersed into 10% sucrose for cryoprotection and frozen coronal sections (12μm) were cut using a cryostat. Sections were washed in 0.01 M PBS containing 0.3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then washed three times in PBS and incubated in PBS containing 0.3% Triton X-100, 0.02% azide and Naip primary antisera (1:1000) for 48 hours at 4°C. The sections were then washed 3 times with PBS and incubated with purified biotin-labelled donkey anti-rabbit secondary antibody (Jackson laboratories; 1:200) for 12 hours. The sections were washed 3 times with PBS and incubated for 3 hours with PBS containing 0.3% Triton X-100 and streptavidin-horseradish peroxidase (Amersham; 1:200). After 3 washes in PBS, the sections were rinsed in 0.1 M acetate buffer (pH 6.0). The reaction was visualized using a glucose oxidase-DAB-nickel method. Washing in acetate buffer terminated the reaction, and the sections were mounted on chromalum-coated slides. After drying, the

sections were dehydrated through a graded series of alcohol, and two changes of xylene, and coverslipped for microscopic observation.

2.10 Western Blotting Analysis

i. Isolation of Cytoplasmic Protein Extracts

Rats were decapitated and the brains were rapidly removed and placed on an ice-cold glass plate. Frontoparietal cortex, about 40 mg, was dissected from the kindled and sham-operated animals. Proteins were extracted from the dissected neocortex according to Doucet et al (1996). Each sample was homogenized in buffer containing 50mM Tris/HCl, 100mM NaCl, 1mM EDTA, 1mM 2-mercaptoethanol and 1mM PMSF, then centrifuged at 12,000 g at 4°C for 10 min (Costar microfuge). Cells were resuspended in 1 ml of 1% Triton X-100 in the above buffer and after 5 min at 4°C and disrupted by douncing (2 ml-Dounce pestle type B, 20 strokes). The extracts were then pelleted by centrifugation at 11,000 g for 20 sec. Each pellet was resuspended in 0.25 ml of high salt buffer containing 0.75 M NaCl, 0.05 M HEPES pH 7.9, 12.5% glycerol, 0.75 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT plus protease inhibitors and incubated on a rotator for 30 min at 4°C. Cytoplasmic extracts were collected by centrifugation at 15,000 g in a Beckman 42.2 rotor. Aliquots (10 µl) were pipetted for protein assay (BioRad) and the extracts were stored at -80°C until further analysis.

ii. SDS-PAGE Electroblotting and Immunostaining

Cytoplasmic protein extracts (20-30 μg) were subjected to electrophoresis in a highly porous SDS-PAGE system (Doucet et al., 1996) which permits rapid and efficient transfer of proteins to nitrocellulose while maintaining a broad degree of resolution. The proteins resolved on 15% running gels (length fixed to 5.5 cm) were transferred to nitrocellulose sheets electrophoretically. The nitrocellulose membranes were first incubated in Tris-HCl-buffeted saline (TBS) containing 5% skim milk for 1 h at 42°C, then incubated overnight at 4°C with Naip antibody at 1:2000 concentration. Blots were then washed in TBS, incubated with a biotin-conjugated anti-rabbit IgG (1:2000; Amersham) for 1 h, streptavidin- horseradish peroxidase (1:2000; Amersham) for 1 h and developed by enhanced chemiluminescent (Amersham). A scanning laser densitometry was used to quantitate the intensity of the bands on the film.

2.11 Experimental Protocol for p53 Studies following Transient Focal Cerebral Ischemia

Animals underwent 90 min MCA-o and followed by reperfusion 1, 4, 8, 12, 24 and 48 hours. Each time point comprised four to six animals that were used for examination of p53 mRNA expression by ISHH. The brains were rapidly frozen in isopentane (-65°C) and coronal sections cut through the fronto-parietal cortical areas using a cryostat. Some frozen sections were used for immunohistochemistry to detect the changes of p53-like protein following MCA-o. Three animals per time point were used for RT-PCR studies. Fresh tissue was dissected from the ischemic penumbra and

the sham cortex of MCA-o animal to compare with p53 mRNA expression detected by RT-PCR.

2.12 Experimental Protocol for Hippocampal Kindling and Naip Induced Neuroprotection Studies

In an initial experiment, we utilised a variety of molecular techniques to examine the expression of *naip* mRNA and protein in the forebrain at several time points following cessation of hippocampal kindling. Four groups, each comprising of 8 kindled and 8 control rats were killed 3 h, 24 h, 7 d or 21 d after their last kindled convulsion. Three of the animals from each of these groups were used for examination of *naip* mRNA expression by ISHH. The brains were rapidly frozen in isopentane (-65°C) and coronal sections cut through the fronto-parietal cortical areas using a cryostat. The effects of kindling on Naip protein levels were assessed in these brain regions by Western blotting using fresh brain tissues dissected from an additional 3 animals from each of the four groups. The final two animals from each of the groups were perfused with saline and 4% paraformaldehyde. The brains were then removed and used for the cellular localization of changes in Naip protein levels by immunohistochemistry.

Experiment 2 was designed to explore the protective effects of kindling on transient focal cerebral ischemia. Three weeks following their last kindled convulsion, kindled (n=8) and associated control (n=6) rats were subjected to 90 min of transient MCA-o. Ischemic brains were rapidly removed and coronal sections cut throughout the cortex using a cryostat. Cortical damage was assessed by the measurement of

infarct volume using the sections stained with cresyl violet using an image analysis system.

2.13 Data Analysis

Analysis of ISHH labelling was performed by digitized image analysis. This process entailed the digitization of sampled areas through a microscope at 100X magnification with a CCD camera to generate a digitized image of 256 X 256 pixel. Each sample area (660 X 800) (as in Figure 15) was first digitized under bright-field illumination and then under dark-field illumination. The camera settings and lighting conditions were held constant for all of the measurements. The matched set of bright-field and dark-field images was then analyzed using a redirected analysis routine. With this routine, neurons in the different regions of the cortex stained with cresyl violet were selected from the bright-field image using a circular field of constant size (400 μm^2), and the average optical density (OD) of silver grains over neurons containing p53 or *naip* mRNA was measured in the corresponding area under dark-field illumination. The average optical density value obtained for each cell was subtracted from 255, which was the maximum optical density value, to provide an inverse optical density, because the silver grains were visualized with dark-field optics. The calculated OD_i value was then used as a measure of label per cell, with higher values indicating greater labelling. A cell was considered to be labeled if its OD_i value was three times greater than background. A total of three measurements, each performed on a separate section, were done for every experimental animal. The cortical areas for quantitative analysis were the same for all the experimental animals in *naip* mRNA

studies. Similar cortical areas were measured for both ischemic and corresponding regions of the contralateral side (non-ischemic) in p53 studies.

2.14 Statistical Analysis

A one-way analysis of variance was performed on the cell counts, average optical densities for data collected from ISHH studies and densitometric data from Western blotting. If the analysis was significant, multiple comparisons were performed using the Newman-Keuls test. An unpaired student t-test was performed on the infarct volumes.

CHAPTER 3

RESULTS

3.1 Transient Focal Cerebral Ischemia Induces p53 mRNA Expression in the Penumbral Region

Changes in neocortex p53 mRNA were quantified by counting the number of silver grain labelled neurons in a 0.3 mm x 0.4 mm area in the penumbra of the MCA territory using an image analysis system. Low basal levels of p53 mRNA were detected by ISHH in non-ischemic brain tissue (Fig 6A, 7A). There was no significant difference between p53 mRNA levels in sham operated animals and the contralateral non-ischemic side of the animals subjected to MCA-o. Transient occlusion of the middle cerebral artery dramatically increased p53 mRNA expression in the ischemic cortex (n=4 per group) (Fig 6C, 6D, 7B). As shown in Figures 6D and 7B, dense labelling for p53 mRNA signal was observed throughout the ischemic penumbral region. This induction was very low at one hour, increased significantly from 4 to 8 hours, peaking at 8-12 hours following reperfusion and was still apparent at 24- 48 h (Fig 8A, 8B). In cresyl violet counterstained sections, the labelling appeared to be neuronal under microscopic observation. No clear labelling was observed in the glial cells. The data demonstrate that neuronal induction of p53 occurs following transient focal ischemia.

Figure 6

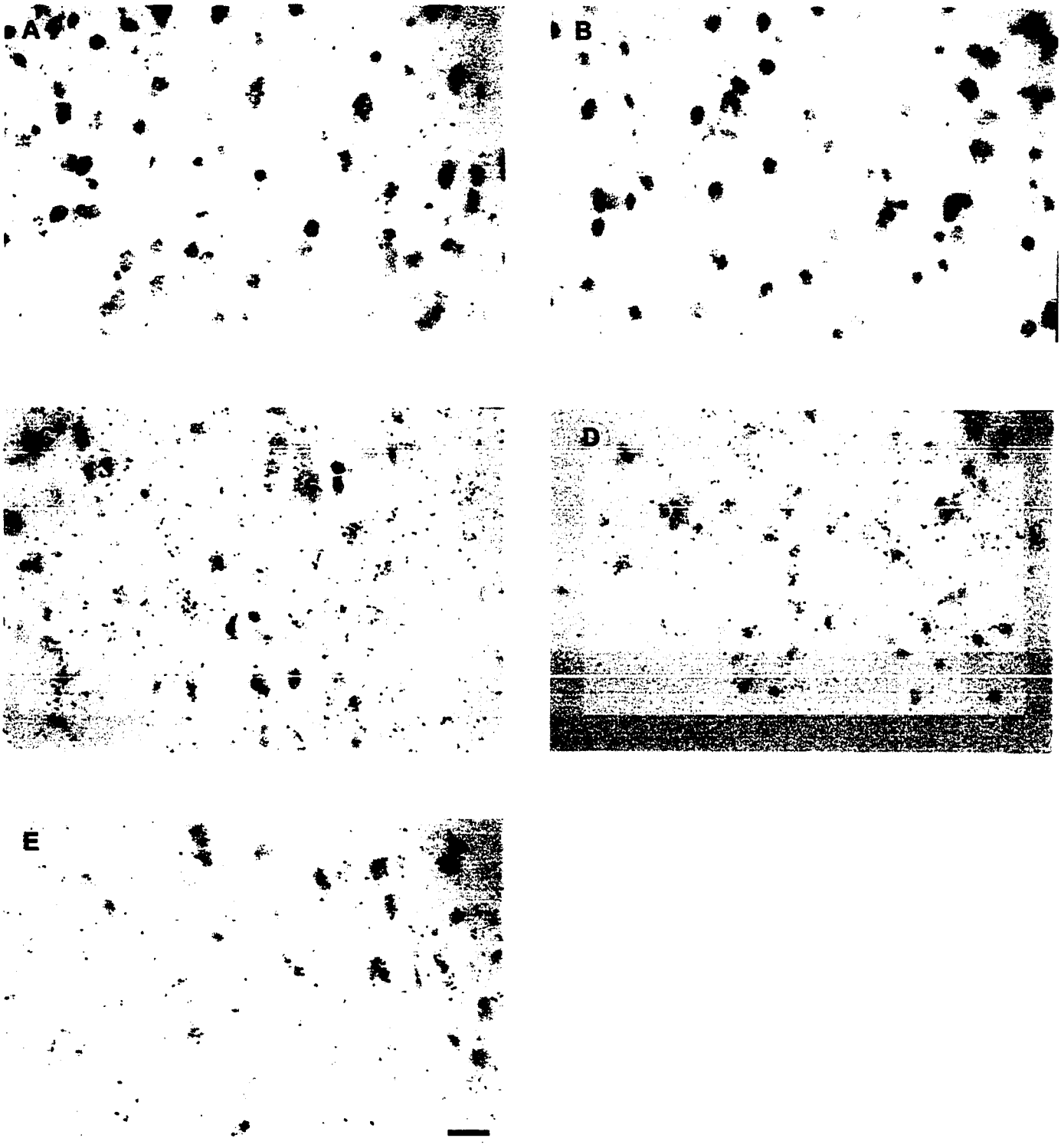


Figure 6 High magnification photomicrographs p53 mRNA-positive neurons in the ipsilateral cortex of animals that underwent either a sham operation (A), or 90 min of MCA-o followed by reperfusion for 1 hr (B), 8 hr (C), 12 hr (D), or 24 hr (E). Notice the elevation of p53 mRNA 8-12 hrs after reperfusion. Scale bar = 50 μm and is the same for all panels.

Figure 7

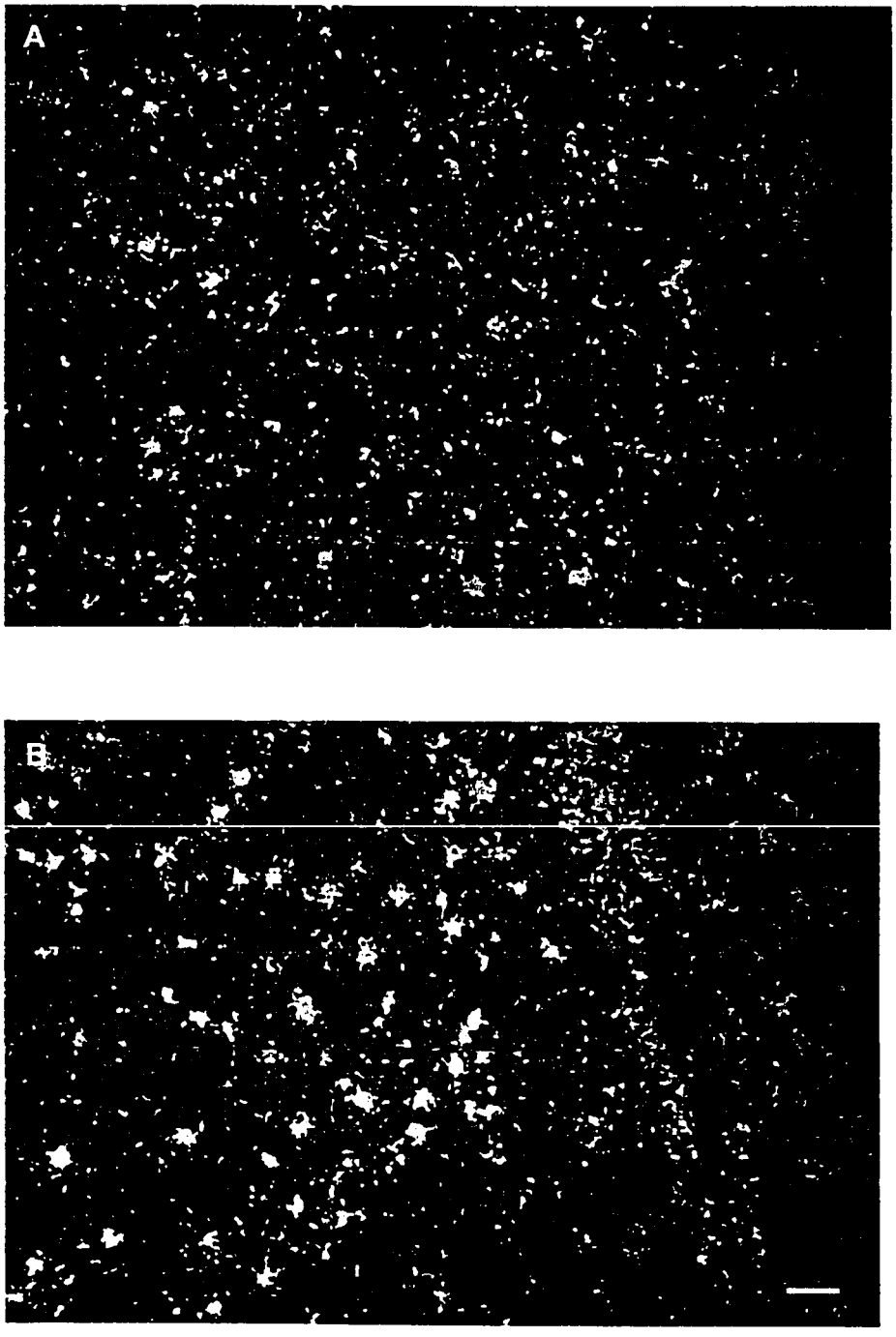
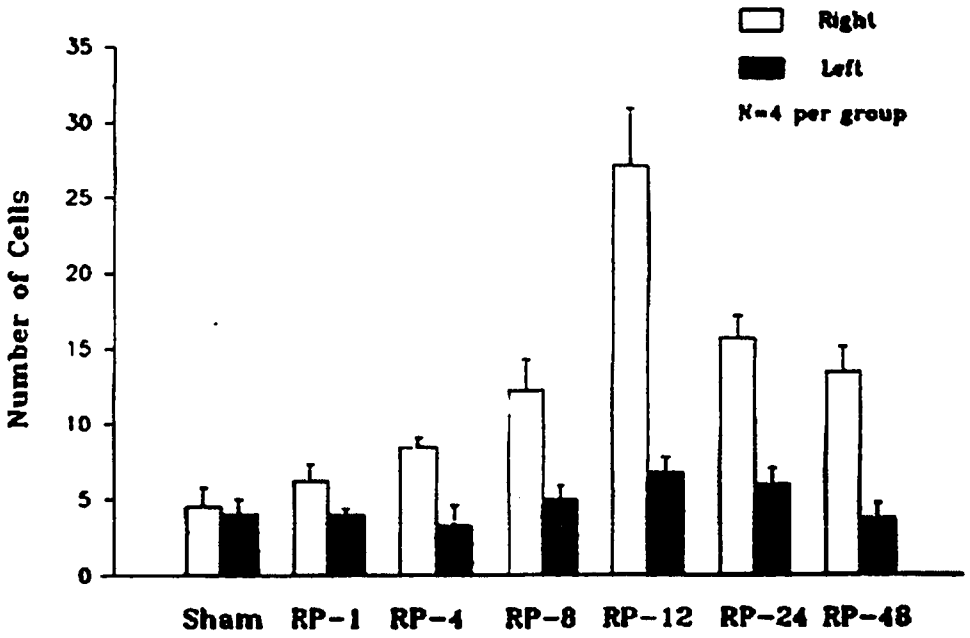


Figure 7 Dark field photomicrographs of p53 mRNA expression in the ipsilateral cortex of sham (A) and MCA-o rats 12 hours after reperfusion (B). Notice the large increase in p53 mRNA-positive cells in (B). Scale bar = 100 μm for both A and B.

Figure 8

A


Right
Left
N=4 per group
Number of Cells
35
30
25
20
15
10
5
0
Sham
RP-1
RP-4
RP-8
RP-12
RP-24
RP-48

B

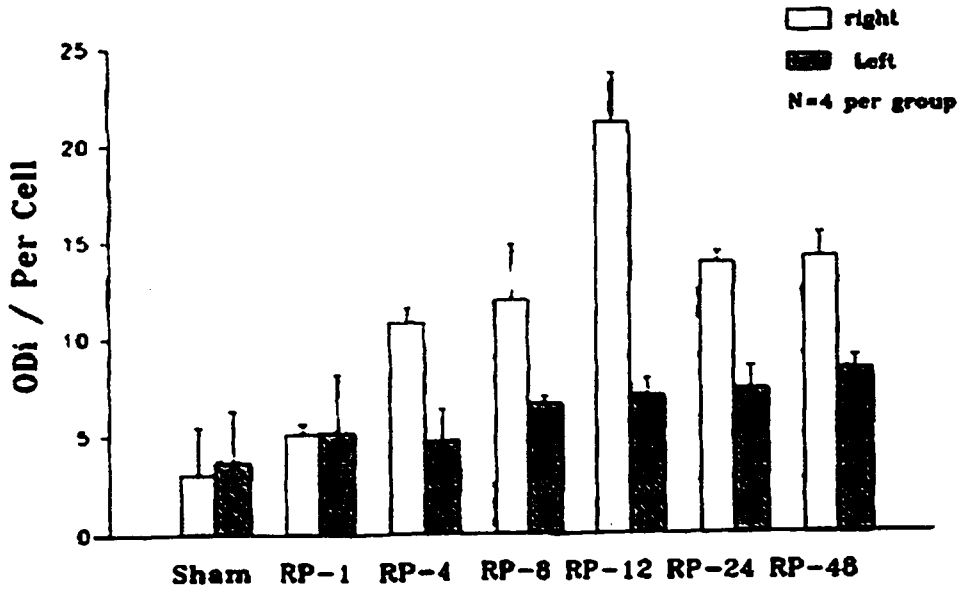

right
Left
N=4 per group
ODi / Per Cell
25
20
15
10
5
0
Sham
RP-1
RP-4
RP-8
RP-12
RP-24
RP-48

Figure 8 Quantitation of p53 mRNA expression in the cortex of sham and MCA-o animals. The top panel shows the effects of these treatments on the number of p53 mRNA-positive cells that were detected in the ipsilateral and contralateral cortex (A). The bottom panel shows the effects of sham and MCA-o on the amount of p53 mRNA expression per cells (ODi/cell) in the ipsilateral and contralateral cortex (B). Each bar represents the average of four animals.

Seven experimental groups, each contained three animals, were used for the RT-PCR. Fresh frozen tissues were dissected from the penumbral area of the ischemic cortex or the corresponding region of the sham-operated. Since ISHH did not reveal any changes of p53 mRNA levels in the core region of the ischemic cortex or in the contralateral side of the ischemic animals, RT-PCR was not performed on tissue containing the ischemic core. Figure 9A shows increased p53 mRNA expression as detected by RT-PCR. The differential intensity of the PCR products stained in the agarose gel at 432 bp clearly reveals that transient focal cerebral ischemia elevated p53 mRNA expression. Although neurons are lost at later time points, the amount of mRNA used for each RT-PCR reaction was the same for all of the groups, as determined by comparing cyclophilin expression in the same tissue extracts (Fig 9B). The basal level of p53 mRNA was very low and remained almost the same at one hour following reperfusion. Thus, the ISHH results were therefore confirmed by RT-PCR which indicated that p53 mRNA expression gradually increased from 4 to 8 h and peaked between 8 to 12 h following reperfusion and was still elevated above basal levels at 48 hours. The consistency of the results obtained by these techniques clearly demonstrates that transient middle cerebral artery occlusion elevates p53 mRNA expression in the ischemic penumbra.

Figure 9

A

p53

Sh 1 4 8 12 24 48

B

Cyclophilin

Sh 1 4 8 12 24 48

Figure 9 p53 transcripts detected by RT-PCR (RT-PCR) in ischemic and control cortices. A. In three experiments, seven time points were sampled for p53 mRNA. A very low level of p53 expression was observed in sham-operated animals. It remained stable at 1h post reperfusion, but slightly elevated 4h later. p53 expression reached a peak between 8 to 12 h following reperfusion and gradually declined 24 h later . Sham, sham operated control; RP, reperfusion. B. Cyclophilin transcripts expressed in the same tissue used for p53 studies. Cyclophilin measurements indicated that same amounts of total RNA were used for the RT-PCR analysis for each of the p53 lanes. Results shown are representative of the findings from three experiments.

3.2 Increased p53-like Immunoreactivities in the Ischemic Penumbral Region

Ischemia-induced changes in p53 protein level were first examined by immunohistochemistry. All of the experimental animals were examined at the same time points as those used for ISHH and RT-PCR studies. A secondary antibody that had been purified against rat antigens was used to minimize the likelihood of cross-reaction between the secondary antibody and serum proteins that may have leaked through the damaged blood-brain barrier. Antibody PAb-240 and Ab-3 that recognize mutant p53 (m-p53), Pab-246 and Ab-5 designed to recognize wild type p53 (wt-p53), respectively, gave similar results. Compared to the very low expression of P53-like immunreactivity (-li) in sham-operated animals, p53-li was elevated by transient focal ischemia. The induction occurred in the ischemic penumbral region (Fig 10C, 10D).

Following only one to four h of reperfusion, neocortical p53-li was unchanged. Moderate p53-li was detected 8 h after blood perfusion was restored, but p53-li was prominent in neurons located at the penumbral region of ischemic cortex 12 h following reperfusion (Fig 10C, 10D). At 24-48 h after initiating reperfusion immunoreactivity in cortical neurons had declined to lower levels (Fig 10E). The positive staining predominantly occurred in cytoplasma. The pattern of p53-li elevation was consistent the mRNA induction following transient focal cerebral ischemia. Omission of primary antibody resulted in the complete absence of p53-li in the ischemic penumbral all time points after reperfusion (10B). Taken together immunohistochemistry demonstrated that p-53-like protein was induced in cortical

neurons of the ischemic penumbral regions by transient focal cerebral ischemia and it was recognized by antibodies raised against both wild and mutant p53.

Figure 10

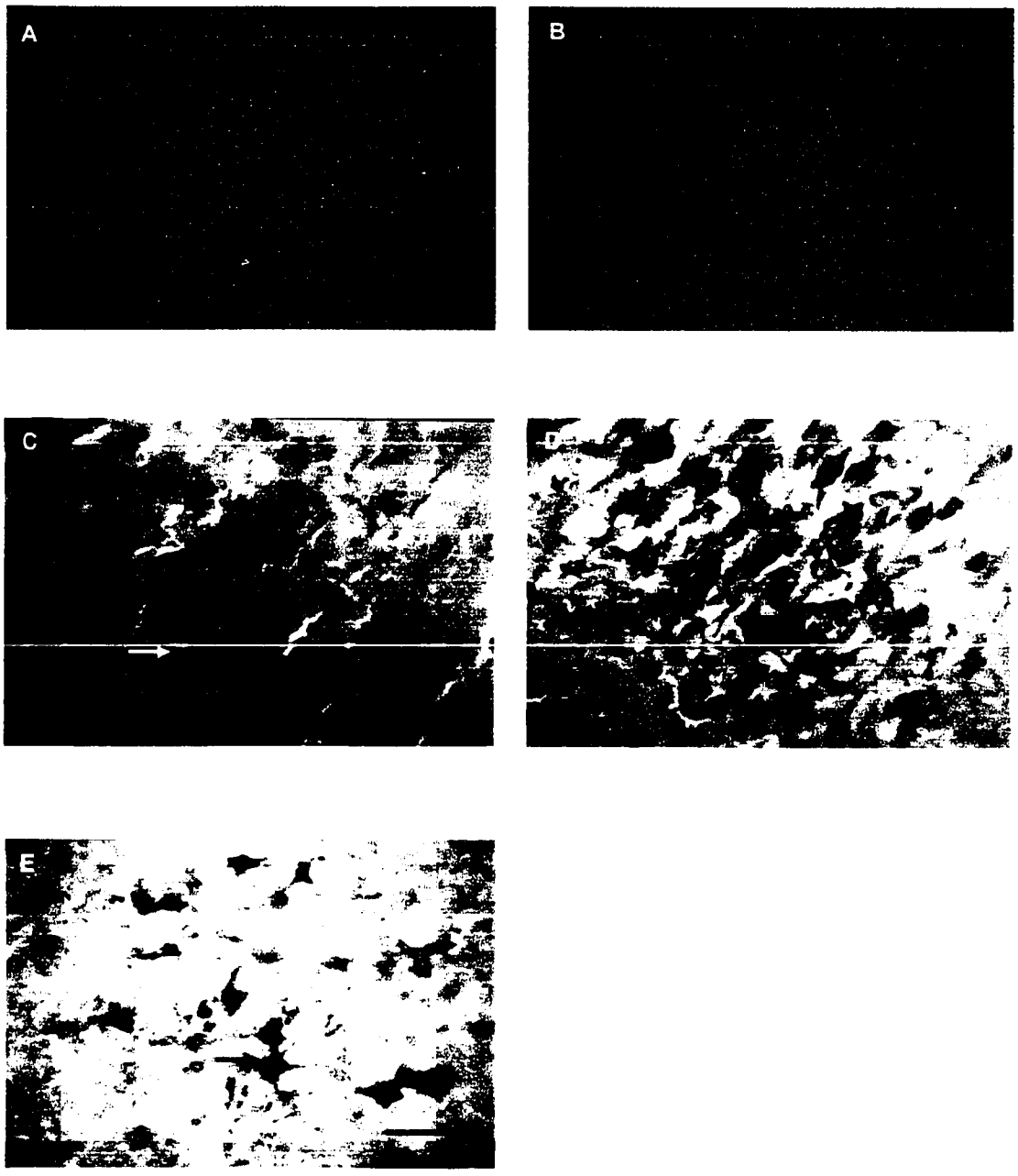


Figure 10. The induction of p53 - like immunoreactivity in the penumbral region of ischemic cortex following 90 min. of middle cerebral artery occlusion. A. Basal levels of p53-Li in sham controls were very low. B. Omission of the primary antibody (PAb-240 or PAb-246) resulted in complete loss of immunoreactivity. C. 12-h reperfusion, dramatic elevations in m-p53-Li were observed in the ischemic penumbra. D. 12-h reperfusion, significant elevations in w-p53-Li were observed in the ischemic penumbra. E. wt-p53-li gradually declined to lower level at 24-h post reperfusion. Scale bar = 50 mm and is the same for all panels.

3.3 Hippocampal Kindling Elevates naip Expression in Several Brain Regions

The specificity of *naip* probe was examined as demonstrated in figure 16. The *naip* sense probe did not show any labelling in neuronal tissue (16 A). Specific *naip* mRNA neuronal labelling detected in the adjacent section of the same animal (16 B). ISHH was used to determine whether *naip* mRNA could be induced by an experimental manipulation, known to be neuroprotective, such as hippocampal kindling. In fact, hippocampal kindling dramatically increased *naip* expression in several brain regions. ISHH revealed that the elevation of *naip* mRNA occurred mainly in the hippocampus and piriform cortex (Fig 11B, 11F; Fig 12B, 12D). Hippocampal kindling did not alter *naip* mRNA expression in the endopiriform cortex and the medial thalamic nuclei compared to baseline levels (Fig 11D, 11H). Kindled groups displayed a significant increase in the numbers of cells which expressed *naip* mRNA in the piriform cortex and hippocampus (Fig 13A, 13b). In addition, the intensity of ISHH labelling expressed, as the average inverse optical density, was two to three folds greater in kindled than control rats (Fig 13E, 13F). Although only one side of the hippocampus was electrically stimulated, *naip* expression was elevated to a comparable degree on the left and right side of the brain. Enhanced *naip* mRNA was detected at 3 h and remained elevated at the 1, 7 and 21 d time points (Fig 13).

