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The effects of multiple ischemia and survival times on hippocampal CA1 neuronal cell loss in a rat model of global ischemia: A long term ischemia maturation study.

by

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A thesis submitted to the

School of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Cellular and Molecular Medicine

University of Ottawa

Ottawa, Ontario

May, 1998

ABSTRACT

It has long been observed that rat CA1 hippocampal neurons die in a delayed fashion following ischemia. It was largely believed that delayed neuronal death was completed after 7 days. Unfortunately, most studies confined investigation to one ischemic severity and histological examination not later than 7 days. Therefore, little information was gathered regarding the effects of milder ischemia with longer survival times. Recent information suggests that the ischemic maturation process extends well beyond 7 days.

We investigated the effects of varying ischemia and survival times on CA1 neuronal loss. Histological analysis of the hippocampus was performed at 2, 7, 14, 28 and 90 days following 3, 5, 7, 10 and 13 minutes of global forebrain ischemia. Our results indicate that the ischemic maturation process extends beyond 7 days. Ten and thirteen minutes of ischemia produced a significant degree of cell loss by 7 days (70.53% and 83.25% respectively), while average cell death at 90 days survival was approximately 12% higher. Most strikingly, seven minutes of ischemia produced approximately 30% CA1 cell loss at 90 days compared to only 3% cell loss at 7 days, a nine-fold increase. No cell loss was observed at 2 and 7 days survival following 5 minutes of ischemia, but an average of 5.6% cell loss was observed at 3 months. Three minutes of ischemia produced no cell damage.

Data collapsed over ischemic serverity suggested that there may 2 rates of cell death evident in this study– 1) a rapid cell loss occurring within the first 7 days of ischemia and 2) a slow progressive cell loss occurring over weeks. Ten and thirteen minutes of ischemia possessed the rapid cell death , while 7 minutes appeared to display only slow progressive cell loss. The fact that the ischemic maturation process extends well beyond 7 days and that mild ischemia

severities can produce significant cell loss at long survival times holds important implications for drug trials and our current knowledge of death mechanisms.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

Firstly, I would like to give thanks to Dr. Howard Lesiuk for his guidance and support over these last years; especially after I scratched his car after only 2 months starting in the lab. I enjoyed our many discussions ranging from ischemia-related topics to discussions on how to put together your own computer. His stories and anecdotes never ceased to amaze and humour me.

Special thanks must go to Kim Barnes. She not only had to put up with my humour and "nutty" episodes, but was always there to help me out whenever I needed it. While I know she is looking forward to a quieter lab☺, I will miss our talks over coffee.

I would also like to thanks doctors Peter Stys, Bin Hu and Leo Renaud for giving me advice, information and suggestions with my thesis. I am truly grateful for all they're help.

Finally, thanks must go to my wife, Shena. There were many....many times over the last 3 years when I was not having much fun and wanted pull my hair out in frustration. Shena was able to motivate, encourage and push me through those tough times.

INTRODUCTION

Stroke remains the third leading cause of death, as well as the most common cause of adult disability in North America. A moderate transient global ischemic insult causes irreversible neuronal degeneration in specific, selectively vulnerable regions of the brain. It is well known that neurons in area CA1 of the hippocampus, striatum, cerebellum and certain layers of the neocortex are particularly sensitive to ischemia (Pulsinelli et.al., 1982; Kirino, 1982;Tomimoto & Yanagihara, 1992;unpublished observation). Research has shown that neurons in these sensitive areas do not die immediately after an ischemic insult. Rather, they regain "normal" functioning manifested by a recovery of transmembrane potentials and signaling activity (Xu & Pulsinelli 1994), and then die 2-4 days after reperfusion. This well-known phenomenon has been termed delayed neuronal death or ischemic maturation. This suggests that there is a "window of opportunity" after an ischemic insult during which therapeutic interventions might still prevent the biochemical and electrical events that ultimately cause neuronal death. Presently, viable drug therapies capable of alleviating the damage induced by a stroke remain elusive. Efforts to prevent ischemia-induced cell loss ultimately will depend upon an understanding of the molecular events initiated by ischemia and their detrimental effect on neurons.

In general, two kinds of animal models are used to study ischemic effects on brain- global ischemia and focal ischemia. Global ischemia models are presumed to mimic the human correlate of a heart attack in the sense that both cause a global, widespread attenuation in blood flow to the brain. In contrast, focal ischemia models mimic what is believed to occur during clinical stroke: cerebral artery thrombosis or embolism. There are essentially three different global models - 2-vessel occlusion (2VO), 4-vessel occlusion (4VO) and induction of cardiac arrest; the first two tend to be the mostly frequently used. Aside from the obvious differences in

surgical methodologies, the primary difference between these global models lies in the severity of cerebral blood flow reduction produced. 4VO or complete ischemia models typically reduced blood flow close to zero percent of control values (Pulsinelli et al., 1982), while 2VO or incomplete models have been shown to lower blood flow less than 20% of control values (Smith et al., 1984). Focal ischemia models focus on occlusion of the middle cerebral artery (MCA), and unlike global ischemia models cause blood flow to be reduced in a specific area of the brain. The word ischemia in the following text will refer to global ischemia unless otherwise stated.

Energy and Ions

A functional mammalian brain requires a continuous supply of oxygen and glucose. Depletion of either or both of these substances results in loss of brain function rapidly (Hansen, 1985; Erecinska & Silver, 1994; Xu & Pulsinelli, 1995), with the potential for cell degeneration if the depletion lasts for more than a few minutes (Pulsinelli et al., 1982; Kirino, 1982). The oxidative metabolism of glucose provides energy in the form of ATP for maintenance of ion homeostasis and basic neuronal function. Local cerebral glucose utilization (LCGU), a measure of cerebral energy metabolism, in the CA1 field of the hippocampus has been found to be markedly reduced during ischemia and also 1 to 3 days post-ischemia (Kozuka et al., 1993; Beck et al., 1995). Erecinska and Silver (1994) calculated that approximately 50-60% of total ATP in the brain is used to support ion movements in order to maintain electrochemical gradients. Maintenance of electrochemical gradients is essential for the brain to generate, process and transmit impulses. Cells in the brain maintain this gradient via pumps, which directly or indirectly require ATP hydrolysis to effectively extrude ions such as sodium and calcium while transporting in potassium. Activation of pumps requires ATP and results in ATP hydrolysis,

increasing the ratio of ADP/ATP. Stimulation of glycolysis and oxidative phosphorylation, the two main energy-producing pathways, increases as the ratio of ADP/ATP increases.

It as been shown that both biochemical techniques and also magnetic resonance spectroscopy that after onset of an ischemic insult, ATP and phosphocreatine (PCr) levels rapidly become negligible, while the inorganic phosphates AMP and ADP concentrations increase within minutes (Erecinska & Silver, 1994; Pulsinelli & Duff, 1983; Ljunggren et al., 1974; Ekholm et al., 1993;Shimizu et al., 1993). Therefore, due to the depletion of oxygen and glucose in the brain during ischemia, energy production through both pathways is attenuated with loss of cell electrochemical gradients. The loss of electrochemical gradients causes ions to move down their electrochemical gradients. Ischemia initiates an increase in extracellular potassium from approximately 3 mM, under normal resting conditions, to approximately 50-80 mM during ischemia (Hansen, 1985; Erecinska & Silver, 1994). Intracellular calcium levels rise from a basal value of approximately 100 nM, under normoxic conditions, to 30.2 ± 11.3 uM during ischemia (Silver & Erecinska, 1992). Likewise, sodium and chloride ions move down their electrochemical gradients into the cell.

These dramatic changes in ionic distribution produce a number of effects. Firstly, the huge efflux of potassium produces a membrane depolarization which has been termed the ischemic depolarization (ID) (Lauritzen & Hansen, 1992; Xu & Pulsinelli, 1994; Mayevsky, 1990; Nedergaard & Hansen, 1993). An ID has been characterized as a large rapid depolarization of approximately 50-60 mV occurring 1-2 minutes into an ischemia insult, and correlates in time with a huge increase in potassium efflux (Mayevsky, 1990; Xu & Pulsinelli, 1994). Xu and Pulsinelli (1994) observed that 5 minutes of ischemia caused the membrane to depolarize to approximately -20 mV, in-vivo. This depolarized state was maintained throughout

the ischemic period. Membrane repolarization did not occur until 1-3 minutes post-ischemia. They also found that the amplitude of the ID was directly proportional to the severity of the ischemic insult (i.e. cerebral blood flow) (Xu & Pulsinelli, 1994). When hippocampal blood flow was reduced to less than 10% of preischemic levels, the membrane potential depolarized to approximately –20 mV. However, when blood flow was reduced to approximately 40% of preischemic levels cells only depolarized about 10 mV from baseline (Xu & Pulsinelli, 1994). This observation supports the view that energy failure is the major mechanism behind the ionic disturbances and subsequent ID. Ionic normalization after an ischemic insult is dependent upon sufficient availability of ATP. The concentrations of high-energy phosphates in the brain have been shown to recover within minutes after anoxia (Ljunggren et al., 1974;Muller et al., 1994), consistent with Xu and Pulsinelli's finding that it takes a few minutes for ionic normalization to occur. However, although ATP levels rebound rapidly after an ischemic insult a secondary decrease in ATP has been observed to occur 1-2 days post ischemia in the hippocampus (Hashimoto et al., 1992; Arai et al. 1986; Pulsinelli & Duff, 1983) , corresponding to when cell loss in this area is known to occur.

Disturbance of ionic distribution also produces electrical silence in the brain during ischemia (Hansen, 1985; Erecinska & Silver, 1994; Xu & Pulsinelli, 1994). As mentioned above, a cell requires specific electrochemical gradient in order to generate impulses. Loss of this gradient results in the attenuation of nerve impulses. EEG recordings during ischemia have been shown to become isoelectric within 20 seconds after ischemic onset, recovering only during the first few minutes post ischemia (Hansen, 1985;Suzuki et al., 1983). Furthermore, synaptic activity in terms of both inhibitory post-synaptic potentials (IPSPs) and excitatory post-synaptic potentials (EPSPs), is greatly reduced during ischemia (Xu & Pulsinelli, 1994; Kirino et al.,

1992;Lobner & Lipton, 1993). Xu and Pulsinelli (1994) observed, in CA1 hippocampal neurons, that IPSPs disappeared shortly after the onset of ischemia while EPSPs were reduced. However, they observed that EPSPs disappeared completely shortly after the onset of the ID. Kirino et al. (1992) found that under normoxic conditions repetitive stimulation of the Schaffer collaterals produced a potentiation of EPSPs leading to long term potentiation (LTP). However, after five minutes of ischemia the amplitude of the EPSPs were reduced and LTP could no longer be induced with Schaffer collateral stimulation 48 hours after ischemia (Kirino et al., 1992). Moreover, by day 4 post-ischemia Schaffer collateral stimulation failed to evoke any potentials at all.

Glutamate

Receptors for glutamate, the major excitatory neurotransmitter in brain, have been divided in two major subtypes – ionotropic and metabotropic glutamate receptors (figure A). Ionotropic glutamate receptors are ligand-gated ion channels and include the kainate, quisqualate, amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and N-methyl-D-aspartic acid (NMDA) receptor subtypes. Metabotropic glutamate receptors are coupled to GTP-binding proteins.

To date, the most established explanation for cell loss caused by ischemia centres around the excitatory neurotransmitter glutamate, and has become known as the glutamate excitotoxicity model. This hypothesis suggests that, during ischemia, large amounts of the neurotransmitter glutamate are released from presynaptic terminals causing hyperexcitation of postsynaptic glutamate receptors (Benveniste et.al., 1984; Rothman and Olney, 1986). Indeed, ischemia has been shown to initiate significant increases in extracellular glutamate concentrations (Mitani et al., 1992; Rothman &Olney, 1986; Benveniste et al., 1984). Subsequently, this activation of

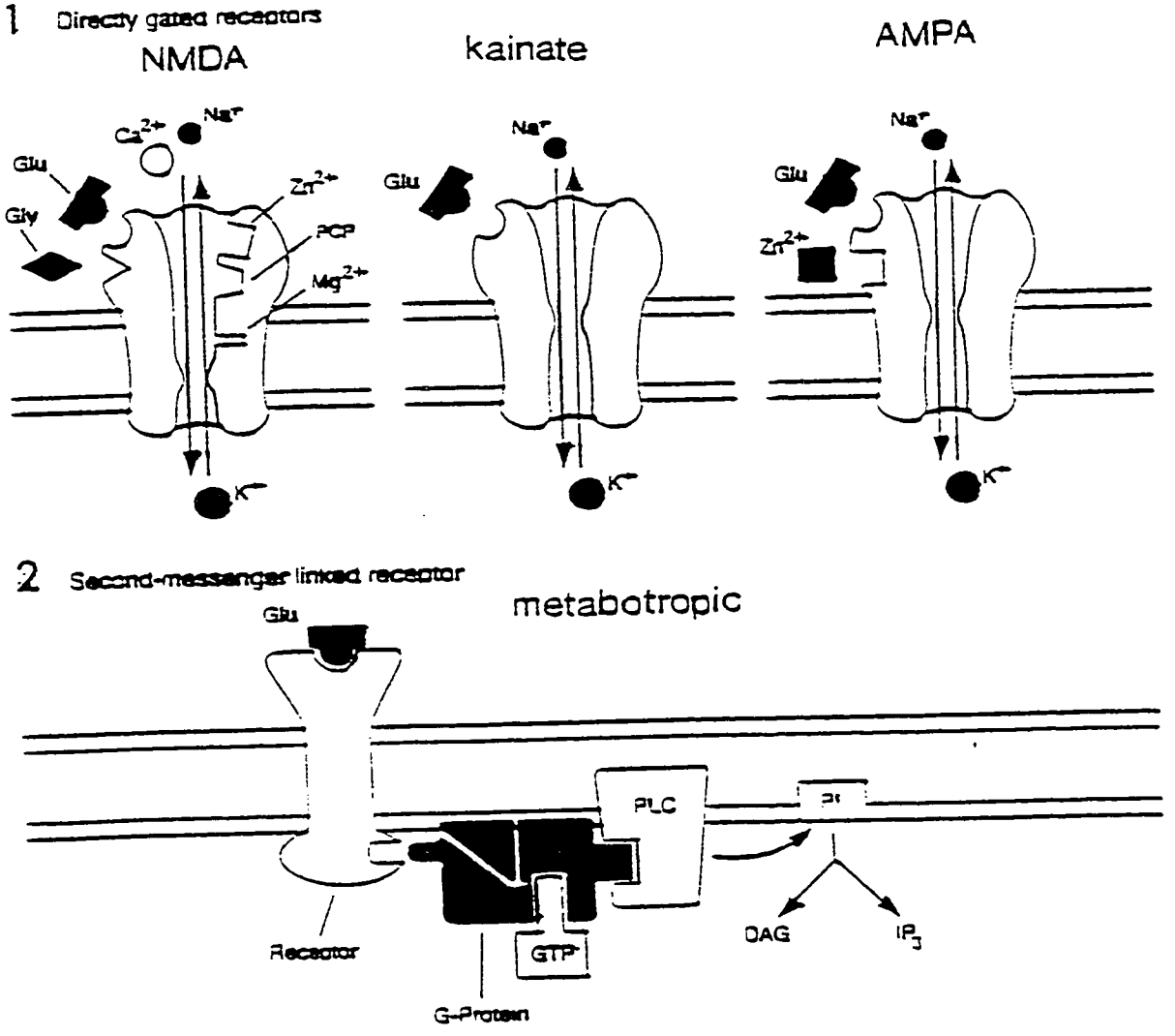
postsynaptic glutamate receptors in turn initiates large increases in intracellular calcium through glutamate receptor agonist-activated calcium channels. As discussed below, the significant increase in intracellular calcium is believed to be critical in causing neuronal death (Choi, 1988; Siejso, 1982).

NMDA

Traditionally, the view had been that the NMDA glutamate receptor subtype was responsible for ischemia-mediated cell loss (Rothman and Olney, 1986; Gill et.al., 1988). This view was based upon the observations that activation of NMDA receptors by glutamate causes significant increases in intracellular calcium and the administration of NMDA antagonists is dramatically neuroprotective, in-vitro (Choi, 1988) and in-vivo (Gill et al., 1988). However, it was later shown that administration of certain NMDA antagonists, in-vivo, induced significant hypothermia and that under normothermic conditions failed to produce any significant protection (Buchan & Pulsinelli, 1990; Nakamura et al., 1993). Notably, hypothermia alone has been frequently observed to provide significant neuroprotection against ischemia (Colbourne & Corbett, 1995; Colbourne et al., 1993; Dietrich et al., 1993; Coimbra & Wieloch, 1994). Nonetheless, in recent years, it has been shown that cell loss following an ischemic insult can be attenuated by antagonists of AMPA (Xue et.al., 1994; Diemer et.al., 1992; Buchan et.al., 1991) receptors and agonists of the metabotropic glutamate receptors (Birrell et.al., 1993; Siliprandi et.al., 1992).

AMPA

Traditionally, it has been observed that AMPA receptor activation initiates influx of sodium ions and efflux of potassium ions resulting in membrane depolarization. The protective effects of AMPA blockers, with the seemingly lack of effect on calcium fluxes, raised concern


1 Directly gated receptors
NMDA
kainate
AMPA
Glu
Gly
Glu
Glu
2 Second-messenger linked receptor
metabotropic
Glu
PLC
DAG
GTP
Receptor
G-Protein

for the role of calcium in delayed cell loss. However, the membrane depolarization produced via sodium influx does open voltage sensitive calcium channels leading to a rise in intracellular calcium. Under ischemic conditions chronic excitation of AMPA receptors by glutamate could be initiated causing a sustained and toxic increase in intracellular calcium. As well, it has been recently observed that AMPA receptors exist which are permeable to calcium (Pellegrini-Giampietro et al., 1992; Hume et al., 1991;Pollard et al., 1993). AMPA receptors missing the GLUR2 subunit are able to pass calcium ions into the cell (Hume et al., 1991). Pellegrini-Giampietro et al (1992) showed a significant reduction in GLUR2 mRNA, but not GLUR 1 and 3 expression, in CA1 neurons of the hippocampus following ischemia, and that this loss persisted for 24 hrs post-ischemia. This decrease in GLUR2 expression would then produce calcium permeable AMPA receptors resulting in a rise in intracellularPage: 9
calcium.

The mechanism behind a reduction in GLUR2 expression is unclear. It is not known whether the reduction occurs at the transcriptional level or whether this subunit is more sensitive to an ischemic insult than the mGLUR1 and 3 subunits (Pellegrini-Giampietro, 1992). Both may also contribute to the reduction in mGLUR1 mRNA. Under the latter scenario, the amount of mGLUR2 RNA, in CA1 neurons, would become smaller relative to that of GLUR1 and 3, and because the transcription machinery is damaged no new mRNA for mGLUR2 is synthesized. Alternatively, Hume et al. (1991) have shown that AMPA receptors expressing GLUR1, 2 and 3 subunits, which normally are impermeable to calcium, are made permeable if one amino acid in the GLUR2 subunit, arginine, is changed to glutamine. Therefore, AMPA receptors could become permeable to calcium due to ischemia-induced RNA editing of the GLUR2 subunit as

opposed to a reduction in its expression. However, Rump et al. (1996), found no evidence of RNA editing of the GLUR2 subunit following ischemia in the gerbil.

