EXPRESSION OF IMMEDIATE-EARLY GENE PROTEINS IN THE RAT HIPPOCAMPUS FOLLOWING TRANSIENT GLOBAL ISCHEMIA

by

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"We cannot teach people anything;
we can only help them discover it
within themselves."

- Galileo Galilei

ABSTRACT

The temporospatial expression patterns of the immediate-early gene (IEG) proteins Fos, FosB, ΔFosB, Jun, JunB, JunD, and NGFI-A were investigated in rat hippocampus by immunohistochemistry 2 h, 12 h, 24 h, and 48 h after forebrain ischemia. Transient global ischemia (20 min), produced by four vessel occlusion (4-VO), elicited different patterns of IEG expression in vulnerable CA1 and more resilient CA3 neurons. Cell counts revealed that initially, ischemia elevated immunoreactivity in both CA1 and CA3 hippocampal subfields for all IEGs examined, with the exception of JunD and NGFI-A. However, distinct patterns of IEG expression became evident in these regions at later time points following recirculation of blood flow. The pivotal difference was the persistence of ischemia-induced elevations of FosB and Jun expression in the CA1 region of the hippocampus. Unlike CA3 neurons where IEG immunoreactivity had subsided to basal levels by 24 h - 48 h after reperfusion, CA1 neurons continued to display increased FosB- and Jun-like immunoreactivity 48 h post-ischemia. Western blot analysis revealed that elevated expression of both FosB and ΔFosB-like proteins were responsible for the immunohistochemical detection of enhanced FosB-like immunoreactivity in CA1 neurons at 48 h. These findings are consistent with recent *in vitro* studies that implicate FosB and Jun in gene signalling pathways responsible for programmed cell death. In contrast to FosB and Jun, JunB expression declined significantly below basal levels in CA1 neurons at 48 h, while JunB-like immunoreactivity remained unaltered in CA3 neurons. Given that JunB has been shown to inhibit the transactivating properties of Jun, decreased JunB levels may contribute to the apoptotic death of CA1 neurons by enhancing the transcriptional regulating activity of Jun. Also notable at 48 h was the complete loss of constitutive NGFI-A expression from CA1 neurons of ischemic animals. In summary, these findings suggest that persistent elevations in FosB and Jun expression coupled with reductions in JunB and NGFI-A levels may play a role in the apoptotic death of CA1 neurons following transient global ischemia.

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II-VI	cortical layers
2-VO	two-vessel occlusion
4-VO	four-vessel occlusion
µm	micrometre
ΔVm	change in membrane voltage
ab	angular bundle
alv	alveus
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	analysis of variance
AP-1	activator protein
ATP	adenosine triphosphate
CA1, CA2, CA3	areas of Ammon's horn (cornu Ammonis)
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium
Ca^{2+}/CM	Ca^{2+}/calmodulin
Ca^{2+}/CM-dep. PK	Ca^{2+}/CM-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CBF	cerebral blood flow
CCA	common carotid artery
CRB	Cambridge Research Biochemicals
CREB	cAMP response binding protein
CVA	cerebrovascular accident
DAB	diaminobenzidine
DAG	diacylglycerol
DG	dentate gyrus
DG_i	infrapyramidal blade of the dentate gyrus

DG_s	suprapyramidal blade of the dentate gyrus
DND	delayed neuronal death
DSE	dyad-symmetry element
DTT	dithiothreitol
EC	entorhinal cortex
ECL	enhanced chemiluminescence
EGTA	ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N',-tetra-acetic acid
ER	endoplasmic reticulum
Fi	fimbria
Fig.	figure
FIRE	*fos* intragenic regulatory element
g	grams
h	hour
HDG	hilus of the dentate gyrus
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hf	hippocampal fissure
Hg	mercury
HRP	streptavidin-horseradish peroxidase
IEG	immediate-early gene
i.p.	intraperitoneal
IP_3	inositol 1,4,5-triphosphate
ITF	inducible transcription factor
kb	kilobases
kDa	kilodalton
kg	kilogram
M	molar
MCA	middle cerebral artery
MK-801	dizocilpine

ml	millilitre
mM	millimolar
mg	milligram
min	minute
Na^+	sodium
NaAz	sodium azide
NGF	nerve growth factor
NGFI-A	nerve growth factor induced-A
nM	nanomolar
NMDA	*N*-methyl-D-aspartate
O_2	oxygen
ODN	oligodeoxynucleotide
PAR	parasubiculum
PBS	phosphate-buffered saline
PC	perirhinal cortex
PCD	programmed cell death
PDGF	platelet-derived growth factor
PKC	protein kinase C
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PRE	presubiculum
ROS	reactive oxygen species
rs	rhinal sulcus
S	subiculum
SCME	*sis*/conditioned medium response element
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second

SEP	somatosensory evoked potential
sm	molecular layer
so	stratum oriens
sr	stratum radiatum
SRE	serum response element
T°	temperature in degrees celsius
TBM	TATA box binding motif
TBP	TATA box binding protein
TBS	Tris-HCl-buffered saline
TBST	Tris-HCl-buffered saline containing tween
TdT	terminal deoxynucleotidyl transferase
TIA	transient ischemic attack
TUNEL	TdT-mediated dUTP-biotin nick end labelling
ve	lateral ventrical
VSCC	L-type voltage-sensitive calcium channel

INTRODUCTION

1. Epidemiology of Strokes

Since the first adequate account by Hippocrates, cerebrovascular accident (CVA), also known as apoplexy, or stroke, has remained one of the most deadly and debilitating of diseases to afflict society (McHenry, Jr., 1978). In Western industrialized countries, cerebrovascular diseases remain the third leading cause of mortality, followed by cardiovascular and malignant diseases (Hacke et al., 1991). There are approximately 100,000 new incidences of stroke across Canada each year, of which, 85% are caused by cerebral ischemia. Roughly 10% of these cases result in death within 2 weeks, while the remainder suffer varying degrees of physical and mental disability. Medical science still has little to offer stoke victims and their families against this major cause of loss of life and cerebral function.

2. Classification of Strokes

According to the epidemiological definition of the World Health organization, there are two major classifications of stroke; haemorrhagic, a result of bleeding from a vessel; and occlusive, which is generally due to closure of a blood vessel.

Haemorrhagic strokes are associated with hypertension or aneurysms. High blood pressure, otherwise known as hypertension, is excessively high systolic pressure above 145 - 160 mm Hg, or diastolic pressure above 90 - 100 mm Hg. Physical causes that contribute to hypertension include obesity, inactivity, smoking, and an overactive adrenal cortex. An aneurysm is a weakening of the arterial wall to the extent that it balloons. In the event that the wall bursts, the resulting haemorrhage may prove fatal.

Occlusive strokes are attributed to atherosclerosis or thrombosis. Atherosclerosis is a form of arteriosclerosis known as hardening of the arteries. It involves the deposition of cholesterol and the growth of fibrous tissue or plaques containing calcium in the vessel wall. Clotting prevents excessive external blood loss, but causes problems when formed inside vessels. Clots may form within 4 - 10 min when thrombocytes encounter rough surfaces such as a hardened or injured artery. Thrombocytes react with the damaged cells to release thromboplastin, which converts the blood protein prothrombin into an active form (thrombin). Thrombin converts the soluble plasma protein fibrinogen into fibrin, an insoluble protein. Fibrin forms a fibrous network which traps erythrocytes and thrombocytes forming a clot. Blood clots forming on the rough inner surface of a cerebral artery can block blood flow to a region of the brain resulting in cerebral thrombosis (Cunningham, 1989). Cases of stroke may be subclassified as a localized cerebral ischemia; an intracerebral hemorrhage; a subarachnoid hemorrhage; or a disturbance of cerebral venous outflow, otherwise known as sinus thrombosis. Infarction in the area of the middle cerebral artery (MCA) is the most frequently encountered stroke syndrome, characterized by language disturbance or weakness in the face and arm, depending on the hemisphere.

3. Cerebral Blood Flow: Normal Regulation and Ischemic Thresholds

The human brain accounts for only 2% of total body weight, but it receives 15% of the cardiac output and uses 20% of the O_2 consumed by the body (Liebman, 1991). The total blood supplied to the brain is approximately 800 ml/min; about 350 ml of which flow through each carotid artery, and 100 - 200 ml flow through the vertebrobasilar system. Energy is supplied to the brain almost exclusively by glucose metabolism. Glucose or glycogen stores in the brain are

sufficient to cover energy requirements for only about 1 min. A delicate balance exists between the O_2 and glucose supply from blood, and the energy requirements of the brain. In the resting state, the human brain consumes approximately 3.35 ml of O_2/min/100 g of brain tissue. The central nervous system regulates arterial blood pressure to maintain an adequate supply of blood to the brain, heart, and vital body organs. Blood pressure is autoregulated by the baroreceptor reflex system; similarly, chemoreceptors monitor O_2, CO_2, and blood pH (Eckert et al., 1988). Cerebral blood flow (CBF) is approximately 50 ml/100 g/min in healthy humans. Variations in brain activity produce corresponding changes in CBF and metabolism. Complex voluntary tasks such as moving a hand result in elevated oxidative metabolism and a higher CBF.

If CBF is temporarily limited to 25 ml/100 g/min, protein synthesis is reduced but no apparent clinical manifestations occur. Insufficiency in blood supply is termed ischemia. It deprives the tissue of both O_2 and glucose, and prevents the removal of potentially toxic metabolites such as lactic acid. If CBF declines to between 20 and 12 ml/100 g/min, neurons display a decreased electrical excitability and somatosensory evoked potential (SEP), but membrane pumps and ion gradients are maintained. Neurons at this stage are time-dependently reversible and comprise a region known as the penumbra. Clinically, at this point in time the patient is undergoing transient ischemic attacks, or TIA's. Cerebral ischemia lasting merely seconds can result in irreversible neuronal damage. If ischemia is sufficiently severe and prolonged, neurons die resulting in an area termed an infarct. When CBF falls below 12 ml/100 g/min, infarction occurs as massive amounts of calcium infiltrate the neurons (Hakim, 1987).

4. Animal Models

In order to better understand and treat human stroke, suitable animal models have been developed which closely resemble clinical syndromes. Two broad categories of cerebral ischemia are available to investigators; focal and global (forebrain) models (Takizawa and Hakim, 1991).

i) Focal Ischemia Model

The focal ischemia model correlates clinically to patients with TIAs due to atherosclerosis or thrombosis. Stroke patients with this syndrome generally have difficulty with their speech. Rats subjected to focal ischemia undergo permanent occlusion of the MCA. In this model, CBF is reduced in a gradient manner. As a result, an infarcted area is formed, surrounded by an intermediate zone where blood flow has been reduced to a level that interrupts SEPs yet permits the maintenance of membrane pumps and ion gradients. This intermediate zone is referred to as the ischemic penumbra. Damage to neurons within this zone is reversible, depending on the duration of ischemic insult and magnitude of reperfusion.

ii) Global Ischemia Model

The global ischemia model more closely correlates to patients that have been successfully resuscitated from cardiac arrest. This case manifests in loss of memory and difficulty in performing tasks involving calculations. Global ischemia may be achieved by 2-vessel occlusion (2-VO) coupled with systemic hypotension, or by 4-vessel occlusion (4-VO).

a) Two-Vessel Occlusion Method

The 2-VO model is accomplished by bilateral occlusion of the common carotid arteries (CCA) together with reducing the systemic arterial pressure to 50 mm Hg. This method is limited by the need to maintain anesthesia throughout the study, by alterations in systemic conditions due to hypotension, and by the inclination toward post ischemic seizures.

b) Four-Vessel Occlusion Method

The 4-VO method was first introduced by Pulsinelli (1979), and provided a reversible model of forebrain ischemia in rats. This technique involves locating and placing loose silk loops around both CCAs. A silk ligature is passed anterior to the cervical and paravertebral muscles but posterior to the trachea, esophagus, external jugular veins and CCAs. The rat's head is tilted downward 30° to horizontal, and the cervical spine is stretched by placing tension on the rat's tail. Through a dorsal, midline neck incision, the vertebral arteries are electrocauterized via the alar foramina of the first cervical vertebra. The following day, the CCAs are occluded for a brief period of time (10 - 30 min) using microaneurysm clips. The cervical suture is slowly tightened to block collateral blood vessels in the neck muscles. Animals which display seizure activity post ischemia are excluded from the study along with those that fail to develop complete loss of righting reflex throughout the ischemic period.

5. Neuronal Damage Following Transient Global Ischemia

Transient forebrain ischemia is accompanied by a delayed loss of neurons from selected regions of the brain. Regional CBF measurements using this model have demonstrated that the

neocortex, hippocampus, and striatum were equally and almost completely ischemic, whereas CBF to the thalamus, cerebellum, and brainstem was less reduced in comparison. Generally, lengthening the duration of the ischemic insult is associated with a greater degree of damage. According to Pulsinelli et al. (1982), 20 min of 4-VO produces moderate to severe damage in the hippocampus; layers 3,5, and 6 of the neocortex; and dorsolateral aspects of the striatum.

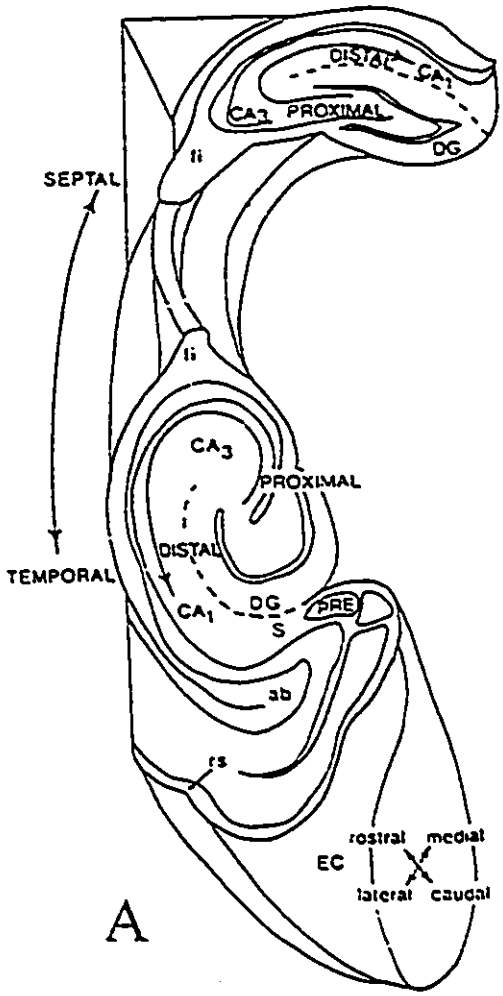
i) Introduction to the Hippocampus

The hippocampal formation is comprised of the hippocampus, dentate gyrus, and closely related cortical areas including the subiculum. The rat hippocampus resembles the shape of a cashew nut, extending from the septum dorsally, to the caudal part of the amygdala ventrally. The hippocampus consists of Ammon's horn, the dentate gyrus, and the subiculum. In the rodent, the dorsal part of Ammon's horn lying ventral to the corpus callosum is referred to as the dorsal hippocampus. The hippocampal structure is composed of two C-shaped, interlocking cell layers: the pyramidal cell layer of Ammon's horn, and the granular cell layer of the dentate gyrus. Ammon's horn has been divided into several subfields based on the morphology of the pyramidal neurons. As cited by Kogure et al. (1985), Lorente de Nó (1934) used the Golgi method to classify the hippocampus into four subfields: CA1, CA2, CA3, and CA4. Each subfield consists of a single layer of neurons with apical dendrites extending into the subiculum, stratum lacunosum-moleculare, stratum radiatum of Ammon's horn, and the stratum moleculare of the dentate gyrus. Underneath the cell layer lies a polymorphic layer which is bordered by a fibrous zone.

The dentate gyrus consists of granule cells tightly packed into a single cell layer. The blade of the dentate gyrus encloses the hilar region. The hilar area contains the polymorphic layer of the dentate gyrus and the proximal part of the CA3 pyramidal layer. The granule cell layer of the dentate gyrus may be subdivided into a suprapyramidal and an infrapyramidal blade which merge at the crest of the dentate gyrus.

Anatomical and electrophysiological data indicate a sequence of unidirectional connections from the dentate gyrus to the subiculum through the CA3, CA2, and CA1 neurons. The dentate gyrus receives major cortical afferents from the entorhinal cortex. Projections to the dentate gyrus and CA3 neurons arise preferentially from layer II of the entorhinal cortex, whereas layer III projects to CA1 neurons. Dentate granule cells extend mossy fibres to the CA3 area. Proximal regions of CA3 are predominantly reached by fibres that originate from the infrapyramidal blade, the crest, and the suprapyramidal blade, while cells at the tip of the suprapyramidal blade project to more distal portions of the CA3 region. Pyramidal cells of the CA3 subfield give rise to Schaffer collaterals that project to the CA1 region. The Schaffer collaterals synapse in the stratum radiatum and stratum oriens with dendrites from CA1 pyramidal neurons. The CA1 neurons extend major projections to the dorsal subiculum (Witter, 1989). A summary of the neuroanatomy and connectivity of the hippocampus is presented in Fig. 1.

Figure 1. Neuroanatomy and connectivity of the hippocampus, adapted from Witter (1989). A) An illustration of the anatomical subfields of the hippocampus and entorhinal cortex of the rat. Abbreviations: II-VI = cortical layers, ab = angular bundle, alv = alveus, CA1,2,3 = subfields of Ammon's horn, DG = dentate gyrus, DG_i = infrapyramidal blade of the dentate gyrus, DG_s = suprapyramidal blade of the dentate gyrus, EC = entorhinal cortex, fi = fimbria, HDG = hilus of the dentate gyrus, hf = hippocampal fissure, PAR = parasubiculum, PC = perirhinal cortex, PRE = presubiculum, rs = rhinal sulcus, S = subiculum, sm = molecular layer, so = stratum oriens, sr = stratum radiatum, ve = lateral ventrical. B) A schematic representation of a transverse section through the hippocampus depicting the trisynaptic circuitry. Dentate granule cells extend mossy fibres to the CA3 area. The CA3 neurons distribute Schaffer collaterals to the CA1 subfield. The CA1 pyramidal neurons extend major projections to the dorsal subiculum.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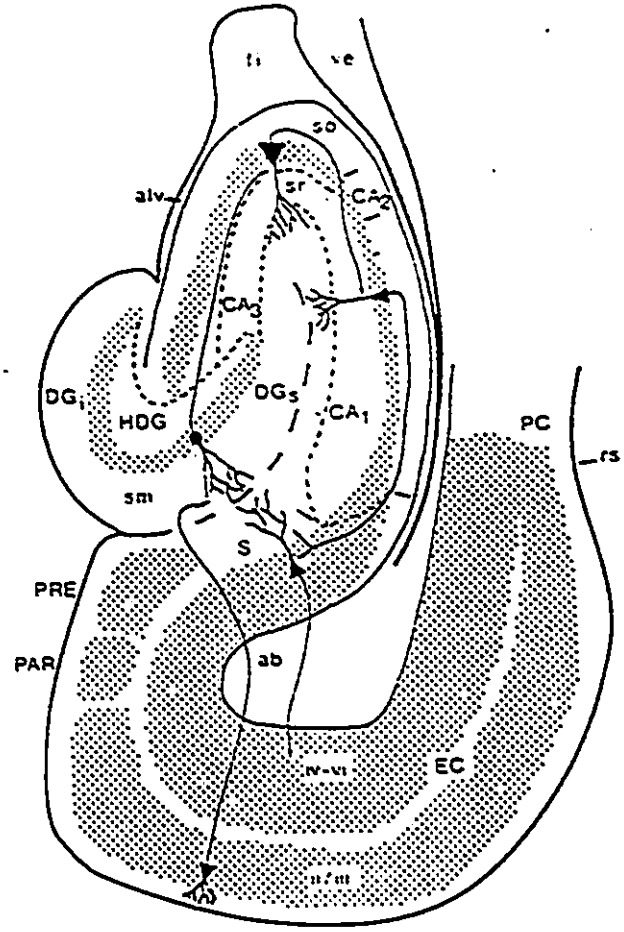
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ii) Selective Neuronal Death Within the Hippocampus

Neurons of the hippocampus are particularly vulnerable to cerebral ischemia. The hippocampus is surrounded dorsally by the cortex, and ventrally by the thalamus. In the hippocampus, 20 min of forebrain ischemia produced by 4-VO selectively damages CA1 pyramidal neurons (Pulsinelli and Brierly, 1979; Jørgensen and Diemer, 1982; Schmidt-Kastner and Freund, 1991). These neurons degenerate slowly following reperfusion with death occurring 24 h - 72 h following the ischemic insult (Pulsinelli et al., 1982). In contrast, CA3 pyramidal neurons are more resistant to ischemic stress. As cited by Kogure et al. (1985), the relationship between hypoxic ischemia and hippocampal damage has received considerable attention since the work of Spielmeyer in 1925. His group suggested that selective vulnerability in the hippocampus was a consequence of local vascular supply, otherwise known as the "vascular theory". In contrast, Vogt (1922), as cited by Kogure et al. (1985), believed that selective resistance in the hippocampus was afforded by differences in physical or chemical characteristics of individual neurons and proposed the term "pathoclisis" to describe differences in susceptibility to ischemic insult. Scholz (1953, 1963), as cited by Pulsinelli (1985), attempted to unify these views by demonstrating that under particular conditions, focal injury could be attributed primarily to vascular inequities, whereas other conditions could only be explained by inherent properties of the neurons themselves. Despite intense investigation, it is still not clear why CA1 neurons are more susceptible to ischemic injury than CA3 neurons.

