DO DENDRITIC SPINES CONTRIBUTE TO ISCHEMIC TOLERANCE?

TINA GILES

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Do Dendritic Spines Contribute To Ischemic Tolerance?

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ABSTRACT

Global ischemia destroys the CA1 pyramidal cells of the hippocampus. This cell loss can be prevented by ischemic preconditioning (IP). IP is a phenomenon whereby brief episodes of ischemia protect against a later more severe insult. Dendritic spines have been suggested to be neuroprotective by regulating toxic calcium levels independently of the parent dendrite. Therefore, dendritic spine formation may play a role in the development of IP. The purpose of the present experiment was to examine the effects of IP on spine densities in the CA1 region in gerbils. Animals received bilateral carotid occlusions of 1.5 min (pre-conditioning) and 5 min (ischemic preconditioning) in duration. Spine densities were calculated from apical and basilar dendrites of CA1 pyramidal cells in ischemic preconditioned animals that survived 3 (IP3), 10 (IP10) or 30 (IP30) days, preconditioned only (PO) animals, and sham animals. Animals were tested on the same days for habituation to a novel open field. Sections were stained using a modified Golgi-Cox procedure and spines were visualized using a Neurolucida® neuron tracing system. Results show that PO, IP10 and IP30 animals have significantly higher spine densities on basilar, proximal and terminal dendrites than all other groups of animals. In the open field, IP animals initially displayed habituation impairments that recovered with time. This apparent recovery coincided with the increase in CA1 spine density. These data may reflect a role for dendritic spines in the neuroprotection and recovery of function associated with ischemic tolerance.

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LIST OF ABBREVIATIONS

AMPA…α-amino-3-hydroxy-5-methyl-4-isoxazol propionate

GABA…gamma-aminobutyric acid

IP…ischemic pre-conditioning

PO…pre-conditioning only

LTP…long term potentiation

MAP2…microtubule associated protein 2

MCAO…middle cerebral artery occlusion

NMDA…N-methly-D-aspartate

TS…triple sham

C…control

N…normal

Ca^{2+}…calcium ion

Na^{2+}…sodium ion

pCREB…phosphorylated cAMP response element protein

cAMP…cyclic adenosine monophosphate

fEPSP…field excitatory postsynaptic potential

ATPase…adenosine triphosphatase

Stroke, as a result of cerebral ischemia, is one of the leading causes of death and chronic disability in North America for which there is currently no effective treatment (Koroshetz and Moskowitz, 1996). It is caused by a blockage of blood flow to the brain leading to a loss of function of specific brain areas. There are a number of risk factors for stroke, including modifiable factors, such as hypertension, cardiac disease, diabetes, cigarette smoking, physical inactivity and hyperlipidemia, which are potentially treatable conditions. There are also non-modifiable risk factors such as age, gender, race, ethnicity and heredity (Elkind and Sacco, 1998) that predispose people to stroke. Drugs that have been found to be neuroprotective in animal models of stroke have failed to improve ischemic outcome when used in clinical trials (De Keyser et al., 1999). Some of these drugs include GABA modulators, (e.g., clomethiazole), and calcium channel blockers, (e.g., nimodipine), which act on specific targets believed to be involved in the ischemic cascade. Researchers are attempting to improve the translation from animal models to the clinical situation by extending survival times, emphasizing functional outcome measures and more carefully controlling physiological variables such as temperature. Arguments have been made that such changes are necessary in order to identify drug treatments that are truly neuroprotective rather than those that only delay or postpone neuronal death (Corbett and Nurse, 1998). Also, much more research is being done on rehabilitation after stroke to improve the functional outcome of patients with physical, cognitive and other deficits.

There are several animal models of ischemic stroke, two of the most common involve global and focal ischemia. Global ischemia occurs when the blood supply to the

forebrain is reduced resulting in damage to selectively vulnerable areas, such as the CA1 region of the hippocampus (Kirino and Sano, 1984). This type of ischemia commonly occurs during cardiac arrest or after hypoxia. Focal ischemia, on the other hand, is an incident whereby the blood supply to a specific brain region is interrupted as a result of blockage of a main artery, such as the middle cerebral artery. Both types of stroke are highly reproducible in rodent models. Global models include the gerbil bilateral carotid occlusion model (Kirino and Sano, 1984), and the two (Smith et al., 1984) and four-vessel occlusion models in rats (Pulsinelli and Brierley, 1979). Focal models involving the middle cerebral artery include vascular application of Endothelin-1 (Sharkey et al., 1993; Sharkey and Butcher, 1995), the intraluminal suture method (Longa et al., 1989) and transient occlusion of the middle cerebral artery with microvascular clips (Buchan et al., 1992).

In the gerbil model of global ischemia there is damage to forebrain structures, in particular, the hippocampus. The hippocampus is an area of the brain involved in learning and memory (Scoville and Milner, 1954), and consequently, these processes are disrupted after global ischemia (Zola-Morgan et al., 1986a) in both animals (Zola-Morgan et al., 1986b) and humans (Squire and Zola, 1996). In the gerbil model, the carotid arteries are bilaterally occluded for 5 - 10 minutes and then blood flow is restored, thus causing a transient ischemic attack. This type of global ischemia predominantly affects the CA1 pyramidal cell layer of the hippocampus, although other areas, such as CA2 and hilar neurons, are also vulnerable (Kirino and Sano, 1984). Since the gerbil has an incomplete circle of Willis (Levine and Payan, 1966) a brief occlusion of the carotid

arteries (approximately 3 - 5 min in duration) causes near total forebrain ischemia and, thus, almost complete destruction of the CA1 pyramidal cell layer. When brain temperature is maintained at normothermic levels during ischemia the loss of CA1 cells is highly consistent, resulting in approximately 75% and 98% cellular death with 3 and 5 min episodes, respectively (Nurse and Corbett, 1994; Colbourne and Corbett, 1994). CA1 pyramidal cell death becomes evident 2 - 4 days following ischemia and, therefore, has been termed delayed neuronal death (Kirino, 1982; Choi, 1990).

The pathophysiology of delayed neuronal death has been extensively studied in order to identify potential treatment regimens (Siesjo, 1988). In normal circumstances the energy supplied by oxygen and glucose fuels the membrane pumps, which regulate ionic homeostasis. In normal synaptic transmission glutamate increases to moderate levels in the synaptic cleft, activates the NMDA and AMPA receptors, which causes a transient rise in intracellular calcium, and eventually glutamate levels return to normal. During an ischemic episode a disruption of ionic balance occurs, which prevents the normal re-uptake of glutamate from the cleft. This causes excessive activation of the glutamate receptors, particularly those of the NMDA type, which leads to a massive increase in intracellular calcium, through both calcium permeable ion channels and release from internal stores (Siesjo and Bengtsson, 1989; Choi, 1992). As the energy supply is depleted (Siesjo, 1988), the membrane pumps, which require ATP to function, are no longer able to transfer calcium, sodium and chloride out of the cell (Mies et al., 1990). This disruption of normal ionic balance also causes the cell to be more excitable and increase its spontaneous firing rate. The increase in calcium is thought to trigger cell

death via a number of destructive pathways, such as mitochondrial dysfunction, free radical formation, calcium activated catabolic enzymes, gene activation, and disintegration of the cytoskeleton and cell membrane (Choi, 1988; Siesjo and Bengtsson, 1989; Koroshetz and Moskowitz, 1996). Thus, calcium entry into cells following an ischemic insult is a major player in the cascade of cell death. Consistent with the findings of elevated intracellular calcium is the abnormal electrophysiology of CA1 cells after 5 min of forebrain ischemia. These cells lose the capacity for long-term potentiation (LTP) and show increased spontaneous firing that can be blocked by the calcium chelator EGTA (Kirino et al., 1992). These findings suggest that vulnerable CA1 cells suffer from abnormal calcium homeostasis, which ultimately may lead to ischemic cell death.

Current research focuses on developing novel treatments to protect the brain against the devastating effects of ischemia. One such approach is ischemic tolerance, whereby the brain develops "tolerance" to ischemia as a result of prior exposure to brief periods of non-injurious ischemia. In the gerbil model of global ischemia two brief episodes of carotid artery occlusion (e.g., 1.5 min in duration), given 24 hours apart, can protect hippocampal CA1 cells from a severe insult (e.g., 5 min in duration) three days later (Kitagawa et al., 1990). This method of CA1 preservation is termed ischemic pre-conditioning (IP) and has been shown in both gerbil (Kirino et al., 1991; Kato et al., 1991) and rat (Liu et al., 1992) models of global ischemia, as well as focal ischemia (Matsushima and Hakim, 1995; Matsushima et al., 1996; Barone et al., 1998). However, the amount of protection declines as survival time increases, such that ten days after IP approximately 80% of CA1 cells remain, but after 30 days this decreases to ~50%

(Corbett and Crooks, 1997). Given that a 5 min occlusion normally produces approximately 95% to 98% CA1 cell loss, this is still a significant amount of cellular preservation. Although ischemic pre-conditioning produces significant histological protection, tolerant animals show behavioural deficits, such as open-field habituation impairments (Corbett and Crooks, 1997), which recover as survival time increases.

