BEHAVIORAL CONSEQUENCES OF CALCIUM/CALMODULIN KINASE II INHIBITION IN RATS

by

Elizabeth Ann Schwartz

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A. Michael Babcock, Ph. D

Approved for the Department of Psychology

Wesley C. Lynch, Ph. D

Approved for the College of Graduate Studies

Bruce R. McLeod, Ph. D

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ABSTRACT

CaM kinase II (CaMKII) comprises 2% of hippocampal protein and plays an important role in learning and models of neural plasticity. Previous studies have employed a variety of techniques to inhibit CaMKII to investigate its role. This includes the use of chemical inhibition, genetic mutation and antisense; all have shown limitations. In the present study, RNA interference (RNAi) was used to inhibit CaMKII in the hippocampus of rats.

The goal of this project was to determine if inhibition of hippocampal CaM kinase would result in behavioral deficits consistent with the role of this kinase. Three behavioral tasks were used to assess behavioral changes associated with a lack of CaMKII in the hippocampus; an open-field task, water maze and T-maze task.

An adeno-associated viral vector was used to deliver α CaMKII specific hairpins into rat hippocampi and cDNA for green fluorescent protein (GFP; marker protein). Control animals received AAV that encodes only GFP.

In the open-field task, it was hypothesized that experimental rats would show changes in behavior consistent with impaired habituation. This hypothesis was supported; behaviors such as escape attempts and direct versus disorganized movement were significantly different between groups.

In the water maze, it was hypothesized that experimental rats would show longer latencies to find the platform in the test phase and spend less time in the target quadrant than control rats during the probe trial. Groups did not differ significantly on latencies to the platform during the test phase but were different during the probe trial. This suggests that experimental rats may be using a non-spatial strategy to locate the platform.

In the T-maze, it was hypothesized that the experimental rats would make more errors than control rats due to working memory deficits. This hypothesis was not supported.

Densities of α and β subunit CaMKII bands were quantified from digitized images using a computerized densitometry program and α CaMKII was significantly reduced. GFP expression was localized to the hippocampus and extended ± 2 mm from the injection site. Intense αCaMKII staining was observed in control tissue, while staining in was markedly reduced in the experimental condition.

INTRODUCTION

The Hippocampus

The hippocampus is part of the limbic system and has a variety of critical functions (Swanson, 1983). It was originally thought to be primarily used for processing olfactory information, but this idea was challenged by Brodal in 1947 (as cited in Swanson, 1983). A decade later, the clinical case of H.M. demonstrated the role of the hippocampus in memory (Scoville & Milner, 1957). H.M suffered from severe seizures and underwent an experimental bilateral-medial temporal lobe resection to reduce the severity. The surgery resulted in damage to the anterior portion of the hippocampal structures. The seizures were reduced, but H.M. experienced severe anterograde amnesia. Although this clinical observation alone did not fully explain the specific role of the hippocampus, it suggested the concept of different types of memory. Since then, the hippocampus has been identified as a structure for declarative memory, memory consolidation, long term potentiation (LTP), and spatial memory (Eichenbaum, Otto & Cohen, 1992).

Lorente de No divided the hippocampus into three fields, the CA1, CA2 and CA3 (Amaral & Witter, 1995). Of these regions, the CA1 region has been demonstrated to have a particularly important role in memory. The CA1 region has a number of anatomical differences from the other two subfields. For example, CA3 pyramidal cells (the principal neuronal cell in the hippocampus) receive input from the dentate gyrus that the CA1 region does not (Amaral & Witter, 1995). In addition, the size and organization

of pyramidal cells in all three fields differ; cells in CA1 are not significantly interconnected (Amaral & Witter, 1995).

Numerous studies have demonstrated the role of the hippocampus in memory (Scoville & Milner, 1957; Morris, 1982; Garrud, Rawlins, & O'Keefe, 1982; reviewed by Eichenbaum, Otto & Cohen, 1992). Zola-Morgan, Squire, and Amaral (1986) described the case of R. B. who suffered an ischemic episode and experienced severe anterograde amnesia. Following his death, his brain was examined to evaluate the extent and regional specificity of damage. A lesion was found in the CA1 region of the hippocampus with little damage found in other areas of the brain. The R. B. case was the first reported case of memory impairment following damage limited to the hippocampus.

Glutamate is a primary excitatory neurotransmitter in the central nervous system and is important for hippocampal function. It is released in the synaptic cleft where it binds to glutamate receptors which include AMPA, kainite and N-methyl-D-aspartate (NMDA). NMDA receptors have a very high conductance for calcium (MacDermott, Mayer, Westbrook, Smith & Barker, 1986). When NMDA receptors are activated, calcium enters the cell and triggers a cascade of events, including the activation of CaMKII. This kinase has been implicated in mediating several processes in the hippocampus.

Calcium/calmodulin kinase II

CaMKII is a ~600-kDa holoenzyme abundantly expressed in the brain and consists of 10-12 subunits each encoded by separate genes (Gaertner, Kolodziej, Wang, Kobayashi, Koomen, Stoops, & Waxham, 2004). It comprises 2% of total hippocampal

protein (Erondu & Kennedy, 1985). Neuronal CaMKII consists primarily of α (~50-kDa) and β (~58-kDa) subunits (Colbran & Soderling, 1990), with the ratio of α to β subunits at 3:1 in the hippocampus (Goldenring, McGuire & DeLorenzo, 1984). Postsynaptic densities (PSD) of neurons are especially enriched with CaMKII (approximately 50%) and its α isoform has been labeled the 'major postsynaptic density protein' (Kennedy, Bennett & Erondu, 1983). CaMKII is activated by calcium binding to calmodulin and can translocate from the cytosol to the PSD (Yoshimura & Yamauchi, 1997; Strack, Choi, Lovinger, & Colbra, 1997). CaMKII reaches a state of partial calcium independence by autophosphorylation at threonine ^{286} (Thr^{286}) (Miller, Patton, & Kennedy, 1988; Hanson, Kaplioff, Lou, Rosendel & Schulman, 1989; Waxham, Aronowski, Westgate & Kelly, 1990). The autophosphorylated state of CaMKII specifically binds to the PSD and can lead to phosphorylation of a number of proteins (Strack, et al., 1997). More than 28 proteins have been identified as CaMKII targets in the PSD (Yoshiyuki, Aoi, & Yamauchi, 2000). CaMKII has a variety of functions including modulating transmitter release, biosynthesis of neurotransmitters, regulation of potassium, voltage-dependent calcium channels and cytoskeletal proteins (Bronstien, Fraber & Wasterlain, 1993). It also plays an important role in LTP and stroke.

Long Term Potentiation

LTP represents a change in synaptic efficacy following stimulation of nerve fibers. It is a model of plasticity and is believed to be the basis of learning and memory (Bliss & Lomo, 1973). In their classic study, Bliss and Lomo (1973) electrically

stimulated the dentate area and perforant path of the hippocampal formation in rabbits. Stimulation of the perforant path showed a brief immediate 'spike' followed by a brief depression. After depression, most of the rabbits (15 out of 18) showed increased potentiation for 30 minutes to 10 hours following conditioning training. The results of this study suggested that LTP is the result of increased synaptic efficiency in the perforant path as well an increased excitability of granule cells of the dentate area.

Abraham & Mason (1988) examined the role of NMDA receptors in LTP by using two different NMDA antagonists; CPP and MK-801. Rats injected with a high dose of either drug demonstrated LTP deficits. In a later study, Ward, Mason & Abraham (1990) used the same compounds to examine behavioral deficits a radial arm maze. Rats trained in a radial arm maze task following injection of MK-801 or CPP. Both CPP and MK-801 injected rats exhibited more errors in the radial arm maze at doses greater than 1 mg/kg. This study suggested that NMDA antagonists given in a dose demonstrated to be an effective block to LTP also can interfere with maze performance.

Tsein, Huerta, and Tonegawa (1996) produced mice lacking the NMDA receptor 1 gene (NMDAR1) which is found in the CA1 region of the hippocampus. NMDAR1 is linked to NMDA receptors and is necessary for normal receptor function. Mutant mice were deficient in LTP induction in the CA1 region and demonstrated impaired spatial learning in the Morris water maze.

