Ca2+/Calmodulin-Dependent Protein Kinase II Activity and Expression in the Aged Mouse Hippocampus

By

James Mitchum

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2018

Approved by

_____

Advisor: Dr. Nicole Ashpole

_____

Reader: Dr. Jamie Stewart

_____

Reader: Dr. Ken Sufka

Acknowledgements

“This study was supported by an Institutional Development Award (IDeA) Grant Number P20GM104932 from the National Institute of General Medical Sciences (NIGMS) and the In Vivo Pharmacology Research Core of the COBRE, a component of the National Institutes of Health (NIH) under the grant number P20GM104932. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NIGMS or NIH. Travel was supported by the Sally McDonnell Barksdale Honors College

ABSTRACT
JAMES MITCHUM: Calmodulin/Ca^{2+}-Dependent Protein Kinase II Activity and Expression in the Aged Mouse Hippocampus
(Under the direction of Nicole Ashpole)

CaM Kinase II (Ca^{2+}/calmodulin-dependent protein kinase II) is a serine/threonine-specific protein kinase enzyme that acts as a critical regulator of learning and memory. It has previously been shown that the inability of CaMKII to maintain activation is connected to memory deficits within the aging brain. While the connection between CaMKII dysfunction and learning and memory impairments is well established, little is known about the changes that cause CaMKII dysfunction. Our research set out to investigate if CaMKII is altered during the process of aging. Specifically, we looked at activity, regulation, and stability by conducting experiments to quantify CaMKII levels of activity and protein expression in both young and aged mouse hippocampi. We hypothesized that when compared to young mouse hippocampi, aged mouse hippocampi would have lower levels of total CaMKII activity, lower levels of phosphorylated CaMKII expression, and increased levels of CaMKII inhibitor expression. The impact of our research lies in the illumination of the mechanisms causing CaMKII dysfunction. By casting light on the process of CaMKII dysfunction, we will help future researchers to establish a possible therapeutic target in the fight against learning and memory impairments.

TABLE OF CONTENTS

LIST OF FIGURES

BACKGROUND

Ca^{2+} Signaling in the Brain

The calcium cation Ca^{2+} is among the most important secondary messengers in cellular signaling, influencing almost every process within the cell. Ca^{2+} signaling is of particular importance in both neuronal growth and survival. Although Ca^{2+} is vital to the livelihood of the cell, it also has the ability to do great cellular harm. Because of the fact that the cation is highly reactive and prolonged exposure to high levels of Ca^{2+} can be toxic to the cell. When intracellular Ca^{2+} levels become too high, both mitochondrial depolarization and over-activation of downstream signaling pathways can occur. In order to avoid the toxic side effects of high levels of Ca^{2+} the cell must use pumps, channels, and proteins to preserve low cellular concentrations of the cation. A family of proteins that play an important role in Ca^{2+} mediation is the calmodulin (CaM) kinase proteins.

The CaM Kinase Family and the Structure of CaMKII

The CaM kinases are a group of serine/threonine protein kinases, which are activated by calcium-bound calmodulin and phosphorylate a serine or a threonine residue in its substrate P-site. The substrate P-site is the target site of phosphorylation. While the CaM kinases all share this defining targeting motif, they each undergo different mechanisms of regulation. Because of the variation in regulation across the family, CaM kinases play roles in a plethora of cellular functions. In this study, we will focus on the neuronal functions of CaMKII. Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) is a mediator of Ca^{2+} signaling that has the ability to phosphorylate a variety of substrates in order to coordinate and regulate cellular functions related to Ca^{2+} signaling (Ashpole