The mechanisms of ischemic tolerance are unknown, but there have been many suggested possibilities. Some of these include: induction of heat shock proteins (Kitagawa et al., 1990; Kato et al., 1993), activation of astroglia (Kato et al., 1994), and interleukin-1 expression (Ohtsuki et al., 1996), decreased inflammation and increased endogenous antioxidant activity (Clemens et al., 1993; Clemens et al., 1994), changes in second messenger systems (Churn et al., 1992; Hu and Wieloch, 1993), and specific expression patterns of immediate early genes involved in cell survival (Kato et al., 1995; Whitfield et al., 1999). While each of these have been implicated in the development of ischemic tolerance it is likely that a combination of factors mediates the neuroprotective effects of IP.

Segal (Segal, 1995) has proposed that morphological changes on the dendrites of cells are neuroprotective in brain injury. Dendritic spines are small protrusions that emanate from dendrites and are the location of over 90% of all excitatory synapses in the central nervous system (Harris and Kater, 1994). They are highly specialized input zones that are connected to the main dendritic shaft by a narrow neck and ending in a bulbous head. The shape of the neck and head defines the numerous types that have been identified, including stubby, thin, sessile, mushroom and branched (Sorra and Harris,

2000). On hippocampal CA1 cells virtually all excitatory synapses are found on dendritic spines and most spines are contacted by only one presynaptic bouton (Andersen et al., 1966). They contain both NMDA and non-NMDA glutamate receptor types, along with calcium/calmodulin dependent protein kinase II, which is activated when calcium flows through the NMDA receptors. The small neck of the spine is thought to restrict the rise in calcium that occurs during synaptic activation, thus, the spine represents an independent biochemical compartment. Some models visualize the spine to be the site at which "long-term memory" is stored (Zador et al., 1990; Bliss and Collingridge, 1993). For example, in the honeybee the first orientation flight leads to spine stem shortening in the retinal ganglion cells (Brandon and Coss, 1982). Indeed this model of spine function deserves some recognition, where recently O'Malley has shown that passive avoidance training in rats produces a transient increase in spine density in the dentate gyrus (O'Malley et al., 1998), again demonstrating a role of spines in learning and memory. However, with the advent of sensitive, high-resolution imaging methods, the emphasis of the role of the spine in memory storage has shifted. Segal hypothesizes a novel function for spines: by isolating the synapse from the parent dendrite the spine may be able to protect the cell from excitotoxicity that occurs as a result of a rise in calcium (Segal, 1995), subsequent to an ischemic episode. Therefore, spines may be able to protect the parent dendrite from the overload of calcium that occurs after brain injury such as in ischemia, thus providing a neuroprotective mechanism. Furthermore, they may be involved in neuroplasticity processes that may be responsible for the recovery of function (e.g., open-field habituation) seen in ischemic tolerant animals.

In models of ischemic tolerance both histological and behavioural measures are used to assess the protection and proper function of the hippocampus. In the gerbil model, IP animals appear histologically normal up to day 10, where approximately 80% of viable CA1 cells are present in the hippocampus. However, these animals show habituation impairments in the open-field. This impairment disappears by day 30 suggesting a recovery of function taking place in these animals that is not reflected in the histological assessment (Dooley and Corbett, 1998; Dowden and Corbett, 1999). Electrophysiology studies have also demonstrated functional disturbances in tolerant hippocampal slices, which recover with time. IP animals show reduced CA1 field excitatory postsynaptic potentials (fEPSP) up to and including day 10, which coincides with the deficits seen in the open-field (Dooley and Corbett, 1998; Dowden and Corbett, 1999). However, these animals have normal amounts of MAP2 (microtubule-associated protein-2), suggesting that dendritic abnormalities may not be responsible for these early functional deficits. The amplitude of the fEPSPs returns to sham levels by day 30, again, correlating temporally with open-field behaviour (Dowden and Corbett, 1999). Kawai (Kawai et al., 1998) has also shown that the functional disturbance seen in IP animals recovers. Hippocampal slices of tolerant gerbils lose the capacity for LTP shortly after a 5 min insult, as well as NMDA receptor-mediated transmission. Both these functional characteristics recovered with time, suggesting that ischemic tolerance produces both functional and morphological protection. The locus of functional preservation seen in ischemic tolerance has been attributed to compensation by other areas of the brain, such

as caudal CA1 or prefrontal cortex (Dooley and Corbett, 1998), but may in fact be the result of neuroplastic mechanisms on surviving CA1 dendritic spines.

The dynamic properties of spines make them a promising player in synaptic plasticity and possibly in neuroprotection. Since the hippocampal formation is closely associated with spatial learning, structural changes in hippocampal synapses that occur as a consequence of learning, are an efficient system to demonstrate the dynamics of spines. Moser (Moser et al., 1994) has shown increased spine density on rat basilar hippocampal CA1 dendrites following spatial learning in a water maze when compared to non-trained counterparts. The increase in spine density is representative of an increase in synapse formation (Andersen et al., 1966) as a result of spatial learning and thus, an altered connectivity within the hippocampus. LTP, the increase in synaptic efficacy believed to be associated with learning and memory mechanisms, has also been shown to involve modifications of synapses and spines. Papa and Segal (Papa and Segal, 1996) have shown that cells respond to an increase in synaptic activity with an increase in spine density, suggesting that spines are likely to have a short-term role in synaptic interaction rather than to constitute a long-term memory storage site. Also, Buchs (Buchs and Muller, 1996) found ultra-structural changes in potentiated CA1 synapses following high-frequency trains of LTP producing stimulation. These changes include perforated postsynaptic densities, larger apposition zone between pre- and postsynaptic structures, longer postsynaptic densities and enlarged spines (Buchs and Muller, 1996). This dynamic nature of spines has also been shown in other situations, such as, environmental

enrichment (Kolb et al., 1991) and throughout the estrous cycle of rodents (Woolley et al., 1990).

Environmental enrichment and its effects on learning and memory have been studied for many years. Exposure to enriched environmental conditions improves learning and problem solving ability and results in plastic changes in the brain (Duffy et al., 2001). Kiyono (Kiyono et al., 1985) found that maternal environmental enrichment during pregnancy in rats exerted a facilitatory effect on postnatal maze learning, where male offspring raised by enriched-housed mothers performed better in a Hebb-Williams maze than the offspring of impoverished and standard-housed mothers. Recently, enrichment has received a lot of attention in brain injury paradigms as well. It has been shown that enrichment improves functional outcome on tasks of spatial learning such as the Morris water maze (Puurunen et al., 1997) following global ischemia in rats. Also, gerbils housed in enriched cages following global ischemia showed improved performance in a T-maze, a test of working spatial memory, as compared to those animals in standard housing (Farrell et al., 2001). Both these studies show the beneficial results of enrichment on brain function following injury. This improvement in memory processes resulting from environmental enrichment is associated with changes in dendritic morphology, that is, an increase in spine density (Globus et al., 1973; Moser et al., 1994). Many groups have shown that rats that are placed in enriched conditions have an increase in dendritic branching/arborization, as well as an increase in spine density on cortical neurons (Greenough et al., 1985; Kolb et al., 1997). Therefore, environmental

enrichment clearly induces changes in dendritic spines that could contribute to neuroprotection and/or synaptic plasticity.

Another area that has received a lot of attention is hormonal effects on dendritic spines. This interest stems from the fact that females are at a lower risk of stroke than their male counterparts during their reproductive years, but this risk increases once they reach menopause. It has been shown that intact female rats have smaller infarct volumes than males and ovariectomized females, in a middle cerebral artery occlusion (MCAO) model of focal stroke (Alkayed et al., 1998; Hurn and Macrae, 2000). Therefore, gonadal hormones are thought to play a role in this protective phenomenon, which may include morphological changes in dendritic spines. Indeed, a number of groups have shown structural changes in hippocampal spines during the estrous cycle of rats and gerbils. During the estrous phase, when estrogen levels are at its highest, spine density is maximal. During the proestrous phase, when estrogen levels are at the lowest, spine density is minimal (Woolley et al., 1990; Woolley and McEwen, 1992). Also, ovariectomy of female rats results in a decrease in spine density on the lateral dendritic branches of CA1 pyramidal cells, which can be prevented (Gould et al., 1990) or reversed (Woolley and McEwen, 1993) by estradiol treatment. This demonstrates the ability of the hippocampus to be extremely plastic, even in response to naturally occurring hormone changes. However, the behavioural significance of this is not clear. Korol (Korol et al., 1994; Korol et al., 1996) has reported changes in spatial behaviour of female rats during the different phases of their cycle. This suggests a role of the spine in hippocampal function. The positive correlation between spine density and estrogen

levels may represent differential regulatory mechanisms of learning and memory processes between the sexes as well as between the different phases of the estrous cycle. How estrogen exerts its effect on dendritic spines is not clearly defined nor is the functional consequence of this change. One possible mechanism is the reduction of GABAergic inhibition in the pyramidal cells to increase the formation of dendritic spines (Murphy et al., 1998). Since global ischemia increases the excitation:inhibition ratio in the hippocampus this is also a potential mechanism of ischemic tolerance in gerbils. Another possibility is the induction of CREB (cAMP response element binding protein)-regulated genes through the increase in calcium (Jin et al., 2001). The plasticity seen in the hippocampus of rats and gerbils during hormone changes, and the protection seen in female animals in models of brain injury, clearly demonstrates the dynamic nature of spines, and their potential role in neuroprotection and functional recovery.