Figure 11

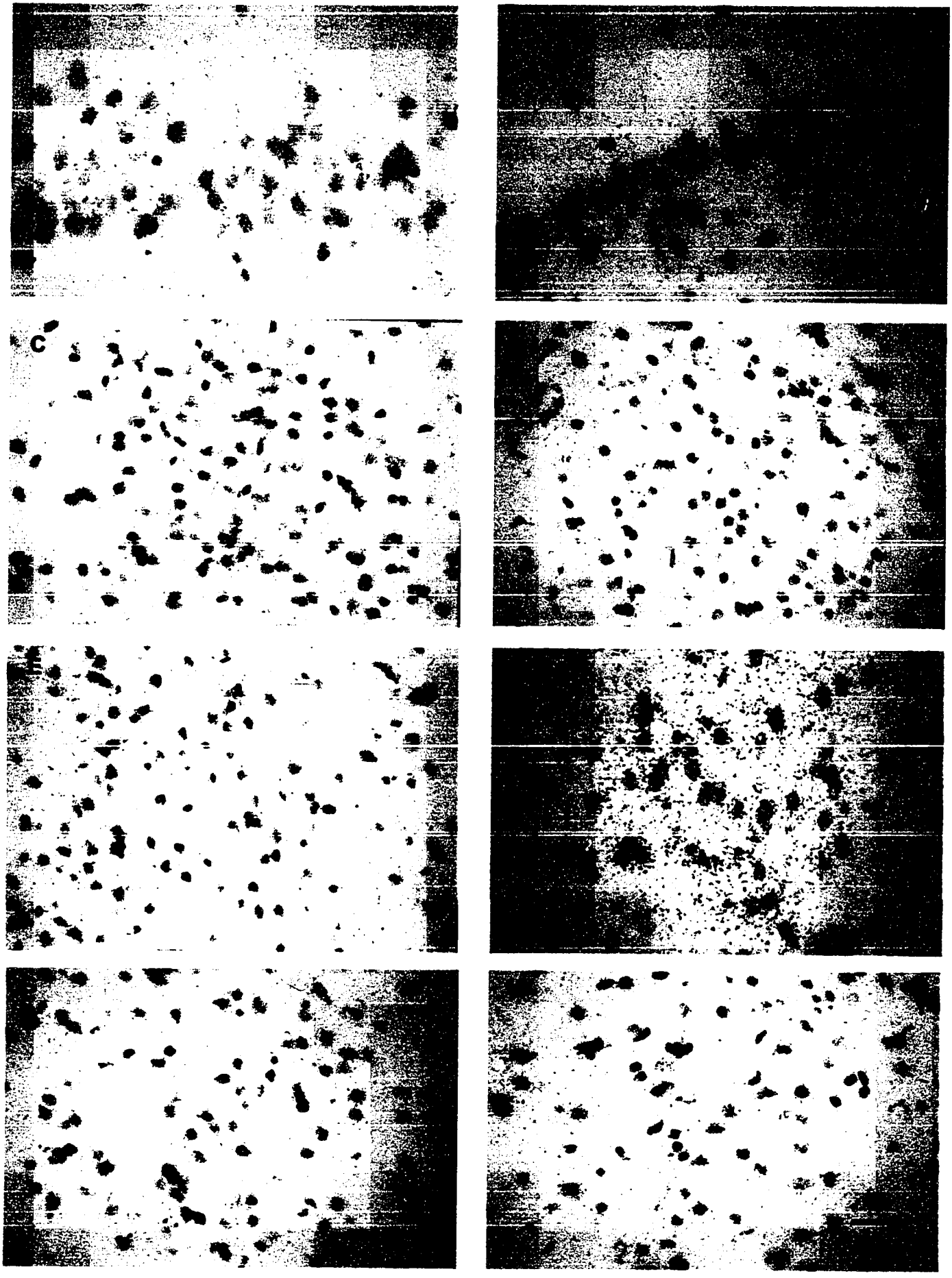


Figure 11. Photomicrographs depicting the effects of hippocampal kindling on expression of *naip* mRNA in several brain structures by *in situ* hybridization histochemistry. Similar baseline levels of *naip* mRNA was present in neurons of hippocampus (A), medial thalamic nuclei (C), piriform cortex (E) and endopiriform cortex (G) in the control animals. *naip* mRNA levels were significantly elevated in neurons of the hippocampus (B) and piriform cortex (F) of kindled animals 3d after the last episode of kindling treatment. The elevation of *naip* mRNA remained increased throughout three weeks period. In contrast, *naip* mRNA was not elevated in the medial thalamic nuclei (D) and endopiriform cortex (H) by kindling. Scale bar = 35μm.

Figure 12

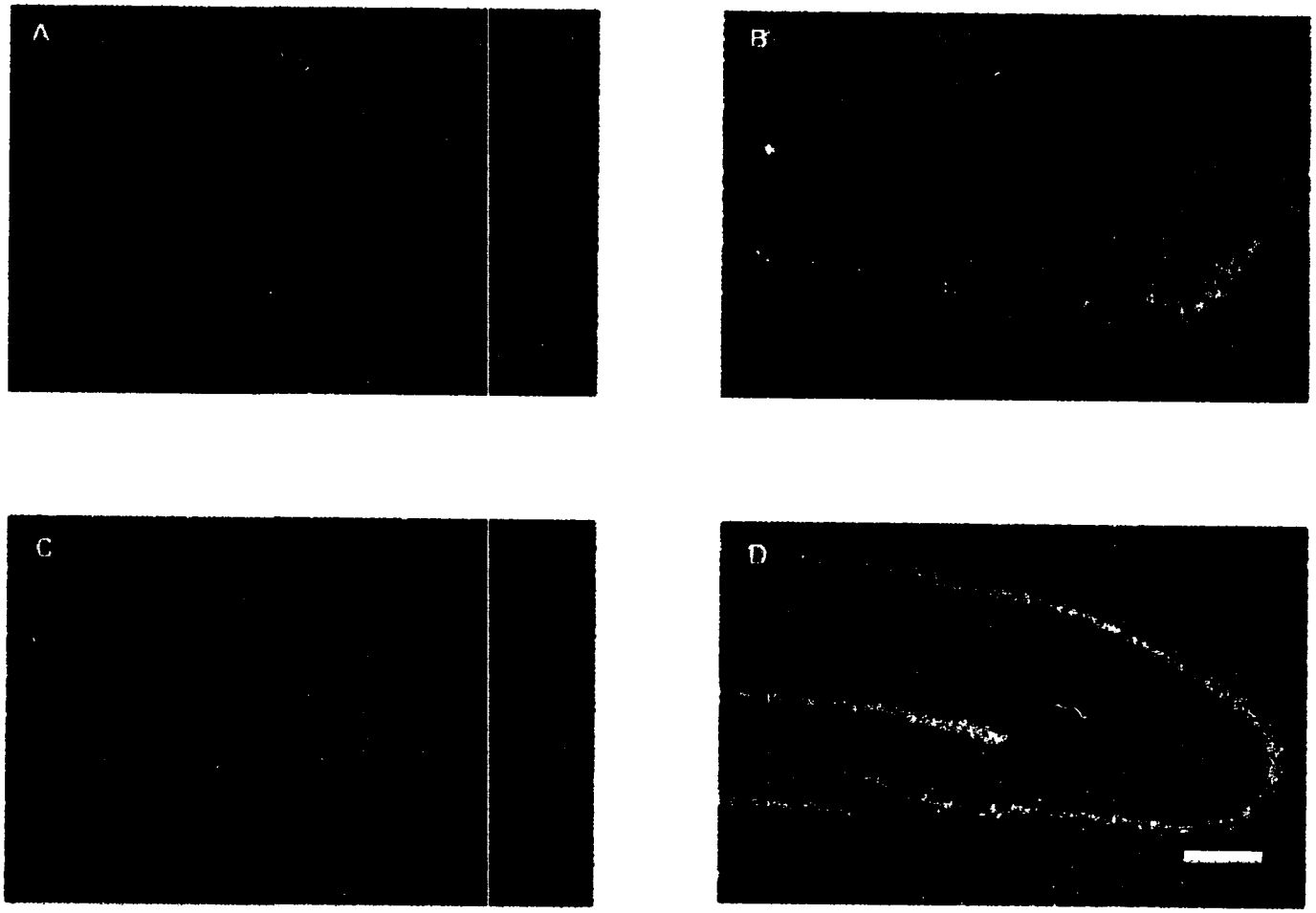


Figure12. Dark field photographs of *naip* mRNA expression in the piriform cortex (A) and hippocampus (C) of control animals, and the piriform cortex (B), hippocampus (D) of hippocampal kindled animals three weeks after the last kindling treatment. Note the large elevation of *naip* mRNA in these brain structures of kindled animals. Scale bar = 15μm.

Figure 13

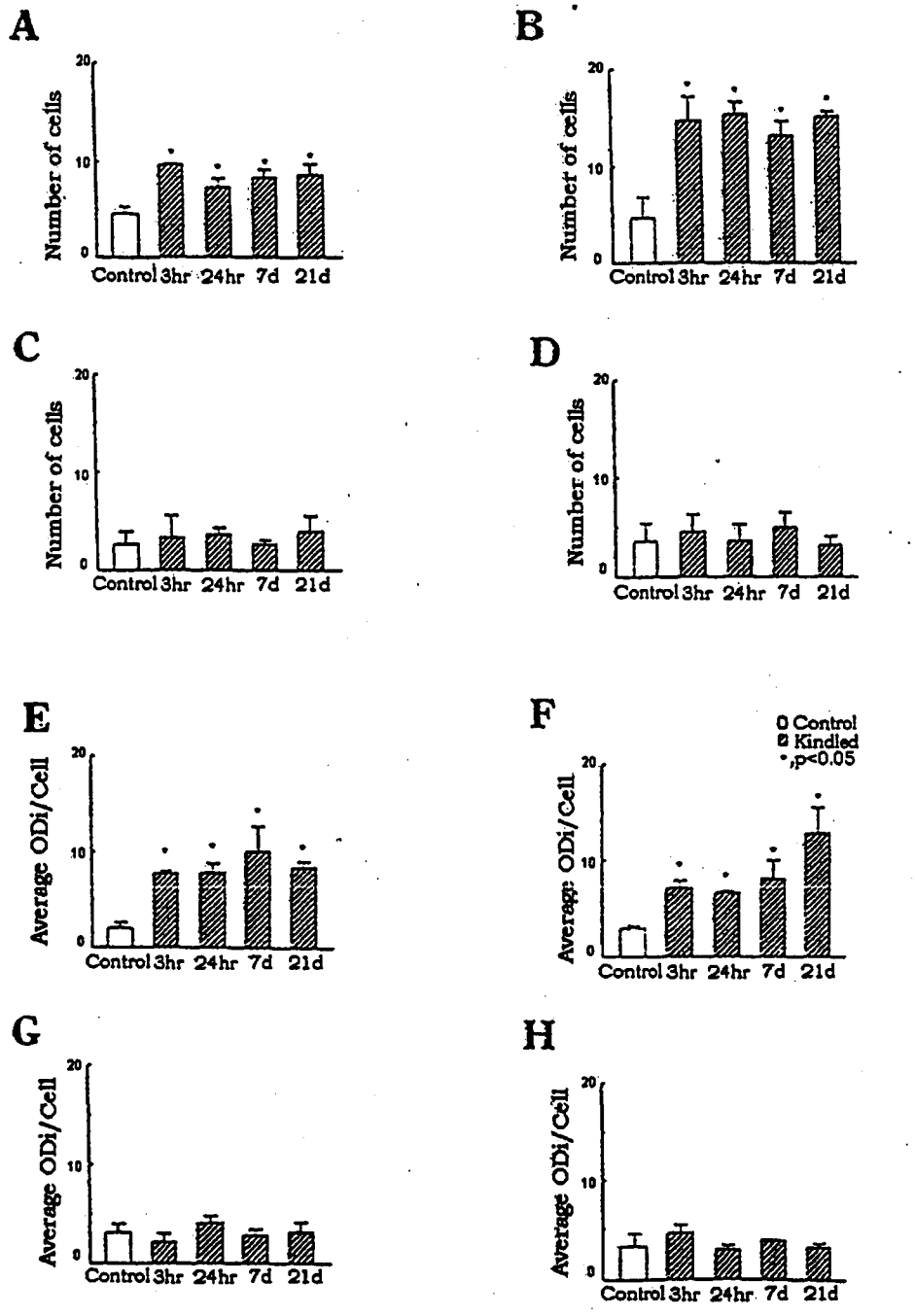

A
B
C
D
E
F
G
H
Number of cells
Average ODi/Cell
20
10
0
Control 3hr 24hr 7d 21d
Control
Kindled
*,p<0.05

Figure 13 Bar graphs depicting the average number of cells and intensity of labelling in different brain structures which exhibited *naip* mRNA after hippocampal kindling treatment. The average number of positive cells labeled and their optical densities were significantly greater than controls in piriform cortex and hippocampus of the kindled animals (13A, 13B, 13E, 13F). Labelling for *naip* mRNA was similar for each of the time points. The average number of positive cells labeled and optical densities of labelled cells were similar in kindled animals and controls in the medial thalamic nuclei and endopiriform cortex (13C, 13D, 13G, 13H). Histograms and bars represent mean and standard error of the mean for three to four animals. Asterisk, significant difference from controls ($p < 0.01$; Newman-Keuls test).

Consistent with the ISHH results, Naip-Li was also elevated in animals that received hippocampal kindling. Enhanced Naip-Li immunoreactivities were observed bilaterally in the hippocampus and piriform cortices (Fig 14B, 14H). This increase in Naip-Li appeared to be specific neurons. Basket cells in the hippocampus and pyramidal cells in layer 3, 4 and 5 of the cortex were among the populations most heavily labelled with Naip antibody. Naip-Li was most intense at 3 and 24 h, but appeared to remain elevated 7 and 21 d later. There were no significant changes of Naip-Li in neurons of the endopiriform cortex and the medial thalamic nuclei of kindled animals at any of the time points relative to controls (Fig 14C, 14D, 14E and 14F).

3.4 The Neuroprotective Effects of Hippocampal Kindling Are Closely Associated with naip Induction

Hippocampal kindling has been shown to impart neuroprotection in certain brain structures (Kelly and McIntyre, 1994). We therefore examined the effects of kindling on levels of Naip protein as determined by immunohistochemistry. If kindling increased resistance to excitotoxic brain injury by increasing Naip levels, a strong correspondence between regions displaying elevated Naip-li and those areas resistant to excitotoxins would be expected. This section examines anatomical mapping correlation to provide further evidence that the neuroprotection by hippocampal kindling is directly related to *naip* induction. ISHH studies were completed on brain tissue from rats that were injected with kainic acid following the kindling procedure.

Figure 14

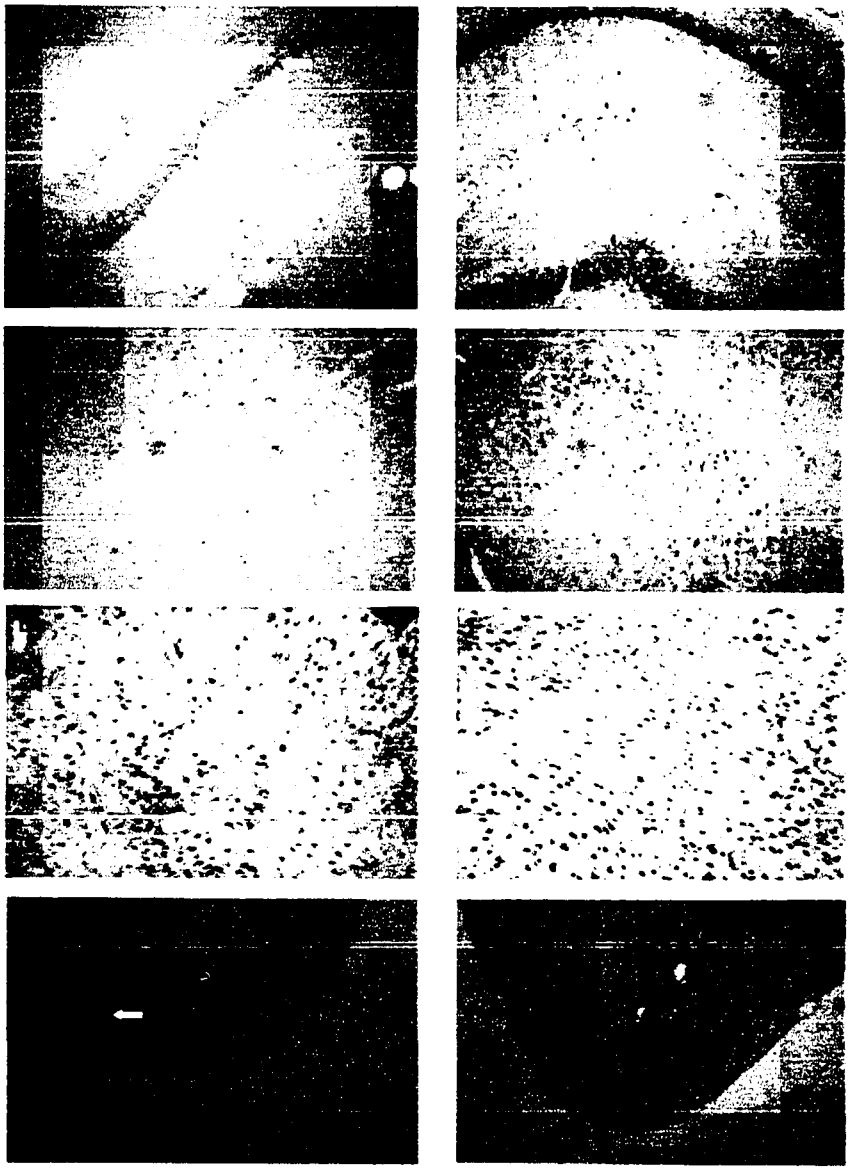


Figure 14. Effects of hippocampal kindling on Naip-like immunoreactivity (Naip-li) in several brain structures. Baseline levels of Naip-li were observed in the control hippocampus (A), endopiriform cortex (C), medial thalamic nuclei (E) and piriform cortex (G). Significant elevation of Naip-li levels were exhibited in neurons of the hippocampus (B) and piriform cortex (H) in kindled animals seven days after last kindling treatment. There was no significant difference between time points. Naip-li remained unchanged in the endopiriform cortex (D) and medial thalamic nuclei (F) of the kindled animals. Scale bar = 15 μm.

Systemic injection of kainic acid has been shown to induce seizures and cause neuronal cell death in the brain. Kindling prior to kainic acid administration offers a remarkable degree of protection against kainic acid induced cell death in certain brain structures (Kelly and McIntyre, 1994). Levels of *naip* mRNA in control animals were compared to those found at four time points following kainic acid injection and to those found at the same times for kindled rats following kainic acid administration. At 30 and 90 minutes following kainic acid injection alone, there was a large degree of variability from animal to animal in *naip* mRNA. However, at 3 hours and 6 hours the results were consistent. Kainic acid administration alone produced a mild elevation of *naip* mRNA (Fig 15D), while kainic acid triggered a tremendous increase in *naip* mRNA induction in kindled animals (Fig 15E) compared to sham kindled animals (Fig 15A). *naip* mRNA levels were especially high in the hippocampus, piriform cortex, and to a lesser degree in the perirhinal and fronto-parietal cortical areas, which have been demonstrated to be resistant to kainic acid injury following hippocampal kindling. n*aip* mRNA expression was unchanged in those regions which fail to display resistance to excitotoxic injury following kindling such as the endopiriform cortex and medial thalamic nuclei.

3.5 Hippocampal Kindling Reduces Infarct Volume following Transient Middle Cerebral Artery Occlusion

The next logical step was to determine if the neuroprotective effects of hippocampal kindling could be extended to other injurious stimuli such as cerebral ischemia. Three weeks after the last kindled convulsion, kindled (n=8) and operated

control (n=6) animals were subjected to 90 min of middle cerebral artery occlusion and permitted to survive for 24 h. The total cortical infarct volume in kindled animals (57.7±4.6 mm^3) was drastically smaller than that observed in controls (156.5±12.6 mm^3) (Fig 17, 18). This demonstrates that hippocampal kindling dramatically reduced the damaging effects of transient neocortical ischemia as well as that induced by kainate.

Figure 15

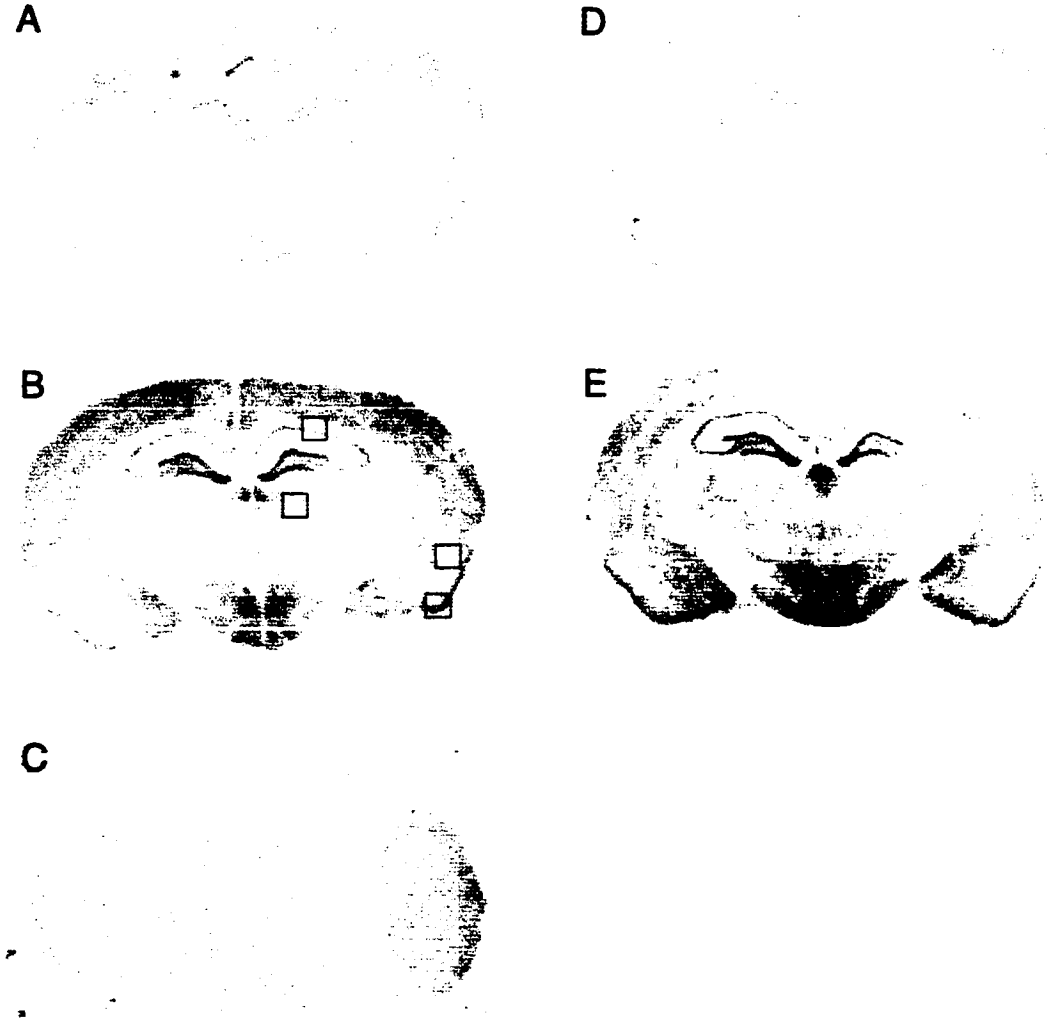
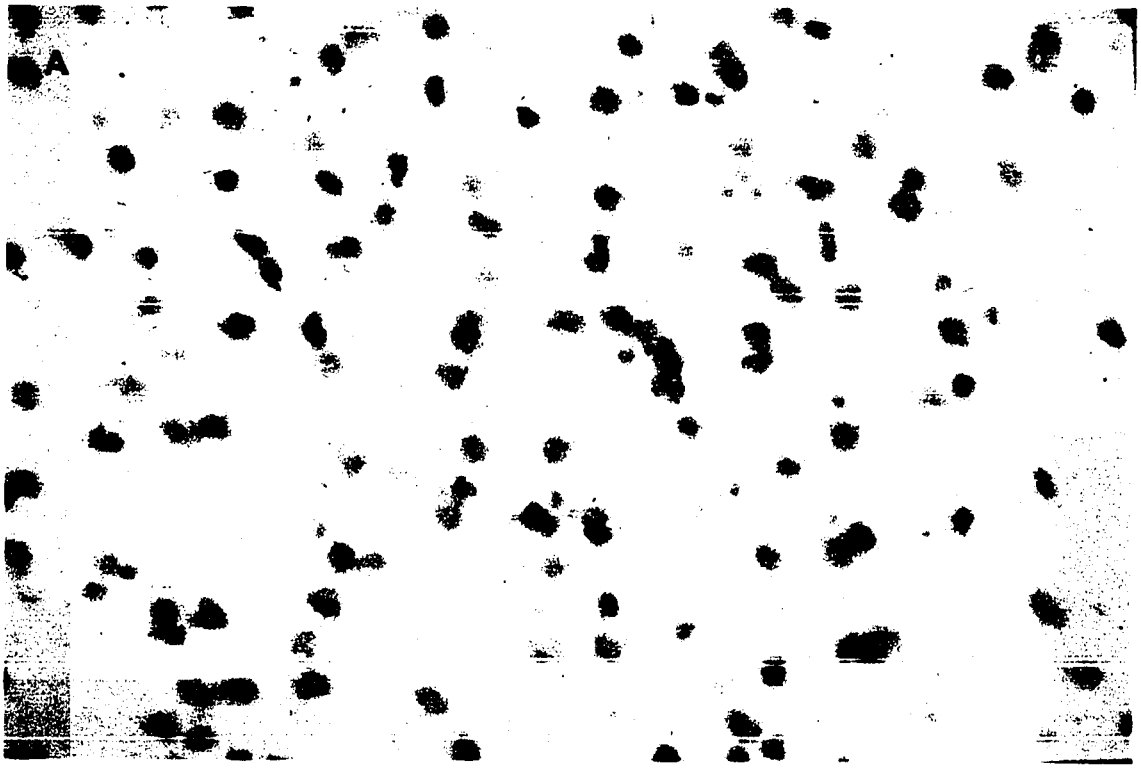


Figure 15 Effects of hippocampal kindling and kainic acid injection on expression of naip mRNA detected by ISHH in the rat brain. Low levels of naip mRNA were present in the control animals (A). Hippocampal kindling alone selectively elevated naip mRNA levels in several brain structures including piriform cortex, hippocampus and fronto-parietal cortex (B). A moderate increase of naip mRNA in similar structures was seen six-hour after Kainic acid injection (D). Hippocampal kindled animals injected with kainic acid produced much greater enhancement of naip mRNA in the similar regions (E). *naip* sense probe did not show any specific labelling (C). The square box illustrates the areas subjected to quantification by image analysis.

Figure 16



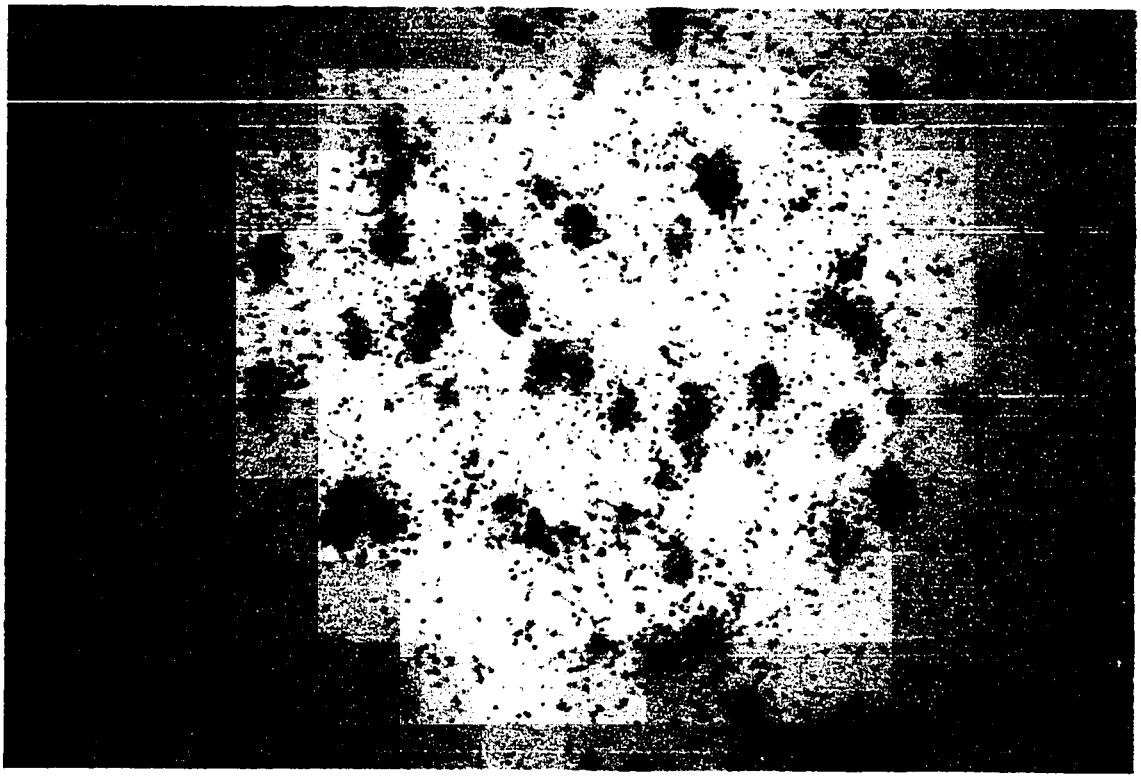


Figure 16 Specific labelling of *naip* mRNA in the piriform cortex of kindled animals detected by *in situ* hybridization histochemistry. The *naip* sense probe did not show any specific labelling in the piriform cortex in a kindled animal 3h after the last kindling seizure (A). Specific *naip* mRNA neuronal labelling detected in the adjacent section of the same animal (B). Scale bar = 35 μm.

