



Antisense oligonucleotide inhibition of calcium/calmodulin-dependent protein kinase II and working memory deficits in the gerbil
by Barry Justin Hoopes

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Psychology
Montana State University
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Abstract:

It has been demonstrated that genetically engineered mice lacking calcium/calmodulin dependent protein kinase II α -subunit (CaM kinase) are impaired in working memory tasks. While this method has been useful in stimulating interest in the relationships, there are limitations to this technique. The relationship between CaM kinase and working memory was investigated by using a T-maze task in the present experiment. It was hypothesized that the AON group would make significantly more errors on a T-maze task than the gerbils injected with missense oligonucleotides. It was also predicted that animals treated with AON's would exhibit a reduction in CaM kinase α -subunit expression, but not CaM kinase β -subunit or actin. To inhibit CaM kinase, pretrained gerbils received intrahippocampal injections of antisense oligonucleotides complimentary to CaM kinase α -subunit (5 μ M; 5'-GGTAGCCATCCTGGACT-3') or missense oligonucleotides (5 μ M; 5'-GGTCGCCATCAGGTC-3'). Animals received three injections separated by 24 hrs. Six hours following the last injection, working memory was assessed by using a T-maze task. Animals were euthanized following behavioral testing and hippocampi were extracted and assayed. Hippocampal proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes. Membranes were probed with a monoclonal antibody against CaM kinase (α and β -subunits) and actin. Animals treated with AON's exhibited a small impairment in working memory and reduction in CaM kinase α -subunit expression. However, these differences were not statistically significant. The data failed to support a role for CaM kinase in working memory. Possible explanations and future implications for these findings are discussed.

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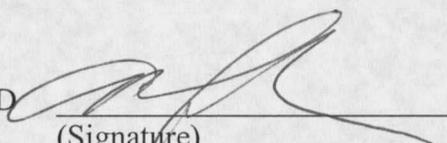
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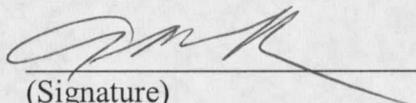
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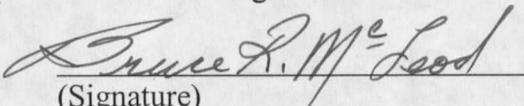
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ABSTRACT

It has been demonstrated that genetically engineered mice lacking calcium/calmodulin dependent protein kinase II α -subunit (CaM kinase) are impaired in working memory tasks. While this method has been useful in stimulating interest in the relationships; there are limitations to this technique. The relationship between CaM kinase and working memory was investigated by using a T-maze task in the present experiment. It was hypothesized that the AON group would make significantly more errors on a T-maze task than the gerbils injected with missense oligonucleotides. It was also predicted that animals treated with AON's would exhibit a reduction in CaM kinase α -subunit expression, but not CaM kinase β -subunit or actin. To inhibit CaM kinase, pretrained gerbils received intrahippocampal injections of antisense oligonucleotides complimentary to CaM kinase α -subunit (5 μ M; 5'-GGTAGCCATCCTGGACT-3') or missense oligonucleotides (5 μ M; 5'-GGTCGCCATCAGGTCCT-3'). Animals received three injections separated by 24 hrs. Six hours following the last injection, working memory was assessed by using a T-maze task. Animals were euthanized following behavioral testing and hippocampi were extracted and assayed. Hippocampal proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes. Membranes were probed with a monoclonal antibody against CaM kinase (α and β -subunits) and actin. Animals treated with AON's exhibited a small impairment in working memory and reduction in CaM kinase α -subunit expression. However, these differences were not statistically significant. The data failed to support a role for CaM kinase in working memory. Possible explanations and future implications for these findings are discussed.

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INTRODUCTION

How do we remember information? Scientists and philosophers have been interested in this question since the times of Plato and Socrates. This fascination continues today in the field of psychology. Herman Ebbinghaus is recognized as the first to study memory "empirically." A disciple of associationism, Ebbinghaus developed an objective scoring method to determine how effectively his memory had retained the learned syllables (Leahey and Harris, 1985). While Ebbinghaus did not employ adequate experimental controls, he introduced the "storehouse metaphor" of memory that is recognized today. His position implied that memory is stored so that it may be recalled at a later date.