One characteristic of spines that may make them so dynamic is their actin cytoskeleton. Spines contain a specialized cytoskeleton, made up of a network of actin filaments, which reflects their need to balance stability and plasticity. Intermediate filaments and microtubules, the other major components of a cell's cytoskeleton, are rare or nonexistent in spines, whereas, actin is extremely enriched in spines relative to the rest of the neuron (Fifkova and Delay, 1982; Cohen et al., 1985). The actin network provides a complex, dynamic structure to cope with the ever changing environment of a neuron. Halpain (Halpain et al., 1998) has suggested that there are two populations of actin in dendritic spines, one that is relatively stable and one that is dynamic. The stable form seems to be involved in the persistence of dendritic spines over a period of hours and

days (Hosokawa et al., 1992; Dailey and Smith, 1996) maintaining the basic shape and profile of spines on dendrites. In fact, low concentrations of actin inhibitors, such as cytochalasins and latrunculins, arrest spine movement but do not disrupt actin filaments in spines (Fischer et al., 1998; Dunaevsky et al., 1999). The dynamic form, however, is presumably responsible for the shape changes seen in a time frame of seconds to minutes (Dailey and Smith, 1996; Fischer et al., 1998; Dunaevsky et al., 1999) and thus, has an essential role in synaptic plasticity. Evidence of this has been shown by Krucker (Krucker et al., 2000), whereby, LTP was selectively blocked by low concentrations of actin assembly inhibitors, demonstrating the role of actin assembly in synaptic plasticity. The two pools of actin filaments may represent the way in which dendritic spines constantly change in the face of an unstable environment and yet still maintain the essential synaptic connections with other areas of the brain, thus, making the spine a worthy candidate in plasticity as well as in neuroprotection.

Calcium has long been thought to be a major player in the ischemic cell death process (Siesjo and Bengtsson, 1989). The ability of spines to change shape and density may provide a mechanism to salvage the cell from damage caused by increasing calcium levels. Intracellular calcium plays a crucial role in a variety of cellular functions in neurons, including neurotransmitter release, activation of ion channels, and growth and plasticity (Choi, 1988; Grover and Teyler, 1990). Calcium-ion concentrations are normally maintained at submicromolar levels within cells by machinery that is responsible for regulating its levels. The dendritic spine is an area of the cell where the local concentration of calcium rises to levels needed to activate biochemical cascades

associated with plasticity (Koch and Zador, 1993). However, like many necessary and beneficial elements too much calcium, as in the case of an ischemic episode, can have damaging effects. Since spines are able to regulate calcium levels independently of the parent dendrite (Muller and Connor, 1991; Guthrie et al., 1991), protecting the main dendritic shaft from a lethal dose of calcium is consistent with the idea that spines may be involved in neuroprotection. This theory is supported by the fact that spines contain a high concentration of protein phosphatases, which regulate calcium dependent phosphorylation of spine proteins (Ouimet et al., 1995). Also, spines are not equipped with organelles that can be damaged by an increase in calcium, such as mitochondria and microtubules. However, they do contain calcium-sequestering agents such as internal calcium stores, endogenous buffers and a Ca^{2+}/Na^{+} exchanger (Segal, 1995).

The shape and/or size of the dendritic spine may also influence the degree of neuroprotection (Sorra and Harris, 2000). Calcium kinetics in spines are different than in the parent dendrite. After synaptic activity there is a larger increase in calcium levels in the spine than in the neighbouring dendrite. Also, the spine shows a slower decay phase than the parent dendrite, suggesting that the spine contains differential mechanisms of calcium decay, such as active extrusion by calcium pumps and diffusion across the spine neck (Majewska et al., 2000). The decay kinetics vary between spines because the shape/length of spines contributes to their calcium regulating abilities. Longer spine necks maintain high calcium levels more so than short spine necks, where calcium can move quickly into the parent dendritic shaft and go on to the cell nucleus. However, a longer spine is more independent from the main dendrite and calcium moves slowly out

of the spine head (Volfovsky et al., 1999). The shorter the spine neck the more similar the intracellular calcium dynamics between the spine head and the parent dendrite (Segal et al., 2000). Therefore, a longer spine may protect the cell from a massive influx of calcium and prevent the potentially devastating effects that this may cause. Because spines can change lengths over a very short time frame spine length serves to fine tune the interaction between the spine head and the parent dendrite on a continuous basis. Also, the differential decay kinetics of longer spines may affect long-term processes such as activation of kinases, phosphatases and mobilization of glutamate receptors to the postsynaptic membrane (Lissin et al., 1999), thus altering the amount of input and synaptic efficacy of the cell.

Consistent with spines being neuroprotective are findings demonstrating the signaling pathways most likely involved in spine formation and alteration. CREB is a transcriptional factor implicated in the control of numerous genes involved in synaptic plasticity pathways and also in cell survival pathways. CREB protein responds to an increase in cAMP and/or calcium levels and is then transformed into its active form: phosphorylated CREB (pCREB). Recent evidence suggests a role for pCREB in the generation of new spines. Segal and Murphy (Segal and Murphy, 1998) have shown that blocking of the cAMP-regulated protein kinase A eliminates estradiol-evoked spine formation (Woolley et al., 1990), as well as the increases in CREB binding protein responses seen with estradiol. Also, bicuculline, which enhances spontaneous firing activity, has been shown to have the same effect on spine density in hippocampal cultured neurons (Papa and Segal, 1996). These results indicate that CREB activation is

a necessary step in the process leading to the generation of new spines, and that the increase in spine density seen in estradiol treated neurons can be accomplished by reduction of GABA input, increased excitation, NMDA-mediated calcium influx and finally CREB phosphorylation. Ischemia also causes an increase in excitation within the hippocampus, where cells of the CA1 pyramidal layer undergo a process of apoptotic cell death and dentate granule cells survive. In the resistant granule cells an increase in pCREB is seen 48 hours after a hypoxic-ischemic episode. However, a dramatic loss of pCREB is seen in the CA1 cells preceding the onset of cell death (Walton et al., 1996). This is consistent with the idea of CREB being important in survival of hippocampal neurons. Therefore, it is logical to suggest that the neuroprotection offered by ischemic pre-conditioning may also be a result of CREB activation and thus, spine formation.

Recently, Segal (Segal, 2001) has proposed a unifying hypothesis of the role of spines in plasticity. He suggests that there is a bimodal relationship between spines and intracellular calcium levels, such that a moderate increase in calcium concentration will cause the formation of novel spines and the elongation of existing ones, whereas, a large and persistent increase in calcium will cause shrinkage and eventual elimination of spines. Furthermore, it is suggested that a local change in calcium levels will change the length of local spines, whereas a central change in calcium will cause the phosphorylation of CREB and formation of novel spines. Thus, both local and central factors play a role in spine morphology, where spine shape and density are thought to affect the ability of spines to regulate calcium.

Therefore, the hypothesis to be tested in this thesis is that an increase in spine density on the CA1 cells of the hippocampus contributes to the development of IP, which supports the survival of these cells and the recovery of function following a severe ischemic insult. The present experiments will examine the effect of IP on hippocampal spine formation to determine its involvement in ischemic tolerance and the time course of the postulated morphological changes. Because spines are extremely dynamic structures, as demonstrated by their response to environmental enrichment, hormonal changes and synaptic activity, and because they have been intimately linked with nuclear mechanisms, such as CREB activity, spines could contribute to the development of ischemic tolerance.

METHODS

Animals

These experiments utilized female (n=64) Mongolian gerbils (*Meriones unguiculatus*), which were purchased from High Oak Ranch Ltd (Baden, On). The gerbils were housed in groups of four in the animal care facility for at least two weeks prior to any experimentation. They were treated with piperazine for 3 days when they arrived to rid them of pinworm infections commonly found in these rodents. They were also kept on a 12-hour light/dark cycle in the animal room. Animals ranged from 4 to 9 months in age.

The gerbils were fed guinea pig pellets, and twice a week they were given a mixture of sunflower seeds and other grains. They were also fed carrots or apples, once a week. Shredded paper and cardboard tubes were in the cages to allow nest building.