6. Biochemical and Molecular Neuronal Responses to Ischemic Insult

i) Short-Term Biochemical Events Associated With Ischemia

Ischemic injury precipitates a multitude of biochemical and molecular neuronal responses in the seconds, hours and days following the ischemic event. At the cellular level, during ischemia there is a reduction in the amount of O_2 supplied to the brain which lowers cellular ATP. Energy depletion leads to the loss of Na^+ gradients normally maintained by the ATP-dependent membrane Na^+-K^+ pump. The resulting accumulation of intracellular Na^+ causes a reversal of the Na^+-Ca^{2+} exchanger which ordinarily extrudes Ca^{2+} from the neuron to maintain a chemical gradient of some 1:10,000 between intracellular (nM range) and extracellular (mM range) free Ca^{2+} (Lipton and Lobner, 1990; Vendrell et al., 1993). The consequent depolarization associated with failure of the Na^+-K^+ pump triggers Ca^{2+} influx through voltage sensitive calcium channels (VSCCs) in the plasma membrane. Large amounts of the excitatory neurotransmitter glutamate are released in response to membrane depolarization and Ca^{2+} influx. Under normal circumstances, surplus extracellular glutamate is returned to presynaptic terminals and glial cells; but during ischemia, glutamate re-uptake is impaired due to lack of energy (Schwarcz, 1982). Impaired glutamate re-uptake results in a prolonged and excessive activation of post-synaptic glutamate receptors. It has been proposed that selective CA1 loss is mediated by enhanced excitatory input from the glutaminergic collaterals of Schaffer (Johansen et al., 1984). Accordingly, lesions of the glutaminergic projections to the CA1 region, or interruption of the intrahippocampal network, afford a protective effect against ischemic injury (Pulsinelli, 1985; Johansen et al., 1987). Conversely, lesions of the inhibitory noradrenergic projection from the locus coeruleus exacerbate hippocampal ischemic damage in the rat (Blomqvist et al., 1985).

Studies have also shown that either blockade of the Na^+-Ca^{2+} exchanger or removal of Na^+ or Ca^{2+} ions from the extracellular space have a protective effect, confirming that infiltration of calcium into the neurons is lethal (Benveniste et al., 1988; Franklin and Johnson, Jr., 1992).

Glutamate activates several subtypes of ligand-gated cation channels which allow further influx of Ca^{2+} (Siesjo et al., 1989; Betz, 1990; Choi, 1990; Manev, 1990). These subtypes of ionotropic glutamate receptors are referred to as *N*-methyl-*D*-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate (KA) receptors (Sommer and Seeburg, 1992). Glutamate also interacts with a metabotropic receptor, linked to phospholipase C (PLC) and G-proteins which causes mobilization of Ca^{2+} from intracellular stores (Sugiyama et al., 1987; Murphy and Miller, 1988). The endoplasmic reticulum (ER) is the main $[Ca^{2+}]_i$ storage compartment, containing two distinct releasable pools. One pool is released upon the action of inositol 1,4,5-triphosphate (IP_3), whereas the other is released by Ca^{2+} directly. Upon activation of the metabotropic receptor by glutamate, IP_3 is formed by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) yielding IP_3 and diacylglycerol (DAG). DAG activates protein kinase C (PKC), while IP_3 precipitates Ca^{2+} release by binding to an intracellular ligand-operated Ca^{2+} channel in the ER membrane. Cytosolic Ca^{2+} may also interact with ryanodine receptors to trigger Ca^{2+} release from a second pool in the ER (Vendrell et al., 1993).

ii) Immediate-Early Gene Activation Following Ischemic Insult

Membrane depolarization triggers multiple second messenger pathways which may activate a class of inducible genes known as immediate-early genes (IEGs) or early response genes, due to their rapid induction kinetics. They are the first set of genes to be activated

following ischemia. Elevations in cAMP or $[Ca^{2+}]_i$ result in phosphorylation of the cAMP response element-binding protein (CREB). Once phosphorylated, CREB interacts with a particular Ca^{2+} response element (CaRE sequence: TGACGTCA) in the promoter region, stimulating the transcription of IEGs containing this sequence in their promoter region (Sheng et al., 1990; Walker and Carlock, 1993). Accumulation of $[Ca^{2+}]_i$ may also promote the formation of a Ca^{2+}/calmodulin complex (Ca^{2+}/CM) and consequently activate a calmodulin-dependent protein kinase (Ca^{2+}/CM-dep. PK). This enzyme can phosphorylate the transcription factor CREB, thereby stimulating transcription of *c-fos*. Therefore, two second messenger pathways converge to activate IEGs, many of which are known to mediate cell death (Colotta et al., 1992; González-Martin et al., 1992; Dragunow et al., 1993; Estus et al., 1994; Ham et al., 1995).

Several regulatory sequences have been shown to regulate *c-fos* in response to a multitude of extracellular stimuli (Sheng et al., 1990). For example, the serum response element (SRE), known as the dyad-symmetry element (DSE), located 300 bases upstream of the transcription start site, mediates *c-fos* induction by serum and other agents including activators of PKC (Rivera and Greenberg, 1990; Curran, 1991). The *sis*/conditioned medium response element (SCME), at -346, confers inducibility by platelet-derived growth factor (PDGF) and functions as an additive manner to the SRE, which is also responsive to PDGF (Wagner et al., 1990). At -60, *c-fos* contains a cyclic AMP/Ca^{2+} response element (CRE/CaRE) which mediates regulation by the second messengers cAMP and Ca^{2+}. All of these *cis*-acting regulatory sequences are located in the 5'-untranslated region of *c-fos*. In contrast, the *fos* intragenic regulatory element (FIRE) responsible for transcriptional elongation lies at the 3'-end of the first exon (Lamb et al., 1990).

Protein products of the *c-fos* and *c-jun* families of IEGs are inducible transcription factors that couple extracellular signals to alterations in phenotype by regulating downstream target genes (Morgan and Curran, 1989). Fos and Jun family proteins contain a common structural motif referred to as a leucine zipper, which permits protein dimerization (Gentz et al., 1989). The inducible nuclear transcription factor complex, AP-1, consisting of heterodimers between Fos and Jun family proteins, regulates the expression of specific downstream target genes containing the consensus sequence TGACTCA (Fanz et al.,1988; Nakabeppu et al., 1988).

7. Death By Any Name ... Is It All The Same?

i) Delayed Neuronal Death by Programmed Cell Death

Selective neuronal loss that evolves over a period of days as observed following moderate ischemia or recurrent seizures is referred to as delayed (secondary) neuronal death (DND). Recent studies suggest that DND may be the result of an active programmed cell death (PCD) or intrinsic suicide system which is commonly observed in developmental neurobiology (Oppenheim, 1991; Steller, 1995). As cited by Schwartz and Osborne, 1993, the term PCD was originally introduced in 1965 by Lockshin and Williams to describe the developmentally-regulated death of specific larval muscles following the emergence of the adult moth. In contrast to PCD, rapid neuronal death resulting in infarction is due to necrosis. PCD may be triggered by a variety of factors, including trophic factor deprivation, hypoxic-ischemic injury, kainic acid lesion, as well as naturally occurring cell death observed during development (Johnson, Jr. et al., 1989; Oppenheim, 1991; Colotta et al., 1992; Martin et al., 1992; Smeyne et al., 1993; Dragunow et al., 1994; Pollard, 1994). However, a potential death-inducing stimulus will only trigger PCD

under specific conditions that occur during differentiation, activation, stage in cell cycle, presence or lack of other signals, and environmental factors. PCD of cultured sympathetic neurons following nerve growth factor (NGF) deprivation can be prevented by blocking protein synthesis with cyclohexamide (Martin et al., 1992). These results suggest that this type of PCD is an active process requiring the synthesis of new or increased levels of pre-existing proteins which mediate the suicide program.

ii) What is Apoptosis? Morphological and Biochemical Criteria

The word apoptosis is derived from a Greek word describing the dropping of foliage from trees or petals falling from flowers (Kerr et al., 1972). There has been much confusion over correct use of the terms apoptosis and PCD. Generally, the terms are used interchangeably. However, PCD is an operational or functional term, whereas apoptosis is a morphological or biochemical description of a mode of cell death (Wick, 1994). Apoptosis is characterized by cell shrinkage, pyknotic nuclei, chromatin condensation, membrane blebbing and the formation of apoptotic bodies (Kerr et al., 1972; Clarke, 1990; Cohen, 1993). As cells undergo apoptosis, the cell membrane balloons out and pinches off, encapsulating whole organelles and portions of the nucleus to form apoptotic bodies. They are then phagocytosed and their contents degraded by heterolysosomes. There is also a loss of ribosomes from polysomes and ER. In some cases, ribosomes crystallize, resulting in cessation of protein synthesis (O'Conner and Wyttenbach, 1974). Many studies have demonstrated that prolonged elevations of intracellular free Ca^{2+} play a key role in the early signalling events leading to apoptosis (Peitsch et al., 1993; Martin et al., 1994). Free $[Ca^{2+}]_i$ activates endonucleases which cleave chromatin between individual

nucleosomes (Zhivotovsky et al., 1994). If DNA is isolated from apoptotic cells and size fractionated by electrophoresis, distinct bands can be detected. These bands are referred to as a DNA ladder and differ in size by 180 base pairs. Oligonucleosomal "ladder-type" fragmentation of nuclear, but not mitochondrial, DNA into mono- or oligomers of approximately 180 base pairs has been widely accepted as a hallmark of apoptosis (Peitsch et al., 1993; Kokileva, 1994). PCD may be induced by receptor-mediated physiological stimuli such as the presence of death signals, absence of survival signals, or combinations of contradictory signals (Kerr et al., 1972; Collins and Lopez Rivas, 1993; Savill, 1994; Steller, 1995; Thompson, 1995). In such a case, alterations in mitochondrial activity and membrane function precede nuclear changes. However, apoptosis can result from genotoxic insults directed at chromatin, in which case nuclear alterations are the initial events. Excessive reactive oxygen species (ROS) or inhibition of antioxidant pathways may also induce apoptosis (Haeker and Vaux, 1994).

iii) Cytoplasmic Mediated Apoptosis ... No Need for a Nucleus!

Recent investigation demonstrates that alterations of the nucleus are not compulsory events for PCD and that processes in the cytoplasm may play an equally important role in apoptosis. Genetic analysis during development of the nematode *Caenohbditis elegans* has identified the protein product of the *ced-3* gene as being required for the execution of 131 superfluous cells from the organism (Yuan et al., 1993). CED-3 appears to be a homolog of the mammalian interleukin-1β (IL-β)-converting enzyme (ICE), a cysteine protease which cleaves the inactive precursor of IL-β at aspartic acid residues to generate the active cytokine (Thornberry et al., 1992). The role of ICE-like proteases in mammalian cell death has been

implied by the ability of CrmA, a known inhibitor of this protease family to block cell death induced by over-expression of ICE-related proteases, or by serum or growth factor deprivation (Fernandes-Alnemri et al., 1994; Gagliardini et al., 1994). Furthermore, peptide inhibitors of the ICE protease family have been demonstrated to arrest PCD of motorneurons *in vitro* as a result of trophic factor deprivation and *in vivo* during a period of naturally occurring cell death (Milligan et al., 1995). Morphological changes associated with nuclear apoptosis are still observed when DNA fragmentation is blocked by nuclease inhibitors (Sun et al., 1994). Certain types of PCD have also been shown to be induced in enucleated cells or cytoblasts, based on non-nuclear parameters such as reduced mitochondrial activity, loss of membrane integrity, and inhibition by the anti-apoptotic gene protein Bcl-2 (Jacobson et al., 1994; Schulze-Osthoff, 1994). In light of these findings, the commonly used definition of apoptosis based on nuclear morphology and DNA degradation does not always correspond to the process itself. Clearly, events occurring independent of nuclear alterations play a major role in regulating and executing PCD.

8. Evidence Suggesting the Delayed Neuronal Death of Hippocampal CA1 Neurons Following Global Ischemia is Apoptosis

In the hippocampus, 20 min of transient global ischemia produced by 4-VO selectively damages CA1 pyramidal neurons. These neurons degenerate slowly following reperfusion with death occurring 48 h - 72 h after the ischemic insult. Several lines of evidence suggest that CA1 neurons may die by apoptosis following forebrain ischemia. First of all, morphological alterations characteristic of apoptosis have been detected in degenerating CA1 neurons by

electron microscopy (Kirino, 1982; Kirino et al., 1985; Nitatori et al., 1995). Secondly, DNA fragmentation indicative of apoptosis has been shown to occur in CA1 neurons of the hippocampus 48 h after forebrain ischemia (Héron et al., 1993; MacManus et al., 1993; Kihara, 1994; Sei et al., 1994). Lastly, ischemia rapidly enhances the neuronal expression of IEGs which encode transcriptional regulating factors known to participate in gene signalling pathways that mediate apoptosis (Colotta et al., 1992; González-Martín et al., 1992; Dragunow et al., 1993; Estus et al., 1994; Ham et al., 1995).

i) Cytoplasmic Morphological Features of CA1 Neurons After Forebrain Ischemia

Changes in the morphology of CA1 neurons after 20 min of global ischemia are slow yet progressive. In the rat hippocampus, forebrain ischemia is accompanied by overt ultrastructural alterations in pyramidal neuron morphology as rapidly as one day post ischemia. At 24 h of recirculation, increased numbers of membranous organelles have been observed by light microscopy (Kirino et al., 1985). Monoribosomes were attached to these membranous arrays as they appeared dispersed randomly throughout the cytoplasm. Darkly stained substances were also observed, scattered throughout the cytoplasm, perikarya, and dendrites. By 48 h of reperfusion, CA1 pyramidal neurons appeared shrunken, with an accumulation of ER cisterns, an increased number of dark granules, and a disaggregation of polyribosomes into monoribosomes. Electron microscopy studies by Nitatori et al. (1995) have demonstrated a significant increase in the number of cathepsin-B immunopositive lysosomes in the CA1 pyramidal layer, suggesting an elevation in the activity of cysteine proteases. After ischemia, a majority of CA1 neurons displayed karyoplasmic condensation, vacuolization, apoptotic bodies,

and contained irregularly shaped nuclei with condensed chromatin masses characteristic of apoptosis (Nitatori et al., 1995). At four days post ischemia, the CA1 region demonstrated extensive gliosis and neuronal degradation (Kirino et al., 1985).

ii) Nuclear Alterations of CA1 Neurons After Ischemic Insult

Apoptosis is characterized by cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and apoptotic bodies. The morphological changes observed in nuclei are attributed to the activation of endogenous endonucleases which cleave DNA into oligonucleosomal sized fragments. These DNA fragments display a laddered pattern in multiples of 180 - 200 base pairs when electrophoresed on a gel and are commonly considered to be a hallmark of apoptosis.

Gavreli (1992) established a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) method to detect DNA strand breaks *in situ* (as cited by Kihara et al., 1994). This method is based on the specific labelling of exposed 3'-hydroxyl termini of DNA with dUTP-biotin by TdT resulting in the synthesis of a biotinylated polydeoxynucleotide polymer that can be detected using peroxidase conjugated avidin.

By combining *in situ* end-labelling with electrophoresis, MacManus et al. (1993) discovered that DNA fragmentation occurs both in the CA1 layer of the hippocampus and in dorsolateral striatum following global ischemia in the rat. The extent of DNA damage was proportional to the duration of the ischemic insult. DNA extracted from hippocampus and striatum 48 h after 16 min of forebrain ischemia, migrated with molecular weight bands characteristic of specific nucleosomal cleavage when subjected to gel electrophoresis.

Densitometric analysis of the gel demonstrated that 25% of hippocampal DNA and 35% of striatal DNA were degraded to fragments below 10 kb. The *in situ* end-labeling procedure revealed that DNA breaks were confined almost exclusively to pyknotic nuclei of hippocampal layers CA1 and CA2. End-labeling *in situ* demonstrates that DNA breaks occur primarily in hippocampal and striatal neurons which undergo delayed death following ischemic injury suggesting that death may occur by apoptosis.

iii) Hippocampal Expression of IEG mRNA In Response to Ischemia

Ischemia rapidly induces the neuronal expression IEGs that encode transcriptional regulating factors which mediate apoptosis (Colotta et al., 1992; González-Martín, 1992; Estus et al., 1994). *In situ* hybridization histochemistry studies have demonstrated that several members of the *fos* and *jun* family of IEGs are induced in the hippocampus following transient global ischemia (Jørgensen et al., 1991; Wessel et al., 1991; Dragunow, 1993; Kiessling et al., 1993). Within the rat hippocampus, 20 min of transient forebrain ischemia produces a bitemporal pattern of *c-fos* and *c-jun* mRNA induction in vulnerable CA1 neurons, with the first peak developing 2 - 3 h post-ischemia, and the second arising 24 - 48 h after recirculation (Wessel et al., 1991).

The first peak of *c-fos* and *c-jun* expression occurs sequentially in the entorhinal cortex, dentate gyrus, CA3 and CA1 pyramidal neurons. This pattern of expression may reflect the trisynaptic circuitry of the hippocampus, which originates in the entorhinal cortex and terminates in the CA1 region. A similar pattern of IEG expression has been previously observed after penetylenetetrazole-induced seizures, where Fos immunoreactivity begins in dentate granule cells at 1 - 2 h, followed by elevations in hippocampal pyramidal neurons at 2 - 3 h (Morgan et al.,

1987). The second peak of *c-fos* and *c-jun* mRNA coincides with the period of CA1 degeneration. This subsequent induction may be a result of calcium ion influx due to pump failure since calcium has been shown to trigger c-fos transcription (Morgan and Curran, 1986).

Conflicting results have been reported, however, with regards to whether these increases in IEG mRNA consistently result in a corresponding elevation of encoded proteins (Jørgensen et al., 1991; Wessel et al., 1991; Kiessling et al., 1993; Neumann-Haefelin et al., 1994). Impaired protein synthesis in CA1 neurons following cerebral ischemia may account partially for these inconsistent findings (Cooper et al., 1977; Dienel et al., 1985; Thilman et al., 1986; Maruno and Yanagihara, 1990; Widmann et al., 1991). This is of critical importance since IEG proteins must be synthesized in order to participate in signal transduction events leading to programmed cell death. Moreover, if IEG proteins contribute to ischemic cell death, cerebral ischemia may elicit different patterns of IEG induction in susceptible CA1 and more resistant CA3 neurons. For example, previous studies suggest that Jun may play an integral role in the cascade of events leading to neuronal death following growth factor withdrawal, status epilepticus, systemic kainic acid treatment, and hypoxic-ischemic brain injury (Colotta et al., 1992; Dragunow et al., 1993, 1994; Estus et al., 1994; Pennypacker et al., 1994). In addition to Jun, Fos and FosB have recently been implicated in gene signalling events that mediate apoptosis, suggesting that these transcriptional regulating factors may also be involved in mediating the delayed death of CA1 neurons following forebrain ischemia (Smeyne et al., 1993; Estus et al., 1994).

9. Objective and Hypothesis of the Present Study

The primary objective of the present study was to determine whether ischemia-induced increases of IEG mRNA are consistently translated into corresponding elevations of encoded proteins. This is of critical importance since IEG proteins must be synthesized in order to participate in signal transduction events leading to PCD. It was hypothesized that protein products of *NGFI-A*, and members of the *c-fos* (Fos, FosB, ΔFosB) and *c-jun* (Jun, JunB, JunD) families of IEGs are differentially induced in vulnerable CA1 and more resistant CA3 neurons in response to transient global ischemia. Alterations in expression of these proteins was assessed at varying times after transient global ischemia using immunohistochemical and Western blotting techniques.

MATERIALS AND METHODS

1. Animals

All animals were treated in accordance with procedures outlined in the *Guide for the Care and Use of Experimental Animals* endorsed by the Medical Research Council of Canada. Adult male Wistar rats (250 - 275 g; Charles River, Montreal) were housed 3 per cage in a humidity and temperature-controlled environment. Animals were acclimated to a 12 h day/12 h night cycle and given free access to water and Purina laboratory chow.