The information-processing theory of memory was developed many years after Ebbinghaus. This theory suggests that memory is a system of related components, which is capable of processing types of representations called cognitive codes (Atkinson and Shiffrin, 1968). The theory states that cognitive codes can be transferred from these related components, called storage, by control processes. Information-processing theorists believe that from an initial sensory register, cognitive codes are next stored in a short-term storage component. From the short-term storage, codes can be transferred to long-term storage by way of rehearsal (Atkinson and Shiffrin, 1968). Researchers have elaborated on distinctions in the information-processing model that extend beyond the parameters of the current study.

Karl Lashley was one of the first to investigate the neurological underpinnings of learning and memory. Initially, Lashley was interested in the hypothetical change in the brain responsible for storing memory. His efforts in "discovering" this change were

unsuccessful. However, from his research Lashley concluded that memories for complex tasks are stored diffusely throughout the neocortex and that all parts of the neocortex play an equal role in the storage of memories (1929). Lashley's work sparked interest in the relationship between the brain and memory while introducing the possibility that learning and memory are integrally related.

Hippocampus and Memory

The hippocampus is a structure located in the temporal lobe at the medial edge of the cerebral cortex. Information from the neocortex enters the dentate gyrus which relays information to the CA3 region of the hippocampus. The CA3 neurons project to the CA1 cells that relay information to the subicular complex. The CA3 neurons relay information back to the neocortex placing the hippocampus in a position to receive and relay information throughout the brain.

It has been demonstrated that the hippocampus is an important structure for learning and memory. H.M. was one of the earliest cases demonstrating the importance of the hippocampus in memory function (Milner, Corkin, and Teuber, 1968). H.M. was a 27-year-old male with an extreme case of epilepsy. Medial portions of his temporal lobes were removed in an attempt to reduce his seizure frequency. Following the procedure, H.M. suffered from severe anterograde amnesia. While his short-term memory storage was within normal range, he could not articulate novel long-term memories. The consolidation of long-term memories was limited to "implicit memory" tasks (i.e. mirror-drawing and rotary-pursuit). He demonstrated improvement on these tasks from one day to the next even though he claimed to have never seen them.

Cohen and Eichenbaum (1993) suggested that the hippocampus supports a declarative memory system that provides a substrate for relational representation of all items in memory. Therefore, humans with hippocampal damage have normal performance on short-term memory tasks (STM) because stimulus relationships are not required for them to remember the information, whereas stimulus relationships are required for long-term memory. An alternative explanation is that STM impairments are a function of the amount of damage to the hippocampus; the greater the damage to the hippocampus, the less information available for STM representations. This is supported by the observation that larger hippocampal lesion had a greater effect on the duration, on the amount of information remembered in STM, or both (Nunn, Polkey, and Morris, 1998; Smith and Milner, 1981).

Animal studies have demonstrated somewhat different patterns of memory deficits. Monkeys with hippocampal lesions show no impairments in STM representations for visual object information even with 40 min delays (Murray and Mishkin, 1998). Although STM deficits have been reported for visual object information after hippocampal lesions (Alvarez, Zola-Morgan, & Squire, 1994), the deficits are most likely due to perirhinal cortex damage (Mishkin and Murray, 1994). In a more recent study, Murray and Mishkin (1998) did not find STM deficits for spatial location information with more restricted lesions of the hippocampus. Hippocampal damage in rats produces no impairment in STM for visual objects, motor responses, odors, or reward value (Kesner, 1998). In contrast, there are profound deficits for spatial information based on memory for a single spatial location, allocentric spatial distance, egocentric spatial distance, or head direction and memory for temporal information based on

duration of exposure and the object (Long and Kesner, 1996, 1998; Jackson, Kesner, and Amann, 1998). It seems that in rats and monkeys the pattern of STM involvement of the hippocampus is a function of the nature of the information that needs to be remembered. Kesner (1998) has suggested that the hippocampus represents both spatial and temporal information.

Working memory is the temporary memory necessary for the successful completion of a task on which one is currently working. This differs from reference memory which involves the consolidation of general skills necessary to perform a certain task. Liu and Bilkey (2001) tested rats in object recognition and spatial memory tasks following hippocampal lesions. Hippocampal lesioned rats were severely impaired in both reference and working memory tasks in water maze and radial arm maze tasks. These findings indicate that the hippocampus plays a role in both working memory and reference memory. Working memory disruption has been demonstrated in various types of hippocampal damage. Kiyoyuki, Yoshinori, Nomura, Masahiko and Yamauchi (2001) examined hippocampal involvement in an operant alternation task with long and short delays. They trained male rats in either a short- or long-delayed alternation task. After training, they injected the GABA_A agonist muscimol into either the ventral or dorsal hippocampus. They found that dorsal hippocampus inactivation impaired rats performance in the long-delay tasks and those that received ventral hippocampus inactivation showed no effects in the alternation task. The results suggest that the dorsal hippocampus is related to performance in working memory (Kiyoyuki et. al., 2001).