Cannula Implantation

Gerbils (50 – 120 g) were anaesthetized with sodium pentobarbital (65 mg/kg i.p.) and given a subcutaneous injection of atropine (0.03 mg/ml, 0.03ml). They were then placed in a stereotaxic instrument and the head immobilized. An incision was made, the skull was exposed and the overlying periosteum was removed with a cotton swab. A small hole (approximately 1mm in diameter) was drilled in the skull approximately 2 mm lateral of the midline suture and 1 mm in front of Bregma. Two plastic screws were glued to the skull to hold dental cement in place. A stainless steel guide cannula (5 mm in length) was held in place in the hole, above the dura with dental cement. A stainless steel stylet was then placed in the cannula to prevent infection. The animal's skin was

then sutured, and it was placed in its cage under a heat lamp until it recovered from the anaesthetic. From this point on the gerbils were housed individually.

Normal Temperature

Three days after the cannula implantation gerbils were anaesthetized with 2.0% halothane (with 30% oxygen and 70% nitrogen) and 8 mm wireless temperature probes (Mini-Mitter Co., Bend, Or, USA) were inserted into the guide cannula. The probe tip terminated in the striatum at a depth that approximates that of the hippocampus. The probe was taped to the cannula arm and the gerbil was removed from anesthesia and placed in a plexiglas cage. These cages were placed on top of AM receivers (model # RA-1010, Data Sciences Inc., St. Paul, MN, USA), which were connected to a computer that records brain temperature every 30 seconds for four hours. This established a baseline temperature. The probes were then removed from the gerbils under halothane and they were placed back in their home cages.

Groups

There were six groups of animals used in this experiment: ischemic pre-conditioned animals (IP) surviving either 3 (IP3), 10 (IP10) or 30 (IP30) days; pre-conditioned only (PO); controls (C); and triple shams (TS). PO animals received two 1.5 min occlusions, separated by 24 hours, and were sacrificed three days after the second occlusion. The IP animals received two 1.5 min occlusions, separated by 24 hours, and followed by a 5 min ischemic insult three days later. Control animals did not receive any surgical treatment. Finally, the TS group received the three surgical procedures consisting of dissection of the arteries, suturing, and temperature monitoring, without any

carotid artery occlusions, to control for the repeated use of anaesthetic. C and TS groups were combined as a normal (N) group for statistical analysis.

Induction of Ischemia

Three days after normal temperature measurement animals were prepared for surgery. Gerbils were anesthetized under 2% halothane (30% oxygen and 70% nitrogen) and brain temperature probes were placed into the cannula. The animal was positioned ventral side up, its neck was shaved, and a midline incision was made. The carotid arteries were isolated from surrounding tissue, using forceps, freed from connective tissue and a silk suture was placed beneath each artery.

When the brain temperature reached 36.5°C the arteries were picked up by the sutures and occluded with mini-aneurysm clips. Occlusions were either 1.5 min in duration (pre-conditioning) or 5 min in duration (ischemia). The arteries were kept moist during occlusion so that the clips would not break the artery upon removal. During the occlusion the animal's brain temperature was maintained at or close to 36.5°C with a hot water blanket (Gaymar heat therapy Mul-T-Pad™model TP-3E, 31/2"x23", Gaymar Industries Inc., Orchard Park, NY, USA) surrounding the head and neck area. Body temperature was also regulated using a homoeothermic heating blanket (Harvard Apparatus, Saint Natick, MA, USA) wrapped around the gerbil's body.

After occlusion the clips were carefully removed from the carotid arteries and reflow was re-established by massaging the arteries with the forceps. The neck incision was then sutured and the brain probe secured to the cannula. The animal was then placed into the plexiglas cage with food and water, and allowed to recover. Brain temperature

was monitored for eight hours following pre-conditioning and 24 hours following the 5 min occlusion. Brain temperature was maintained at/or above 36.5°C for one hour after ischemia with heating lamps located above the cages. Generally, after one hour the animals recovered and self-regulation of temperature was restored. Following the temperature monitoring period the animals were reanaesthetized and brain probes removed. They were then placed back into their home cages.

Behavioral Testing

All gerbils were tested in the open-field, which is a sensitive measure of hippocampal dysfunction (Wang and Corbett, 1990; Babcock et al., 1993). This testing was done on days 3, 7, 10 and 30 after the last ischemic episode, depending on survival time. The animals were brought to the testing area approximately 30 min prior to testing and were disturbed as little as possible during this time.

The gerbils were tested individually in a soundproof room using an open-field measuring 72 x 76 x 57 cm^3. The floor of the open-field was electronically divided into 25 squares. A visual tracking system (HVS Systems, Kingston, UK) recorded the number of squares entered per 10 min test session. At the end of the 10 min session the gerbil was removed from the apparatus and placed back in its home cage. The floor of the apparatus was washed between test sessions to eliminate any odours that may interfere with the next animal's behaviour. Gerbils were weighed after every open-field session.

Histology

At the end of the experiment the animals were given an overdose of sodium pentobarbital and perfused transcardially with 0.9% saline. The brains were removed immediately after perfusion and placed in a modified Golgi-Cox solution (Glaser and Van der Loos, 1981) for 14 days. The brains were then immersed in a 30% sucrose solution for a minimum of 3 days. The brains were sliced on a vibratome at 200μm and mounted on gelatin-immersed slides. Sections were kept moist with sucrose to prevent excessive drying of the sections. They were then blotted with bibulous paper and put in a slide holder that was covered with a damp paper towel. This was then put in an airtight container with deionized water on the bottom, and placed in a dark cupboard. Twenty-four hours later the slides were removed from the container and blotted again in the same manner. They were then stained using a modified Golgi-Cox staining procedure (Gibb and Kolb, 1998). Absolute alcohol used in this staining procedure was dehydrated with a molecular sieve (type 5A, BDH Inc., Toronto, On) to minimize moisture in the tissue. After the slides were cover slipped they were placed in a container filled with desiccant.

Image-Analysis

Brain tissue was analyzed using the Neurolucida® neuron tracing system and Neuroexplorer® (Microbrightfield Inc., Colchester, VT, USA) programs. CA1 spine densities were sampled from the rostral level of the hippocampus. Only cells/dendrites reaching the following criteria were analyzed (Gibb and Kolb, 1998): 1) the cell had to be well impregnated with stain and not obscured by blood vessels, astrocytes or other dendrites; 2) the apical and basilar branches had to be mostly intact and within the plane

of section. Fifteen dendritic branches were analyzed from each brain. This consisted of five basilar, 5 proximal and 5 terminal branches (Fig. 1). Proximal branches were considered to be first and second order dendrites that projected laterally from the main apical dendrite. Terminal branches were third, fourth and higher order dendrites that projected more randomly and posterior from the main apical dendrite. Branch order was determined for the apical dendrites such that branches arising from the primary apical dendrite were first order; after one bifurcation, second order; and so on. Branch order was determined for the basilar dendrites such that branches arising from the cell body were first order, and so on. Ideally, each cell would contain an adequate basilar, proximal and terminal branch to analyze, but this wasn't possible for all brains. The minimum number of cells used was 5 and the maximum was 9. Cells were taken from both hemispheres depending upon staining and availability.

Chosen dendritic branches were divided into 3 parts, the inner part being closest to the bifurcation node, middle, and outer being the end of the branch. Only middle portions were traced and were between 33.3 - 86.5 μm long. Tracings were made of dendrites visualized at 1000 X magnification. Spine densities were calculated using Neuroexplorer®, and were expressed as the number of spines per 10 μm. No attempt was made to correct for spines hidden beneath or above the dendritic segment, therefore, the spine densities are likely an underestimate of the actual density of the dendritic spines.

Statistics

One-way ANOVAs were used to analyze open-field and spine density data. Newman-Keuls post-hoc tests were used to compare treatment means. The significance level was set at $p<0.05$.

RESULTS

Temperature

The mean brain temperature recorded for the IP groups and the TS group following the 5 min occlusion was 36.47°C ± 0.08 S.D. (n=39). The group temperature means did not differ significantly from each other (p=0.205) throughout the 24 hour post-ischemic monitoring period (Table 1).