Metabotropic glutamate receptor (mGLUR)

More recently, researchers have begun to investigate the mGLUR receptor subtypes involvement in ischemia. Expression of mGLURs have revealed that they are a 7 transmembrane domain receptor linked to a g-protein, and to date 7 subtypes (mGLUR1-7) of mGLURs have been identified (Scheopp & Conn, 1993). Functional expression of mGLUR1 to mGLUR7 has shown differential responses from activation of phospholipase C to inhibition of cAMP to activation of cAMP (Scheopp & Conn, 1993; Nakanishi, 1992). While blockade of the ionotropic glutamate receptors, such as AMPA, has been observed to provide neuroprotection, *activation* of the metabotropic glutamate receptor has been shown to be neuroprotective in vivo (unpublished data in our lab) and in vitro (Siliprandi et al., 1992; Birrell et al., 1993). Activation of the mGLUR with a mGLUR agonist, 1S, 3R-1-aminocyclopentane-1,3-dicarboyxlic acid (t-ACPD), has been shown to provide significant protection to hippocampal CA1 neurons against ischemia in vivo in our lab (unpublished observations). t-ACPD has also been observed to provide neuroprotection against NMDA-induced toxicity to retinal (Siliprandi et al., 1992) and cortical cultures (Birrell et al., 1993). Application of t-ACPD on its own to cultured cortical neurons has been shown to lack any cytotoxic effects (Thomsen et al., 1993). However, unlike Birrell et al. (1993), Thomsen et al. (1993) found that t-ACPD did not prevent NMDA-induced toxicity to cortical neurons (Thomsen et al., 1993).

Evidence suggests that the neuroprotective effects of mGLUR agonists may be related to their effects on calcium fluxes. Birrell et al. (1993) measured calcium accumulation in cortical cultures exposed to NMDA toxicity and observed that application of NMDA resulted in

concentration dependent increases in calcium accumulation. However, co-application of t-ACPD reduced calcium accumulation and provided significant neuroprotection. Activation of mGLURs with t-ACPD can inhibit voltage-gated calcium currents in visceral sensory neurons (Hay & Kunze, 1994) and striatal neurons (Stefani et al., 1994). Garaschuk et al. (1992) studying hippocampal neurons, in vitro, observed that t-ACPD selectively inhibited extracellularly recorded EPSPs accompanied by depression of electrical excitability of CA1 neurons, but not CA3 neurons. This finding is particularly relevant in that CA1 but not CA3 neurons (Ito et al., 1975; Kirino, 1982; Pulsinelli et al., 1982) are selectively vulnerable to ischemia. mGLUR agonist, such as t-ACPD, therefore, could inhibit the hyperexcitability seen during ischemia and thus attenuate the cytotoxic effects of glutamate on CA1 neurons. Further investigation is required to confirm the neuroprotective effects of mGLUR agonists and to decipher what mechanism(s) they initiate which produce protection against ischemia.

While it seems clear that glutamate plays, at the very least, a partial role in ischemic cell loss, the excitotoxic hypothesis is likely an over simplification of the post-ischemic mechanisms which ultimately cause delayed neuronal death in the CA1 field of the hippocampus. The following is a further elaboration of the possible complexity of events leading to cell loss following ischemia.

Calcium

Excitable cells use cytosolic calcium as a first and second messenger and regulator of metabolic pathways to transform external stimuli into an appropriate cellular response (Carafoli, 1987;Miller, 1987; Berridge, 1987). Increases in cytosolic calcium, under normal physiologic conditions, can be brought about via ligand-gated and voltage-dependent calcium channels, as

well as release of calcium from internal stores (Ehrlich et al., 1994). Once intracellular calcium levels have risen within the cell providing a signal and cellular response, calcium itself then triggers extrusion and sequestration mechanisms in order to maintain calcium homeostasis and cell excitability. Extrusion mechanisms include the calcium-ATPase and the sodium/calcium exchanger (Carafoli, 1987). Sequestration mechanisms include buffering compounds and/or sequestration of calcium into the endoplasmic reticulum (ER) and mitochondria (Carafoli, 1987).

Despite its physiological function, it has also been observed that an increase in cytosolic calcium can lead to severe neuronal damage in many cell lines (Nicotera et al., 1986;Smith et al., 1991; Michaels and Rothman, 1990). Moreover, treatment with calcium chelators, such as BAPTA or EGTA (Tsubokawa et al., 1992; Tymianski et al., 1993), and the snail conotoxin N-type calcium blockers (Valentino et al., 1993; Adams et al., 1993) have been shown to protect neurons from ischemic/anoxic cell loss. However, Buchan et al. (1994) noted that the neuroprotective effects of the conotoxin SNX-111 were confounded with a drug-induced hypotensive effect that reduced rCBF during and after the ischemic insult. Drugs such as NBQX block ionotropic glutamate receptors that allow a massive influx of calcium into the cell during ischemia, and therefore provide significant neuroprotection supposedly due to attenuation of calcium influx (Xue et.al., 1994; Diemer et.al., 1992; Buchan et.al., 1991). Rises in intracellular calcium appear to occur during the post-ischemic period, before any histological signs of cell loss occur (Choi, 1988; Hasimoto et al., 1992;Andine et al., 1992;Nakamura et al., 1993) and intracellular calcium accumulation in the ischemic brain correlates with regions that are vulnerable to ischemia (Simon et al., 1984; Dienel, 1984;Andine et al., 1992). These findings lend support to the theorized role of calcium-induced anoxic/ischemic brain damage (Siesjo, 1988;Choi, 1988).

Under pathological conditions, such as ischemia, it is believed that abnormal rises in intracellular calcium influx could occur via a variety of different mechanisms. First, the extrusion and sequestration mechanisms for calcium fail due to ATP depletion. Extrusion of calcium by the calcium-ATPase is ATP dependent and since ATP is lost during ischemia ATPases ability to extrude calcium is lost. Similarly, sequestration of calcium by the ER is compromised due its dependence on ATP. Secondly, the electrogenic sodium/calcium exchanger which normally extrudes one calcium ion for every three sodium ions might operate in reverse (Nachsen et al., 1986) causing an inward movement of calcium under elevated cytosolic sodium and/or membrane depolarization. Both have been observed to occur during ischemia (Choi, 1988; Siesjo, 1990). Thirdly, intracellular calcium levels could rise due to calcium influx via voltage sensitive calcium channels which open when sodium enters the cell causing membrane depolarization, as occurs during ischemia (Valentino et al., 1993; Adams et al., 1993).

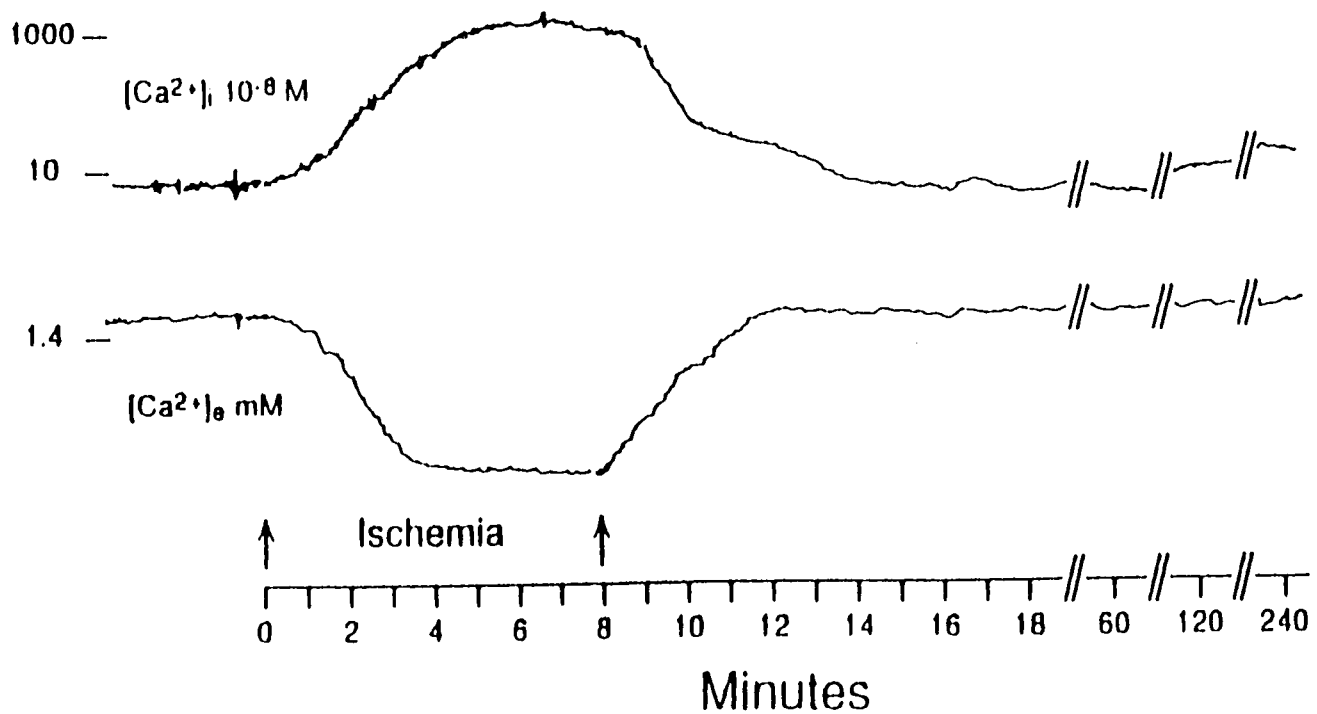
Fourthly, as mentioned above, calcium could enter via glutamate-receptors linked to calcium channels such as the NMDA receptor subtype (Rothman and Olney, 1986; Gill et.al., 1988 Choi, 1988) or via AMPA receptors missing the GLUR2 subunit (Pellegrini-Giampietro et al., 1992; Hume et al., 1991). The fifth possible route for calcium influx is via a non-specific membrane leak due to cell swelling occurring during ischemia (Choi, 1988). Finally, calcium could enter cytoplasm through release from internal calcium stores such as the endoplasmic reticulum. Calcium can be mobilized from internal stores, like the ER, by inositol 1,4,5-triphosphate (Taylor & Traynor, 1995) which is activated via the metabotropic glutamate receptor subtype (Schoepp & Conn, 1993). Indeed, recently a potentiation of the phosphoinositide signalling pathway was observed in hippocampal slices after 30 minutes of in-vivo ischemia (Lu et al., 1993).

Many studies have looked at changes in calcium concentrations as well as the relationship between extra- and intracellular calcium levels and neuronal functioning during and after ischemia (Lobner & Lipton, 1993; Tsubokawa et al., 1992; Silver & Erecinska, 1992;Hashimoto et al., 1992). Silver & Erecinska (1992) using the rat 4VO ischemic model observed a significant decrease in extracellular calcium concentrations over the 8 min ischemic period. Intracellular calcium levels reached physiological concentration after 20 minutes of recirculation and were maintained for approximately an hour (Figure B). After 1-hour reperfusion intracellular calcium levels rose approx. five fold. This biphasic change in intracellular calcium concentration was seen in CA1 neurons but not in CA2 or CA3 neurons, cells known to be relatively resistant to ischemia (Ito et al., 1975; Kirino, 1982; Pulsinelli et al., 1982). One of the many effects of ischemia on a CA1 hippocampal neuron is loss of synaptic transmission capabilities (Lobner & Lipton, 1993;Xu & Pulsinelli, 1994; Kirino et al., 1992). Lobner and Lipton (1993) using a hippocampal slice treated to 5 min of in-vitro ischemia observed long term synaptic failure which did not return until after 60 minutes reoxygenation. Reducing intracellular calcium levels with a buffer profoundly attenuated the loss of synaptic transmission (Lobner & Lipton, 1993).

Reasons why sustained elevations in intracellular calcium are cytotoxic include activation of proteases, lipases and endonucleases, generation of free radicals and impairment of mitochondrial function.

Proteases

One of the many important biochemical mechanisms initiated by calcium in neurons is the activation of calcium-sensitive proteases or calpains. Important cytoskeletal proteins which


1000
$[Ca^{2+}]_i$ 10^{-8} M
10
1.4
$[Ca^{2+}]_e$ mM
Ischemia
0
2
4
6
8
10
12
14
16
18
60
120
240
Minutes

are preferred substrates for calpain include spectrin, microtubule-associated protein (MAP2), and neurofilament proteins (Wang & Yuen, 1994; Matesic & Lin, 1994; Kumar & Wu, 1995; Yokota et al., 1995; Robert-Lewis et al., 1994; Hong et al., 1994). Increased proteolysis of one or all of these proteins by calpain could have deleterious effects on the integrity of neuronal structure and function. Several findings suggest that calpain has a role in initiating neuronal death. Firstly, calpain is found in neuronal populations known to be exquisitely sensitive to an ischemic insult (Siman et al., 1985; Hamakubo et al., 1986; Manev et al., 1991). Secondly, calcium activated proteolysis has been observed in the neocortex following focal ischemia (Hong et al., 1994) and in the CA1 field of the hippocampus following global ischemia, as measured by a significant increase in spectrin breakdown products (BDPs) (Roberts-Lewis et al., 1994;Lee et al., 1991), immunohistochemial loss of MAP2 (Matesic & Lin, 1994; Miyazawa et al., 1995), and loss of neurofilaments (Yokota et al., 1995; Kumar & Wu, 1995). Five minutes of global ischemia in the gerbil has been shown to result in a complete loss of MAP2 4-7 days after the ischemic insult (Kitagawa et al., 1989). Finally, protease inhibitors which block calpain have been shown to be neuroprotective (Lee et al., 1991; Arai et al., 1990;Matesic & Lin, 1994;Hong et al., 1994;Bartus et al., 1994). Moreover, application of these protease inhibitors not only provide neuroprotection to CA1 neurons following global ischemia but also greatly reduce the amount of spectrin BDPs (Lee et al., 1991) and loss of MAP2 (Matesic & Lin, 1994) seen in control animals. This suggests that the neuroprotective action of these inhibitors is presumably due to their direct blockade of calcium-activated calpains.

Until recently, direct evidence showing calpain activation during ischemia had not been shown. However, Neumar et al. (1996) using a global ischemia model observed increased calpain autolysis during global ischemia. Autolysis of calpain has been consistently observed to be

accompanied by increased proteolytic activity in vivo (Hayashi et al., 1991;Molinari et al., 1994) demonstrating that measurement of calpain autolysis is a direct way of measuring its proteolytic activity. Direct evidence of calpain activation *in ischemic neurons* specifically, and its subsequent involvement in causing neuronal death has yet to be shown. While calpains role in ischemic cell loss is encouraging, protease inhibitors such as AK295 and leupeptin are known to target proteases other than calpain. Furthermore, calpain inhibition following toxic-induced cell loss has been shown to lack neuroprotective effects (Manev et al., 1991). Manev et al (1991) showed that glutamate neurotoxicity was independent of calpain inhibition in primary cerebellar granule cell cultures.

Calpain has been shown to cause proteolysis of PKC and CaM Kinase II (Kishimoto et al., 1983;Young et al., 1987;Kwiatkowski & King, 1989) and is involved in membrane signalling. PKC is a Ca^{2+}-activated enzyme possessing a number of regulatory roles, including modulation of neurotransmitter release, neuronal plasticity, gene regulation, and membrane permeability (Nishizuka, 1986; Nichols et al., 1987; Routtenberg , 1987). Compared to normoxic conditions changes in PKC have been reported following hypoxic / ischemic insults. Yamaoka et. al. measured PKC isozymes following 30 minutes of transient hypoxia in rat hippocampus and found a significant decrease in both PKC-α and PKC-γ isoforms. Aronowski et al. (1993) also observed a reduction in PKC following 20 minutes of ischemia, in the rat. However, application of dextrorphan before ischemia provided significant neuroprotection to CA1 neurons and reduced the loss of the PKC compared to control animals (Aronowski et al., 1993). In addition to a loss of PKC isoforms, others have observed a translocation of cytosolic PKC to the cell membrane with a subsequent reduction in kinase activity (Cardell & Wieloch,

1993; Busto et al., 1994). Thus it appears that cerebral ischemia is associated with a modification of PKC activity prior to cell loss. A causal relationship between PKC changes and cell loss has yet to be established.

CaMKII is another protein kinase affected by ischemia, and may play a role in the observed cell loss. In hippocampal pyramidal neurons it accounts for at least 2% of total protein (Erondu & Kennedy, 1985). It phosphorylates a plethora of proteins, and like PKC is highly expressed in excitatory postsynaptic densities (Colbran et al., 1989). Thus, loss of CAMKII could result in deleterious functional consequences . It has been shown that CaM kinase II activity is significantly reduced immediately following transient ischemia (Hiestand et al., 1992; Yamamoto et al., 1992). Yamamoto et al. (1992) reported cytosolic CaMKII was completely lost in the hippocampus and cortex following just 2 minutes of ischemia. Particulate CaMKII disappeared more slowly, but was substantially reduced 2 hours following 10 minutes of bilateral occlusion. Similarly, Churn et al. (1990) have reported that five minutes of cerebral ischemia in the gerbil significantly reduces the activity of CaMKII, however they did not report a reduction in the kinase itself.

Free Radicals/Nitric Oxide (NO)

Free radicals are highly reactive toxic molecules which react with and damage proteins, nucleic acids, lipids, and other molecules (Schmidley, 1990). The brain possesses relatively poor antioxidant defences and brain tissue is especially sensitive to oxidative damage (Cao et al., 1988). During and after an ischemic insult free radical generation may occur via many different mechanisms.

Nitric Oxide

One potential mechanism for neuronal degeneration following an ischemic insult is the production of the free radical nitric oxide (NO). Nitric oxide is a diffusible gas that is synthesized through the conversion of arginine to citrulline. Nitric oxide synthase (NOS) is activated via an increase in intracellular calcium and is the enzyme responsible for catalyzing the conversion. The target for NO in the brain is cytoplasmic guanylate cyclase. Increased calcium inhibits guanylate cyclase which means for NO to have an effect it must act upon other cells. It does this by diffusing through the plasma membrane and enters adjacent cells. NO-activated guanlyate cyclase produces guanosine $3^{1},5^{1}$- cyclic monophosphate (cGMP) that can initiate multiple actions within the cell.

Excessive glutamate release, as seen in ischemia, stimulates the NMDA receptor subtype resulting in increased intracellular calcium that is able to activate (NOS), in vitro (Dawson et al., 1991;Dawson et al., 1993;Garthwaite et al., 1989). In vivo studies have shown that focal ischemia can initiate NOS production within the first hour of ischemia (Kader et al., 1993;Sato et al., 1994; Malinski et al., 1993), as well as during reperfusion (Sato et al., 1994). Furthermore, immunohistochemistry and in situ hybridization methodologies have demonstrated a calcium-dependent form of NOS in CA1 hippocampal neurons (Maiese et al., 1994). Therefore, the possibility exists that calcium-activated NOS may contribute to neuronal death. Maiese et al. (1994) demonstrated, in vitro, that the calcium antagonist HA1077 protected hippocampal neurons during anoxia, but failed to provide significant neuroprotection when exposed concurrently to NO. They imply from this that calcium is the initial cellular messenger in the ischemic cascade, but the eventual cell loss is dependent upon the NO pathway. Furthermore, it has been shown that inhibition of NO is neuroprotective against both hypoxic neuronal injury

(Wallis et al, 1992), as well as NMDA-induced toxicity in cultured neurons (Dawson et al., 1991; 1993). Moreover, Dawson et al. (1993) demonstrated that enhancing the phosphorylation of NOS with the immunosuppressant FK506, thereby decreasing the production of NO, protected against NMDA-induced neurotoxicity in cell cultures. These findings suggest that excessive release of glutamate leads to increases in intracellular calcium subsequently activating NOS and NO production and then cell loss at some point down the cascade. However, Buchan et al. (1994) failed to prevent neuronal loss of CA1 hippocampal neurons following cerebral ischemia following inhibition of NOS with L-arginine.

However, it should be pointed out that NO has been shown be neuroprotective rather than neurotoxic. Application of L-Arginine, a precursor of NO, enhances NO and decreases infarct size following focal ischemia (Morikawa et al., 1992;Yamamoto et al., 1992) and possibly in global ischemia (Zhang et al., 1995). These seemingly contradictory effects of NO have led to the notion of a dual effect of nitric oxide in focal ischemia (Dalkara et al., 1994; Sato et al., 1992;Lipton et al., 1993). Dalkara et al. (1994) suggest that a dual role of NO in focal ischemia makes sense depending upon where in the brain NO is acting. They observed that 7-NI, which selectively inhibits the neuronal isoform of NOS, was significantly neuroprotective following focal ischemia. However, enhancement of NO via intravenous administration of L-Arginine was shown to enhance cerebral blood flow within the perinfarct zone and provided significant neuroprotection. Dalkara et al. (1994) concluded, therefore, that *neuronal* overproduction of NO plays a role in the development of ischemia necrosis, while endothelial and perivascular NO may protect against ischemia by increasing regional cerebral blood flow. Finally, a recent study by Huang et al. (1994) showed that genetically knocking out the neuronal isoform of NOS, in mice, provided a significant reduction in infarct size following focal ischemia.