Figure 17

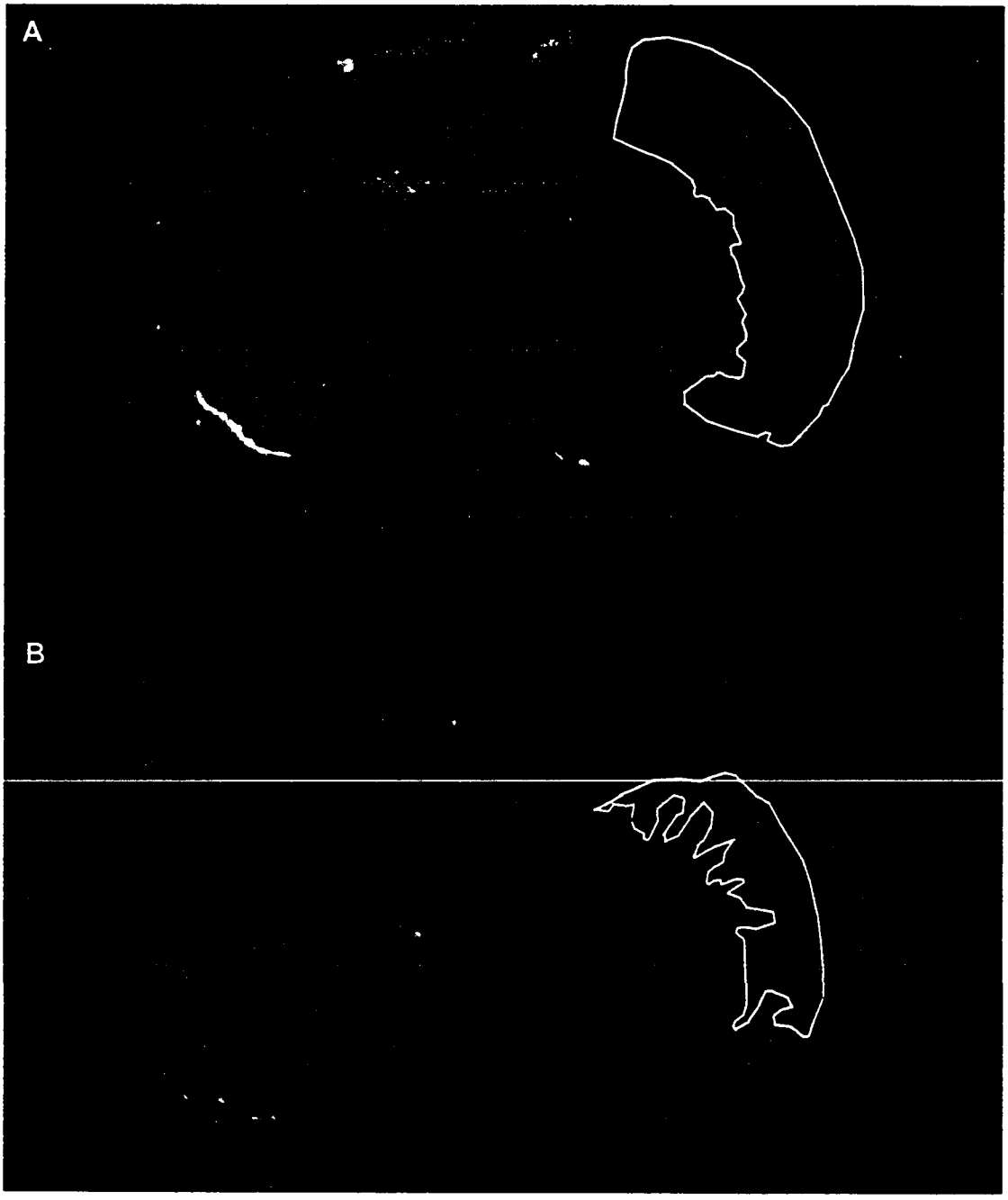


Figure 17. Protective effects of hippocampal kindling on the infarct volume following transient focal ischemia. A large area of the infarct can be seen in the control animal subjected to 90 min of MCA-o (A). Hippocampal kindling dramatically reduced the area of infarct produced by 90 min of MCA-o (B).

Figure 18

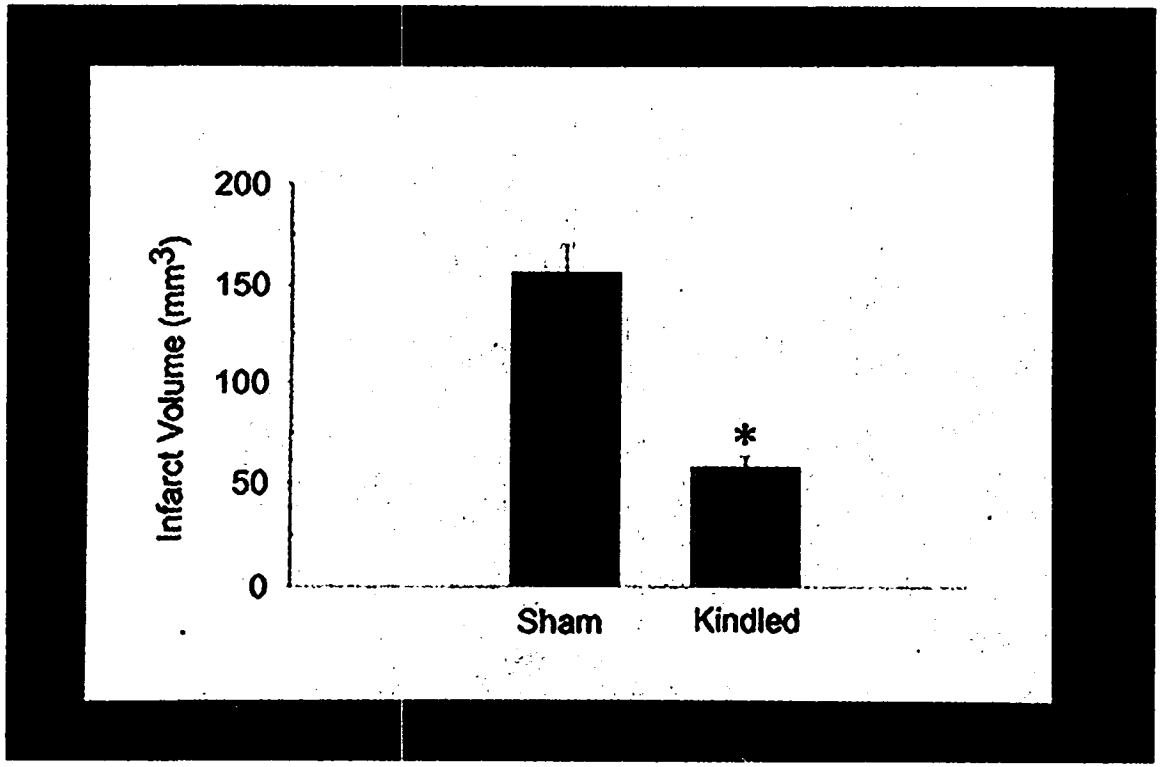
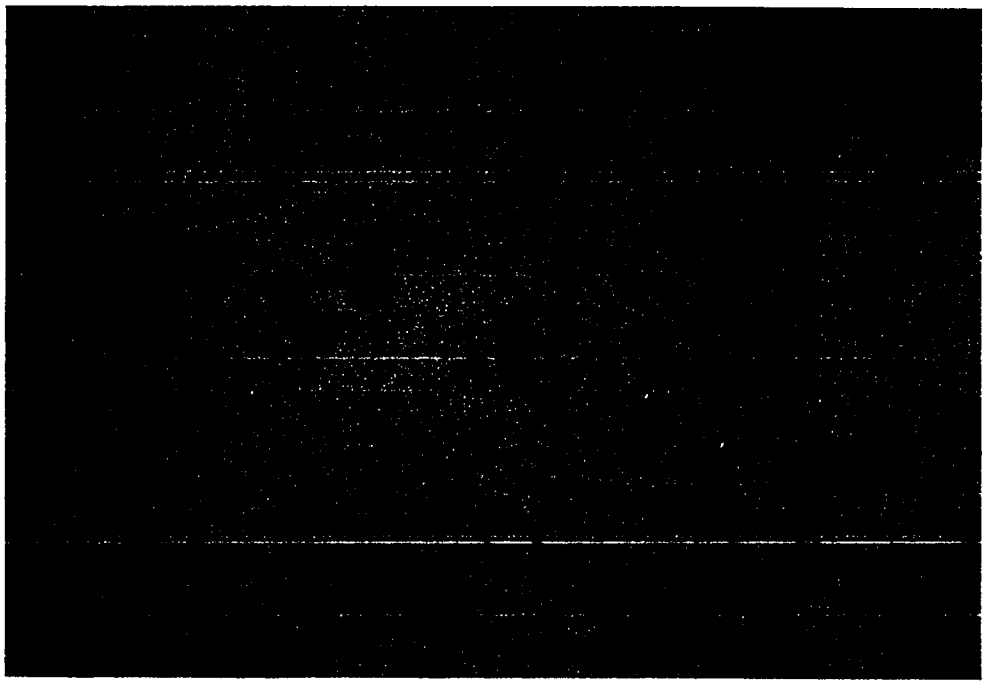


Figure 18. Bar graphs depicting the average infarct volume in the cerebral cortex 24 hr following transient MCA-o. The cortical damage in the control animal was 156.5 ± 12.6mm3. Kindling reduced the volume of the infarction by about 60% to 57.7± 4.6mm3. Histograms and bars represent mean and the standard error of mean for 6 to 8 animals. Asterisk, significantly different from control ($p<0.01$, t-test).

Figure 19



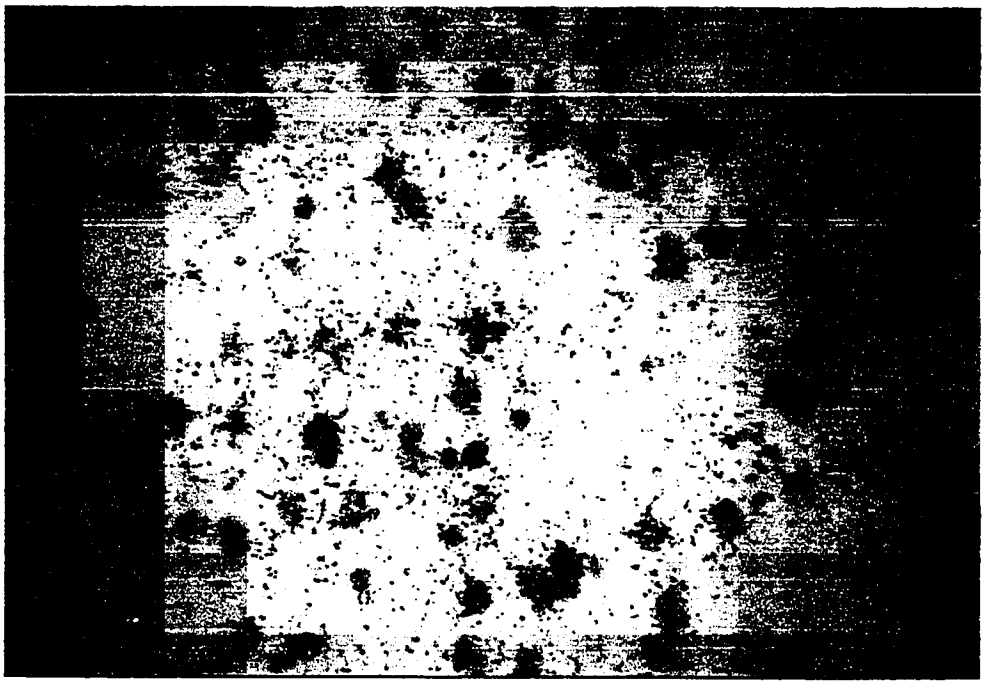


Figure 19 Photomicrographs depicting the effects of hippocampal kindling on expression of *naip* mRNA in the cortex detected by *in situ* hybridization histochemistry. Low levels of ***naip*** mRNA were present in the control animals (A). *naip* mRNA levels were dramatically elevated in cortex 21 d after the last kindled seizure (B). Scale bar = 35 μm.

3.6 Hippocampal Kindling does not Alter Regional Cerebral Blood Flow

Direct measurement of regional cerebral blood flow (rCBF) revealed that the baseline levels of blood flow were similar in both control (108 ± 7.8, n=6) and kindled animals (109.8 ± 4.4, n=8). A dramatic fall of the rCBF was detected during the ischemic period in which the rCBF levels were all below 12% of the baseline in both groups. This rCBF level remained persistent in the control animals for the 90 min artery occlusion, while kindled animals showed a slight increase in rCBF that were not significantly different from control values (Table 2). The levels of rCBF fully recovered after removal of the clip in both control (111 ± 6.3) and kindled animals (108.4 ± 3.2). This data suggested that hippocampal kindling did not exert its protective effects through changes in rCBF, indicating that the reduction of infarct volume in kindled animals was based on some other factor.

3.7 Hippocampal Kindling Elevates naip mRNA Expression in the Neocortex

MCA occlusion produces ischemia mainly in the fronto-parietal cortex. In the previous section I showed that *naip* mRNA elevation induced by hippocampal kindling occurred primarily in the piriform cortex and hippocampus, areas protected from kainic acid induced injury. This section was designed to study the effects of hippocampal kindling on areas injured by ischemia, such as the fronto-parietal cortex. Apparently hippocampal kindling also dramatically increased *naip* mRNA expression in these neocortical areas (Fig 19B). ISHH revealed that the kindled groups displayed

Table 2 Effects of the hippocampal kindling on the regional cerebral blood flow during focal ischemia

Baseline	MCA-o	Pre-RP	Post-RP	24 h
	(Mean ± SEM) (ml/100g/min)			
Control (n = 6)	108.0 ± 7.8	11.7 ± 1.7	10.6 ± 0.6	111.0± 6.3
Kindled (n = 8)	109.8 ± 4.4	10.9 ± 0.6	13.6 ± 0.9	108.4± 3.2

Data represents the mean and standard error of the mean for 6 to 8 animals.

rCBF, regional cerebral blood flow = ml/100g/min; MCA-o, middle cerebral artery occlusion; pre-RP, pre-reperfusion; post-RP, post-reperfusion.

a significant increase in the numbers of cells which expressed *naip* mRNA, i.e. the kindled animals had significantly more positive cell counts per field compared to control animals. The elevation of *naip* mRNA expression persisted for as long as 21 days after the last kindled treatment (Fig 20A). Also the intensity of ISHH labelling, expressed as the average inverse optical density, was two fold greater in kindled than control rats (Fig 20B). Although only one side of the hippocampus was electrically stimulated, *naip* expression was elevated to a comparable degree on the left and right side of the brain. Enhanced *naip* mRNA was detected in the neocortex of the contralateral side of the brain in hippocampal kindled animals at 3 h and remained elevated at the 1, 7 and 21 d time periods.

3.8 Hippocampal Kindling Elevates Naip-li Expression in the Neocortex

Consistent with the ISHH results, Naip-li was also elevated in animals that received hippocampal kindling. Enhanced Naip-li was observed bilaterally in the frontoparietal cortex. The increase appeared to be in certain neuronal populations (Fig 21B). Some pyramidal cells in the layer 3 and 5 of the cortex were among the populations most heavily labelled with the Naip antibody. Naip-li was mostly intense at 3 and 24 h, but appeared to remain elevated at 7 and 21 d relative to nonkindled controls.

Western blot analysis was used to determine relative Naip levels in the frontoparietal cortical regions. Consistent with the immunohistochemistry findings, kindling was found to elevate Naip levels in the cortex (Fig 22A top panel). Some tissue extracts were used to detect the neuronal marker NeuN that served as a loading

control marker. Figure 22A bottom panel did not show any significant difference of the NeuN band between kindled and control animals. A quantitative value was available by measuring the relative optical density of the positive bands. Naip levels were elevated at 3 h, 1 and 7 d with an average volume of optical density of 1200 to 1500 compared to an average value of 800 observed in the control group. Naip protein levels were still elevated 21 d after the last kindled seizure (Fig 22B).

Figure 20

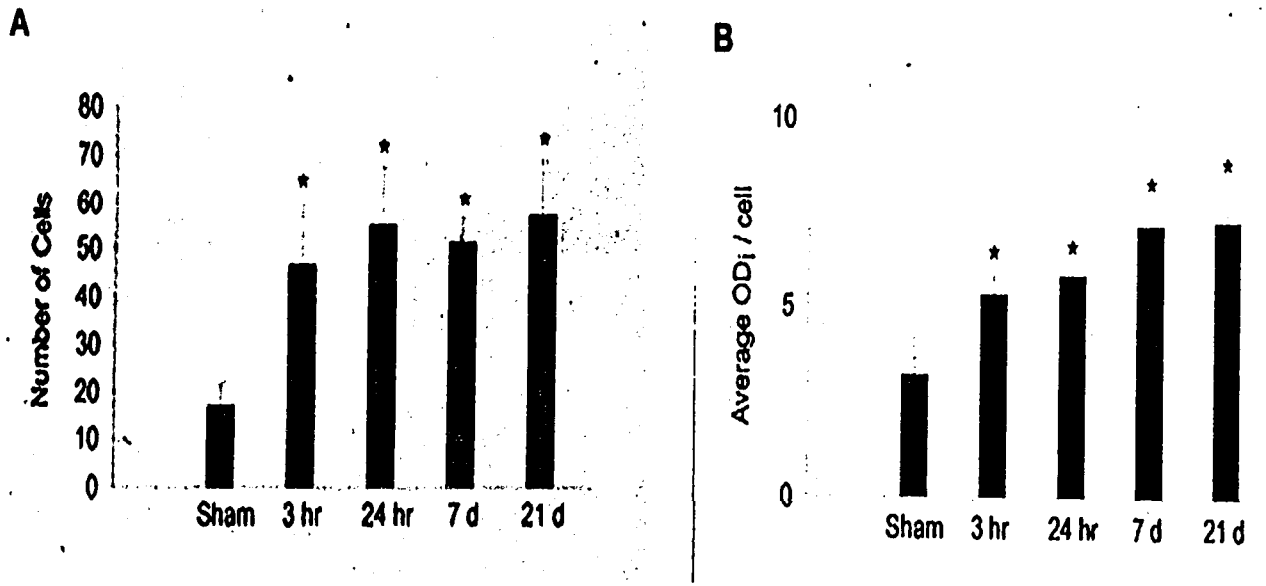

A
Number of Cells
80
70
60
50
40
30
20
10
0
Sham
3 hr
24 hr
7 d
21 d
B
Average OD$_i$ / cell
10
5
0
Sham
3 hr
24 hr
7 d
21 d

Figure 20. Bar graphs depicting the average number of cortical neurons which exhibited *naip* mRNA after hippocampal kindling. The average number of positive cells labelled in the kindled animals was two to three fold greater than the controls (A). The average optical density of cells was two fold greater in kindled than in control tissue (B). Histograms and bars represent mean and the standard error of the mean for 3-4 animals. Asterisk, significantly different from control animals ($p<0.01$; Newman-Keuls test).

Figure 21

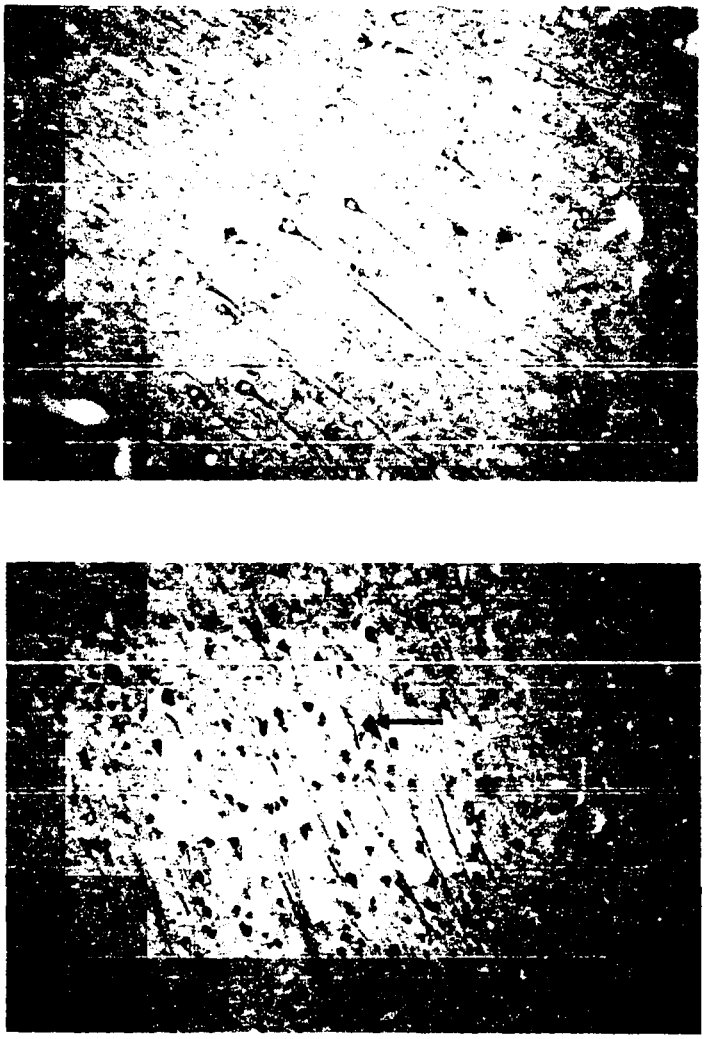


Figure 21 Effects of hippocampal kindling on Naip-like immunoreactivity (Naip-li) in cortical neurons. Moderate levels of Naip-li were present in the control animal (A). High levels of Naip-li were exhibited by large pyramidal neurons primarily in layers 3 and 5 of the cortex of hippocampal kindled animal (B). Scale bars = 100 μm.

Figure 22

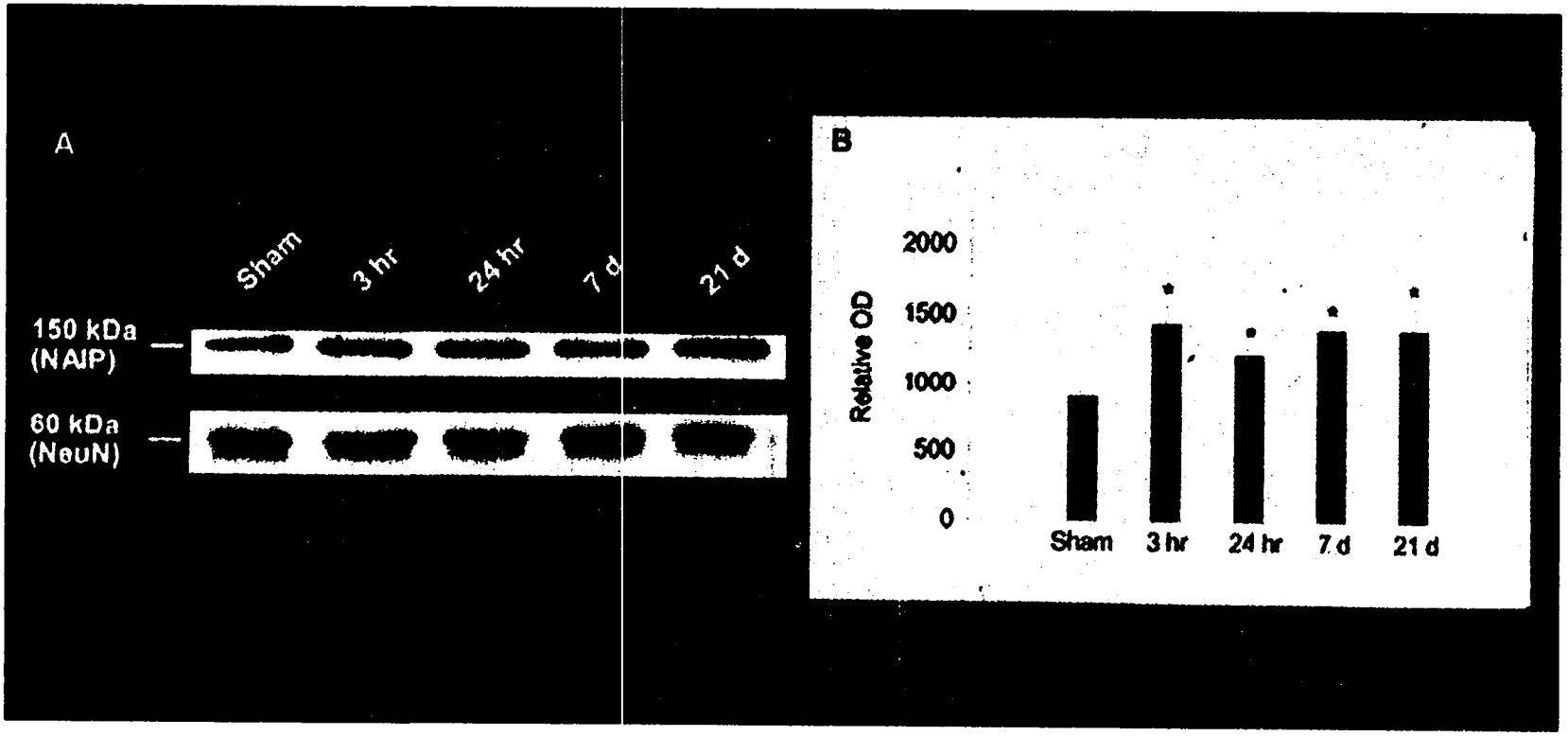

A
Sham
3 hr
24 hr
7 d
21 d
150 kDa
(NAIP)
60 kDa
(NeuN)
B
Relative OD
2000
1500
1000
500
0
Sham
3 hr
24 hr
7 d
21 d

Figure 22 Effects of hippocampal kindling on Naip levels in cortical areas. A. Immunoblotting analysis of Naip levels in the fronto-parietal cortex after hippocampal kindling (until five stage-six seizures). Kindling elevated Naip levels at all time points, with the highest occurring at 3h after the last episode of seizure, and remains high 21d later (A top panel). The same membrane was immunoblotted for NeuN. NeuN levels were similar in control and treated conditions (A bottom panel), indicating that each of these lanes was loaded with an equivalent amount of protein. B. Measurements of Naip levels following hippocampal kindling. Densitometric analysis of Naip immunoblots demonstrated that hippocampal kindling increased Naip levels by approximately 60 to 95 %. Histograms and bars represent mean and the standard error of the mean for 3 animals. Asterisk, significantly different from control animals ($p<0.01$; Newman-Keuls test).

CHAPTER 4

DISCUSSION

Various studies have shown that cerebral ischemia modifies the expression of genetic information in the brain (An et al., 1993; Dragunow et al., 1994; 1995; Guegan and Sola, 2000; Jordan et al., 1996; Kiessling et al., 1993; Hou et al., 1997; Tu et al., 1998). The alterations are proposed to represent selective synthesis of proteins specialized to either augment or to overcome post-ischemic processes because these specific proteins are produced when overall protein synthesis in the brain is severely depressed (Araki, et al., 1990; Krause and Tiffany, 1993). Induction of some genes may be associated with cell death (Benchoua et al., 2001; Colotta et al., 1992; Jordan et al., 1996; Kiessling et al., 1993; Li et al., 1994; Morgan and Curran, 1991; Smeyne et al., 1993; Velier et al., 1999). Others believe that induction of certain genes may participate in the process for neuronal survival (Akins et al., 1996; Herzog and Morgan, 1996; Hou et al., 1997; Hughes and Dragunow, 1995; Tseng et al., 1997; Tu et al., 1998).

Immediate early genes (IEGs) are well-studied gene products that are elevated following a variety of cellular challenges including cerebral ischemic events. Most prominent among the IEGs are the FOS and JUN families (Morgan and Curran, 1991; Hughes and Dragunow, 1995). The expression of IEGs, such as c-fos and c-jun in both neuronal and non-neuronal cells is elicited by a variety of stimuli, both *in vivo* and *in vitro*. Glutamate receptor activation can induce c-fos/jun expression (Gorman et al.,

1995; Herrera et al., 1993). Global (Kiessling et al., 1993) and focal cerebral ischemia (An et al., 1993) rapidly increases neuronal expression of IEGs. Increased intracellular calcium is believed to activate their transcription (Morgan and Curran, 1986). It is possible that calcium/calmodulin kinase phosphorylates one or more proteins that directly interact with the transcription regulatory elements of c-fos/jun, resulting in an induction of these genes. A DNA sequence located upstream of the transcription initiation site of c-fos is essential for induction by a calcium dependent agent (Sheng et al., 1988).

