Effect of glutamate neurotoxicity on CaM kinase immunoreactivity in vivo
by Hui Liu

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences
Montana State University

Abstract:
It has been hypothesized that glutamate released during ischemia causes delayed neuronal cell death in vulnerable brain regions including the hippocampus. It has also been observed that calcium/calmodulin-dependent protein kinase II (CaM kinase) activity and immunoreactivity are decreased following ischemia. Recently, it was reported that glutamate induced reduction of CaM kinase immunoreactivity preceded neuronal cell death in cell culture. The present study was conducted to test the effect of glutamate neurotoxicity on CaM kinase immunoreactivity in vivo.

In Experiment I, 9 µl L-glutamate at a concentration of 34 µg/µl was bilaterally injected into the dorsal hippocampus of 10 gerbils. Five animals were perfused at 12 hr after infusion while the remaining 5 animals were perfused at 24 hr. Significant cell death was observed at 24 hr, but not at 12 hr following injection. The result demonstrated that injection of L-glutamate at this concentration caused cell death that was delayed.

Gerbils in Experiment II were injected with 9 µl L-glutamate (n=5) or D-glutamate (n=5) each at a concentration of 34 pg/pl. Twenty-four hrs following infusion, the animals were perfused. Significant cell death was observed in L-glutamate injected animals, but not D-glutamate injected animals. The results suggest that at this time point, damage to the pyramidal cell was a result of L-glutamate toxicity rather than alterations in osmotic pressure. Thus, the damage to pyramidal cells appeared to be mediated by stimulation of glutamate receptors.

In Experiment III, 10 gerbils received bilateral glutamate injections at a concentration of 34 pg/pl while a second group of 10 received saline injections. At 12 hr following glutamate injection, ten gerbils (5 glutamate-injected and 5 saline-injected) were behaviorally tested followed by perfusion and subsequent immunocytochemistry processing. The remaining 10 gerbils (5 glutamate injected and 5 saline injected) were euthanized at 12 hr following injection and hippocampi removed for immunoblot processing. During behavior testing, gerbils that received L-glutamate were not significantly more active than saline controls. However, gerbils injected with L-glutamate had a significant reduction in CaM kinase immunoreactivity than gerbils injected with saline.

These findings suggest that reduction of CaM kinase immunoreactivity post injection is mediated by glutamate and supports the notion that CaM kinase plays a role in glutamate-dependent, delayed neuronal cell death.

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Hui Liu

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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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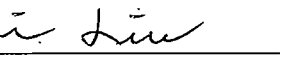
Signature 

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ABSTRACT

It has been hypothesized that glutamate released during ischemia causes delayed neuronal cell death in vulnerable brain regions including the hippocampus. It has also been observed that calcium/calmodulin-dependent protein kinase II (CaM kinase) activity and immunoreactivity are decreased following ischemia. Recently, it was reported that glutamate induced reduction of CaM kinase immunoreactivity preceded neuronal cell death in cell culture. The present study was conducted to test the effect of glutamate neurotoxicity on CaM kinase immunoreactivity *in vivo*.

In Experiment I, 9 μl L-glutamate at a concentration of 34 μg/μl was bilaterally injected into the dorsal hippocampus of 10 gerbils. Five animals were perfused at 12 hr after infusion while the remaining 5 animals were perfused at 24 hr. Significant cell death was observed at 24 hr, but not at 12 hr following injection. The result demonstrated that injection of L-glutamate at this concentration caused cell death that was delayed.

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In Experiment III, 10 gerbils received bilateral glutamate injections at a concentration of 34 μg/μl while a second group of 10 received saline injections. At 12 hr following glutamate injection, ten gerbils (5 glutamate-injected and 5 saline-injected) were behaviorally tested followed by perfusion and subsequent immunocytochemistry processing. The remaining 10 gerbils (5 glutamate injected and 5 saline injected) were euthanized at 12 hr following injection and hippocampi removed for immunoblot processing. During behavior testing, gerbils that received L-glutamate were not significantly more active than saline controls. However, gerbils injected with L-glutamate had a significant reduction in CaM kinase immunoreactivity than gerbils injected with saline.

These findings suggest that reduction of CaM kinase immunoreactivity post injection is mediated by glutamate and supports the notion that CaM kinase plays a role in glutamate-dependent, delayed neuronal cell death.

INTRODUCTION

The American Heart Association reported that stroke killed 149,740 people in 1993, accounting for about one of every 15 U.S. deaths. Stroke is the third leading cause of death, ranking behind diseases of the heart and cancer (Heart and Stroke Facts: 1996 Statistical Supplement, American Heart Association). About one-third of all strokes are preceded by one or more "mini-strokes" known as transient global ischemia. This type of ischemic attack often occurs during cardiac arrest or anoxia, and can result in delayed neuronal cell death in certain vulnerable brain regions including the hippocampus (Cummings et al., 1984; Petito et al., 1990). Damage to the hippocampus can cause impaired learning and memory of certain types of events after ischemia (Davis et al., 1985; Imamura et al., 1991; Petito et al., 1987). Previous studies have shown that glutamate increases severalfold in hippocampus following ischemia (Choi, 1990; Rothman and Olney, 1986). Thus, it is hypothesized that glutamate released during ischemia causes neuronal death in vulnerable brain regions including the hippocampus (Jorgensen and Diemer, 1982). One of the most important distinctions between the hippocampus and other areas in the brain is the high density of glutamate receptors (Benveniste et al., 1989; Shigemoto, 1992). Once glutamate receptor channels are opened, Ca^{2+} influx into neuronal cells increases causing a cascade of events that lead to cell death (Benveniste et al., 1989; Shigemoto, 1992).