Free radical generation: Mitochondria

Another possible mechanism for free radical generation involves the electron transport chain in the mitochondria. Mitochondria provide ATP for cellular function by way of the electron transport chain by reducing oxygen completely to water. It has been estimated that approximately 90% of all oxygen in the brain is accounted for in this reaction. However, 1 or 2 % of this oxygen is not reduced to water but is generated into a reactive oxygen species (ROS) such as superoxide (Piantadosi et al., 1996; Halliwell, 1992). The rate of superoxide production increases as mitochondrial calcium increases or pH decreases. Uptake of calcium by mitochondria is predominantly mediated by a uniporter that is driven by the mitochondrial membrane potential (Gunter & Pfeiffer, 1990). Zaidan and Sims (1994) observed that 30 minutes of ischemia initiated a 2-fold increase in mitochondrial calcium. Indeed, Piantadosi and Zhang (1996) observed a five fold increase in mitochondrial generated superoxide radicals following 15 minutes of ischemia. Superoxide radicals may also be generated during reperfusion via by-products of arachidonic acid reduction and by xanthine oxidase. Both arachidonic acid and xanthine oxidase accumulate significantly during ischemic reperfusion.

Superoxide anions, however produced, can then rapidly dismutate to hydrogen peroxide via manganese superoxide dismutase (Halliwell, 1992). Hydrogen peroxide can easily cross cell membranes and in the presence of transition metals, such as iron, can be reduced to produce highly reactive hydroxyl radicals (Halliwell, 1992). Hydroxyl radicals are severely toxic to neurons reacting with and causing DNA damage, and they can initiate lipid peroxidation of membrane lipids causing changes in membrane fluidity and permeability and compromising membrane function (Schimdley, 1990).

Application of free radical scavengers or antioxidants have been used to discern whether they provide any significant neuroprotection and thus lend support to the role they play in ischemia mediated cell loss. Calapai et al. (1993) found that application of the antioxidant IRF-016 provided significant, dose-dependent neuroprotection to CA1 hippocampal neurons following 5 minutes of global ischemia in the gerbil. Other antioxidants have also proven to be effective in providing neuroprotection following both global and focal ischemia (Clemens & Panetta, 1994;Panetta & Clemens, 1994), as well as attenuating cell damage in a liver ischemia model (Paroni et al., 1995). Application of superoxide dismutase, which inhibits peroxide production, has also been shown to ameliorate damage in CA1 neurons (Kitagawa et al., 1990), and transgenic mice with over expression of superoxide dismutase had a reduction in infarct size following focal ischemia (Yang et al., 1994).

Apoptosis

While virtually all authors agree that an ischemic insult initiates delayed neuronal death to CA1 hippocampal neurons, the nature of the cell loss observed is under considerable debate; do these cells die due to necrotic or apoptotic cell loss? Necrosis is characterized by a marked inflammatory reaction, organelle swelling and cytoplasmic condensation that subsequently leads to cell disintegration and cell loss. Apoptosis, or programmed-cell loss, is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, apoptotic bodies and membrane blebing (Iwai et al., 1995;Wyllie et al., 1984;Gerschenson & Rotello, 1992;Nitatori et al., 1995). Until recently, death of neurons following both focal and global ischemia has been most frequently explained as necrotic rather than apoptotic cell loss. However, recently morphological evidence of apoptosis following ischemia has been reported.

Controversy exists due to the fact that not all researchers have found morphological evidence of apoptosis in ischemically injured CA1 hippocampal neurons (Deshpande et al., 1992). Apoptosis has been implied to involve activation of genes that would code a protein(s) having a lethal effect in the same cells containing that gene. This concept has been supported with the findings that RNA and/or protein synthesis inhibitors have been shown to suppress or delay apoptosis in non-ischemia models (Cohen and Duke, 1984; McConkey et al., 1988). If ischemia initiates apoptotic cell loss, one would assume that inhibition of RNA and/or protein synthesis would be neuroprotective. However, protein synthesis inhibitors following 10 minutes of ischemia (Desphande et al., 1992) and glutamate-induced toxicity (Leppin et al., 1992) failed to prevent cell loss. Finally, excitotoxin-induced nerve cell loss by kainate and quinolinate was devoid of DNA fragmentation, a common indicator of apoptotic cell loss (Ignatowicz et al., 1991; Masters et al., 1989).

However, despite some researchers failing to observe evidence for apoptotic cell loss, there are an equal number who have shown support for involvement of apoptosis in both global (Nitatori et al., 1995; MacManus et al., 1993; Kihara et al., 1994; Iwai et al., 1995) and focal (Li et al., 1995; Chen et al., 1995) ischemic cell loss. Proponents for the role of apoptosis in ischemic cell loss primarily have used DNA fragmentation or laddering as the standard by which to show and conclude that apoptosis exits in ischemically injured cells. DNA fragmentation has been observed to occur starting between 2-4 days post-ischemia exclusively in CA1 and some CA2 neurons of the hippocampus (Nitatori et al., 1995; Iwai et al., 1995; Kihara et al., 1994; MacManus et al., 1993). In addition to DNA laddering Nitatori et al. (1995) observed other morphological signs of apoptosis consisting of dense chromatin masses, apoptotic bodies and heterophagocytosis by microglial cells, starting at 3 days post ischemia.

Recently, a gene has been found which acts as a death suppressor gene, rather than a death effector gene as seen with apoptosis. Bcl-2 is a proto-oncogene which as been reported to block apoptotic death (Hockenbery et al., 1990;Vaux et al., 1988; Fanidi et al., 1992). Using immunocytochemistry and Western blot analysis, Chen et al. (1995) recently reported that bcl-2 is expressed in non-infarcted neurons which survived 60 or 120 minutes of focal ischemia, in the rat. The authors suggest that bcl-2 protein could be an endogenous neuroprotectant to neurons during an ischemic insult.

It should be noted however, there is generally not always a 100% correspondence between injured/dead CA1 neurons and manifestation of DNA fragmentation (MacManus et al., 1993). MacManus et al. (1993) observed that 25-35% of the DNA in the hippocampus was degraded apoptotically at 48 hours following 16 minutes of ischemia. While this is a significant proportion of observed cell loss, 65-75% of the remaining dead cells are presumably characteristic of necrotic cell loss. MacManus et al. (1993) propose that a continuum of damage may exist from apoptosis to necrosis depending on the severity of ischemia and on the amount of reperfusion time following ischemia. Interestingly, such a continuum between apoptosis and necrosis has been observed in the ischemic kidney (Schumer et al., 1992). Finally, it is important to mention that necrosis can also produce DNA fragmentation due to lysosomal DNAases which are activated in necrotic cells (Kihara et al, 1994). This obviously complicates conclusions that delayed neuronal death following ischemia is apoptotic, which are based solely on observations of DNA fragmentation. This shows the importance of finding alternative methodologies in addition to DNA fragmentation in order to distinguish apoptotic from necrotic cell loss.

Ischemic maturation: Rationale for study

A fascinating phenomenon seen in ischemia is the observation that hippocampal CA1 neurons do not die immediately after an ischemic insult but retain morphological as well as functional integrity, dying days after the insult (Ito et al., 1975; Araki et al., 1993; Pulsinelli et al., 1982; Kirino & Sano, 1984; Xu & Pulsinelli, 1994). This phenomenon has been termed delayed neuronal death or ischemic maturation. To date an explanation for why neurons die in a delayed fashion is yet to be elucidated, and therefore this area of ischemia has received much attention in past years. It is postulated that the maturation process involves an ischemia-initiated cascade of biochemical and structural changes requiring days to become lethal to neurons.

To date, the vast majority of rat and gerbil ischemia models have followed a strict experimental protocol. They typically consist of a given duration of ischemia, typically 5 min in gerbil and 10 min in rat models, followed by histological analysis at 7 days survival. Virtually nothing is known of the interaction between different ischemia severity's and survival times. A mapping out of a dose-response curve for the ischemic maturation process is important because: a) it would possibly elucidate the ischemic-switch (see below), b) dictate drug therapy regimes, and c) there is existing evidence indicating that the maturation process extends far beyond 7 days.

The idea of an ischemic switch refers to an ischemic threshold where ischemia times at and/or exceeding threshold induce delayed neuronal death, while times below this threshold fail to initiate the cascade of events leading to cell loss. At present no clear threshold has been solidly elucidated. For example, if a hypothetical experiment observed 7 minutes of ischemia at 7 days produced cell loss while 6 minutes did not, it is wrong to conclude that the ischemic threshold lies between six and 7 minutes. An incorrect conclusion would be drawn without

further information because evidence exists suggesting that the maturation process extends beyond 7 days survival. Therefore, 6 minutes of ischemia could realistically initiate the same cascade of chemical events as 7 minutes. It is postulated that the difference between six and 7 minutes of ischemia is that the maturation process, and subsequent cell loss, for 6 minutes is slower than for 7 minutes. Indeed it is known that ischemia times greater than 10 minutes speed up the maturation process and cell loss in CA1 neurons (Ito et al., 1975; Pulsinelli et al., 1982; Kirino & Sano, 1984; Smith et al., 1984). Therefore, intuitively one could argue that ischemia times less than 10 minutes could slow down the maturation process and subsequent cell loss in CA1.

Researchers in the focal ischemia field have more thoroughly investigated the concept of ischemic maturation and threshold than the global ischemia field. In the past, it was proposed that a blood flow of 25 ml/100g/min (15% of control) represented the threshold for ischemic damage to occur, in the rat (Tyson et al., 1984). However, more recently it was found that blood flows of 25-50 ml/100g/min produced infarction (Jacewicz et al., 1992). Jacewicz et al. (1992) explain the infarct produced with blood flow markedly higher than 25ml/100g/min as possibly due to the fact that they measured cell loss histologically at 24 hrs, while Tyson et al. (1984) looked only at 4 hours, post-ischemia. The evolving infarction may not be as readily detectable at 4 hours as it is at 24 hours. Kirino et al. (1988) concluded that the evolution of infarct injury was complete 12-24 hours after focal ischemia. However, Du et al. (1996) observed that neuronal injury persisted greater than 3 days after the focal insult and noted that researchers must be cautious in selecting a temporal endpoint for measurement of focal and global ischemic damage. Similarly, Sekhon et al. (1994) observed that just reducing blood flow to just below 40-50% of control values resulted in impaired neuronal function manifested by impaired long-term

potentiation 6 months after focal ischemia. Clinically, 15 minutes of MCA occlusion is defined as a transient ischemic attack (TIA) and has thought to be a non-lethal ischemic episode. However, Nakano et al. (1990) observed that 15 minutes of MCA occlusion in rats resulted in cell loss 1 week after the insult and at 4 weeks the presence of cell loss was still evident. Nakano et al. (1990) stated that a few months may be required to fully appreciate the extent of the damage. Finally, Kiyota et al. (1993) showed that dimethylthiourea, a free radical scavenger, only delayed the eventual infarct; in other words the drug only slowed the maturation process.

The fact that dimethylthiourea only provided transient neuroprotection supports the importance of investigating the potential therapeutic effects of drugs past just a few days during the post-ischemic period. Hypothermia has been shown by many to provide significant neuroprotection against ischemia (Colbourne & Corbett, 1994; Colbourne & Corbett, 1995; Dietrich et al., 1993). However, it has been observed that postischemic hypothermia protected CA1 neurons at 3 and 7 days but not at 2 months post-ischemia, suggesting that it only delayed the eventual death (Dietrich et al., 1993). Colbourne and Corbett (1994) observed that 12 hours of hypothermia initiated 1 hour after 3 or 5 minutes of global ischemia offered more protection at 10 versus 30 days post-ischemia; again, suggesting there is a progression of cell loss up to at least 30 days. More recently, Colbourne and Corbett (1995) looked at the effects of hypothermia at 30 days and 6 months post ischemia. They observed that 24 hours of hypothermia rescued 90% of CA1 neurons compared to controls (95% loss) at 30 days, but hypothermia rescued only 70% of CA1 neurons at 6 months. While it is clear that hypothermia provided persistent and effective neuroprotection at 6 months, the number of neurons protected was significantly less compared to earlier survival times. This indicates again that the ischemic maturation process is capable of extending to at least 6 months post-ischemia.

Luhmann et al. (1995) using the 2VO ischemia model investigated the long-term effects of ischemia on the electrical properties of rat neocortical neurons. After six months, they found a profound down-regulation of inhibitory function with a concurrent NMDA receptor-mediated hyperexcitability occurred in the neocortex. This study showed that while neurons may appear morphologically healthy, functionally they are impaired.

There are a few papers which have looked at the effects of long animal survival times on CA1 hippocampal neurons following global ischemia (Murdick & Baimbridge, 1989; Bonnekoh et al., 1990; Onodera et al., 1990,1993; Hsu, et al., 1994; Fukuda et al., 1993; Kirino, 1982), but only for a single ischemia time. A common observation at survival times greater than one month was - a) severe degeneration of CA1 pyramidal neurons, b) a significant shrinkage in the stratum radiatum and oriens of the CA1 subfield of the hippocampus, not seen at earlier survival times, c) astrocytic and microglial proliferation and d) preservation of presynaptic terminal boutons. However, some researchers found neuronal loss of supposedly ischemia resistant CA3 spiny neurons past 100 days survival (Onodera et al., 1993; 1990; Hsu et al., 1994), while others found CA3 neurons to remain resistant to ischemia up to 6 and 10 months post ischemia (Bonnekoh et al., 1990;Fukuda et al., 1993). This difference in CA3 ischemic vulnerability versus resistance could be due to the different animals and ischemia models used. Onodera et al. (1993) and Hsu et al. (1994) used the 4-vessel occlusion in the rat, while Bonnekoh et al. (1990) and Fukuda et al. (1993) used the 2-vessel occlusion model in the gerbil.

Fukuda et al. (1993) studied the effects of 5 min of global ischemia on CA1 non-pyramidal neurons at survival times up to 6 weeks post-ischemia. No changes or damage were seen in the non-pyramidal neurons up to 3 weeks survival, but at 6 weeks rough endoplasmic reticulum (RER) was fragmented and neurons shrunken with hyperchromatic nuclei and

cytoplasm. Immunostaining revealed a significant decrease in parvalbumin staining suggesting that these non-pyramidal neurons lacked normal neuronal activity (Fukuda et al., 1993). Murdick and Baimbridge (1989) also found loss of parvalbumin staining in CA1 region at 6 months following 20 minutes of global ischemia. They also observed that these GABAergic interneuronal terminal networks were reduced with dendrites retracted. It should be noted that in all these studies looking at long term survival times, with one exception (Fukuda et al., 1993), no temperature monitoring was considered (Murdick & Baimbridge, 1989; Hsu et al., 1994; Kirino, 1982; Onodera et al., 1993;1990; Bonnekoh et al., 1990). Animals lose thermoregulation during and after an ischemic insult and if normothermia is not maintained animals will become hypothermic. As mentioned earlier, hypothermia is a potent neuroprotector. Failure to maintain normothermia could produce results that do not reflect actual damage produced by the insult.

To our knowledge there is only one study that investigated a combination of different survival times and long survival times (Araki et al., 1993). Araki et al. (1993) used the gerbil 2-V0 model. They induced ischemia for three or 10 minutes combined with a survival time of 8 months. At 8 months, 3 minutes of ischemia resulted in severe neuronal damage specifically in the CA1 subfield of the hippocampus, while 10 minutes produced neuronal damage in the CA1 and CA3 regions of the hippocampus. Unfortunately, they did not cite quantified values of damage for three or 10 minutes of ischemia. Nor did they state the extent of cell loss at earlier survival times to see if cell loss progressed or matured from 7 days to 8 months.

It is clear that evidence exists suggesting that the maturation process continues well beyond 7 days of survival. Hsu et al (1994) went so far as to suggest that the events initiated by ischemia cause progressive changes in the brain throughout the organism's life. While this may or may not be true, investigating the interaction between ischemic severity and survival times is

required. Mapping out a dose-response curve for ischemic maturation is very important for two reasons. Firstly, if it is direct evidence can be shown that indeed the maturation process continues months after an ischemic insult, neuroprotective drugs may have to be given chronically to a patient versus a one time single bolus to avoid just delaying the eventual death. As mentioned earlier, a single bolus of dimethylthiourea only delayed cell loss (Kiyota et al., 1993). Second, it hopefully will elucidate a quantified ischemic threshold. This would allow researchers to focus on the threshold value which they know will induce cell loss and by comparing the biochemical, structural, and genetic differences initiated at threshold versus sub-threshold ischemia times, the mechanism(s) which ultimately cause delayed neuronal death could be deduced. This would of course help lead to the development of new therapeutic agents.

Objectives and hypotheses

Therefore, we set out to investigate the interaction between ischemic severity and survival times on hippocampal CA1 neurons using the 2-VO model of ischemia in the rat. To achieve a characterization of this interaction, ischemic severity's of 3, 5, 7, 10 and 13 minutes and survival times of 2, 7, 14, 28, and 90 days were used. Three hypothetical results were considered.

1. The first hypothetical result is the most static and sets 7 days as the time point when the evolution of damage is complete for all ischemic severity's. For example, 10 minutes of ischemia at 7 days produces 80% damage and 7 minutes produces 40% damage in CA1 hippocampal neurons. The extent of the damage in this model would not change at any time point past 7 days. In other words, the maturation process remains confined to the first 7 days. This hypothetical model appears unrealistic considering the evidence to the contrary mentioned above.

2. A second model that would more accurately reflect the current body of evidence suggesting that the maturation process extends well beyond 7 days. In this model once the ischemic threshold has been reached, whether that be at three or 13 minutes, it triggers the biochemical, genetic and/or structural changes that occur during the maturation process. The greater the ischemic insult is above the threshold the faster the maturation process, and subsequent cell loss; vice versa for those ischemic times closest to the threshold. Recall, that ischemic times greater than 10 minutes speed up the maturation process and time to cell loss in CA1 neurons (Ito et al., 1975; Pulsinelli et al., 1982; Kirino & Sano, 1984; Smith et al., 1984). Hypothetically, once the ischemic switch has been turned on, the resultant degree of cell loss in CA1 will be the same regardless of the severity of the insult. The effect of longer versus shorter ischemia times on CA1 neurons in this model is manifested in how long it takes to achieve the same degree of cell loss.

3. This model incorporates the characteristics of models one and two. In this model, the amount of cell loss observed depends upon the severity of the ischemic insult and on the amount of time a given animal survives following the insult. For example, 13 minutes of ischemia results in 95% cell loss at 7 days of ischemia, while 3 minutes of ischemia results in 0% cell loss at 7 days following ischemia. However, histological investigation of the CA1 subfield 3 months following 3 minutes of ischemia reveals 20% neuronal loss.

METHODS

Treatment of the rats were be in accordance with the Animals for Research Act, the Guidelines of the Canadian Council on Animal Care and relevant University and Loeb Institute policies.

Experimental protocol:

Male white Sprague Dawley rats (225-300 g) were used. All rats were housed in a climatically controlled environment and placed on a 12-hr day/night cycle (day starting at 7:00 am). Food and water were given ad libitum. Two variables were manipulated - (i) the severity of the ischemic insult and (ii) survival times. Ischemia was induced for 3,5,7,10 and 13 minutes followed by survival times of 2, 7, 14, 28 and 90 days following the ischemic insult for a total of 25 independent experimental groups. Five animals per group were used as a minimum.