The immediate early genes (IEGs) may modify the outcome following cerebral ischemia because they encode transcription factors that couple early cytoplasmic events to long-term changes by regulating gene expression and cellular phenotype. The induction of the IEGs and other stress proteins is thought to be essential for converting short-term stimuli into long lasting intracellular changes in neurons that require protein synthesis (Morgan and Curran, 1986; Yee et al., 1993). The protein products of IEGs, known as transcription factors, can influence the expression of various target genes, by binding specific DNA sites. Activator protein-1 (AP-1) is a transcription factor formed by members of the JUN and FOS families of proteins (Dragunow and Preston, 1995). JUN family members can form either homo- or heterodimers, whereas FOS members can only form heterodimers with JUN family members (Angel and Karin, 1991). This dimeric protein complex subsequently binds to the AP-1 site (Morgan and Curran, 1989) influencing basal and elevated expression of various target-genes (Vendrell et al., 1993).

Specificity of IEG response is dictated by the stoichiometry of the various members and the available target genes. Cell type- and stimulus-dependent post-translational modification of IEG products may contribute to their biological activity as well as adding a further level of specificity to their response (Herzog and Morgan, 1996). In the normal CNS, these proteins are present at relatively low levels but correlations have been made between increased expression of FOS and JUN, and the subsequent delayed neuronal death induced by ischemia or a variety of disease states. A causal link of IEG expression to subsequent cell death was made in cultured neurons by using blocking antibodies against JUN or dominant-negative JUN transfections that prevented the ultimate demise of treated cells (Herzog and Morgan, 1996).

Despite suggestive evidence for a role in cell death for the IEGs, an alternate view maintains that FOS and JUN may be components of an adaptive or repair/regeneration process, because many cells that have increased expression actually survive insults such as excitotoxicity (Akins et al., 1996; Herzog and Morgan, 1996; Hughes and Dragunow, 1995). This idea is supported by the finding of normal cell death in FOS-null mice, and the lack of effect on neuronal cell death in the spinal cord after sciatic nerve resection in FOS knockout animals (Roffler-Tarlov et al., 1996). A prolonged and enhanced expression of FOS after focal ischemia in hypoglycemic animals is correlated with diminished damage to cortical neurons (Lin et al., 1997). Further support comes from studies showing increased FOS and JUN D mRNA in hippocampal dentate neurons that are resistant to global ischemia (Tseng et al., 1997). Thus, FOS and JUN may variously activate genes in a positive, negative or neutral manner. The increased expression of IEGs following ischemia could be influencing

pathways that mediate apoptosis directly (Colotta et al., 1992; Smeyne et al., 1993) and/or via DNA repair (Bruccoleri et al., 1997; Dosch and Kaina, 1996).

4.1 p53 Induction following Cerebral Ischemia May Promote Growth Arrest and DNA Repair via Activating the Downstream Genes

i. Induction of p53 following Cerebra Ischemia

The present study demonstrated an elevation of p53 mRNA expression in cortical tissue of rats subjected to transient focal ischemia using *in situ* hybridization histochemistry and RT-PCR. Increased p53 mRNA was present at the inner boundary of the ischemic region. The induction occurred gradually and peaked at 8-12 h, but persisted for as long as 48 h. p53 positive immunoreactive neurons were also detected in the same cortical areas. p53-like positive staining was induced at 8 h reperfusion, peaked at 12 hs and then gradually declined to basal levels 24 h later. In the previous reports (Chopp et al., 1992; Li et al., 1994), only mutant p53 was induced following transient cerebral ischemia. The induction was scattered throughout the ischemic cortex including the core and penumbral regions. In contrast, a recent study demonstrated an increased wild type p53 induction correlated with DNA fragmentation in the penumbral region following MCA-o (Watanabe et al.,1999). Interestingly, our study showed that there was no difference in the levels of induction detected between wild and mutant type p53 immunoreactivity following transient focal

cerebral ischemia. Consistent with later reports, the induction of p53 occurred predominantly at the ischemic penumbra.

p53 is a transcriptional factor predominantly located in the nucleus. Interestingly, p53 positivity is primarily localized within the cytosol of the cortical neurons in this study, which is consistent with the findings of other reports following cerebral ischemia (Li et al., 1994; Watanabe et al., 1999). p53 translocation also occurs in other conditions. In about 37% of the breast cancer tissue, p53 is also found in the cytoplasm as opposed to the nucleus (Moll et al., 1992). The mechanism of p53 accumulation in the cytoplasm is far from clear. Subsequent studies have shown that a dysfunctional nuclear trasport system, such as importin α, was the main reason for the translocation (Kim et al., 2000). The prototype of the nuclear transport signal is the classical nuclear localization signal (Koand Prives, 1996), and nuclear import of proteins bearing a signal is dependent on two cellular factors termed importin α and importin β (Gorlich et al., 1995; Nigg, 1997; Mattaj and Englmeier, 1998). Importin α provides the signal-biding site and then interacts with importin β, which in turn interacts with the nuclear pore complex (Weis et al., 1996; Kutay et al., 1997). It is postulated that p53 cytoplasmic accumulation may secondary to the impaired nuclear transport system following cerebral ischemia. Moreover, Sansome and colleagues (2001) discovered that a fraction of induced p53 protein rapidly translocates to the mitochondria during hypoxia-induced apoptosis. Importantly, mitochondrially localized p53 reproducibly induced apoptosis at least as efficiently as nuclear p53, indicating that p53 is sufficient to launch an apoptotic pathway from mitochondrial level (Merchenko et al., 2000).

p53 is normally expressed at low levels and has a short half-life in most, if not all, normal cells (Levin et al, 1991). Furthermore, transgenic mice bearing nonfunctional p53 alleles develop normally but have a higher incidence of certain cancer (Donehower, 1992). Low- level p53 expression per se must not be detrimental under most circumstances, nor is p53 essential for development. Wild-type p53 is, however, a potent inhibitor of cell proliferation. Reintroduction of wild-type p53 into transformed cells that have lost it suppresses cell growth, as does up-regulation of endogenous p53 levels in normal cells (Diller, 1990; Mercer et al, 1990; Martinez et al, 1991). Exactly how p53 suppresses cell growth is not clear, although the protein is capable of modulating transcription, both positively and negatively (Fields and Jung, 1990; Ginsberg et al, 1991; Farmer et al, 1992; Zambetti et al, 1992), and also of regulating DNA synthesis (Bargonetti et al, 1991)

Normal cells respond to DNA damage by cell-cycle arrest concomitant with transient p53 accumulation, followed by repair and re-entry into the cell cycle (Kastan et al, 1992; Kuerbitz et al, 1992). Transformed cells with either mutant p53 or which is p53 null do not arrest after DNA damage but, instead, continue cycling (Kastan et al, 1992; Kuerbitz et al, 1992). Thus, p53 could function as an SOS-like response, similar to the rad9 cell cycle control checkpoint gene of yeast, to sense DNA damage, prevent entry into S phase or mitosis until repairs have been affected, and thereby minimize transmission of genetic alteration (Kastan et al, 1992; Kuerbitz et al, 1992).

One mechanism by which the transcriptional factor p53 may function involves the ability of p53 to induce G1 cell cycle arrest that is largely mediated by p21WAF1/CIP1 (cyclin-dependent kinase interacting protein 1), and some other

transcription target genes of p53 (El-Deiry et al., 1993) (see later discussion). P21 WAF1/CIP1 inhibits G1 cyclin-dependent kinase (cyclin/cdk) activity and prevents cells from entering into S phase (Harper et al., 1993; Gu et al., 1993; Xiong et al., 1993). This would allow the cell to repair genetic damage and thereby reduce the likelihood of propagation of a cell with DNA damage (Jacks and Weinberg, 1996).

Consistent with this line of reasoning, the pattern of p53 induction observed in this study is consistent with the expression of its downstream genes, Gadd45 and Mdm2 that serve as anti-apoptotic function in the same ischemic model (Shen et al., 1997; Tu et al., 1998). The data argues for a role for p53 in growth arrest and DNA repair following transient ischemia (Figure 24).

p53 may function differently in cells which DNA damage is so severe as to be irreparable. This alternative pathway may involve activation of cell suicide programs to permit selective elimination of irreparably damaged cells. Thus, p53 may represent a control point either for cell cycle arrest, followed by repair, or for induction of cell death in cells that have sustained substantial genetic damage (Lowe et al., 1993). (Figure 23).

The first indication of a role for p53 in apoptosis came from a study in which myeloid leukemic cells overexpressing a temperature-sensitive p53 mutant underwent apoptosis when they were shifted to the permissive temperature in the absence of IL-6 (Yonish-Rousch et al., 1991; Yonish-Rousch et al., 1993). It was subsequently demonstrated that p53-dependent apoptosis can be triggered by a variety of signals, such as DNA damage (Lowe et al., 1993), inappropriate oncogene activation (Lowe et

al., 1993; Wagner et al., 1994), cytokine deprivation (Caman et al., 1995), and hypoxia (Graeber et al., 1996; Schmaltz et al., 1998). p53-induced apoptosis can be triggered in different types of cells, including thymocytes, peripheral lymphocytes, and neurons (Bellamy, 1997; Schimke and Mihich, 1994). p53 expression has been shown to be elevated in damaged neurons that display fragmented DNA following systemic administration of the excitotoxic kainic acid (Sakhi et al., 1994). Recent studies show that glutamate and kainate elevate p53 DNA binding activity, and blockade of p53 induction by a specific p53 antisense oligonucleotide, produced a complete inhibition of EAA induced apoptosis (Uberti et al., 1998). Consistent with this line of reasoning, p53 may also play a role during cerebral ischemia. p53 expression has been shown to be elevated in the injured neocortex in a focal ischemic model (Li et al., 1994; Watanabe et al., 1999) and in hippocampal pyramidal culture neurons hypoxia (Jordan et al., 1997). Moreover, mice null for p53 are more resistant to ischemic injury. p53 knockout mice display a significant reduction in infarct volume in a permanent middle cerebral ischemic model (Crumrine et al., 1994). Taken together, these findings suggest that p53 induction play a role in apoptosis following cerebral ischemia.

Figure 23

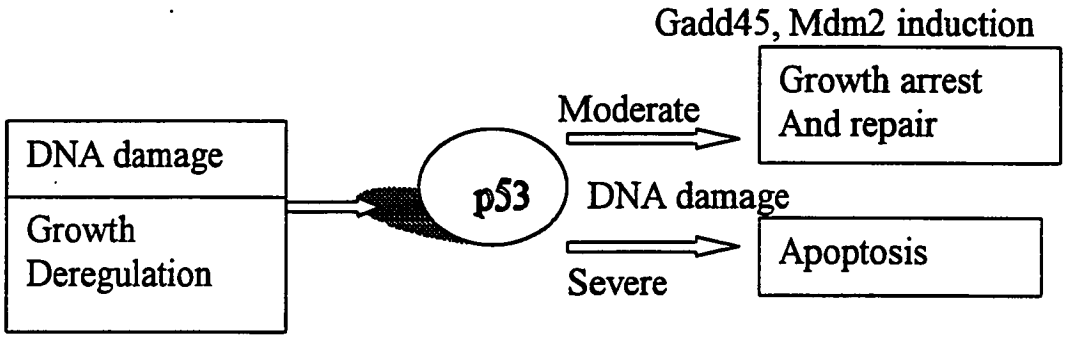


Figure 23. Possible mechanisms by which the p53 gene induces growth arrest or apoptosis.

Wild-type p53 is usually considered to be associated with apoptosis (Lowe et al., 1993; Aldridge et al., 1998). The positive staining in mutant p53 certainly raised a question about the apoptotic role of p53 following cerebral ischemia. However, studies showed that apoptosis was mediated by p53 mutant lacking transcriptional activities (Haupt et al., 1995). Importantly, the p53 mutation in human colon carcinoma cell SW480 has lost its ability to bind non-specific DNA while retaining the ability to bind specific DNA (Liu and Kulesz-Martin, 2001). A positive mutant p53 immunoreactivity colocalized with TUNEL labeling neurons following focal cerebral ischemia (Li et al., 1994), indicating certain mutant p53 may also pose an apoptotic function. Identifying specific mutations in p53 and associated apoptotic potential following cerebral ischemia would certainly be very helpful to address this issue.

p53 induced apoptosis is thought to be associated the activation of caspase-3 and related caspases (Lotem and Sachs, 1996; Chandler et al., 1997; Fuchs et al., 1997) (see below discussion for caspases). Although far from clear, recent studies shed substantial light on how apoptotic signals might be transduced from p53 to caspases. p53 is also involved in regulating bcl-2 and bax expression. The first clue that bax may be a p53 target gene came from studies showing that induction of bax depends on the presence of functional p53 (Selvakumaran et al., 1994: Miyashita et al., 1994; Zhan et al., 1994). Subsequently, it was found that the bax promoter contains p53-responsive elements that can direct p53-dependent transcription from a heterologous promoter (Miyashita and Reed, 1995). Induction of p53 reduces expression of the anti-apoptosis gene bcl-2, while simultaneously increasing expression of bax, whose protein product inhibits Bcl-2 function (Selvaknmaran et al., 1994; Miyashita et al., 1994). Consistent

with this finding, mice deficient in p53 exhibit increased levels of Bcl-2 and decreased amounts of Bax in a variety of brain structures (Miyashita et al., 1994). The mechanism by which Bax promotes apoptosis remains obscure. Bax heterodimerizes with Bcl-2, suggesting that Bax may function by inactivating Bcl-2 and related antiapoptotic proteins (see below discussion).

Taken together, elevation of p53 at the ischemic penumbra may indicate that neurons undergo growth arrest and/or DNA repair following cerebral ischemia. However, the induction of p53 may also be the trigger for neuronal apoptosis, which contributes to the exaggeration of the ischemic infarct if ischemic insult is too severe.

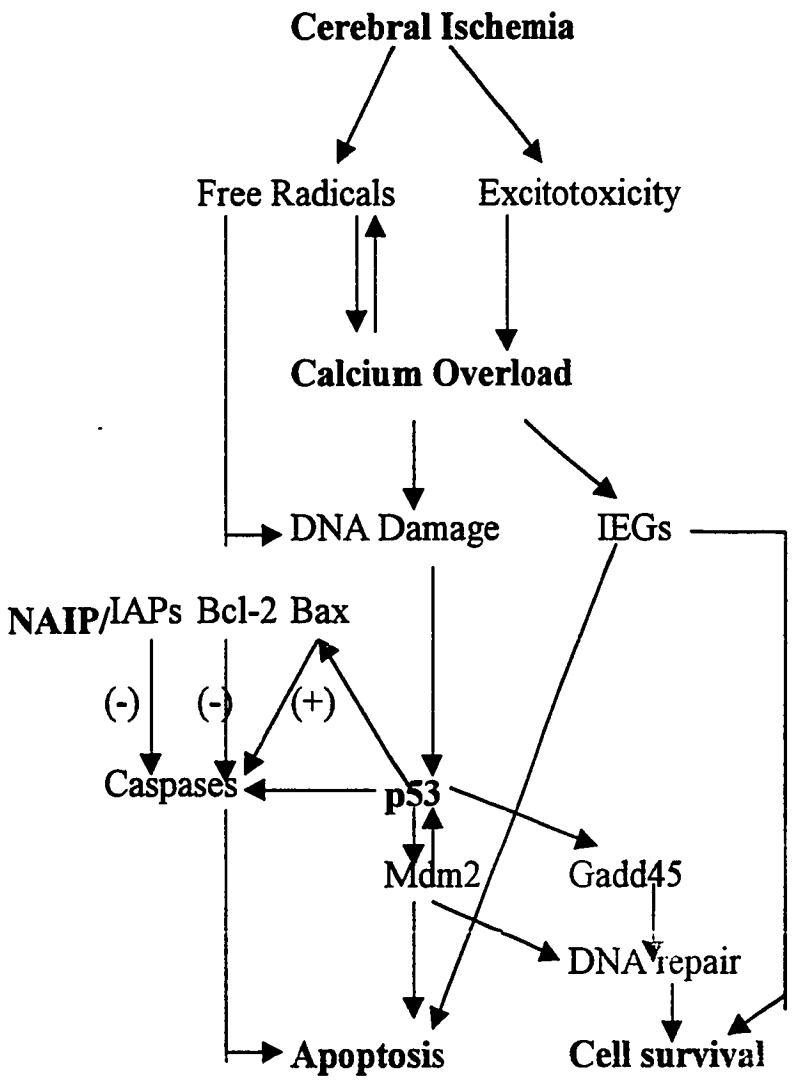


Figure 24. Genetic alterations following cerebral ischemia involved in neuronal death or survival.

ii p21WAF1/CIP1 and Gadd45 Induction following Cerebral Ischemia as an Indicator for Growth Arrest and/or DNA Repair

As a transcriptional factor, p53 is likely to activate the transcription of target genes. p53 target genes with potential roles in cell cycle control, DNA repair and apoptosis induction include: p21WAF1/CIP1, gadd45, mdm2, cyclin G and bax (MacManus and Linnik, 1997). The p21 protein was identified as an inhibitor of the cyclin dependent kinases which control cell cycle progression by preventing phosphorylation of proteins such as the retinoblastoma tumour suppressor protein p (Rb) (Hartwell and Kastan, 1994). It is also now well recognized that p21 is able to interact directly with proliferative cell nuclear antigen (PCNA) that is a subunit of a polymerase involved in DNA replication and repair (Waga et al., 1994). Thus increases in p21 induced by p53 may lead to growth arrest and DNA repair (Reinke et al., 1997). A recent study has demonstrated that elevated p21WAF1/CIP1 expression is evident in neurons that have survived an episode of transient a cerebral ischemia (Van Lookeren Campagne and Gill, 1998).

Another p53 target gene, which may be involved in growth arrest and DNA repair, is gadd45, originally found to be induced by DNA-damaging agents and radiation (Smith and Fornace, 1996; Yoshida et al., 1996). Similar to p21, gadd45 is capable of binding directly to PCNA (Smith and Fornace, 1996) and has been shown to be decreased in vulnerable neurons compared to those resistant to cerebral ischemic injury (Tomasevic et al., 1996). A study performed in collaboration with Dr. John MacManus (Hou et al 1997) demonstrated that an elevated gadd45 mRNA in the neocortical tissue following transient focal cerebral ischemia as detected by RT-PCR.

Furthermore, *in situ* hybridization histochemistry revealed that increased gadd45 mRNA occurred principally within the ischemic penumbra. The presence of gadd45 protein was confirmed by immunohistochemical detection of this protein. When gadd45 immunoreactivity was combined with in situ detection of DNA breaks (TUNEL), the protein was absent from frankly damaged cells in the ischemic core, but was increased in TUNEL-negative cells in the penumbra (Hou et al, 1997). The induction of gadd45 appears greatest at earlier time points compared to later time points when massive neuronal death occurs. These data suggest that a DNA repair process have been initiated as evidenced by the upregulation of gadd45 following cerebral ischemia. Growth arrest and DNA repair appear concentrated at the ischemic penumbra, while massive neuronal destruction occurs predominantly at the ischemic core region.

iii Induction of Anti-apoptotic Gene Mdm2 following Transient Focal Cerebral Ischemia

The mdm2 proto-oncogene was originally discovered as one of three genes that are amplified in a tumorigenic cell line derived from non-transformed Balb/c cells (Cahilly-Snyder et al., 1987). Consistent with the expression pattern of mdm2 in these cells, it was later shown that the transforming potential of mdm2 can be activated by experimental overexpression (Perry et al., 1993; Price and Park, 1994; Kondo et al., 1995). Overexpression of mdm2 protein has been detected in a number of diverse malignancies, indicating that this oncogene may play a key role in carcinogenesis. The mdm2 gene is also the transcriptional target of p53. DNA damage-induced p53

expression leads to increases in mdm2 mRNA and protein (Levin, 1997). Mdm2 in turn binds the transcriptional activation domain of p53 and blocks its ability to regulate target genes and to exert antiproliferative effects (Chen et al., 1993; Momand and Zambetti, 1997). Thus, it appears that an auto-regulatory feedback loop exists between these two proteins which keeps the growth suppressive function of p53 in check during normal cell cycling. Apparently, p53 regulates mdm2 at the transcriptional level, while mdm2 regulates p53 at the functional level. Pathological changes leading to elevated levels of mdm2 likely impair the ability of p53 to orchestrate the expression of genes controlling cell cycle progression during cellular insults. Mdm2 may also promote the rapid degradation of p53 under conditions in which p53 is otherwise stabilized (Haupt et al., 1997).

Expression of mdm2 is increased in response to many DNA damaging agents, e.g., ultraviolet light (Perry et al., 1993), gamma-radiation (Price and Park, 1994), and cisplatin treatment (Kondo et al., 1995). Cerebral ischemia induced DNA damage has been discussed in the introduction, along with injury promoting agents such as increased glutamate, intracellular calcium overload and free radical formation. Tu et al, (collaboration, 1998) demonstrated increased mdm2 expression following transient focal cerebral ischemia. The expression of mdm2 mRNA was increased in the ischemic ipsilateral cortex between 6 to 8 hr, and remained increased at 18 to 24 hr in animals subjected to 90 min MCA-o followed by reperfusion, with no evidence of preferential expression in either the core or penumbra. Immunohistochemistry and Western blotting confirmed elevated mdm2 protein following transient focal cerebral ischemia.

One of the mechanisms by which the caspases induce apoptosis is via modifying mdm2. Caspase-3 cleaves the COOH-terminal RING finger domain of mdm2, results in the loss of RNA binding activity of mdm2 and possible apoptosis (Chen et al., 1997). Treatment with an antisense oligonucleotide targeted against mdm2 increased the susceptibility of tumor cells to apoptosis (Kondo et al., 1995). In contrast, overexpression of mdm2 confers resistance of tumor cell to cytoxin induced apoptosis. Taken together, these data suggest that the upregulation of mdm2 may have the potential to inhibit apoptotic processes following cerebral ischemia. But the occurrence of the mdm2 in the ischemic core region is somewhat in disagreement with an anti-apoptotic function in ischemic neuronal death.

4.2 Caspases Play a Role in Ischemic Cell Death by Inducing Apoptosis

The genetic control of apoptosis has been partially elucidated by work on PCD in the nematode *Caenorhabditis elegans*. During normal development, 131 of 1090 somatic cells undergo PCD in *C elegans*. The ced-3 (c elegans death gene) and ced-4 are required for cell death in *C. elegans* (Yuan and Horvitz 1990, 1992), whereas the overexpression of ced-9 prevents cell death (Garrieli et al., 1992). The product of ced-3 has sequence and functional similarities to the mammalian cysteine proteases ICE (Yuan et al., 1993), and the cysteine protease nedd-2 in mouse (Kumar et al., 1994) or ich-1 in human (Wang et al., 1994). Some functional data in mammalian cells support a role for these molecules, or perhaps unknown members of the same family. For example, expression of ced-3, ICE, ich-1 and nedd-2 induces apoptosis in several cell types (Miura et al., 1993; Kumar et al., 1994; Wang et al., 1994). In addition, a known

inhibitor of ICE, the cowpox protein crmA, reduces apoptosis caused by ICE and ich-1 (Miura et al., 1993; Wang et al., 1994), more importantly, crmA inhibits PCD in cells after growth factor withdrawal (Gagliardini et al., 1994).

Elevated caspase activity is also associated with increased apoptotic cell death in neurons. Exposure to 6-hydroxydopamine induces apoptosis of cerebellar granule neurons in cell culture. The rate of apoptosis induction was paralleled by an activation of caspase-3 (Dodel et al., 1999). Intrastriatal administration of the excitotoxin quinolinic acid results in the death of medium-sized neurons in the striatum. Intrastriatal administration of 6-hydroxy dopamin results in the death of dopamine neurons in the substantia nigra pars compacta. It has been shown that apoptotic profiles occurs in neurons injured in both of these and that the cell death was apoptotic. Natural developmental neuron death and induced developmental death following either striatal target injury with quinolinic acid or dopamine terminal lesion with intrastiatal injection of 6-hydroxydopamine. Using an antibody to the large (p17) subunit of activated caspase-3, experiments were able to demonstrate that apoptotic profiles in all models were associated with an increased caspase activity (Jeon et al., 1999). Inhibition of the caspase activities was capable of preventing neuronal death against various stimuli (Dodel et al., 1999; Schierle et al., 1999; Tenneti et al., 1998). Caspase inhibitors Ac-DEVD-CHO and zVAD-FMK can attenuate neuronal death induced by 6-hydroxydopamine in cerebellar granule neurons (Dodel et al., 1999). Treatment of the embryonic nigral cell suspension with caspase inhibitor, Ac-YVAD-cmk mitigated DNA fragmentation and reduced apoptosis in nigral transplants (Schierle et al., 1999). Recent data indicate increased caspase activity within vulnerable neurons following

cerebral ischemia. Immunoblot and immunohistochemical analyses using antibodies against caspase substrates such as spectrin and actin have provided evidence that caspases are activated in neurons in the ischemic penumbra prior to their death. Caspase-8 is activated within 6 h of focal isch-emia induced by permanent MCA-o in rats (Velier et al., 1999). Active caspase-8 was located mainly in pyramidal neurons of layer V of cerebral cortex. Active caspase-3 was observed in neurons in layers II and III, beginning 24 h after the onset of ischemia, and in microglia. Intraventricular administration of caspase inhibitors prior to or 6 h postreperfusion, resulted in a significant decrease in cerebral infarct size (Hara et al., 1997; Endres et al., 1998). The caspases that may mediate ischemic neuronal death probably include caspase-1 and caspase-3. Indeed, ischemic brain injury was decreased in mice lacking caspase-1 (Schielke et al., 1998).

Chen and coworkers (Chen et al., 1998) showed that caspase-3 activation precedes death of CA1 neurons, and studies demonstrated that administration of the broad-spectrum caspase inhibitor zVAD-fmk protects neurons agaist ischemic injury (Chen et al., 1998; Noshita et al., 2001; Schulz et al., 1999). Intraventricular administration of the pseudosubstrate caspase inhibitor N-tosyl-L-phenylalanyl chloromethyl ketone reduced damage to CA1 neurons following transient global ischemia in rats (Hara et al., 1998). Caspase-9 is released from isolated mitochondria on treatment with calcium or Bax, stimuli implicated in ischemic conditions that are known to induce cytochrome *c* release from mitochondria (Cao et al., 2001). In neuronal cell culture models, apoptosis-inducing agents trigger translocation of caspase-9 from mitochondria to the nucleus. Similarly, in an animal model of transient

forebrain ischemia, caspase-9 release from mitochondria and accumulation in nuclei was observed in vulnerable neurons such as hippocampal CA1 neurons exhibiting early postischemic changes preceding apoptosis (Krajewski et al., 1999). Caspase-3 mRNA and protein are induced in the hippocampus in an animal model of transient forebrain ischemia. The caspase-3 activity is increased predominantly in degenerating CA1 pyramidal neurons (Chen et al., 1998). Caspase-3 protein is also induced in neuronal perikarya within the middle cerebral artery territory at the time of reperfusion and 1-12 hr after focal cerebral ischemia (Namura et al., 1998). DNA laddering is correlated with increased caspase activity in the ischemic region (Chen et al, 1998; Namura et al., 1998).

Caspases, a family of cytoplasmic proteases, have a cysteine residue at their active site and cleave their substrates after aspartate residues (Alnemri et al., 1996). At least ten family members are now known in mammals and all of them are present in the cytoplasm as inactive *zymogens* called-pro-caspases. Activation of pro-caspases requires cleavage adjacent to aspartate to generate one large (p17) and one small subunit (p12), which in turn associate into a *tetramer* (two large and two small subunits) to form the active caspase. Once activated, caspases can autocatalytically cleave and activate themselves as well as other members of the caspase family, leading to an amplifying of the proteolytic cascade (Nicholson and Thornberry, 1997).

Activation of the caspase cascade occurs via a two-stage process in which the so-called "initiator" caspases are first activated. Initiator caspases are activated by autoproteolysis, which is induced by binding to specific activators. Each initiator caspase is activated in response to a subset of cytotoxic stimuli. For example, caspase-

8 is activated by binding to DISC, a protein complex, which is formed after stimulation of the death receptors such as Fas and TNFR (Ashkenazi and Dixit 1998). Caspase-9 is activated by binding to a complex containing APAF-1 and cytochrome *c*, which is formed after a number of stimuli, including DNA damage. Importantly, cytochrome *c* has to be released from mitochondria to participate in caspase-9 activation. The activated initiator caspases activate effector caspases by proteolytic processing. While initiator caspases are specific for each pathway of apoptosis, the effector caspases are shared.

The "executioner" caspases are responsible for the proteolytic cleavage of many intracellular proteins, leading to the morphological and biochemical changes associated with apoptosis (Salvesen and Dixit, 1997). This model is best exemplified by apoptosis induced by the cell-death receptor Fas. Binding of Fas ligand (FasL) to Fas induces conformational changes of the Fas receptor, which recruits pro-caspase-8 via an adapter, FADD/MORT1. The oligomerization of pro-caspase-8 induces autoproteolytic generation of active caspase-8 (Yang et al., 1998), which then directly or indirectly activates caspase-3. Similarly, cytochrome *c* can form a complex with apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9. This leads to the cleavage of pro-caspase-9, converting it to an active protease which in turn cleaves and activates caspase-3 (Liu et al., 1996; Zou et al., 1997; Li et al., 1997).