Since NMDA receptors have a high conductance for calcium (McDermott et al., 1986), the role of CaMKII in LTP has been examined. Silva, Stevens, Tonegawa and Wang (1992) demonstrated LTP deficits in α CaMKII mutant mice. Using field potential and whole cell recordings, the study demonstrated increased synaptic strength in wild

type mice and unchanged synaptic strength in mutant mice. Nine of eleven wild type mice exhibited LTP, whereas only two of sixteen mutants showed LTP. The study suggests that CaMKII plays an important role in LTP.

Silva, Paylor, Wehner and Tonegawa (1992) conducted a subsequent study to examine behavioral deficits in the same type of mutant mice. Mice were tested in a visible and hidden platform version of a water maze task. In this type of task animals must locate a hidden platform by using spatial cues in the room (Morris, 1981). All mice performed similarly on the visible platform task by the end of training; demonstrating that both groups of mice had the motor skills necessary to swim and find the platform. In the hidden platform version, mutant mice took longer to find the platform than wild-type mice, but improved across days. During the probe trial, mutant mice spent equal amount of time in each quadrant. Since mice improved during test days, but showed impairment during the probe trial, it was concluded that mutant mice did not use a spatial strategy to locate the platform and may have developed other strategies to solve the task.

A water filled plus maze task was also used to assess whether performance in the water maze could be explained by an inability to see and attend to distal cues. The plus maze was clear so mice could use distal cues in the room to find the correct arm. To make a correct choice in this task, the mice need to only learn one relationship since the platform was always in the same arm. There was no significant difference between wild-type and mutant mice on the plus maze. These data confirm that differences found in the previous hidden platform water maze task were not due to inability to see distal cues or learn a simple relationship. Mutant mice were also studied in an open-field task and Y-maze to test for changes in exploratory behavior. Mutant mice displayed higher activity

than wild-type mice on both tasks. This study suggested learning deficits in CaMKII mutant mice.

Giese, Fedorov, Filipkowski and Silva (1998) explored the hypothesis that autophosphorylation of CaMKII at Thr^{286} was necessary for LTP. As mentioned previously, autophosphorylation at Thr^{286} allows CaMKII to become independent of calcium (Waxham et al., 1990). Alanine was substituted for Thr^{286} in mice so that CaMKII was unable to reach its CaM-independent state. It was confirmed that the mutation affected the autophosphorylated state of CaMKII, but not the CaM dependent activity. LTP was tested by using extracellular field recordings in hippocampal slices in the CA1 region. The mutant mice showed decreased LTP and deficits in the Morris water maze. It was concluded that the autophosphorylated state of CaMKII is necessary for spatial learning. These studies (Silva et al., 1992; Geise, Fedorov, Filipkowski & Silva, 1998) support the notion that CaMKII is necessary for LTP and certain types of learning.

Cerebral Ischemia

In addition to learning, several studies have suggested a role for CaMKII in hippocampal cell death during cerebral ischemia (Waxham, Grotta, Silva, Strong & Aronowski, 1995; Shackelford, Yeh, Buzaski & Zivin, 1995). Following the transient flow of blood to the brain, there is delayed, selective neuronal cell death of hippocampal cells (Kirino, 1982). Kirino (1982) subjected gerbils to bilateral carotid occlusion for 5 minutes and observed patterns of cell death in subfields of the hippocampus. The CA1

region showed very slow changes in pyramidal cells, only being noticeable two days following occlusion. At four days, almost all of the pyramidal cells in CA1 were destroyed. Areas of delayed cell death have alterations in CaMKII function, but the specific role of this protein remains unknown.

A crucial event in this process is a six fold increase in glutamate (Bienveniste, Jorgensen, Sandberg, Christensen, Haberg & Diemer, 1989; Hagberg, Lehmann, Sandberg, Bystrom, Jacobson & Hamberger, 1985). As mention previously, NMDA receptors are a type of glutamate receptor with a high conductance for calcium (McDermott et al., 1986). During ischemia, intra-cellular calcium levels rise rapidly (Silver & Erecinska, 1992). Calcium enters hippocampal cells (via NMDA receptors) and binds to calmodulin triggering CaMKII autophosphorylation and translocation to the PSD (Strack, 1997).

Hewitt and Corbett (1991) used the NMDA antagonist, MK-801, and a calcium channel blocker, nicardipine, to further evaluate the mechanisms of hippocampal cell death. Gerbils were subjected to bilateral carotid occlusion and injected with MK-801 fifteen minutes after occlusion. Nicardipine was given by injection and through an osmotic pump for 3 days. Treatment with MK-801 alone resulted in reduction of cell death by 27%. Nicardipine treatment resulted in a 13% reduction in cell death. Use of these drugs together resulted in 44.5% reduction in CA1 cell death. The study suggests that MK-801 and nicardipine could be used as an effective treatment against ischemic insult. More importantly, it supported a role for NMDA receptors and calcium in the cascade of events leading to cell death.

Two opposing hypotheses have been suggested regarding the role of CaMKII in ischemic cell death. One idea is that decreased CaMKII activity results in cell death. The other suggests that activation of CaMKII is responsible for cell death. A study conducted by Waxham, Grotta, Silva, Strong and Aronowski (1996) supports the inhibition hypothesis. In this study, CaMKII knock out mice exhibited nearly twice the infarct size as control animals following ischemic insult. The study concluded that lack of CaMKII predisposes neurons to ischemic damage. Additionally, baclofen, a drug that inhibits glutamate release *in vivo* (Babcock, Everingham, Paden & Kimura, 2002) and MK-801 have been shown to be neuroprotective (Hewitt & Corbett, 1991). These compounds prevent a decrease in CaMKII activity, which is associated with ischemic insult.

Numerous studies have supported the CaMKII activation hypothesis. For example, a CaMKII inhibitor KN-62, is neuroprotective (Hajimohammadreza, Probert, Coughenour, Borosky, Maroux, Boxer & Wang 1995). In addition, CaMKII translocates to synaptic structures where cell die for up to 24 hours following ischemia (Aronowski, Grotta & Waxham, 1992). 72 hours following ischemia AMPA receptors (a target for CaMKII) allow calcium to enter the cell where CA1 pyramidal cells later die (Mammen, Kameyama, Roche, Huganir, 1997). These findings suggest that the activation of CaMKII plays a role in cell death.

RNA Interference

Modeling changes in CaMKII could be helpful in studying the varied role of this protein. A number of methods have been used to inhibit CaMKII, including antisense,

cell permeable inhibitors, and knock-out/mutant models, but each of these methods has limitations (Gerlai, 1996; Hajimohammedrza et al., 1995, Kurreck, 2003). An alternative approach, which was employed in the present study, is RNA interference (RNAi). This technique was first used by Fire, Xu, Montgomery, Kostas, Driver, and Mello (1998) in the nematode *Caenorhabditis elegans.* Although relatively new, RNAi has been used to successfully knock-down the expression of a variety of genes in mammalian cells in a wide range of species (Shi, 2003).

RNAi is the inhibition of specific gene expression by the introduction of double-stranded RNA's (dsRNA). Small interfering RNA's (siRNA) are created by RNase III endonuclease dicer, which degrades the complementary RNA. The siRNA unwinds, and one strand is incorporated into RNA-induced silencing complex (RISC). RISC directs cleavage of homologous RNA and RISC is free from cleaved mRNA and recycled for multiple catalysis (Zang & Hua, 2004).

siRNA can produce greater than 90% reduction in the target RNA (Zhang & Hua, 2004). Researchers have generated siRNA chemically and *in vivo* with expression vectors. Chemically synthesized siRNA are synthetic and expensive. *In vivo* expression of siRNA has a number of advantages to chemically synthesized siRNA. For example, *in vivo* siRNA is more stable, the effects are long-term, and the method is cost-effective (Zhang & Hua, 2004). One viral vector that shows promise is the adeno-associated virus (AAV). AAV can promote long-term *in vivo* gene expression and transfects many types of cells. In addition, AAV is replicant deficient and non-toxic (Conlon & Flotte, 2004).

An AAV vector was successfully used by Hommel, Sears, Georgescu, Simmons and DiLeone (2003) to express both GFP and siRNA targeting *Th*. *Th* is a gene that

encodes the dopamine synthesis enzyme tyrosine hyroxylase. AAV mediated RNAi was also successfully demonstrated by Babcock, Poulsen, Allen, Knisely, Paden (2003) to inhibit CaMKII. Unfortunately, this study did not evaluate the behavioral consequences of suppressing CaMKII. This is the focus of the present series of experiments.