Behaviour

The data from the open-field scores are shown in Fig. 2. The scores of the animals in the IP groups did not differ with survival time (Day 3, p=0.27; Day 7, p=0.22; Day 10, p=0.14) and were pooled for analysis (see Table 2). Analysis of variance indicated a significant treatment effect on Day 3 ($F_{3,53}$=8.426, p<0.0001), Day 7 ($F_{2,30}$=30.908, p<0.0001), and Day 10 ($F_{2,30}$=8.764, p<0.01). All groups displayed a moderate amount of activity on their first exposure (Day 3) to the open-field, where the IP group exhibited heightened levels of activity compared to the other groups (p<0.01). All groups displayed habituation to the open-field, as shown by the declining activity levels on Day 7, Day 10 and Day 30. However, the IP animals did not show the same amount of habituation. Their activity levels decreased with each test day but their activity scores were significantly higher than the C and TS group on Day 7 (p<0.01) and higher than the C animals on Day 10 (p<0.05). By Day 30, however, the IP groups' scores returned to sham levels (p=0.93)

Spine Densities

Fig. 1 is a schematic diagram of a typical CA1 cell, showing the different types of dendritic branches examined in the present experiment. The basilar branches are those

emanating directly from the cell body. Proximal and terminal dendrites stem from the main apical branch and are also depicted in this drawing. Fig. 3 shows a representative CA1 pyramidal cell dendrite used to calculate spine density. Spine densities were calculated as the number of spines per 10 μm of dendrite. Unpaired t-tests showed that TS and C groups' spine densities did not differ on basilar (TS=5.98 ± 0.7; C=5.64 ± 1.1; p=0.461), proximal (TS=6.42 ± 2.4; C=6.14 ± 1.3; p=0.5186) or terminal (TS=6.14 ± 0.9; C=6.16 ± 1.1; p=0.97) dendrites. Therefore, these groups were combined into an N (normal) group. Table 3 shows the increase in spine density expressed as a percentage of the N group.

Basilar Dendrites

Five basilar branches were traced, from which a mean spine density was calculated, for each animal (Fig. 4). Analysis of variance indicated that there was a significant treatment effect for basilar spine densities ($F_{4,60}$=8.754, p<0.0001). Newman-Keuls post hoc tests indicated that the PO animals had a significantly higher spine density than the normal (N) animals (p<0.01), showing an increase of almost 30% (Table 3). IP animals that survived 10 and 30 days after the 5 min insult also displayed a significantly higher spine density than the N group (p<0.01) reaching approximately 30 and 27%, respectively. However, IP animals that survived just 3 days after the last insult had spine densities that were significantly lower than the PO, IP10 and IP30 groups (p<0.01), and were decreased from N by 3%.

Proximal Dendrites

Significant treatment effects were also found ($F_{4,60}=4.669$, $p<0.01$) for proximal dendrites. Animals that survived 10 and 30 days after the 5 min ischemic insult (IP groups) displayed significantly higher spine densities than the normal animals (N) ($p<0.05$, Newman-Keuls) of approximately 30 and 27%, respectively. However, PO animals did not have a higher spine density than any of the other groups on these dendritic branches (Fig. 5).

Terminal Branches

Again, analysis of variance showed a significant treatment effect on terminal dendrites ($F_{4,60}=9.369$, $p<0.001$). On these branches spine densities were significantly higher in the PO, IP10 and IP30 groups ($p<0.05$) relative to the N group (23, 29 and 21% increase, respectively). Also, IP animals that survived 10 and 30 days had a higher spine density ($p<0.01$ and $p<0.05$, respectively) than animals that only survived 3 days following the last insult (Fig. 6).

Table 1: Intraischemic brain temperatures

	Mean Temperature (°C)	**±SD**
PO (n=9)	36.4	0.16
IP3 (n=10)	36.4	0.16
IP10 (n=13)	36.4	0.16
IP30 (n=10)	36.6	0.18

Table 2. Mean open-field scores for the individual IP groups on days 3, 7 and 10. No significant differences exist between the IP groups and therefore, they were pooled for further analysis.

Group	Day 3	Day 7	Day 10
IP3 (n = 10)	823 ± 115	---	---
IP10 (n = 13)	918 ± 77	794 ± 69	614 ± 125
IP30 (n = 10)	891 ± 206	742 ± 128	541 ± 94

Table 3. Increase in spine density as a percentage of N group values.

	Basilar	**Proximal**	**Terminal**
PO (n=9)	30%	20%	23%
IP3 (n=10)	-3%	6%	3%
IP10 (n=13)	30%	29%	29%
IP30 (n=10)	27%	27%	21%

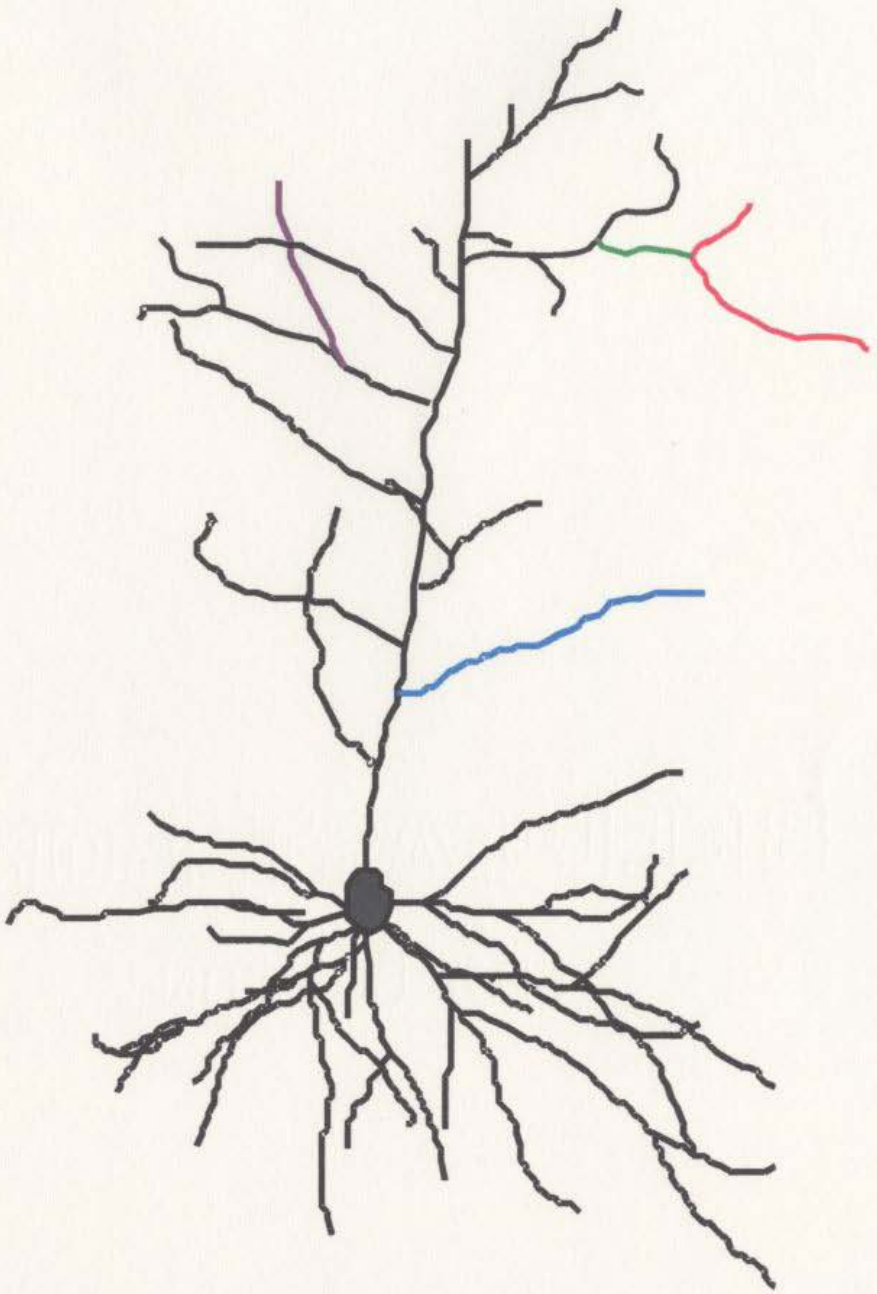

Fig 1: Schematic drawing of hippocampal CA1 pyramidal cell, demonstrating proximal (first and second order, shown in blue and purple), terminal (third and fourth order, shown in green and red), and basilar (those emanating directly from cell body) dendrites.