2011). The mediation functionality of CaMKII arises from the enzyme's unique structure. CaMKII has the form of a multimeric holoenzyme with a C-terminal association domain creating a central hub and N-terminal kinase domains protruding outward. In the space between these two domains, CaMKII has a $Ca^{2+/}$CaM-binding autoregulatory region as well as a variable region in which splicing can occur (Coultrap 2011). Because of CaMKII's multimeric structure and autoregulatory domain, it is able to play an active role in the location, timing, and sensitivity of its action. CaMKII can: (1) be activated in a manner dependent on Ca^{2+} concentration, (2) achieve independence from its initial $Ca^{2+/}$CaM activators, and (3) function as a molecular switch, which is a function vital to certain types of learning and memory (Hudmon and Schulman 2002). The four CaMKII isoforms are α, β, γ, and δ. All CaMKII isoforms have a catalytic domain, an autoregulatory domain, a variable region, as well as a self-association domain. There are binding sites for adenosine triphosphate (ATP) and other substrate proteins inside the catalytic domain. Moreover, it is at this domain that a phosphate is transferred from ATP onto a Ser or Thr substrate residue (Hudmon and Schulman 2002). When phosphorylation occurs at Thr-286, CaMKII is constitutively activated due to the fact that the autoregulatory domain is blocked and can no longer bind to the p-site. When this circumstance occurs, CaMKII remains activated, even when Ca^{2+} and calmodulin are absent (Yang 1999). The variable and self-associative domains are responsible for the sensitivity of CaMKII to Ca^{2+} and calmodulin. The activation state of the enzyme will be dictated by CaMKII's level of sensitivity. Initially, the CaMKII is in an active state; autophosphorylation is not possible at this time due to the lack of Ca^{2+} or calmodulin, which are necessary to facilitate binding to neighboring subunits. As Ca^{2+} and calmodulin

levels increase, autophosphorylation takes place resulting in persistent activation of CaMKII for a brief period. The autophosphorylation of CaMKII will be discussed later in detail. When dephosphorylation of Thr-286 occurs, CaMKII becomes inactivated. It is the features of the variable and self-association domains that determine the identity of the different CaMKII isoforms. It is important to note that under ischemic conditions, CaMKII forms extra-synaptic clusters by way of the CaMKII holoenzyme self-aggregation mechanism (Dosemeci 2000; Tao-Cheng 2002; Hudmon 2005; Vest 2009).

Prevalence of CaMKII in Neurons

While CaMKII is found in the majority of tissues, it has particularly high concentration within neurons. The two splice variants which make up the majority of isoforms in neurons are α (50 kDa) and β (60 kDa) (Bayer 1998; Schulman 1978). CaMKIIβ functions as a targeting or docking module, and CaMKIIα is crucial to neuronal plasticity, spatial memory, and hippocampal long-term potentiation (LTP). The γ and δ isoforms are highly expressed in astrocytes and are also expressed throughout the body (Tobimatsu and Fujisawa 1989; Takeuchi 2000)

Functionality of α-CaMKII in Neuronal Plasticity and Spatial Memory

α-CaMKII is expressed exclusively in glutamatergic neurons: glutamatergic neurons produce glutamate, which is among the most common excitatory neurotransmitters in the central nervous system (Institute of Medicine (US) Forum on Neuroscience and Nervous System Disorders 1970). In the hippocampus, CaMKII is not localized in the nucleus. Thus, α-CaMKII is not directly involved in gene transcription in the hippocampus (Giese

2013). It has been established that α-CaMKII plays a part in hippocampal neuronal plasticity; for example, it has been shown that CaMKII inhibition induces neuronal apoptosis (Ashpole 2012). Studies have also shown that α-CaMKII mutations impair spatial learning in mice (Silva 1992; Ohno 2006). Moreover, α-CaMKII$^{\text{Thr-286A+/-}}$ mutant mice exhibit impaired new learning induced by extinction training, while extinction related unlearning was unaffected (Kimura 2008).