Behavioral Tasks

A variety of tasks have been used to evaluate hippocampal function. These include an open-field task, Morris water maze and T-maze.

Open-Field task

An open-field task can be used to assess changes in habituation, exploratory and locomotor behavior. Increased exploratory behavior is observed in animals with hippocampal damage (Foreman, 1983; Trond, 1988). As mentioned previously, Silva et al., (1992) demonstrated increased locomotor behavior in an open-field task and Y-maze task in CaMKII mutant mice. Previous studies have shown that ischemic damage to the hippocampus results in alterations in locomotor activity (Babcock, Baker, & Lovec, 1993).

Babcock et al., (1993) examined the impact of changing the test environment on locomotor activity following ischemic insult. Locomotor activity was evaluated in an open-field apparatus for 5 minutes across 16 days. Ischemic gerbils demonstrated increased locomotor activity when compared to control animals, and this difference decreased with repeated exposures to the apparatus. On the final 2 days of testing, the apparatus was moved to a novel environment. Activity significantly increased in

ischemic animals when tested in the novel environment. The results of the study suggest that increase in locomotor activity following ischemia represented in part a deficit in habituation or spatial mapping.

Morris Water Maze

The Morris water maze (Morris, 1981) is used to examine place learning in rodents. In this task, animals are placed in a large pool filled with opaque water. The animal is allowed to swim freely until finding a platform hidden beneath the water. Rats can learn to use distal cues in the environment in order to effectively find the hidden platform. A probe trial is generally used following training with the hidden platform to assess whether subjects show a preference for the area that previously had the platform (increased amount of time spent in the target quadrant). Differences between groups often become apparent during the probe trial since animals cannot rely on non-spatial strategies to find the platform as during training. This has been used to examine navigation ability in rats with hippocampal lesions and in CaMKII mutant mice (Morris, 1982; Silva et al., 1992; Geise et al., 1998).

T-Maze

A T-maze task can be used to measure working memory. In this task, rats are trained to alternate between baited arms of the maze (Douglas, 1966). This alternation between arms is a natural foraging behavior of rats that requires a memory of the previous choice (Lalonde, 2002). Typically, this task involves placing an animal in the start box and presenting pairs of forced and choice trials. For a forced choice trial, one arm is blocked off so the animal must enter the opposite baited arm. After it has entered the arm and consumed the reward, it is placed back in the start box and presented with a

choice trial. A choice trial is when both arms are opened and the rat must choose an arm to enter. Some maze paradigms will use a delay between the forced and choice trial to make the task more difficult. The correct response is for the rat to choose the arm which was not entered (Douglas, 1966). It has been demonstrated that rats will alternate arms of the maze naturally, unless there has been damage to the limbic pathways including the basal ganglia, hippocampus, thalamus, prefrontal cortex, dorsal striatum and cerebellum (Lalonde, 2002).

T-maze tasks have been used to demonstrate working memory deficits in animals who have received various treatments. For example, Hagan and Beughard (1990) used a T-maze to demonstrate impaired place navigation in ischemic rats. Babcock and Graham-Goodwin (1997) examined the effect preoperative training on T-maze performance in ischemic gerbils.

STATEMENT OF PUPOSE

The goal of this project was to determine if inhibition of hippocampal α CaMKII would result in behavioral deficits consistent with the role of this kinase. An adeno-associated virus (AAV) vector was used to deliver a α CaMKII-specific siRNA hairpin sequences in rat hippocampi. The vector used in this study also carried the cDNA for GFP. This was used as a marker of transfection efficacy. This design was successfully used in the project presented by Babcock et al., (2003).

Three behavioral tasks were used to assess deficits associated with a lack of α CaMKII in the hippocampus. An open-field task was used to evaluate locomotor activity and habituation. Behaviors including escape attempts, and direct versus disorganized movement were studied. It was hypothesized that experimental rats would show consistently higher activity across five days and behavioral changes consistent with a lack of habituation to the apparatus. A hidden platform version of the Morris water maze task was used to examine place learning. For this study, it was hypothesized that experimental rats would show longer latencies to find the hidden platform during the training phase and spend less time in the target quadrant than control rats during the probe trial. Finally, a T-maze task was used to examine working memory and it was hypothesized that the experimental rats would make more errors than control rats due to memory deficits.

METHODS

Subjects

Subjects were 14 female rats housed individually in temperature and light controlled environment. They were give commercial rat pellets and water *ad libitum* except during the T-maze task when rats were deprived to 85% of their free-feeding weight. Experimental procedures involving these animals were approved by the Institutional Animal Care and Use Committee

Materials

The open-field apparatus consisted of a metal screen floor 77 x 77 cm with clear Plexiglas walls 15 cm high. The apparatus was elevated 60 cm and the screen floor was 8 cm from that surface. The floor was divided into nine equal squares. The water maze was a circular pool 1.37 m diameter x 60 cm high filled to a depth of 30 cm with water temperature of 24ºC. A Plexiglas platform (11.43 cm x 11.43 cm) was placed in the center of one quadrant for all training sessions. The top of the platform was 2 cm below the surface of the water. Black Tempra paint was added to the water to make it opaque. The T-maze apparatus was constructed of masonite with all walls measuring 50 x 10 cm (Yadin, Friedman & Bridger, 1991). Food cups were placed at the end of each arm. Doors were designed to fit tightly in the maze and were slid in from the top of the maze. The viral vectors were a generous gift from Dr. Dave Poulson at the University of Montana (Figure 1).

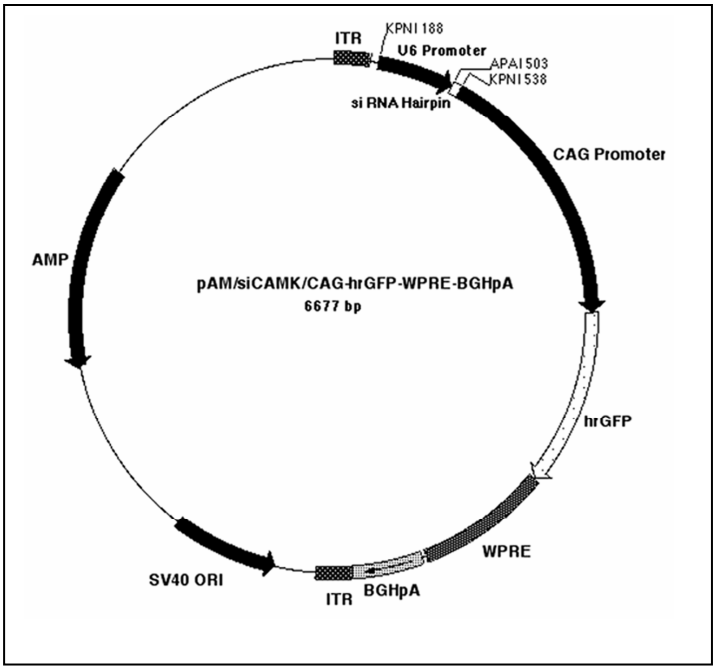


Figure 1. AAV vector design for expression of GFP and a U6 promoter-driven shRNA. Complementary oligonucleotides were synthesized to create an α CaMKII specific small hairpin (shRNA) sequence containing an Apa I compatible overhang at the 5' end and a KpnI restriction site and an EcoRI compatible overhang at the 3' end.

The vector was designed to express both GFP and a U6 promoter-driven siRNA. Complementary oligonucleotides were synthesized to create α CaMKII specific siRNA sequence which contained an Apa I compatible overhang at the 5' end and a KpnI restriction site and an EcoRI compatible overhang at the 3' end. The sense oligonucleotide sequence was: 5'TCCTCTGAGAGCACCAACATTCA AGAGATGTTGGTGCTCTCAGAGGATTTTTTGGTACC 3' and the antisense oligonucleotide sequence was: 5' AATTGGTACCAAAAAATCCTCTGAGAG CACCAACATCTCTTGAATGTTGGTGCTCTCAGAGGAGGCC 3'. The sense and antisense oligonucleotides were annealed and ligated into the Apa I and EcoRi sites

downstream of the U6 promoter in pSilencer (Ambion, Austin, TX). Constructs were confirmed by double stranded sequencing. A 373 bp Kpn I fragment containing the U6 promoter-siRNA hairpin sequence was excised and subcloned into the KpnI site of the AAV vector pAM-CBA-GFP.