2. Experimental Design

i) Immunohistochemical Analysis of Immediate Early Gene Proteins

Effects of global cerebral ischemia on the temporospatial expression patterns of the immediate-early gene (IEG) proteins Fos, FosB, ΔFosB, Jun, JunB, JunD, and NGFI-A were investigated in the rat hippocampus by immunohistochemistry. Rats were randomly allocated to ischemic or sham conditions. Four groups, containing 4 animals each, were subjected to 20 min of global ischemia and anaesthetized with pentobarbital (100 mg/kg, i.p.) 2, 12, 24, and 48 h following recirculation. Two additional groups, composed of 4 animals each, were sham manipulated and anaesthetized with pentobarbital (100 mg/kg, i.p.) 2 and 48 h later. Once deeply anesthetized, all animals were perfused transcardially with 200 ml of saline (0.9%) followed by 150 ml of 0.1 M phosphate buffer containing 4% paraformaldehyde. The brains were post-fixed for at least 12 h and 30 μm thick coronal sections (approximately 3.60 - 4.52 mm caudal to bregma, according to the atlas of Paxinos and Watson, 1986) were then cut through the

hippocampus using a vibratome. Sections from sham and experimental animals were processed simultaneously for either NGFI-A, Fos_{2-16}, $FosB_{4-338}$, FosB(N), FosB(C), Jun, JunB, or JunD-like immunoreactivity.

ii) Western Analysis of FosB-like Proteins in Hippocampal CA1 Neurons

Rats were randomly divided into ischemic and sham groups consisting of 2 rats each. All animals were deeply anaesthetized with pentobarbital (100 mg/kg, i.p.) and sacrificed by cervical decapitation 48 h after four-vessel occlusion (4-VO) or sham manipulation. The brains were rapidly removed and CA1 regions of both hippocampi dissected on an ice-cold glass plate with the aid of a dissecting microscope (Zeiss). Western analysis was performed on proteins extracted from CA1 regions of ischemic and sham control animals using the FosB(C) and FosB(N) antibodies.

3. Drugs, Western Blotting and Immunostaining Reagents

Each drug, immunoreagent and antibody employed in these studies is catalogued below with the respective source, vehicle or diluent.

i) Drugs

DRUG	SUPPLIER	VEHICLE
Pentobarbital	MTC Pharmaceuticals, Cambridge, Ontario	sodium salt in aqueous propylene glycol base

ii) Immunoreagents

IMMUNOREAGENT	SUPPLIER	DILUENT
Biotin-SP-conjugated AffiniPure Donkey Anti-rabbit IgG (H+L)	Jackson ImmunoResearch West Grove, PA	0.01 M sodium phosphate
Biotinylated whole antibody, Donkey Anti-sheep Ig Affinity purified	Amersham Oakville, Ont.	0.01 M sodium phosphate
Normal Rat Serum	Jackson ImmunoResearch West Grove, PA	0.01 M sodium phosphate
Rabbit anti-$FosB_{4-338}$ Polyclonal	Dr. R. Bravo Bristol-Myers Squibb, Princeton, N.J.	0.01 M sodium phosphate
Rabbit anti-FosB(C) Polyclonal	Dr. Y. Nakabeppu Kuyshu University 69, Fukuoka 812, Japan	0.01 M sodium phosphate
Rabbit anti-FosB(N) Polyclonal	Dr. Y. Nakabeppu Kuyshu University 69, Fukuoka 812, Japan	0.01 M sodium phosphate
Rabbit anti-c-Jun Polyclonal	Dr. R. Bravo Bristol-Myers Squibb, Princeton, N.J.	0.01 M sodium phosphate
Rabbit anti-JunB Polyclonal	Dr. R. Bravo Bristol-Myers Squibb, Princeton, N.J.	0.01 M sodium phosphate
Rabbit anti-JunD Polyclonal	Dr. R. Bravo Bristol-Myers Squibb, Princeton, N.J.	0.01 M sodium phosphate
Rabbit anti-NGFI-A Polyclonal	Dr. R. Bravo Bristol-Myers Squibb, Princeton, N.J.	0.01 M sodium phosphate
Sheep anti-Fos_{2-16} Affinity purified Polyclonal	Cambridge Research Biochemicals, Cheshire, UK	0.01 M sodium phosphate

IMMUNOREAGENT	SUPPLIER	DILUENT
Streptavidin-horseradish peroxidase conjugate	Amersham Oakville, Ont.	0.01 M sodium phosphate

4. Antibodies

Jun-, JunB-, JunD-, NGFI-A-, and $FosB_{4-338}$-like immunoreactivity were detected using antisera generously provided by R. Bravo (Bristol-Myers Squibb, Princeton, N.J.). These antibodies were generated in rabbits immunized with bacterially expressed fusion proteins (Kovary and Bravo, 1991). The following sequences were used for fusion: Jun, amino acids 80 - 334; JunB, amino acids 46 - 344; JunD, amino acids 1 - 102; NGFI-A, amino acids 15 - 322; FosB, amino acids 4 - 338. NGFI-A, JunD and FosB were expressed and fused to MS2 polymerase. Jun and JunB were expressed in bacteria fused to ß-galactosidase. The specificity of these antibodies have been established by immunoprecipitation and Western blot experiments (Kovary and Bravo, 1991). Fos_{2-16}-like immunoreactivity was detected with an affinity-purified sheep antibody [OA-11-823; Cambridge Research Biochemicals (CRB), Cambridge] raised to a synthetic peptide corresponding to amino acids 2 - 16 in the NH_2-terminal region of the Fos protein. This region is conserved in mouse and human Fos. Immunoblot analysis (CRB technical data sheet for batch 03078, 10 October 1989) indicates that this antibody recognizes Fos (55 kDa). ΔFosB- and FosB-like immunoreactivity were detected using two different affinity purified rabbit polyclonal antibodies (Nakabeppu and Nathans, 1991; Nakabeppu et al., 1993). One antibody, FosB(N), raised against amino acids 79 - 131 of the N-terminus of FosB, recognizes both FosB and ΔFosB. The second antibody, FosB(C), directed against a portion of

the C-terminus of FosB that is absent from ΔFosB (amino acids 245 - 315), selectively recognizes the long form of FosB (Nakabeppu and Nathans, 1991).

i) Primary Antibodies

PRIMARY ANTIBODY	ANTIGENIC SEQUENCE	SPECIFICITY
Rabbit anti-FosB$_{4\text{-}338}$ Polyclonal	purified bacterially expressed fusion proteins containing amino acids 4-338 of the FosB protein	⇒ preabsorbed against other Fos family members ⇒ co-precipitation assays exhibit no cross-reactivity
Rabbit anti-FosB(C) Polyclonal	amino acids 245-315 of the C-terminus exclusively inherent to FosB	⇒ no cross-reactivity when tested against extracts from COS-1 cells transfected with Fos expression plasmids
Rabbit anti-FosB(N) Polyclonal	amino acids 79-131 of the N-terminus, common to both ΔFosB and FosB proteins	⇒ no cross-reactivity when tested against extracts from COS-1 cells transfected with Fos expression plasmids
Rabbit anti-c-Jun Polyclonal	purified bacterially expressed fusion proteins containing amino acids 80-334 of the Jun protein	⇒ preabsorbed against other Jun family members ⇒ coprecipitation assays exhibit no cross-reactivity
Rabbit anti-JunB Polyclonal	purified bacterially expressed fusion proteins containing amino acids 46-344 of the JunB protein	⇒ preabsorbed against other Jun family members ⇒ coprecipitation assays exhibit no cross-reactivity
Rabbit anti-JunD Polyclonal	purified bacterially expressed fusion proteins containing amino acids 1-102 of the JunD protein	⇒ preabsorbed against other Jun family members ⇒ coprecipitation assays exhibit no cross-reactivity
Rabbit anti-NGFI-A Polyclonal	purified bacterially expressed fusion proteins with amino acids 15-332 of NGFI-A	⇒ coprecipitation assays exhibit no cross-reactivity

PRIMARY ANTIBODY	ANTIGENIC SEQUENCE	SPECIFICITY
Sheep anti-Fos_{2-16} Affinity purified Polyclonal	amino acids 2-16 of Fos conserved in both mouse and human Fos protein	⇒ immunoblot analysis detects a 55-60 kDa band indicative of Fos protein

5. Transient Forebrain Ischemia Model

Transient forebrain ischemia was performed using published modifications (Pulsinelli and Duffy, 1983; Pulsinelli and Buchan, 1988) of the four-vessel occlusion (4-VO) method (Pulsinelli and Brierley, 1982). Male Wistar rats (Charles River, Quebec) weighing 250 g were anaesthetized under 1.5% halothane anesthesia one day prior to ischemia. The alar foraminae of the first cervical vertebra were then exposed through a dorsal midline neck incision. The vertebral arteries were permanently occluded by inserting an electrocautery needle through the foraminae. Carotid arteries were isolated through a ventral midline neck incision and silk ligatures were placed loosely around them. A silk suture was passed through the cervical region, posterior to the trachea, esophagus, external jugular veins, and common carotid arteries. The ends of the ligature were secured loosely to the nape of the neck using adhesive tape. Incisions were then closed with topical xylocaine jelly.

The following day, rats were randomly divided into two groups and subjected to either 20 min of forebrain ischemia (experimental) or manipulation without carotid occlusion (sham). Forebrain ischemia was achieved by lifting the common carotid arteries using the silk ligatures placed around them and occluding the vessels with micro aneurysm clips (#160-863, George Tiemann & Co., Plainview, N.Y.). The ligature encircling the neck muscles was then tightened in order to block collateral circulation. Brain temperature was measured indirectly via a

thermocouple probe placed in the temporalis muscle (Busto et al., 1987). Body temperature was maintained between 36 - 37 °C by external warming. Ischemia was terminated by removal of the clips and loosening of the neck ligature. Animals that convulsed following reperfusion were omitted from the study along with those that did not develop fully dilated pupils or complete loss of righting reflex during ischemia. Sham animals were subjected to vertebral occlusion and nonocclusive manipulation of the carotid arteries.

6. Immunohistochemical Analysis of Immediate Early Gene Products

i) Immunohistochemistry

Immunohistochemistry was performed using a standard procedure described previously by Robertson and Fibiger (1992). Free floating hippocampal sections (30 µm thick) were washed in 0.01 M phosphate buffered saline (PBS) containing 0.3% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase activity. Sections were then washed three times (10 min per wash) in PBS. Tissue sections were incubated for 48 h at 4 °C in 0.01 M PBS containing 0.3% Triton X-100, 0.02% sodium azide (NaAz), and one of the following primary antisera: Fos_{2-16} (1:2500), $FosB_{4-338}$ (1:8000), Jun (1:2000), JunB (1:2000), JunD (1:2000), NGFI-A (1:20000), FosB(C) (1:2000) and FosB(N) (1:2000). In order to suppress non-specific background staining, normal rat serum (1:100) was included in the primary incubation medium when staining for JunD-like immunoreactivity. Sections were then washed three times in 0.01 M PBS (10 min per wash), and incubated for 24 h in PBS containing the appropriate biotin labelled secondary antisera (1:200) and 0.3% Triton X-100. The sections were washed 3 times with PBS and

incubated for 3 hours at room temperature with PBS containing 0.3% Triton X-100 and streptavidin-horseradish peroxidase (HRP) (Amersham; 1:100). After 3 washes in PBS, the sections were rinsed in 0.1 M acetate buffer (pH 6.0). Immunohistochemical staining was visualized by the avidin-biotin technique, using diaminobenzidine (DAB) as the peroxidase substrate (according to the *Handbook of Immunohistochemical Staining Methods*, 1989). Sections were immersed in 1.32 M DAB solution and 20 µl of glucose oxidase was added to every 3 ml of reaction mixture to catalyze the conversion of glucose to hydrogen peroxide. HRP forms a complex with H_2O_2 which then oxidizes DAB, converting it into a coloured precipitate which can be visualized under a light microscope (Fig. 2). Immunostaining resulting from the oxidation of DAB was enhanced by the inclusion of 1.32 M nickel ammonium sulfate in the reaction mixture. The reaction was terminated after 5 min by washing the sections three times in 0.1 M acetate buffer (10 min per wash). Finally, tissue sections were mounted on gelatin coated slides, defatted in a series of alcohols, and coverslipped with permount. Controls for the specificity of immunostaining included omission of the primary antibody (c-Jun, JunB, JunD, NGFI-A, $FosB_{4-338}$), or preabsorption of the antibody with the antigenic sequence (Fos_{2-16}, FosB(C), FosB(N)). Immunoreactivity was lost under both of these conditions.

ii) Quantification of Immunoreactive Nuclei

Coronal sections through the hippocampus approximately 3.6 mm caudal to bregma were selected for quantification. Changes in IEG protein were quantified by counting the number of immunoreactive nuclei in hippocampal CA1 and CA3 regions using an image analysis system

equipped with Image 1.47 software (Wayne Rasband, NIMH). Digitization of the sampled areas (200 X 800 μm; Fig. 3) was performed at 100X magnification using a CCD camera linked to a microscope. Variations in the intensity of immunostaining produced with different antisera necessitated the use of a standardization criterion for thresholding of the digitized image. Nuclear size was used as the standardization criterion for selecting the appropriate threshold value, since pyramidal neuronal nuclei (approximately 10 - 15 μm in diameter) were stained. Before each count, the threshold was eroded until the average particle size (labelled nuclei) was about 8 - 10 μm in diameter. A single determination was performed on separate sections from each animal.

iii) Statistical Analysis

Statistical analysis of regional changes in IEG expression over the sampled time points was performed by one-way analysis of variance (ANOVA). If the ANOVA was significant, multiple comparisons were performed using the Neuman-Keuls test ($p \leq 0.01$). Subsequent analysis of alterations in IEG expression between regions was determined by performing an ANOVA on normalized CA1/CA3 ratios across the sampled time points ($p \leq 0.01$).

Figure 2. Flow chart illustrating the glucose oxidase-3,3'-diaminobenzidine (DAB)-nickel enhanced method used to detect immunoreactivity for immediate early gene proteins.

IMMUNOHISTOCHEMICAL PROTOCOL

[glucose oxidase-3,3'-diaminobenzidine (DAB)-nickel enhanced method]

Wash free floating (30 µm) coronal hippocampal sections with 0.01 M PBS containing 0.3% H_2O_2 (10 min)

⬇

Wash sections 3 times with 0.01 M PBS (10 min per wash)

⬇

Incubate sections with 0.01 M PBS containing 0.3% Triton X-100, 0.02% NaAz, and primary antisera for 48 h at 4 °C

⬇

Wash sections 3 times with 0.01 M PBS (10 min per wash)

⬇

Incubate sections with 0.01 M PBS containing appropriate biotin labelled secondary antisera (1:200) and 0.3% Triton X-100 for 24 h at 4 °C

⬇

Wash sections 3 times with 0.01 M PBS (10 min per wash)

⬇

Incubate sections with 0.01 M PBS containing 0.3% Triton X-100, and 1% streptavidin-horseradish peroxidase for 3 h at room T°

⬇

Wash sections 3 times with 0.01 M PBS (10 min per wash)

⬇

Rinse sections with 0.1 M acetate buffer (pH 6.0)

⬇

Immerse sections in diaminobenzidine and visualize hydrogen peroxide reaction upon addition of glucose oxidase

$HRP + H_2O_2 \Rightarrow [HRP/H_2O_2] + DAB \Rightarrow$ oxidized $DAB + H_2O + {}^{\cdot}O_2 + HRP$

⬇

Terminate reaction after 5 min by washing sections 3 times with 0.1 M acetate buffer (pH 6.0) (10 min per wash)

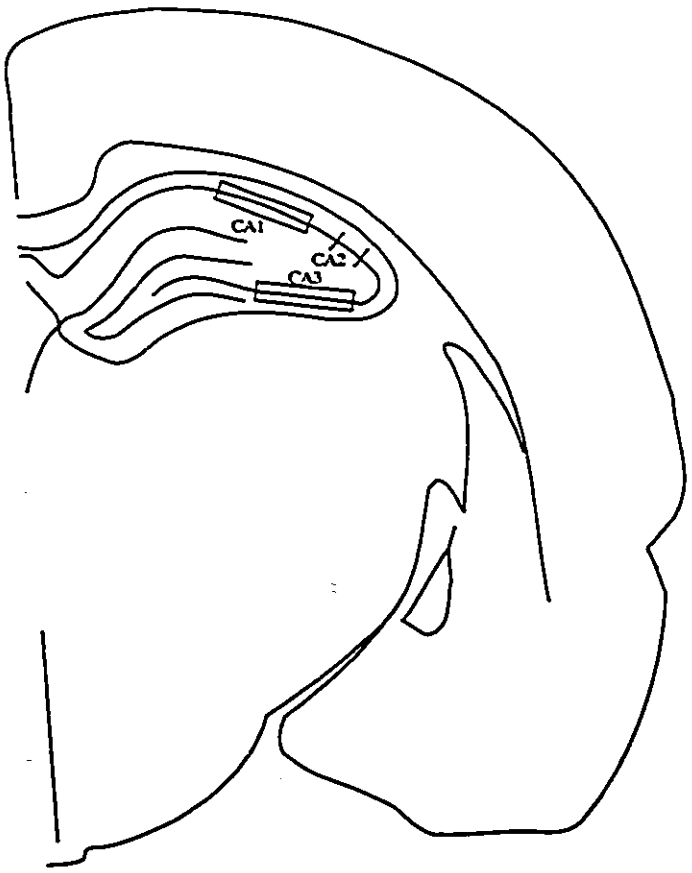
⬇

Mount sections on gelatin coated slides, defat, and coverslip

⬇

Quantify immunoreactive nuclei in designated 200 X 800 µm areas of CA1 and CA3 hippocampal subfields using an image analysis system equipped with Image 1.47 software

Figure 3. Camera lucid drawing of a representative section used to quantify immunoreactive neurons in hippocampal subfields by computer-assisted image analysis. Boxes (200 X 800 μm) indicate the areas which were sampled in CA1 and CA3 regions.


CA1
CA2
CA3

7. Western Analysis of FosB-like Proteins in Hippocampal CA1 Neurons

i) Isolation of Nuclear Extracts

CA1 regions were homogenized (2 ml-Dounce pestle type A, 15 strokes) in 1 ml of 0.25 M sucrose, 0.05 M Tris-HCl (pH 6.8), 0.025 M NaCl, 4 mM $MgCl_2$, and the following protease inhibitors: 1 mM PMSF, 2 µg/ml leupeptin and 5 µg/ml aprotinin. EGTA was also added to this buffer at a concentration of 1 mM to prevent calcium-dependent protease activity. Cells were collected by centrifugation at 11,000 g for 20 sec. Cells were resuspended in 1 ml of 1% Triton X-100 in the above buffer and after 5 min at 4 °C, they were disrupted by douncing (2 ml-Dounce pestle type B, 20 strokes). The nuclei 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 including protease inhibitors and set on ice for 30 min. Nuclear extracts were collected by centrifugation at 11,250 g in a Beckman 42.2 rotor. Aliquots (10 µl) were pipetted for protein assay (Bio Rad) and the extracts were stored at -80 °C until further analysis. Protein concentrations were determined using the Bio-Rad protein assay.

ii) SDS-PAGE, Electroblotting and Immunostaining

Nuclear protein extracts (10 - 20 µg) were subjected to electrophoresis in a highly porous SDS-PAGE system (Doucet et al., 1990) which permits rapid and efficient transfer of proteins to nitrocellulose while maintaining high resolution. The proteins resolved on 15% running gels (length fixed to 5.5 cm) were transferred to nitrocellulose sheets electrophoretically as described

by Towbin et al. (1979) with the exception of omitting methanol from the transfer buffer. The nitrocellulose membranes were first incubated in Tris-HCl-buffered saline (TBS) containing 5% skim milk for 1 h at 42 °C, then incubated overnight at 4 °C with FosB(N) (1:1500), or FosB(C) (1:1500) antisera. Blots were then washed three times (10 min each) with TBS containing 0.05% tween (TBST), and incubated with a biotin-conjugated anti-rabbit IgG (1:2000; Amersham) for 1 h at room temperature. Following washing with TBST, blots were incubated in streptavidin-horseradish peroxidase (1:2000; Amersham) for 1 h and developed by enhanced chemiluminescence (ECL, Amersham). A schematic summary of the techniques used to perform Western analysis is presented in figure 4.

Figure 4. Schematic summary of techniques utilized to perform Western analysis on CA1 regions of 48 h occluded and 48 h sham animals.