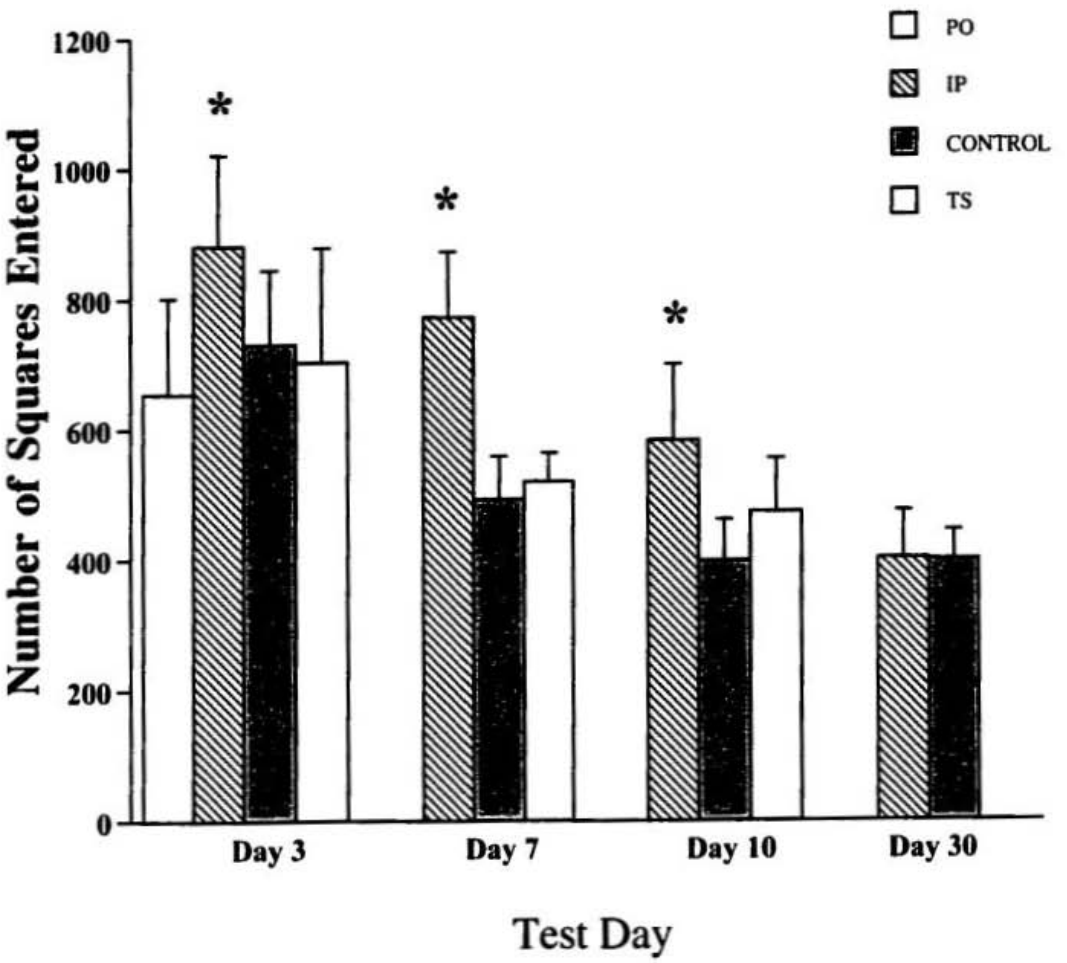

Fig 2: Open-field activity scores (mean ± SD). Scores for IP animals were pooled as one group. On day 3, PO (n=9), TS (n=7) and control (n=8) animals had similar scores, however, the IP animals (n=33) had a significantly higher score than all the other groups (*$p < 0.05$). On days 7 and 10 the IP animals (n=23) exhibited significantly higher activity scores than the controls (n=8) and the TS animals (n=3) ($p < 0.05$). On day 30 IP animals (n=10) were not different than controls (n=7).

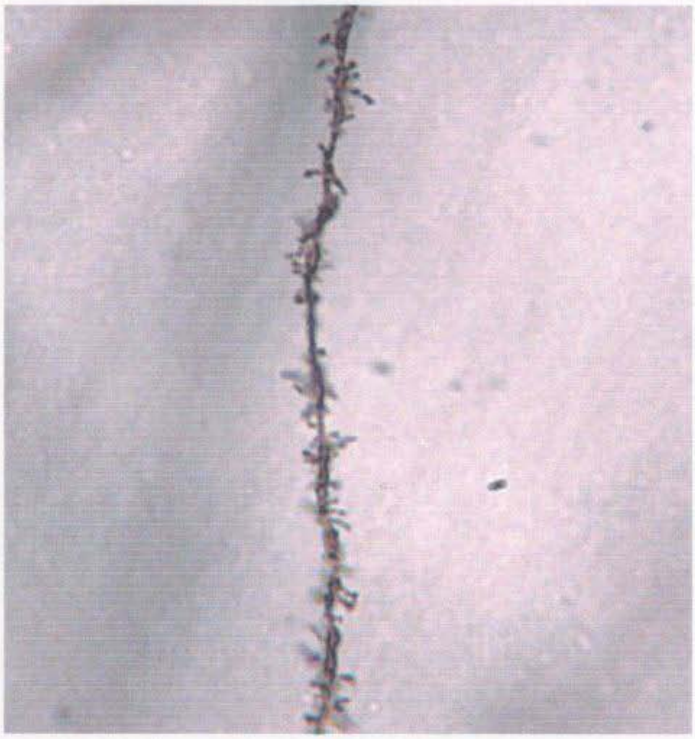

Fig 3: Representative photograph (magnification 1000X) of a terminal dendrite from a CA1 pyramidal cell showing dendritic spines.

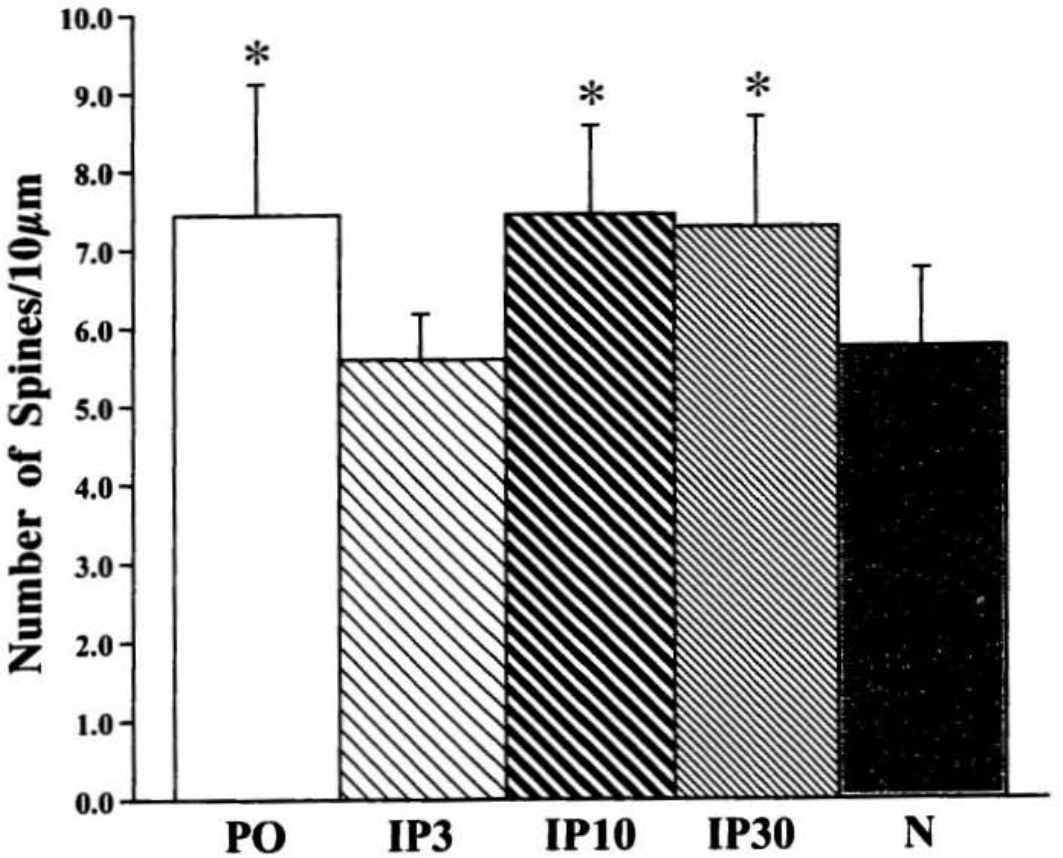

Fig 4: Spine densities of CA1 basilar dendrites (mean ± SD) from PO (n=9), IP3 (n=10), IP10 (n=13), IP30 (n=10) and N (n=22) animals. (*$p < 0.01$ versus N).