Recombinant AAV1 was packaged in cultures of HEK 293T cells. Approximately 1.5 X 10^7 293T cells were seeded into 150 cm dishes in complete DMEM (Cellgro; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 1mM MEM sodium pyruvate, 0.1 mM MEM nonessential amino acids solution, and 0.05% Penicillin-Streptomycin (5,000 units/ml). At 24 hours after seeding, cells were transferred to culture media containing 5% fetal bovine serum and transfected with three separate plasmids: Adeno helper plasmid (pF6), AAV helper (H21), and the AAV transgene vector containing the CBA-GFP expression cassette either alone (control) or in combination with the U6 α CaMKII specific siRNA (experimental). In both cases, expression cassettes were flanked by AAV2 inverted terminal repeats. Plasmids were transfected into HEK293T cells using Polyfect according to the manufacturer's conditions (Qiagen, Valencia, CA). Cultures were incubated in 5% CO_2 for 72 hours (37^o C). Cells were then harvested and pelleted by centrifugation. The pellet was resuspended in 10 mM Tris, pH 8.0 and chilled on ice. Cells were lysed by three freeze-thaw cycles followed by treatment with 50U benzonase (Novagen, San Diego, CA) and 0.5% sodium deoxycholate for 30 minutes at 37^oC. Virus was purified by density gradient centrifugation in iodixinol according to the method of Zolotukhin, Byrne, Mason, Zolotukhin, Potter, Chesnut, Summerford, Samulski, and Muzyczka (1999). Purified virus preparations were concentrated and desalted in sterile PBS by centrifugation in Ultrafree-15 filter devices

(Millipore, Bedford, MA). The titer of each virus (genomic particles/ml) was determined by quantitative RT-PCR using an ABI Prism 7700 with primers and a probe specific for the WPRE sequence. Each viral vector was diluted 1:1 with mannitol prior to infusion.

Procedure

All surgeries were conducted by Dr. Mike Babcock at Montana State University. Eight animals were infused with rAAV carrying the cDNA for α CaMKII-specific siRNA hairpin sequences and GFP. The remaining six animals received rAAV carrying only cDNA encoding GFP. Animals were anesthetized with isoflorane and mounted into a stereotaxic device. A midline incision was made and small holes drilled in the skull 4.1 mm posterior to bregma, and ± 2.0 mm from the midline (flat skull). The tip of a 22-gauge injection cannula (Plastics One, Roanoke, VA) was lowered 3.7 mm from the skull surface into the dorsal hippocampus. Injections were made using a Hamilton microsyringe mounted in a programmable infusion pump. The cannula was connected to the microsyringe with clear PE 20 tubing. Rats received bilateral infusions of AAV with GFP or AAV with siRNA hairpins at a rate of 0.4 µl/minutes for 20 minutes (4×10^9 genomic particles/hippocampi). The injector remained in place for an additional 2 minutes following infusion. Scalp incisions were sutured and animals returned to their home cages following the infusion procedure.

Behavioral testing commenced 21-24 days following surgery since previous reports indicate that this is the optimal interval for vector expression (Hommel, et al., 2003). Tasks were conducted in the order presented. Handling the rodents prior to

conducting behavioral tasks minimize stress which can affect performance (Gerlai, 2001). Thus, all animals were handled each day for 5 consecutive days prior to testing. All behavioral test sessions were videotaped and animals were identified only by number. Behavior coding was conducted without knowledge of group assignment.

Open-field task data were collected for five consecutive days by two independent observers. Subjects were placed in the center square and the number of squares entered was recorded each minute for a total of 10 minutes. Behaviors observed in the open-field task included number of non-wall hugging movements (movement taking place where the rat was not touching or very close to touching the wall), direct movements (straight, quick motion with slight variation in direction) disorganized movements (slower movement, sometimes stopping, with direction change), escape attempts (trying to leave the apparatus), rearing, grooming and latencies to platform. Subjects were brought into the test room in groups of 3-4 animals and placed in the apparatus individually. Animals were chosen randomly each day for order of testing, and time of day was counterbalanced (morning/afternoon). When an animal tried to leave the apparatus they were gently pushed back down by one of the observers. Some animals would stand in the corners with paws on the wall to look out; the animals were only pushed back into the apparatus when their hind legs made contact with the wall as an attempt to escape.

The water maze task was conducted following the open-field task. Test sessions lasted for 4 consecutive days with the probe trial occurring 2 days later. During each training day, rats were place into the water facing the wall at 4 different locations in random order. Trials lasted until the animal had found the platform or 60 seconds elapsed. If the animal did not find the platform after 60 seconds, it was guided there by

the experimenter. Once on the platform, animals were left there for 10 seconds before it was removed from the pool. For the probe trial, the platform was removed and time spent in each quadrant was recorded. Rats were released in the quadrant opposite to the one where the platform was initially located. All distal cues remained constant during test session and probe trial.

Following the water maze task, rats were habituated to the T-maze for seven days before testing. The habituation phase consisted of allowing the rat to freely explore the maze with no experimenter interaction for 3-5 minutes. The food reward used was Cocoa Krispies cereal since chocolate is a preferred food for rats (Yadin, Friedman & Bridger, 1991; Dudchenko & Davidson, 2002; Seibell, Demarest & Rhoads, 2003). The reward was scattered throughout the maze for the first two days and then was placed in food cups only for the remaining days. Most rats ate the Cocoa Krispies in the maze, but not with enough regularity to be an effective reward for maze training. To increase motivation, rats were deprived to 85% of free feeding weight (Volpe, Waczek, & Davis, 1988; Johnson, Zambon & Gibbs, 2002: Kirby & Rawlins, 2003).

Rats were placed in the start box of the T-maze with an arm blocked off so the subject could only enter one arm (forced choice). At the end of the arms was a food cup baited with food. After the rat ate the food it was placed back in the start box. Next, both arms were opened and the rat was allowed to choose between them. Once the rat had made a choice it was allowed to eat the food and was put in its cage until the next trial. Each animal received six trials each day for five days. If the rat failed to choose an arm after one minute, it was removed from the apparatus. After animals had completed six trials in the T-maze, they were weighed and fed up to their 85% free-feeding weight.

Immunohistochemistry

On the day following behavioral testing, a random subset of animals (n=2/group) was deeply anaesthetized with isoflorane prior to intracardial perfusion with PBS followed by 500 ml of 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde (1 hour) prior to sectioning. One randomly selected brain from the experimental group was frozen and 50 μm sections collected anterior and posterior to the injection site to determine the anatomical boundaries of hippocampal GFP expression. These sections were evaluated using a Bio-Rad DVC-250 confocal microscope equipped with an argon-krypton laser and Optronics DE1-470 integrating CCD camera. To determine the anatomical location of GFP expression on these sections, adjacent sections were stained with cresyl violet and compared to a standard stereotaxic atlas using an Olympus BH-2 microscope. The remaining brains were sectioned (15 μm; vibratome) near the injection site and processed for CaMKII immunoreactivity. For this procedure, sections were washed with PBS and incubated in normal horse serum containing 0.3% Triton X-100 for 1 hour. Next, tissue was incubated for 48 hour with a monoclonal antibody against α CaMKII (1:1,000; Chemicon, Temecula, CA). After washes with PBS, sections were incubated with biotinylated anti-mouse IgG and avidin-biotin-horseradish peroxidase complex (Vector, Burlingame, CA). Tissue was reacted with 0.05% diaminobenzidene and 0.01% hydrogen peroxide for 5-10 min. Sections were dehydrated, cleared with xylene, and coverslipped. Sections from both conditions were processed simultaneously to minimize non-experimental staining variation. Adjacent

sections from each animal were stained with cresyl violet to confirm that pyramidal cells were histologically intact.