WESTERN ANALYSIS OF CA1 HIPPOCAMPAL REGIONS

i) Isolation of Nuclear Extracts

Homogenize CA1 regions (2 ml-Dounce pestle type A, 15 strokes)
in 1 ml isotonic buffer containing protease inhibitors + EGTA

⬇

Collect cells by centrifugation (11,000 g for 20 sec)

⬇

Resuspend cells in 1 ml of isotonic buffer
containing Triton X-100 (5 min at 4 °C)

⬇

Disrupt cells by douncing
(2 ml-Dounce pestle type B, 20 strokes)

⬇

Pellet cells by centrifugation (11,000 g for 20 sec)

⬇

Resuspend pellet in high salt buffer
containing protease inhibitors (incubate on ice 30 min)

⬇

Collect nuclear extracts by centrifugation
(11,000 g, Beckman 42.2 rotor)

⬇

Perform protein assay (BioRad) on 10 µl aliquots

ii) SDS-PAGE and Electroblotting

Electrophorese (10 - 20 µg) nuclear protein extract
through highly porus SDS-PAGE system

⬇

Resolve proteins on 15% running gels (fixed length 5.5 cm)

⬇

Transfer proteins to nitrocellulose
membranes by electrophoresis

WESTERN ANALYSIS OF CA1 HIPPOCAMPAL REGIONS

iii) Immunoblotting Protocol

Incubate nitrocellulose membranes in TBS containing 5% skim milk (1 h at 42 °C)

⬇

Incubate nitrocellulose membranes in TBS containing
FosB(C) (1:1500) or FosB(N) (1:1500) antisera (12 h at 4°C)

⬇

Wash blot 3 times with TBS containing 0.05% tween (TBST)
(10 min per wash)

⬇

Incubate blot with biotin-conjugated anti-rabbit IgG (1:200)
(1 h at room T°)

⬇

Wash blot 3 times with TBST containing 0.05% tween

⬇

Incubate blot in streptavidin-horseradish peroxidase (1:2000)
(1 h at room T°)

⬇

Develop by enhanced chemiluminescence (ECL)

RESULTS

1. Immunohistochemical Analysis of Immediate Early Gene Proteins in the Hippocampus Following Global Ischemia

i) Fos_{2-16}-like Immunoreactivity

In both the 2 h and 48 h sham-operated controls, hippocampal Fos_{2-16}-like immunoreactivity was detected in only a few dispersed nuclei located primarily in dentate granule cells and CA3 pyramidal neurons (Fig. 5). At 2 h of recirculation, there was a striking increase in the number of neurons that displayed Fos_{2-16}-like immunoreactivity in both CA1 and CA3 regions of the hippocampus (Figs. 5 and 13). Although not quantified, Fos_{2-16}-like immunoreactivity also seemed to be elevated in the dentate gyrus at this time. At 12 h following reperfusion, Fos_{2-16}-like immunoreactivity had returned to basal levels in both CA1 and CA3 hippocampal regions. However, 24 h after ischemia, there was a subsequent elevation of Fos_{2-16}-like immunoreactivity (Figs. 5 and 13). Although this increase appeared to occur in both CA1 and CA3 neurons, only the increase in CA1 neurons reached statistical significance (Figs. 5 and 13). In contrast, it seemed that Fos_{2-16}-like immunoreactivity had subsided to basal levels in dentate granule cells at this time. At 48 h of survival, the number of CA1 neurons which displayed Fos_{2-16}-like immunoreactivity was not significantly different from that observed in sham controls.

ii) $FosB_{4-338}$-like Immunoreactivity

In sham animals, $FosB_{4-338}$-like immunoreactivity was seldom found in CA1 or CA3 neurons, and was observed in only a few scattered dentate granule cells (Fig. 6). At 2 h of reperfusion, the number of $FosB_{4-338}$-like immunoreactive nuclei in both the CA1 and CA3 regions remained at control levels (Figs. 6 and 13). Although not quantified, $FosB_{4-338}$-like immunoreactivity appeared to be elevated in the dentate gyrus at this time. At 12 h following recirculation, there was a large increase in the number of $FosB_{4-338}$-like immunoreactive neurons in both the CA1 and CA3 hippocampal subfields. This increase remained apparent in CA1 neurons 24 and 48 h following the ischemic insult. In contrast, $FosB_{4-338}$-like immunoreactivity declined to basal levels in CA3 neurons at 48 h of survival (Fig. 6 and 13). Although not quantified, $FosB_{4-338}$-like immunoreactivity also seemed to have declined to basal levels in the dentate gyrus at this time.

iii) FosB(N)-like Immunoreactivity

FosB(N)-like immunoreactivity was present in a few CA3 pyramidal neurons and dentate granule cells, but was seldom observed in the CA1 region of 2 h or 48 h sham controls (Figs. 7 and 13). Although not quantified, FosB(N)-like immunoreactivity was dramatically elevated in dentate granule cells 2 h following reperfusion. At 12 h of recirculation, there was a significant increase in the number of neurons which expressed FosB(N)-like immunoreactivity in both the CA1 and CA3 regions of the hippocampus. These increases were still apparent in both CA1 and CA3 neurons 24 h following the ischemic insult. However, 48 h post-ischemia,

the number of FosB(N)-like immunoreactive neurons in the CA1 region remained statistically elevated; whereas FosB(N)-like immunoreactivity in the CA3 region had subsided to control levels (Figs. 7 and 13). Elevated FosB(N)-like immunoreactivity was also still apparent in dentate granule cells at this time.

iv) FosB(C)-like Immunoreactivity

FosB(C)-like immunoreactivity was seldom observed in CA1 or CA3 hippocampal subfields of sham animals (Fig. 8). There was an immense increase in the number of FosB(C)-like immunoreactive neurons in both the CA1 and CA3 regions 2 h following reperfusion (Figs. 8 and 13). Although not quantified, FosB(C)-like immunoreactivity appeared to be substantially elevated in dentate granule cells at this time. The elevations of FosB(C)-like immunoreactivity observed in both CA1 and CA3 neurons persisted 12 h and 24 h after the ischemic insult. At 48 h of survival, FosB(C)-like immunoreactivity had returned to basal levels in the CA3 region, while it remained elevated in the CA1 neurons (Figs. 8 and 13). Although not quantified, FosB(C)-like immunoreactivity in the dentate gyrus seemed to have subsided to control levels at this time.

v) Jun-like Immunoreactivity

Basal levels of Jun-like immunoreactivity were very low in both CA1 and CA3 regions of 2 h and 48 h sham-operated control animals. At 2 h of reperfusion, the number of Jun-like immunoreactive nuclei in these regions did not differ significantly from control levels. However,

there was a large increase in the number of CA1 neurons which displayed Jun-like immunoreactivity both 12 h and 24 h following the ischemic insult (Figs. 9 and 14). In contrast, significant elevations of Jun-like immunoreactivity did not occur in the CA3 region until 24 h of survival. Increases in Jun-like immunoreactivity were still apparent in CA1 neurons 48 h after the ischemic insult, whereas Jun-like immunoreactivity had declined to basal levels in CA3 neurons (Figs. 9 and 14).

vi) JunB-like Immunoreactivity

In both 2 h and 48 h sham controls, JunB-like immunoreactivity was detected in a substantial number of CA1 and CA3 neurons. In comparison to sham animals, JunB-like immunoreactivity was not significantly altered in either area 2 h following reperfusion. However, at 12 h of recirculation, there was a significant increase in the number of neurons which displayed JunB-like immunoreactivity in both CA1 and CA3 regions (Figs. 10 and 14). Although not quantified, JunB-like immunoreactivity also appeared to be elevated in the dentate gyrus at this time. JunB-like immunoreactivity had declined to basal levels in CA1 and CA3 neurons at 24 h of survival, and also appeared to have diminished in the dentate gyrus. However, 48 h following reperfusion, the number of JunB-like immunoreactive neurons in the CA1 region was significantly lower than that observed in sham controls (Figs. 10 and 14).

vii) JunD-like Immunoreactivity

Basal JunD-like immunoreactivity was abundant in CA1 and CA3 neurons of both 2 h and 48 h sham control animals. Ischemia did not significantly alter JunD-like immunoreactivity in either the CA1 or the CA3 region of the hippocampus at any time point examined following reperfusion (Figs. 11 and 14).

viii) NGFI-A-like Immunoreactivity

Constitutive NGFI-A-like immunoreactivity was high in both CA1 and CA3 neurons. At 2 h following reperfusion, there was a significant increase in the number of NGFI-A-like immunoreactive nuclei in the CA3 region of the hippocampus (Figs. 12 and 14). Although not quantified, there also appeared to be an elevation of NGFI-A-like immunoreactivity in the dentate gyrus at this time. NGFI-A-like immunoreactivity was still elevated in CA3 neurons 12 h after the ischemic episode; however, it had subsided to basal levels by 24 h of survival. At 48 h following reperfusion, constitutive NGFI-A-like immunoreactivity was completely lost from CA1 neurons. Similarly, the number of CA3 neurons which expressed NGFI-A-like immunoreactivity had also diminished below basal levels at 48 h (Figs. 12 and 14).

Figure 5. Photomicrographs of ischemia-induced Fos_{2-16}-like immunoreactivity in the dorsal hippocampus in response to 20 min of global ischemia. In 2 h and 48 h sham control animals, Fos_{2-16}-like immunoreactivity was present in only a few scattered CA3 neurons and dentate granule cells (SHAM-2h; SHAM-48h). Fos_{2-16}-like immunoreactivity was elevated in CA1 and CA3 regions, as well as dentate granule cells 2 h after four-vessel occlusion (4VO-2h). At 12 h, Fos_{2-16}-like immunoreactivity in CA1, CA3 and dentate granule cells had returned to basal levels (4VO-12h). Fos_{2-16}-like immunoreactivity was elevated exclusively in the CA1 region 24 h after recirculation (4VO-24h). By 48 h, Fos_{2-16}-like immunoreactivity had declined to basal levels in the CA1 neurons. Scale bar = 500 μm.

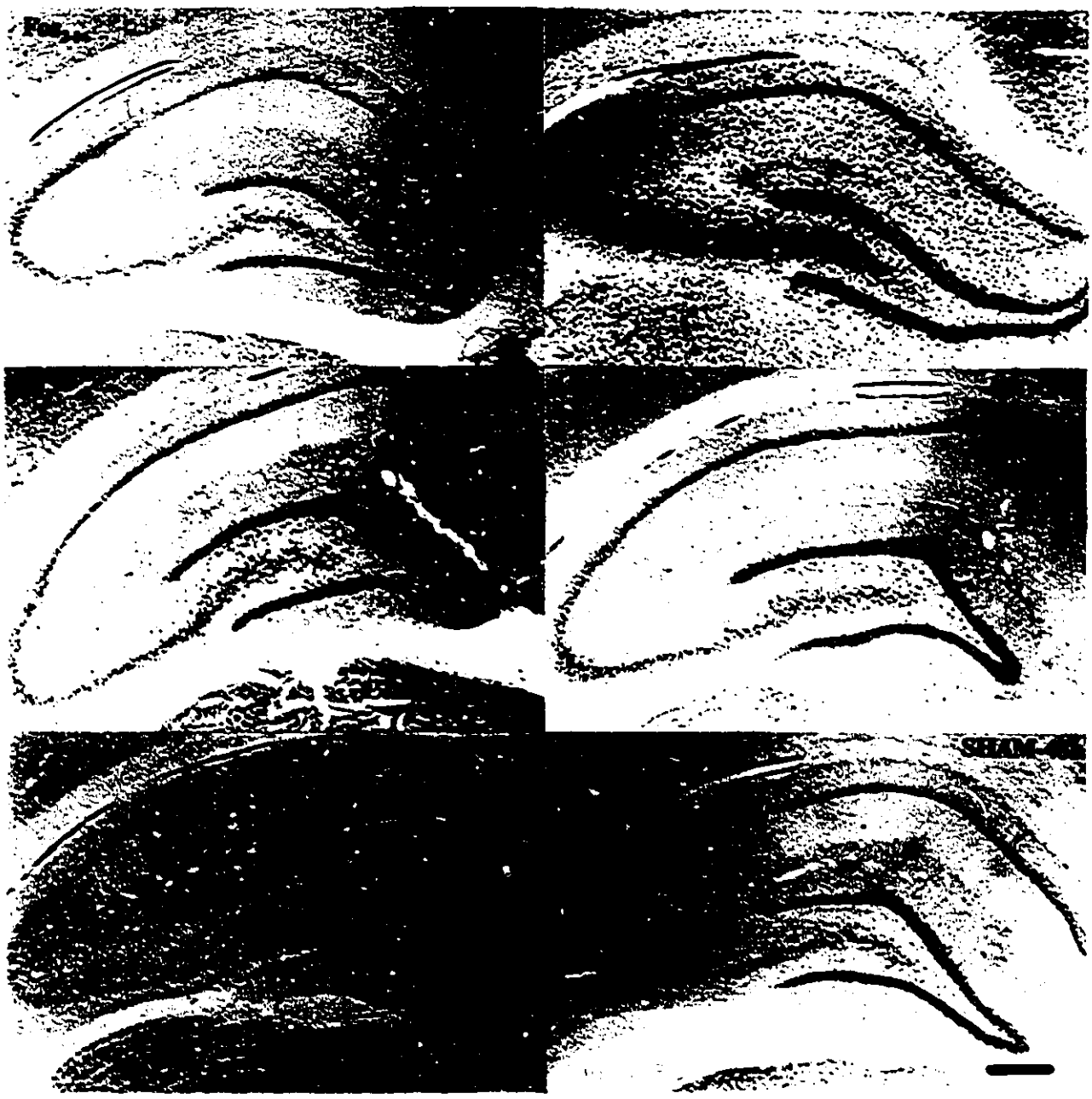


Figure 6. Photomicrographs of $FosB_{4\text{-}338}$-like immunoreactivity induced in the dorsal hippocampus by 20 min of forebrain ischemia. In control animals, $FosB_{4\text{-}338}$-like immunoreactivity was present in only a few dispersed dentate granule cells (SHAM-2h; SHAM-48h). $Fos_{4\text{-}338}$-like immunoreactivity was selectively elevated in the dentate gyrus 2 h after ischemia (4VO-2h). At 12 h, $Fos_{4\text{-}338}$-like immunoreactivity was detected not only in dentate granule cells but CA1 and CA3 neurons as well (4VO-12h). $Fos_{4\text{-}338}$-like immunoreactivity remained elevated in CA1, CA3 and dentate neurons 24 h following recirculation (4VO-24h). At 48 h of survival, $Fos_{4\text{-}338}$-like immunoreactivity remained elevated exclusively in CA1 neurons (4VO-48h). Scale bar = 500 μm.

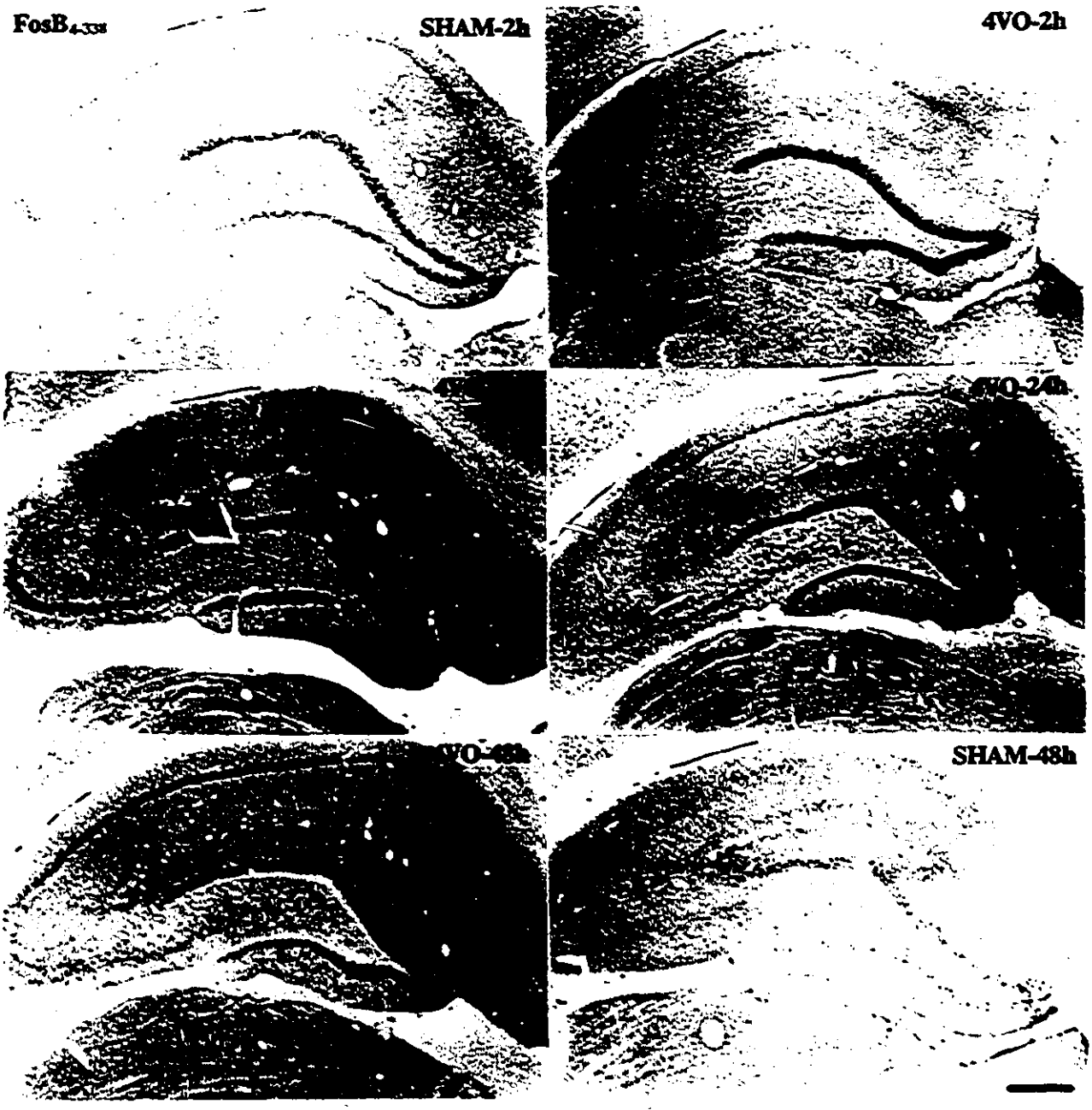

FosB4-33
SHAM-2h
4VO-2h
SHAM-48h

Figure 7. Photomicrographs depicting the time course of ischemia-induced FosB(N)-like immunoreactivity in the hippocampus by 20 min of global ischemia. In sham control animals, FosB(N)-like immunoreactivity was present in a few scattered CA3 pyramidal neurons and dentate granule cells (SHAM-2h; SHAM-48h). FosB(N)-like immunoreactivity was selectively enhanced in the dentate gyrus 2 h after four-vessel occlusion (4VO-2h). At 12 h of reperfusion, FosB(N)-like immunoreactivity had declined in the dentate gyrus but was significantly enhanced in CA1 and CA3 neurons (4VO-12h). FosB(N)-like immunoreactivity remained elevated in CA1, CA3 and dentate neurons 24 h after recirculation (4VO-24h). At 48 h, FosB(N)-like immunoreactivity remained elevated selectively in CA1 neurons (4VO-48h). Scale bar = 500 μm.

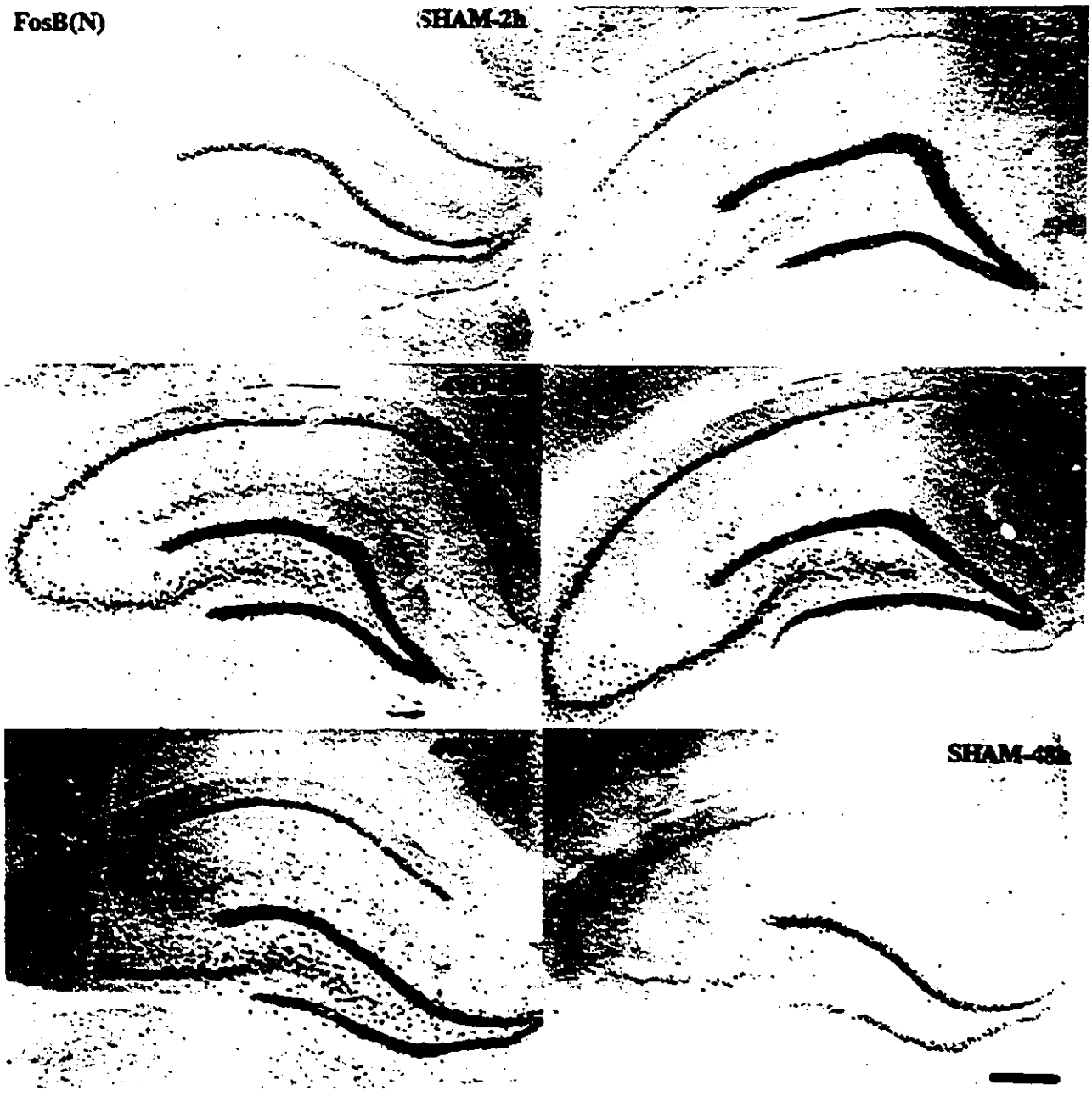

FosB(N)
SHAM-2h
SHAM-48h

Figure 8. Photomicrographs of FosB(C)-like immunoreactivity induced in the dorsal hippocampus in response to 20 min of global ischemia. In 2 h and 48 h shams, FosB(C)-like immunoreactivity was detected in only a few scattered neurons within the CA3 region and the dentate gyrus (SHAM-2h; SHAM-48h). FosB(C)-like immunoreactivity was dramatically elevated in CA1 and CA3 neurons as well as dentate granule cells 2 h after four-vessel occlusion (4VO-2h). FosB(C)-like immunoreactivity remained elevated in the CA1–CA3, and dentate granule cells 12 h after reperfusion (4VO-12h). Elevations of FosB(C)-like immunoreactivity persisted in the dentate gyrus as well as CA1 and CA3 neurons 24 h after recirculation (4VO-24h). By 48 h, FosB(C)-like immunoreactivity remained enhanced exclusively in CA1 neurons (4VO-48h). Scale bar = 500 μm.