Hippocampal-dependent memory can also be investigated by alternation tasks in a T-maze that involves spatial memory to obtain reinforcement or avoid punishment. Farr,

Banks, La Scola, Flood, and Morley (2000) found that electrolytic lesions or temporary inactivation of the hippocampus significantly impaired acquisition and retention for T-maze footshock avoidance in mice. Following transient cerebral ischemia, gerbils demonstrate working memory impairment in an alternation T-maze task which is correlated with the loss of hippocampal neurons (Andersen and Sams-Dodd, 1998). It has also been demonstrated that seizure kindling of the hippocampal field CA1 impairs spatial learning and retention in the Morris water maze (Gilbert, McNamara, and Corcoran, 1996).

Deacon, Bannerman, and Rawlins (2001) demonstrated that other tasks may not be hippocampal-dependent. They compared rats with axon-sparing cytotoxic hippocampal lesions against controls on a variety of instrumental conditioning paradigms. The lesioned animals did not differ from the controls in their ability to choose objects that correlated with their respective "internal states" (hunger or thirst). They also did not differ from the control group in their ability to acquire a conditional visuospatial discrimination with black and white boxes. However, lesioned rats were impaired on a T-maze task when cued by their "internal state" (hunger or thirst) or their previous response (working memory) (Deacon et. al., 2001).

Ohno, Yamamoto, and Watanabe (1994) studied the effects of intrahippocampal injections of the M₁ muscarinic receptor antagonist pirenzepine and the M₂ muscarinic receptor antagonist methoctramine on working and reference memory in male rats. Bilateral injections of pirenzepine significantly increased the working memory error rate in rats. The results of the experiment suggested that processes mediated by M₁ muscarinic receptors are involved in working memory but not in reference memory

(Ohno et. al., 1994). The literature indicates the important role of hippocampus in learning and memory. An important molecular phenomenon that explains hippocampal learning is long-term potentiation

Long-Term Potentiation

Long-term potentiation (LTP) is an enduring facilitation of synaptic transmission that occurs following activation of a synapse by intense high-frequency stimulation of the presynaptic neurons (Bliss and Gardener-Medwin, 1973). LTP has been studied most extensively at the synapse where the NMDA (N-methyl-D-aspartate) receptor is predominant. LTP is one of the most widely studied neuroscientific phenomena and dates back to work done by D.O. Hebb (Bliss and Gardener-Medwin, 1973). Hebb believed that experiences triggered unique patterns of neural activity which consolidate through cerebral circuits. This, in turn, would lead to long-term changes in the synapses and would facilitate subsequent transmission across the respective synapses and store the memory of the initial experience. LTP has been deemed the molecular basis for learning and memory (Fukunaga and Miyamoto, 2000).

LTP has two properties that correlate with Hebb's hypothesized characteristics of the neurological mechanism of learning and memory. LTP can last a long time (for many weeks) and develops only if the firing of the presynaptic neuron is followed by the firing of the postsynaptic neuron (Racine & deJonge, 1988, Kelso, Ganong, & Brown, 1986). Without the co-occurrence of firing of the presynaptic and postsynaptic cells, LTP does not develop.

There are two important properties associated with the NMDA receptor and how it applies to LTP. First, in order for it to respond maximally, the neuron must be partially

depolarized (Malinow, Madison, & Tsien, 1988). This depolarization is responsible for the ejection magnesium from the NMDA receptor allowing for the influx of calcium. Calcium triggers a cascade of events that induces LTP (Malinow, Schulman, & Tsien, 1989). Calcium/calmodulin protein dependent kinase II (CaM kinase) is an important target for calcium and plays a central role in triggering and maintaining LTP which will be discussed in a later section. In a study that investigated LTP and working memory, van Hulzen and van der Staay (1991) trained rats to an intermediate level of performance on a radial maze task. The experimental group received a series of high-frequency trains of electrical pulses applied to the right perforant path of the hippocampus. Twenty-four hours after the experimental treatment, the animals were tested in a radial maze. Following a retention interval of two months, one radial maze trial was administered on each of three consecutive days. The analysis of field potential data showed that periodic LTP stimulation produced a state of hippocampal LTP confined to the initial portion of the acquisition phase. I found that a significant improvement of working memory performance in the experimental group during the rising phase (the increased amplitude of the population spike created by the firing of a greater number of granule cells) of hippocampal LTP. Since LTP was generally maintained over the retention interval and performance in the memory task remained stable, it seems that LTP correlates with the consolidation of memory.