Western Analysis

Levels of α CaMKII hippocampal protein were evaluated using Western blot analysis. One day following behavioral testing, a subset of randomly selected animals were deeply anaesthetized with isoflorane and euthanized by decapitation. Hippocampi were removed and frozen within 60 seconds. Tissue was homogenized in an ice-cold buffer containing 500 mM 3-(N-mopholino) propanesulfonic acid (pH 7.6), 20 mM DTT, 1.0 mM sodium orthovandate, 30 mM EGTA, 1.07 mM magnesium acetate, 3.2 mM sucrose, phenylmethylsulfonyl fluoride (0.017 mg/ml), leupeptin (20 μg/ml), aprotinin (5 μg/ml), and pepstatin (10 μg/ml). Protein content was determined using the Pierce BCA protein assay kit, with BSA as the standard. Protein mixtures were boiled for 3 min and subjected to SDS-PAGE as described previously in Babcock, Liu, Paden, Churn, and Pittman, (1999). Samples from each condition (n = 5/group) were loaded on the same gel and following separation were transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Next, membranes were incubated in a blocking solution for 30 minutes followed by an antibody against α CaMKII (1:10, 000) for 2 hours. After washes, membranes were incubated with an alkaline phosphatase-conjugated secondary antibody (Sigma, St. Louis, MO) for 1 hour prior to reaction with the appropriate substrate. Membranes were digitized and than reprobed with an antibody against β CaMKII (1:5,000) (generous gift from S.B. Churn) using the identical procedure. Optical densities of the separate α and β CaMKII bands (same membrane) were quantified from digitized images using a computerized densitometry program (Silk Scientific, Orem, Utah).

RESULTS

Open-field task

All five days of locomotor activity were analyzed since the data was collected in real time. The results for the remaining behaviors include only the last four days of testing as there was some data missing from the tape for day one. Data were analyzed using 2x5 mixed-model ANOVA with repeated measures on the second factor to determine if groups differed on number of squares entered across days. Means (94.2 vs. 76.6 squares) were not significantly different between groups, $F(1, 11)= 2.08$, $p>.05$. Escape attempt data were analyzed using 2x4 mixed -model ANOVA with repeated measures on the second factor and the number of escape attempts across days was significantly different between groups $F(1, 11)=6.38$, $p =.03$. siRNA animals showed significantly higher frequency of escape attempts than control animals. Proportion of direct versus disorganized behaviors was analyzed using a Mann Whitney U-test and these data were significantly different between groups, $p <.02$. siRNA animals exhibited more direct behaviors than control rats. Total number of non-wall hugging movements between groups were compared using 2x4 mixed-model ANOVA, these were not significantly different, $F(1, 11)=.296$, $p>.05$. Number of rearing behaviors between groups were compared using 2x4 mixed-model ANOVA and this was not significantly different, $F(1, 11)=.34$, $p=.572$. Grooming behaviors were also examined between groups with 2x4 mixed-model ANOVA, these data were not significantly different, $F(1, 11)=3.64$, $p>.083$. Locomotor, escape, and movement data are summarized in Figure 2.

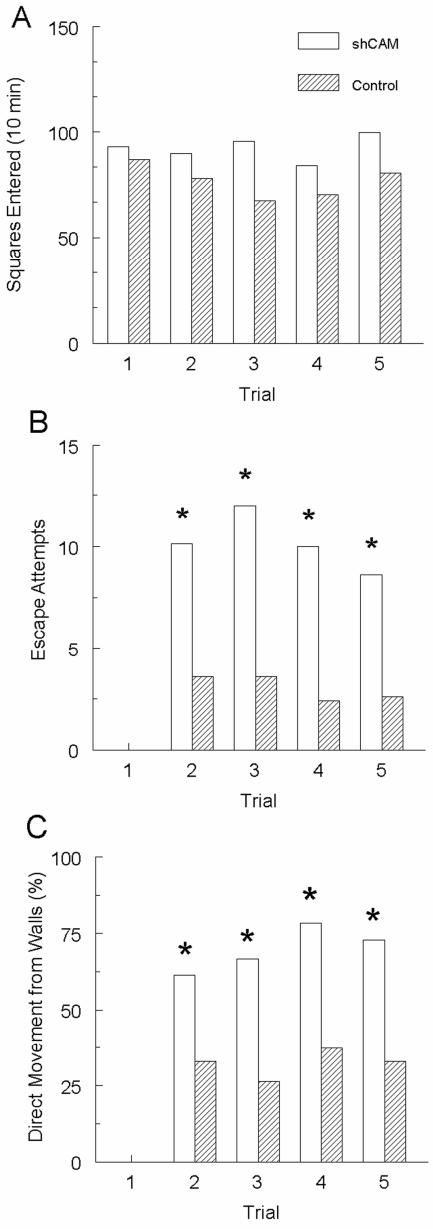


Figure 2. Exploratory behaviors 19 days following hippocampal microinfusion of AAV-GFP or AAV-siRNA (shCAM). Number of squares entered did not significantly differ between AAV-siRNA and controls conditions (Panel A). Analysis of remaining behaviors was limited to trials 2-5 because of equipment problems on trial 1. The frequency of escape attempts was significantly greater for AAV-siRNA animals in trails 2-5 (*p = .03; Panel B). Movements away from the apparatus wall terminating at a different wall were categorized as direct or disorganized. AAV-siRNA animals exhibited a higher proportion of direct movements (Panel C), and this difference in exploratory behavior was significant for each testing day (trials 2-5; *p < .02).

Morris Water Maze

Tapes for all 4 test days were coded for swim speed and latency to platform. Swim speed did not significantly differ between groups, (5.1 vs 4.7 cm sec^{-1}; $p > .05$). A significant decrease in the latency to locate the hidden platform across the training sessions was observed (F (3,30)= 29.1, $p < .001$), however latencies between groups did not differ ($p > .05$). Groups differed significantly on the probe trial, t (11) = 2.6, $p<.001$. siRNA animals spent significantly less time in the correct quadrant than control animals with the platform removed (36% vs 48%; see Figure 3). Wall hugging behavior was also evaluated and this frequency was not significantly different between groups $p > .05$.

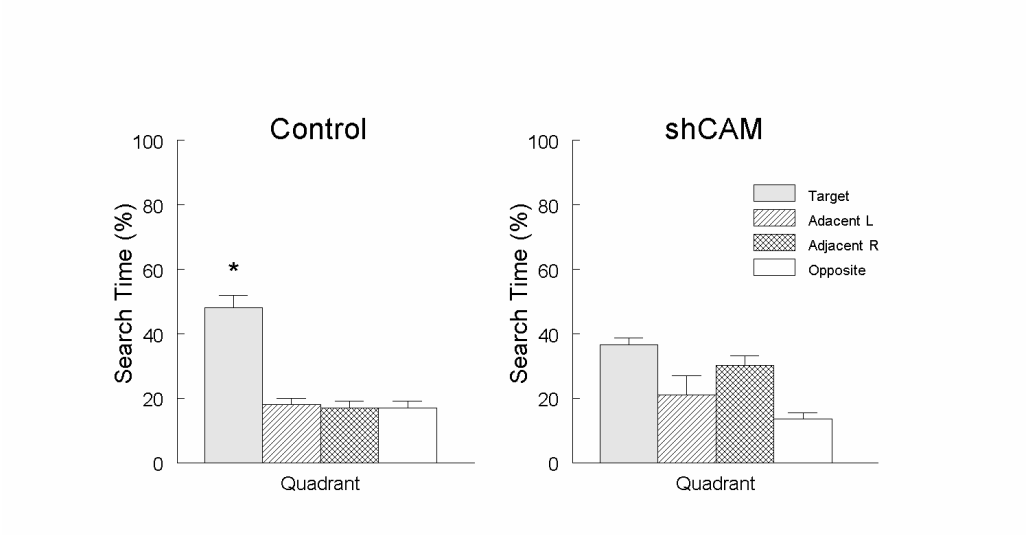


Figure 3. Time spent in the four quadrants of the water maze during the probe trial (platform removed). Animals received a bilateral microinfusion of AAV-GFP or AAV-siRNA (shCAM) into the hippocampus 24 days prior to hidden platform training and testing for place learning (probe trial). Control animals spent significantly more time in the target quadrant compared to α CaMKII knock-down rats, and compared to non-target quadrants (* $p < 0.05$).

T-Maze

T-maze tapes were analyzed for all 5 days. A correct response was defined as correctly alternating arms after consuming the food reward. Proportion of correct responses across days was analyzed using the Mann Whitney U-test. This data was not significantly different, $p > 05$. Data are summarized in Figure 4.