Activity and immunoreactivity of Calcium/calmodulin-dependent protein kinase II (CaM kinase), an enzyme important for numerous cellular functions, have been shown to be reduced following ischemia (Churn et al., 1990; Churn et al., 1992a and b). Recently, it was reported that glutamate could reduce CaM kinase immunoreactivity *in vitro* (Churn et al., 1995). Taken together, these data suggest that glutamate released during ischemia is responsible for the reduction of CaM kinase activity and immunoreactivity, which may in turn mediate ischemic cell death. The following sections review the classification of stroke and the current theories regarding the mechanisms of selective ischemic cell death in the hippocampus.

Classifications of Stroke

A stroke occurs when blood vessels carrying oxygen and other nutrients to a specific part of the brain rupture or become blocked. Strokes fall into several major categories, based on whether the disruption in blood supply is caused by ischemia or hemorrhage (Smith, 1967). Ischemic stroke results from a blocked blood vessel, and includes both thrombotic and embolic stroke. Thrombotic stroke (or cerebral thrombosis) is the most common type of stroke. Here, a blood clot (thrombus) forms inside an artery in the brain, blocking blood flow. Typically the clot occurs in one of the carotid or vertebral arteries. Blood clots can form in arteries damaged by atherosclerosis. Embolic stroke (or cerebral embolism) is also

caused by a clot; however, unlike cerebral thrombosis, the clot originates somewhere other than the brain. This stroke occurs when a piece of a clot (an embolus) breaks loose and is carried by the blood stream to the brain. Traveling through the arteries as they branch into smaller vessels, the clot reaches a point where it can go no further and plugs the vessel, cutting off the blood supply.

Hemorrhagic stroke occurs when a blood vessel in or around the brain ruptures, spilling blood into the brain tissue (Smith, 1967). When this occurs, the cells nourished by the artery fail to get their normal supply of nutrients and cease to function properly. Furthermore, the accumulated blood from the ruptured artery soon clots, displacing normal brain tissue and disrupting brain function. Cerebral hemorrhage is most likely to occur in people who suffer from a combination of atherosclerosis and high blood pressure. There are two main types of hemorrhagic strokes: subarachnoid and intracerebral, which refer to the parts of the brain affected by the bleeding.

About one-third of all strokes are preceded by one or more "mini-strokes" known as transient ischemic attacks (TIA's). TIA's usually result from cardiac arrest or anoxia where blood flow to the entire brain is disrupted for a short amount of time. Depending upon the area of the brain involved, various deficits may occur. Some patients may not be able to move a limb, or even one side of the body. The most common symptom in TIA patients is a deficit in cognitive abilities

such as confusion, difficulties in memory and judgment, and dementia (Ullman, 1962). It has been reported that TIA's can cause delayed neuronal cell death to certain vulnerable brain regions including the hippocampus (Cummings et al., 1984; Davis et al., 1985; Imamura et al., 1991; Petito et al., 1987).

The Hippocampus and TIA's

The hippocampus, a structure located deep within the temporal lobes, is named for its resemblance to a seahorse. Hippocampus has been differentiated into subfields CA1 through CA4, largely on the basis of the cytoarchitecture of the pyramidal neurons. Afferents to the hippocampus enter mainly from the entorhinal cortex by the perforant path. The incoming information reaches the dentate gyrus and sends mossy fibers to the CA3 region. The axons of CA3 pyramidal neurons give rise to the Schaffer collaterals, which synapse with CA1 pyramidal cells. The efferent projections of the hippocampus are from the pyramidal neurons of CA1, and their axons send messages through the fornix to the mammillary bodies, which ultimately sends axons to the cingulate cortex. The hippocampal formation also sends a set of efferent fibers to the septal region.

After a TIA, patients can suffer an amnesia syndrome which is characterized by impaired learning and memory of events after injury (Cummings et al., 1984; Davis et al., 1985; Imamura et al., 1991; Petito et al., 1987). In recent years, studies have shown that these types of memory impairments reflect damage

limited to the hippocampus. In one early study, the brains of fourteen cardiopulmonary arrest patients were evaluated. Eight patients dying eighteen hours or less after cardiac arrest had minimal damage in hippocampus and moderate damage in cerebral cortex and putamen. Six patients surviving twenty-four hours or more had severe damage in all three regions. The increase in damage with time post-arrest was significant only in the hippocampus (Petito et al., 1987).

Other studies have shown a relationship between damage to the hippocampus and memory impairment. Among the reported cases, patient R.B. provided valuable information about the organization of memory functions (Zola-Morgan et al., 1986). Patient R.B. suffered amnesia in 1978 as the result of an ischemic event that occurred after cardiac arrest. This individual survived for five years after the ischemic event. He was evaluated for his cognitive functions and found to have a severe anterograde memory impairment. Examination of R.B.'s brain after his death showed a lesion limited to the CA1 sector of the hippocampus. The lesion was bilateral and extended the full rostrocaudal extent of the hippocampus and no other major damage was found in other brain areas. This case established a significant relationship between damage limited to the hippocampus and memory impairment. In 1990, a similar case of memory impairment associated with a bilateral lesion of the hippocampus was reported (Victor and Agamanolis, 1990).