CaM kinase

CaM kinase is an enzyme that is highly expressed in the brain where it is composed primarily of α and β subunits. Throughout the brain each subunit has similar catalytic attributes, but the composition varies (Bronstein, Farber, and Wasterlain, 1993).

The ratio of α and β subunits in the hippocampus is 3:1, respectively. CaM kinase is localized in the postsynaptic density directly adjacent to the channels that mediate synaptic transmission. CaM kinase is activated when calcium binds with calmodulin and then combines with the enzyme. After activation, CaM kinase can undergo autophosphorylation, becoming partially calcium and calmodulin independent, and targets a variety of substrates including tyrosine hydroxylase, microtubule associated protein 2 (MAP 2), tau, and synapsin I (Bronstein, et. al., 1993). CaM kinase regulates the synthesis of neurotransmitters by phosphorylating tyrosine hydroxylase which mediates catecholamine synthesis. CaM kinase is also involved in the regulation of cellular processes such as potassium current, cyclic nucleotide phosphodiesterase, the inositol triphosphate receptor, voltage dependent calcium channels, and transcriptional induction by calcium (Bronstein, et. al. 1993).

A direct role for CaM kinase in triggering and perhaps maintaining LTP is supported by studies in which CaM kinase activity was acutely increased either with viral transfection or injection of calcium and calmodulin (Lisman, Malenka, Nicoll, and Malinow, 1997). The initial triggering of LTP begins with a brief rise in postsynaptic calcium that results in the phosphorylation of AMPA receptors. It has been suggested that CaM kinase influences AMPA receptors directly, because the phosphorylation of AMPA receptors after LTP induction occurs at a site that can be targeted by CaM kinase. Indeed, the phosphorylation of AMPA receptors during LTP is blocked by an inhibitor of CaM kinase (Lisman, et. al., 1997). Evidence suggests that the maintenance of the AMPA receptor phosphorylation may be due to the ability of CaM kinase to maintain its activity for long periods after its initial activation by calcium. It accomplishes this by the

autophosphorylation of the threonine residue at position 286, which renders its activity independent of calcium. CaM kinase remains phosphorylated at the 286 site for at least one hour after LTP induction (Barria, et. al. 1997). The role that CaM kinase plays in LTP is useful in explaining the relationship between CaM kinase inhibition and working memory deficits. Avoidance learning is impaired by hippocampal CaM kinase inhibition (Cammarota, Bernabeu, Levi, Izquierdo, and Medina, 1998). One method to directly test the relationship between CaM kinase and working memory is to inhibit the autophosphorylation of CaM kinase. The development of specific CaM kinase inhibitors has become a useful strategy to directly test the role of CaM kinase in memory.

KN-62 (1-[NO-bis-1,5-isoquinolinesulfonyl]-N-methyl-L-tyrosyl-4-phenylpiperazine) is a cell permeable CaM kinase inhibitor that prevents calmodulin binding (Tokumitsu, Chijiwa, Hagiwara, Mizutani, Terasawa, & Hidaka, 1990). KN-62 is highly selective at preventing the autophosphorylation of both the α and β subunits of CaM kinase, but has no effect on myosin light chain kinase, protein kinase C, or cAMP-dependent kinase II activity (Tokumitsu, et. al., 1990). Intrahippocampal injection of KN-62 impairs the acquisition of a Morris water maze (spatial task), but not when the target platform is visible (non-spatial task) (Tan and Liang, 1996). This compound prevents the induction of LTP when microinfused into the hippocampus (Ito, Hidaka, and Sugiyama, 1991). Avoidance learning is also impaired when KN-62 is microinfused into the hippocampus and this behavioral deficit is correlated with alterations in AMPA receptor binding (Cammarota, et. al., 1998). These studies suggest a relationship between CaM kinase and working memory.