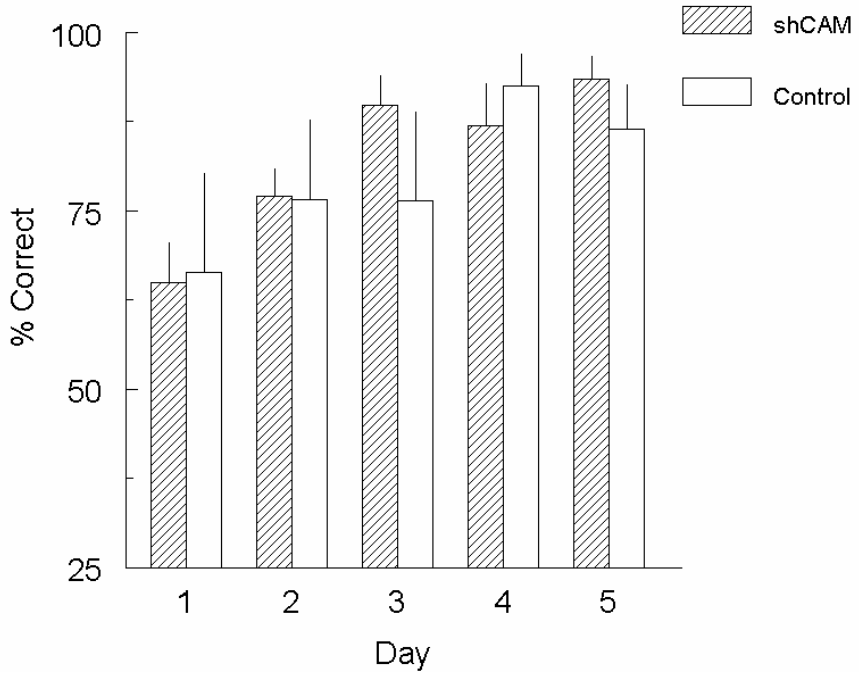


Figure 4. Proportion of correct responses in T-maze. A correct response was when the animal chose the arm it has not already entered in a 'choice' trial. Groups did not differ significantly, but did perform at greater than 50% correct indicating that animals learned the task and were performing better than chance.

Immunohistochemistry

Tissue sections were examined 26-29 days following AAV microinfusion into the hippocampus. GFP expression was observed in all hippocampal regions. Pyramidal cells in both the CA1 and CA3 regions exhibited GFP-positive cells with dendritic projects into stratum radiatum and lacunosum moleculare. The granular cell layer of the dentate gyrus also contained GFP-positive cells. It is likely that some of the GFP-positive fibers in the stratum lacumosum moleculare originated from contralateral CA3 commissural fibers since preliminary studies involving unilateral infusion of this AAV vector resulted in contralateral GFP expression. To investigate the boundaries of GFP expression following AAV infusion, sections rostral and caudal to the injection site were examined. GFP expression in hippocampal pyramidal and granule cells was observed ± 2 mm from the infusion region (Figure 5). Areas outside of the hippocampal region did not exhibit detectable levels of GFP expression. Sections from control and experimental animals were processed for α CaMKII immunoreactivity. Intense α CaMKII staining was observed in the pyramidal cell layers (CA1-CA3) and dentate gyrus of control tissue, while staining in these regions was markedly reduced in the experimental condition. Comparable staining for α CaMKII was observed in cortical neurons and in structures proximal to the hippocampus indicating a selective regional loss of immunoreactivity in tissue of experimental animals. Examination of adjacent sections revealed no detectable loss of hippocampal neurons. These data are summarized in Figures 6 and 7.

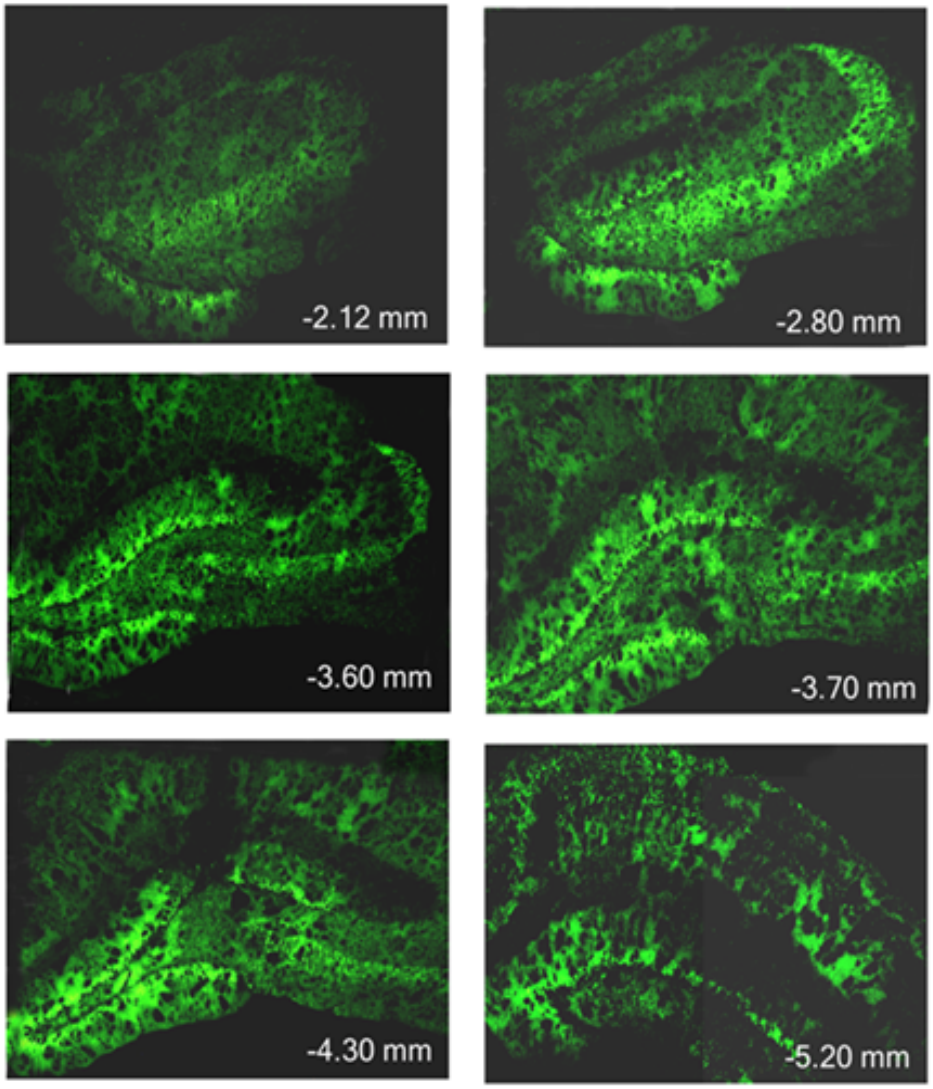


Figure 5. GFP expression in the hippocampus. GFP expression is observed in each section ranging ± 2 mm rostral and caudal to the injection site. Pyramidal cells in both the CA1 and CA3 regions exhibited GFP-positive cells with dendritic projects into stratum radiatum and lacunosum moleculare. The granular cell layer of the dentate gyrus also contained GFP-positive cells. Areas outside of the hippocampal region did not exhibit detectable levels of GFP expression