Functionality of α-CaMKII in Long-Term Potentiation

Long-term potentiation (LTP) and long-term depression (LTD) are synaptic processes that take place over an extended period during the learning process. LTP and LTD work in opposition to each other: LTP functions to strengthen synapses, and LTD works to decrease synaptic strength. The length of time in which LTP takes place makes it a likely mechanism of memory storage. When LTP induction occurs, calcium is able to enter the cell. Once Ca^{2+} enters, CaMKII is activated. Upon activation CaMKII translocates to the synapse, where it binds to N–methyl-D-aspartate (NMDA) receptors and brings about potentiation via the phosphorylation of glutamate receptor subunits (Lisman 2012). NMDA receptors are a type of ionotropic glutamate receptor; the flux of Ca^{2+} through NMDA receptors is believed to be critical in synaptic plasticity. Likewise, When CaMKII is inhibited, memory formation is substantially impaired (Giese 2013; Lisman 2002; Elgersma 2004; Irvine 2006; Wayman 2008; Lucchesi 2011; Coultrap and Bayer 2012; Ashpole 2012). Long-term depression (LTD) works in opposition to LTP in order to diminish synaptic signaling for an extended period of time.

Autophosphorylation of α-CaMKII

The role of CaMKII in transducing synaptic activity as a means to cause change in synaptic strength and facilitate learning and memory is dependent upon the autophosphorylation of Thr-286 (Hudmon and Schulman 2002). Upon autophosphorylation at Thr-286, CaMKII becomes autonomous, meaning that it acts independently from Ca^{2+} stimulation. It is the autonomy achieved by autophosphorylated CaMKII that allows the kinase to control the induction and maintenance of the synaptic plasticity underlying learning and memory (Coultrap 2012). Likewise, CaMKII autonomy is known to be of importance in neuronal plasticity as well (Ashpole, Song, Brustovetsky, Engleman, Brustovetsky, Cummins, & Hudmon 2012). It is important to note that Thr-286 autophosphorylation has a function in the mediation of LTP induction and memory formation rather than LTP maintenance and memory storage as evidenced by previous inhibitor studies (Coultrap 2012). Introduction of an α-CaMKII$^{T286A\text{-}129B6F2}$ point mutation, which blocks Thr-286 autophosphorylation, results in severe impairments to hippocampus-dependent memory formation: this result suggests that autophosphorylation is a process of critical importance (Giese 1998; Need and Giese 2003; Irvine 2005 and 2011).

Dysfunction of CaMKII and Its Connection to Dementia

The progressive loss of synaptic elements preceding neuronal death has been correlated with the advancement of cognitive decline in humans and rodents alike (Terry 1991; Moolman 2004). The hippocampal and cortical regions show the most loss of these features in patients exhibiting symptoms across the cognitive dementia spectrum (Ghosh

2015). While the precise mechanism by which synaptic dysfunction occurs in the aging brain remains unclear, it has previously been shown that Aβ oligomers are able to bind to synaptic sites and reduce LTP while also promoting LTD (Lambert 1998; Walsh 2002; Lacor 2004; Shankar 2008; Li 2009). It is thought that Aβ oligomers may target post synaptic elements (Marcello 2008) such as CaMKII among other signaling pathways. Building upon this thought, it has been shown that Ca^{2+} concentration homeostasis is dysregulated in Alzheimer's disease (LaFerla 2002; Bezprozvanny 2008). Furthermore, Aβ oligomers can intrude on Ca^{2+} signaling pathways and impair cognitive function (Mattson 1991; Demuro 2010). Because of its important role in post-synaptic LTP induction, CaMKII is likely a binding target for Aβ oligomers. It has been shown that CaMKII is dysfunctional in Alzheimer's disease hippocampi (Ghosh 2015). Likewise, it has been shown that subcellular localization of α-CaMKII autophosphorylation is altered in Alzheimer's diseased brains (Reese 2011). It has been established that treating hippocampal neurons with Aβ oligomers impairs CaMKIIα activation (Wang 2013; Townsend 2007). Furthermore, redistribution of phosphorylated Thr-286 in CaMKIIα could act as a contributor to cognitive impairment in Alzheimer's disease.