An extensive review by McManus and Linnik (1997) outlines in detail the roles of caspases in apoptosis. Caspase-mediated cleavage of poly (ADP-ribose) polymerase (PARP) blocks DNA repair and prevents the reduction in the cellular pool of nicotinamide adenine dinucleotide associated with PARP activity (Schwartz and

Milligan, 1996; Ashkens and Werb, 1996). A caspase-mediated loss of DNA-dependent protein kinase activity would also inhibit the cells ability to repair DNA damage (Casciola-Rosen et al., 1996; Song et al., 1996). Caspase mediated-cleavage of the structural protein actin, contributes to cellular and nuclear collapse and leads to the activation of DNase I (Kayalar et al., 1996). Caspase also mediates the cleavage of cytoskeleton lamin which can trigger the progression to nuclear shrinkage and fragmentation into apoptotic bodies (Rao et al., 1996; Takahashi et al., 1996). Caspase-mediated degradation of hnRNA may compromise the ability of cells to recover from an apoptotic-initiating event by altering protein synthesis (Waterhouse et al., 1996). Although it is not clear how the processing of caspase substrates leads to cell death, it appears that caspases target key components of cell structures and signaling pathways. For example, during apoptosis, DNA is degraded by CAD, a DNAse, which is constitutively expressed but is bound to its inhibitor ICAD/DFF45 in live cells (Liu et al., 1997; Enari et al., 1998). Caspase-3 releases active CAD by cleaving ICAD/DFF45 at only two sites (Sakahira et al., 1998). In another example, caspases disassemble the nuclear lamina, which is formed by polymers of lamins, by cleaving these proteins at a single site (Lazebnik et al., 1995; Orth et al., 1996; Takahashi et al., 1996). Finally, caspases cleave mdm2 thus inhibits the apoptotic action by mdm2 (Chen et al., 1997).

4.3 Alteration of Bcl-2 Family Expression Contributes Neuronal Cell Death following Cerebral Ischemia

The overexpression of the DNA repairing gene gadd45 and the negative p53 regulator mdm2 suggests that neuronal cells struggle for survival under stress insults such as cerebral ischemia. This indicates a role of the anti-apoptotic genes in the ischemic setting. The prototypical and most well studied gene is bcl-2, originally identified as an oncogene in B cell lymphoma. Examination of the primary structure of Bcl-2 reveals several interesting features (Alnemri et al., 1992). There are 239 amino acids in human Bcl-2; the predicted relative molecular mass is 26.3kD. Although there is a hydrophobic carboxyl terminus, no other regions are likely membrane-spanning domains. Deletion of this hydrophobic carboxyl terminus at amino acid 189 results in a loss of apoptosis inhibition in nonneuronal cells (Alnemri et al., 1992).

Bcl-2 inhibits apoptosis in a variety of cell types in response to diverse insults, including several which induce oxidative stress (Buttke and Sandstrom, 1994; Hockenbery et al., 1990; Hockenbery et al., 1993; Zhong et al., 1993). Specifically, Bcl-2 protects cells against death from ionizing radiation, heat shock, inhibition of glutathione (GSH) synthesis and GSH depletion, and treatment with tumor necrosis factor (TNF), hydrogen peroxide, or t-butylhydroperoxide (Hennet et al., 1993; Hockenbery et al., 1990; Hockenbery et al., 1993; Kane et al., 1993; Zhong et al., 1993). Bcl-2 also inhibits neuronal cells from death induced by the calcium ionophore A23187 (Mah et al., 1993; Zhong et al., 1993), an agent which increases intracellular calcium to a cytotoxic level. Overexpression of the proto-oncogene bcl-2 can protect against cell death resulting from exposure to reactive oxygen species (Kane et al.,

1993), glutamate (Behl et al., 1993), and withdrawal of serum or growth factors (Allsopp et al., 1993). Bcl-2 also prevents PCD of sympathetic neurons deprived of NGF (Garcia et al., 1992). Therefore, cells may undergo apoptosis if synthesis protein of key antiapoptotic proteins, such as bcl-2, has been hindered. Isenmann and colleagues (1998) demonstrated that DNA damage and nuclear fragmentation seen in cells located in the lesion core in focal cerebral ischemia were often associated with increased levels of Bax, but not with elevated Bcl-2 or Bcl-x protein levels, suggesting a role for Bax in the induction of apoptotic death in these cells. The upregulation of Bcl-2 and Bcl-x expression in surviving neurons close to the penumbra might reflect an active survival mechanism that protects these neurons from cell death following focal cerebral ischemia. Moreover, expression of Bcl-2 from a defective herpes simplex virus-1 vector reduces cortical infarct following focal cerebral ischemia (Lawrance et al., 1996; Linnik et al., 1995). Transgenic mice overexpressing bcl-2 demonstrate a dramatic attenuation in neuronal damage together with DNA fragmentation in the hippocampus of a transient forebrain ischemia (Kitagawa et al., 1998) and cortex following focal ischemia (Martinou et al., 1994).

The mechanism of action for bcl-2 is unclear. Accumulating evidences indicate that bcl-2 protects cell death through antioxidant activity (Hockenbery et al., 1993; Kane et al., 1993). Kane et al (1993) demonstrated that the neuronal cell line, GT1-7, is protected by bcl-2 expression from death induced by GSH depletion.

Bcl-2 overexpressing cells demonstrate lower levels of reactive oxygen species (ROS) and lipid peroxides, suggesting that bcl-2 decrease the generation of free radicals. Hockenbery et al (1993) have provided evidence that lymphocytes expressing

bcl-2 are protected from the damaging results of hydrogen peroxide and menadione (an agent that generates $O2^{\cdot}$). Dexamethasone treated cells overexpressing bcl-2 demonstrated lower levels of lipid peroxides, suggesting that bcl-2 also operate by detoxifying free radical species. It was also determined that hypothalamic neurons expressing bcl-2 had a 2.5 to 5-fold higher level of reduced GSH. However, when GSH levels were reduced by treatment with diethylmaleate or ethacrynic acid, both the control and bcl-2-expressing cells had similar very low levels of GSH as measured by the monochlorobimane method, yet the inhibition of cell death associated with bcl-2 expression remained. Dichlorofluorescein diacetate studies indicated a rapid rise of reactive oxygen species in the control cells, and this was associated with rapid onset of cell death. Cells expressing bcl-2 did not show an increase in reactive oxygen species or in cell death, suggesting that the mechanism by which bcl-2 inhibits neuronal cell death is not by increasing GSH directly (Sarafian et al., 1994).

The Bcl-2 protein is known to be membrane-bound, but its primary localization is somewhat controversial. Studies have found it localized mainly to the inner mitochondrial membrane (Hockenbery et al., 1990), or the endoplasmic reticulum (ER), nuclear envelope, and outer mitochondrial membrane (Chen-Levy et al., 1989; Jacobson et al., 1993; Krajewski et al., 1993). However, bcl-2 can still rescue cells from apoptotic death in cells lacking a nucleus (Jacobson et al., 1994), or lacking functional mitochondrial DNA (Jacobson et al., 1993). The latter study does not preclude a mitochondrial function of bcl-2, because such cells may still produce functional respiratory chain complexes and may produces $O2^{\cdot}$. Therefore, the intracellular location of bcl-2 protein is consistent with an antioxidant mechanism, as it

is localized to membranes, which contain electron-transporting systems, which as a result have a capacity for $O2^{\cdot}$ generation.

Other mechanisms for the Bcl-2 families have also been suggested in recent years. The dimerization of Bcl-2 families determines the functional action. The formation of Bax homodimers is dominant for cell death, and Bcl-2/Bcl-xL negatively regulates cell death by inhibiting the formation of the toxic Bax homodimer. Alternatively, Bcl-2/Bcl-xL dimers may be dominant repressors of cell death (Yang and Korsmeyer, 1996). Furthermore, studies have demonstrated that when Bcl-2 is expressed in the outer mitochondrial membrane, it inhibits the PT pore in response to several apoptosis inducers (Zamzami et al., 1996; Marchetti et al., 1996), such as cytochrome *c* which is a known to induce caspase activation (Liu et al., 1996). Bax has been implicated as a key member of the PT pore as discussed previously. In addition, Bcl-2 also enhances the calcium uptake capacity of the mitochondria, thereby reducing intracellular free calcium that poses toxic effect to the cells (Murphy et al., 1996). Recent evidence links anti-apoptotic activity of Bcl-2 to caspases inhibition as well. Bcl-2 has been shown to directly interact with Ced-4 and inhibit the cell killing effect of Ced-4 (Chinnaiyan et al., 1997; Wu et al., 1997).

4.4 The Neuroprotective Effects of Hippocampal Kindling Correlates with Increased Expression of Neuronal naip

The observation that Bcl-2 levels are very low in the adult central nervous system coupled with the finding that Bcl-2 concentrations do not change appreciably

after transient cerebral ischemia suggests that the protein may not be a key determinant of susceptibility to neuronal death following ischemia (Krajewski et al., 1995).

A candidate gene for spinal muscular atrophy (SMA) was isolated (Roy et al., 1995). This gene, encoding naip, is homologous to two baculovirus inhibitor of apoptosis proteins (Cp-Iap and Op-Iap) and is partly deleted in individuals with type I SMA. A clear link between an hereditary neurodegenerative disorder and failed caspase inhibition has recently been proposed for spinal muscular atrophy (SMA). In severe SMA, naip is often dysfunctional due to missense and truncation mutations. Iaps such as naip potently block the enzymatic activity of caspases 3 and 7 suggesting that naip mutations may permit unopposed developmental apoptosis to occur in CNS resulting in lethal muscular atrophy. Consistent with this line of reasoning, overexpression of naip protects several different cell lines from death induced by a variety of apoptotic triggers (Liston et al., 1996). A role for naip in SMA is also supported by the close correspondence between the distribution of naip-like immunoreactivity (naip-li) in the central nervous system and the pattern of neuronal degeneration observed in acute SMA (Xu et al., 1997b). Recent studies suggest that naip may be relevant for neurodegenerative disorders other than SMA. For instance, upregulation of naip expression following transient global cerebral ischemia is associated with increased resistance to damage in certain neuronal populations such as CA3 pyramidal neurons in the hippocampus, ventral/lateral thalamic neurons, and cholinergic neurons in the striatum (Xu et al., 1997b). That naip induction actually mediates resistance to ischemic damage is supported by the ability of adenovirally-

mediated naip overexpression to reduce CA1 neuronal loss following a brief period of global cerebral ischemia (Xu et al., 1997a).

i Hippocampal Kindling Elevates Neuronal naip mRNA Overexpression in Several Brain Structures

Naip protects hippocampal CA1 neurons against brief ischemia. Does the neuroprotection applies to all the neuronal populations injured by various insults? A very first step in this study was to anatomically correlate naip expression with neuroprotection. Kelly and McIntyre (1994) have demonstrated that hippocampal kindling is capable of protecting brain structural damage resulting from kainate acid induced-status epilepticus. The present study clearly demonstrated that the neuroprotective effects of hippocampal kindling were closely associated with naip induction. Abundant *naip* mRNA overexpression occurred in the brain regions demonstrating resistance to ischemic injury, such as the piriform cortex and hippocampus (Kelly and McIntyre, 1994). Hippocampal kindling failed to induce *naip* mRNA expression in those areas that lack neuroprotection such as midline thalamic nuclei and endopiriform cortex (Kelly and McIntyre, 1994). A more striking finding of the current study was that the induction of both *naip* mRNA and protein remains consistently high three weeks after kindling treatment. The sustained elevation of *naip* expression following hippocampal kindling is also found in other brain structures, such as frontoparietal cortex.

ii Hippocampal Kindling Elevates NAIP Expression, Which May Protect against Focal Cerebral Ischemia

Hippocampal kindling may produce more widespread neuroprotection than adenoviral vector, which only induces local naip expression due to the limited penetration of virus within the brain. This allows us to assess whether naip upregulation is associated with protections against a more severe, prolonged duration of cerebral ischemia, such as focal cerebral ischemia. The neuroprotective effects of hippocampal kindling was demonstrated in the transient focal ischemic model. A nearly 60% reduction of cortical infarction in hippocampal kindled animals was observed, and the greatest protection was within the ischemic penumbra, suggested a reduction in lesion expansion (Chopp and Li, 1996). The reduction of cortical infarct in the MCA-O model by hippocampal kindling was independent of rCBF changes. Since hippocampal kindling has no effect on cerebral blood flow during subsequent ischemia, the neuroprotective effect of kindling is thought to occur via a long-term up-regulation of the ability of brain cells to resist ischemic injury. Thus, the close anatomical relationship between naip overexpression and the reduction of cortical infarction following hippocampal kindling strongly suggests that the neuroprotective effects of hippocampal kindling may be related to the induction of naip.

iii Neuroprotection by NAIP May Be Mediated by Caspase Inhibition

The anti-apoptotic action of naip is thought to be similar to the inhibitor of apoptosis proteins (Iaps) (Clem and Miller, 1993). Naip shows significant homology

with two-baculoviral gene products, Cplap and orgyia pseudotsugata nuclear polyhedrosis virus (Oplap). There are three additional human complementary DNAs and a Drosophila melanogaster sequence that are homologous to the baculovirus Iaps. The four open reading frames (ORFs) possess three baculoviral inhibition of apoptosis protein repeat domains (BIR) and a carboxyl-terminal RING zinc-finger (Liston et al., 1996). The BIR motif is a conserved sequence of approximately seventy amino acids that was identified originally in the Iap family. BIR-containing protein (BIRPs) are found in virus, yeast and metazoans. Recent genetic analysis of a nematode BIRP demonstrated an essential role in cytokinesis (Fraser et al., 1999). It is likely that BIRs originated in eukaryotes to serve a role in cytokinesis and/or mitotic spindle function during cell division and that, with gene duplication, the more recent adaptation of some BIRPs to the regulation of apoptosis was possible (Clem and Miller, 1993; Duckett et al., 1996).

The BIR domain is essential for the anti-apoptotic activity of the Iaps (Takahashi et al., 1998). *In vivo* binding studies demonstrated that both baculovirus and Drosophila Iaps physically interact with an apoptosis-inducing protein of Drosophila, Reaper (RPR), through their BIR region (Vucic et al., 1997). By analyzing the effects of various fragments of the Iaps on caspases activity *in vitro* and on apoptosis suppression in cells, Takahashi et al (1998) demonstrated that only the second of the three BIR domains was capable of binding and inhibiting caspases. Expression of the BIR2 domain in cells also partially suppressed Fas-induced apoptosis.

CpIap and OpIap are capable of inhibiting insect cell apoptosis (Clem and Miller, 1993). Iaps can also prevent cell death in mammalian cells (Duckett et al., 1996). Transiently expressed OpIap strongly inhibited ICE-induced cell death (Deveraux et al., 1997; Hawkins et al., 1996). A similar inhibition was observed in Drosophila (Kaiser et al., 1998). Human malignant glioma cell lines expressing Iaps are resistant to CD95 ligand-induced apoptosis (Wagenknecht et al., 1999). Ectopic expression of Iaps impared neuronal survival after potassium withdrawal and glutamate exposure (Simons et al., 1999). Recent study showed that loss of CA1 neurons induced by transient global forebrain ischemia is preceded by caspase-3 activation, and overexpression of X chromosome-linked inhibitor of apoptosis protein (Xiap) via virus-mediated transfection prevented caspase-3 activation, death of CA1 neurons, and spatial memory deficits (Xu et al., 1999).

It has been documented that Iaps and their homologs are able to bind to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2 in yeast two-hybrid assays, suggesting that Iap proteins that inhibit apoptosis may do so by regulating signals required for activation of ICE-like proteases (Uren et al., 1996).

Caspases are the principal effectors of apoptosis. They are also involved in the final common pathway for neuronal cell death following cerebral ischemia (Chen et al, 1998; Endres et al, 1998). Caspase-3 activity is increased predominantly in degenerating CA1 pyramidal neurons in global ischemia (Chen et al., 1998; Nuwa et al., 2001), and in neuronal perikarya within the middle cerebral artery territory in a focal ischemic model (Namura et al., 1998; Harrison et al., 2001; Benchoua et al., 2001). DNA laddering is correlated with increased caspase activity in the ischemic

region (Chen et al, 1998; Namura et al., 1998). More importantly, caspase inhibitors have been proved to be neuroprotective against hypoxia (Cheng et al., 1998), global (Chen et al., 1998; Zhan et al., 2001; Hayashi et al., 2001) and focal cerebral ischemia (Endres et al., 1998) accompanied with decreased DNA laddering. Thus, new strategies that inhibit caspase activity can be explored to block cell death in experimental models of cerebral ischemia and preserve neurological function.

Studies found that the Iaps are able to bind specifically to the caspases that function in the distal portions of the proteolytic cascades involved in apoptosis (Lacasse et al., 1998; Vucic et al., 1997). Accumulating evidences demonstrated that the apoptotic inhibition of Iaps is associated with inhibiting the activity of caspases (mainly caspases 3, 7 and 8) *in vitro* (Roy et al., 1997; Simons et al., 1999; Wagenknecht et al., 1999), in Drosophila (Kaiser et al., 1998; McCarthy and Dixit, 1998) and *in vivo* studies (Hara, 1999). The data suggest that one of the mechanisms by which naip inhibits apoptosis is similar as Iaps via regulating caspases (Figure 24), since naip shows significant homology with Iaps with the conserved BIR domains. A recent report confirmed that naip protected neuronal death against cerebral ischemia by inhibiting the elevated caspase activity (Hara, 1999).

iv other neuroprotective mechanisms by kindling

It is far from clear what accounts for the neuroprotective mechanism in hippocampal kindling A close anatomical, spatial correlation of sustained naip overexpression induced by kindling and reduction of cortical infarct is highly suggestive that naip is at least partially attributed to neuroprotection following

hippocampal kindling. There may be other mechanisms involved. An increased SOD activity that is long lasting in whole brain has been reported in kindled animals (Mori et al., 1991; Itoh et al., 1992; Mori et al., 1993). Cumulative evidence supports the notion that induceable SOD protects against neuronal injury. A delayed neuroprotective effect of low doses of lipopolysaccharide-induced brain ischemic tolerance is mediated by an increased synthesis of brain SOD (Bordet et al., 2000).

Reactive oxygen superoxide or free radicals are thought to be one major contributor to the neuronal cell death following cerebral ischemia. In experimental studies, an increased free radical generation during cerebral ischemia/reperfusion injury has been shown in vivo using several techniques such as microdialysis, salicylate spin trapping, and electron paramagnetic resonance (Zini et al., 1992; Globus et al., 1995; Dugan et al., 1995; Kil et al., 1996). An increase of lipid peroxidation products (Sakamoto et al., 1991; White et al., 1993) and a decrease in tissue antioxidant levels in the brain during ischemia (Kinuta et al., 1989) have been reported as indirect evidence of oxidative stress. One of the mechanisms that living cell exists in spite of the occurrence of the free radical is that free radical scavenging, such as SOD in the mitochondria, is capable of clearing free radicals. Pharmacological studies in animals showed that antioxidant molecules able to cross the blood-brain barrier, such as polyethylene glycol–conjugated SOD and catalase (Liu et al., 1989) and lazaroids (hall et al., 1994), reduce ischemic cerebral damage. Experimental study demonstrates that SOD overexpression markedly attenuates free radical generation and prevents cell death in culture. Membrane lipid peroxidation, protein nitration and neuronal cell death after focal cerebral ischemia are significantly reduced in transgenic mice

overexpression SOD (Keller et al., 1998; Kinouchi et al., 1991). Conversely, SOD mutant animals have greater cerebral infarction following focal cerebral ischemia (Kondo et al., 1997; Murakami et al., 1998).

One may postulate that the kindling phenomenon is similar to cortical spreading depression (CSD). CSD is classically characterized as a slowly propagating wave of neuronal and astrocytic depolarization that results in a transient depression of synaptic transmission (Leao, 1944; Somjen et al., 1992). CSD propagates radially from its initiation site and is associated with a redistribution of ions (K+ and Ca 2+) and an increase in local blood flow and energy metabolism (Dietrich et al., 1994; Hansen et al., 1980; Shinohara et al., 1979), most of which are rapidly reversed (Martins-Ferreira et al., 1966). Despite these marked changes, which are similar to those found during ischemia, experimental CSD does not result in irreversible neuronal damage, even when elicited repetitively for 4– 5 h (Kraig et al., 1991; Nedergaard and Hansen, 1988) whereas a prior episode of CSD can reduce the lesion area produced by subsequent ischemia in rats (Kobayashi et al., 1995; Matsushima et al., 1998; Taga et al., 1997; Yanamoto et al., 1998). Since CSD has no effect on cerebral blood flow during subsequent ischemia, the neuroprotective effect of CSD is thought to occur via a long-term up-regulation of the ability of brain cells to resist ischemic injury (Matsushima et al., 1995). In this regard, CSD has been reported to induce a range of short- and long-term neurochemical changes, including up-regulation of transcription and trophic factors (c-fos, brain-derived neurotrophic factor), neuropeptides (neuropeptide Y, cholecystokinin, enkephalin), transmitter receptors (adrenoreceptors),

and synaptic proteins (chromogranins) (Arabia et al., 1998; Jacobs et al., 1994; Kokaia et al., 1993; Rangel et al., 2001; Shen et al., 1998; Shen and Gundlach, 1998).

A recent study demonstrated that ischemic preconditioning attenuated both the early and late phases of cytochrome c redistribution (Zhan et al., 2001). Examination of neuronal survival revealed that ischemic preconditioning prevents ischemic hippocampal CA1 neuronal death. DNA fragmentation detected by TUNEL *in situ* was largely attenuated by ischemic preconditioning (Zhan et al., 2001). These results indicate that the loss of cytochrome *c* from mitochondria correlates with hippocampal CA1 neuronal death after transient cerebral ischemia and neuroprotective effects of ischemic preconditioning occur at the level of cytochrome *c* release.

Hippocampal kindling leads to a marked and transient increase in mRNA for NGF and BDNF in the dentate gyrus, the parietal cortex, and the piriform cortex (Ernfors et al., 1991). These neurotrophic factors are believed to be involved in modulating synaptic efficacy (Morimoto et al., 1998). The overexpression of these neurotrophic factors in the brain regions of kindled animals may also offer neuroprotective effects. Exogenous NGF has been reported to rescue injured neurons (Altar et al., 1992) and the atrophy of certain neuronal population such as basal forebrain cholinergic neurons can be rescued by NGF (Fisher et al., 1987). The majority of brain neurons do not respond to NGF. The newly discovered neurotrophic factors, BDNF, NT-3, NT4/5 affect a wider trophic activity for neurons. *In vitro*, BDNF enhances the survival and differentiation of mesencephalic dopaminergic neurons (Hyman et al., 1991; Knusel et al., 1991). *In vivo*, BDNF and NT4/5 have been shown to be able to prevent motorneuronal cell death following axotomy

(Koliatsos et al., 1993). NGF, BDNF and NT4/5 have also been used to prevent damage caused by excitotoxicity. NGF protects hippocampal neurons against glutamate toxicity (Cheng and Mattson, 1991) *in vitro*. *In vivo*, NGF reduces the size of the infarct caused by quinolinic acid striatum (Frim et al., 1993). NGF-transgenic mice are more resistant to focal cerebral ischemia by reducing cortical infarct and impairment of apoptosis (Guegan et al., 1998). Moreover, BDNF rescues cultured neurons exposed to 6-hydroxydopamine (Spina et al., 1992). Intracranial infusion of BDNF has been proven to reduce cortical infarction following focal ischemic insult. However, to date, there is no reports supporting a long lasting induction of the neurotrophic factors (more than a week) following hippocampal kindling, and there is no data suggesting that this transient induction of the neurotrophic factors can provide a long lasting protective effect.

Downregulation of glutamate receptors has also been reported in kindling studies. Wong et al (1993) examined the levels of GluR1 mRNA in hippocampal sections from kindled animals using *in situ* hybridization histochemistry. A transient downregulation of GluR1 in the brain was noticed 24 h after the last kindled seizure. GluR2 was also found to be reduced in limbic cortex and piriform cortex in kindled animals. The decrease in GluR2 that was observed 24 h after the last kindled seizure, but was not present one week and one month later (Prince et al., 1995). A similar study also found a 22-58% decreases in AMPA/kainate receptor mRNA in the hippocampus, amygdala, entorhinal cortex and in frontoparietal cortex 24 h but not 30 days after the last kindled seizure (Lee et al., 1994). The NMDA receptor has also been demonstrated to undergo transient downregulation in the brain of kindled

animals, and this decrease no longer present 7 days later (Akiyama et al., 1992). Down regulation of the glutamate receptor resulted in decreased calcium influx, prevent the intracellular calcium overload and eventually prevent neuronal damage against excitotoxicity. Again the studies reviewed above only reveal a transient downregulation of the glutamate receptors, which may hardly explain the protective effects lasting month after the last kindled seizure activity.

Although hippocampal kindling induces transient growth factor expression (Morimoto et al., 1998) and promotes free radical scavenger generation (Itoh et al., 1992; Mori et al., 1993), which might partially contribute to neuroprotection as well. However, the close correlation between the naip overexpression in the frontoparietal cortex and the reduction of cortical infarct in the kindled animals, strongly suggests that naip at least might partially contribute to the protective effects against cerebral ischemia by inhibiting neuronal apoptosis at the ischemic penumbra and limiting infarct expansion. Further studies in transgenic animals that overexpressing naip shall provide more direct evidences for the role of naip protecting against ischemic neuronal cell death.

CHAPTER 5

CONCLUSION

Altered gene expression may be associated with apoptosis following cerebral ischemia , which might contribute to the expansion of cortical infarction. This thesis provides further evidence to support the notion of apoptotic elements involving in ischemic neuronal injury from molecular basis. An increased expression of p53 and its downstream DNA genes gadd45, and mdm2 indicate that a growth arrest, DNA repairing or/and apoptosis process has occurred following transient cerebral ischemia. Futhermore, overexpression in anti-apoptotic gene naip by hippocampal kindling is evident in several selective brain regions, which correlate with a significant infarct volume reduction following transient focal cerebral ischemia. These data suggest that naip might be capable of inhibiting some apoptotic components and limiting the infarct expansion following focal cerebral ischemia. This study provides a basis for strategies of design of possible genetic therapies for cerebral vascular disease.

REFERENCES

R. F. Abercrombie and C. E. Hart. Calcium and proton buffering and diffusion in isolated cytoplasm from Myxicola axons. *Am.J.Physiol* 250 (3 Pt 1):C391-C405, 1986.

S. G. Agrawal and R. H. Evans. The primary afferent depolarizing action of kainate in the rat. *Br.J.Pharmacol.* 87 (2):345-355, 1986.

P. T. Akins, P. K. Liu, and C. Y. Hsu. Immediate early gene expression in response to cerebral ischemia. Friend or foe? *Stroke* 27 (9):1682-1687, 1986.

K. Akiyama, Y. Yoneda, K. Ogita, T. Itoh, A. Daigen, I. Sora, I. Kohira, H. Ujike, and S. Otsuki. Ionotropic excitatory amino acid receptors in discrete brain regions of kindled rats. *Brain Res.* 587 (1):73-82, 1986.

D. R. Aldridge and I. R. Radford. Explaining differences in sensitivity to killing by ionizing radiation between human lymphoid cell lines. *Cancer Res.* 58 (13):2817-2824, 1998.

T. E. Allsopp, S. Wyatt, H. F. Paterson, and A. M. Davies. The proto-oncogene bcl-2 can selectively rescue neurotrophic factor- dependent neurons from apoptosis. *Cell* 73 (2):295-307, 1993.

E. S. Alnemri, N. M. Robertson, T. F. Fernandes, C. M. Croce, and G. Litwack. Overexpressed full-length human BCL2 extends the survival of

baculovirus-infected Sf9 insect cells. *Proc.Natl.Acad.Sci.U.S.A* 89 (16):7295-7299, 1992.

E. S. Alnemri, D. J. Livingston, D. W. Nicholson, G. Salvesen, N. A. Thornberry, W. W. Wong, and J. Yuan. Human ICE/CED-3 protease nomenclature. *Cell* 87 (2):171, 1996.

C. A. Altar, M. Armanini, M. Dugich-Djordjevic, G. L. Bennett, R. Williams, S. Feinglass, V. Anicetti, D. Sinicropi, and C. Bakhit. Recovery of cholinergic phenotype in the injured rat neostriatum: roles for endogenous and exogenous nerve growth factor. *J.Neurochem.* 59 (6):2167-2177, 1992.

G. Ambrosini, C. Adida, and D. C. Altieri. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat.Med.* 3 (8):917-921, 1997.

G. An, J. Tesfaigzi, D. M. Carlson, and R. Wu. Expression of a squamous cell marker, the spr1 gene, is posttranscriptionally down-regulated by retinol in airway epithelium. *J.Cell Physiol* 157 (3):562-568, 1993.

P. Angel and M. Karin. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim.Biophys.Acta* 1072 (2-3):129-157, 1991.

B. Antonsson, F. Conti, A. Ciavatta, S. Montessuit, S. Lewis, I. Martinou, L. Bernasconi, A. Bernard, J. J. Mermod, G. Mazzei, K. Maundrell, F.

Gambale, R. Sadoul, and J. C. Martinou. Inhibition of Bax channel-forming activity by Bcl-2. *Science* 277 (5324):370-372, 1997.

A. M. Arabia, P. J. Shen, and A. L. Gundlach. Increased striatal proenkephalin mRNA subsequent to production of spreading depression in rat cerebral cortex: activation of corticostriatal pathways? *Brain Res.Mol.Brain Res.* 61 (1-2):195-202, 1998.

H. Arai, J. V. Passonneau, and W. D. Lust. Energy metabolism in delayed neuronal death of CA1 neurons of the hippocampus following transient ischemia in the gerbil. *Metab Brain Dis.* 1 (4):263-278, 1986.

T. Araki, H. Kato, T. Inoue, and K. Kogure. Regional impairment of protein synthesis following brief cerebral ischemia in the gerbil. *Acta Neuropathol.(Berl)* 79 (5):501-505, 1990.