Animal Preparation and Surgical Procedure:

Animals were denied access to food overnight prior to anesthesia. General anesthesia was induced with sodium pentobarbital (65 mg/kg i.p.). Atropine (35 mg/kg) was given to attenuate tracheal secretions prior to tracheal intubation. The trachea was intubated via a #16 Teflon iv. catheter under direct vision with a laryngoscope. Animals were then placed on mechanical ventilatory support (Model 665 small animal ventilator, Harvard apparatus). A thermocouple probe was placed through the tympanic membrane into the middle ear to permit continuous

monitoring and adjustment of head temperature. Temperature was regulated as necessary with a heating lamp to maintain normothermia (37.5 +/- 0.2 C)

Tail arteries were surgical exposed and cannulated with a #24 Teflon iv catheter (heparinized saline) to allow for - continuous monitoring of blood pressure, withdrawal of blood to induced controlled hypotension (see below), and arterial blood sampling for AGB, and glucose measurements. Blood pressure monitoring was achieved by connecting the cannula to a pressure transducer which was connected to a PC computer containing software (DataQ Instruments Inc., CODAS) able to display and record blood pressure. ABG samples were extracted from the cannulated tail artery into a heparinized syringe and analyzed (Blood Gas Manager Model 1312, pH/Bloodgas Analyzer, Instrumentation Laboratory). Pre-ischemic ABGs were taken in order to ensure normocapnia (pCO_2 = 35-45 torr) and normoxia (pO_2 = 90 -120 torr) were present prior to induction of the ischemic insult. Post-ischemic ABGs were taken to compare with pre-ischemic values. Post-ischemic blood-glucose samples were taken via the tail cannulation to observe whether or not animals were hyper or hypoglycemic (One Touch II Glucometer, Lifescan Canada Ltd.)

Once normocapnia and noromoxia were achieved, ischemia was induced through temporary occlusion of the carotid arteries using cerebral aneurysm clips coupled with controlled hypotension (BP = 40 mmHG) induced by the temporary partial exsanguination of blood via the arterial cannulation into a continuously warmed syringe contained 0.5 ml of heparinzed saline. All rats had both carotid arteries exposed. Experimental groups were exposed to 3, 5, 7, 10 or 13 minutes of ischemia, while sham operations (carotids exposed but not occluded) were performed on control animals. After ischemia the carotids were unoccluded and extracted blood re-infused over 30 seconds. Ventilatory support was continued until the animal was breathing well and

moving its extremities spontaneously. Animals recovered under normothermic conditions using a rectal probe and a thermistor controlled heating lamp.

Histology

Following ischemia, all animals were allowed to recover and given free access to food and water until the time of sacrifice. Animals were then re-anethetized and perfusion-fixed with a transcardiac infusion of 10% formalin solution. Brains were then left in the head for 2 days at which time the brains were removed from the skull and stored in 10% formalin solution for an additional 2 days. Coronal sections 7 um thick were cut, paraffin-embedded, and the sections were stained with haematoxylin and eosin at several levels of bregma.

Each animal was assigned a code so that what experimental group they belonged to was concealed during histological examination. Damage to the CA1 region of the hippocampus was assessed by counting both the number of histologically normal and abnormal appearing neurons under 40X light microscopy. The results were then expressed as a percentage of dead neurons [total CA1 neurons - normal CA1 neurons/total CA1 neurons x 100]. A cell count of total number of pyramidal cells in the CA1 field was taken from non-ischemic animals to achieve an average baseline. This was done because microscopic investigation of the CA1 field at survival times of 28 and 90 days revealed a significant reduction in the total number of CA1 pyramidal neurons. It was assumed that those cells that were "lost" were dead cells. Therefore, if the total number of CA1 neurons in any given animal was two standard deviations (S.D.) away from the mean of total CA1 neurons in control animals, it was concluded that there were missing cells. Cells that were missing under this criteria were counted as dead cells. The actual total number of CA1 neurons for that animal was then subtracted from the baseline total number minus 2

standard deviations. This difference was then added to the counted number of dead neurons [i.e. actual total – (baseline total – 2 s.d.) + counted dead neurons].

Statistical Analysis:

The Kolmogorov-Smirnov test for normality was performed on the percentage data to confirm a non-normal distribution. All percentage data were normalized via an arc-sine transformation method (Zar, 1984). A statistical comparison was performed on the transformed data using a 2-way between factor analysis of variance (ANOVA). Any significant results (alpha = 0.05) were further investigated using a standard intergroup comparision test (Tukey post-hoc tests for unequal N) (Spjotvoll/Stoline test). Data for the physiological variables, blood- glucose, oxygen, carbon dioxide, and pH were combined across survival times, and pre- and post-ischemic values were compared using a 2-way-between-within factor ANOVA. Any differences between the groups were further investigated using a post-hoc Tukey test for unequal N (Spjotvoll/Stoline test).

Results

Mortality

23 animals died during this study and were removed from the data set. All 23 died within the first 6 days following surgery. The vast majority of these animals died during recovery from the surgical procedure and never regained consciousness. Within this group of animals almost all required additional barbiturate to achieve complete anesthesia. It is possible that too much

barbiturate was given to these animals resulting in respiratory failure and death. Those animals that died during recovery and were not given additional anesthetic belonged to the 10 and 13-minute ischemia groups. Animals that survived the recovery period but died due to euthanasia over the next 6 days primarily due to sickness. These animals also belonged to the 10 and 13-minute ischemia animals.

Physiologic parameters:

Arterial blood pH:

Mean +/- standard error (S.E.M.) pH values for all ischemia severities can be seen in Table 1. There was a significant interaction between ischemic severity and time of measurement [$F(4,139) = 3.78$; $p<0.01$]. Pre-ischemic pH values were not significantly different between ischemic groups. Pre-ischemic pH was significantly different from post-ischemic pH for all ischemic groups. As the severity of the ischemic insult increased pH became more acidic. Thirteen minutes of ischemia resulted in a mean post-ischemic pH of 7.21 +/- 0.21. This proved to be significantly different from three, five and 7 minute post-ischemic pH values [$F(4,139) = 3.78$; $p<0.00005$; $p<0.005$;$p<0.005$ respectively]. Ten minutes of ischemia had a slightly more basic pH than thirteen minutes of 7.23 +/- 0.17. This difference was not significantly different [$F(4,139) = 3.78$; $p>0.05$]. The post-ischemic pH for ten minutes of ischemia was significantly different from three minutes of ischemia [$F(4,139) = 3.78$; $p<0.00005$, but was not statistically different from five and 7 minutes of ischemia. The post-ischemic pH values for three, five, and 7 minutes of ischemia were not significantly different from each other.

TABLE 1. Physiological variables [mean +/-S.E.M. (n)]

ISCH-TIME	*PreO2 (mm Hg)*	*PostO2 (mm Hg)*	*PreCO2 (mm Hg)*	*PostCO2 (mm Hg)*	*Pre-pH*	*Post-pH*	*Glucose (mmol/L)*
3 min.	111.3+/-1.70 (27)	93.7+/-4.10 (27)	34.4+/-0.64 (27)	38.6+/-1.50 (27)	7.36+/-0.0065 (27)	7.31+/-0.011 (27)	3.9+/-0.19 (26)
5 min.	111.6+/- 1.94 (39)	92.7+/-3.81 (39)	37.9+/-0.89 (39)	42.0+/-1.42 (39)	7.36+/-0.0077 (34)	7.27+/-0.014 (34)	4.4+/-0.16 (38)
7 min.	108.1+/-1.86 (33)	89.9+/-5.25 (33)	38.2+/-1.21 (32)	45.4+/-1.94 (32)	7.35+/-0.016 (31)	7.27+/-0.013 (31)	4.4+/-0.18 (31)
10 min.	106.6+/-1.90 (30)	76.2+/-4.04 (30)	36.3+/-0.73 (30)	43.3+/-1.70 (30)	7.33+/-0.0094 (26)	7.23+/-0.020 (26)	4.7+/-0.20 (28)
13 min.	106.4+/-2.26 (23)	75.7+/-4.25 (23)	36.8+/-0.78 (26)	45.7+/-1.42 (26)	7.34+/-0.017 (26)	7.21+/-0.027 (26)	4.7+/-0.34 (26)

Table 2. MABP for rats measured before, immediately after and 1-3 minutes after ischemia [mean +/- SEM (n)]

ISCH-TIME	*BEFORE*	*IMMEDIATELY AFTER*	*AFTER*
3 min.	126.06 +/-3.04 (24)	112.19 +/-1.91 (24)	139.92 +/-3.06 (24)
5 min.	119.35 +/-2.19 (31)	104.19 +/-2.24 (31)	134.52 +/-2.37 (31)
7 min.	124.44 +/-2.37 (31)	102.52 +/-2.82 (31)	136.37 +/-3.00 (31)
10 min.	124.14 +/-3.55 (18)	103.62 +/-3.24 (18)	129.03 +/-6.23 (18)
13 min.	122.63 +/-3.42 (20)	105.00 +/-3.87 (20)	126.13 +/-3.67 (20)

TABLE 3. Percent neuronal damage in CA1 field of the hippocampus [mean +/- SEM (n)]

ISCH-TIME	*2 DAYS*	*7 DAYS*	*14 DAYS*	*28 DAYS*	*90 DAYS*
3 min	0 (5)	0 (6)	0 (5)	0 (5)	0 (6)
5 min	0 (5)	0 (7)	3.80+/-1.89 (9)	3.03+/-2.01 (9)	5.36+/-3.53 (10)
7 min	0 (5)	3.30+/-2.20 (7)	14.22+/-5.29 (5)	15.24+/-8.20 (7)	27.95+/-8.33 (10)
10 min	12.91+/-9.19 (7)	70.53+/-2.44 (7)	70.66+/-3.37 (4)	79.16+/-6.15 (5)	82.39+/-6.99 (4)
13 min	31.0+/-4.29 (7)	83.25+/-0.81 (4)	89.26+/-2.71 (6)	91.21+/-4.96 (3)	93.44+/-3.18 (6)

Arterial blood glucose:

Post-ischemic glucose values can be seen in table 1. The severity of the ischemic insult had no significant effect glucose concentration taken 5 minutes after the ischemic insult [$F(4, 144) = 2.02$; $p>0.05$]. While there wasn't a significant difference in glucose values between the different ischemia times, the concentration of glucose increased as the severity of the ischemic insult increased. However, despite this mild increase all glucose values remained well within normal physiological values.

Arterial blood oxygen tension:

Pre- and post-ischemic oxygen values for three, five, seven, ten and thirteen minutes of ischemia are presented in table 1. The ANOVA resulted in a significant main effect of ischemic severity on oxygen [$F(4,147) = 4.49$; $p<0.005$]. The partial pressure of oxygen decreased as the severity of the ischemic insult increased. For example, three minutes of ischemia produced an average partial pressure of oxygen of 102.54 mmHg compared to 90.59 mmHg following thirteen minutes of ischemia. Overall, the partial pressure for oxygen dropped significantly after ischemia compared to pre-ischemic levels, regardless of the severity of the ischemic insult [$F(1,147) = 140.96$; $p<0.000001$]. It should be noted that while there was an ischemic caused a significant drop in oxygen, the post-ischemic values remained within physiological normal ranges and adequate to maintain normal saturation of heamogoblin.

Arterial carbon dioxide tension:

ANOVA results show a significant main effect of ischemia time on carbon dioxide (CO_2) levels [$F(4,149) = 3.13$; $p<0.05$], and revealed a significant difference between pre- and post

ischemic carbon dioxide values [F (1, 149) = 87.68; p<0.00001]. Pre- versus post-ischemic CO2 levels for all ischemic groups can be seen in table 1. There was no significant difference in pre-CO2 values between all ischemic groups. There was a rise in the partial pressure of CO2 following all ischemia times. However, this rise in post-ischemic CO2 was statistically different for only the 7, ten and thirteen minute ischemia times. In general, the post-ischemic partial pressure of CO2 increased as the severity of the ischemic insult increased. The only exception was for ten minutes of ischemia that had a lower CO2 value than 7 minutes of ischemia; this difference was not significant however. The post-ischemic CO2 value for three minutes of ischemia was significantly different from 7, ten, and thirteen minutes of ischemia, but not five minutes of ischemia. All other post-ischemic CO2 values were not significantly different from each other. Again it should be noted that, like for oxygen, while there was a significant increase in carbon dioxide after ischemia, the post-ischemic values remained within physiological normal ranges.

Arterial blood pressure:

The values for mean +/- S.E.M. for mean arterial blood pressure (MABP) before immediately after and after ischemia can be seen in Table 2. The 2-factor between-within ANOVA revealed no effect of ischemic severity on BP [F (4, 119) = 1.833; p>0.05]. BP was significantly affected depending upon when BP was measured (before ischemia, immediately after ischemia, after 1-3 minutes of reperfusion) [F(2, 238) = 183.18; p<0.00001]. Mean BP before ischemia was 123.32 mmHg, 105.5 mmHg immediately after the clips were removed and 133.19 mmHg, approximately three minutes into the post-ischemia period. Pre-ischemic BP was significantly different from both BP measured immediately after and 1-3 minutes after the

ischemic insult. BP measured immediately after attenuation of the ischemic insult was also significantly different from BP measured approximately three minutes later.

In addition there was a significant interaction between the severity of the ischemic insult and time of measurement [$F(8,238) = 1.99$; $p<0.05$]. There was no significant difference in pre-ischemic BP for all ischemia times. Pre-ischemic BP was significantly different from post-ischemic BP following three, five, and 7 minutes of ischemia. However, there was not a significant difference between pre- and post-ischemic BP following 10 and 13 minutes of ischemia. However, there was a significant difference between pre-ischemic BP and BP measured immediately after 10 and 13 minutes of ischemia. All ischemic groups produced a significant difference between BP measured immediately after ischemia and BP measured 1-3 minutes later. There was no statistical difference between BPs measured immediately after ischemia for all ischemia groups. All post-ischemic BP values were not statistical different from each other with the exception of a statistical difference between 13 and 3 minutes of ischemia [$F(8,238) = 1.99$; $p<0.005$].

Histology: CA1 pyramidal cell death

Control animals

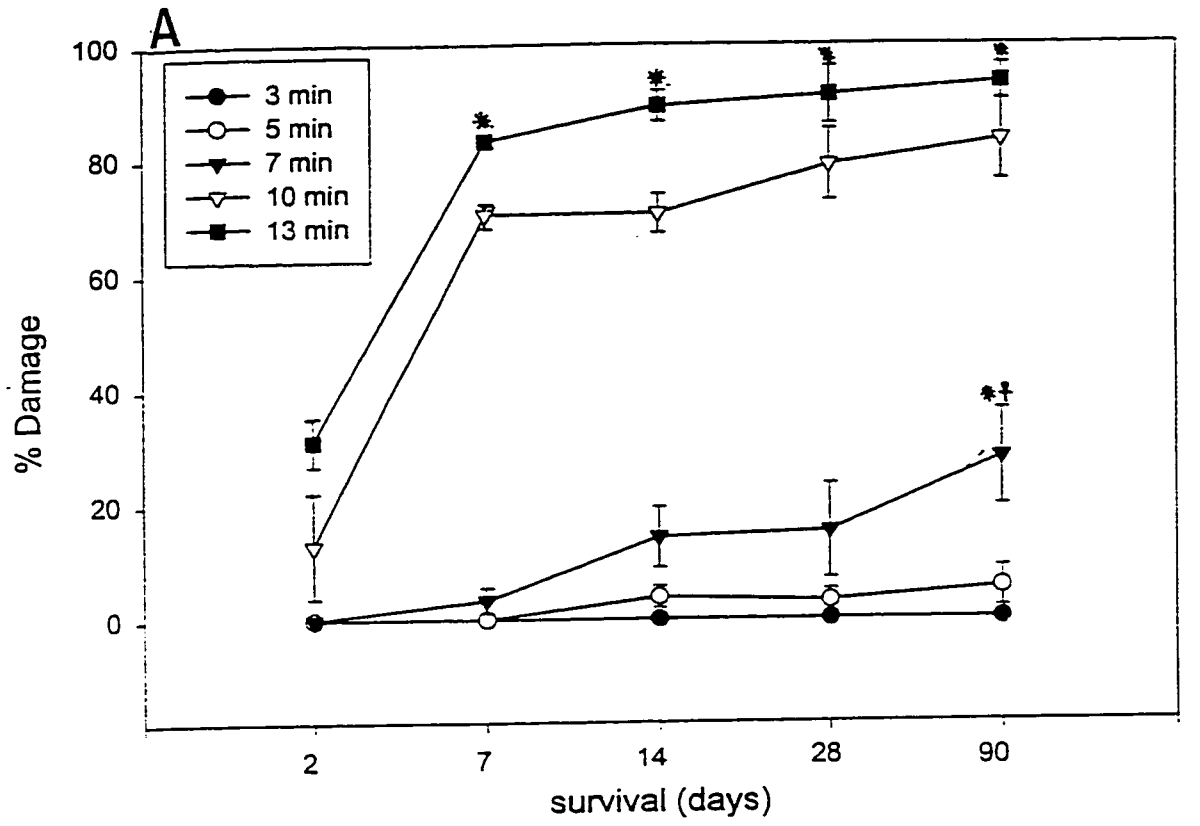
Coronal sections of the hippocampus at 7 days exhibited clear differentiation of CA1-CA4 sectors (Figure 2A). There was no apparent neuronal damage throughout any of the hippocampal fields. As can be seen in figure 3A, healthy hippocampal neurons were round, had intact membranes and the nucleus was clearly visible under 40X magnification. Histological investigation of the CA1 subfield was also performed in control animals allowed to survive 3 months. No cell loss was seen. This was performed to ensure that CA1 neurons did not die due to

age-related effects. In contrast, figure 3B represents the result from 10 minutes of ischemia (7-day survival). These neurons are shrunken (pyknotic), irregularly shaped, lack a clearly defined nucleus and are eosinophyllic (pinkish-red in colour). Bilateral cell counts of control CA1 pyramidal neurons yielded an average of 1145 cells, under 40X magnification. Cell counting was performed on slices between Bregma -3.20 to -3.80 mm.

Ischemic groups

Except for a dragging of the hind limbs during the first hour of the recovery period the rats exhibited no obvious signs of altered neurological behaviour at any ischemic duration. Over time,

animals continued to gain weight, drank, and behaved normally. The Kolmogorov-Smirnov test for normality on the percentage data for neuronal damage confirmed a non-normal distribution ($p<0.01$), thus requiring an arc-sine transformation. Collapsing the data across ischemic groups produced a significant main effect of survival time [$F(4, 129) = 24.88; p<0.00001$]. Figure 1 demonstrates that the longer the animals survive after an ischemic insult, the greater is the degree of cell loss to CA1 pyramidal neurons. Post-hoc Tukey tests confirmed that the degree of damage at three months survival was significantly greater than at two and 7 days survival. In addition, the degree of damage at 7 days survival was significantly greater than at two days. Statistical analysis also revealed a significant interaction between the degree of ischemic severity and the length of survival [$F(16,129) = 4.72; p<0.00001$]. This interaction is graphically depicted in figure 1. CA2, CA3, CA4 and dentate lacked cell loss following all ischemia and survival times with the exception of 2 animals that manifested a few dead cells in the CA4 subfield following 13 minutes of ischemia.