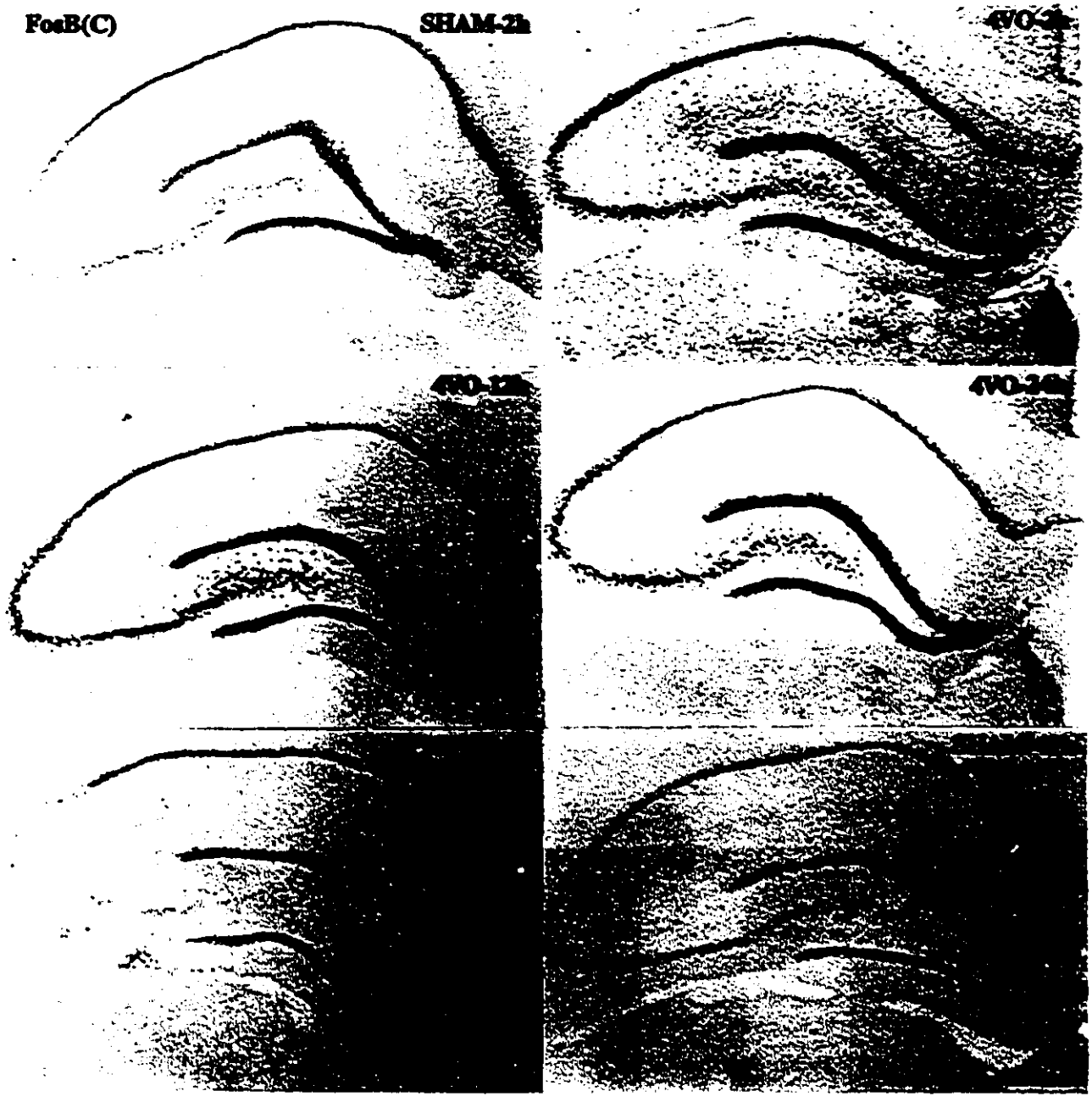

FosB(C)
SHAM-2h

Figure 9. Photomicrographs of Jun-like immunoreactivity induced in the hippocampus in response to 20 min of forebrain ischemia. In control animals, 2 h and 48 h shams, Jun-like immunoreactivity was present in only a few neurons within the CA3 region and the dentate gyrus (SHAM-2h; SHAM-48h). Jun-like immunoreactivity was not significantly altered in the dorsal hippocampus 2 h after four-vessel occlusion (4VO-2h). Jun-like immunoreactivity was selectively enhanced in CA1 neurons 12 h after reperfusion (4VO-12h). Elevated levels of Jun-like immunoreactivity were detected in both CA1 and CA3 neurons 24 h after recirculation (4VO-24h). By 48 h of survival, increased Jun-like immunoreactivity persisted exclusively in the CA1 region (4VO-48h). Scale bar = 500 μm.

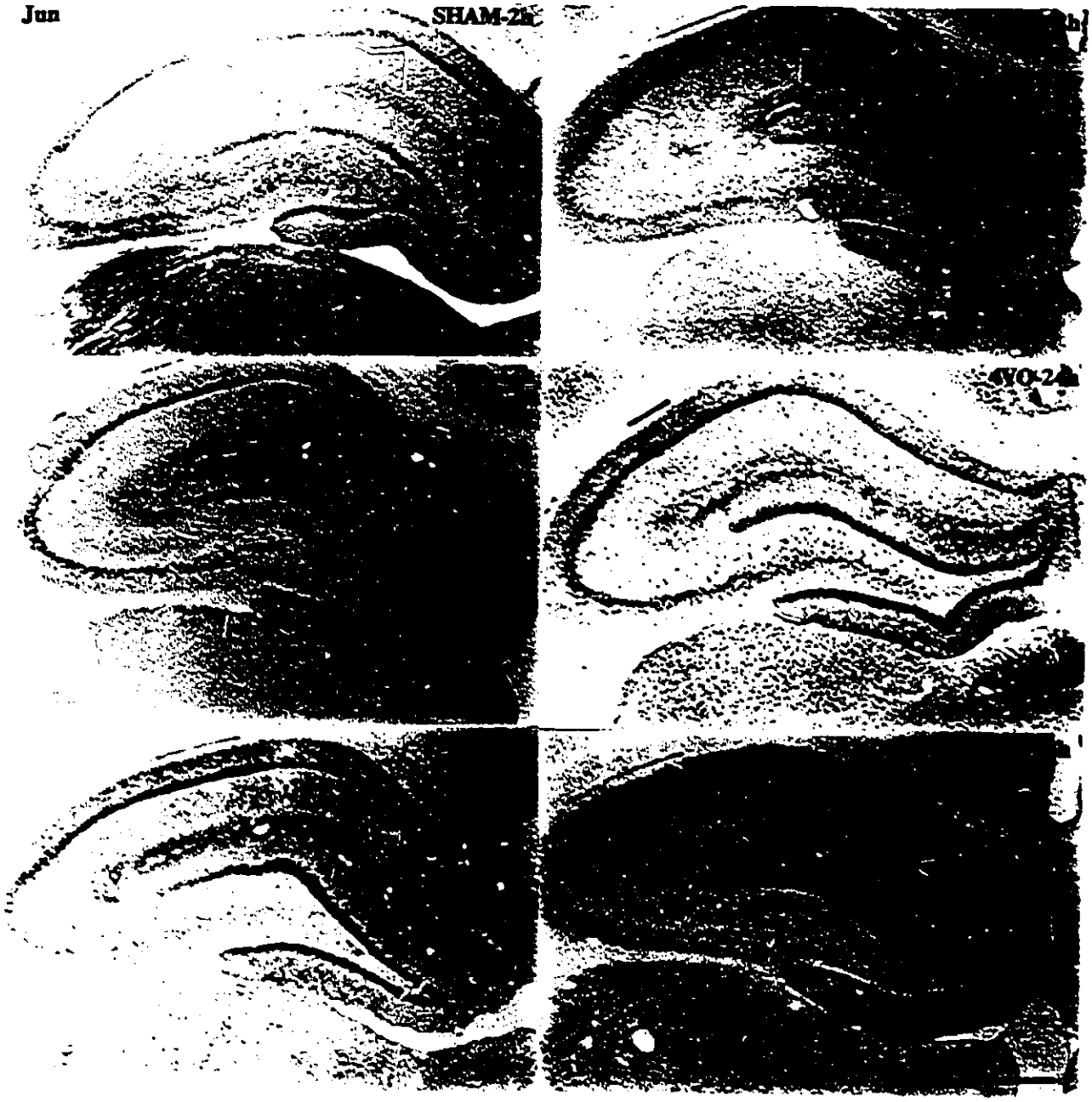

Jun
SHAM-2h
VO-24

Figure 10. Photomicrographs depicting the effect of 20 min of global ischemia on JunB-like immunoreactivity in the dorsal hippocampus. Basal JunB-like immunoreactivity was present in a significant number of CA1 and CA3 neurons of 2 h and 48 h sham controls (SHAM-2h; SHAM-48h). JunB-like immunoreactivity was selectively elevated in the dentate gyrus 2 h after four-vessel occlusion (4VO-2h). At 12 h of recirculation, JunB-like immunoreactivity was enhanced in both CA1 and CA3 neurons (4VO-12h). By 24 h, JunB-like immunoreactivity had subsided to control levels in the CA1 and CA3 region as well as the dentate gyrus (4VO-24h). At 48 h post-ischemia, the number of JunB-like immunoreactive neurons in the CA3 region had declined significantly below control levels (4VO-48h). Scale bar = 500 μm.

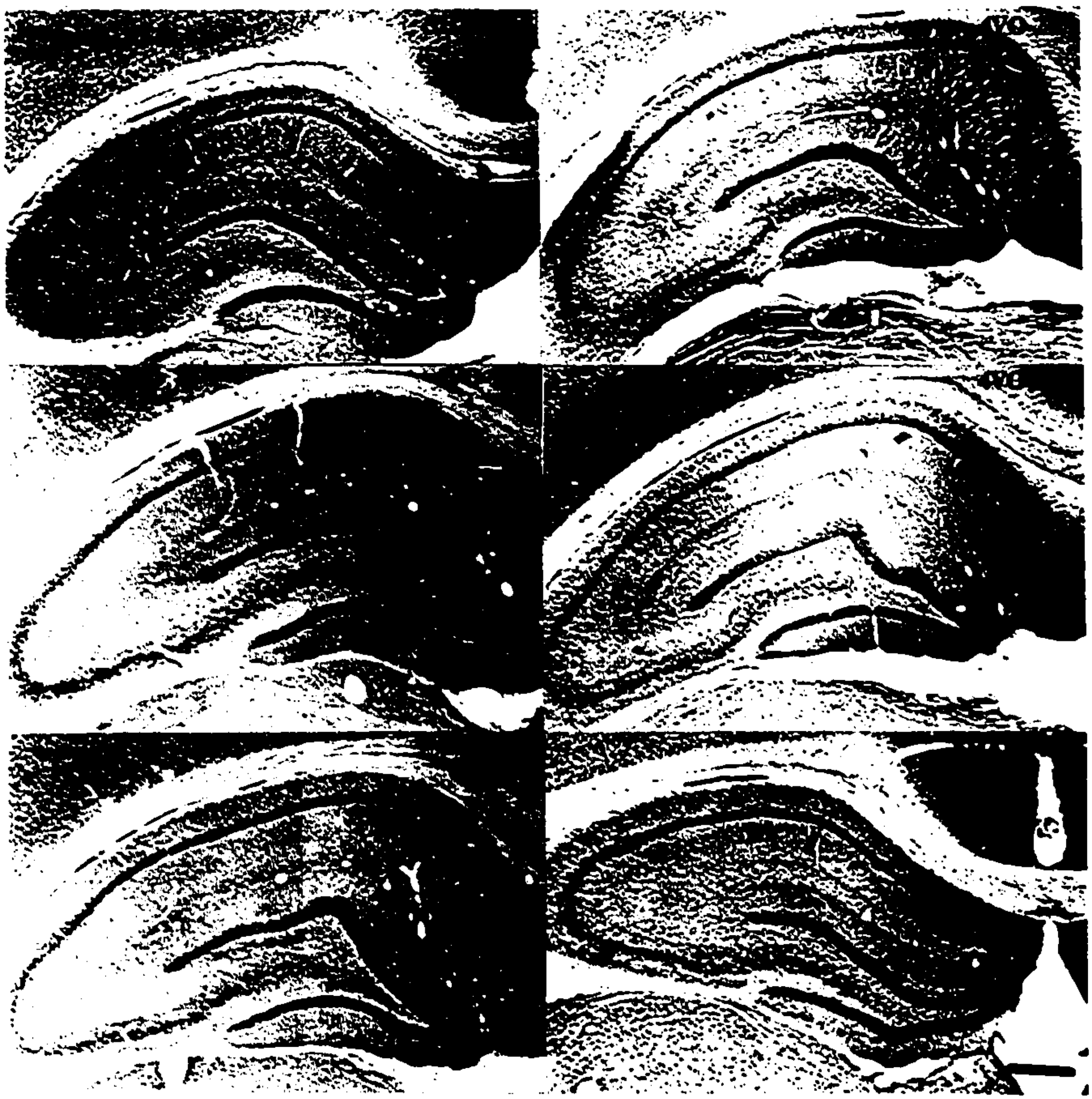


Figure 11. Photomicrographs of JunD-like immunoreactivity in the dorsal hippocampus following 20 min of forebrain ischemia. Basal JunD-like immunoreactivity was detected in a substantial number of neurons in the CA1 and CA3 regions of control animals (SHAM-2h; SHAM-48h). JunD-like immunoreactivity remained unaltered 2 h (4VO-2h), 12 h (4VO-12h), 24 h (4VO-24h) or 48 h (4VO-48h) after reperfusion. Scale bar = 500 μm.

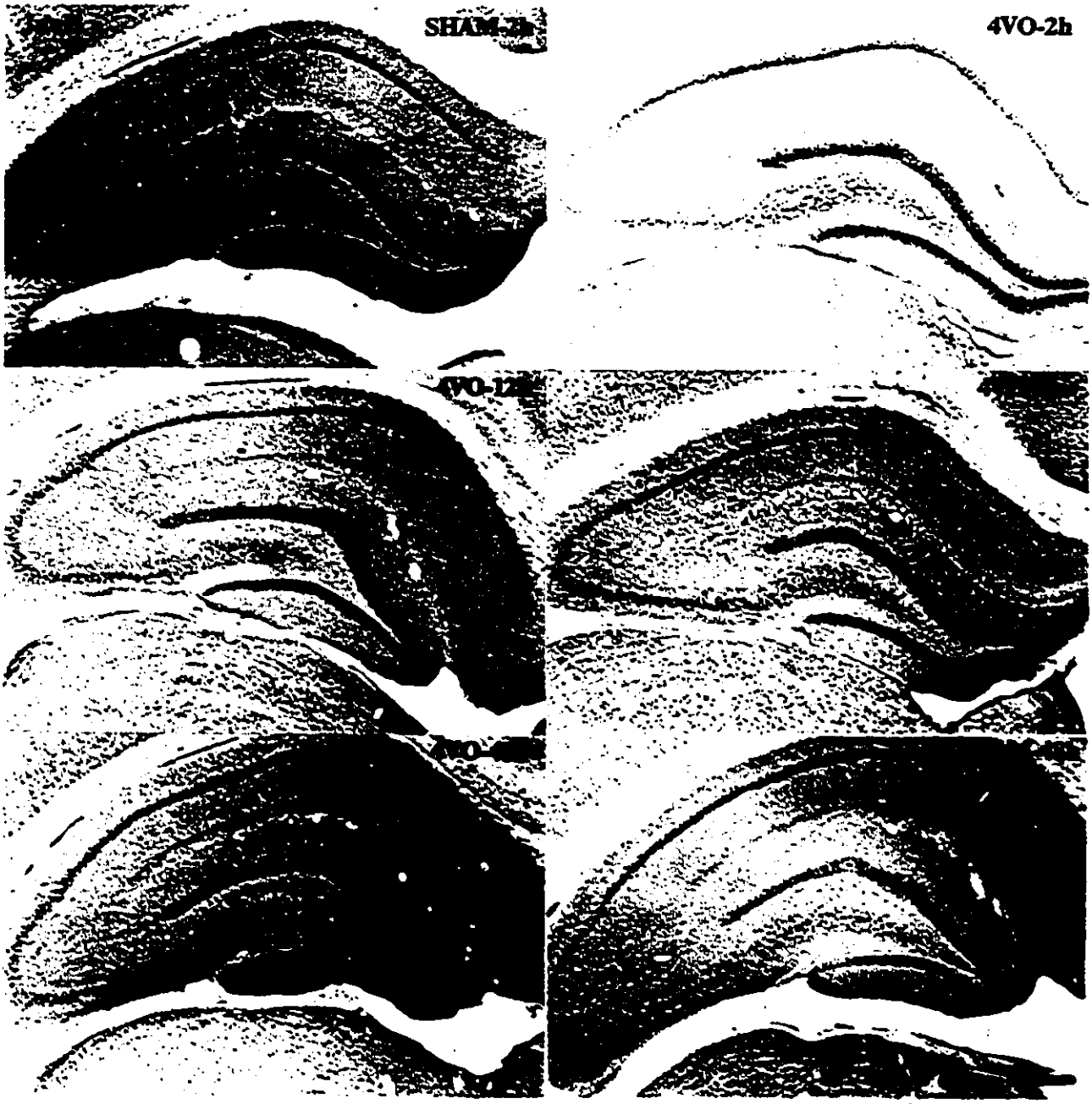

SHAM-2h
4VO-2h

Figure 12. Photomicrographs demonstrating the effect of 20 min of global ischemia on NGFI-A-like immunoreactivity in the dorsal hippocampus. Constitutive NGFI-A-like immunoreactivity was very high in the CA1 and CA3 hippocampal regions of 2 h and 48 h sham controls (SHAM-2h; SHAM-48h). At 2 h after recirculation, NGFI-A-like immunoreactivity was elevated in CA3 neurons and dentate granule cells (4VO-2h). NGFI-A-like immunoreactivity remained enhanced in the CA3 region and the dentate gyrus 12 h after reperfusion (4VO-12h). By 24 h of survival, NGFI-A-like immunoreactivity had declined to control levels in the CA3 region as well as the dentate gyrus (4VO-24h). At 48 h post-ischemia, NGFI-A-like immunoreactivity had declined below basal levels in both CA1 and CA3 pyramidal neurons (4VO-48h). Scale bar = 500 μm.