While KN-62 is a useful inhibitor of CaM kinase, it is difficult to demonstrate the *in vivo* inhibition of the enzyme using this compound. KN-62 inhibits the autophosphorylation of CaM kinase by blocking the binding of calmodulin to the enzyme. Since the action of KN-62 was characterized in cell culture, it is widely accepted that this same mechanism occurs following *in vivo* injections (Tokumitsu et. al., 1990). As cited previously, Tan and Liang (1996) demonstrated that intrahippocampal injections of KN-62 retarded the acquisition of spatial task in the Morris water maze. Upon *in vitro* assay examination, they found no difference in total CaM kinase activity between the two groups. However, the KN-62 significantly lowered in the percentage of Ca^{2+} - independent activity of hippocampal CaM kinase II suggesting that CaM kinase activity had been inhibited. How accurately an *in vitro* assay represents CaM kinase activity following *in vivo* administration of KN-62 is not known. It is possible that the process of homogenization of the tissue and assay conditions could alter the interaction of KN-62 and the target kinase.

A second approach that has been used to study the role of CaM kinase in learning and memory is to genetically engineer mice that lack the protein (knock-out model). Silva, Stevens, Tonegawa, and Wang (1992) have demonstrated that mice genetically deficient in the CaM kinase α -subunit were impaired in their ability to produce LTP. In addition, they are impaired in various spatial learning tasks (Silva, Paylor, Wehner, and Tonegawa, 1992). Although knockout models represent a valuable research tool, this approach is limited to murine models. In addition, it is possible that some adaptation has occurred in the CaM kinase-deficient mice. This model precludes site specific inhibition since the knockout models are totally deficient in the target protein.

Antisense oligonucleotides (AON) represent an innovative method for investigating cell-signaling pathways. AON can inhibit protein expression by binding specifically to mRNA targets. Inhibitory effects of AON may occur by disturbing ribosome assembly and/or promoting endogenous RNase H degradation (Koller, Gaarde, and Monia, 2000). Nicot, Ogawa, Berman, Carr, and Pfaff (1997) utilized AON's to inhibit preproenkephalin expression in the ventromedial nucleus of the hypothalamus which resulted in a significant reduction in lordosis quotient compared to the control (reverse oligonucleotide). Churn, Sombati, Jakoi, Sievert, and DeLorenzo (2000) utilized AON to inhibit CaM kinase α -subunit in cultured hippocampal neurons. As a control, they employed a missense oligonucleotide. They demonstrated a 53.4% reduction in the α -subunit protein levels. Protein expression of the CaM kinase β -subunit or structural proteins including tubulin, MAP, or synapsin was not significantly altered.

Converging evidence suggest that there is a link between CaM kinase activation and hippocampal dependent memory. Given the limitations of KN-62 and knockout models, the present series of experiments explored the use of AON to inhibit CaM kinase to investigate the role of CaM kinase in working memory.

Experiment Hypotheses

Hypothesis 1

It was hypothesized that intrahippocampal microinfusion of AON complimentary to CaM kinase II α -subunit mRNA would result in a significant reduction in protein expression. To test this hypothesis, animals were injected with AON complimentary to the mRNA of CaM kinase α -subunit or missense oligonucleotides. Hippocampi were extracted and probed for protein expression.

Hypothesis 2

It was hypothesized that gerbils injected with AON would have significantly more errors in the T-maze task than the gerbils injected with the control oligonucleotides. This was tested by injecting pretrained animals with either AON or missense oligonucleotides and examining working memory performance in a standard T-maze task.

METHOD

Introduction to Experiments

Gerbils were trained in a T-maze task over a two-week period prior to the implantation of bilateral cannulae aimed at the dorsal hippocampus. Following a recovery period missense or AON⁷ were microinfused into the hippocampus over a three-day period. After the final injection, gerbils were tested in a T-maze. Next, animals were euthanized and hippocampi extracted. Tissue was homogenized and proteins separated using SDS-PAGE. Proteins were transferred to Immobilon-P membranes and probed with antibodies against the CaM kinase II α -subunit. As a control, other membranes were probed for the expression of the β -subunit of CaM kinase and actin. A detail description of the methodology used in this study is presented in the following sections.

Subjects

Adult male Mongolian gerbils were used as subjects. They were housed individually within a temperature (23° C) and light (12-hr light/dark cycle) controlled room. Rodent pellets and water were provided ad libitum except during training when animals were limited to 4-5 gms of food/ day.

Behavioral Procedure

The apparatus was a T-shaped maze. The stem of the T was 10 cm long and the choice arms were 50 cm each. A start box with a door was attached to the stem of the T-maze. Doors were also at the entrance of each choice arm. A food cup was fitted into a hole in the floor at the end of each choice arm.