A
3 min
5 min
7 min
10 min
13 min
% Damage
100
80
60
40
20
0
2
7
14
28
90
survival (days)

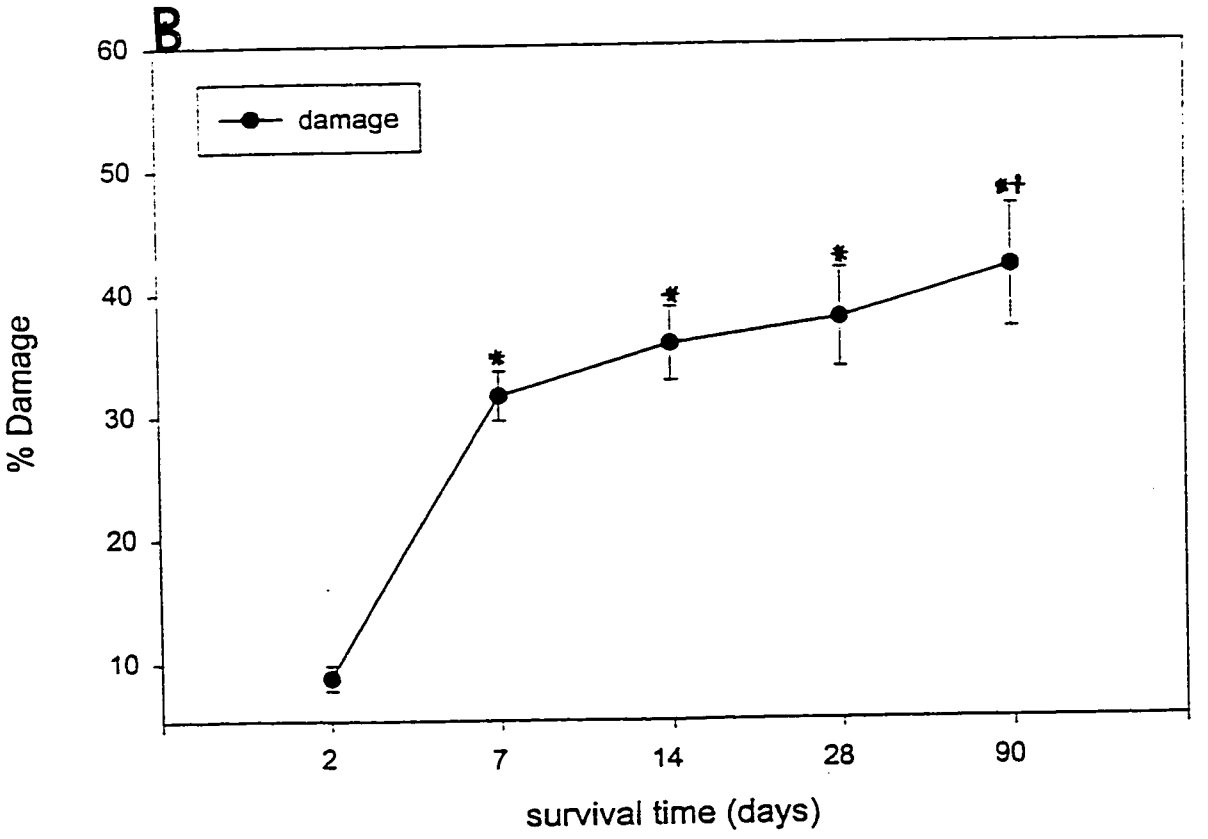
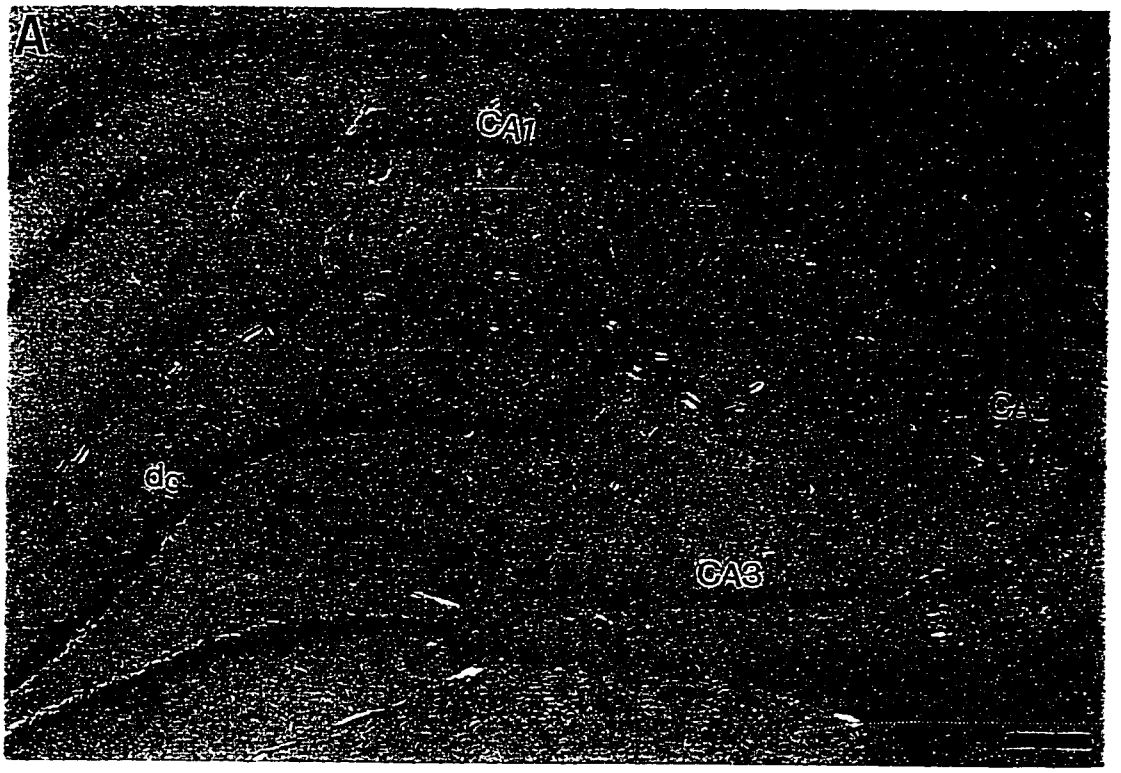
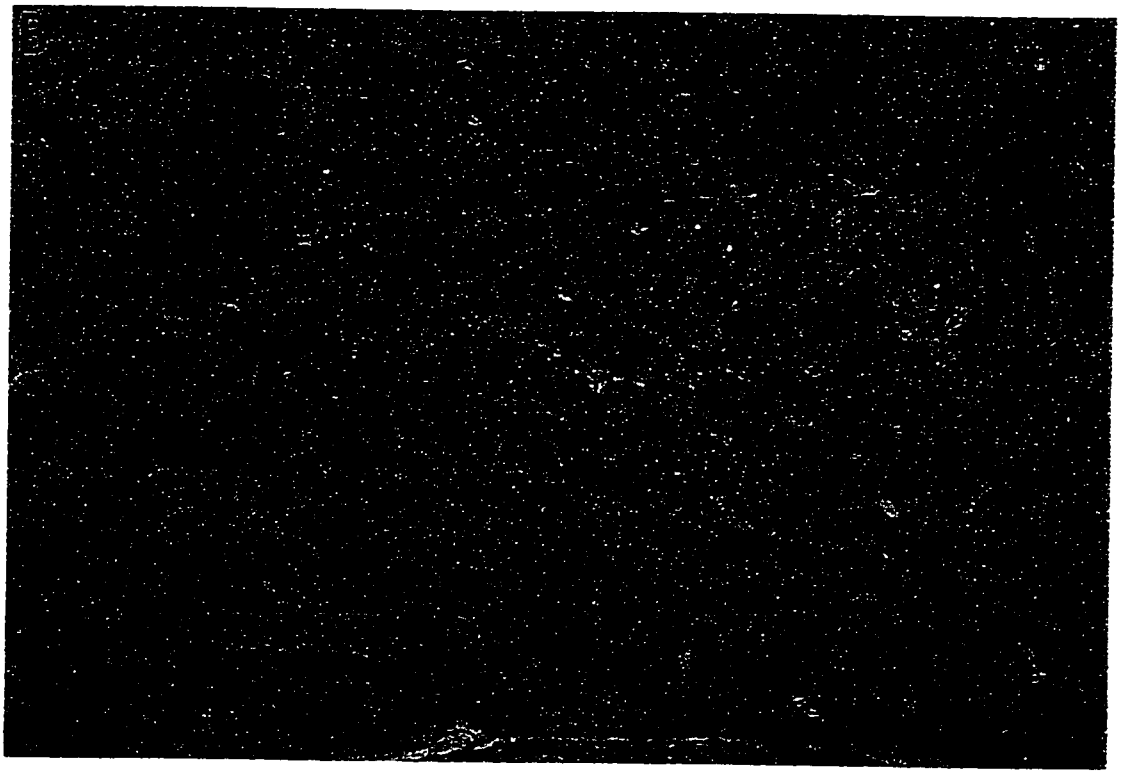

B
damage
% Damage
60
50
40
30
20
10
2
7
14
28
90
survival time (days)

Figure 2. H–E coronal sections of rat hippocampus showing hippocampal fields CA1, CA2, CA3 and dentate gyrus (dg). Arrows indicate CA1/CA2 subfield border. **A**: sham section illustrating healthy hippocampal formations. **B**: hippocampus after 7 days survival following 10 minutes of ischemia. At this magnification level neuronal state is difficult to evaluate other than the observation that CA1 in 2B appears to be sparsely populated compared with 2A. Scale bar = 187μm.


A
CA1
dg
CA3



3 minutes

Three minutes of ischemia did not produce cell loss in any field of the hippocampus at any of the survival times (Figure1). This can be seen in figure 4A-E. These photomicrographs illustrate healthy CA1 pyramidal neurons at all survival times.

5 minutes

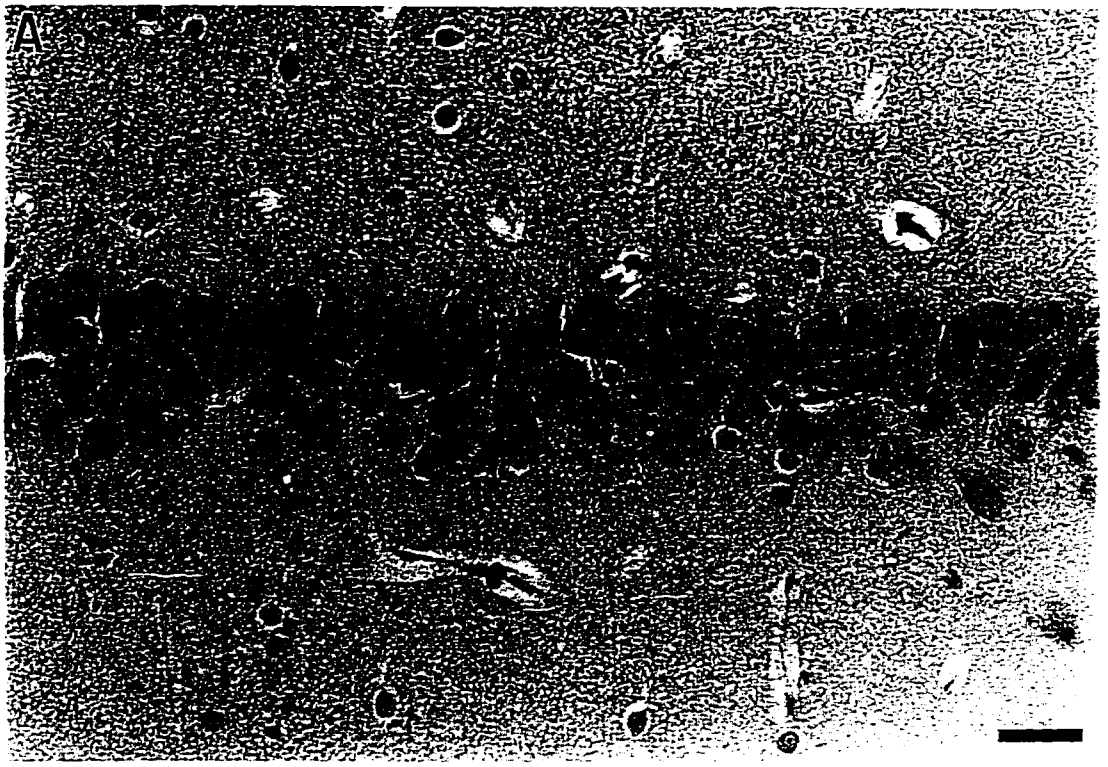
Post-hoc tests comparing the effects of five minutes of ischemia at 2, 7, 14, 28 and 90 days revealed no statistically significant differences. There was no cell loss observed in any animal, at any survival time in the other hippocampal fields.

Not all animals in the 14, 28 and 90 survival groups manifested neuronal death of CA1 neurons. In fact, 5 minutes of ischemia resulted in a lack of neuronal damage in the majority of animals. Four out of 9, 2 out of 9 and 3 out of 10 animals in the 14, 28 and 90 day survival groups displayed CA1 neuronal death. Figure 5E depicts eosinophyllic CA1 neurons 90 days, mixed with healthy pyramidal neurons 90 days after the ischemic insult.

7 minutes

Post-hoc analysis exposed a significant difference in CA1 pyramidal cell loss between two and ninety days survival following 7 minutes of ischemia. In figure 1A a progressive rise in CA1 neuronal death is seen with increasing survival time. The increase in neuronal death, compared with 2 days survival, did not achieve statistical significance until 90 days had elapsed. As seen with 5 minutes, 7 minutes of ischemia did not produce neuronal death in all animals. Four out of 7, 4 out of 5, 3 out of 7 and 7 out of 10 animals

Figure 3. H+E coronal sections of CA1 subfield of the hippocampus. **A**: sham section illustrating healthy pyramidal neurons. Neurons are round with pale cytoplasm and a distinct nucleus. **B**: Section illustrates damaged CA1 pyramidal neurons after 7 days survival following 10 minutes of ischemia. Compared with A, these neurons are shrunken, irregularly shaped, pyknotic, eosinophyillic (pinkish-red colour) and lack a clearly defined nucleus.
Scale bar = 23μm.


A

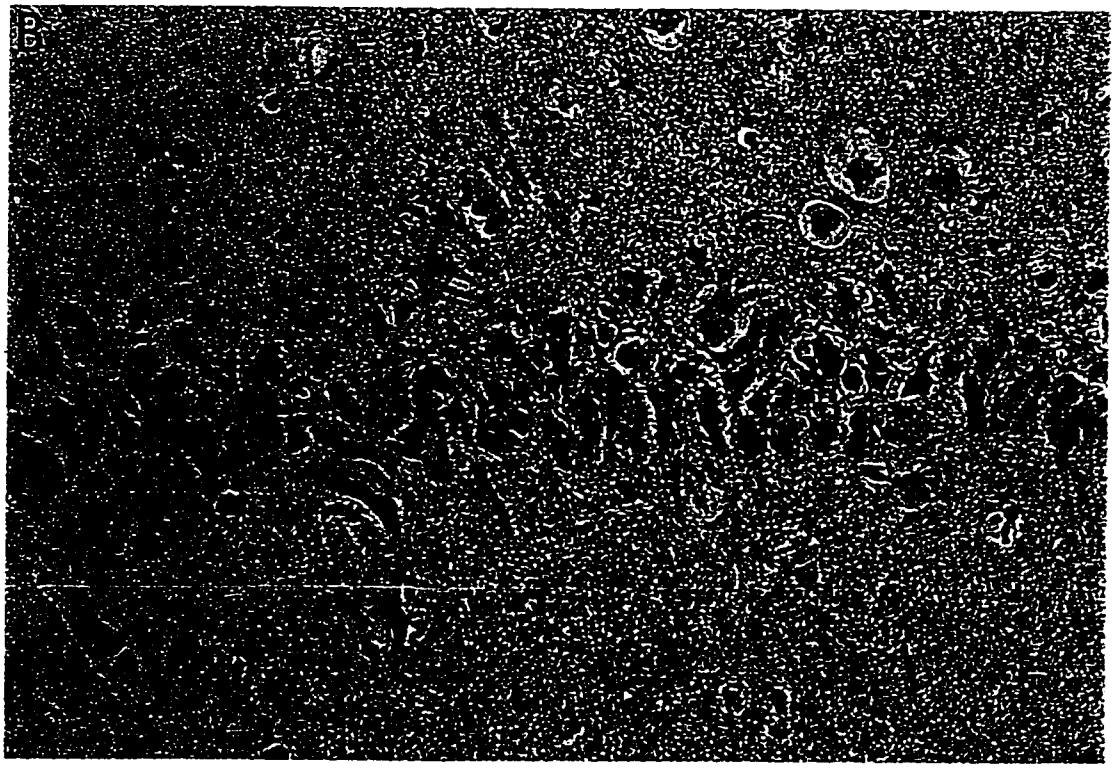
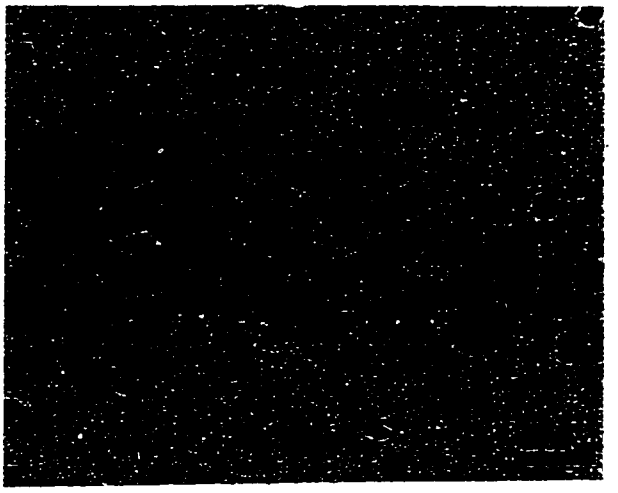
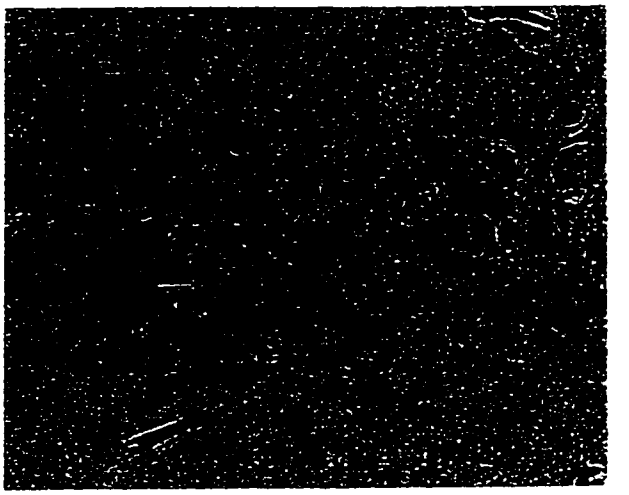
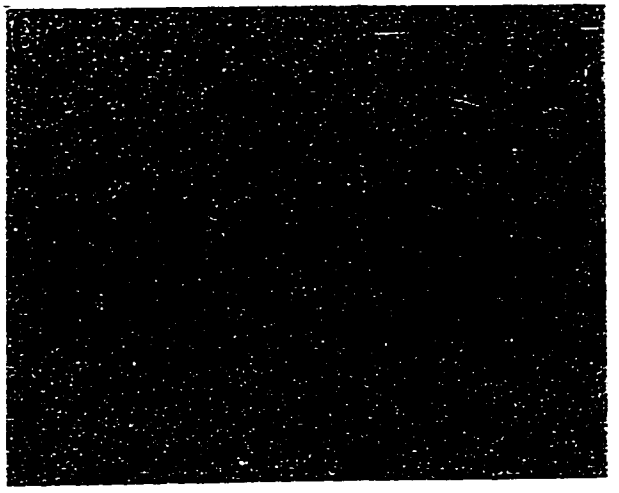
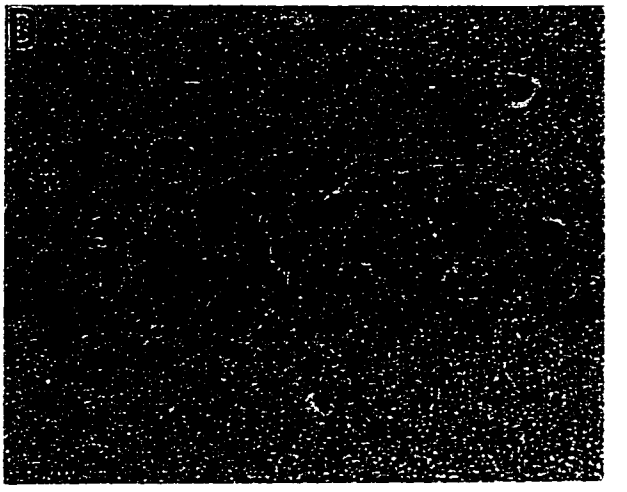

B

Figure 4. H+E coronal sections of CA1 subfield of the hippocampus following 3 minutes of ischemia. **A-E**: 2, 7, 14, 28 and 90 day's survival after ischemic insult. All pictures show healthy CA1 neurons that are round with pale cytoplasm, with a distinct nucleus. Scale bar = 23μm