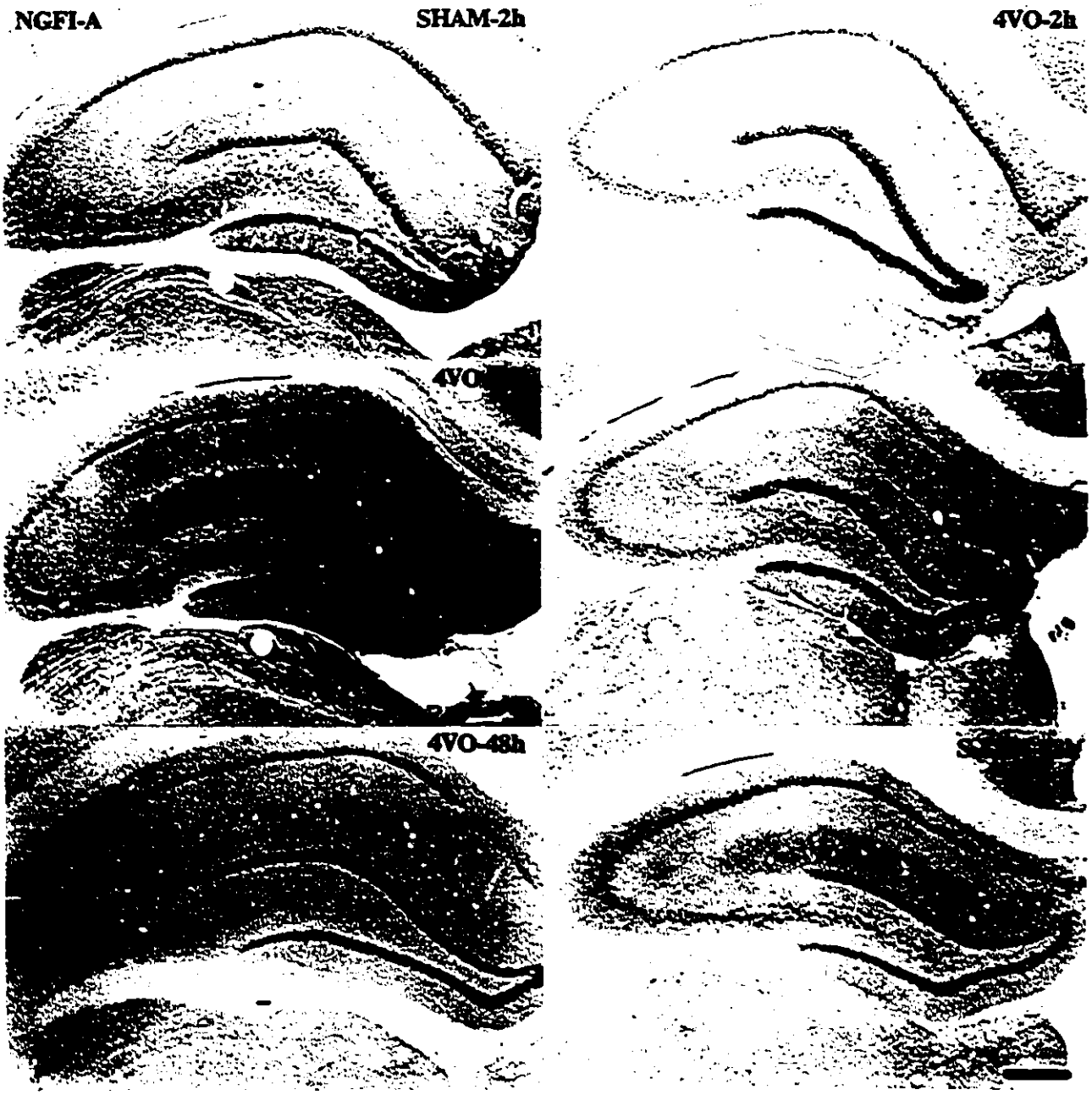

NGFI-A
SHAM-2h
4VO-2h
4VO
4VO-48h

Figure 13. Bar graphs depicting the average number of CA1 and CA3 neurons which exhibited $Fos_{2\text{-}16}$-, $FosB_{4\text{-}388}$-, FosB(N)- and FosB(C)-like immunoreactivity 2 h, 12 h, 24 h and 48 h after 20 min of global ischemia. Each bar represents the average ± SEM of 4 animals. SHAM-2h, animals sacrificed 2 h after sham manipulation; 4VO-2h, 4VO-12h, 4VO-24h, 4VO-48h, animals sacrificed 2 h, 12 h, 24 h, and 48 h, respectively, after 20 min of four-vessel occlusion; SHAM-48h, animals sacrificed 48 h after sham manipulation. Asterisk, significantly different from SHAM-2h, Newman-Keuls test ($P<0.01$).

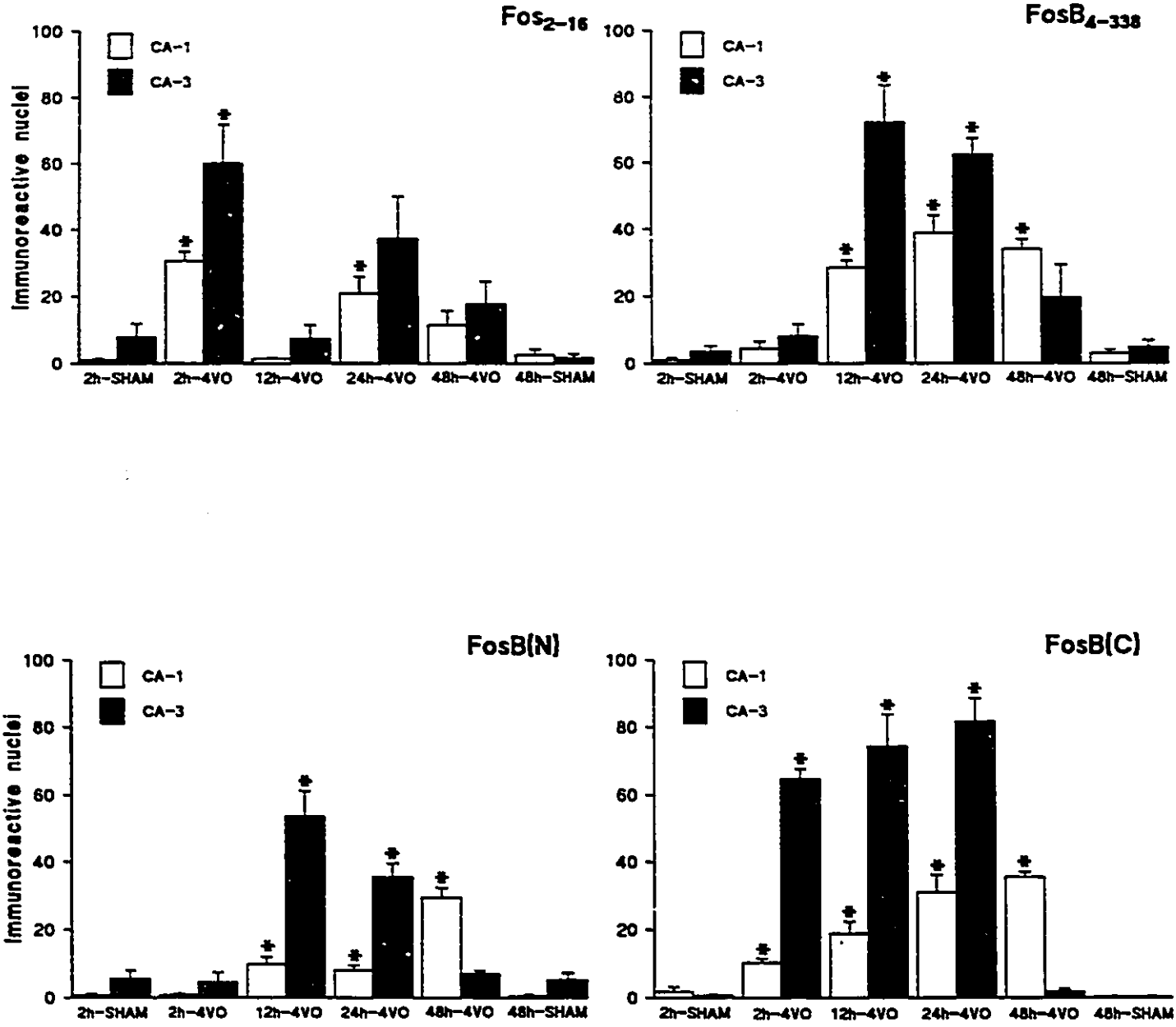

Fos2–16
FosB4–338
FosB(N)
FosB(C)
CA-1
CA-3
Immunoreactive nuclei
0
20
40
60
80
100
2h-SHAM
2h-4VO
12h-4VO
24h-4VO
48h-4VO
48h-SHAM

Figure 14. Bar graphs depicting the average number of CA1 and CA3 neurons which exhibited Jun-, JunB-, JunD- and NGFI-A-like immunoreactivity 2 h, 12 h, 24 h and 48 h after 20 min of global ischemia. Each bar represents the average ± SEM of 4 animals. SHAM-2h, animals sacrificed 2 h after sham manipulation; 4VO-2h, 4VO-12h, 4VO-24h, 4VO-48h, animals sacrificed 2 h, 12 h, 24 h, and 48 h, respectively, after 20 min of four-vessel occlusion; SHAM-48h, animals sacrificed 48 h after sham manipulation. Asterisk, significantly different from SHAM-2h, Newman-Keuls test ($P<0.01$).

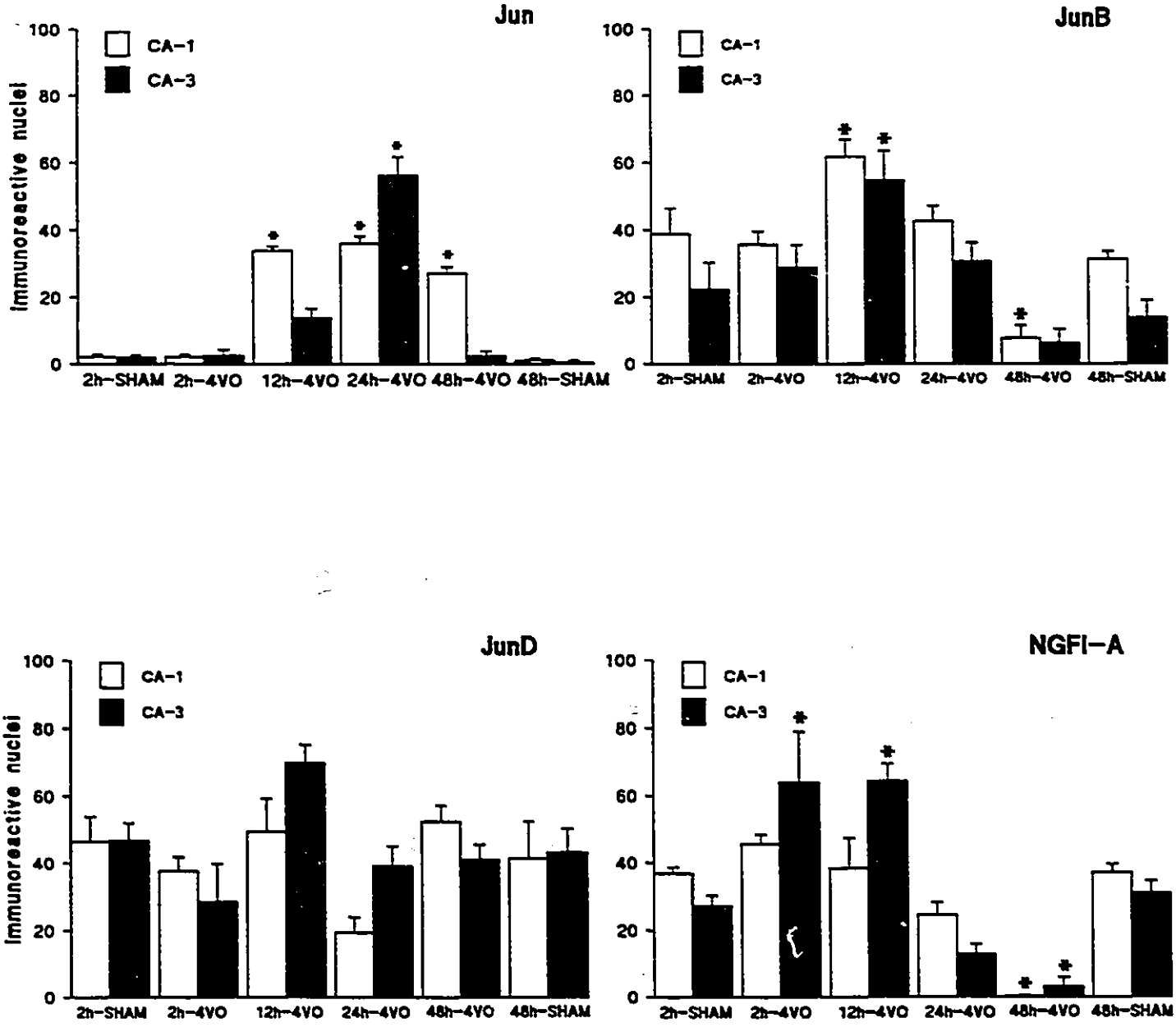

Jun
JunB
JunD
NGFI-A
CA-1
CA-3
Immunoreactive nuclei
0
20
40
60
80
100
2h-SHAM
2h-4VO
12h-4VO
24h-4VO
48h-4VO
48h-SHAM
*

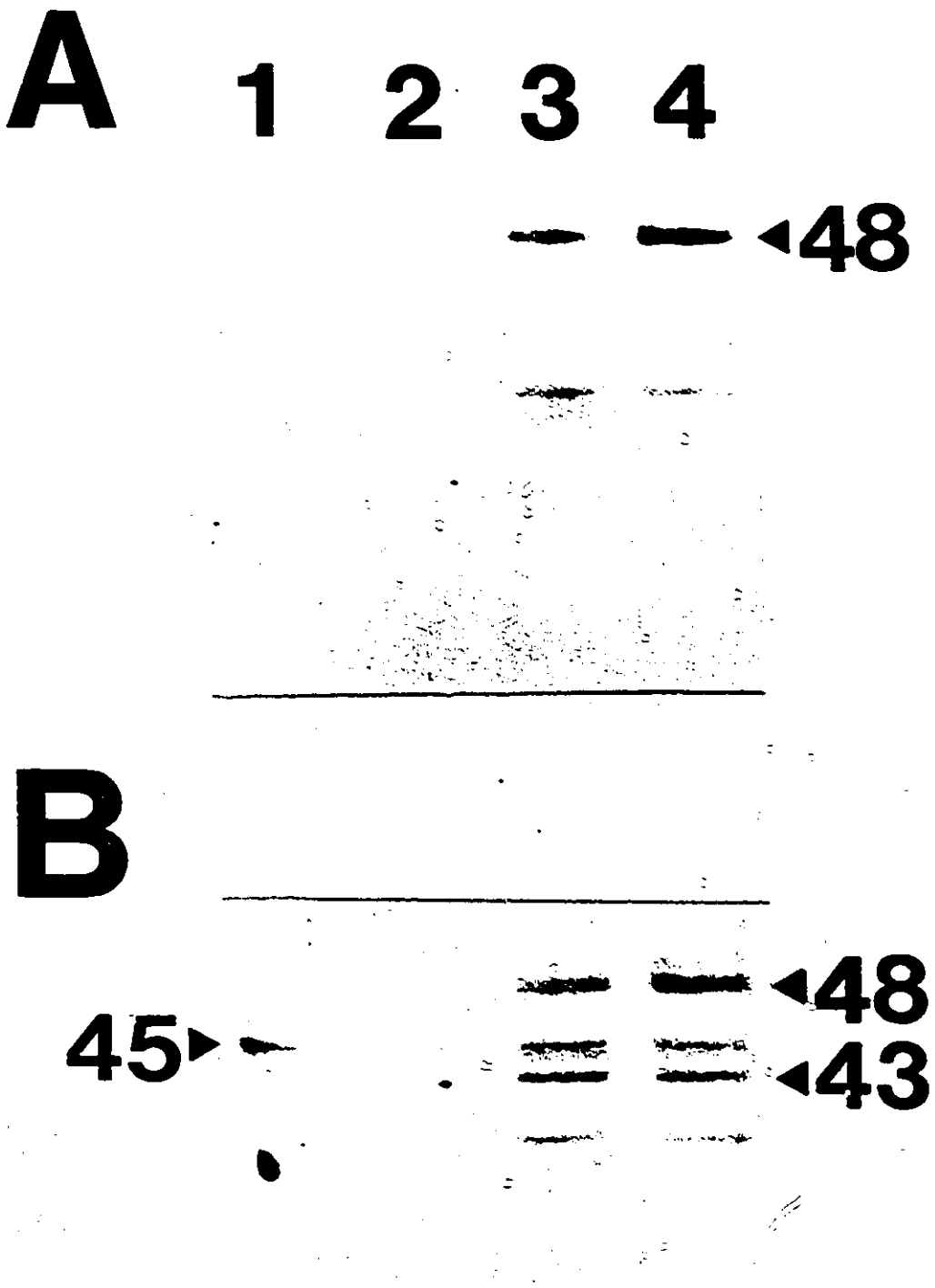
Figure 15. Analysis of alterations in IEG expression between regions as determined by performing ANOVA on normalized CA1/CA3 ratios across the sampled time points. Asterisk denotes differential IEG protein expression between regions, ANOVA ($P \leq 0.01$).

IEG PROTEIN	ANOVA OF NORMALIZED CA1/CA3 RATIOS OVER TIME (P≤0.01)			
	2 h 4-VO	12 h 4-VO	24 h 4-VO	48 h 4-VO
JUN	0.970	0.700	0.800	0.008*
FOSB(C)	0.360	0.405	0.459	0.001*
FOSB(N)	0.992	0.587	0.670	0.0001*
$FOSB_{4-338}$	0.357	0.957	0.773	0.005*
FOS_{2-16}	0.920	0.689	0.411	0.438
JUNB	0.141	0.074	0.253	0.070
JUND	0.075	0.296	0.112	0.424
NGFI-A	0.475	0.188	0.108	0.201

2. Western Analysis of FosB-like Proteins in Hippocampal CA1 Neurons

The FosB(C) and FosB(N) antibodies were used to assess the effect of ischemia on the expression of FosB-like proteins in CA1 neurons of 48 h ischemic and 48 h sham control animals. The FosB(C) antibody selectively recognizes the long form of FosB since it is targeted against the C-terminus of FosB which is absent from ΔFosB. Western blots performed using the FosB(C) antibody on nuclear proteins extracted from CA1 neurons 48 h after ischemia, revealed a dramatic elevation of the single (48 kDa) immunoreactive band corresponding to FosB (Fig. 16A; lanes 3 and 4). In order to determine whether ΔFosB expression was also elevated by cerebral ischemia, Western blots were performed using the FosB(N) antibody which is raised against the N-terminus common to both FosB and ΔFosB. Western blots performed using the FosB(N) antibody indicated that both FosB (48 kDa) and ΔFosB-like protein(s) (43 and 45 kDa doublet) were elevated in CA1 neurons 48 h following transient global ischemia (Fig. 16B; lanes 3 and 4). Contrary to that found in ischemic animals, Western blots performed on nuclear proteins extracted from CA1 neurons 48 h after sham manipulation, revealed barely detectable amounts of FosB (48 kDa band) and ΔFosB-like protein(s) (43 and 45 kDa doublet) in sham controls (Fig. 16A,B; lanes 1 and 2).

Figure 16. Western analysis of FosB and ΔFosB immunoreactive proteins in hippocampal CA1 neurons. A) FosB immunoreactive proteins in CA1 nuclear extracts from animals 48 h after sham (lanes 1 and 2) or 20 min of four-vessel occlusion (lanes 3 and 4). Each lane corresponds to a separate animal. Immunoreactivity was detected using FosB(C), an affinity purified polyclonal antibody that selectively recognizes FosB (48 kDa). B) FosB and ΔFosB immunoreactive proteins in nuclear extracts from animals 48 h after sham (lanes 1 and 2) or 20 min of four-vessel occlusion (lanes 3 and 4). Each lane corresponds to a single animal. Immunoreactivity was detected using FosB(N), an affinity purified polyclonal antibody that recognizes FosB (48 kDa) and ΔFosB (43-45 kDa doublet). There was a dramatic elevation in both FosB and ΔFosB immunoreactive proteins in CA1 neurons 48 h after transient global ischemia.


A
1 2 3 4
48
B
48
45
43

DISCUSSION

Immunohistochemical and Western Analysis of Immediate Early Gene Proteins in the Hippocampus Following Transient Global Ischemia

The results of the present study demonstrate that 20 min of transient global ischemia produced by 4-VO elicits different patterns of immediate-early gene expression in vulnerable CA1 and more resistant CA3 neurons. Cell counts of immunopositive neurons revealed that initially, ischemia evoked increased immunoreactivity in both CA1 and CA3 hippocampal subfields for all IEGs examined, with the exception of JunD and NGFI-A. However, distinct patterns of IEG expression became apparent in these regions at later time points following recirculation of blood flow.

1. Hippocampal IEG-immunoreactivity in Sham-Manipulated Control Animals

The hippocampal IEG-immunoreactivity detected in sham-operated control animals resembles previously reported immunohistochemical analysis of constitutive Jun, JunB, JunD, Fos, FosB, and NGFI-A expression patterns investigated in untreated rat brain (Hughes et al., 1992; Herdegen et al., 1995). Basal expression of JunB-, JunD- and NGFI-A-like immunoreactivity in the hippocampus of sham animals supports the hypothesis that constitutively expressed IEGs may be involved in maintaining the expression of so called "housekeeping genes" tonically stimulated by excitatory transmitters such as glutamate (Hughes et al., 1992; Herdegen et al., 1995). Basal NGFI-A-like expression is prominent in the dorsal horn of the spinal cord, colliculi, lateral geniculate nucleus, striatum, and limbic associated structures such as the cortex,

amygdala, and hippocampal CA1 neurons (Herdegen et al., 1995). Basal NGFI-A-like expression is thought to be a consequence of normal excitatory synaptic activity mediated by NMDA receptor activation. Consistent with this proposal, blockade of the NMDA receptor channel with MK-801 abolishes basal NGFI-A-like expression within hours of administration (Worely et al., 1991; Gass et al., 1993).