J. Ashkenas and Z. Werb. Proteolysis and the biochemistry of life-or-death decisions. *J.Exp.Med.* 183 (5):1947-1951, 1996.

A. Ashkenazi and V. M. Dixit. Death receptors: signaling and modulation. *Science* 281 (5381):1305-1308, 1998.

J. Astrup, B. K. Siesjo, and L. Symon. Thresholds in cerebral ischemia - the ischemic penumbra. *Stroke* 12 (6):723-725, 1981.

J. Bargonetti, P. N. Friedman, S. E. Kern, B. Vogelstein, and C. Prives. Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. *Cell* 65 (6):1083-1091, 1991.

J. S. Beckman, T. W. Beckman, J. Chen, P. A. Marshall, and B. A. Freeman. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc.Natl.Acad.Sci.U.S.A* 87 (4):1620-1624, 1990.

C. Behl, L. Hovey, III, S. Krajewski, D. Schubert, and J. C. Reed. BCL-2 prevents killing of neuronal cells by glutamate but not by amyloid beta protein. *Biochem.Biophys.Res.Commun.* 197 (2):949-956, 1993.

C. O. Bellamy. p53 and apoptosis. *Br.Med.Bull.* 53 (3):522-538, 1997.

A. Benchoua, C. Guegan, C. Couriaud, H. Hosseini, N. Sampaio, D. Morin, and B. Onteniente. Specific caspase pathways are activated in the two stages of cerebral infarction. *J.Neurosci.* 21 (18):7127-7134, 2001.

P. Bernardi, K. M. Broekemeier, and D. R. Pfeiffer. Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. *J.Bioenerg.Biomembr.* 26 (5):509-517, 1994.

B. Bettler, J. Boulter, I. Hermans-Borgmeyer, A. O'Shea-Greenfield, E. S. Deneris, C. Moll, U. Borgmeyer, M. Hollmann, and S. Heinemann. Cloning of a

novel glutamate receptor subunit, GluR5: expression in the nervous system during development. *Neuron* 5 (5):583-595, 1990.

B. Bettler, J. Egebjerg, G. Sharma, G. Pecht, I. Hermans-Borgmeyer, C. Moll, C. F. Stevens, and S. Heinemann. Cloning of a putative glutamate receptor: a low affinity kainate- binding subunit. *Neuron* 8 (2):257-265, 1992.

R. Bordet, D. Deplanque, P. Maboudou, F. Puisieux, Q. Pu, E. Robin, A. Martin, M. Bastide, D. Leys, M. Lhermitte, and B. Dupuis. Increase in endogenous brain superoxide dismutase as a potential mechanism of lipopolysaccharide-induced brain ischemic tolerance. *J.Cereb.Blood Flow Metab* 20 (8):1190-1196, 2000.

I. Brabet, S. Mary, J. Bockaert, and J. P. Pin. Phenylglycine derivatives discriminate between mGluR1- and mGluR5- mediated responses. *Neuropharmacology* 34 (8):895-903, 1995.

D. S. Bredt, P. M. Hwang, and S. H. Snyder. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 347 (6295):768-770, 1990.

D. S. Bredt and S. H. Snyder. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc.Natl.Acad.Sci.U.S.A* 87 (2):682-685, 1990.

D. S. Bredt and S. H. Snyder. Nitric oxide, a novel neuronal messenger. *Neuron* 8 (1):3-11, 1992.

S. Brint, M. Jacewicz, M. Kiessling, J. Tanabe, and W. Pulsinelli. Focal brain ischemia in the rat: methods for reproducible neocortical infarction using tandem occlusion of the distal middle cerebral and ipsilateral common carotid arteries. *J.Cereb.Blood Flow Metab* 8 (4):474-485, 1988.

J. R. Brorson, P. A. Manzolillo, and R. J. Miller. Ca2+ entry via AMPA/KA receptors and excitotoxicity in cultured cerebellar Purkinje cells. *J.Neurosci.* 14 (1):187-197, 1994.

A. Bruccoleri, R. Gallucci, D. R. Germolec, P. Blackshear, P. Simeonova, R. G. Thurman, and M. I. Luster. Induction of early-immediate genes by tumor necrosis factor alpha contribute to liver repair following chemical-induced hepatotoxicity. *Hepatology* 25 (1):133-141, 1997.

A. J. Bruce-Keller, J. G. Begley, W. Fu, D. A. Butterfield, D. E. Bredesen, J. B. Hutchins, K. Hensley, and M. P. Mattson. Bcl-2 protects isolated plasma and mitochondrial membranes against lipid peroxidation induced by hydrogen peroxide and amyloid beta- peptide. *J.Neurochem.* 70 (1):31-39, 1998.

A. J. Bruce-Keller, J. W. Geddes, P. E. Knapp, R. W. McFall, J. N. Keller, F. W. Holtsberg, S. Parthasarathy, S. M. Steiner, and M. P. Mattson. Anti-death properties of TNF against metabolic poisoning: mitochondrial stabilization by MnSOD. *J.Neuroimmunol.* 93 (1-2):53-71, 1999.

A. M. Buchan, D. Xue, and A. Slivka. A new model of temporary focal neocortical ischemia in the rat. *Stroke* 23 (2):273-279, 1992.

N. Burnashev, H. Monyer, P. H. Seeburg, and B. Sakmann. Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* 8 (1):189-198, 1992.

T. M. Buttke and P. A. Sandstrom. Oxidative stress as a mediator of apoptosis. *Immunol.Today* 15 (1):7-10, 1994.

L. Cahilly-Snyder, T. Yang-Feng, U. Francke, and D. L. George. Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. *Somat.Cell Mol.Genet.* 13 (3):235-244, 1987.

K. Cain, S. B. Bratton, C. Langlais, G. Walker, D. G. Brown, X. M. Sun, and G. M. Cohen. Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4-MDa apoptosome complexes. *J.Biol.Chem.* 275 (9):6067-6070, 2000.

C. E. Canman, T. M. Gilmer, S. B. Coutts, and M. B. Kastan. Growth factor modulation of p53-mediated growth arrest versus apoptosis. *Genes Dev.* 9 (5):600-611, 1995.

G. Cao, M. Minami, W. Pei, C. Yan, D. Chen, C. O'Horo, S. H. Graham, and J. Chen. Intracellular Bax translocation after transient cerebral ischemia:

implications for a role of the mitochondrial apoptotic signaling pathway in ischemic neuronal death. *J.Cereb.Blood Flow Metab* 21 (4):321-333, 2001.

L. Casciola-Rosen, D. W. Nicholson, T. Chong, K. R. Rowan, N. A. Thornberry, D. K. Miller, and A. Rosen. Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. *J.Exp.Med.* 183 (5):1957-1964, 1996.

P. H. Chan and R. A. Fishman. Transient formation of superoxide radicals in polyunsaturated fatty acid-induced brain swelling. *J.Neurochem.* 35 (4):1004-1007, 1980.

P. H. Chan. Oxygen radicals in focal cerebral ischemia. *Brain Pathol.* 4 (1):59-65, 1994.

P. H. Chan. Role of oxidants in ischemic brain damage. *Stroke* 27 (6):1124-1129, 1996.

J. M. Chandler, E. S. Alnemri, G. M. Cohen, and M. MacFarlane. Activation of CPP32 and Mch3 alpha in wild-type p53-induced apoptosis. *Biochem.J.* 322 (Pt 1):19-23, 1997.

C. Charriaut-Marlangue, I. Margaill, M. Plotkine, and Y. Ben Ari. Early endonuclease activation following reversible focal ischemia in the rat brain. *J.Cereb.Blood Flow Metab* 15 (3):385-388, 1995.

Z. Chen-Levy, J. Nourse, and M. L. Cleary. The bcl-2 candidate proto-oncogene product is a 24-kilodalton integral- membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. *Mol.Cell Biol.* 9 (2):701-710, 1989.

J. Chen, V. Marechal, and A. J. Levine. Mapping of the p53 and mdm-2 interaction domains. *Mol.Cell Biol.* 13 (7):4107-4114, 1993.

J. Chen, X. Wu, J. Lin, and A. J. Levine. mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. *Mol.Cell Biol.* 16 (5):2445-2452, 1996.

J. Chen, K. Jin, M. Chen, W. Pei, K. Kawaguchi, D. A. Greenberg, and R. P. Simon. Early detection of DNA strand breaks in the brain after transient focal ischemia: implications for the role of DNA damage in apoptosis and neuronal cell death. *J.Neurochem.* 69 (1):232-245, 1997.

J. Chen, T. Nagayama, K. Jin, R. A. Stetler, R. L. Zhu, S. H. Graham, and R. P. Simon. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J.Neurosci.* 18 (13):4914-4928, 1998.

Z. Chen, M. Naito, S. Hori, T. Mashima, T. Yamori, and T. Tsuruo. A human IAP-family gene, apollon, expressed in human brain cancer cells. *Biochem.Biophys.Res.Commun.* 264 (3):847-854, 1999.

B. Cheng and M. P. Mattson. NGF and bFGF protect rat hippocampal and human cortical neurons against hypoglycemic damage by stabilizing calcium homeostasis. *Neuron* 7 (6):1031-1041, 1991.

Y. Cheng, M. Deshmukh, A. D'Costa, J. A. Demaro, J. M. Gidday, A. Shah, Y. Sun, M. F. Jacquin, E. M. Johnson, and D. M. Holtzman. Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. *J.Clin.Invest* 101 (9):1992-1999, 1998.

P. L. Chin, J. Momand, and G. P. Pfeifer. In vivo evidence for binding of p53 to consensus binding sites in the p21 and GADD45 genes in response to ionizing radiation. *Oncogene* 15 (1):87-99, 1997.

A. M. Chinnaiyan, K. O'Rourke, B. R. Lane, and V. M. Dixit. Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science* 275 (5303):1122-1126, 1997.

D. W. Choi. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1 (8):623-634, 1988.

D. W. Choi. Excitotoxic cell death. *J.Neurobiol.* 23 (9):1261-1276, 1992.

D. W. Choi. Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci.* 18 (2):58-60, 1995.

D. W. Choi. Ischemia-induced neuronal apoptosis. *Curr.Opin.Neurobiol.* 6 (5):667-672, 1996.

M. Chopp, Y. Li, Z. G. Zhang, and S. O. Freytag. p53 expression in brain after middle cerebral artery occlusion in the rat. *Biochem.Biophys.Res.Commun.* 182 (3):1201-1207, 1992.

M. Chopp and Y. Li. Apoptosis in focal cerebral ischemia. *Acta Neurochir.Suppl (Wien.)* 66:21-26, 1996.

R. J. Clem and L. K. Miller. Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus. *J.Virol.* 67 (7):3730-3738, 1993.

F. Colbourne, G. R. Sutherland, and R. N. Auer. Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. *J.Neurosci.* 19 (11):4200-4210, 1999.

F. Colotta, N. Polentarutti, M. Sironi, and A. Mantovani. Expression and involvement of c-fos and c-jun protooncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines. *J.Biol.Chem.* 267 (26):18278-18283, 1992.

M. Crompton. The mitochondrial permeability transition pore and its role in cell death. *Biochem.J.* 341 (Pt 2):233-249, 1999.

M. J. Croucher, J. F. Collins, and B. S. Meldrum. Anticonvulsant action of excitatory amino acid antagonists. *Science* 216 (4548):899-901, 1982.

R. C. Crumrine, A. L. Thomas, and P. F. Morgan. Attenuation of p53 expression protects against focal ischemic damage in transgenic mice. *J.Cereb.Blood Flow Metab* 14 (6):887-891, 1994.

T. M. Dawson, V. L. Dawson, and S. H. Snyder. A novel neuronal messenger molecule in brain: the free radical, nitric oxide. *Ann.Neurol.* 32 (3):297-311, 1992.

R. M. Denton and J. G. McCormack. Ca2+ as a second messenger within mitochondria of the heart and other tissues. *Annu.Rev.Physiol* 52:451-466, 1990.

J. Deshpande, K. Bergstedt, T. Linden, H. Kalimo, and T. Wieloch. Ultrastructural changes in the hippocampal CA1 region following transient cerebral ischemia: evidence against programmed cell death. *Exp.Brain Res.* 88 (1):91-105, 1992.

Q. L. Deveraux, R. Takahashi, G. S. Salvesen, and J. C. Reed. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 388 (6639):300-304, 1997.

Q. L. Deveraux, N. Roy, H. R. Stennicke, T. Van Arsdale, Q. Zhou, S. M. Srinivasula, E. S. Alnemri, G. S. Salvesen, and J. C. Reed. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J.* 17 (8):2215-2223, 1998.

Q. L. Deveraux and J. C. Reed. IAP family proteins--suppressors of apoptosis. *Genes Dev.* 13 (3):239-252, 1999.

W. D. Dietrich, Z. C. Feng, H. Leistra, B. D. Watson, and M. Rosenthal. Photothrombotic infarction triggers multiple episodes of cortical spreading depression in distant brain regions. *J.Cereb.Blood Flow Metab* 14 (1):20-28, 1994.

L. Diller, J. Kassel, C. E. Nelson, M. A. Gryka, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S. J. Baker, B. Vogelstein, and . p53 functions as a cell cycle control protein in osteosarcomas. *Mol.Cell Biol.* 10 (11):5772-5781, 1990.

R. C. Dodel, Y. Du, K. R. Bales, Z. Ling, P. M. Carvey, and S. M. Paul. Caspase-3-like proteases and 6-hydroxydopamine induced neuronal cell death. *Brain Res.Mol.Brain Res.* 64 (1):141-148, 1999.

L. A. Donehower, M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel, and A. Bradley. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356 (6366):215-221, 1992.

J. Dosch and B. Kaina. Induction of c-fos, c-jun, junB and junD mRNA and AP-1 by alkylating mutagens in cells deficient and proficient for the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) and its

relationship to cell death, mutation induction and chromosomal instability. *Oncogene* 13 (9):1927-1935, 1996.

J. P. Doucet, Y. Nakabeppu, P. J. Bedard, B. T. Hope, E. J. Nestler, B. J. Jasmin, J. S. Chen, M. J. Iadarola, M. St Jean, N. Wigle, P. Blanchet, R. Grondin, and G. S. Robertson. Chronic alterations in dopaminergic neurotransmission produce a persistent elevation of deltaFosB-like protein(s) in both the rodent and primate striatum. *Eur.J.Neurosci.* 8 (2):365-381, 1996.

M. Dragunow, E. Beilharz, E. Sirimanne, P. Lawlor, C. Williams, R. Bravo, and P. Gluckman. Immediate-early gene protein expression in neurons undergoing delayed death, but not necrosis, following hypoxic-ischaemic injury to the young rat brain. *Brain Res.Mol.Brain Res.* 25 (1-2):19-33, 1994.

M. Dragunow and K. Preston. The role of inducible transcription factors in apoptotic nerve cell death. *Brain Res.Brain Res.Rev.* 21 (1):1-28, 1995.

J. Drejer, H. Benveniste, N. H. Diemer, and A. Schousboe. Cellular origin of ischemia-induced glutamate release from brain tissue in vivo and in vitro. *J.Neurochem.* 45 (1):145-151, 1985.

C. Du, R. Hu, C. A. Csernansky, C. Y. Hsu, and D. W. Choi. Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? *J.Cereb.Blood Flow Metab* 16 (2):195-201, 1996.

C. S. Duckett, V. E. Nava, R. W. Gedrich, R. J. Clem, J. L. Van Dongen, M. C. Gilfillan, H. Shiels, J. M. Hardwick, and C. B. Thompson. A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *EMBO J.* 15 (11):2685-2694, 1996.

T. E. Duffy, S. R. Nelson, and O. H. Lowry. Cerebral carbohydrate metabolism during acute hypoxia and recovery. *J.Neurochem.* 19 (4):959-977, 1972.

L. L. Dugan, S. L. Sensi, L. M. Canzoniero, S. D. Handran, S. M. Rothman, T. S. Lin, M. P. Goldberg, and D. W. Choi. Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J.Neurosci.* 15 (10):6377-6388, 1995.

E. Dux, G. Mies, K. A. Hossmann, and L. Siklos. Calcium in the mitochondria following brief ischemia of gerbil brain. *Neurosci.Lett.* 78 (3):295-300, 1987.

J. A. Dykens, A. Stern, and E. Trenkner. Mechanism of kainate toxicity to cerebellar neurons in vitro is analogous to reperfusion tissue injury. *J.Neurochem.* 49 (4):1222-1228, 1987.

J. A. Dykens. Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated CA2+ and Na+: implications for neurodegeneration. *J.Neurochem.* 63 (2):584-591, 1994.

J. Egebjerg, B. Bettler, I. Hermans-Borgmeyer, and S. Heinemann. Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. *Nature* 351 (6329):745-748, 1991.

J. Egebjerg and S. F. Heinemann. Ca2+ permeability of unedited and edited versions of the kainate selective glutamate receptor GluR6. *Proc.Natl.Acad.Sci.U.S.A* 90 (2):755-759, 1993.

W. S. el Deiry, T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein. WAF1, a potential mediator of p53 tumor suppression. *Cell* 75 (4):817-825, 1993.

M. Enari, H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391 (6662):43-50, 1998.

M. Endres, S. Namura, M. Shimizu-Sasamata, C. Waeber, L. Zhang, T. Gomez-Isla, B. T. Hyman, and M. A. Moskowitz. Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family. *J.Cereb.Blood Flow Metab* 18 (3):238-247, 1998.

P. Ernfors, J. Bengzon, Z. Kokaia, H. Persson, and O. Lindvall. Increased levels of messenger RNAs for neurotrophic factors in the brain during kindling epileptogenesis. *Neuron* 7 (1):165-176, 1991.

S. Estus, W. J. Zaks, R. S. Freeman, M. Gruda, R. Bravo, and E. M. Johnson, Jr. Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. *J.Cell Biol.* 127 (6 Pt 1):1717-1727, 1994.

J. L. Farber and E. E. Young. Accelerated phospholipid degradation in anoxic rat hepatocytes. *Arch.Biochem.Biophys.* 211 (1):312-320, 1981.

G. Farmer, J. Bargonetti, H. Zhu, P. Friedman, R. Prywes, and C. Prives. Wild-type p53 activates transcription in vitro. *Nature* 358 (6381):83-86, 1992.

S. Fields and S. K. Jang. Presence of a potent transcription activating sequence in the p53 protein. *Science* 249 (4972):1046-1049, 1990.

R. K. Filipkowski, M. Hetman, B. Kaminska, and L. Kaczmarek. DNA fragmentation in rat brain after intraperitoneal administration of kainate. *Neuroreport* 5 (12):1538-1540, 1994.

W. Fischer, K. Wictorin, A. Bjorklund, L. R. Williams, S. Varon, and F. H. Gage. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature* 329 (6134):65-68, 1987.

E. Fontaine, O. Eriksson, F. Ichas, and P. Bernardi. Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation By electron flow through the respiratory chain complex i. *J.Biol.Chem.* 273 (20):12662-12668, 1998.

A. G. Fraser, C. James, G. I. Evan, and M. O. Hengartner. Caenorhabditis elegans inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis. *Curr.Biol.* 9 (6):292-301, 1999.

H. Friberg, M. Ferrand-Drake, F. Bengtsson, A. P. Halestrap, and T. Wieloch. Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. *J.Neurosci.* 18 (14):5151-5159, 1998.

D. M. Frim, T. A. Uhler, M. P. Short, Z. D. Ezzedine, M. Klagsbrun, X. O. Breakefield, and O. Isacson. Effects of biologically delivered NGF, BDNF and bFGF on striatal excitotoxic lesions. *Neuroreport* 4 (4):367-370, 1993.

E. J. Fuchs, K. A. McKenna, and A. Bedi. p53-dependent DNA damage-induced apoptosis requires Fas/APO-1- independent activation of CPP32beta. *Cancer Res.* 57 (13):2550-2554, 1997.

M. Fujimura, Y. Morita-Fujimura, K. Murakami, M. Kawase, and P. H. Chan. Cytosolic redistribution of cytochrome c after transient focal cerebral ischemia in rats. *J.Cereb.Blood Flow Metab* 18 (11):1239-1247, 1998.

V. Gagliardini, P. A. Fernandez, R. K. Lee, H. C. Drexler, R. J. Rotello, M. C. Fishman, and J. Yuan. Prevention of vertebrate neuronal death by the crmA gene. *Science* 263 (5148):826-828, 1994.

I. Garcia, I. Martinou, Y. Tsujimoto, and J. C. Martinou. Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. *Science* 258 (5080):302-304, 1992.

Y. Gavrieli, Y. Sherman, and S. A. Ben Sasson. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J.Cell Biol.* 119 (3):493-501, 1992.

T. A. Gilbertson, R. Scobey, and M. Wilson. Permeation of calcium ions through non-NMDA glutamate channels in retinal bipolar cells. *Science* 251 (5001):1613-1615, 1991.

D. Ginsberg, F. Mechta, M. Yaniv, and M. Oren. Wild-type p53 can down-modulate the activity of various promoters. *Proc.Natl.Acad.Sci.U.S.A* 88 (22):9979-9983, 1991.

S.R. Glaum and R.J. Miller RJ. In The metabotropic glutamate receptors: Acute regulation of synaptic transmission by metabotropic glutamate receptors. Eds. Conn PJ and Patel J. pp.147-72. *Humana Press*, 1995.

M. Y. Globus, R. Busto, B. Lin, H. Schnippering, and M. D. Ginsberg. Detection of free radical activity during transient global ischemia and recirculation: effects of intraischemic brain temperature modulation. *J.Neurochem.* 65 (3):1250-1256, 1995.

S. A. Goldman, W. A. Pulsinelli, W. Y. Clarke, R. P. Kraig, and F. Plum. The effects of extracellular acidosis on neurons and glia in vitro. *J.Cereb.Blood Flow Metab* 9 (4):471-477, 1989.

P. Golstein, D. M. Ojcius, and J. D. Young. Cell death mechanisms and the immune system. *Immunol.Rev.* 121:29-65, 1991.

D. Gorlich, F. Vogel, A. D. Mills, E. Hartmann, and R. A. Laskey. Distinct functions for the two importin subunits in nuclear protein import. *Nature* 377 (6546):246-248, 1995.

A. M. Gorman, M. P. Scott, P. C. Rumsby, C. Meredith, and R. Griffiths. Excitatory amino acid-induced cytotoxicity in primary cultures of mouse cerebellar granule cells correlates with elevated, sustained c-fos proto-oncogene expression. *Neurosci.Lett.* 191 (1-2):116-120, 1995.

K. Goto, A. Ishige, K. Sekiguchi, S. Iizuka, A. Sugimoto, M. Yuzurihara, M. Aburada, E. Hosoya, and K. Kogure. Effects of cycloheximide on delayed neuronal death in rat hippocampus. *Brain Res.* 534 (1-2):299-302, 1990.

T. G. Graeber, C. Osmanian, T. Jacks, D. E. Housman, C. J. Koch, S. W. Lowe, and A. J. Giaccia. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 379 (6560):88-91, 1996.

D. R. Green and J. C. Reed. Mitochondria and apoptosis. *Science* 281 (5381):1309-1312, 1998.

Y. Gu, C. W. Turck, and D. O. Morgan. Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature* 366 (6456):707-710, 1993.

C. Guegan, B. Onteniente, Y. Makiura, M. Merad-Boudia, I. Ceballos-Picot, and B. Sola. Reduction of cortical infarction and impairment of apoptosis in NGF-transgenic mice subjected to permanent focal ischemia. *Brain Res.Mol.Brain Res.* 55 (1):133-140, 1998.

T. E. Gunter and D. R. Pfeiffer. Mechanisms by which mitochondria transport calcium. *Am.J.Physiol* 258 (5 Pt 1):C755-C786, 1990.

T. E. Gunter, K. K. Gunter, S. S. Sheu, and C. E. Gavin. Mitochondrial calcium transport: physiological and pathological relevance. *Am.J.Physiol* 267 (2 Pt 1):C313-C339, 1994.

A. M. Hakim. Ischemic penumbra: the therapeutic window. *Neurology* 51 (3 Suppl 3):S44-S46, 1998.

E. D. Hall, J. M. McCall, and E. D. Means. Therapeutic potential of the lazaroids (21-aminosteroids) in acute central nervous system trauma, ischemia and subarachnoid hemorrhage. *Adv.Pharmacol.* 28:221-268, 1994.

A. J. Hansen, B. Quistorff, and A. Gjedde. Relationship between local changes in cortical blood flow and extracellular K+ during spreading depression. *Acta Physiol Scand.* 109 (1):1-6, 1980.

H. Hara. Involvement of caspase on apoptosis in ischemia-induced neuronal cell death: usefulness of caspase inhibitors for stroke therapy. *Nippon Yakurigaku Zasshi* 113 (2):97-111, 1999.

J. W. Harper, G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin- dependent kinases. *Cell* 75 (4):805-816, 1993.

D. C. Harrison, R. P. Davis, B. C. Bond, C. A. Campbell, M. F. James, A. A. Parsons, and K. L. Philpott. Caspase mRNA expression in a rat model of focal cerebral ischemia. *Brain Res.Mol.Brain Res.* 89 (1-2):133-146, 2001.

L. Hartwell. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* 71 (4):543-546, 1992.

L. H. Hartwell and M. B. Kastan. Cell cycle control and cancer. *Science* 266 (5192):1821-1828, 1994.

Y. Haupt, S. Rowan, E. Shaulian, K. H. Vousden, and M. Oren. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes Dev.* 9 (17):2170-2183, 1995.

Y. Haupt, R. Maya, A. Kazaz, and M. Oren. Mdm2 promotes the rapid degradation of p53. *Nature* 387 (6630):296-299, 1997.

C. J. Hawkins, A. G. Uren, G. Hacker, R. L. Medcalf, and D. L. Vaux. Inhibition of interleukin 1 beta-converting enzyme-mediated apoptosis of mammalian cells by baculovirus IAP. *Proc.Natl.Acad.Sci.U.S.A* 93 (24):13786-13790, 1996.

Y. Hayashi, I. Jikihara, T. Yagi, M. Fukumura, Y. Ohashi, Y. Ohta, H. Takagi, and M. Maeda. Immunohistochemical investigation of caspase-1 and effect of caspase-1 inhibitor in delayed neuronal death after transient cerebral ischemia. *Brain Res.* 893 (1-2):113-120, 2001.

W. D. Heiss. Flow thresholds of functional and morphological damage of brain tissue. *Stroke* 14 (3):329-331, 1983.

T. Hennet, G. Bertoni, C. Richter, and E. Peterhans. Expression of BCL-2 protein enhances the survival of mouse fibrosarcoid cells in tumor necrosis factor-mediated cytotoxicity. *Cancer Res.* 53 (6):1456-1460, 1993.

A. Herb, N. Burnashev, P. Werner, B. Sakmann, W. Wisden, and P. H. Seeburg. The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. *Neuron* 8 (4):775-785, 1992.

A. Heron, H. Pollard, F. Dessi, J. Moreau, F. Lasbennes, Y. Ben Ari, and C. Charriaut-Marlangue. Regional variability in DNA fragmentation after global ischemia evidenced by combined histological and gel electrophoresis observations in the rat brain. *J.Neurochem.* 61 (5):1973-1976, 1993.

D. G. Herrera, D. Maysinger, R. Gadient, C. Boeckh, U. Otten, and A. C. Cuello. Spreading depression induces c-fos-like immunoreactivity and NGF mRNA in the rat cerebral cortex. *Brain Res.* 602 (1):99-103, 1993.

J. M. Herrmann and W. Neupert. Protein transport into mitochondria. *Curr.Opin.Microbiol.* 3 (2):210-214, 2000.

K. H. Herzog and J. I. Morgan. Cellular immediate-early genes and cell death in the nervous system. *Neuropathol.Appl.Neurobiol.* 22 (6):484-488, 1996.

L. Hillered, M. L. Smith, and B. K. Siesjo. Lactic acidosis and recovery of mitochondrial function following forebrain ischemia in the rat. *J.Cereb.Blood Flow Metab* 5 (2):259-266, 1985.

D. Hockenbery, G. Nunez, C. Milliman, R. D. Schreiber, and S. J. Korsmeyer. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348 (6299):334-336, 1990.

D. M. Hockenbery, Z. N. Oltvai, X. M. Yin, C. L. Milliman, and S. J. Korsmeyer. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 75 (2):241-251, 1993.

M. Hollmann, A. O'Shea-Greenfield, S. W. Rogers, and S. Heinemann. Cloning by functional expression of a member of the glutamate receptor family. *Nature* 342 (6250):643-648, 1989.

M. Hollmann, M. Hartley, and S. Heinemann. Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science* 252 (5007):851-853, 1991.

S. Hortelano, B. Dallaporta, N. Zamzami, T. Hirsch, S. A. Susin, I. Marzo, L. Bosca, and G. Kroemer. Nitric oxide induces apoptosis via triggering mitochondrial permeability transition. *FEBS Lett.* 410 (2-3):373-377, 1997.

S. T. Hou, Y. Tu, A. M. Buchan, Z. Huang, E. Preston, I. Rasquinha, G. S. Robertson, and J. P. MacManus. Increases in DNA lesions and the DNA damage indicator Gadd45 following transient cerebral ischemia. *Biochem.Cell Biol.* 75 (4):383-392, 1997.

C. Y. Huang, M. Fujimura, N. Noshita, Y. Y. Chang, and P. H. Chan. SOD1 down-regulates NF-kappaB and c-Myc expression in mice after transient focal cerebral ischemia. *J.Cereb.Blood Flow Metab* 21 (2):163-173, 2001.