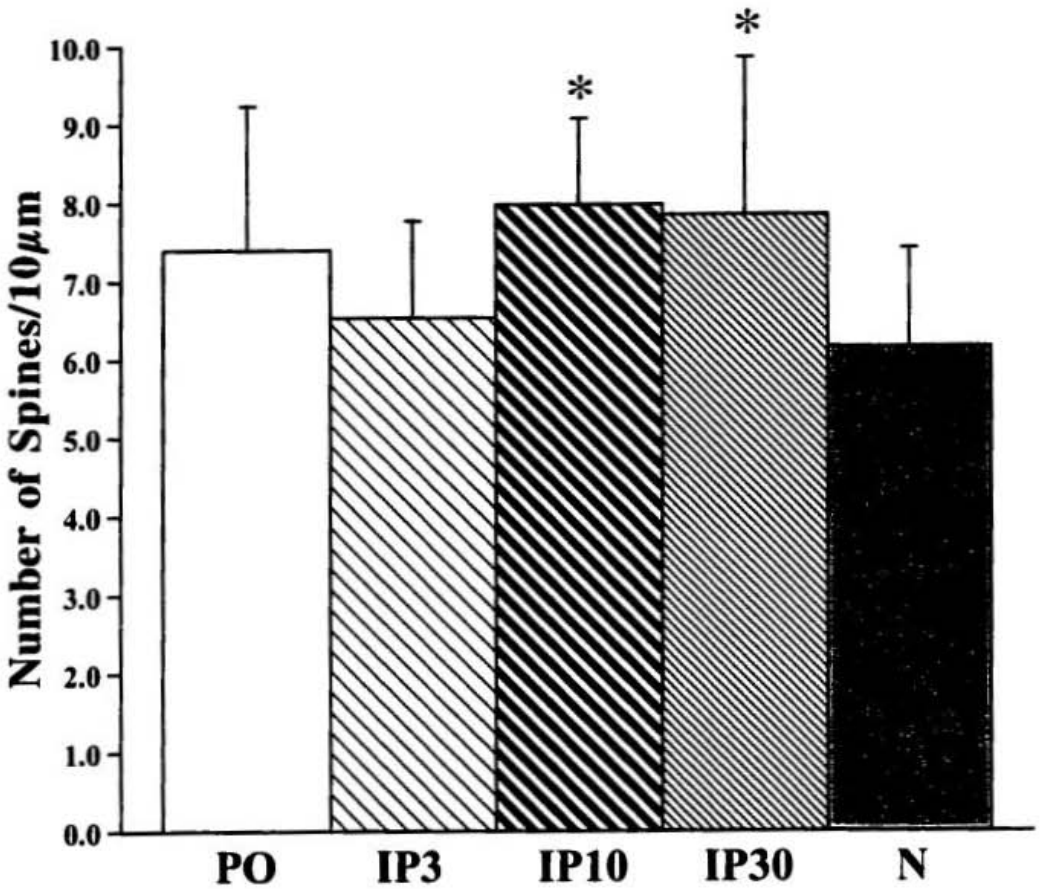

Fig 5: Spine densities of CA1 proximal dendrites (mean ± SD) from same groups as previous figure. (*$p < 0.05$ versus N).

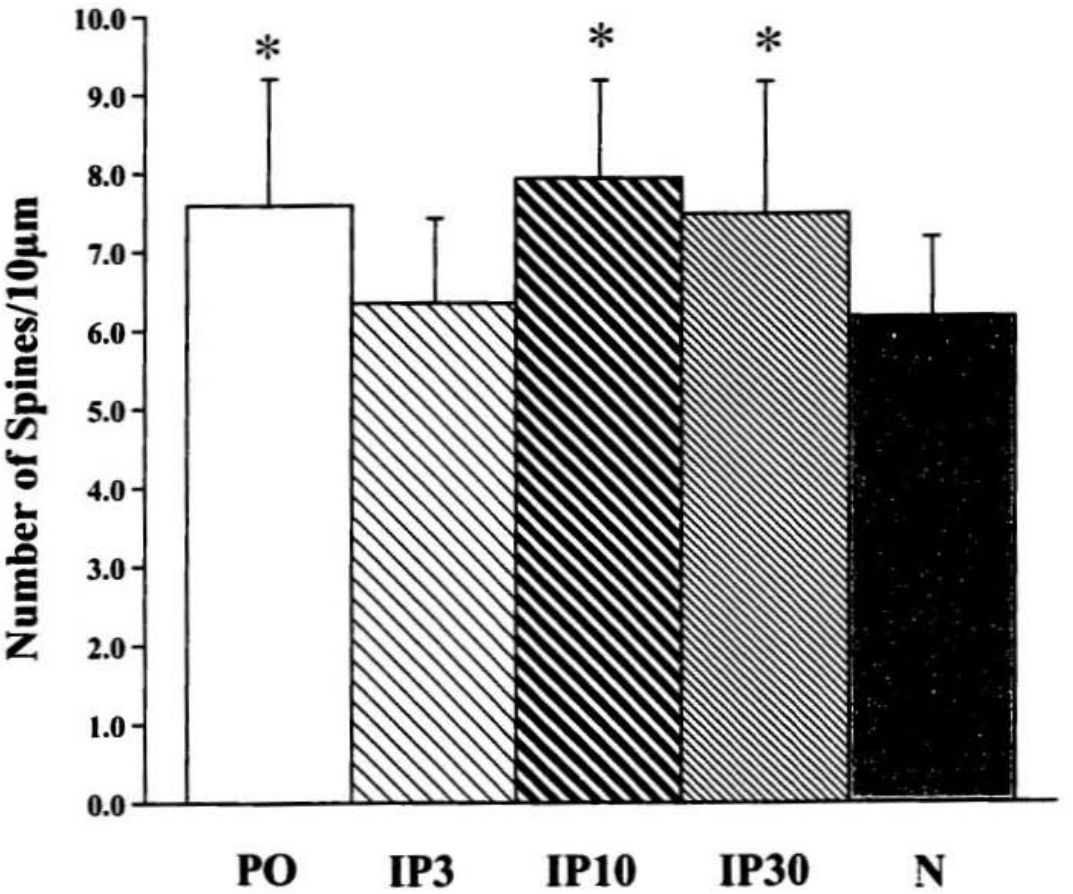

Fig 6: Spine densities of CA1 terminal dendrites (mean ± SD) from same groups as previous figures (*$p < 0.05$ versus N).

DISCUSSION

The present experiment has demonstrated that there are differences in spine densities between PO, IP and normal animals. These differences are in correlation with the hypothesis that dendritic spines contribute to the development of ischemic tolerance. Therefore, the present experiment suggests that morphological dendritic changes may be a possible mechanism of ischemic tolerance. An increase in spine density was seen 10 and 30 days following IP, and, 3 days following PO treatment. In addition, it was shown that open-field behaviour in IP animals was initially abnormal but began to recover to normal sometime between 10 and 30 days following IP, a time when spine density was elevated.

The PO animals had a higher spine density than normal animals on basilar, proximal and terminal dendrites. These results suggest that the observed increase in spine formation, which was seen 3 days after the last preconditioning episode, might be neuroprotective because this elevation coincides with the greatest degree of protection/ischemic tolerance. Normally with IP, the 5 min insult is given 3 days following the last preconditioning episode. Previous studies have shown that this 3 day interval seems to provide the greatest amount of protection against the more severe insult (i.e., 5 min) since shorter or much longer intervals results in less CA1 protection (Kitagawa et al., 1990; Kirino et al., 1991). Therefore, 3 days after preconditioning is an optimal time to detect mechanism(s) of ischemic tolerance. The observed increase in spine density seen in the PO animals supports the idea that dendritic spines may have a

neuroprotective role in brain injury (Segal, 1995; Segal, 2001). Segal proposes that small, transient increases in intracellular calcium are sufficient to cause elongation of existing spines and the formation of novel spines, whereas, large, more sustained increases in intracellular calcium may cause shrinkage and possibly collapse of existing spines (Segal, 2001). This may be the case in preconditioning, where the brief episodes of ischemia increase calcium to concentrations sufficient to cause an increase in spine density. Therefore, after the two PO episodes the dendrites have more spines, which may be able to buffer excess amounts of calcium if needed. During the 5 min ischemic episode calcium levels increase significantly, which would normally cause cell death (Choi, 1992). However, the CA1 cells, as a result of pre-conditioning, are better equipped to handle excess calcium. The extra spines take up the calcium and prevent it from reaching the parent dendrite through unique calcium buffering systems, including the presence of smooth endoplasmic reticulum, which takes up excess calcium as an internal store, and polyribosomes that are capable of synthesizing calcium-buffering proteins locally (Harris and Kater, 1994). This is supported by the finding that ischemic-tolerant hippocampal CA1 cells have differential calcium dynamics compared to non-tolerant neurons (Ohta et al., 1996). In the hippocampus of ischemic tolerant gerbils plasma membrane Ca^{2+} ATPase levels (i.e., plasma membrane calcium pumps) were significantly elevated before the 5 min episode and remained at a higher level throughout the following episode. The levels in these cells were similar to those displayed by ischemia resistant CA3 neurons. The increased calcium-buffering abilities reduced calcium toxicity following the 5 min insult and prevented delayed neuronal death.

Therefore, an increase in spine density may be responsible for the increase in calcium buffering systems seen in these ischemic-tolerant CA1 cells, thus providing a mechanism of neuroprotection.