2. Differential Induction of FosB- and Jun-like Immunoreactivity in CA1 and CA3 Pyramidal Neurons Following Transient Global Ischemia

The pivotal difference between IEG expression in CA1 and CA3 regions was the persistence of ischemia-induced elevations of FosB and Jun expression in CA1 neurons. In both CA1 and CA3 neurons, increased numbers of FosB- and Jun-like immunoreactive neurons were initially detected 2 h and 12 h, respectively, after reperfusion. However, by 24 h - 48 h following recirculation, IEG-immunoreactivity had declined to basal levels in CA3 neurons, whereas CA1 neurons continued to display increased FosB- and Jun-like immunoreactivity 48 h after the ischemic insult.

i) Effects of Cerebral Ischemia on Hippocampal Jun Expression

Previous reports regarding the effect of ischemia on *c-jun* expression at different time points after reperfusion have yielded inconsistent findings. *In situ* hybridization histochemistry studies by Neumann-Haefelin et al. (1994) demonstrated a sustained induction of *c-jun* mRNA throughout the hippocampus 3 h - 48 h following 30 min of forebrain ischemia in the rat. In

contrast, Wessel et al. (1991) reported a bitemporal pattern of *c-jun* mRNA induction in CA1 neurons after 20 min of global ischemia in the rat; with the first peak developing 1 h - 2 h post-ischemia, and the second peak arising 24 h - 48 h following recirculation. Taken together, these *in situ* hybridization histochemistry studies indicate that global ischemia induces *c-jun* at the transcriptional level. Indeed, we have found that transient forebrain ischemia induces a long lasting elevation of Jun-like immunoreactivity in vulnerable CA1 neurons, indicating that ischemia-induced elevations of *c-jun* mRNA are translated into protein. This is an important finding because transient global ischemia has been shown to impair protein synthesis in CA1 neurons (Cooper et al., 1977; Dienel et al., 1985; Thilman et al., 1986; Maruno et al., 1990; Widmann et al., 1990). In contrast, Kiessling et al. (1993) have reported that transient forebrain ischemia tends to elevate Jun-like immunoreactivity in the CA1 neurons of gerbils. This discrepancy may be attributed to interspecies differences as well as variations in the duration of the ischemic insult. Unlike rats, gerbils possess a functionally incomplete circle of Willis, and are therefore more sensitive to ischemia since communicating arteries may not function as anastomoses when a major vessel becomes occluded (Pulsinelli et al., 1979). Furthermore, gerbils are genetically predisposed to convulsions induced by mild stimuli. Generalized seizures elevate cerebral energy metabolism and may thereby further injure neurons during ischemic and post-ischemic periods (Howse et al., 1974). Seizures are a rare complication in human stroke, hence it is important to omit animals which have experienced seizures from pathophysiological studies (Louis and McDowell, 1967).

Previous studies suggest that Jun may play an integral role in the signal transduction events that mediate delayed neuronal death following growth factor withdrawal, status epilepticus, systemic kainic acid treatment, and hypoxic-ischemic brain injury (Colotta et al., 1992; Dragunow et al., 1993, 1994; Estus et al., 1994; Pennypacker et al., 1994). Over-expression of the full-length c-Jun protein has been shown sufficient to induce apoptosis in sympathetic neurons. Furthermore, sympathetic neurons are protected against NGF withdrawal-induced death when microinjected with an expression vector for a c-Jun dominant mutant (Ham et al., 1995). Since the delayed neuronal death observed in the CA1 pyramidal layer of the hippocampus following transient cerebral ischemia has been suggested to occur by an apoptotic mechanism (Héron et al., 1993; MacManus et al., 1993; Kihara et al., 1994; Sei et al., 1994; Nitatori et al., 1995), it is tempting to speculate that prolonged Jun expression may be a critical component of the gene signalling cascade which mediates the delayed death of CA1 neurons after forebrain ischemia.

ii) Ischemia-induced $FosB_{4-338}$-like Immunoreactivity in the Hippocampus

Analogous to Jun-like immunoreactivity, transient global ischemia evoked a persistent elevation of $FosB_{4-338}$-like immunoreactivity in the CA1 sector of the hippocampus. Significantly increased numbers of $FosB_{4-338}$-like immunoreactive neurons were detected in both CA1 and CA3 hippocampal subfields 12 h and 24 h post-ischemia. Enhanced $FosB_{4-338}$-like immunoreactivity was still apparent in CA1 neurons 48 h following recirculation, whereas $FosB_{4-338}$-like immunoreactivity had subsided to basal levels in CA3 neurons at this time.

These results which demonstrate that ischemia elevates $FosB_{4-338}$-like immunoreactivity in the hippocampus are also inconsistent with previous studies by Kiessling et al. (1993) who reported that $FosB_{4-338}$-like immunoreactivity in CA1 neurons remained indistinguishable from control levels at all post-ischemic time points. In contrast, our data illustrated a persistent elevation of $FosB_{4-338}$-like immunoreactivity in CA1 neurons 12 h - 48 h after reperfusion. This inconsistency may be attributed to interspecies differences as well as variations in the duration of the ischemic insult.

In addition to Jun, FosB has been implicated in gene signalling pathways that mediate neuronal apoptosis following hypoxic-ischemic injury and NGF deprivation (Dragunow et al., 1994; Estus et al., 1994). Hence, the long lasting display of FosB-like immunoreactivity by CA1 neurons suggests that FosB may play an critical role in the signal transduction events which mediate the apoptotic death of these neurons following global ischemia.

IEG expression is not only triggered by the induction of cell proliferation, protein members of the *c-fos* and *c-jun* families are also required for cell cycle progression (Kovary and Bravo, 1991a). Microinjection of anti-Fos and anti-Jun family antibodies have been shown sufficient to arrest serum-stimulated or asynchronously growing cells at the entrance to the S phase of the cell cycle (Kovary and Bravo, 1991b). Prolonged FosB and Jun expression by CA1 neurons may reflect an inappropriate attempt of post-mitotic neurons to re-enter the cell cycle and replace irreversibly injured neurons. This hypothesis is further supported by more recent immunohistochemical data demonstrating dramatic and prolonged increases in hippocampal p53- and Myc-like immunoreactivity in CA1 neurons after 4-VO (McGahan et al., 1995). The proto-

oncogene *c-myc*, and the tumor suppressor gene *p53*, encode proteins that function as transcriptional regulating factors which govern cell proliferation, differentiation, and apoptosis (Evan et al., 1992; Vogelstein and Kinzler, 1992; Levine, 1993; Prives, 1993; Freeman et al., 1994; Hermeking and Eick, 1994; Levine et al., 1994).

Protein products of the *c-fos* and *c-jun* families of IEGs are inducible transcriptional factors (ITFs) which couple extracellular signals to alterations in phenotype by regulating downstream target genes (Morgan and Curran, 1989). Fos and Jun family proteins share a common structural motif referred to as a leucine zipper which permits protein dimerization (Gentz et al., 1989). The inducible nuclear transcription factor complex, AP-1, consisting of heterodimers between Fos and Jun family proteins, regulates the expression of specific target genes containing the consensus sequence TGACTCA (Franz et al., 1988; Nakabeppu et al., 1988). Jun family proteins (Jun, JunB, and JunD) may form dimers and bind with varying affinities to AP-1 sites (Nakabeppu et al., 1988). However, the DNA binding and transactivating properties of Jun are enhanced significantly by heterodimerization with Fos family proteins (Fos, FosB, Fra-1, and Fra-2), particularly FosB (Zerial et al., 1989). This suggests that persistent FosB and Jun expression following global ischemia may promote the delayed death of CA1 neurons by activating AP-1 regulated genes that mediate apoptosis.

iii) Induction of FosB(C)- and FosB(N)-like Immunoreactivity in the Hippocampus Following Cerebral Ischemia

Two different forms of *fos*B mRNA are generated by alternative splicing of the transcript from a single gene (Mumberg et al., 1991; Nakabeppu and Nathans, 1991; Yen et al., 1991). The longer transcript (*fos*B) encodes a protein of 338 amino acids in length known as FosB. The shorter transcript (Δ*fos*B) codes for a 237-amino acid long protein termed ΔFosB. Δ*fos*B mRNA results from deletion of 140 bases from the *fos*B transcript. This deletion shifts the reading frame by a single base creating the in-frame stop codon TGA. This alternative splicing event results in a truncated protein ΔFosB, which retains the bipartite DNA-binding domain common to Fos family members yet lacks 101 C-terminal amino acids found in FosB. Consequently, ΔFosB is capable of forming heterodimers with members of the Jun family, and subsequently binding to AP-1 sites of target genes. As shown previously (Metz et al., 1994), the transactivating properties of FosB have been attributed to two independent domains located in the C-terminal 95 amino acids. The first region, termed the TATA box binding protein motif (TBM), is specified by the last 55 C-terminal amino acids of FosB. The TBM supplements transcriptional activation by interacting directly with the TATA box binding protein (TBP). The second region is a proline-rich sequence (amino acids 243 - 283) which constitutes the major activating region of FosB. Hence, since ΔFosB is deficient in 101 C-terminal amino acids, it lacks the potent trans-activating properties of FosB (Mumberg et al., 1991; Metz et al., 1994).

In the current study, FosB was selectively detected using an antibody [FosB(C)] raised against amino acids 245 - 315 of the C-terminus of FosB which is absent from ΔFosB

(Nakabeppu and Nathans, 1991). FosB and ΔFosB were recognized by a second antibody, FosB(N), targeted at amino acids 79 - 131 of the N-terminus of FosB which is common to both proteins (Nakabeppu and Nathans, 1991). Immunohistochemical analysis using the FosB(N) and FosB(C) antibodies yielded similar results; in CA1 neurons, both FosB(N)- and FosB(C)-like immunoreactivity were elevated 12 h - 48 h following transient global ischemia. Similarly, both FosB(N)- and FosB(C)-like immunoreactivity were enhanced in CA3 neurons 12 h - 24 h after recirculation. However, the number of FosB(C)- and FosB(N)-like immunoreactive CA3 neurons subsided to basal levels by 48 h of survival. Western blotting indicated that increases in both ΔFosB- and FosB-like proteins occurred after forebrain ischemia. Western analysis using the FosB(C) antibody demonstrated a striking induction of the 48 kDa band pertaining to the long form of FosB in CA1 neurons of 48 h ischemic animals. Western blots performed with the FosB(N) antibody revealed that in addition to FosB, levels of ΔFosB-like proteins (43 - 45 kDa doublet) were also enhanced in CA1 neurons 48 h post-ischemia. Like other members of the Fos family, ΔFosB contains a leucine zipper motif which enables it to heterodimerize with members of the Jun family (Mumberg et al., 1991; Nakabeppu and Nathans, 1991; Yen et al., 1991). However, since ΔFosB lacks a proline rich activator region, it possesses much weaker trans-activating properties than FosB (Metz et al., 1994). Consequently, enhanced ΔFosB production reduces the transcriptional activity of FosB by competing for available Jun proteins (Nakabeppu and Nathans, 1991). The induction of ΔFosB-like proteins in CA1 neurons following forebrain ischemia may reflect an attempt to antagonize the transcriptional activity of FosB.

3. Differential Induction of Fos_{2-16}-like Immunoreactivity in CA1 and CA3 Neurons in Response to Forebrain Ischemia

Continuous *c-fos* expression has been demonstrated to precede numerous types of naturally occurring and excitotoxic cell death (Gunn et al., 1990; Popovici et al., 1990; González-Martín et al., 1992; Smeyne et al., 1993). The destruction of CA1 neurons after systemic administration of the excitotoxin kainic acid is preceded by two peaks of c-fos induction (Schreiber et al., 1993; Smeyne et al., 1993). In the case of kainic acid-induced excitotoxicity, the second wave of *c-fos* induction occurs immediately before and during the death of CA1 neurons, suggesting that it may play a role in the processes leading to their apoptotic demise.

Several previous studies examining the transcription and translation of *c-fos* following global ischemia in the rat have proven contradictory. Jørgensen et al. (1989), failed to detect any early transcription of *c-fos*, but demonstrated a delayed induction of *c-fos* mRNA and protein in CA1 neurons (Jørgensen et al., 1991). Other studies (Wessel et al., 1991; Neumann-Haefelin, 1994) report a rapid, transient *c-fos* induction in widespread regions of the brain followed by delayed, selective *c-fos* induction in vulnerable CA1 neurons of the hippocampus. The present immunohistochemical analysis confirms that biphasic ischemia-induced increases in *c-fos* mRNA are translated into protein despite compromised energy conditions unconducive to protein synthesis (Cooper et al., 1977; Dienel, 1985; Thilmann et al., 1986; Maruno et al., 1990; Widmann et al., 1990). Examination of Fos_{2-16}-like immunoreactivity following forebrain ischemia revealed a biphasic induction in vulnerable CA1 neurons. The first wave of enhanced Fos_{2-16}-like immunoreactivity was detected in CA1 and CA3 neurons at 2 h of survival. At 12

h following recirculation, Fos_{2-16}-like immunoreactivity had declined to control levels in both CA1 and CA3 hippocampal regions. A second peak of Fos expression occurred exclusively in CA1 neurons 24 h post-ischemia, however, immunoreactivity had dissipated to basal levels by 48 h. Consequently, there was not a strong temporal correlation between the expression of Fos and the loss of CA1 neurons which occurs 48 h - 72 h following transient global ischemia.

The early post-ischemic induction of *c-fos* closely resembles that observed following seizures or hypoxic-ischemic injury (Popovici et al., 1990; Gass et al., 1993; Dragunow et al., 1994). This similarity may be attributed to the fact that both ischemia and seizures evoke membrane depolarization which triggers Ca^{2+} influx through L-type VSCCs in the plasma membrane (Morgan and Curran, 1986; Siesjo, 1988; Murphy et al., 1991; Morley et al., 1994). During ischemia, lack of O_2 diminishes cellular ATP levels. Energy depletion leads to the loss of Na^+ gradients normally maintained by the ATP-dependent membrane Na^+-K^+ pump. Increasing intracellular Na^+ results in reversal of the Na^+-Ca^{2+} exchanger which normally functions to extrude Ca^{2+} from the cell (Seisjö et al, 1989; Lipton and Lobner, 1990; Khodorov et al., 1993). The subsequent membrane depolarization also causes these neurons to release large amounts of the excitatory neurotransmitter glutamate (Griffiths et al., 1982; Benveniste et al., 1988). Normally excess glutamate is returned to presynaptic neurons or glial cells (Schwarcz et al., 1982). However, during ischemia, glutamate reuptake is impaired due to lack of energy, resulting in excessive activation of post-synaptic glutamate receptors. Glutamate triggers further Ca^{2+} infiltration though several subtypes of ligand-gated cation channels as well as mobilization from intracellular stores (Betz, 1990; Murphy and Miller, 1988). Elevations in cAMP or $[Ca^{2+}]_i$ result

in phosphorylation of CREB. Phosphorylated CREB interacts with the transcriptional machinery to promote transcription of the *c-fos* gene (Sheng et al., 1990; Walker and Carlock, 1993). Since cerebral ischemia has been associated with increased cystolic free calcium, elevations in $[Ca^{2+}]_i$ during ischemia are likely to be responsible for the induction of *c-fos* observed following transient global ischemia (Uematsu et al., 1988). Sustained elevations of $[Ca^{2+}]_i$ also contribute to cell death by generating free radicals in addition to activating various Ca^{2+}-dependent proteases, phospholipases and endonucleases (Siesjo et al., 1989; Siesjo, 1992).

4. Cerebral Ischemia-induced Alterations of Hippocampal JunB-like Immunoreactivity

Cerebral ischemia evoked only modest elevations of hippocampal JunB-like immunoreactivity, in comparison to the more robust increases observed for Jun-like immunoreactivity. Evaluation of JunB-like immunoreactivity following forebrain ischemia revealed moderate increases of JunB-like immunoreactivity in CA1 and CA3 neurons 12 h after recirculation. These results confirm that previously described ischemia-induced increases in *jun*B mRNA observed during the early reperfusion period are translated into protein despite compromised energy conditions associated with ischemia (Neumann-Haefelin et al., 1994). At 24 h of survival, JunB-like immunoreactivity has subsided to basal levels in both CA1 and CA3 hippocampal regions. However, at 48 h following reperfusion, the number of JunB-like immunoreactive CA1 neurons was significantly less than that observed in sham controls.

JunB expression tends to be influenced to a greater extent by pharmacological and physiological treatments which modify synaptic neurotransmission without actually inflicting

neural damage (Herdegen et al., 1991; Carter, 1992; Vaccarino et al., 1992). Transient co-transfection studies performed using non-neuronal cell lines demonstrate that JunB is a negative regulator of Jun (Chiu et al., 1989; Schutte et al., 1989). Hence, diminished JunB levels may enhance the trans-activating properties of Jun. It is therefore important to note that JunB-like immunoreactivity was significantly suppressed in CA1, but not CA3 neurons 48 h post-ischemia. Depressed JunB levels in the CA1 region may have contributed to the DND of CA1 neurons following ischemia by enhancing the ability of FosB/Jun dimers to activate AP-1 regulated genes that mediate apoptosis.

5. The Effect of Transient Forebrain Ischemia on JunD Expression in the Hippocampus

Consistent with previous *in situ* hybridization histochemistry and immunohistochemistry studies depicting high basal JunD expression in the hippocampus, the current analysis detected a significant number of JunD-like immunoreactive neurons in CA1 and CA3 hippocampal regions of sham animals (Wisden et al., 1990; Schlingensiepen et al., 1991; Herdegen et al., 1995). High levels of *jun*D mRNA are also found in quiescent fibroblasts, hence due to its ubiquitous presence, *jun*D is presumed to have transcriptional influence in controlling basic cell metabolism (Bravo, 1990). Cerebral ischemia did not significantly alter JunD-like immunoreactivity in either the CA1 or CA3 region of the hippocampus at any time point examined.

6. Alterations in Hippocampal NGFI-A Expression in Response to a Transient Ischemic Insult

Basal NGFI-A-like immunoreactivity was high in both CA1 and CA3 hippocampal sectors of 2 h and 48 h sham control animals. This is consistent with previous *in situ* hybridization histochemistry and immunohistochemistry studies which depict abundant constitutive NGFI-A expression in the hippocampus (Hughes et al., 1992; Herdegen et al., 1995). High constitutive NGFI-A expression in rat brain is thought to be mediated by natural synaptic activity that is driven by NMDA receptor activation (Wisden et al., 1990; Worley et al., 1991). There was a significant increase in the number of CA3 neurons which displayed NGFI-A-like immunoreactivity 2 h and 12 h post-ischemia that subsided to control levels 24 h after reperfusion. This observation is in agreement with previous immunohistochemical detection demonstrating elevated NGFI-A-like immunoreactivity in CA3 neurons following hypoxic-ischemic injury (Dragunow et al., 1994). The present immunohistochemical analysis also demonstrated that constitutive NGFI-A-like immunoreactivity had dissipated entirely from CA1 neurons by 48 h of survival. Complete loss of constitutive NGFI-A expression from CA1 neurons most likely reflects a suppression of protein synthesis typically associated with ischemia. Persistent inhibition of protein synthesis has been demonstrated to precede the delayed neuronal death of CA1 neurons following cerebral ischemia in the gerbil (Thilmann et al., 1986). Consequently, prolonged suppression of NGFI-A and JunB synthesis in CA1 neurons may contribute to the apoptotic death observed in this region following ischemic insult. Similarly, the number of CA3 neurons which expressed NGFI-A-like immunoreactivity had diminished below

control levels at 48 h. Depressed NGFI-A-like immunoreactivity in CA3 neurons indicates that despite their ability to survive forebrain ischemia, the gene signalling capacity of these neurons is at least temporally perturbed by ischemic insult. Since NGFI-A expression has been correlated with neuronal activity, reduced NGFI-A-like immunoreactivity in CA3 neurons likely reflects a reduction in their synaptic activity (Worely et al., 1991).

In summary, the results of the present immunohistochemical and Western analysis indicate that IEG proteins were differentially expressed in both susceptible CA1 and more resilient CA3 neurons in response to transient forebrain ischemia produced by 4-VO.

CONCLUSIONS

This work has been devoted to further investigating changes which may contribute to the selective neuronal death observed in the dorsal hippocampus following transient global ischemia. In the rat hippocampus, 20 min of forebrain ischemia accomplished by 4-VO is accompanied by the delayed death of CA1 pyramidal neurons 48 h - 72 h after the ischemic insult. In contrast, CA3 neurons are more resilient to ischemic injury (Pulsinelli et al., 1982; Kirino et al., 1984). DND of CA1 neurons has also been demonstrated to occur in human brain following cardiac arrest (Petito et al., 1987). This study attempted to explore why CA1 neurons are more susceptible to ischemic injury than CA3 neurons.