In recent years, with the development of magnetic resonance imaging, it has become possible to acquire anatomical information in living patients. Using this technology, Squire et al. (1990) found abnormalities of hippocampal structures in four patients with circumscribed memory impairment. Thus, visual evidence has been presented in support of the essential role of hippocampus in memory function.

Animal Models of Ischemia

It is difficult to study the effects of ischemia in humans because the occurrence and severity of TIA's are unpredictable. It has been necessary to use animal models to understand the underlying mechanism of human brain damage due to ischemia. Mishkin (1978) used monkeys to develop an animal model of human amnesia by making large medial temporal lobe lesions, which were intended to mimic the surgical lesion sustained by a stroke patient. Before the lesion, monkeys made 90 correct choices out of 100 trials in a object-pair test after a only brief (a few seconds) familiarization. After the lesion, when the monkeys' performance returned to criterion, their recognition ability was examined further. A delay between stimuli presentation was lengthened in stages from the original 10 sec delay to 30 sec, then to 60 sec, and finally to 120 sec. In addition, the number of objects given for familiarization before pairing each one with a novel object was increased in stages from the original 1 object to 3 objects presented

successively, then to 5 objects, and finally to 10 objects. Since the animals had already regained the ability to perform the basic task, their sharp decrease in performance with the longer delays and increase in number of objects given for familiarization before pairing represented a true memory loss rather than some other difficulty such as a problem with visual perception. Thus, this monkey lesion model demonstrated that the damage of the hippocampus caused the same memory impairment found in humans.

Rat models of cerebral ischemia have also been used to demonstrate a similar pattern of delayed cell death that occurred selectively in the CA1 area of the hippocampus (Aggleton et al., 1986; Sutherland and McDonald, 1990). In these models, a common carotid artery occlusion is preceded by electrocoagulation of the vertebral arteries. This is needed since the connections between the vertebral arteries and the common carotid arteries can provide blood to the forebrain. One of drawbacks to this model is that about 25% of the animals die during electrocoagulation of the vertebral arteries or exhibit respiratory failure due to lack of blood flow to the brain stem (Ginsberg and Busto, 1989). A variety of behavioral tests have been used with rats to assess hippocampal functions. For example, an eight to twelve radial arm maze can be used to examine the working and reference memory (Davis et al., 1985; Olton et al., 1979). In a typical radial maze, the same 5 arms may be baited on all trials. Reference memory performance

refers to entering baited arms only, while working memory performance refers to not re-entering a baited arm after the food has been taken. In other words, reference memory performance requires the animal to learn that baited arms remain constant relative to room cues for all trials. Working memory performance requires the animal to remember from which arms food has been taken so that it will not re-enter that arm during a particular trial. The reference memory component represents invarant material that is useful over many trials, while working memory involves retention of trial-specific information that varies among trials. After hippocampal lesioning, rats consistently exhibit working memory errors.

Gerbils have also been used in ischemic research because they lack a complete Circle of Willis, a structure that connects carotid arteries to vertebral arteries. Based on this feature, a simple surgical operation has been developed to produce damage restricted to the hippocampus. A marked loss of pyramidal cells of CA1 area is produced with 5 minutes of bilateral occlusion of the common carotid arteries (Kirino, 1982). As in rats, ischemic gerbils exhibit permanent working memory errors following cerebral ischemia (Amano et al., 1993; Babcock and Graham-Goodwin, 1997; Imamura et al., 1991; Katoh et al., 1992). Although an eight-arm radial maze task has been commonly used for studying working memory, the data obtained in this task reflect not only working memory but also

reference memory (Watts et al., 1981). A delayed non-matching to position task can be used to evaluate working memory in isolation (Imamura et al., 1991). Typically, a T-maze task is a tool for the delayed non-matching to position task (Amano et al., 1993; Babcock and Graham-Goodwin, 1997; Imamura et al., 1991; Katoh et al., 1992). In this task, each trial consists of a pair of forced and choice trials. A forced trial is when the gerbil is allowed to enter an open arm to receive food reinforcement. In the choice trial, the animal must enter the arm opposite to the forced run in order to obtain a reinforcer (Babcock and Graham-Goodwin, 1997). Studies have shown that bilateral occlusion of the common carotid arteries in gerbils for 5 min significantly decreased the percentage of correct responses (Amano et al., 1993; Babcock and Graham-Goodwin, 1997; Imamura et al., 1991; Katoh et al., 1992). These data suggested that the working memory is highly vulnerable to the cerebral ischemia in the gerbils.