J. E. Huettner. Glutamate receptor channels in rat DRG neurons: activation by kainate and quisqualate and blockade of desensitization by Con A. *Neuron* 5 (3):255-266, 1990.

P. Hughes and M. Dragunow. Induction of immediate-early genes and the control of neurotransmitter- regulated gene expression within the nervous system. *Pharmacol.Rev.* 47 (1):133-178, 1995.

C. Hyman, M. Hofer, Y. A. Barde, M. Juhasz, G. D. Yancopoulos, S. P. Squinto, and R. M. Lindsay. BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature* 350 (6315):230-232, 1991.

S. R. Ikeda, D. M. Lovinger, B. A. McCool, and D. L. Lewis. Heterologous expression of metabotropic glutamate receptors in adult rat sympathetic neurons: subtype-specific coupling to ion channels. *Neuron* 14 (5):1029-1038, 1995.

S. Isenmann, G. Stoll, M. Schroeter, S. Krajewski, J. C. Reed, and M. Bahr. Differential regulation of Bax, Bcl-2, and Bcl-X proteins in focal cortical ischemia in the rat. *Brain Pathol.* 8 (1):49-62, 1998.

T. Itoh, K. Akiyama, M. Hiramatsu, and S. Otsuki. Synaptosomal membrane fluidity, lipid peroxidation and superoxide dismutase activity in the brain of amygdala-kindled rats. *Jpn.J.Psychiatry Neurol.* 46 (4):957-966, 1992.

K. Izumiyama, K. Kogure, S. Kataoka, and T. Nagata. Quantitative analysis of glucose after transient ischemia in the gerbil hippocampus by light and electron microscope radioautography. *Brain Res.* 416 (1):175-179, 1987.

T. Jacks and R. A. Weinberg. Cell-cycle control and its watchman. *Nature* 381 (6584):643-644, 1996.

O. Jacobs, L. Van Bree, P. Mailleux, F. Zhang, S. N. Schiffmann, P. Halleux, N. Albala, and J. J. Vanderhaeghen. Homolateral cerebrocortical increase of immediate early gene and neurotransmitter messenger RNAs after minimal cortical lesion: blockade by N-methyl-D-aspartate antagonist. *Neuroscience* 59 (4):827-836, 1994.

M. D. Jacobson, J. F. Burne, M. P. King, T. Miyashita, J. C. Reed, and M. C. Raff. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature* 361 (6410):365-369, 1993.

M. D. Jacobson, J. F. Burne, and M. C. Raff. Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.* 13 (8):1899-1910, 1994.

B. J. Jasmin, R. K. Lee, and R. L. Rotundo. Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction. *Neuron* 11 (3):467-477, 1993.

B. S. Jeon, N. G. Kholodilov, T. F. Oo, S. Y. Kim, K. J. Tomaselli, A. Srinivasan, L. Stefanis, and R. E. Burke. Activation of caspase-3 in developmental models of programmed cell death in neurons of the substantia nigra. *J.Neurochem.* 73 (1):322-333, 1999.

J. W. Johnson and P. Ascher. Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 325 (6104):529-531, 1987.

D. P. Jones, D. J. McConkey, P. Nicotera, and S. Orrenius. Calcium-activated DNA fragmentation in rat liver nuclei. *J.Biol.Chem.* 264 (11):6398-6403, 1989.

J. Jordan, M. F. Galindo, J. H. Prehn, R. R. Weichselbaum, M. Beckett, G. D. Ghadge, R. P. Roos, J. M. Leiden, and R. J. Miller. p53 expression induces apoptosis in hippocampal pyramidal neuron cultures. *J.Neurosci.* 17 (4):1397-1405, 1997.

M. B. Jorgensen, J. Deckert, D. C. Wright, and D. R. Gehlert. Delayed c-fos proto-oncogene expression in the rat hippocampus induced by transient global cerebral ischemia: an in situ hybridization study. *Brain Res.* 484 (1-2):393-398, 1989.

J. M. Jurgensmeier, Z. Xie, Q. Deveraux, L. Ellerby, D. Bredesen, and J. C. Reed. Bax directly induces release of cytochrome c from isolated mitochondria. *Proc.Natl.Acad.Sci.U.S.A* 95 (9):4997-5002, 1998.

W. J. Kaiser, D. Vucic, and L. K. Miller. The Drosophila inhibitor of apoptosis D-IAP1 suppresses cell death induced by the caspase drICE. *FEBS Lett.* 440 (1-2):243-248, 1998.

H. Kalimo, S. Rehncrona, B. Soderfeldt, Y. Olsson, and B. K. Siesjo. Brain lactic acidosis and ischemic cell damage: 2. Histopathology. *J.Cereb.Blood Flow Metab* 1 (3):313-327, 1981.

D. J. Kane, T. A. Sarafian, R. Anton, H. Hahn, E. B. Gralla, J. S. Valentine, T. Ord, and D. E. Bredesen. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* 262 (5137):1274-1277, 1993.

M. B. Kastan, Q. Zhan, W. S. el Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett, B. Vogelstein, and A. J. Fornace, Jr. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* 71 (4):587-597, 1992.

C. Kayalar, T. Ord, M. P. Testa, L. T. Zhong, and D. E. Bredesen. Cleavage of actin by interleukin 1 beta-converting enzyme to reverse DNase I inhibition. *Proc.Natl.Acad.Sci.U.S.A* 93 (5):2234-2238, 1996.

K. Keinanen, W. Wisden, B. Sommer, P. Werner, A. Herb, T. A. Verdoorn, B. Sakmann, and P. H. Seeburg. A family of AMPA-selective glutamate receptors. *Science* 249 (4968):556-560, 1990.

J. N. Keller, M. S. Kindy, F. W. Holtsberg, D. K. St Clair, H. C. Yen, A. Germeyer, S. M. Steiner, A. J. Bruce-Keller, J. B. Hutchins, and M. P. Mattson. Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J.Neurosci.* 18 (2):687-697, 1998.

M. E. Kelly and D. C. McIntyre. Hippocampal kindling protects several structures from the neuronal damage resulting from kainic acid-induced status epilepticus. *Brain Res.* 634 (2):245-256, 1994.

J.F. Kerr and B.V. Harmon. Definition and incidence of apoptosis: An historical perspective. In apoptosis: the molecular basis of cell death, Eds: LD Tomei and FO Cope, pp5, *Cold spring harbor laboratory press*, Cold spring harbor, NY, 1991.

J. F. Kerr, A. H. Wyllie, and A. R. Currie. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br.J.Cancer* 26 (4):239-257, 1972.

M. Kiessling, G. Stumm, Y. Xie, T. Herdegen, A. Aguzzi, R. Bravo, and P. Gass. Differential transcription and translation of immediate early genes in the gerbil hippocampus after transient global ischemia. *J.Cereb.Blood Flow Metab* 13 (6):914-924, 1993.

H. Y. Kil, J. Zhang, and C. A. Piantadosi. Brain temperature alters hydroxyl radical production during cerebral ischemia/reperfusion in rats. *J.Cereb.Blood Flow Metab* 16 (1):100-106, 1996.

I. S. Kim, D. H. Kim, S. M. Han, M. U. Chin, H. J. Nam, H. P. Cho, S. Y. Choi, B. J. Song, E. R. Kim, Y. S. Bae, and Y. H. Moon. Truncated form of importin alpha identified in breast cancer cell inhibits nuclear import of p53. *J.Biol.Chem.* 275 (30):23139-23145, 2000.

H. K. Kimelberg, K. D. Barron, R. S. Bourke, L. R. Nelson, and E. J. Cragoe. Brain anti-cytoxic edema agents. *Prog.Clin.Biol.Res.* 361:363-385, 1990.

H. Kinouchi, C. J. Epstein, T. Mizui, E. Carlson, S. F. Chen, and P. H. Chan. Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing CuZn superoxide dismutase. *Proc.Natl.Acad.Sci.U.S.A* 88 (24):11158-11162, 1991.

Y. Kinuta, H. Kikuchi, M. Ishikawa, M. Kimura, and Y. Itokawa. Lipid peroxidation in focal cerebral ischemia. *J.Neurosurg.* 71 (3):421-429, 1989.

T. Kirino. Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res.* 239 (1):57-69, 1982.

K. Kitagawa, M. Matsumoto, Y. Tsujimoto, T. Ohtsuki, K. Kuwabara, K. Matsushita, G. Yang, H. Tanabe, J. C. Martinou, M. Hori, and T. Yanagihara. Amelioration of hippocampal neuronal damage after global ischemia by neuronal overexpression of BCL-2 in transgenic mice. *Stroke* 29 (12):2616-2621, 1998.

N. W. Kleckner and R. Dingledine. Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. *Science* 241 (4867):835-837, 1988.

T. Knopfel, R. Kuhn, and H. Allgeier. Metabotropic glutamate receptors: novel targets for drug development. *J.Med.Chem.* 38 (9):1417-1426, 1995.

B. Knusel, J. W. Winslow, A. Rosenthal, L. E. Burton, D. P. Seid, K. Nikolics, and F. Hefti. Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. *Proc.Natl.Acad.Sci.U.S.A* 88 (3):961-965, 1991.

L. J. Ko and C. Prives. p53: puzzle and paradigm. *Genes Dev.* 10 (9):1054-1072, 1996.

S. Kobayashi, V. A. Harris, and F. A. Welsh. Spreading depression induces tolerance of cortical neurons to ischemia in rat brain. *J.Cereb.Blood Flow Metab* 15 (5):721-727, 1995.

M. Kohler, N. Burnashev, B. Sakmann, and P. H. Seeburg. Determinants of Ca2+ permeability in both TM1 and TM2 of high affinity kainate receptor channels: diversity by RNA editing. *Neuron* 10 (3):491-500, 1993.

Z. Kokaia, G. Gido, T. Ringstedt, J. Bengzon, M. Kokaia, B. K. Siesjo, H. Persson, and O. Lindvall. Rapid increase of BDNF mRNA levels in cortical neurons following spreading depression: regulation by glutamatergic mechanisms independent of seizure activity. *Brain Res.Mol.Brain Res.* 19 (4):277-286, 1993.

V. E. Koliatsos, R. E. Clatterbuck, J. W. Winslow, M. H. Cayouette, and D. L. Price. Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. *Neuron* 10 (3):359-367, 1993.

S. Kondo, G. H. Barnett, H. Hara, T. Morimura, and J. Takeuchi. MDM2 protein confers the resistance of a human glioblastoma cell line to cisplatin-induced apoptosis. *Oncogene* 10 (10):2001-2006, 1995.

T. Kondo, A. G. Reaume, T. T. Huang, E. Carlson, K. Murakami, S. F. Chen, E. K. Hoffman, R. W. Scott, C. J. Epstein, and P. H. Chan. Reduction of CuZn-superoxide dismutase activity exacerbates neuronal cell injury and edema

formation after transient focal cerebral ischemia. *J.Neurosci.* 17 (11):4180-4189, 1997.

R. P. Kraig, W. A. Pulsinelli, and F. Plum. Heterogeneous distribution of hydrogen and bicarbonate ions during complete brain ischemia. *Prog.Brain Res.* 63:155-166, 1985.

R. P. Kraig and M. Chesler. Astrocytic acidosis in hyperglycemic and complete ischemia. *J.Cereb.Blood Flow Metab* 10 (1):104-114, 1990.

R. P. Kraig, L. M. Dong, R. Thisted, and C. B. Jaeger. Spreading depression increases immunohistochemical staining of glial fibrillary acidic protein. *J.Neurosci.* 11 (7):2187-2198, 1991.

R. P. Kraig and C. Nicholson. Profound acidosis in presumed glia during ischemia, in Cerebrovascular Dis, Eds: Powers WJ and Raichle ME, pp. 97-102. *Raven Press*, NY, 1987.

S. Krajewski, S. Tanaka, S. Takayama, M. J. Schibler, W. Fenton, and J. C. Reed. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res.* 53 (19):4701-4714, 1993.

S. Krajewski, J. K. Mai, M. Krajewska, M. Sikorska, M. J. Mossakowski, and J. C. Reed. Upregulation of bax protein levels in neurons following cerebral ischemia. *J.Neurosci.* 15 (10):6364-6376, 1995.

S. Krajewski, M. Krajewska, L. M. Ellerby, K. Welsh, Z. Xie, Q. L. Deveraux, G. S. Salvesen, D. E. Bredesen, R. E. Rosenthal, G. Fiskum, and J. C. Reed. Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. *Proc.Natl.Acad.Sci.U.S.A* 96 (10):5752-5757, 1999.

G. S. Krause and B. R. Tiffany. Suppression of protein synthesis in the reperfused brain. *Stroke* 24 (5):747-755, 1993.

I. Kruman, A. J. Bruce-Keller, D. Bredesen, G. Waeg, and M. P. Mattson. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J.Neurosci.* 17 (13):5089-5100, 1997.

S. J. Kuerbitz, B. S. Plunkett, W. V. Walsh, and M. B. Kastan. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc.Natl.Acad.Sci.U.S.A* 89 (16):7491-7495, 1992.

S. Kumar, M. Kinoshita, M. Noda, N. G. Copeland, and N. A. Jenkins. Induction of apoptosis by the mouse Nedd2 gene, which encodes a protein similar to the product of the Caenorhabditis elegans cell death gene ced-3 and the mammalian IL-1 beta-converting enzyme. *Genes Dev.* 8 (14):1613-1626, 1994.

U. Kutay, E. Izaurralde, F. R. Bischoff, I. W. Mattaj, and D. Gorlich. Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. *EMBO J.* 16 (6):1153-1163, 1997.

E. C. LaCasse, S. Baird, R. G. Korneluk, and A. E. MacKenzie. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. *Oncogene* 17 (25):3247-3259, 1998.

M. S. Lawrence, D. Y. Ho, G. H. Sun, G. K. Steinberg, and R. M. Sapolsky. Overexpression of Bcl-2 with herpes simplex virus vectors protects CNS neurons against neurological insults in vitro and in vivo. *J.Neurosci.* 16 (2):486-496, 1996.

J. W. Lazarewicz, J. T. Wroblewski, M. E. Palmer, and E. Costa. Activation of N-methyl-D-aspartate-sensitive glutamate receptors stimulates arachidonic acid release in primary cultures of cerebellar granule cells. *Neuropharmacology* 27 (7):765-769, 1988.

Y. A. Lazebnik, A. Takahashi, R. D. Moir, R. D. Goldman, G. G. Poirier, S. H. Kaufmann, and W. C. Earnshaw. Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. *Proc.Natl.Acad.Sci.U.S.A* 92 (20):9042-9046, 1995.

A. A. P. Leao. Spreading depression of activity in the cerebral cortex, *J Neurophysiol* 7:359-390, 1944.

S. Lee, J. Miskovsky, J. Williamson, R. Howells, O. Devinsky, E. Lothman, and S. Christakos. Changes in glutamate receptor and proenkephalin gene expression after kindled seizures. *Brain Res.Mol.Brain Res.* 24 (1-4):34-42, 1994.

A. J. Levine, J. Momand, and C. A. Finlay. The p53 tumour suppressor gene. *Nature* 351 (6326):453-456, 1991.

A. J. Levine. p53, the cellular gatekeeper for growth and division. *Cell* 88 (3):323-331, 1997.

D. E. Levy, J. B. Brierley, and F. Plum. Ischaemic brain damage in the gerbil in the absence of 'no-reflow'. *J.Neurol.Neurosurg.Psychiatry* 38 (12):1197-1205, 1975.

D. E. Levy and T. E. Duffy. Cerebral energy metabolism during transient ischemia and recovery in the gerbil. *J.Neurochem.* 28 (1):63-70, 1977.

F. Li, A. Srinivasan, Y. Wang, R. C. Armstrong, K. J. Tomaselli, and L. C. Fritz. Cell-specific induction of apoptosis by microinjection of cytochrome c. Bcl-xL has activity independent of cytochrome c release. *J.Biol.Chem.* 272 (48):30299-30305, 1997.

P. Li, D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91 (4):479-489, 1997.

Y. Li, M. Chopp, Z. G. Zhang, C. Zaloga, L. Niewenhuis, and S. Gautam. p53-immunoreactive protein and p53 mRNA expression after transient middle cerebral artery occlusion in rats. *Stroke* 25 (4):849-855, 1994.

Y. Li, V. G. Sharov, N. Jiang, C. Zaloga, H. N. Sabbah, and M. Chopp. Ultrastructural and light microscopic evidence of apoptosis after middle cerebral artery occlusion in the rat. *Am.J.Pathol.* 146 (5):1045-1051, 1995.

T. N. Lin, J. Te, H. C. Huang, S. I. Chi, and C. Y. Hsu. Prolongation and enhancement of postischemic c-fos expression after fasting. *Stroke* 28 (2):412-418, 1997.

M. D. Linnik, R. H. Zobrist, and M. D. Hatfield. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 24 (12):2002-2008, 1993.

M. D. Linnik, P. Zahos, M. D. Geschwind, and H. J. Federoff. Expression of bcl-2 from a defective herpes simplex virus-1 vector limits neuronal death in focal cerebral ischemia. *Stroke* 26 (9):1670-1674, 1995.

P. Liston, N. Roy, K. Tamai, C. Lefebvre, S. Baird, G. Cherton-Horvat, R. Farahani, M. McLean, J. E. Ikeda, A. MacKenzie, and R. G. Korneluk. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* 379 (6563):349-353, 1996.

P. K. Liu, C. Y. Hsu, M. Dizdaroglu, R. A. Floyd, Y. W. Kow, A. Karakaya, L. E. Rabow, and J. K. Cui. Damage, repair, and mutagenesis in nuclear genes after mouse forebrain ischemia-reperfusion. *J.Neurosci.* 16 (21):6795-6806, 1996.

T. H. Liu, J. S. Beckman, B. A. Freeman, E. L. Hogan, and C. Y. Hsu. Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. *Am.J.Physiol* 256 (2 Pt 2):H589-H593, 1989.

X. Liu, C. N. Kim, J. Yang, R. Jemmerson, and X. Wang. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86 (1):147-157, 1996.

X. Liu, H. Zou, C. Slaughter, and X. Wang. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 89 (2):175-184, 1997.

Y. Liu and M. Kulesz-Martin. p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. *Carcinogenesis* 22 (6):851-860, 2001.

R. A. Lockshin. Cell death in metamorphosis, In cell death in biology and pathology, Eds: ID Bowen and RA Lockshin, *Chapman and Hall*, pp 79. NY, 1981.

J. Lotem and L. Sachs. Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. *Blood* 82 (4):1092-1096, 1993.

S. W. Lowe, E. M. Schmitt, S. W. Smith, B. A. Osborne, and T. Jacks. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 362 (6423):847-849, 1993.

J. P. MacManus, A. M. Buchan, I. E. Hill, I. Rasquinha, and E. Preston. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci.Lett.* 164 (1-2):89-92, 1993.

J. P. MacManus, I. E. Hill, Z. G. Huang, I. Rasquinha, D. Xue, and A. M. Buchan. DNA damage consistent with apoptosis in transient focal ischaemic neocortex. *Neuroreport* 5 (4):493-496, 1994.

J. P. MacManus and M. D. Linnik. Gene expression induced by cerebral ischemia: an apoptotic perspective. *J.Cereb.Blood Flow Metab* 17 (8):815-832, 1997.

S. P. Mah, L. T. Zhong, Y. Liu, A. Roghani, R. H. Edwards, and D. E. Bredesen. The protooncogene bcl-2 inhibits apoptosis in PC12 cells. *J.Neurochem.* 60 (3):1183-1186, 1993.

N. D. Marchenko, A. Zaika, and U. M. Moll. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J.Biol.Chem.* 275 (21):16202-16212, 2000.

P. Marchetti, S. A. Susin, D. Decaudin, S. Gamen, M. Castedo, T. Hirsch, N. Zamzami, J. Naval, A. Senik, and G. Kroemer. Apoptosis-associated derangement of mitochondrial function in cells lacking mitochondrial DNA. *Cancer Res.* 56 (9):2033-2038, 1996.

D. P. Martin, R. E. Schmidt, P. S. DiStefano, O. H. Lowry, J. G. Carter, and E. M. Johnson, Jr. Inhibitors of protein synthesis and RNA synthesis prevent

neuronal death caused by nerve growth factor deprivation. *J.Cell Biol.* 106 (3):829-844, 1988.

S. J. Martin, A. M. Bonham, and T. G. Cotter. The involvement of RNA and protein synthesis in programmed cell death (apoptosis) in human leukaemia HL-60 cells. *Biochem.Soc.Trans.* 18 (4):634-636, 1990.

J. Martinez, I. Georgoff, J. Martinez, and A. J. Levine. Cellular localization and cell cycle regulation by a temperature- sensitive p53 protein. *Genes Dev.* 5 (2):151-159, 1991.

J. C. Martinou, M. Dubois-Dauphin, J. K. Staple, I. Rodriguez, H. Frankowski, M. Missotten, P. Albertini, D. Talabot, S. Catsicas, C. Pietra, and . Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* 13 (4):1017-1030, 1994.

H. Martins-Ferreira and G. O. de Castro. Light-scattering changes accompanying spreading depression in isolated retina. *J.Neurophysiol.* 29 (4):715-726, 1966.

K. Matsushima and A. M. Hakim. Transient forebrain ischemia protects against subsequent focal cerebral ischemia without changing cerebral perfusion. *Stroke* 26 (6):1047-1052, 1995.

K. Matsushima, M. J. Hogan, and A. M. Hakim. Cortical spreading depression protects against subsequent focal cerebral ischemia in rats. *J.Cereb.Blood Flow Metab* 16 (2):221-226, 1996.

K. Matsushima, R. Schmidt-Kastner, M. J. Hogan, and A. M. Hakim. Cortical spreading depression activates trophic factor expression in neurons and astrocytes and protects against subsequent focal brain ischemia. *Brain Res.* 807 (1-2):47-60, 1998.

S. Matsuyama, S. L. Schendel, Z. Xie, and J. C. Reed. Cytoprotection by Bcl-2 requires the pore-forming alpha5 and alpha6 helices. *J.Biol.Chem.* 273 (47):30995-31001, 1998.

I. W. Mattaj and L. Englmeier. Nucleocytoplasmic transport: the soluble phase. *Annu.Rev.Biochem.* 67:265-306, 1998.

J. V. McCarthy and V. M. Dixit. Apoptosis induced by Drosophila reaper and grim in a human system. Attenuation by inhibitor of apoptosis proteins (cIAPs). *J.Biol.Chem.* 273 (37):24009-24015, 1998.

D. J. McConkey, P. Hartzell, S. K. Duddy, H. Hakansson, and S. Orrenius. 2,3,7,8-Tetrachlorodibenzo-p-dioxin kills immature thymocytes by Ca2+- mediated endonuclease activation. *Science* 242 (4876):256-259, 1988.

D. J. McConkey, P. Nicotera, P. Hartzell, G. Bellomo, A. H. Wyllie, and S. Orrenius. Glucocorticoids activate a suicide process in thymocytes through

an elevation of cytosolic Ca2+ concentration. *Arch.Biochem.Biophys.* 269 (1):365-370, 1989.

T. J. McDonnell, N. Deane, F. M. Platt, G. Nunez, U. Jaeger, J. P. McKearn, and S. J. Korsmeyer. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 57 (1):79-88, 1989.

W. E. Mercer, M. T. Shields, M. Amin, G. J. Sauve, E. Appella, J. W. Romano, and S. J. Ullrich. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc.Natl.Acad.Sci.U.S.A* 87 (16):6166-6170, 1990.

M. Miura, H. Zhu, R. Rotello, E. A. Hartwieg, and J. Yuan. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. *Cell* 75 (4):653-660, 1993.

T. Miyashita, S. Krajewski, M. Krajewska, H. G. Wang, H. K. Lin, D. A. Liebermann, B. Hoffman, and J. C. Reed. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* 9 (6):1799-1805, 1994.

T. Miyashita and J. C. Reed. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 80 (2):293-299, 1995.

U. M. Moll, G. Riou, and A. J. Levine. Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. *Proc.Natl.Acad.Sci.U.S.A* 89 (15):7262-7266, 1992.

J. Momand and G. P. Zambetti. Mdm-2: "big brother" of p53. *J.Cell Biochem.* 64 (3):343-352, 1997.

J. I. Morgan and T. Curran. Role of ion flux in the control of c-fos expression. *Nature* 322 (6079):552-555, 1986.

J. I. Morgan and T. Curran. Calcium and proto-oncogene involvement in the immediate-early response in the nervous system. *Ann.N.Y.Acad.Sci.* 568:283-290, 1989.

J. I. Morgan and T. Curran. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. *Annu.Rev.Neurosci.* 14:421-451, 1991.

N. Mori, J. A. Wada, M. Watanabe, and H. Kumashiro. Increased activity of superoxide dismutase in kindled brain and suppression of kindled seizure following intra-amygdaloid injection of superoxide dismutase in rats. *Brain Res.* 557 (1-2):313-315, 1991.

N. Mori and H. Yokoyama. Role of superoxide dismutase in a kindling model of epilepsy. *Comp Biochem.Physiol C.* 104 (3):373-376, 1993.

K. Morimoto, K. Sato, S. Sato, N. Yamada, and T. Hayabara. Time-dependent changes in neurotrophic factor mRNA expression after kindling and long-term potentiation in rats. *Brain Res.Bull.* 45 (6):599-605, 1998.

Y. Morita-Fujimura, M. Fujimura, T. Yoshimoto, and P. H. Chan. Superoxide during reperfusion contributes to caspase-8 expression and apoptosis after transient focal stroke. *Stroke* 32 (10):2356-2361, 2001.

K. Murakami, T. Kondo, M. Kawase, Y. Li, S. Sato, S. F. Chen, and P. H. Chan. Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *J.Neurosci.* 18 (1):205-213, 1998.

A. N. Murphy, D. E. Bredesen, G. Cortopassi, E. Wang, and G. Fiskum. Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. *Proc.Natl.Acad.Sci.U.S.A* 93 (18):9893-9898, 1996.

M. Muzio, B. R. Stockwell, H. R. Stennicke, G. S. Salvesen, and V. M. Dixit. An induced proximity model for caspase-8 activation. *J.Biol.Chem.* 273 (5):2926-2930, 1998.

S. Nakanishi. Molecular diversity of glutamate receptors and implications for brain function. *Science* 258 (5082):597-603, 1992.

S. Namura, J. Zhu, K. Fink, M. Endres, A. Srinivasan, K. J. Tomaselli, J. Yuan, and M. A. Moskowitz. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J.Neurosci.* 18 (10):3659-3668, 1998.

M. Nedergaard and A. J. Hansen. Spreading depression is not associated with neuronal injury in the normal brain. *Brain Res.* 449 (1-2):395-398, 1988.

D. G. Nicholls. A role for the mitochondrion in the protection of cells against calcium overload? *Prog.Brain Res.* 63:97-106, 1985.

D. W. Nicholson and N. A. Thornberry. Caspases: killer proteases. *Trends Biochem.Sci.* 22 (8):299-306, 1997.

F. Nicoletti, V. Bruno, A. Copani, G. Casabona, and T. Knopfel. Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? *Trends Neurosci.* 19 (7):267-271, 1996.

E. A. Nigg. Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature* 386 (6627):779-787, 1997.

T. Nitatori, N. Sato, S. Waguri, Y. Karasawa, H. Araki, K. Shibanai, E. Kominami, and Y. Uchiyama. Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J.Neurosci.* 15 (2):1001-1011, 1995.

M. Niwa, A. Hara, T. Iwai, S. Wang, K. Hotta, H. Mori, and T. Uematsu. Caspase activation as an apoptotic evidence in the gerbil hippocampal CA1 pyramidal cells following transient forebrain ischemia. *Neurosci.Lett.* 300 (2):103-106, 2001.

N. Noshita, T. Sugawara, M. Fujimura, Y. Morita-Fujimura, and P. H. Chan. Manganese Superoxide Dismutase Affects Cytochrome c Release and Caspase- 9 Activation After Transient Focal Cerebral Ischemia in Mice. *J.Cereb.Blood Flow Metab* 21 (5):557-567, 2001.

L. Nowak, P. Bregestovski, P. Ascher, A. Herbet, and A. Prochiantz. Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* 307 (5950):462-465, 1984.

C. W. Olanow. A radical hypothesis for neurodegeneration. *Trends Neurosci.* 16 (11):439-444, 1993.

H. Onodera, K. Iijima, and K. Kogure. Mononucleotide metabolism in the rat brain after transient ischemia. *J.Neurochem.* 46 (6):1704-1710, 1986.

R. W. Oppenheim. Cell death during development of the nervous system. *Annu.Rev.Neurosci.* 14:453-501, 1991.

K. Orth, A. M. Chinnaiyan, M. Garg, C. J. Froelich, and V. M. Dixit. The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J.Biol.Chem.* 271 (28):16443-16446, 1996.

M. A. Packer and M. P. Murphy. Peroxynitrite formed by simultaneous nitric oxide and superoxide generation causes cyclosporin-A-sensitive mitochondrial calcium efflux and depolarisation. *Eur.J.Biochem.* 234 (1):231-239, 1995.

K. M. Partin, D. K. Patneau, C. A. Winters, M. L. Mayer, and A. Buonanno. Selective modulation of desensitization at AMPA versus kainate receptors by cyclothiazide and concanavalin A. *Neuron* 11 (6):1069-1082, 1993.

D. E. Pellegrini-Giampietro, R. S. Zukin, M. V. Bennett, S. Cho, and W. A. Pulsinelli. Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proc.Natl.Acad.Sci.U.S.A* 89 (21):10499-10503, 1992.

D. E. Pellegrini-Giampietro, W. A. Pulsinelli, and R. S. Zukin. NMDA and non-NMDA receptor gene expression following global brain ischemia in rats: effect of NMDA and non-NMDA receptor antagonists. *J.Neurochem.* 62 (3):1067-1073, 1994.