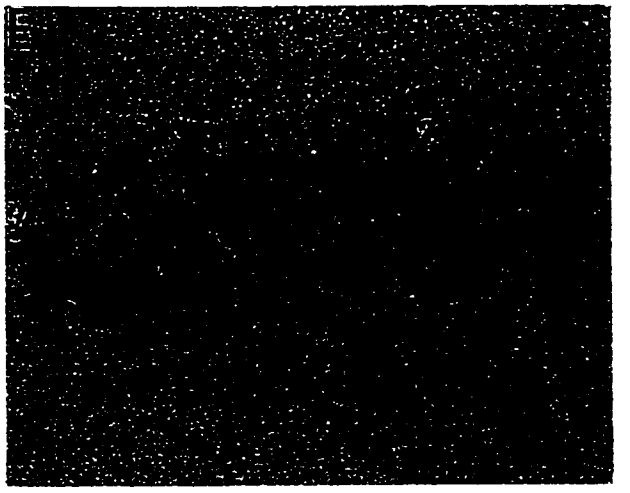
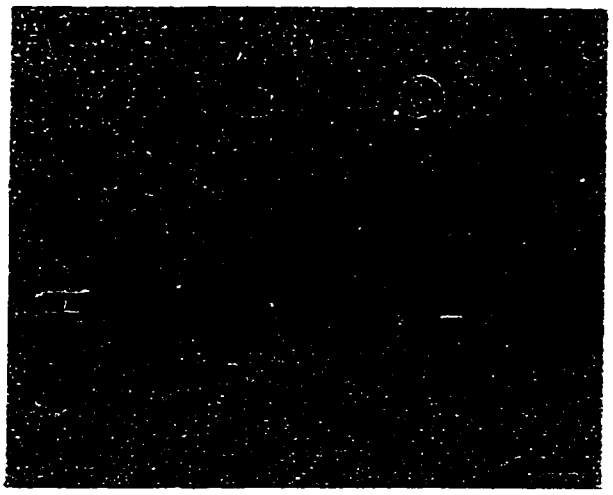
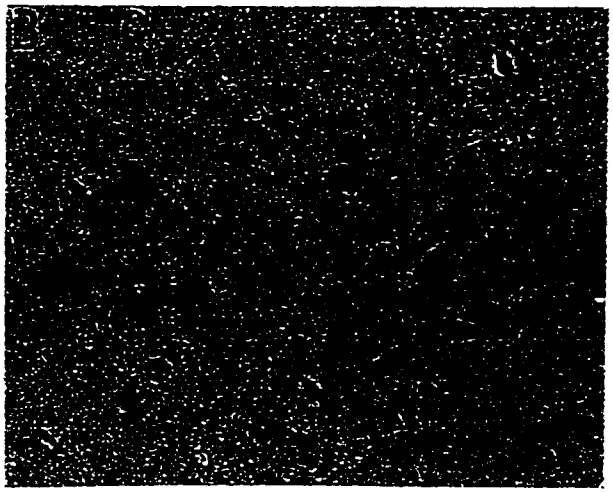
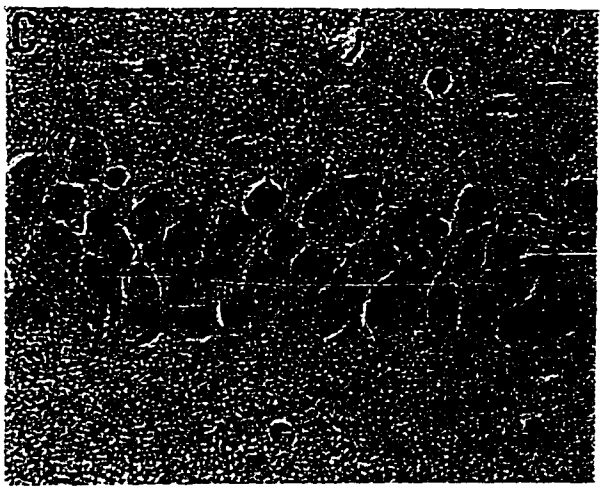
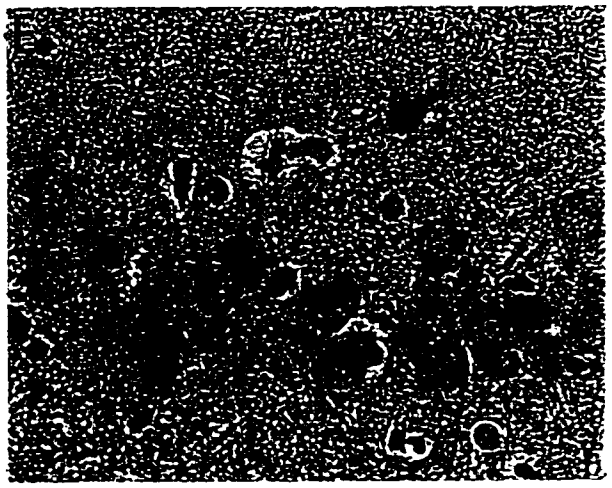


Figure 5. H+E coronal sections of CA1 subfield of the hippocampus following 5 minutes of ischemia. **A-E**: 2, 7, 14, 28 and 90 day's survival after ischemic insult. **A-D**: No damaged neurons are seen between 2 and 28 days survival. **E**: arrowhead points to a dead pyramidal neuron in this animal. The cell is eosinophyllic, no nucleus is visible and is dramatically shrunken compared to the neighbouring healthy neurons. Scale bar = 23μm.









were observed to have neuronal damage. No cell loss was ever observed in the CA2, CA3 and/or the CA4 subfields of the hippocampus.

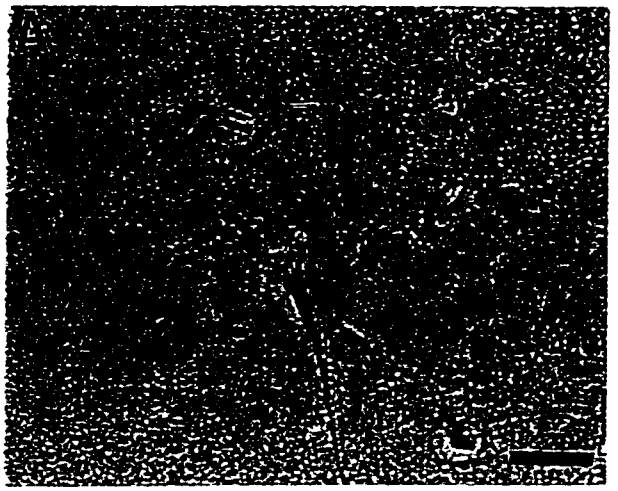
At 90 days survival 7 minutes of ischemia produced significantly more CA1 pyramidal damage (27.95%) than three or five minutes of ischemia (0 and 5.4% respectively) at the same survival time. There was no statistically significant difference in CA1 damage following 7 minutes versus three and five minutes of ischemia at all other survival times. There was an absence of eosinophyllic neurons in some of the animals 90 days after 7 minutes of ischemia (Figure 6E). However, as can be seen in figure 6E there is a significant reduction in the number of healthy CA1 neurons compared to control animals. In this animal there is a total of approximately 11 healthy neurons (figure 6E) compared to approximately 25 healthy neurons in figure 6A.

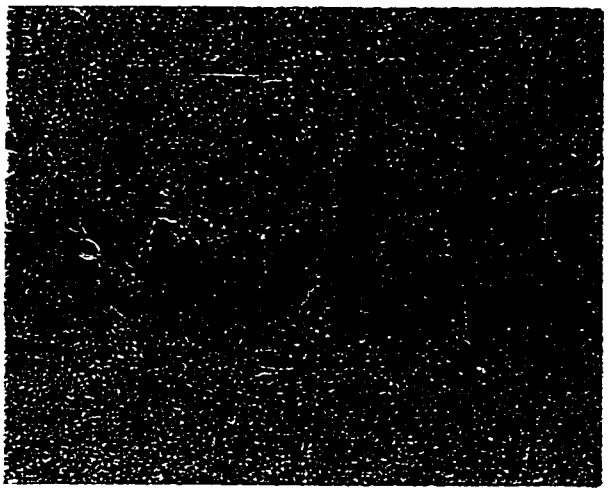
10 minutes

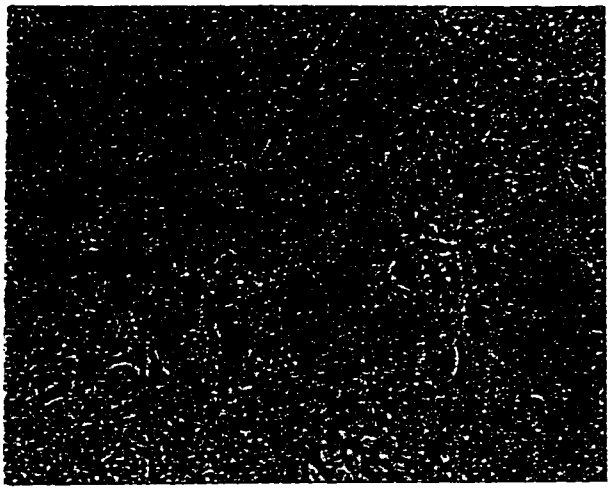
Post-hoc analysis resulted in a significant difference between cell loss at 2 days compared with that seen at 7, 14, 28 and 90 days. No other statistically significant difference was observed. Although not significant, average cell loss after 7 days was 70.5% but was 82.4% at 90 days survival; a difference of 11.9%.

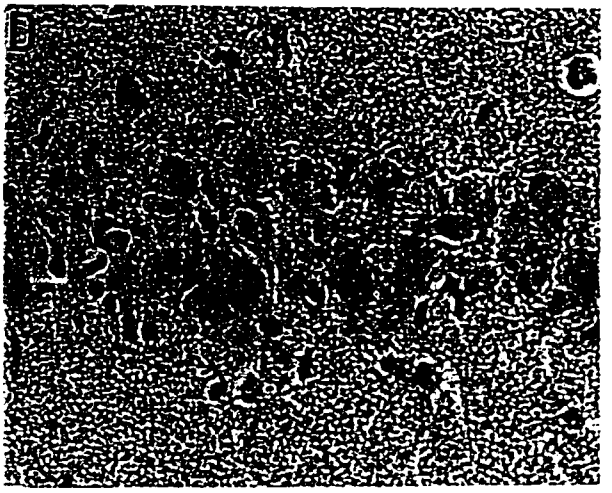
Quantification of cell loss of CA1 pyramidal neurons at 14, 28 and 90 days was complicated by the fact there was a significant reduction in the total number of cells compared to control values (i.e. 1145 cells). While there were morphologically normal looking cells in CA1, the vast majority of dead cells that are normally present in the CA1 field at earlier survival times following ten minutes of ischemia, had disappeared (Figure 7D&E). Percent neuronal death

Figure 6. H+E coronal sections of CA1 subfield of the hippocampus following 7 minutes of ischemia. **A-E**: 2, 7, 14, 28 and 90 days survival after ischemic insult. **A**. All neurons at 2 days survival appear to be healthy with the exception of one neuron in the middle of the picture. **B+C**. The appearance of a few eosinophyllic neurons with neighbouring healthy neurons. **D**. The presence of more damaged neurons compared to B+C. In addition to eosinophyllic neurons, this picture depicts small, irregularly shaped, darkly stained cells, appearing to surround damaged pyramidal neurons. **E**. An absence of eosinophyllic neurons at 90 days survival. Notice however the minimal amount of total neurons in CA1 compared to A and even B+C. Scale bar = 23μm.









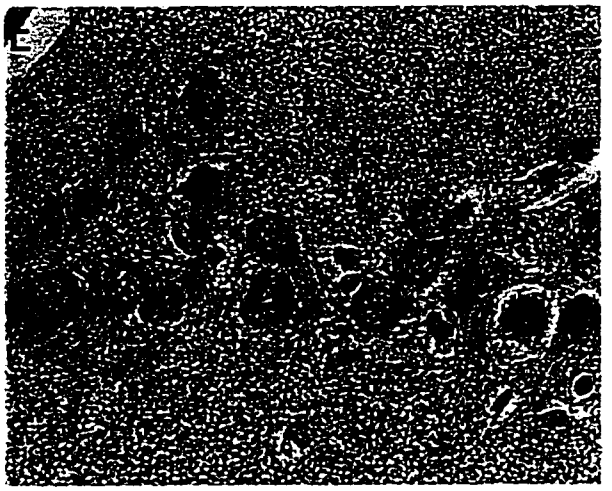
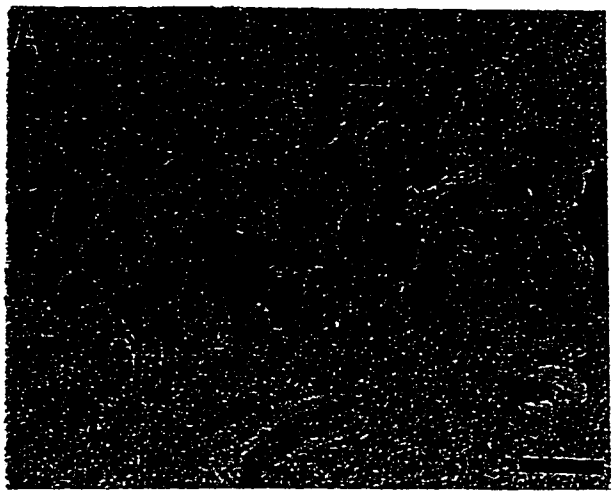
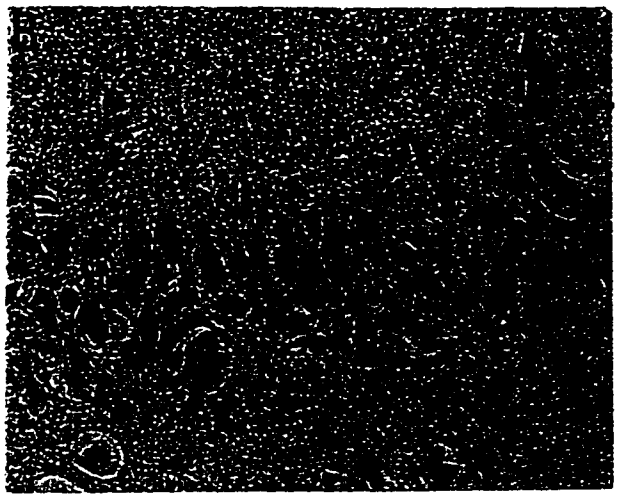
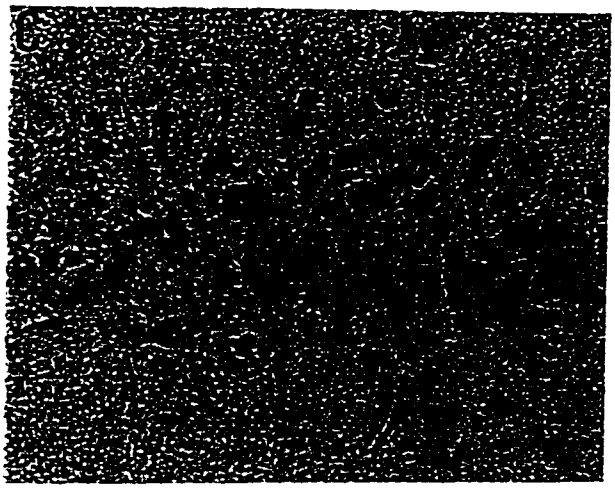
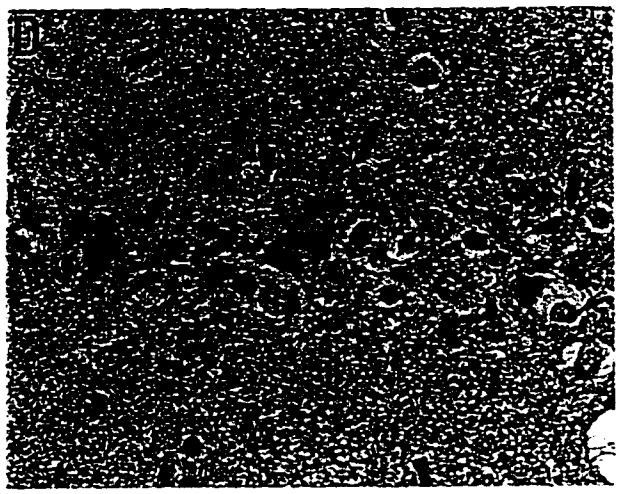


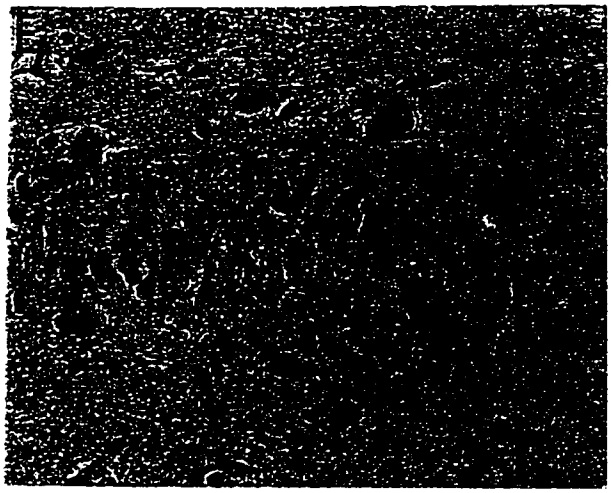
Figure 7. H+E coronal sections of CA1 subfield of the hippocampus following 10 minutes of ischemia. **A-E**: 2, 7, 14, 28 and 90 day's survival after ischemic insult. **A**. The appearance of damaged neurons mixed with healthy neurons at 2 days survival. Notice however that these damaged neurons are not as shrunken and eosinophyllic as those in B+C. **B+C**. severely damaged CA1 subfield. Many eosinophyllic neurons with few neighbouring healthy neurons. Notice the appearance in Figure 7C of small, dark, irregularly shaped cells as seen in Figure 6D. **D+E**. Well-defined healthy or damaged CA1 neurons are difficult to distinguish in these two micrographs. As in Figure 7C, D+E illustrate the same dark, irregularly shaped cells within the CA1 area. This is dramatically seen in E. Scale bar = 23μm.











because of 10 minutes of ischemia was statistically different from percent cell loss following 3, 5 and 7 minutes of ischemia at all survival times (Figure 1).

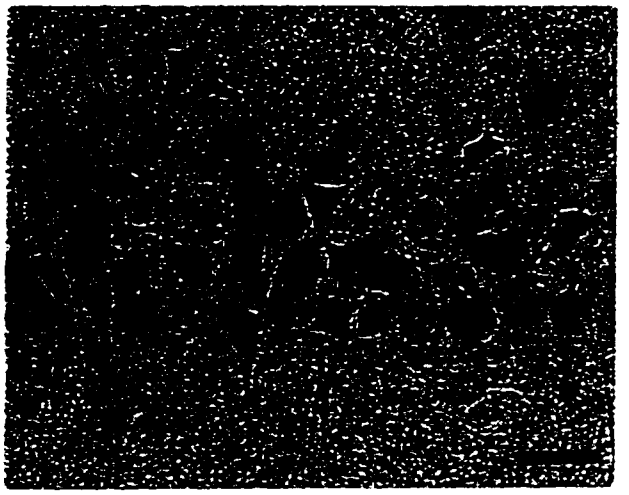
13 minutes

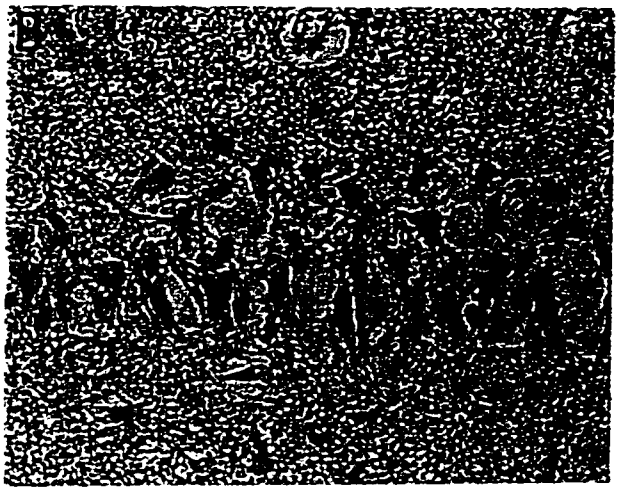
Post-hoc tests comparing percent cell loss at the five survival times exposed a significant difference between 2 and all other survival times (31.0 and 93.4 % respectively). While not statistically significant, cell loss of CA1 neurons at 90 days was 10.2% more then the amount of cell death seen at 7 days (83.3 and 93.4% respectively). As with 10 minutes of ischemia, the vast majority of eosinophyllic CA1 neurons, normally present in abundance at shorter survival times, were missing at 28 and 90 days following 13 minutes of ischemia (Figure 8D&E). Thirteen minutes of ischemia resulted in a significantly greater degree of cell loss of CA1 pyramidal neurons compared to 3, 5 and 7 minutes of ischemia at all survival times. The degree of cell loss between 13 and 10 minutes of ischemia was not statistically significant at all survival times. However, as can be seen in Figure 1, 13 minutes of ischemia did produce qualitatively more cell loss than 10 minutes at all survival times. 31.0 versus 12.9, 83.3 versus 70.5, 89.3 versus 70.7, 91.2 versus 79.1 and 93.4 versus 82.4 % at 2, 7, 14, 28 and 90 days respectively. There was a minimum of 11.0% difference in percent neuronal death between 13 and 10 minutes of ischemia at any measured survival time point.