Another behavioral marker of ischemic damage is increased locomotor activity (Babcock et al., 1993b; Chandler et al., 1985; Mileson and Schwartz, 1991; Wang and Corbett, 1990). In a study by Babcock et al. (1993b), gerbils were tested in an open field apparatus for 10 minutes following ischemic insult. Ischemic gerbils exhibited a large increase in locomotor activity when tested at 24hr or 14 days post-occlusion. These data suggested that the effects of ischemia on locomotor activity are not limited to a brief period after stroke and may

represent a long-term, or permanent, deficit (Babcock et al., 1993b). It has been proposed that the deficit in spatial mapping or habituation induced by ischemia is due to an impairment in the ability to transfer information from short-term memory to long-term memory (Chandler et al., 1985; Mileson and Schwartz, 1991; Wang and Corbett, 1990).

Excitotoxic Glutamate Hypothesis

Why the hippocampus is so sensitive to ischemia is not completely understood. One anatomical distinction of the hippocampus from other areas in the brain is the high density of glutamate receptors (Benveniste et al., 1989; Shigemoto, 1992). The receptors that mediate the diverse processes elicited by glutamate have been classified into two major categories: ionotropic receptors that have glutamate-gated cation channels, and metabotropic receptors that are linked to the inositol phosphate/Ca^{2+} intracellular signaling pathway (Monaghan, 1989). Pharmacological and electrophysiological studies have further defined three subtypes of ionotropic glutamate receptors: N-methyl-D-asparate (NMDA), alpha-amino-3-hydroxy-5-methyl-ioxyzole-4-propionic acid (AMPA) and kainate. The existence of multiple subtypes for metabotropic glutamate receptors has also been suggested pharmacologically by stimulating phosphoinositol turnover and mobilizing intracellular calcium in various mammalian CNS preparations (Schoepp et al., 1990). Relatively high levels of NMDA receptors are found in

CA1 stratum oriens and stratum radiatum, and inner molecular layer. High levels of kainate and AMPA receptors are found in CA1 and CA3 pyramidal cell layers. It has also been shown that metabotropic receptors are found in the stratum oriens and pyramidal cell layer of CA1, and the stratum oriens and stratum radiatum of CA3 (Benveniste et al., 1989; Shigemoto, 1992).

Olney (1986) used the term "excitotoxicity" to refer to the ability of glutamate and structurally related excitatory amino acids to mediate the death of neurons. Microdialysis studies have shown that the interstitial glutamate increases severalfold in the hippocampus following ischemia (Benveniste, 1991; Choi, 1990; Rothman and Olney 1986). Normally, a glutamate concentration of 138 μM is observed and represents an average of widely fluctuating concentrations that occur during and between transmission in the synaptic cleft. During ischemia, the glutamate concentration in interstitial space is increased significantly to 968 μM. Thus, it was hypothesized that glutamate released during ischemia may cause neuronal death in vulnerable brain regions (Jorgensen and Diemer, 1982).

Support for this idea has come from a number of studies. Benveniste et al. (1989) assessed the neurotoxic property of glutamate associated with ischemia by injecting the same concentration of glutamate (0.17 μg/μl) into the CA1 region as is observed during ischemia. They found that glutamate, at the concentration of 0.17 μg/μl could destroy CA1 pyramidal cells in the area of the injection site.

It has also been reported that brain regions vulnerable to ischemia can be protected when glutamatergic afferents are removed prior to the ischemic insult (Johansen et al., 1986). In the areas where axons from CA3 to CA1 were lesioned, the concentration of glutamate measured during ischemia was significantly lower than that obtained in nonlesioned rats. No CA1 pyramidal cell loss was demonstrated in these CA3-lesioned animals (Benveniste, 1991).

Glutamate antagonists can also be neuroprotective by blocking the activation of glutamate receptors. NMDA antagonists can be competitive and noncompetitive. Competitive NMDA antagonists directly block the glutamate recognition site (Foster and Fagg, 1984; Kurumaji et al., 1989; Schoepp et al., 1989). This group includes 2-amino-5-phosphonovalerate (APV) and 2-amino-7-phosphonoheptanoate (APH) which do not readily cross the blood brain barrier (Foster and Fagg, 1984), while others such as 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (Kurumaji et al., 1989) and cis-4-phosphonomethyl-2-piperidine-carboxylic acid (CGS 19755) (Schoepp et al., 1989) can. Noncompetitive NMDA antagonists do not compete for binding at the glutamate receptor site, but bind at the phencyclidine site within the NMDA receptor complex (Wrong et al., 1986). This group of antagonists includes ketamine, phencyclidine (PCP), benzomorphan, and the dibenzoxycloheptenine, (+)-5-methyl-10, 11-dihydro-5H-dibenzo (a, d) cyclohepten-5, 10-imine (MK-801). One

of the beneficial effects of glutamate receptor antagonists during ischemia could be their ability to limit excessive intracellular calcium accumulation. However, glutamate is a mixed agonist, acting on kainate, AMPA, NMDA and metabotropic receptors. Thus, the NMDA receptor antagonists only partially inhibited calcium influx by blocking the receptor-gated calcium channel during ischemia; whereas other calcium channels are still open by metabotropic receptors. This could explain the ineffectiveness of NMDA antagonists in the global ischemia model of the rat and the complete prevention of ischemia-induced calcium influx by deafferentation (Benveniste et al., 1989; Block and Pulsinelli, 1987).