Unknown Mechanism of CaMKII Dysfunction in the Naturally Aging Brain

Although it has previously been shown that the inability of CaMKII to maintain activation is connected to memory deficits within the diseased brain, very little is known about the mechanism of CaMKII dysfunction in the naturally aging brain. In this study, our central aim was to determine if CaMKII activity, regulation, and or stability are altered during the natural process of aging in the wild type mouse hippocampus. In order

to accomplish our aim, experiments were done to quantify activity, gene expression, and protein expression in both young and aged wild-type mouse hippocampal supernatant and aggregate samples.

METHODS

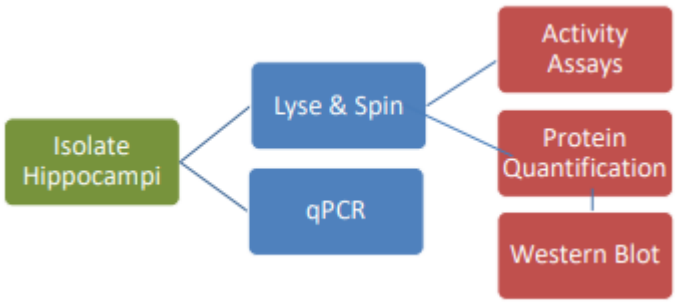


Figure 1: **Methodology Flow Chart**: first, the hippocampi of young and aged mice were isolated. The hippocampal samples were then split evenly into two groups. The first group of samples were lysed and centrifuged prior to being utilized in protein quantification and western blot analysis. The second group of samples underwent qPCR. Activity Assays were also performed

Animals and Hippocampus Isolation

The animals used in this study were young and aged wild type C57Bl6 mice purchased from Jackson Laboratories. The mice were aged in house and given access to food and water *ad libidum*. All procedures were approved by the Institutional Animal Care and Use Committee. The young mice were between 3 and 4 months old at the time of euthanasia, and the aged mice were 24 months old at the time of euthanasia. It is important to note that the aged mice used in this study had previously exhibited impaired

learning and memory in behavioral studies. In accordance with our IACUC approved standard operating procedures, mice were euthanized by rapid decapitation, and the brain and dorsal hippocampus was dissected out.

Sample and Buffer Preparation

In order to prepare the samples, lysis buffer was made using the following recipe: 50 mM HEPES buffer (pH 7.4), 4 mM EGTA buffer, 10 mM EDTA buffer, 15 mM $Na_4P_2O_7$-$10H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, and protease inhibitor were combined in order to yield lysis buffer. After lysis buffer was added to the tissue samples, the pellets were sonicated for 3 sec. Next, 200 µl of RIPA buffer was added to each supernatant sample; then the supernatant samples were centrifuged for 30 min at 1200 xg. The RIPA buffer was made using the following recipe: 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% NP-40,), 25% Na-Deoxycholate, 1 mM PMSF, 1X Roche complete mini protease inhibitor cocktail, and 1X Pierce phosphatase inhibitor cocktail. At this point, samples were either used in activity assays or underwent protein quantification followed by western blot analysis.

Protein Quantification

A detergent compatible protein assay (DC protein assay) was performed on both resuspended pellet and supernatant samples using the microplate assay protocol from the BioRad DC Protein Assay instruction manual. 20 µl of Reagent S was added to 1 ml of Reagent A to make Reagent A'. Serial solutions of BSA standard were used to establish a BSA concentration curve. 1:10 dilutions of each sample were made. On a clean dry

microtiter plate, 5 µl of standards as well as diluted and undiluted samples were added. 25 µl of Reagent A' was added to each well. Then 200 µl of reagent B was added into each well. The microplate was incubated for 15 min and then absorbance was read at 750 nm. In order to prepare the protein samples for gel electrophoresis, the volume of protein sample necessary to yield 10 µg of protein was calculated. Then specific amounts of ddH_2O and 4X LDS buffer were added to each protein sample. The LDS buffer was made by adding 20 µl of BME to 1 ml of 4X LDS. The mixed samples were then heated for 5 min at 95 ºC, centrifuged, and cooled. The mixed samples were then loaded on to the gel.