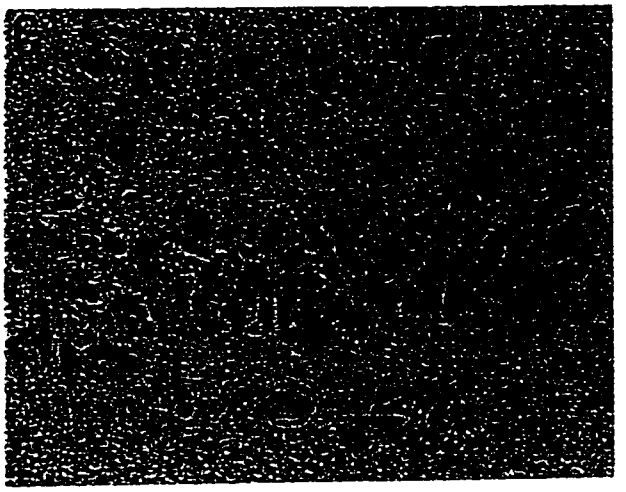
Ischemic Maturation

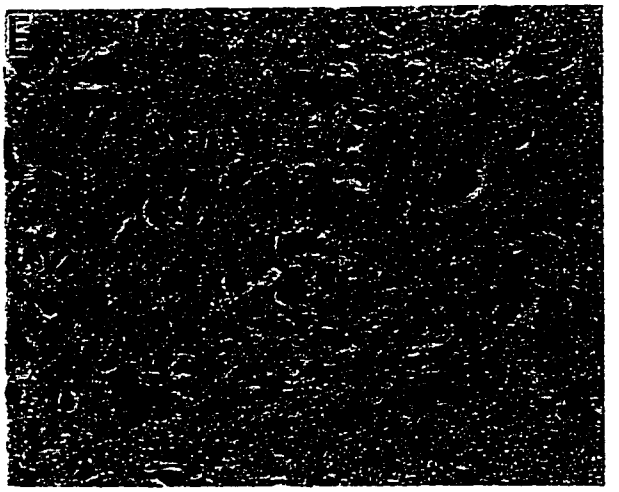
Figure 1B represents data collapsed over the severity of ischemia looking at the main effect of survival time on ischemic cell death in this study. Collapsing data across an

Figure 8. H+E coronal sections of CA1 subfield of the hippocampus following 13 minutes of ischemia. **A-E**: 2, 7, 14, 28 and 90 day's survival after ischemic insult. **A**. Damaged and healthy neurons are illustrated. Notice however that the damaged neurons depicted in A are not as shrunken or eosinophyllic as those seen in figures 8B+C. **B+C**. Severely damaged CA1 field with no healthy neurons visible in this micrograph. In figure 8C there is the occurrence of some small, dark, irregularly shaped cells. **D+E**. Difficult to distinguish damaged neurons, especially in D. In both pictures there is also a large number of small, dark, irregularly shaped cells throughout CA1. Scale bar = 23μm.





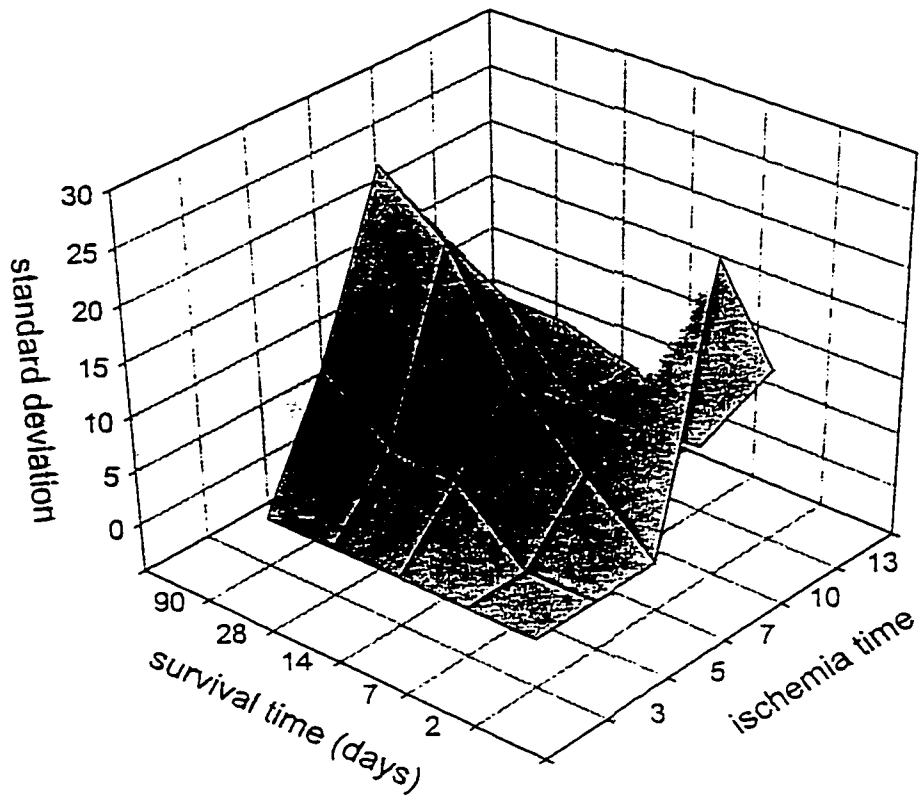




independent variable (i.e. ischemic severity), in order to investigate the main effect of another independent variable, is valid statistically if it can be shown that important information would be derived from such an analysis (Keppel, 1991). Average cell death at 90 days survival was significantly greater from that seen at 7 and 2 days survival. Moreover, cell death at 7,14,28 and 90 day survival were all significantly greater than that seen at 2 days survival. The slope of the line from 2 to 7 days appears to be much steeper than the slope from 7 to 90 days, possibly depicting 2 rates of cell death.

Variability

Figure 9 represents a 3-dimensional graph plotting the variability (standard deviation) of cell loss for all experimental groups. Most strikingly were the two peaks. One peak at seven minute – 90 and 28 days, the other at 10 and 13 minutes – 2 days. The variability of 7 minutes of ischemia clearly increased as survival time increased, peaking at a standard deviation over 25, at 90 days survival. Furthermore, 7 minutes of ischemia clearly had the largest amount of variability compared to all other ischemia times. On the other hand, the variability for 10 and 13 minutes of ischemia was largest at 2 days survival (standard deviations over 20) which subsequently leveled off to standard deviations of approximately 10 at all other survival times. Five minutes of ischemia followed a similar pattern as 7 minutes of ischemia in that variability increased as survival time increased.


standard deviation
30
25
20
15
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5
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28
14
7
2
survival time (days)
3
5
7
10
13
ischemia time

DISCUSSION

Ischemic maturation: CA1 pyramidal cell loss

In general, the vast majority of global ischemia studies have utilized a rigid protocol of ten minutes of ischemia followed by histological examination after 7 days of reperfusion. Studies that have investigated outside this protocol have either looked at the effects of a) one ischemia time with long survival times or b) different ischemia times within 7 days survival. To our knowledge this is the first attempt to examine the effects and interactions of multiple ischemic durations and multiple survival times. This study indicates that ischemic maturation progresses beyond 7 days (Figure 1B). Furthermore, it appears that both the duration of the ischemic insult and the duration of the survival period influence the proportion of CA1 cell loss (Figure 1B). The most striking example of this was the ischemic maturation seen following 7 minutes of ischemia. While a significant amount of cell loss at 7 days post-ischemia is consistently seen following 10 minutes of ischemia (see Table 3), a very small amount was produced following 7 minutes of ischemia at 7 days. If this were a typical study, histological examination would have been performed at 7 days with the conclusion that 7 minutes of ischemia produced a relatively weak insult. However, extending survival time past 7 days revealed a slow progressive neuronal degeneration such that by 3 months the degree of cell loss had increased approximately 9 fold. Interestingly, a similar interaction between duration of ischemia and length of survival has been observed in retinal ganglion cell (RGC) death following transient pressure-induced ischemia (Selles-Navarro et al., 1996). It should be emphasized that this model is methodologically distinct from our model. Retinal ischemia is produced by applying intraocular pressure (Selles-Navarro et al., 1996).

Ten and thirteen minutes of ischemia qualitatively produced a different pattern of cell loss compared to that seen following seven minutes (Figure 1A). Firstly, cell loss of CA1 neurons was observed starting at two days following ten and thirteen minutes of ischemia; no cell loss was observed two days following seven minutes of ischemia. Secondly, ten and thirteen minutes produced a "rapid" neuronal degeneration of CA1 neurons during the first seven days of survival. Seven minutes of ischemia lacked this "rapid" neuronal degeneration seen with the higher ischemia times. For purposes of the following discussion "rapid" onset of cell loss will refer to cell loss which occurs within the first seven days of reperfusion.

Unfortunately, increase in cell death after 7 days survival following 10 and 13 minutes of ischemia was qualitative only, lacking statistical significance. Statistical significance may have been achieved if it weren't for the variability in this study (i.e. cell death at 90 days survival being significantly different from 7 days survival). This could be a realistic prediction considering that average cell loss at 90 days survival was approximately 12 and 13 percent greater than that at seven days following ten and thirteen minutes of ischemia, respectively. In the event of a significant finding , it would suggest that a slow progressive loss of an additional 12-13 percent of CA1 neurons could represent a mechanism distinct from the mechanism(s) that caused the initial rapid cell loss manifested at seven days survival. Such a possibility would indicate that seven minutes of ischemia is not severe enough to cause a "rapid" onset in cell loss, but instead slowly activates a slow progressive cell loss pathway. However, Figure 1B does appear to show two rates of cell death when collapsed over ischemic severity. The possibility exists that the gentler slope in cell loss between 7 and 90 days is due to mechanism(s) distinct from that producing the steep cell loss within seven days. More experiments are required before an accurate assessment concerning this issue can be made.

If a slow progressive loss in cell death is a real event, its characteristics and mechanism are yet to be discovered. This question is not easily answered considering the fact that it remains uncertain, and controversial, what causes delayed neuronal death within the first seven days. Apoptosis could be one candidate that accounts for slow progressive cell loss observed in this study. Recall that MacManus et al. (1993) observed a combination of apoptosis and necrosis following global ischemia and proposed that a continuum of damage may exist from apoptosis to necrosis depending on the severity of the insult and on the length of time allowed for reperfusion. This continuum has also been observed in the ischemic kidney (Schumer et al., 1992). Schumer et al. (1992) found that five minutes of ischemia resulted in the appearance of apoptotic bodies and DNA fragmentation with no sign of cellular necrosis. However, as the severity of the ischemia was increased necrotic cell loss became more prominent.

Such a continuum between apoptotic and necrotic cell loss may differentiate the slow progressive cell loss from the faster cell loss observed in this study. Necrotic cell loss could be responsible for the fast rise in cell loss occurring during the first seven days, followed by slow the slow progressive apoptotic death over weeks. This continuum, like that for the ischemic kidney (Schumer et al., 1992), could be dependent upon the severity of the insult. Hypothetically, ten and thirteen minutes of ischemia could produce both apoptotic and necrotic cell loss which is manifested by both a slow and fast rate of death. Seven minutes of ischemia, however, appears to only produce a slow progressive death over weeks. Possibly, seven minutes of ischemia is not potent enough to produce the fast cell loss during the first seven days, but is sufficient to initiate the slow progressive cell loss, perhaps due to apoptotic mechanisms. One problem with this explanation is the observation that markers for apoptosis are seen within seven days following

ischemia (MacManus et al., 1993; Nitatori et al., 1995; Iwai et al., 1995; Schumer et al., 1992) and whether programmed cell loss continues weeks after its initiation is uncertain and awaits testing.

Another possible explanation for slow progressive cell loss is the development of focal seizure activity (Bonnekoh et al., 1990) within the hippocampal area. Bonnekoh et al. (1990) suggest that surviving CA1 neurons, in association with reactive glial proliferation, may contribute to the development of epileptogenic foci. It has been observed that patients suffering from chronic epilepsy suffer CA1 neuronal damage (Scholz, 1951;cited in Bonnekoh et al., 1990), and the mechanism behind this cell loss is believed to be the result of increased intracellular calcium (Plum et al., 1968). The similarity of epileptogenic-induced CA1 neuronal cell loss to ischemia-induced cell loss is intriguing. Bonnekoh et al. (1990) observed the presence of glial and phagocytes 3 weeks, six months and even up to ten months in the CA1 sector of the hippocampus following five minutes of ischemia in the gerbil. Therefore, it is possible that the slow progressive cell loss seen in this study is the result of an epileptogenic mechanism that is initiated, potentially, weeks post-ischemia.

Nakano et al. (1990) briefly mentioned the possibility of focal seizure activity leading to the slowly progressive cell loss they observed following focal ischemia. However, they discounted this as a possible explanation since they reported that epileptic seizure activity was rarely if ever observed. Similarly, we also did not observe clinically obvious signs of seizure activity in any of the animals following all ischemia times. However, animals that survived greater than seven days were not observed on a daily basis leaving the possibility that seizure activity was present in some animals. It is also possible that there was seizure activity within the

hippocampus of these animals in the absence of any overt behavioural signs epileptogenic activity.

A slow progressive cell loss has been reported in the optic tract and superior colliculus following ischemia in the rat (Gallyas et al., 1992). Gallyas et al. (1992) failed to observe cell loss in the optic tract and superior colliculus during the first five days of survival (short-term group). However, cell loss appeared in these regions 1-5 months following fifteen minutes of ischemia. Apparently, research investigating ischemia-induced optic tract cell loss has followed a similar trend to hippocampal cell loss in that previous histological investigations had been devoted to short-term ischemic neuronal damage. While they comment that the reason for such a long delay in cell loss is unclear, they found evidence-suggesting ischemia might initially cause damage to retinal ganglion cells or their presynaptic elements with the subsequent degeneration of the optic tract a consequence of this initial insult.

Variability:

This study disclosed areas of variability within the 2-VO paradigm. The source of this variability was primarily from two populations of animals –: 1) ten and thirteen minute animals at two days survival, 2) seven-minute ischemia animals at twenty-eight and ninety days survival. Delayed neuronal death of CA1 hippocampal neurons has been consistently reported to begin approximately 48-72 hours post-ischemia (Kirino, 1982; Pulsinelli et al., 1982). The large variability in degree of cell loss at two days survival, following ten and thirteen minutes of ischemia, is probably due to this approximate time frame in which cell loss is believed to start. Since brain fixation was performed at 48 hours, a variation in the extent of damage could be expected. This explanation is supported given that the variability in cell loss for ten and thirteen

minutes is reduced approximately 50% by seven days survival. There was not any variability at two days following three, five, and seven minutes of ischemia as no damage was ever seen at this survival time point.

Seven minutes of ischemia produced the highest variability in cell loss compared to all ischemic groups. The amount of variability increased as survival time increased. The source of this variability was the result of an increase in the range of cell damage seen as survival time increased. At three months survival, for example, the degree of cell loss ranged from as low as no damage observed to as high as 80% damage. The interesting question is why do some animals following seven minutes of ischemia acquire zero damage, across all survival times tested, while some acquire 80% damage by three months? This may be because seven minutes of ischemia is close to the ischemic threshold required to produce neuronal death. Ischemia times at or close to threshold could result in increased variability because animal variability may influence ischemic outcome. Animal variability for the purposes of this discussion refers to the notion that some animals may possess better endogenous neuroprotective mechanisms than others may. Presumably, this diversity becomes less and less of a contributing factor as the severity of the ischemic insult increases overriding any endogenous protective mechanisms.

A number of ideas have been put forward suggesting the existence of a variety of endogenous neuroprotective mechanisms and these could contribute to variability in ischemic outcome. First, some researchers have suggested that animals possess variations in their cerebrovascular anatomy (Berry et al., 1975). Therefore, during incomplete ischemia (i.e. 2-vessel occlusion model) this would potentially allow for greater perfusion to ischemically vulnerable areas in some animals compared to others. Further support for the brain possessing endogenous neuroprotectants stems from ischemia tolerance studies. Briefly, induction of a non-

lethal ischemic insult has been shown to provide neuroprotection from a subsequent lethal insult (Matsushima & Hakim, 1995; Perez-Pinzon et al., 1996; Kato et al., 1995). An explanation for this protective effect is unknown, but evidence suggests that the non-lethal insult "activates" stress proteins, such as heat-shock protein, that protects CA1 neurons from the subsequent lethal insult (Liu et al., 1992;Nowak, 1985). Therefore, the influence of endogenous neurprotectants in the brain could account for the variability seen at lower ischemia times in this study. These protectants do not influence ischemic outcome to the same degree at longer ischemia times presumably because they fail to offset the severity of the insult.

Two other factors that could also produce variability in ischemic outcome are temperature and/or anesthesia effects. Hypothermia is a potent neuroprotectant (Coimbra & Wieloch, 1994; Colbourne &Corbett, 1994), while hyperthermia has been shown to exacerbate damage following both focal (Kim et al., 1996; Morimoto et al., 1997) and global ischemia (Baena et al., 1997). Moreover, fluctuations of just 2-3 degrees from physiological body temperature are required to produce these temperature effects. Most studies, including our own, that try to maintain normothermic body temperature, tend to use rectal probes during the recovery period after an ischemic insult. While, this system keeps the core body temperature of the animal at approximately 37 degrees Celsius, Colbourne et al. (1993) showed that rectal temperature monitoring is not a reliably represent intra-ischemic brain temperature. Dale Corbett who uses a temperature monitoring system that measures intra-ischemic brain temperature in real time, found that intra-ischemic brain temperature fell 1.5 degrees from normal even though rectal and even skull temperatures remained at 37 degrees. Therefore, variation in our study could arise from some animals having more hypothermic intra-ischemic brain temperatures than others. This of course would reduce the damage seen in these animals.

The use of barbiturates (e.g. phenobarbital, pentobarbital), have been shown to provide neuroprotection following both global (Nordstrom & Rehncrona, 1977;Smith et al., 1980;Ishimaru et al., 1995) and focal ischemia (Warner et al., 1996). Kawagoe et al. (1993) observed that application of 40mg/kg of pentobarbital attenuated CA1 neuronal death and inhibited the induction of heat shock proteins HSP70 and HSC70, indicators of stress. They concluded that pentobarbital's neuroprotective actions could be due to a reduction in ischemia-induced stress. However, they also reported hypothermic effects of pentobarbital after 30 and 60 minutes of reperfusion that could also account for neuroprotection of CA1 neurons. Interestingly, Murase et al. (1993) reported neuroprotective effects of pentobarbital limited to short ischemia times following ischemia in the gerbil. The protective effects of the barbiturate were lost at longer ischemia times presumably because the insult was too severe. This study also used pentobarbital. Therefore, use of this anesthetic could account for some of the variability seen in this study, especially the variability following shorter ischemia times (i.e. five and seven minutes). . However, in defense of our model it must be noted that although intraischemic divergence between core and cerebral temperature is well described, no variation between core and cerebral temperature has been documented in the recovery phase. This study used tympanic temperature monitoring during ischemia that correlates well with brain parenchymal temperature (unpublished observations).