Role of Ca^{2+} in Ischemic Neuron Death

Once glutamate is released into the synaptic cleft, it binds to its receptors causing increased intracellular calcium (Benveniste, 1991; Shigemoto, 1992). The normal level of intracellular free Ca^{2+} is about 100 nM in contrast to the interstitial concentration of 1 mM (Benveniste, 1991). During ischemia however, intracellular Ca^{2+} levels rapidly rise to 1 μM or more in response to incoming signals (Hanson and Schulman, 1992). There are two main pathways for the intracellular level of calcium to increase during ischemic insult: first, via influx from the interstitial compartment through calcium channels, and second, via release from intracellular stores. The NMDA receptor gates a cation channel that is permeable to Ca^{2+} and Na^{+}, and is gated by Mg^{2+} in a voltage-dependent manner. Glutamate binding to

AMPA and kainate receptors allows Na^+ to enter and K^+ to leave the cell, resulting in the depolarization of the postsynaptic membrane. Mg^{2+} is then ejected from the NMDA receptor permitting the entry of Ca^{2+} into the cell.

Calcium entry can also be caused by glutamate binding to a metabotropic receptor. This receptor is linked to the inosital phosphate/Ca^{2+} intracellular signaling pathway coupled to G-protein. After glutamate binding, G-protein is activated causing the hydrolysis of phospholipids into inositol-phospholipid 3 (IP_3) and diacylglycerol (DAG) by phospholipase C. IP_3 induces calcium mobilization. DAG can be degraded into arachidonic acid which inhibits glutamate reuptake, increasing the short-term efficacy of the synapse (Barinaga, 1993). It is well established that IP_3 has a crucial role in Ca^{2+} mobilization. Evidence for this theory has been obtained by studying permeabilized pancreatic cells (Streb et al, 1983). When added to a low calcium medium, IP_3 results in increased Ca^{2+} level. It has been hypothesized the IP_3 can access intracellular Ca^{2+} stores and stimulate its release. The rough endoplasmic reticulum and Golgi appear to be the sites of the intracellular Ca^{2+} stores (Berridge and Irvine, 1984) since IP_3 receptors are found on their membranes (Mignery et al., 1989; Ross et al., 1989)

Neurons normally maintain an extremely low intracellular level of Ca^{2+} and utilize transient intracellular increases as a second messenger system. Post-synaptic responses at glutamatergic synapses are terminated by re-uptake of the

neurotransmitter and by extrusion of Ca^{2+} and Na^{+}. During ischemia, a lack of ATP inhibits transmitter re-uptake, which results in large increases in extracellular glutamate. This in turn causes prolonged receptor activation and channel opening with an exaggerated influx of calcium (Benveniste, 1991). In addition, due to energy failure, Ca^{2+} sequestering mechanisms are greatly reduced and extrusion of this cation is stopped by reversal of the Na^{+}/Ca^{2+} exchange mechanisms (Carafoli, 1987). All these pathological responses result in an excessive overload of calcium that appears to play a central role in ischemic neuronal death.

In addition, glutamate neurotoxicity has also been thought to induce excessive cell swelling caused by cellular entry of Na^{+} and Cl^{-} (Olney et al., 1986). Although this may be important *in vitro*, it may not play a major role in the intact brain because of the limited amount of extracellular space which prevents excessive swelling (Benveniste, 1991). More attention has been paid to several catabolic enzymes that are activated by the increased Ca^{2+} following ischemia. These enzymes include calpain (protease), nitric oxide (NO) synthase, phospholipase A_2 (PLA_2) and CaM kinase.

Catabolic Enzymes in Ischemic Neuron Death

Calpain

Calpain is a calcium-dependent intracellular protease that is important in modulating both normal and pathological cellular metabolism. The participation of

calpain in normal neuronal functioning has been proposed by Lasek and colleagues (1977), who suggested that the apparent degradation of neurofilaments in active, non-growing axon terminals is accomplished by the activation of calpain in response to the synaptically evoked influx of Ca^{2+}. Under normal conditions, spectrin, microtubule-associated protein 2 (MAP2), and tubulin are thought to be primary components of the submembraneous protein skeleton that share structural and functional homology with the cytoskeleton. These components may control cell shape, membrane protein, and lipid organization (Shoeman and Traub, 1990). Once activated, calpain breaks down spectrin and MAP2. However, sustained increased intracellular Ca^{2+} may cause a number of pathogenic conditions mediated by calpain (Lee et al., 1991; Siman et al., 1989; Seubert et al., 1989). The degradation of proteins could bring about localized collapse of the protein skeleton and overlying membrane, thereby initiating neuronal structural disintegration (Arai et al., 1991; Johnson et al., 1991; Siman et al., 1985; Siman et al., 1989; Seubert et al., 1989). Calpains have also been implicated in the formation of free radicals. Free radicals are considered to cause lipid peroxidation as well as the oxidation of proteins and nucleic acids. Consequently, this could lead to the dysfunction of the membrane and cellular enzymes contributing to the process of cell death (Sies, 1986). Cheng and Sun (1994) found free radical formation in the gerbil brain after administration of kainic acid (KA). It was

shown that calpain inhibitor I as well as allopurinol, a selective xanthine oxidase (XO) inhibitor, significantly protected cortical neurons from KA-induced cell death. These data suggest that calpain-induced XO activation may play an important role in neuronal excitotoxicity.