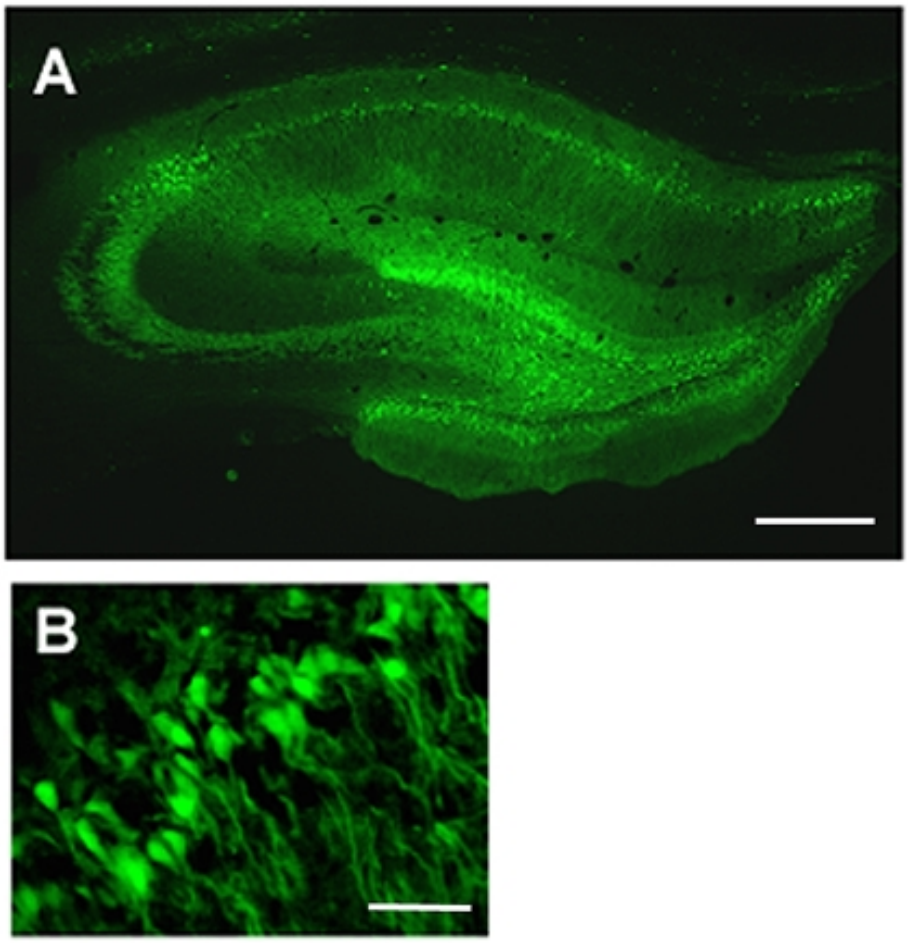


Figure 6. Confocal photomicrographs of GFP expression 29 days following microinfusion of AAV-siRNA into the hippocampus. GFP expression was observed in the pyramidal cell layers and dentate gyrus (panel A, low magnification; scale bar = 500 μm). Dendritic projections into the *s. radiatum* and *l. moleculare* regions of the hippocampus were also visible. High magnification of the pyramidal cell layer shows that GFP expression is restricted to neuronal somata and processes (panel B, scale bar = 50 μm).

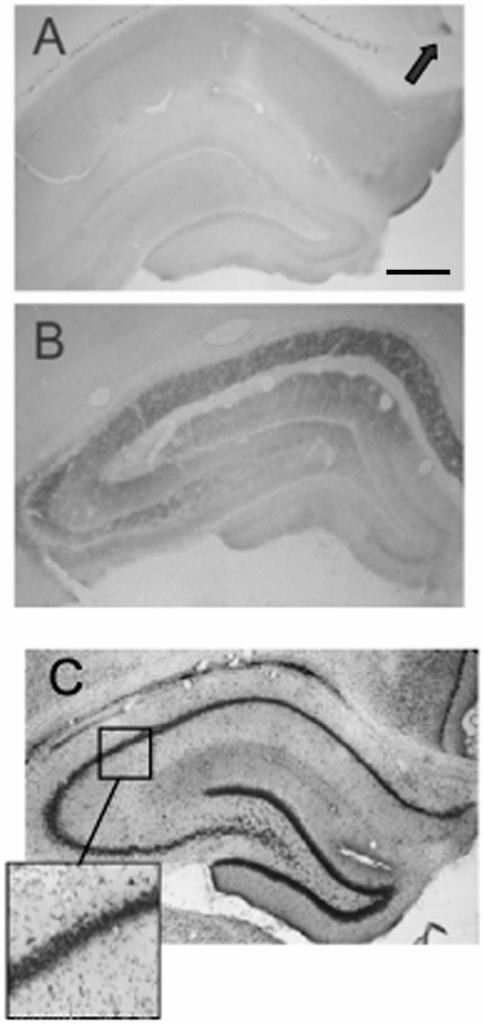


Figure 7**.** Hippocampal α CaMKII immunoreactivity 29 days following microinfusion of AAV-siRNA (A) or AAV-GFP (B) into the hippocampus. Vibratome sections of each condition were processed with an antibody against α CaMKII. Immunoreactivity was present in the hippocampus of the AAV-GFP animal and absent in the hippocampal region of the AAV-siRNA animal. Comparable staining was observed in the indusium griseum of the AAV-siRNA and AAV-GFP animals (arrow; A), indicating a regional suppression of immunoreactivity within the hippocampus. Panel C shows a cresyl violet stained section from an AAV-siRNA treated animal. No histological damage was observed in the hippocampus; intact pyramidal cells in the CA1 region are visible at higher magnification (inset). Scale bar = 500 μm.

Western Analysis

Hippocampal protein homogenates from control and experimental animals were collected 21-24 days following surgery and subject to Western analysis. Treatment with AAV-siRNA produced a 46% reduction in α CaMKII optical density relative to controls (Figure 7). Analysis of these density values revealed a significant difference ($t(8) = 2.3$, $p < .05$). As an additional control, the identical membrane was reprobed with an antibody against β CaMKII. Analysis revealed that the optical density of α CaMKII for the two groups did not differ significantly ($p > .05$).

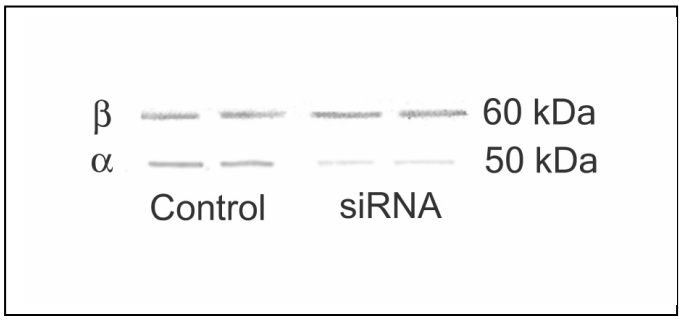


Figure 8. Western analysis of hippocampal protein homogenates from animals receiving bilateral microinfusion of AAV-GFP or AAV-siRNA. Representative lanes from each group are shown (n=5/condition). Hippocampal proteins were separated using SDS-PAGE and transferred to a membrane. The membrane was probed sequentially with antibodies to α and β CaM kinase. Treatment with AAV-siRNA resulted in a significant reduction in α CaMKII immunoreactivity ($p < 0.05$), while there was no change in immunoreactivity of the non-targeted β CaMKII.

DISCUSSION

Behavioral Tasks

Open-field task

Based on previous reports (Silva et al., 1992), animals were expected to differ significantly on general locomotor behavior. The mean squares entered across five days by siRNA animals were greater, but this difference was not significant. A possible explanation for the very small effect is that these results may have been affected by escape behavior. Since animals often spent the majority of the ten minute session in one square trying to escape, this could represent a conflicting response to entering different squares. Escape attempt data was significantly different, and could be interpreted as either increased activity or exploratory behavior in siRNA animals.

The types of movement when the animal was not on the wall of the apparatus were also analyzed. Non-wall hugging movements were defined as any movement that began when the rat left a wall and ended when the rat reached a wall again. All non-wall hugging movements were classified as either a 'direct' or 'disorganized' movement. A direct movement was a straight, quick motion with minimal variation in direction. A disorganized movement was slower, with pauses and more than one direction change. siRNA rats demonstrated a significantly greater frequency of direct behaviors relative to control rats. This movement pattern continued throughout testing, even following repeated exposures to the testing environment. One interpretation of this data is the siRNA animals exhibited an inability to habituate to the apparatus. This supports the initial hypothesis that siRNA rats would experience impaired habituation in the open-field task.

Previous studies on α CaMKII deficient animals have not noted differences in escape attempts and movement type. This is likely because the present study used an open-field apparatus that permitted the expression of different behaviors. Additionally, since all sessions were taped, more detailed analysis of these behaviors was possible.

Rearing behaviors were defined as when the front paws were off the ground, most often resting on wall. This does not include escape, unless both rear paws are on ground for at least five seconds before escape attempt occurs. Grooming was defined as when the front paws are off the ground cleaning the face and head. This was also not expected to be different between groups as differences in grooming are not consistent with CaMKII inhibition literature.

Silva et.al, (1992) showed increased general locomotor activity in α CaMKII mutant mice, but the results of the present study imply that reduction of CaMKII does not necessarily increase activity. There are several notable differences between these studies. First, Silva et al., (1992) used a knock-out model which reduces CaMKII in all brain structures, not just the hippocampus. It is possible that reduction of α CaMKII limited to the hippocampus does not affect general locomotor activity. However, the present study demonstrates increased escape attempts as well changes in exploratory behavior in animals lacking hippocampal α CaMKII. Differences in exploratory behavior could be another way of quantifying increased activity; but it seems more likely that experimental animals have a greater need to explore, that may not always lead to increased activity.