Ischemic injury causes a multitude of biochemical and molecular neuronal responses ranging from short-term modifications such as membrane depolarization to long-term alterations in gene expression. Incorporating these results with previously published literature, it is possible to hypothesize that a cascade of events may occur, during and following cerebral ischemia, which may lead to altered functional integrity and the eventual apoptotic demise of CA1 neurons (Figure 17).

1. Biochemical Prelude to IEG Induction

At the cellular level, lack of O_2 during ischemia results in lowered cellular ATP levels. Energy depletion culminates in the loss of sodium gradients which are normally maintained by the ATP-dependent membrane Na^+-K^+ pump. The resulting accumulation of Na^+ reverses the Na^+-Ca^{2+} exchanger which normally functions to extrude Ca^{2+} ions out of the neuron (Seisjö et

al., 1989; Khodorov et al., 1993). Subsequent membrane depolarization triggers Ca^{2+} influx through L-type VSCCs in the plasma membrane (Seisjö, 1992). These neurons release large amounts of the excitatory neurotransmitter glutamate in response to depolarization (Griffiths et al.,1982; Benveniste et al., 1988). Excess glutamate is normally actively returned to presynaptic terminals or surrounding glial cells in an energy dependent manner (Schwarcz et al., 1982). However, during ischemia glutamate re-uptake is impaired resulting in excessive glutamate receptor stimulation. Glutamate activates three types of ligand-gated cation channels causing further infiltration of Ca^{2+}. These subtypes of inotropic glutamate receptors are defined on the basis of the effects of the agonists *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate (KA). Glutamate may also activate a metabotropic receptor liked to PLC and G-proteins. This leads to the subsequent generation of IP_3) and DAG, culminating in the release of Ca^{2+} from intracellular stores and activation of PKC (Morley et al, 1994). Glutamate also induces endonucleases responsible for the laddering of DNA within nuclei (Kure et al., 1991). The discovery that laddered DNA fragmentation occurs in CA1 neurons 48 h after transient global ischemia suggests that these neurons die by an apoptotic mechanism (MacManus et al., 1993). Accumulation of intracellular Ca^{2+} may activate Ca^{2+}-dependent enzymes which promote cell death as well as the formation of a Ca^{2+}/CM complex and consequently activate Ca^{2+}/CM-dep. PK. This enzyme can phosphorylate the transcription factor cAMP response element binding protein. Phosphorylation of CREB leads to the induction of IEGs which contain a particular CaRE (sequence: TGACGTCA) in their promoter region (Vendrell et al., 1993).

2. IEG Expression and Long-Term Modification of Gene Expression

IEGs have been proposed to play a "third messenger" role in the cascade of signalling events linking extracellular stimulation to long-term changes in gene expression (Morgan and Curran, 1989). The presence of laddered DNA in hippocampal CA1 neurons 48 h after forebrain ischemia suggests that these neurons die by an apoptotic mechanism (MacManus et al., 1993). Cerebral ischemia rapidly increases neuronal expression of IEGs which encode transcriptional regulating factors known to participate in gene signalling pathways that mediate apoptosis (Jørgensen et al., 1988; Wessel et al., 1991). Figure 18 illustrates the temporal trends in IEG-immunoreactivity observed in hippocampal CA1 neurons following global ischemia. Cell counts of immunopositive CA1 neurons revealed that immunoreactivity for all examined IEG proteins was initially elevated by forebrain ischemia, with the exception of JunD and NGFI-A. Enhanced $Fos_{2\text{-}16}$-like immunoreactivity was detected in CA1 neurons at 2 h of survival. At 12 h of survival, $Fos_{2\text{-}16}$-like immunoreactivity had subsided to basal levels in the CA1 region. At 24 h of recirculation, JunB-like immunoreactivity had subsided to basal levels in CA1 pyramidal neurons, while Jun- and FosB-like immunoreactivity remained elevated. A second peak of Fos expression occurred exclusively in vulnerable CA1 neurons 24 h after reperfusion. At 48 h post-ischemia, CA1 neurons continued to display elevated FosB- and Jun-like immunoreactivity. In contrast, $Fos_{2\text{-}16}$-like immunoreactivity had subsided to control levels while JunB- and NGFI-A-like expression had declined below basal levels by 48 h of survival. Both Fos and Jun have been

implicated in the signal transduction events which mediate the apoptotic death of sympathetic neurons following NGF withdrawal (Estus et al., 1994). Hence, persistent elevations of FosB and Jun expression in CA1 neurons may contribute to the neuronal apoptosis observed in this region after forebrain ischemia. Jun may heterodimerize with FosB by way of a leucine zipper, consisting of an α helix containing 4 leucines spaced at every seventh amino acid (Stuhl, 1989; McMahon and Monroe, 1992). FosB/Jun dimers may subsequently bind AP-1 sites of target genes involved in gene signalling pathways which mediate apoptosis. The induction of different combinations of IEGs may have diverse effects on gene expression. The leucine zipper motif permits versatility in the binding of *fos* (Fos, FosB, Fra-1, and Fra-2) and *jun* (Jun, JunB, and JunD) family proteins, allowing the formation of a wide variety of transcriptional regulating complexes (Herschman, 1989; Lamb and McKnight, 1991). Other IEGs encoding zinc finger proteins (NGFI-A, Krox20, NGFI-C and NGFI-B) allow even greater variation by binding to different DNA motifs. A variety of DNA-binding sites may coexist on a particular target gene to allow neuron-specific responses to extracellular stimuli. Hence, the induction of differential IEG combinations in vulnerable CA1 and more resilient CA3 neurons following forebrain ischemia may result in diverse effects on target genes that could determine neuronal fate. The close temporal correlation between these changes in IEG expression and the loss of CA1 neurons suggests that alterations in IEG expression may participate in the delayed death of CA1 neurons following transient global ischemia.

Transcription factors from both *jun* and *fos* families of IEGs are thought to be required for cell cycle progression (Kovary and Bravo, 1991a). The proto-oncogene *c-myc* and the tumor

suppressor gene *p53* also encode proteins which function as transcriptional regulating factors governing cell proliferation, differentiation, and apoptosis (Evan et al., 1992; Freeman et al., 1994; Hermeking and Eick, 1994; Levine et al., 1994). Forebrain ischemia elicited persistent increases in FosB and Jun expression in vulnerable CA1 neurons. Further immunohistochemical analysis revealed a dramatic increase in p53- and Myc-like immunoreactivity exclusively in 48 h occluded animals (McGahan et al., 1995). Taken together, these results may reflect an inappropriate attempt of post-mitotic neurons to re-enter the cell cycle and replace irreversibly injured CA1 neurons.

Figure 17. Schematic diagram illustrating stimulus-transcription coupling events which may occur in response to cerebral ischemia and account for the altered functional integrity and eventual apoptotic demise of CA1 neurons.

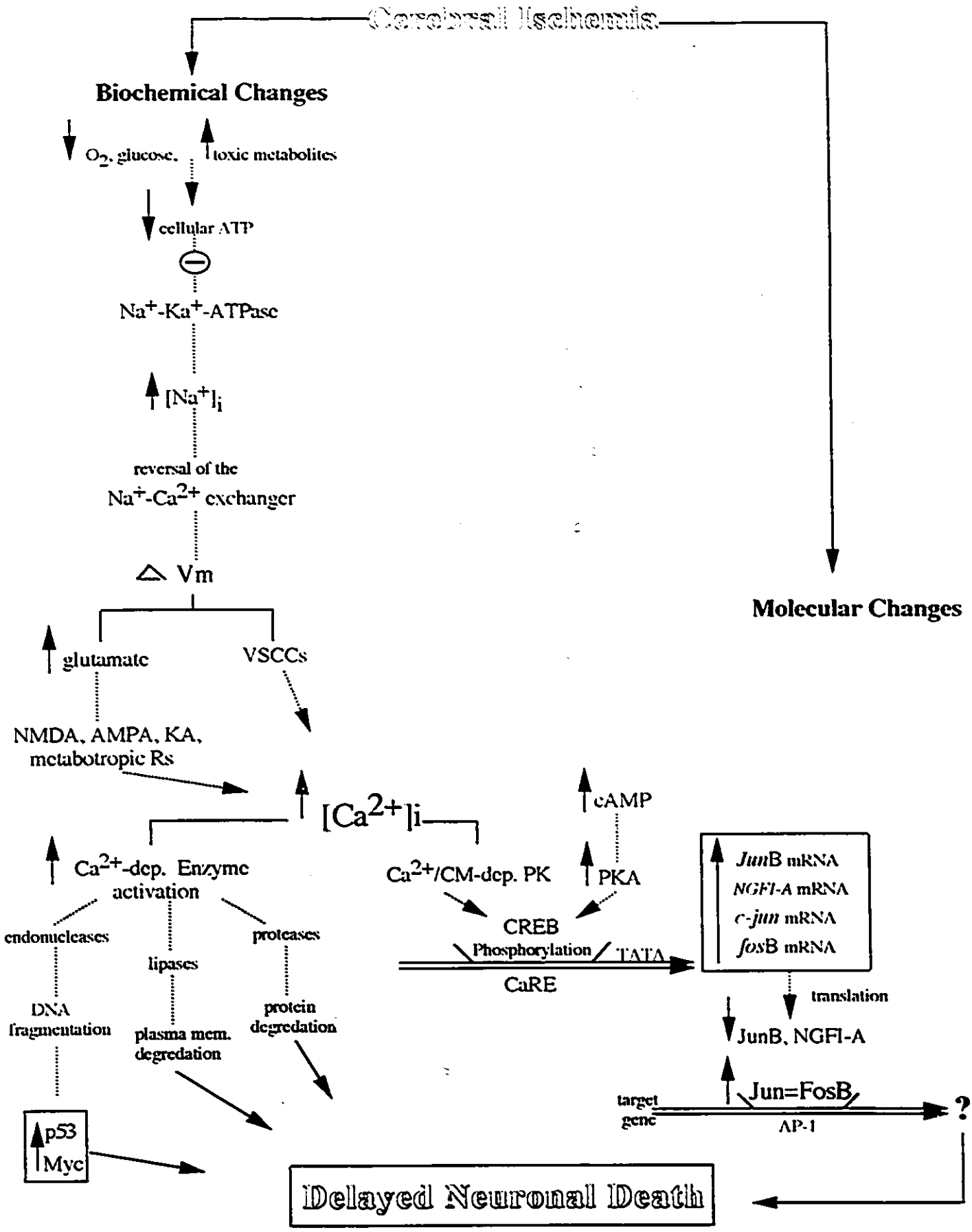
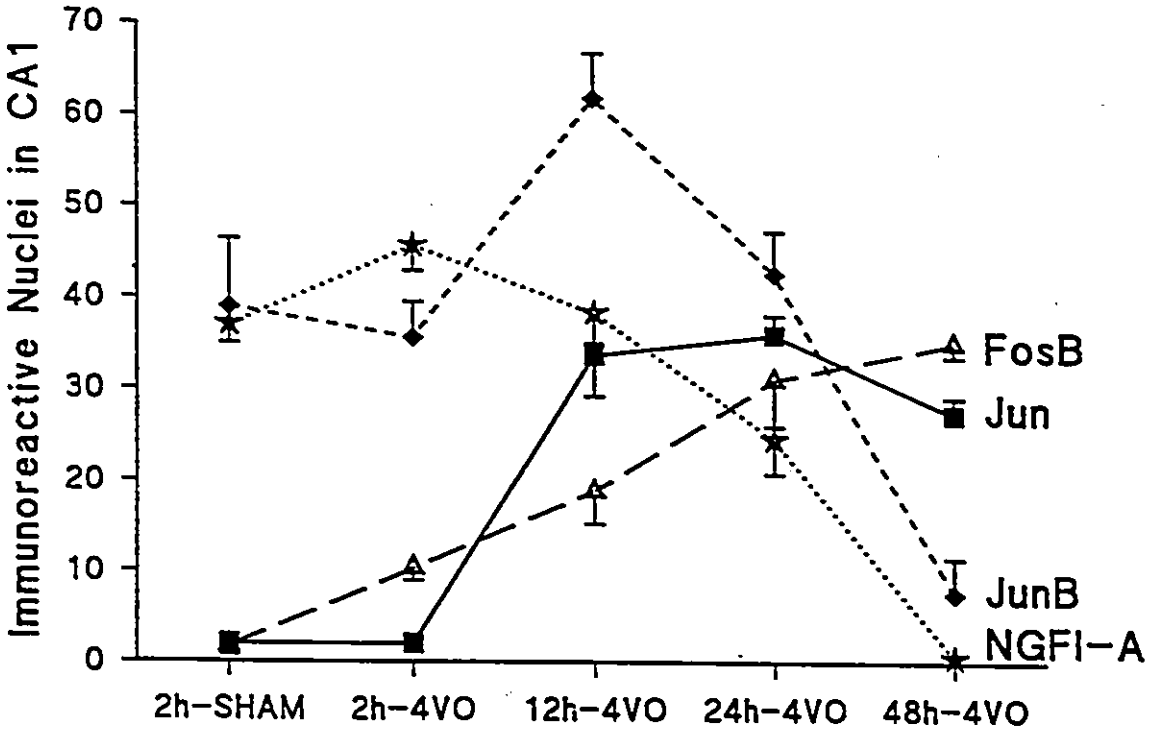

Cerebral Ischemia
Biochemical Changes
O_2, glucose,
toxic metabolites
cellular ATP
Na^+-Ka^+-ATPase
$[Na^+]_i$
reversal of the
Na^+-Ca^{2+} exchanger
Vm
Molecular Changes
glutamate
VSCCs
NMDA, AMPA, KA,
metabotropic Rs
$[Ca^{2+}]i$
cAMP
Ca^{2+}-dep. Enzyme
activation
Ca^{2+}/CM-dep. PK
PKA
endonucleases
lipases
proteases
CREB
Phosphorylation
TATA
CaRE
*Jun*B mRNA
NGFI-A mRNA
c-jun mRNA
*fos*B mRNA
translation
DNA
fragmentation
plasma mem.
degredation
protein
degredation
JunB, NGFI-A
target
gene
Jun=FosB
AP-1
?
p53
Myc
Delayed Neuronal Death

Figure 18. Line graph of quantitative analysis depicting temporal trends in Jun-, JunB-, FosB(C)-, NGFI-A-like immunoreactivity observed in hippocampal CA1 neurons 2 h, 12 h, 24 h, and 48 h following 20 min of global ischemia. Each data point represents the average ± SEM for 4 animals. 2h-SHAM, animals sacrificed 2 h after sham manipulation; 2h-4VO, 12h-4VO, 24h-4VO, 48h-4VO, animals sacrificed 2 h, 12 h, 24 h, and 48 h, respectively after 20 min of four-vessel occlusion. At 48 h following transient forebrain ischemia, both Jun- and FosB(N)-like immunoreactivity were elevated while both JunB- and NGFI-A-like immunoreactivity were depressed in CA1 pyramidal neurons.


Immunoreactive Nuclei in CA1
70
60
50
40
30
20
10
0
2h-SHAM
2h-4VO
12h-4VO
24h-4VO
48h-4VO
FosB
Jun
JunB
NGFI-A

FUTURE INVESTIGATION

The profile of IEG protein expression patterns presented in this thesis provide a fundamental time course of the alterations in IEG expression which accompany both susceptibility and resistance to ischemic cell death as observed in CA1 and CA3 neurons, respectively. Based on these findings, it may be possible to determine the precise role of these genes in conferring either susceptibility or resistance to cerebral ischemia by selectively inhibiting their expression. Previous studies indicate that both Jun and FosB play critical roles in gene signalling pathways that mediate neuronal apoptosis following NGF deprivation (Estus et al., 1994). Similarly, in the present study, incessant display of Jun- and FosB-like immunoreactivity by CA1 neurons suggests that these genes may play a crucial role in mediating the apoptotic death of CA1 neurons following transient global ischemia. To further test this hypothesis one could attempt to attenuate the loss of CA1 neurons by selectively blocking ischemia-induced Jun and FosB expression using antisense phosphorothioate oligodeoxynucleotides (ODN) to these IEGs either singularly or in combination.

Implementing this approach, a pilot study was conducted using antisense cDNA to block the expression FosB in CA1 neurons and hence possibly intercede the cascade of events leading to neuronal death (Cohen and Hogan, 1994; Wagner, 1994). However, the results of injecting antisense to FosB directly into the CA1 region of the hippocampus prior to carotid occlusion remain inconclusive. There was much difficulty in discerning whether antisense offered any protection from ischemic injury since conventional cannulation resulted in extensive damage to the CA1 region. The antisense approach may require a less intrusive method of oligonucleotide

administration in order to confer a neuroprotective effect. The most efficient and less injurious means of administration may be by using phospholipid vesicles termed liposomes to deliver oligoneucleotides (Akhtar and Juliano, 1992). The use of liposomes offers the potential to control and sustain the release of oligonucleotides as well as protect them from enzymatic degradation. In support of this proposal, the inhibition of NMDA-R1 synthesis by treatment with antisense ODNs has previously been demonstrated to selectively reduce the expression of NMDA receptors, thereby preventing neurotoxicity and reducing the infarct volume associated with focal cerebral ischemia (Wahlestedt et al., 1993). Similarly, Liu et al., 1994, have successfully suppressed Fos expression and AP-1 activity following focal ischemia by intraventricular infusion of an antisense ODN (anti-rncfosr$_{115}$) to *c-fos* mRNA. However, no histological assessment of infarct size was mentioned.

To better understand the underlying principles of IEG expression following global ischemia, it would be beneficial to examine ischemia-induced IEG expression under the effect of therapies which confer some neuronal protection. In such a way, it may be possible to identify IEGs involved in bestowing neuronal resistance to ischemic injury. The Na^+ channel blocker, tetrodotoxin (TTX), has been shown to be protective in organotypic hippocampal culture models of ischemia when administered immediately following metabolic inhibition (Vornov et al. 1994). A non-competitive NMDA receptor antagonist, dizocilpine (MK-801) has been demonstrated to attenuate *c-fos* expression associated with injury and global ischemia (Dragunow et al., 1990; Woodburn et al.,1993). However, the neuroprotective activity of MK-801 following transient global ischemia seems to be attributed largely to its hypothermic effects rather than a

direct action on NMDA receptor channels (Buchan and Pulsinelli, 1990; Buchan et al., 1991). Moreover, MK-801 has been shown to cause dose-dependent vacuolarization (Olney et al., 1989). AMPA receptor activation has been demonstrated to play an important role in mediating ischemic cell death. Li and Buchan (1993), have shown that the selective AMPA receptor antagonist NBQX is capable of reducing CA1 neuronal injury when administered as late as 12 h following forebrain ischemia. Combined administration of the L-type calcium channel blocker, nimodipine, and the noncompetitive NMDA receptor antagonist MK-801 have conferred some protection against neuronal death following forebrain ischemia (Rod et al., 1992). Future investigative studies are needed to assess whether IEG expression is affected by such pharmacological interventions.

Many recent studies have demonstrated that various growth factors are neuroprotective following hypoxic-ischemia, status epilepticus, axotomy, and neurotoxin administration (Otto and Unsicker, 1990; Hyman et al., 1991; Hagg and Varon, 1993; MacMillan et al., 1993; Schnell et al., 1994). Another question to be addressed is whether neuroprotective growth factors work by suppressing a constitutive apoptotic pathway or by one requiring *de novo* protein synthesis. Recent studies by Mattson et al. 1993a,b, showed that growth factors can alter NMDA receptor expression, interfere with free radical effects, and maintain calcium homeostasis in neurons.

Neurons subjected to mild stress, sufficient to perturb cellular metabolism, can acquire tolerance to subsequent more lethal ischemic stress *in vivo*. Both brief ischemic insult, and cortical spreading depression have been demonstrated to confer neuroprotection upon subsequent ischemic insult (Kitagawa et al., 1990; Glazier et al., 1994; Kobayashi et al., 1995). It would

be advantageous to determine whether IEG proteins are induced upon application of this initial stress factor, and whether they play a role in mediating protection from subsequent lethal ischemic episodes.

The profile of IEG protein expression patterns presented in this thesis provide a fundamental basis for determining the precise role of these genes in conferring susceptibility or resistance to cerebral ischemia. Once elucidated, further studies could be performed in attempt to downregulate genes which may be involved in mediating the apoptotic cascade by using antisense strategies or knockout (Chiasson et al., 1992; Gillardon, 1994). Similarly, genes which are thought to confer protection could possibly be upregulated. In support of this hypothesis, Linnik et al. 1995, have demonstrated that expression of Bcl-2 from a defective herpes simplex virus-1 vector limits neuronal death following focal cerebral ischemia. This notion is further supported by the fact that transgenic mice which constitutively over-express Bcl-2 had smaller infarct volumes following cerebral ischemia (Martinou et al., 1994). In conclusion, further investigation is required to elucidate the biochemical and molecular events leading to apoptosis. The redox state, dimerization partner, and phosphorylation state of ITFs probably determine and regulate their functions. The ability of a gene to mediate apoptosis likely depends upon environmental conditions such as the availability of kinases, peptides, and growth factors. Based on recent studies, the future holds much promise for the development of an effective treatment for stroke and other neurodegenerative diseases.

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