Nitric oxide synthase

It would seem likely that excitotoxicity involves more than a mere increase in glutamate release since under normal conditions, cell-surface proteins known as glutamate transporters can mop up a flood of glutamate and save neurons from damage (Cooper et al., 1991). It has been suggested that the transporters somehow lose their function during excitotoxicity. It was reported that NO is produced by NO synthase when glutamate binds to the NMDA receptor (Pogun et al., 1994; Pogun and Kuhar, 1994). NO in turn may destroy the transporters, leaving the destructive flood of glutamate to accumulate in the synapse. It has also been suggested that NO plays other roles in mediating excitotoxicity. When a glutamate-stimulated cell makes more of the gas, NO could diffuse back to the glutamate-producing cell, boosting glutamate release. NO may be converted into an even more reactive free radical molecule such as peroxynitrate which can also damage cells.

Phospholipase A_2

Although arachidonic acid (AA) can be produced from DAG by the action of DAG lipase, it is mainly derived from phospholipids by the activation of G-protein regulated PLA_2, which is ubiquitously present in mammalian tissues (Channon and Leslie, 1990; Waite, 1985). These enzymes are active in the presence of Ca^{2+} concentrations less than 1 μM, and appear to translocate to membranes in response to agonists such as bradykinin, histamine, ATP, and thrombin (Channon and Leslie, 1990). There seem to be AA-selective and non-selective PLA_2 within cells (Schalkwijk et al, 1990). The AA is produced from phospholipids by the action of a nonselective type of PLA_2 (Chen et al, 1992; Khan et al, 1992). The three major groups of AA are prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs). These AA are not stored in tissue but are synthesized on demand, particularly in pathophysiological conditions (Cooper et al., 1991). After produced, these AA are metabolized to free radicals.

Calcium / calmodulin kinase II

CaM kinase is a multifunctional enzyme that plays an important role in calcium second messenger systems (Yamamoto et al., 1985). It typically contains 50-54 kDa (α) and 58-60 kDa (β) subunits in a ratio of 3:1 (Hanson and Schulman, 1992). CaM kinase is highly expressed in brain, about 20-50 fold more than in non-neuronal tissues. It comprises 1% of total forebrain protein and about

2% of total hippocampal protein. The α subunit of CaM kinase constitutes 1.4%, while β subunits about 0.6% of hippocampal protein (Kennedy et al., 1983b; Erondu and Kennedy, 1985). CaM kinase is expressed primarily in neurons (Erondu and Kennedy, 1985). Within the hippocampus, the highest level of CaM kinase immunoreactivity is observed in the molecular and CA pyramidal cell layers and in granule cell layers of the dentate gyrus (Kennedy et al., 1983b; Erondu and Kennedy, 1985). CaM kinase is found on both sides of the synapse suggesting that this enzyme is important for normal synaptic function (Kennedy et al., 1983a). In response to Ca^{2+} signals, CaM kinase is activated and controls a series of cellular functions including metabolism of carbohydrates, lipids, and amino acids, neurotransmitter release, neurotransmitter synthesis, ion channels, and gene expression (Hanson and Schulman 1992).

Calcium activates CaM kinase by binding to calmodulin, which in turn binds to the enzyme. The binding domain of calmodulin is located between residues 296 and 309 in the α-subunit and 297 and 310 in the β-subunit. Once activated, CaM kinase undergoes autophosphorylation. The α- and β-subunits can autophosphorylate independently of each other. Initial rapid autophosphorylation converts a completely calcium/calmodulin-dependent enzyme to a partially calcium/calmodulin-independent kinase. At this state of incomplete autophosphorylation, CaM kinase is still sensitive to calcium/calmodulin and in its

presence, the majority of maximal activity can be obtained (Bronstein et al, 1993). After this state of autophosphorylation, the activation of CaM kinase is no longer dependent on calcium/calmodulin. Activity of CaM kinase in the absence of calcium/calmodulin increased dramatically from about 5% to 74% compared to that observed in the presence of calcium/ calmodulin (Saitoh and Schwartz, 1985). The concentration of ATP has an effect on CaM kinase activity during autophosphorylation. With 5 μM of ATP, 75% of the total kinase activity is lost, while at 500 μM of ATP, the total activity of the enzyme shows much less decrease (Lou et al., 1986). After autophosphorylation, CaM kinase is activated to phosphorylate protein substrates that are important to neural functioning (Bronstein et al., 1993).

Rapid changes in CaM kinase activity and immunoreactivity have been observed following ischemia (Churn et al., 1990; Churn et al., 1992a and b; Taft et al., 1988; Wasterlain and Powell, 1986). CaM kinase activity and immunoreactivity disappear after 5 min of ischemia. Reductions in CaM kinase activity and immunoreactivity are typically demonstrated using an assay that measures autophosphorylation of the enzyme and immunocytochemistry respectively. The sudden change in CaM kinase activity and immunoreactivity has the potential to adversely affect many cellular processes. Ischemia has been shown to change almost every aspect of neuronal metabolism, such as ion homeostasis,

ATP levels, metabolic products, toxin levels and membrane potential. These changes could be initiated by the reduction of CaM kinase. However, these important physiological alterations can return to preischemic levels on recirculation, while the decrease in CaM kinase activity and immunoreactivity after ischemia is an early and long-lasting phenomenon that precedes the development of delayed neuron death.