The behavioral procedure used in the study was adapted from that of Andersen and Sams-Dodd (1998). The procedure consisted of three phases: habituation, alternation

training, and testing. During habituation, food-deprived gerbils were placed in the maze with all doors open once a day for five successive days (7-10 min/ day). For alternation training, gerbils were trained to alternate in the T-maze. For this phase, gerbils were trained for ten trials/day over five days. Each pair of trials consisted of a force trial (FT) and a choice trial (CT). On FT, one arm was blocked and the gerbils were allowed to enter the opposite arm to obtain a food reward. The position of the FT arm was random, but the number of left and right positions was equal in each session. After entering the arm, the animals were returned to the start box and the door closed and both arms were immediately available but only the formerly closed arm contained the food reward. Gerbils were permitted 30 seconds to enter either of the arms (CT). If there was no response after thirty seconds, the paired trial was repeated. When the arm was entered, the door was closed and the gerbil was allowed to consume the reward. If the wrong arm was chosen, the gerbil was confined to that arm for 10 seconds. Following the injection phase, the gerbils were tested with 10 paired trials of the task (testing phase). Scores were recorded in terms of number of correctly performed CT's.

Implantation Surgery

Cannula aimed at the dorsal hippocampus were implanted bilaterally in each animal following maze training. For implantation, gerbils were anesthetized with isoflurane. An incision was made on the dorsal surface of the scalp and two burr holes drilled at 2.2 mm posterior to bregma and 1.5 mm lateral to both sides of the midline. A double cannula (Plastics One, 3.0 mm distance between cannula) was lowered to a depth immediately dorsal to the hippocampal CA1 region; 1.1 mm ventral to cortical surface (Babcock, Liu, Paden, Churn, and Pittman, 1997). To assure that the cannula would

remain intact, a screw was placed in the skull and attached to the cannula with dental acrylic. Following the implantation procedure, gerbils were allowed 1 day of recovery.

Oligonucleotides (both missense and antisense groups) were injected over three days. The antisense (5'-GGTAGCCATCCTGGCACT-3') and missense (5'-GGTCGCCATCAGGTCCT-3') oligonucleotides were identical to those previously described in Churn et. al. (2000). The AON is complementary at the 33 to 50 portion of the mRNA. The oligonucleotides were injected under isoflurane anesthesia daily over three days at a concentration of 5 μ M (5 μ l/side over 2 min). Approximately six hours following the final injection, the gerbils were tested in the alternation task. Immediately following testing, the animals were deeply anesthetized and euthanized by cervical dislocation. The hippocampi were rapidly extracted and frozen.

Western Analysis

Hippocampal tissues were homogenized in an ice cold buffer containing 500 mM 3-(N-morpholino) propanesulfonic acid (pH 7.6), 20 mM DTT, 1.0 mM sodium orthovanadate, 30 mM EGTA, 1.07 mM magnesium acetate, 3.2 mM sucrose, phenylmethylsulfonyl fluoride (0.017mg/ml), leupeptin (20 μ g/ml), aprotinin (5 μ g/ml), and pepstatin (10 μ g/ml). The protein content of samples was determined using the Pierce BCA Protein Assay kit. The samples were mixed with varying concentrations of 5 X SDS sample buffer consisting of 0.3 mol/l Tris-HCl (pH 6.8), 25% beta-mercaptoethanol, 12% SDS, 25 mmol/l EDTA, 20% glycerol and 0.1% bromo phenol blue, boiled for 3 min and subjected to SDS-PAGE (25 μ g protein/lane). Proteins were electrotransferred onto Immobilon-P membranes at a constant voltage of 14 V overnight. The membranes were washed with a protein solution (I-Block: Western Lights) for 30 min at room temperature

prior to incubation at 4°C overnight with a monoclonal antibody against the α -subunit CaM kinase II (1: 10,000; Sigma). As a control, selected samples were probed with monoclonal antibodies against the β -subunit of CaM kinase (1: 10,000; Generous gift from S.B. Churn) or actin (1: 50; Sigma).

Following incubation with the primary antibody, membranes were washed two times (5 min) with I-Block at a room temperature and incubated with goat anti-mouse secondary antibody (conjugated to alkaline phosphatase, 1:20,000; Sigma). Membranes were reacted with a luminescent substrate (Western Lights Buffer) and exposed to Kodak X-Omat film at various exposures to obtain optimal images following which they were developed for 5 min in developer and 5 min in fixer solutions. Optical densities were measured with an MCID imaging system. The values were defined as the larger score signifying more protein.