M. A. Perez-Pinzon, G. P. Xu, W. D. Dietrich, M. Rosenthal, and T. J. Sick. Rapid preconditioning protects rats against ischemic neuronal damage after 3 but not 7 days of reperfusion following global cerebral ischemia. *J.Cereb.Blood Flow Metab* 17 (2):175-182, 1997.

M. E. Perry, J. Piette, J. A. Zawadzki, D. Harvey, and A. J. Levine. The mdm-2 gene is induced in response to UV light in a p53-dependent manner. *Proc.Natl.Acad.Sci.U.S.A* 90 (24):11623-11627, 1993.

M. E. Peter and P. H. Krammer. Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. *Curr.Opin.Immunol.* 10 (5):545-551, 1998.

P. X. Petit, S. A. Susin, N. Zamzami, B. Mignotte, and G. Kroemer. Mitochondria and programmed cell death: back to the future. *FEBS Lett.* 396 (1):7-13, 1996.

C. A. Piantadosi and J. Zhang. Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. *Stroke* 27 (2):327-331, 1996.

H. Pollard, C. Charriaut-Marlangue, S. Cantagrel, A. Represa, O. Robain, J. Moreau, and Y. Ben Ari. Kainate-induced apoptotic cell death in hippocampal neurons. *Neuroscience* 63 (1):7-18, 1994.

A. G. Porter. Protein translocation in apoptosis. *Trends Cell Biol.* 9 (10):394-401, 1999.

B. D. Price and S. J. Park. DNA damage increases the levels of MDM2 messenger RNA in wtp53 human cells. *Cancer Res.* 54 (4):896-899, 1994.

H. K. Prince, P. J. Conn, C. D. Blackstone, R. L. Huganir, and A. I. Levey. Down-regulation of AMPA receptor subunit GluR2 in amygdaloid kindling. *J.Neurochem.* 64 (1):462-465, 1995.

W. Pulsinelli. Pathophysiology of acute ischaemic stroke. *Lancet* 339 (8792):533-536, 1992.

W. A. Pulsinelli, J. B. Brierley, and F. Plum. Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann.Neurol.* 11 (5):491-498, 1982.

W. A. Pulsinelli, D. E. Levy, and T. E. Duffy. Regional cerebral blood flow and glucose metabolism following transient forebrain ischemia. *Ann.Neurol.* 11 (5):499-502, 1982.

W. A. Pulsinelli and T. E. Duffy. Regional energy balance in rat brain after transient forebrain ischemia. *J.Neurochem.* 40 (5):1500-1503, 1983.

Y. M. Rangel, K. Kariko, V. A. Harris, M. E. Duvall, and F. A. Welsh. Dose-dependent induction of mRNAs encoding brain-derived neurotrophic factor and heat-shock protein-72 after cortical spreading depression in the rat. *Brain Res.Mol.Brain Res.* 88 (1-2):103-112, 2001.

L. Rao, D. Perez, and E. White. Lamin proteolysis facilitates nuclear events during apoptosis. *J.Cell Biol.* 135 (6 Pt 1):1441-1455, 1996.

S. Rehncrona, H. N. Hauge, and B. K. Siesjo. Enhancement of iron-catalyzed free radical formation by acidosis in brain homogenates: differences in effect by lactic acid and CO2. *J.Cereb.Blood Flow Metab* 9 (1):65-70, 1989.

V. Reinke and G. Lozano. The p53 targets mdm2 and Fas are not required as mediators of apoptosis in vivo. *Oncogene* 15 (13):1527-1534, 1997.

I. J. Reynolds and T. G. Hastings. Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. *J.Neurosci.* 15 (5 Pt 1):3318-3327, 1995.

C. Richter, V. Gogvadze, R. Schlapbach, M. Schweizer, and J. Schlegel. Nitric oxide kills hepatocytes by mobilizing mitochondrial calcium. *Biochem.Biophys.Res.Commun.* 205 (2):1143-1150, 1994.

G. S. Robertson, S. R. Vincent, and H. C. Fibiger. D1 and D2 dopamine receptors differentially regulate c-fos expression in striatonigral and striatopallidal neurons. *Neuroscience* 49 (2):285-296, 1992.

S. Roffler-Tarlov, J. J. Brown, E. Tarlov, J. Stolarov, D. L. Chapman, M. Alexiou, and V. E. Papaioannou. Programmed cell death in the absence of c-Fos and c-Jun. *Development* 122 (1):1-9, 1996.

D. M. Rosenbaum, M. Michaelson, D. K. Batter, P. Doshi, and J. A. Kessler. Evidence for hypoxia-induced, programmed cell death of cultured neurons. *Ann.Neurol.* 36 (6):864-870, 1994.

R. J. Rotello, M. B. Hocker, and L. E. Gerschenson. Biochemical evidence for programmed cell death in rabbit uterine epithelium. *Am.J.Pathol.* 134 (3):491-495, 1989.

M. Rothe, M. G. Pan, W. J. Henzel, T. M. Ayres, and D. V. Goeddel. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* 83 (7):1243-1252, 1995.

N. Roy, M. S. Mahadevan, M. McLean, G. Shutler, Z. Yaraghi, R. Farahani, S. Baird, A. Besner-Johnston, C. Lefebvre, X. Kang, and . The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell* 80 (1):167-178, 1995.

N. Roy, Q. L. Deveraux, R. Takahashi, G. S. Salvesen, and J. C. Reed. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J.* 16 (23):6914-6925, 1997.

H. Sakahira, M. Enari, and S. Nagata. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 391 (6662):96-99, 1998.

A. Sakamoto, S. T. Ohnishi, T. Ohnishi, and R. Ogawa. Relationship between free radical production and lipid peroxidation during ischemia-reperfusion injury in the rat brain. *Brain Res.* 554 (1-2):186-192, 1991.

S. Sakhi, A. Bruce, N. Sun, G. Tocco, M. Baudry, and S. S. Schreiber. p53 induction is associated with neuronal damage in the central nervous system. *Proc.Natl.Acad.Sci.U.S.A* 91 (16):7525-7529, 1994.

S. Sakhi, N. Sun, L. L. Wing, P. Mehta, and S. S. Schreiber. Nuclear accumulation of p53 protein following kainic acid-induced seizures. *Neuroreport* 7 (2):493-496, 1996.

K. Sakimura, T. Morita, E. Kushiya, and M. Mishina. Primary structure and expression of the gamma 2 subunit of the glutamate receptor channel selective for kainate. *Neuron* 8 (2):267-274, 1992.

G. S. Salvesen and V. M. Dixit. Caspases: intracellular signaling by proteolysis. *Cell* 91 (4):443-446, 1997.

C. Sansome, A. Zaika, N. D. Marchenko, and U. M. Moll. Hypoxia death stimulus induces translocation of p53 protein to mitochondria. Detection by immunofluorescence on whole cells. *FEBS Lett.* 488 (3):110-115, 2001.

T. A. Sarafian, L. Vartavarian, D. J. Kane, D. E. Bredesen, and M. A. Verity. bcl-2 expression decreases methyl mercury-induced free-radical generation and cell killing in a neural cell line. *Toxicol.Lett.* 74 (2):149-155, 1994.

G. S. Schierle, O. Hansson, M. Leist, P. Nicotera, H. Widner, and P. Brundin. Caspase inhibition reduces apoptosis and increases survival of nigral transplants. *Nat.Med.* 5 (1):97-100, 1999.

R. T. Schimke and E. Mihich. Fifth annual Pezcoller symposium: apoptosis. *Cancer Res.* 54 (1):302-305, 1994.

C. Schmaltz, P. H. Hardenbergh, A. Wells, and D. E. Fisher. Regulation of proliferation-survival decisions during tumor cell hypoxia. *Mol.Cell Biol.* 18 (5):2845-2854, 1998.

J. W. Schmidley. Free radicals in central nervous system ischemia. *Stroke* 21 (7):1086-1090, 1990.

J. B. Schulz, M. Weller, and M. A. Moskowitz. Caspases as treatment targets in stroke and neurodegenerative diseases. *Ann.Neurol.* 45 (4):421-429, 1999.

L. M. Schwartz and C. E. Milligan. Cold thoughts of death: the role of ICE proteases in neuronal cell death. *Trends Neurosci.* 19 (12):555-562, 1996.

M. Schweizer and C. Richter. Nitric oxide potently and reversibly deenergizes mitochondria at low oxygen tension. *Biochem.Biophys.Res.Commun.* 204 (1):169-175, 1994.

M. Selvakumaran, H. K. Lin, T. Miyashita, H. G. Wang, S. Krajewski, J. C. Reed, B. Hoffman, and D. Liebermann. Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. *Oncogene* 9 (6):1791-1798, 1994.

P. J. Shen and A. L. Gundlach. Differential spatiotemporal alterations in adrenoceptor mRNAs and binding sites in cerebral cortex following spreading depression: selective and prolonged up-regulation of alpha1B-adrenoceptors. *Exp.Neurol.* 154 (2):612-627, 1998.

P. J. Shen and A. L. Gundlach. Differential increases in chromogranins, but not synapsin I, in cortical neurons following spreading depression: implications for functional roles and transmitter peptide release. *Eur.J.Neurosci.* 10 (7):2217-2230, 1998.

M. Sheng, S. T. Dougan, G. McFadden, and M. E. Greenberg. Calcium and growth factor pathways of c-fos transcriptional activation require distinct upstream regulatory sequences. *Mol.Cell Biol.* 8 (7):2787-2796, 1988.

G. M. Shepherd. In Neurobiology (3rd edition), *Oxford University Press*, NY, 1994.

C. B. Shields, J. P. Miles, L. Albright, D. J. Beasley, and H. D. Garretson. Early management of head injuries. *J.Ky.Med.Assoc.* 78 (1):9-13, 1980.

T. Shigeno, Y. Yamasaki, G. Kato, K. Kusaka, T. Mima, K. Takakura, D. I. Graham, and S. Furukawa. Reduction of delayed neuronal death by inhibition of protein synthesis. *Neurosci.Lett.* 120 (1):117-119, 1990.

M. Shinohara, B. Dollinger, G. Brown, S. Rapoport, and L. Sokoloff. Cerebral glucose utilization: local changes during and after recovery from spreading cortical depression. *Science* 203 (4376):188-190, 1979.

B. K. Siesjo. Acidosis and ischemic brain damage. *Neurochem.Pathol.* 9:31-88, 1988.

B. K. Siesjo and F. Bengtsson. Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: a unifying hypothesis. *J.Cereb.Blood Flow Metab* 9 (2):127-140, 1989.

B. K. Siesjo, A. Ekholm, K. Katsura, and S. Theander. Acid-base changes during complete brain ischemia. *Stroke* 21 (11 Suppl):III194-III199, 1990.

B. K. Siesjo. Pathophysiology and treatment of focal cerebral ischemia. Part II: Mechanisms of damage and treatment. *J.Neurosurg.* 77 (3):337-354, 1992.

I. A. Silver and M. Erecinska. Ion homeostasis in rat brain in vivo: intra- and extracellular [Ca2+] and [H+] in the hippocampus during recovery from short-term, transient ischemia. *J.Cereb.Blood Flow Metab* 12 (5):759-772, 1992.

R. Siman and J. C. Noszek. Excitatory amino acids activate calpain I and induce structural protein breakdown in vivo. *Neuron* 1 (4):279-287, 1988.

G. Simic, D. Seso-simic, P. J. Lucassen, A. Islam, Z. Krsnik, A. Cviko, D. Jelasic, N. Barisic, B. Winblad, I. Kostovic, and B. Kruslin. Ultrastructural analysis and TUNEL demonstrate motor neuron apoptosis in Werdnig-Hoffman disease. *J. Neuropathol. Exp. Neurol.* 59 (5): 398-407, 2000.

R. P. Simon, J. H. Swan, T. Griffiths, and B. S. Meldrum. Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* 226 (4676):850-852, 1984.

N. A. Simonian, R. L. Getz, J. C. Leveque, C. Konradi, and J. T. Coyle. Kainic acid induces apoptosis in neurons. *Neuroscience* 75 (4):1047-1055, 1996.

M. Simons, S. Beinroth, M. Gleichmann, P. Liston, R. G. Korneluk, A. E. MacKenzie, M. Bahr, T. Klockgether, G. S. Robertson, M. Weller, and J. B. Schulz. Adenovirus-mediated gene transfer of inhibitors of apoptosis protein delays apoptosis in cerebellar granule neurons. *J.Neurochem.* 72 (1):292-301, 1999.

R. S. Sloviter, A. L. Sollas, E. Dean, and S. Neubort. Adrenalectomy-induced granule cell degeneration in the rat hippocampal dentate gyrus: characterization of an in vivo model of controlled neuronal death. *J.Comp Neurol.* 330 (3):324-336, 1993.

R. J. Smeyne, M. Vendrell, M. Hayward, S. J. Baker, G. G. Miao, K. Schilling, L. M. Robertson, T. Curran, and J. I. Morgan. Continuous c-fos expression precedes programmed cell death in vivo. *Nature* 363 (6425):166-169, 1993.

M. L. Smith, I. T. Chen, Q. Zhan, I. Bae, C. Y. Chen, T. M. Gilmer, M. B. Kastan, P. M. O'Connor, and A. J. Fornace, Jr. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science* 266 (5189):1376-1380, 1994.

M. L. Smith and A. J. Fornace, Jr. Mammalian DNA damage-inducible genes associated with growth arrest and apoptosis. *Mutat.Res.* 340 (2-3):109-124, 1996.

G. G. Somjen, P. G. Aitken, G. L. Czeh, O. Herreras, J. Jing, and J. N. Young. Mechanism of spreading depression: a review of recent findings and a hypothesis. *Can.J.Physiol Pharmacol.* 70 Suppl:S248-S254, 1992.

B. Sommer, M. Kohler, R. Sprengel, and P. H. Seeburg. RNA editing in brain controls a determinant of ion flow in glutamate- gated channels. *Cell* 67 (1):11-19, 1991.

B. Sommer, N. Burnashev, T. A. Verdoorn, K. Keinanen, B. Sakmann, and P. H. Seeburg. A glutamate receptor channel with high affinity for domoate and kainate. *EMBO J.* 11 (4):1651-1656, 1992.

Q. Song, S. R. Burrows, G. Smith, S. P. Lees-Miller, S. Kumar, D. W. Chan, J. A. Trapani, E. Alnemri, G. Litwack, H. Lu, D. J. Moss, S. Jackson, and M. F. Lavin. Interleukin-1 beta-converting enzyme-like protease cleaves DNA-dependent protein kinase in cytotoxic T cell killing. *J.Exp.Med.* 184 (2):619-626, 1996.

T. Soussi, de Fromentel Caron, C. Breugnot, and E. May. Nucleotide sequence of a cDNA encoding the rat p53 nuclear oncoprotein. *Nucleic Acids Res.* 16 (23):11384, 1988.

M. B. Spina, S. P. Squinto, J. Miller, R. M. Lindsay, and C. Hyman. Brain-derived neurotrophic factor protects dopamine neurons against 6- hydroxydopamine and N-methyl-4-phenylpyridinium ion toxicity: involvement of the glutathione system. *J.Neurochem.* 59 (1):99-106, 1992.

N. E. Stagliano, M. A. Perez-Pinzon, M. A. Moskowitz, and P. L. Huang. Focal ischemic preconditioning induces rapid tolerance to middle cerebral artery occlusion in mice. *J.Cereb.Blood Flow Metab* 19 (7):757-761, 1999.

R. A. Swanson, M. T. Morton, G. Tsao-Wu, R. A. Savalos, C. Davidson, and F. R. Sharp. A semiautomated method for measuring brain infarct volume. *J.Cereb.Blood Flow Metab* 10 (2):290-293, 1990.

K. Taga, P. M. Patel, J. C. Drummond, D. J. Cole, and P. J. Kelly. Transient neuronal depolarization induces tolerance to subsequent forebrain ischemia in rats. *Anesthesiology* 87 (4):918-925, 1997.

A. Takahashi, E. S. Alnemri, Y. A. Lazebnik, T. Fernandes-Alnemri, G. Litwack, R. D. Moir, R. D. Goldman, G. G. Poirier, S. H. Kaufmann, and W. C. Earnshaw. Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc.Natl.Acad.Sci.U.S.A* 93 (16):8395-8400, 1996.

R. Takahashi, Q. Deveraux, I. Tamm, K. Welsh, N. Assa-Munt, G. S. Salvesen, and J. C. Reed. A single BIR domain of XIAP sufficient for inhibiting caspases. *J.Biol.Chem.* 273 (14):7787-7790, 1998.

I. Tamm, Y. Wang, E. Sausville, D. A. Scudiero, N. Vigna, T. Oltersdorf, and J. C. Reed. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res.* 58 (23):5315-5320, 1998.

L. Tenneti, D. M. D'Emilia, C. M. Troy, and S. A. Lipton. Role of caspases in N-methyl-D-aspartate-induced apoptosis in cerebrocortical neurons. *J.Neurochem.* 71 (3):946-959, 1998.

R. Thilmann, Y. Xie, P. Kleihues, and M. Kiessling. Persistent inhibition of protein synthesis precedes delayed neuronal death in postischemic gerbil hippocampus. *Acta Neuropathol.(Berl)* 71 (1-2):88-93, 1986.

N. A. Thornberry and Y. Lazebnik. Caspases: enemies within. *Science* 281 (5381):1312-1316, 1998.

G. Tomasevic, P. Stubberod, T. Wieloch. DNA damage and repair. Changes in the tumor suppressor protein p53 and the proliferative nuclear antigen (PCNA) in the rat brain following transient cerebral ischemia, In pharmacology of cerebral ischemia, Eds: J Krieglstein and H Oberpichler-Schwenk, pp1-5, *Wissenschaftliche Verlagsgesellschaft mbh*, Stuttgart, Germany, 1996.

T. Tominaga, S. Kure, K. Narisawa, and T. Yoshimoto. Endonuclease activation following focal ischemic injury in the rat brain. *Brain Res.* 608 (1):21-26, 1993.

D. Torbati, J. H. Greenberg, and C. J. Lambertsen. Regional cerebral glucose metabolic rate during thirty minutes hypoxia of 7% oxygen in adult conscious rats. *Neurosci.Lett.* 65 (3):253-258, 1986.

B. F. Trump and I. K. Berezesky. The role of cytosolic Ca2+ in cell injury, necrosis and apoptosis. *Curr.Opin.Cell Biol.* 4 (2):227-232, 1992.

M. T. Tseng, S. A. Chan, K. H. Reid, and V. G. Iyer. Fos-like immunoreactivity in rat hippocampal neurons following transient global ischemia. *Neurol.Res.* 19 (1):89-91, 1997.

Y. Tu, S. T. Hou, Z. Huang, G. S. Robertson, and J. P. MacManus. Increased Mdm2 expression in rat brain after transient middle cerebral artery occlusion. *J.Cereb.Blood Flow Metab* 18 (6):658-669, 1998.

D. Uberti, M. Belloni, M. Grilli, P. Spano, and M. Memo. Induction of tumour-suppressor phosphoprotein p53 in the apoptosis of cultured rat cerebellar neurones triggered by excitatory amino acids. *Eur.J.Neurosci.* 10 (1):246-254, 1998.

A. G. Uren, M. Pakusch, C. J. Hawkins, K. L. Puls, and D. L. Vaux. Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit

apoptosis and/or bind tumor necrosis factor receptor-associated factors. *Proc.Natl.Acad.Sci.U.S.A* 93 (10):4974-4978, 1996.

Campagne M. van Lookeren and R. Gill. Increased expression of cyclin G1 and p21WAF1/CIP1 in neurons following transient forebrain ischemia: comparison with early DNA damage. *J.Neurosci.Res.* 53 (3):279-296, 1998.

M. G. Vander Heiden, N. S. Chandel, P. T. Schumacker, and C. B. Thompson. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol.Cell* 3 (2):159-167, 1999.

M. Vendrell, T. Curran, and J. I. Morgan. Glutamate, immediate-early genes, and cell death in the nervous system. *Ann.N.Y.Acad.Sci.* 679:132-141, 1993.

T. A. Verdoorn, N. Burnashev, H. Monyer, P. H. Seeburg, and B. Sakmann. Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* 252 (5013):1715-1718, 1991.

D. Vucic, W. J. Kaiser, A. J. Harvey, and L. K. Miller. Inhibition of reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). *Proc.Natl.Acad.Sci.U.S.A* 94 (19):10183-10188, 1997.

S. Waga, G. J. Hannon, D. Beach, and B. Stillman. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 369 (6481):574-578, 1994.

B. Wagenknecht, T. Glaser, U. Naumann, S. Kugler, S. Isenmann, M. Bahr, R. Korneluk, P. Liston, and M. Weller. Expression and biological activity of X-linked inhibitor of apoptosis (XIAP) in human malignant glioma. *Cell Death.Differ.* 6 (4):370-376, 1999.

A. J. Wagner, J. M. Kokontis, and N. Hay. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21wafl/cipl. *Genes Dev.* 8 (23):2817-2830, 1994.

L. Wang, M. Miura, L. Bergeron, H. Zhu, and J. Yuan. Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* 78 (5):739-750, 1994.

H. Watanabe, S. Ohta, Y. Kumon, S. Sakaki, and M. Sakanaka. Increase in p53 protein expression following cortical infarction in the spontaneously hypertensive rat. *Brain Res.* 837 (1-2):38-45, 1999.

N. Waterhouse, S. Kumar, Q. Song, P. Strike, L. Sparrow, G. Dreyfuss, E. S. Alnemri, G. Litwack, M. Lavin, and D. Watters. Heteronuclear ribonucleoproteins C1 and C2, components of the spliceosome, are specific targets of interleukin 1beta-converting enzyme-like proteases in apoptosis. *J.Biol.Chem.* 271 (46):29335-29341, 1996.

J. C. Watkins. The NMDA receptor concept: Origins and development, In The NMDA Receptor, Eds GL Collingridge and JC Watkins,pp 1-30. *Oxford University Press*, NY, 1994.

K. Weis, U. Ryder, and A. I. Lamond. The conserved amino-terminal domain of hSRP1 alpha is essential for nuclear protein import. *EMBO J.* 15 (8):1818-1825, 1996.

B. C. White, A. Daya, D. J. DeGracia, B. J. O'Neil, J. M. Skjaerlund, S. Trumble, G. S. Krause, and J. A. Rafols. Fluorescent histochemical localization of lipid peroxidation during brain reperfusion following cardiac arrest. *Acta Neuropathol.(Berl)* 86 (1):1-9, 1993.

R. J. White and I. J. Reynolds. Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. *J.Neurosci.* 16 (18):5688-5697, 1996.

G. T. Williams. Programmed cell death: apoptosis and oncogenesis. *Cell* 65 (7):1097-1098, 1991.

G. T. Williams and C. A. Smith. Molecular regulation of apoptosis: genetic controls on cell death. *Cell* 74 (5):777-779, 1993.

P. A. Wolf and R. B. D'Agostino. Epidemiology of stroke. In Stroke. Eds: Barnett HJM, Mohr JP, Stein BM and Yatsu FM, *Churchill Livingston*, NY, 1998.

D. Wu, H. D. Wallen, and G. Nunez. Interaction and regulation of subcellular localization of CED-4 by CED- 9. *Science* 275 (5303):1126-1129, 1997.

X. Wu, J. H. Bayle, D. Olson, and A. J. Levine. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.* 7 (7A):1126-1132, 1993.

A. H. Wyllie, J. F. Kerr, and A. R. Currie. Cell death: the significance of apoptosis. *Int.Rev.Cytol.* 68:251-306, 1980.

Y. Xiong, G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. p21 is a universal inhibitor of cyclin kinases. *Nature* 366 (6456):701-704, 1993.

D. Xu, Y. Bureau, D. C. McIntyre, D. W. Nicholson, P. Liston, Y. Zhu, W. G. Fong, S. J. Crocker, R. G. Korneluk, and G. S. Robertson. Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. *J.Neurosci.* 19 (12):5026-5033, 1999.

D. G. Xu, S. J. Crocker, J. P. Doucet, M. St Jean, K. Tamai, A. M. Hakim, J. E. Ikeda, P. Liston, C. S. Thompson, R. G. Korneluk, A. MacKenzie, and G. S. Robertson. Elevation of neuronal expression of NAIP reduces ischemic damage in the rat hippocampus. *Nat.Med.* 3 (9):997-1004, 1997.

D. G. Xu, R. G. Korneluk, K. Tamai, N. Wigle, A. Hakim, A. MacKenzie, and G. S. Robertson. Distribution of neuronal apoptosis inhibitory protein-like immunoreactivity in the rat central nervous system. *J.Comp Neurol.* 382 (2):247-259, 1997.

H. Yanamoto, N. Hashimoto, I. Nagata, and H. Kikuchi. Infarct tolerance against temporary focal ischemia following spreading depression in rat brain. *Brain Res.* 784 (1-2):239-249, 1998.

E. Yang and S. J. Korsmeyer. Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood* 88 (2):386-401, 1996.

J. Yang, X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 275 (5303):1129-1132, 1997.

X. Yang, H. Y. Chang, and D. Baltimore. Autoproteolytic activation of pro-caspases by oligomerization. *Mol.Cell* 1 (2):319-325, 1998.

W. M. Yee, D. M. Frim, and O. Isacson. Relationships between stress protein induction and NMDA-mediated neuronal death in the entorhinal cortex. *Exp.Brain Res.* 94 (2):193-202, 1993.

E. Yonish-Rouach, D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* 352 (6333):345-347, 1991.

E. Yonish-Rouach, D. Grunwald, S. Wilder, A. Kimchi, E. May, J. J. Lawrence, P. May, and M. Oren. p53-mediated cell death: relationship to cell cycle control. *Mol.Cell Biol.* 13 (3):1415-1423, 1993.

T. Yoshida, T. Okazaki, P. E. Hughes, E. L. Schneider, and N. Mori. Cloning of rat GADD45 gene and induction analysis following ionizing radiation in vivo. *FEBS Lett.* 380 (1-2):87-92, 1996.

J. Yuan and H. R. Horvitz. The Caenorhabditis elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development* 116 (2):309-320, 1992.

J. Yuan, S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* 75 (4):641-652, 1993.

J. Y. Yuan and H. R. Horvitz. The Caenorhabditis elegans genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. *Dev.Biol.* 138 (1):33-41, 1990.

E. Zaidan and N. R. Sims. The calcium content of mitochondria from brain subregions following short-term forebrain ischemia and recirculation in the rat. *J.Neurochem.* 63 (5):1812-1819, 1994.

G. P. Zambetti, J. Bargonetti, K. Walker, C. Prives, and A. J. Levine. Wild-type p53 mediates positive regulation of gene expression through a specific DNA sequence element. *Genes Dev.* 6 (7):1143-1152, 1992.

N. Zamzami, S. A. Susin, P. Marchetti, T. Hirsch, I. Gomez-Monterrey, M. Castedo, and G. Kroemer. Mitochondrial control of nuclear apoptosis. *J.Exp.Med.* 183 (4):1533-1544, 1996.

H. U. Zeilhofer, T. H. Muller, and D. Swandulla. Inhibition of high voltage-activated calcium currents by L-glutamate receptor-mediated calcium influx. *Neuron* 10 (5):879-887, 1993.

Q. Zhan, S. Fan, I. Bae, C. Guillouf, D. A. Liebermann, P. M. O'Connor, and A. J. Fornace, Jr. Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. *Oncogene* 9 (12):3743-3751, 1994.

Q. Zhan, C. K. Bieszczad, I. Bae, A. J. Fornace, Jr., and R. W. Craig. Induction of BCL2 family member MCL1 as an early response to DNA damage. *Oncogene* 14 (9):1031-1039, 1997.

R. Z. Zhan, C. Wu, H. Fujihara, K. Taga, S. Qi, M. Naito, and K. Shimoji. Both caspase-dependent and caspase-independent pathways may be involved in hippocampal CA1 neuronal death because of loss of cytochrome c From mitochondria in a rat forebrain ischemia model. *J.Cereb.Blood Flow Metab* 21 (5):529-540, 2001.

L. X. Zhang, M. A. Smith, X. L. Li, S. R. Weiss, and R. M. Post. Apoptosis of hippocampal neurons after amygdala kindled seizures. *Brain Res.Mol.Brain Res.* 55 (2):198-208, 1998.

L. T. Zhong, T. Sarafian, D. J. Kane, A. C. Charles, S. P. Mah, R. H. Edwards, and D. E. Bredesen. bcl-2 inhibits death of central neural cells induced by multiple agents. *Proc.Natl.Acad.Sci.U.S.A* 90 (10):4533-4537, 1993.

I. Zini, A. Tomasi, R. Grimaldi, V. Vannini, and L. F. Agnati. Detection of free radicals during brain ischemia and reperfusion by spin trapping and microdialysis. *Neurosci.Lett.* 138 (2):279-282, 1992.

H. Zou, W. J. Henzel, X. Liu, A. Lutschg, and X. Wang. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90 (3):405-413, 1997.

APPENDIX 1

I would like to include here as an appendix other works that I carried out while I was a graduate student at the university of Ottawa. These publications had not been included as part of my thesis submission.

1. Sheng-Tao Hou et al., 1997, Increases in DNA lesions and the DNA damage indicator Gadd45 following transient cerebral ischemia in Biochem. Cell Biol. 75:383-392.
2. Yahong Tu et al., 1998, Increased Mdm2 expression in rat brain after transient middle cerebral artery occlusion, J CBF & Met 18:658-669.

My contribution to both papers included:

1) I carried out all the work involving in MCA-o ischemia, blood flow monitor and tissue preparation.
2) I partially participated in situ hybridization histochemistry studies and data analysis.