How CaM kinase activity and immunoreactivity are reduced following ischemia is not completely understood. Several explanations have been proposed. First, it has been suggested that following ischemic insult, a posttranslational modification of CaM kinase occurs. This change of CaM kinase structure is responsible for the loss of the enzyme immunoreactivity (Churn et al., 1992a). Since the total amount of enzyme is unchanged, the decreased immunoreactivity of the enzyme might result from the posttranslational alteration of the enzyme. CaM kinase from ischemic brain tissue shows a decreased affinity for ATP while calcium and calmodulin binding are unaffected. An alternative explanation is that ischemia causes a down-regulation of CaM kinase expression (Hiestand and Kindy, 1992). CaM kinase mRNA levels in hippocampus of ischemic tissue were reported to decrease by 26% (Hiestand and Kindy, 1992). However, Babcock et al. (1995) demonstrated a disappearance of CaM kinase immunoreactivity in hippocampal regions that are vulnerable to ischemic insult without changes in

mRNA levels. This finding suggests that the rapid disappearance of CaM kinase activity is not mediated by a change in mRNA expression. A third hypothesis is that CaM kinase rapidly declines in the supernatant fraction while increasing in the particulate fraction (Aronowski et al., 1992). This study suggested that ischemia may cause a translocation of CaM kinase. A final possible explanation suggests that changes in ATP levels might influence CaM kinase activity (Churn et al., 1990; Lou et al., 1985). At low levels of ATP, loss of CaM kinase activity is more pronounced (Churn et al., 1990; Lou et al., 1985). In support of this proposal, it has been reported that mild hypothermia during or shortly after an ischemic insult preserves CaM kinase activity and ischemia-induced damage both in gerbils and rats (Churn et al., 1990; Busto et al., 1989). The possible mechanism is that hypothermia can preserve ATP, and may even protect against reduction of CaM kinase activity (Churn et al., 1990; Busto et al., 1989; Lou et al., 1985).

STATEMENT OF PURPOSE

It has been previously demonstrated that transient global ischemia results in delayed neuronal cell death in certain vulnerable brain regions including the hippocampus (Cummings et al., 1984; Davis et al., 1985; Imamura et al., 1991; Petito et al., 1987). It has also been shown that extracellular glutamate levels are high during ischemia and that CaM kinase activity and immunoreactivity decrease prior to cell death (Choi, 1990; Churn et al., 1990; Churn et al., 1992a and b; Rothman and Olney 1986). Although each of these events is correlated, it is not clear how glutamate causes cell death following ischemia and whether it plays its role by causing a reduction of CaM kinase activity. Recently, it was reported that glutamate causes reduction of CaM kinase immunoreactivity in cultured hippocampal neurons (Churn et al., 1995). In this study, the glutamate-induced reduction of CaM kinase immunoreactivity preceded neuronal cell death, supporting the hypothesis that change of this enzyme may play a role in glutamate-dependent, delayed neuronal cell death. However, this research was conducted *in vitro,* which can not model the complex factors of an *in vivo* system which includes a blood supply, changing levels of ATP, glutamate re-uptake mechanisms, calcium mobilization, calcium sequestering and calcium extrusion processes. To our knowledge, there is no *in vivo* data showing a relationship between glutamate excitotoxicity and a reduction of CaM kinase

immunoreactivity. Thus, the present series of experiments was designed to evaluate the effect of glutamate neurotoxicity on CaM kinase immunoreactivity *in vivo*.

EXPERIMENTS

Experiment I

Introduction

Infusion of glutamate into the rat hippocampus, at concentrations observed during ischemia, destroys hippocampal CA1 pyramidal cells in the vicinity of the injection site (Benveniste et al., 1989). The goal of the present study was to determine the dose of L-glutamate capable of producing delayed cell death in the gerbil hippocampus. These data were critical to subsequent experiments aimed at studying the relationship between glutamate and CaM kinase *in vivo*. In a series of preliminary studies, the effects of L-glutamate at doses ranging from 0.17 μg/μl to 170 μg/μl were evaluated (Data not shown). Histological examination of the hippocampal pyramidal cells was conducted at 12 hours and 24 hours after glutamate injection. Result indicated that 34 μg/μl was the lowest concentration which reliably causes the damage of pyramidal cells of CA1 in hippocampus at 24 hour with histologically viable cells still present at 12 hour after injection. The following experiment was a replication of this finding.

Methods

Subjects

Ten adult male and female Mongolian gerbils (*Meriones unguiculatus*) weighing between 70 and 80 gms were used as subjects. Ten animals were injected with L-glutamate. After injection, all animals were housed individually in a temperature (23°C) and light (12-hr light/dark cycle) controlled environment with commercial rodent pellets and water provided *ad libitum*.