Morris Water Maze

The hypothesis that experimental animals would exhibit longer latencies to locate the platform on test days was not supported. This is not uncommon in water maze tasks,

and is often interpreted as rats using a non-spatial strategy to locate the platform (Morris, 1982). Hodges (1996) reported that normal animals will hug the walls of the maze initially, and then will venture further out as trials continue. In time, they will spend a majority of the trial in the target quadrant and eventually swim directly to the platform. In impaired animals, wall hugging increases toward the end of the trial as the animal gives up and waits to be guided to the platform. Fixed distance wall hugging and circling are other examples of this phenomenon, and may represent an alternative spatial strategy.

The probe trial can distinguish between animals using spatial and non-spatial strategies. In the present study, experimental animals spent less time in the target quadrant than controls, supporting the hypothesis that decreasing α CaMKII results in place learning deficits. Morris (1982) noted that rats with hippocampal damage could improve on test trials and are using alternative non-spatial strategies. During the probe trial, however, differences between groups were pronounced. Normal animals frequently swam directly over the former location of the platform; lesioned animals spent very little time in the target quadrant.

T-Maze

The T-maze did not reveal any deficits in working memory and there are a number of possible explanations for this. One possibility is that a T-maze is not sensitive to differences in α CaMKII levels. A T-maze task is considered to be a test of working memory (WM), and it is possible that a reduction of α CaMKII does not alter WM. The T-maze task has not been used with CaMKII deficient animals previously, but has been used with ischemic animals and hippocampal damaged animals. Results of these other studies imply a role for CaMKII in WM, but has it not been explicitly demonstrated.

Another possibility is that the protocol used in the present experiment affected their performance. One rat was removed from testing due to illness, and some other animals were 'timing out' during the later days of testing. Since all animals were performing at better than 50% correct, it would appear that they knew how to do the task. Timing out would imply they were not motivated to consume the food reward and illness or fatigue may have been a confounding factor.

Some animals demonstrated aggressive behavior toward the experimenter that was not observed during the first two tasks. This may have been related to CaMKII inhibition. Chen, Rainier, Greene & Tonegawa (1994) demonstrated that α CaMKII mutant mice show more aggressive behavior than normal mice. The mice displayed two types of aggressive behavior more that control animals; attacks towards intruders and defense attacks. The number of rats demonstrating these behaviors in the present study was minimal, but still notable due to its potential impact on the outcome of the task and it's implications about the role of CaMKII.

Western Analysis and Immunohistochemistry

A previous study by Babcock et al., (2003) demonstrated a significant decrease in α CaMKII from using RNAi. As revealed by Western analysis and immunohistochemistry, the present study confirms this previous observation. Treatment with siRNA produced a significant reduction in α CaMKII optical density when compared to controls. Failing to see a reduction in the β subunit of CaMKII immunoreactivity suggests siRNA hairpins were specific to the α subunit. Probing for

both the α and β subunits of CaMKII on the same membrane also served as a loading control. Widespread (> 4 mm) expression of GFP was observed in the hippocampus 26 days following AAV microinfusion. Because the α CaMKII shRNA construct was packaged with this GFP reporter gene, it is likely that the distribution of shCAM and GFP expression overlap. This is supported by the observation that the reduction of α CaMKII immunoreactivity was limited to hippocampal regions that also had GFP expression.

RNAi

Using RNAi to inhibit CaMKII could lead to a number of important findings concerning the role of this kinase in stroke, LTP and other neuronal processes. RNAi has a number of methodological advantages over previous techniques including chemical inhibition, antisense and knock-out models.

KN-62 is a CaMKII specific inhibitor that is cell permeable. It competitively inhibits CaMKII by preventing CaM from binding, and is only effective in inhibiting the enzyme prior to autophosphorylation (Tokumitsu, Chiwaja, Hagiwara, Miztutani, Terasawa & Hidaka, 1990). With KN-62, it can be difficult to identify the extent of inhibition in a living animal since the hippocampus is examined after homogenization. During homogenization, KN-62 may gain access to cells and promote a profile of inhibition that differs in the non-living brain. In the present study, siRNA is expressed via AAV, and this will only happen in living cells.

Antisense models have problems with *in vivo* stability, cellular uptake, and toxicity (Kurreck, 2003). On average, only 1 of 8 antisense oligonucleotides binds

properly to the target mRNA (Stein, 2001). Long RNA molecules are often based on computer representations which are unlikely to represent actual structure and can lead to inefficient oligonucleotides designs (Kurreck, 2003). Additionally, the protocols used for antisense can be quite tedious since it requires multiple injections. Since the present study uses an expression vector, siRNA is being manufactured by the cell rather than being repeatedly injected.

Knockout models, like that of Silva et al., (1992), have recently been criticized because of the possible influence of background genotype. It has been suggested that genotype may actually be responsible for behavioral differences in mutant mice than the gene being targeted (Gerlai, 1996). By targeting a specific gene, it becomes easy to ignore the effect of other genes. This could lead to a misinterpretation of results, especially behavioral differences (Gerlai, 1996). Another advantage over a knockout model is the inhibition of the target protein with siRNA can be localized to a specific structure rather than the entire animal. For example, in α CaMKII knock-out models, the entire animal is deficient, making it difficult to determine the specificity of the effect.

Limitations

The open-field task is traditionally carried out with higher walls so the animals do not interact with the edges of the apparatus. In this study, the walls were low enough that animals could jump and hang on the top of the wall edge. This had both benefits and limitations in the present study. If the walls would have been higher, there would be no conflicting response and amount of horizontal locomotor behaviors would have been

likely elevated as in the Silva et al., (1992) study. However, since the groups were different on number of escape attempts, it can be interpreted in different ways that have not been discussed in prior studies.

In literature describing Morris water maze procedures, use of a visible platform task is often mentioned. The visible platform task is non-spatial and is used to assess if the animals have the physical ability to reach the platform (Gerlai, 2001). Without this portion of the test, there can be question as to whether the groups have the same ability to swim to the platform. Since experimental rats were only impaired on the probe trial, it likely that the difference was a result of CaMKII inhibition and not a physical disadvantage. However, the inclusion of a visible platform test would allow for more confident conclusions.

Future Directions

This study could be replicated using different behavioral tasks or variations of the ones used in the present study. For example, it would be interesting to conduct the open-field task with higher walls to see if activity would be increased with out issues of competing behavior. Also, using a Y-maze task with or in place of the nine square gird could be useful to compare activity between the two tasks. The water maze task would be improved with the addition of a visible platform. It would also be useful to use a radial-arm maze in place of the T-maze as the memory processes involved are similar.

Even more important than characterizing the behavioral differences, is the utility of siRNA to further study the role of CaMKII. Now that siRNA has been successfully used to inhibit CaMKII in the hippocampus, it can be used to further our knowledge of its

role in LTP and stroke. For example, it would be possible to use siRNA to inhibit hippocampal CaMKII and employ field potential recording and whole cell recording to study synaptic strength in rats. This would be similar to Silva et al., (1992), but CaMKII inhibition would be hippocampal specific. It would allow for examination of another species since CaMKII mutants are always mice. Mutants in that study were LTP deficient, so it could be useful to compare results.

In stroke research, using RNAi to inhibit CaMKII could directly test the hypothesis that decreased α CaMKII leads to cell death. A previous study (Waxham et al., 1996) induced stroke in CaMKII mutant mice, but the limitations of knockout models made it difficult to generalize to selective cell death in the hippocampus. However, with RNAi, stroke could be induced in rats lacking α CaMKII in only the hippocampus. Regardless of the results, a study of this nature would further our knowledge of CaMKII's role in CA1 region cell death.

Conclusion

The present study successfully inhibited CaMKII in the hippocampus by using RNAi. Some of the behavioral tasks used were sensitive to these changes. The open-field task did not show group differences on locomotor activity, but differences on some behaviors not previously mentioned in the CaMKII literature. Escape attempt data were different between groups as well as proportion of direct and disorganized behaviors. Animals did not show differences in latency to the platform during the test phase of the

Morris water maze, but did on the probe trial. In the T-maze task, no differences were seen between groups on proportion of correct responses.

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