This discussion of variability raises the important issue concerning the consistency of animal models of stroke. Obviously it is very important that any animal model of ischemia provide a consistent degree of neuronal damage for a given ischemic severity. The ability to provide such consistency is especially important when the models are used to test potential neuroprotective agents. The amount of apparent "neuroprotection" provided by the drug is

suspect if the control group lacks consistent damage. This study suggests that using ischemia times less than ten minutes would be inappropriate for investigating the effects of potentially therapeutic agents. However, investigating the differences between animals that manifest resistance to ischemia at seven minutes, for example, versus those that acquire CA1 cell loss could provide important answers.

Implications:

Three important implications can be derived from the observation that the ischemic maturation process extends beyond seven days. The first regards the use of potentially therapeutic agents. One ultimate aim of ischemic research is the discovery of potentially therapeutic agents that could be used clinically to alleviate neuronal damage due to stroke. Our results suggest that a single bolus of "anti-ischemic" medication given to a stroke victim 24-48 hours following an ischemic attack may not be effective in the long term. Recall that Kiyota et al. (1995) showed that dimethylthiourea did not protect neurons, but only delayed the eventual neuronal death. Therefore, our results suggest that patients who have suffered from an ischemia attack be *chronically* medicated with the "anti-ischemic" drug. With this in mind, animal models of stroke should evaluate the efficacy of drugs well beyond seven days to ensure long lasting neuroprotection. Drugs may need to be given at least up to six months post-insult. Colbourne & Corbett (1995) showed a 20% decrease in neuroprotection, provided by hypothermia, at six months compared to that at one month. Dietrich et al (1993) reported significant neuroprotection provided by hypothermia to CA1 neurons at seven days, but found that this protection was absent by two months. Finally, these results concur with Nurse and Corbett's (1996) conclusions that costly clinical trials evaluating potentially therapeutic agents are confounded if short survival time protocols are used.

Secondly, the results of this study have important implications in the way in which we think about mechanisms of cell loss following an ischemic insult. The brain may possess mechanisms that continue or are turned on weeks after the insult and result in cell loss in a very slow and progressive manner. Hsu et al. (1994) has suggested the effects of ischemia might endure the entire life of the animal. While this assertion of a life-long maturation process may be considered an extreme position which does not find much support in conventional long term clinical observations of stroke victims, the notion in the past that delayed neuronal death was complete seven days post-ischemia should now be considered equally extreme.

Thirdly, the large variability seen in this study appears to be an inherent property of this model *at mild ischemia times* and indicates that we are close to the threshold required to produce cell loss. Interestingly, Smith et al. (1984) also reported large variability using mild ischemia times compared to the standard ten minutes. Defining a specific threshold such as 6.5 minutes, for example, becomes difficult knowing that animal differences could significantly influence ischemic outcome following ischemia times close to threshold. Using the term "ischemic switch" (see introduction), therefore, is incorrect. A better term is "ischemic range". For example, in this study the ischemic range would be between five and seven minutes of ischemia. Using a range of values more accurately describes our results because it takes into consideration that the ischemic threshold fluctuates from animal to animal depending upon how sensitive or resistant they are to an ischemic insult.

An important variable that must be considered when defining an ischemia range is survival time. Consider seven minutes of ischemia in this study. Seven minutes of ischemia produced zero percent damage in all animals at two days and mild insults at 7 days survival. In the past histological analysis would have probably stopped at seven days post-ischemia. Under

this protocol, this study would have wrongly concluded that seven minutes of ischemia produces a weak ischemic insult, missing the appearance of a significant amount of damage by 90 days of reperfusion. While it seem improbable that three minutes of ischemia would not have resulted in CA1 cell loss past three months survival in this study, this possibility still exists. In other words, while the amount of cell loss incurred is dependent upon the severity of the ischemic insult, the amount of time allowed for survival is equally important when determining if a given ischemia time meets the criterion for threshold.

Selective vulnerability within hippocampus:

Only CA1 hippocampal neurons were selectively vulnerable to an ischemic insult in this study. The other hippocampal fields were resistant to an ischemic attack up to 3 months of reperfusion. The only exceptions were two animals that manifested a few dead cells in the CA4 subfield following 13 minutes of ischemia. It is striking to see extensive cell loss throughout the CA1 field which abruptly stops at the CA1/CA2 border (see figure 2). What biological differences exist between the hippocampal fields to account for this phenomenon? At present a definitive answer to this question remains elusive. However a significant amount of research has been performed investigating the differences between vulnerable CA1 neurons to the resistant neurons of the other hippocampal fields. Most research has focused on investigating biological differences between CA1 and CA3 subfields. Silver and Erecinska (1992) observed a rise in intracellular calcium after approximately 2 hours of reperfusion in CA1 pyramidal neurons following 8 minutes of ischemia. This effect was not seen in CA3 neurons. Moreover, changes in intracellular calcium in CA3 were, in general, much smaller and slower than in the CA1 subfield. Due to the fact that calcium is postulated to be of prime importance in ischemia-induced cell

loss, a difference in calcium fluxes between CA1 and CA3 neurons could account for their differing susceptibilities. Hashimoto et al. (1992) reported preservation of energy metabolism in CA3 but not CA1 neurons, notwithstanding increased intracellular calcium levels, following ischemia. They suggest that a CA3 neurons ability to retain energy metabolism renders them resistant to an ischemic insult as they are able to cope with the large rise in intracellular calcium.

As mentioned above, it has been observed that ischemia can cause a reduction in the GLUR2 subunit of the AMPA receptor making the receptor permeable to calcium ions (Pellegrini-Giampietro et al., 1992; Hume et al., 1991). This phenomenon is thought to be one potential mechanism that could result in cell loss. Support for this theory is strengthened with the observation that the reduction in the GLUR2 AMPA-receptor subunit was only seen in the CA1 field of the hippocampus prior to CA1 neuronal death (Pellegrini-Giampietro et al., 1992). The reduction was not seen in the ischemically resistant CA3 or dentate regions. Since glutamate is believed to be a key player in ischemic-induced cell loss, regional differences in glutamate concentrations could help explain CA1 field vulnerability versus CA3 resistance. Mitani et al. (1992) measured the levels of released glutamate during ischemia in the CA1 and CA3 fields. They reported that extracellular glutamate levels were increased in both fields of the hippocampus. Mitani et al. (1992) concluded that an increase in glutamate during ischemia does not play a pivotal role in the subsequent cell loss of CA1 neurons. However, an increase in glutamate could still be pivotal despite the finding that glutamate levels increase in both fields. Extracellular glutamate levels may increase in both fields but differences in glutamate receptor subunits, second messenger systems and calcium buffering and sequestering abilities, for example, between CA1 and CA3 fields could account for CA1 vulnerability versus CA3 resistance.

Ischemic-induced cell loss could also arise from a reduction in GABAergic inhibition. For example, Luhmann et al. (1995) reported a significant reduction in inhibition of rat cortical neurons 6 months after ischemia. Obviously, a reduction in inhibition would lead to an imbalance between excitation and inhibition with the net result being hyperexcitability. It is interesting then that Li et al. (1993) observed an initial reduction in GABAA receptor alpha 1 and 2 mRNA expression in all fields of the hippocampus. However, mRNA levels returned to normal in the CA3 and dentate fields after 4 to 12 hours of reperfusion, but continued to decline in the CA1 field over the next 3 days as the cells degenerated. Therefore, loss of GABAergic inhibition could contribute to the development of neuronal degeneration in CA1 whereas the other fields remain intact due to preservation of a balance between excitation and inhibition.

Other differences between CA1 and CA3/dentate regions include gene expressions of acetylcholine receptor subtypes (Hsu et al., 1996) and heat-shock protein (Ferrer et al., 1995;Kato et al., 1993), levels of brain-derived-neurotrophic factor (BDNF) (Kokaia et al., 1996), nerve-growth factor (NGF) (Lee et al., 1995) and superoxide dismutase (Kato et al., 1995;Liu et al., 1993) and stimulation of tyrosine phosphorylation (Hu & Wieloch, 1994) and protein synthesis (Bergstedt et al., 1993). Clearly many biochemical and structural differences exist between CA1 compared to the other hippocampal fields. Whether or not these differences translate into definitive explanations for CA1 vulnerability versus CA3/4 dentate resistance remains to be elucidated.

Lateral versus medial sensitivity

Just as there are regional differences in the degree of vulnerability to an ischemic insult within the hippocampus, the same phenomenon holds true within the CA1 field. In this study the medial region of the CA1 and the subiculum were far more sensitive to ischemia than the lateral

CA1 region. This observation has been observed by others (MacManus et al., 1995;Iwai et al., 1995;Baena et al., 1997). MacManus et al. (1995) reported DNA damage started in the medial hippocampus and over the next 7 days progressed laterally. At 2 days survival there was a greater degree of cell loss within the medial CA1 area compared to the lateral region, following ten and thirteen minutes of ischemia. Furthermore, any CA1 neurons that survived seven, ten and thirteen minutes of ischemia, at longer survival times, were primarily located within the lateral CA1 region. This phenomenon suggests one of two possibilities. Firstly, CA1 neurons are more sensitive to ischemia than lateral CA1 neurons. What accounts for this difference in sensitivity is presently unknown. Presumably, it could suggest that there is a gradual change in CA1 medial to lateral cells make up (e.g. genes, metabolic machinery) that results in a gradual change in pyramidal sensitivities to ischemia medially to laterally. To our knowledge a gradient in sensitivities to ischemia from medial to lateral CA1 has not been documented. Alternatively, the pyramidal population isn't heterogeneous but rather the density of afferents innervating medial CA1 neurons could be greater than those neurons innervated more laterally. If the effects of these afferents upon hippocampal CA1 neurons were responsible for CA1 cell loss, then a greater density of afferents medially versus laterally could translate into a more potent "death" signal medially versus laterally. This possibility could be tested via deafferentation studies and or pharmacological blockade of potential synaptic inputs.

The second possibility is whatever death mechanisms are initiated by ischemia begin in the medial CA1/subiculum region and progress laterally towards the CA2 field of the hippocampus, abruptly stopping at the CA1/CA2 border. This however would be in the opposite direction suggested by experiments that cut the Schaffer Collateral's before induction of ischemia. CA3 neurons project to the lateral CA1 via the Schaffer Collateral's. Some

investigators have reported protection of CA1 neurons if the Schaffer Collateral's are cut, effectively disconnecting CA1 from CA3 (Diemer et al., 1993;Kaplan et al., 1989). This would suggest that the progression of damage would move from lateral to medial CA1. However, histologically the progression of cell loss clearly proceeds from medial to lateral CA1. The discrepancy in these findings is unclear. Although it should be noted that not all researchers have shown successful neuroprotection to CA1 neurons with cutting of the Schaffer Collateral's (Buchan & Pulsinelli, 1990).

If ischemia initiates mechanisms of cell loss beginning in the medial CA1/subiculum region, it could suggest that a substance(s) is released which slowly diffuses laterally in CA1. A substance that meets this criterion is nitric oxide. As noted above, evidence exists which suggests that nitric oxide could be involved in delayed neuronal death. Interestingly, Kato et al. (1995) reported that the CA3 and dentate fields of the hippocampus initially lost and then recovered immunostaining for superoxide dismutase, an enzyme that scavenges nitric oxide, while immunostaining never recovered in CA1 neurons. This could account for both the progression of cell loss from medial to lateral CA1 and why cell loss abruptly stops at CA3/CA1 border.

Future experiments

Our results provide intriguing questions to be answered in future experiments. The most obvious experiment to be performed is focusing on ischemia times between three and five minutes. Three minutes of ischemia produced zero percent damage in all animals, while five minutes produced a very mild degree of cell in approximately 30% of animals. Looking at ischemia times between three and five minutes would determine if three minutes of ischemia represents the longest ischemia time that *does not* result in any cell loss. Once this is

accomplished the more interesting question involves comparing the biochemical, genetic and /or structural differences between those ischemia times above versus below threshold could be tackled. The information gained from this analysis would be of tremendous benefit towards discovering potential neuroprotective agents. For example, theoretically assume that ischemia time A produces cell loss, while ischemia time B does not. A comparison of protein expression between these ischemia times shows that ischemia time A resulted in activation of a particular protein, while ischemia time B did not. A pharmaceutical agent could then be used to block the expression of that protein in an effort to see if any protection was offered. If no protection was offered, or the drug provided partial protection, other differences could then be explored and targeted with pharmaceuticals.

Our study showed that seven minutes of ischemia resulted in a slow progressive cell loss over three months. Referring back to figure one, it appears that the amount of cell loss following seven minutes of ischemia has not plateaued at three months survival. This suggests that the amount of cell loss may have continued to increase if survival time was extended beyond three months. The same could also be true for five minutes of ischemia. Moreover, the few cells that were remaining in CA1 following ten and thirteen minutes of ischemia at three months could also eventually have met their death if survival time was extended beyond three months. There is every reason to believe that the maturation process could extend beyond three months survival considering that the effects of ischemia have been reported at 6-10 months post-ischemia (Luhmann et al., 1995; Bonnekoh et al., 1990), and remarkably even up to twenty-seven months post-insult (Hsu et al., 1994). Therefore, a future study should be performed that extends the survival period well past three months. Survival times of six months to a year would hopefully answer these questions.

An important study that needs to be performed is a determination of the types of cell loss initiated at different ischemia times and over time. Specifically, testing for signs of apoptotic and necrotic cell loss. Recall one of the explanations for observation of the fast delayed neuronal death within 7 days post-ischemia versus the slow progressive cell loss seen over weeks is one primarily results from necrotic cell loss, while the other from apoptotic cell loss. This gradient between apoptotic and necrotic cell loss has been observed in the ischemic kidney (Schumer et al., 1992). Furthermore, MacManus et al. (1993) concluded that both modes of cell loss comprise CA1 neuronal death, and proposed that a continuum between apoptotic and necrotic cell loss exists that is dependent upon both the severity of the insult and the length of survival. Interestingly, Nosseri et al. (1994) observed that U937 human myeloid leukemia cells underwent apoptosis with application of hydrogen peroxide and hyperthermia. However, when the hydrogen peroxide or hyperthermic treatment was prolonged the mode switched to necrotic cell loss. Again, this study emphasizes the notion that a gradient between apoptotic and necrotic cell loss exists depending upon the severity of the insult.

Summary

In conclusion, we have shown that ischemia initiates a cascade of events that have long lasting effects within the rat hippocampus at least three months after the insult. Our data suggest that ischemia may initiate two rates of cell loss - a rapid cell loss occurring within seven days following ischemia followed by a slower, progressive cell loss over the next few months. Qualitatively, ten and thirteen minutes of ischemia appear to possess both rates of cell loss but further study is required to come to a definitive conclusion. Seven minutes of ischemia lacked a fast onset in cell death , but instead was characterized by a slow progressive cell loss. A

significant degree of variability, we feel, is an inherent property of this ischemia model at ischemia times close to threshold. The ischemic threshold is best described as an ischemic range, probably influenced by many factors. While this study offers interesting and novel findings, it also leaves us with more questions to be answered in future experiments.

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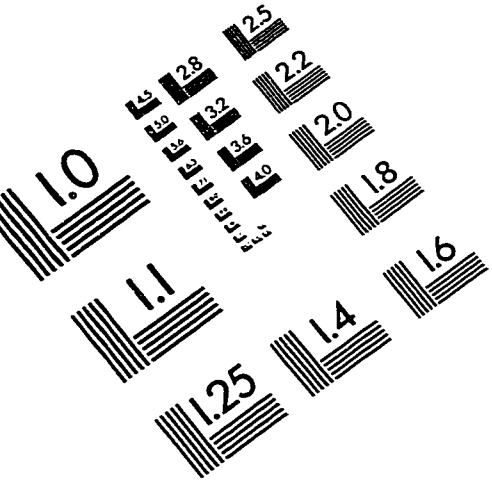
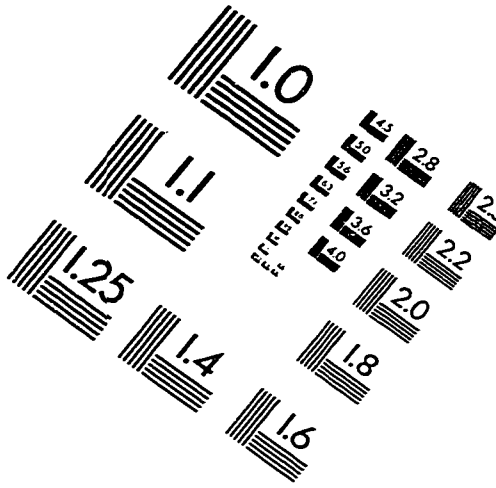
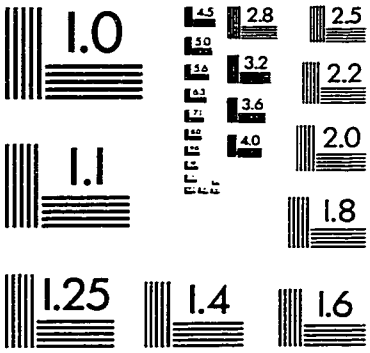
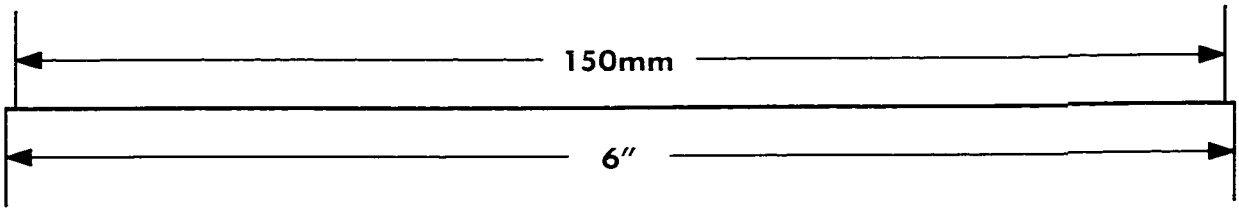
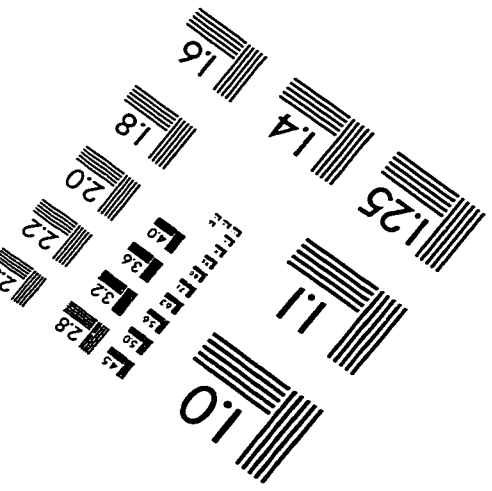
IMAGE EVALUATION TEST TARGET (QA-3)

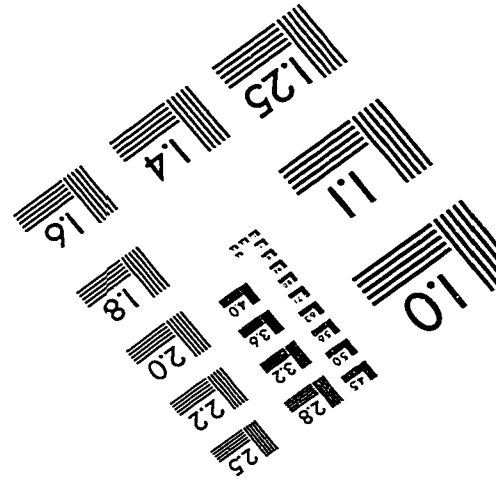












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