Spine density was also elevated in IP animals that survived 10 and 30 days following the last ischemic insult. This effect was seen on basilar, proximal and terminal dendrites. However, those animals that survived only 3 days following the last ischemic insult did not show any change in spine density at this time point, which is seemingly due to the impact of the 5min episode. Following an ischemic insult hippocampal pyramidal cell dendrites show varicosity formation and collapse of spines, which may be occurring to reduce the synaptic efficacy of afferent fibres. Both of these characteristic changes are reversible following a return to standard conditions (Park et al., 1996). An increase in spine density in the IP animals following the 5 min episode may not be seen until 10 days later because the cell may initially retract dendritic spines in order to prevent excess incoming excitatory (potentially excitotoxic) input. Therefore, the IP3 animals do not show an increase in spine density, although spine densities are assumed to have been elevated prior to the 5min episode to the same level as the PO animals. This insult may then have caused a significant decease in spine number, that is, a possible retraction of spines. Between 10 and 30 days later the cells may attempt to recover lost synaptic connections, due to the death of neighbouring CA1 cells, by increasing the number of spines on the dendrites of surviving cells. In fact, the majority of incoming excitatory synaptic connections in the hippocampus are on dendritic spines (Andersen et al., 1966). Therefore, this may represent compensatory mechanisms that are taking place due to the

loss of surrounding CA1 neurons. If so, this could contribute to the recovery of function seen in the open-field behaviour at 30 days following IP. The open-field data show the IP animals had higher scores than normal animals up to and including 10 days after the last ischemic insult. This implies an impairment of habituation, a function of the hippocampus (Wang and Corbett, 1990; Babcock et al., 1993), which disappears by day 30, thus correlating temporally with the increase in spine density. Therefore, the increase in the number of spines evident at 10 and 30 days following IP may account for the subsequent return to normal open-field behaviour. In support of this interpretation, rats that were given frontal cortical lesions demonstrated recovery on a spatial learning task, which was associated with an increase in spine density on remaining cortical neurons (Kolb et al., 1997), suggesting that recovery from cortical injury may be mediated by dendritic changes in the remaining cortex.

Unlike the PO group, the increase in spine density seen in the IP animals is less likely to be attributed to neuroprotection, but is more likely a mechanism of neuroplasticity. It is possible that a neuroprotective response may still be in effect since the cell death process can continue for more than a month after ischemic pre-conditioning (Dooley and Corbett, 1998). However, the majority of the evidence suggests that the increase in spine density is a compensatory mechanism (Humm et al., 1997).
Dendritic injury following an ischemic insult has been studied intensively in recent years, although Ramon Y Cajal's description of cellular injury almost 100 years ago (Segal and Andersen, 2000) closely resembles what modern researchers have observed. The pattern of dendritic changes, that includes focal swelling (varicosities) and beading, has been

characterized both *in vivo* (Hsu and Buzsaki, 1993; Hori and Carpenter, 1994; Matesic and Lin, 1994) and *in vitro* (Park et al., 1996; Hasbani et al., 2001). After an ischemic or hypoxic event dendrites show segmental swelling with periodic spherical beading joined by thin regions of membrane. This is also accompanied by a loss of dendritic spines, which is mediated by glutamate release and NMDA receptor activation (Goldberg et al., 1987; Goldberg and Choi, 1993; Park et al., 1996). The nature of the swellings may possibly be caused by an excessive influx of ions and water, or, disruption of the cytoskeleton (Park et al., 1996). It is possible that the structural changes that occur in response to hypoxia or ischemia lead to early changes in synaptic efficacy and transmission failure, where focal constrictions seen between varicosities may cause electrical isolation of dendrites from the cell body, and a loss of dendritic spines would limit ongoing neuronal damage by excitotoxic mechanisms (Park et al., 1996). Therefore, the pathological process of dendritic swelling and beading may actually be a mechanism of cellular preservation. These dendritic changes have been observed in other models of global ischemia (Hori and Carpenter, 1994) and also noted in the present experiment. Although dendritic beading and swelling are characteristics of pathological processes, their recovery back to normal dendritic shape may contribute to functional recovery, such as that demonstrated in the present experiment. Neurons are capable of reorganizing synapses in response to injury. For example, Hasbani (Hasbani et al., 2001) has shown that despite widespread dendritic injury and spine loss following hypoxia, cultured cortical cell dendrites recovered from extensive spine loss after removal of the hypoxic situation. In addition they observed a re-emergence of spines from the same location

from which they disappeared, as well as the formation of dendritic filopodia in new locations along the dendritic shaft after dendritic recovery. Furthermore, throughout spine loss and recovery, presynaptic and postsynaptic elements remained intact (Hasbani et al., 2001). These findings suggest that the re-establishment of dendritic spine synapses in surviving neurons and the formation of novel spines may be a mechanism of functional recovery after an ischemic insult. Another study has demonstrated that striatal medium spiny I neurons go through a process of loss and replacement of dendritic spines after unilateral decortication, suggesting that these neurons are capable of forming new synaptic circuitry following deafferentation (Cheng et al., 1997). Since other neuronal cell types have the capacity for functional plasticity following injury, it is likely that hippocampal CA1 cell also possess the ability to restructure functional connections after ischemia. The present study has shown an increase in spine density on surviving neurons in the IP animals which can be seen up to one month after ischemia and which correlates temporally with a recovery of habituation in the open-field. Previous results from this lab have demonstrated recovery of function following ischemia. Following ischemic pre-conditioning the amplitude of fEPSPs are initially attenuated but return to sham levels 30 days following the last ischemic episode (Dooley and Corbett, 1998; Dowden and Corbett, 1999), and remain stable as long as 120 days (Farrell et al., 2001). As stated earlier, this coincides with a recovery of normal open-field behaviour. Therefore, the change in spine density in the IP10 and IP30 groups may be attributed to neuroplastic changes taking place to compensate for the loss of other CA1 cells, and thus restore the learning and memory functions associated with the hippocampus.

The signaling mechanisms leading to an increase in spine formation are still being investigated. One of the most promising candidates involved in this pathway is CREB (cAMP response element binding protein). CREB is known to be involved in long-term synaptic plasticity, more specifically, LTP, where the calcium-dependent phosphorylation of CREB (pCREB) is critical for maintaining the late-phase of LTP (Nguyen and Kandel, 1996). Imperative to the study of long-term neuronal plasticity are dendritic spines, since they are the primary targets of excitatory synaptic inputs and have been intimately linked with the morphological changes associated with LTP and behavioural plasticity (Lee et al., 1980; Chang and Greenough, 1984). Although it has been well established that both CREB and dendritic spines play pivotal roles in neuronal plasticity the molecular events linking them together in the regulation of new spine formation is still unknown. In CA1 cells, which are highly vulnerable to ischemia, there is a dramatic loss of CREB, whereas, in the resistant dentate granule cells there is a delayed increase in the levels of pCREB, suggesting that pCREB may be important for cell survival (Walton and Dragunow, 2000). Segal's (Segal and Murphy, 1998) study of the involvement of CREB in the estradiol-evoked increase in spine formation on cultured hippocampal neurons has demonstrated that an influx of calcium via NMDA-dependent synaptic channels leads to the activation of a cAMP cascade, which in turn, leads to the phosphorylation of CREB and the subsequent formation of new dendritic spines. In fact, the estradiol-induced increase in spine density (Woolley and McEwen, 1992) can be attributed to CREB activation through a GABA inhibitory mechanism, which has been linked to calcium influx (Murphy et al., 1998). Therefore, ischemic pre-conditioning may work through a

similar pathway to cause the formation of new spines on the CA1 cells of the hippocampus.

Another aspect of spines that may contribute to their neuroprotective/neuroplastic properties is their shape and size. Unfortunately, in the present experiment, spine lengths were unable to be appropriately analyzed due to the limitations of the tracing system used. However, according to these spine density results and Segal's unifying hypothesis (Segal, 2001), it would be suspected that the PO animals would show an increased spine length as compared to the other groups. Spines that are longer are more biochemically and electrically isolated from the parent dendrite and can maintain and regulate calcium levels independently from the parent dendrite, thereby, adding to the neuroprotective efficacy of dendritic spines (Volfovsky et al., 1999). This may also explain why a majority of the CA1 neurons in IP animals survive over time. Spine shape may be involved in neuroplastic mechanisms, as well. Novel or existing spines may develop bulbous heads, longer necks and even multiple spine heads, thereby increasing the surface area and the number of synaptic connections that they can accommodate. Hippocampal CA1 cells have varying ranges of spine dimensions; for example, spine neck diameter can range from 0.04 – 0.5 μm; neck length from 0.1 – 2 μm; maximum number of boutons per spine is 3; and maximum number of branches per spine is 3. These differences in spine parameters can be seen on the same cell and even on the same dendritic segment (Harris and Kater, 1994). Therefore, the dynamic nature, shape, and size of CA1 dendritic spines may contribute to the neuroprotective and neuroplastic mechanisms characteristic of the gerbil model of ischemic tolerance.

In conclusion, the present experiment supports previous claims that dendritic spines are involved in protecting the brain from injury, and also suggests that they are involved in compensatory mechanisms to recover from brain injury. Future work in this laboratory will examine the time course of CREB expression in the ischemic tolerant brain as it may correlate with the observed increase in spine density seen in this study. Another interesting experiment would be to investigate spine lengths and shape using electron microscopy in the IP model, since these structural features may contribute to the protective effects of dendritic spines. Also, measuring calcium influx during or after a pre-conditioning ischemic episode, as compared to the 5 min insult, would further shed light on the idea that alterations of dendritic morphology can be neuroprotective. The present experiment provides additional insights into the true mechanism(s) of ischemic tolerance. Once these endogenous mechanisms have been identified, they may provide a basis for novel treatment of stroke that is relatively free of undesirable side effects.

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