Injection Procedure

Gerbils were mounted in a Kopf stereotaxic frame after being anesthetized with methoxyflurane. Methoxyflurane and oxygen were continuous administered during the procedure via a modified nose cone. Subjects were placed on a homeothermic control blanket (Harvard Homeothermic Unit) and body temperature was maintained between 37-38 °C. An opening was cut on the dorsal surface of the scalp exposing the cranium and bregma was determined. Based on a standard gerbil brain atlas, the injection site was 2.0 mm posterior to bregma, 1.6 lateral to midline, and 1.1 mm below the cortical surface. A burr hole was drilled at this mark and the injection cannula lowered to a depth immediately dorsal to the hippocampal CA1 pyramidal cell layer on both sides. Ten gerbils were injected with 9 µl of L-glutamic acid (34 µg/µl) dissolved in saline, over a 5 min period as

described in a previous study (Benveniste et al., 1989). At the end of the injection, the cannula was removed and the incision closed under aseptic conditions.

Histology

Gerbils were euthanized with CO_2 and perfused with 0.9% saline followed by 10% buffered formalin at 12 (n=5) or 24 (n=5) hours following infusion. Brains were postfixed in 10% formalin for at least 48 hours before cryoprotecting with 30% sucrose. The tissue was frozen and cut using a Reichert-Jung 2800 Frigocut N cryostat. Sections (20μm) were collected at the injection site. Cresyl violet was used to stain sections for the evaluation of hippocampal damage in the region of the injection site. Sections were dehydrated with a series of alcohols and cleared with xylenes before being cover-slipped. Damage to the CA1 hippocampal region was evaluated without knowledge of treatment condition. Viable cells (those which were symmetrical and in which the nuclei could be seen) in the CA1 region of the hippocampus were counted in a standard grid under 10 X magnification. The grid size was 28900 μm^2 at the magnification used. A random site in the CA1 region of both the left and right sides for each animal was sampled and the number of viable cells was obtained by averaging the two sides. An independent t-test was used to evaluate difference between the two time points.

Results

Ten gerbils were injected with L-glutamate at concentration of 34 μg/μl. Five of these gerbils were perfused at 12 hour while the remaining five gerbils were perfused at 24 hour after injection. At 12 hours, gerbils exhibited a mean of 32.3 viable cells/grid square. The mean number of cells at 24 hour following injection was 7.4 viable cells/grid square (Figure 1). Analysis revealed that this difference was significant [$t(8)=13.03$, $p<0.001$]. Representative photomicrographs of the hippocampus at 12 and 24 hours following glutamate injection are shown in Figure 2 and 3.

Discussion

Gerbils evaluated at 24 hours following glutamate injection were found to have significantly fewer viable cells than that at 12 hours. To assure that the number of viable cells observed in the 12 hour group was not less than that of normal animals, slides from sham gerbils that served in a previous study were evaluated for comparisons (n=5). No significant difference between the number of viable cells of these two groups was observed (Data not shown). In our preliminary studies, we were unable to show that L-glutamate, at a concentration of 0.17 μg/μl, caused neuronal damage as previously reported (Benveniste et al., 1989).

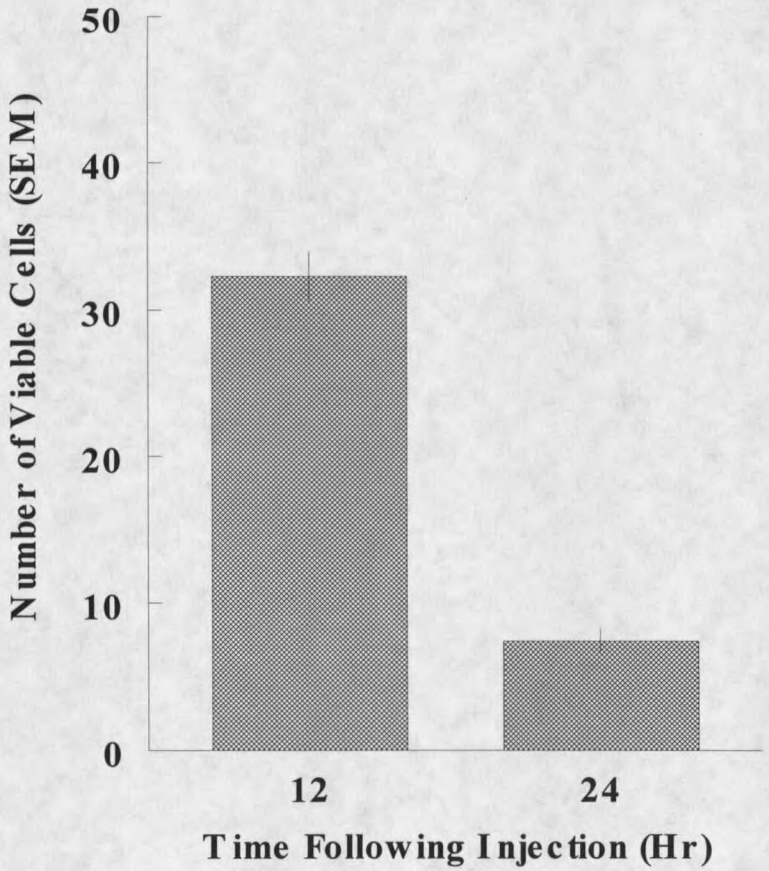

Figure 1. Number of viable cells counted in a randomly sampled region of the CA1 area. Gerbils evaluated at 12 hours after glutamate injection were found to have significantly more viable cells than those perfused at 24 hours after injection ($p< 0.05$).