SDS-PAGE Protein Electrophoresis

The precast gel cassette was prepared by removing the comb and removing the strip of tape at the bottom of the cassette. Next, the gel was placed inside the electrophoresis cassette holder, and the well and the front reservoirs were filled with 1X MOPS running buffer. At this point two important checks were done: it was confirmed that the lip of the gel faced the buffer and it was made sure that there were no leaks from the assembly. Also, the wells were rinsed 3 times with 1X MOPS running buffer before the samples were loaded. Next, the samples were slowly added into their corresponding wells as was the appropriate molecular weight marker. The electrodes were connected and the gel was run at 80 V until the dye was fully integrated into the gel, which usually took 20 min. The voltage was increased to 120 V and the gel ran until loading dye was in the bottom fifth of the gel: this step usually took 90 min. The protein samples were now ready to be transferred from gel to nitrocellulose membranes. Having presoaked all necessary membranes, pads, and filter paper in ice cold 1X NuPAGE transfer buffer, the sandwich

assembly was made. Next the assembly was secured in the transfer electrophoresis holder, and the outside system was filled with ice cold NuPAGE transfer buffer. The system was run at 30 V for a minimum of 1 hr. Once transfer was completed, the membranes were soaked in 5% BSA blocking buffer and were shaken for 1 hr. The membranes were rinsed 3 times for 5 min with TBST. Next, the membranes were incubated with the primary antibody. The primary antibodies used in this study were purchased from Cell Signaling Technology. They were CaMKII-Alpha mouse monoclonal antibody, P-CaMKII (T286) rabbit monoclonal antibody, CaMKII Nβ mouse monoclonal antibody, and GAPDH rabbit monoclonal antibody. The primary antibody dilutions used in this study ranged in volume (7-10 ml per gel) and concentration (1:1000 – 1:5000). The gels were incubated with the diluted primary antibody and TBST on a shaker for 1 to 2 hrs at room temperature or overnight at 4 ºC. After having been incubated with the primary antibody, the gel was now ready to be incubated with the secondary antibody. The secondary antibodies used in this study were purchased from Invitrogen and ThermoScience. They were ThermoScience goat anti-mouse IgG (H+L) secondary antibody (α-CaMKII, CaMKII Nβ, GAPDH) and Invitrogen Goat antiRabbit IgG (H+L) cross absorbed secondary antibody (P-CaMKII), The secondary antibody dilutions used in this study ranged in volume (7-10 ml per gel) and concentration (1:10000-1:20000). The gels were incubated in darkness with the diluted secondary antibody in TBST buffer for 1 hr at room temperature. After 1 hr, the secondary antibody was discarded and the membranes were washed quickly 3 times in TBST and then were washed 3 times for 5 min in TBST. Each of the secondary antibodies used in our study

were fluorescently tagged secondary antibodies. The antibodies were visualized using Licor Odyssey scanners in the labs of Dr. Jason Paris and Dr. David Pasco.

CaMKII Activity Assay

The catalytic activity of CaMKII in lysed hippocampi was evaluated by measuring phosphorylation of the CaMKII autoregulatory domain autocamtide-2 (AC2). As mentioned previously, neuronal cultures were lysed in lysis buffer containing 50 mM HEPES, pH 7.4, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$-$10H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, 1% Triton X-100, and protease inhibitor mixture, sonicated, and incubated with 50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 100 M ATP, 2 mM $CaCl_2$, 5 M CaM, 50 M AC2 (KKALRRQETVDAL), and [γ-32P]ATP (3 μCi per reaction) for 3 min at 30 °C. The linear range of the reaction extended from 30 sec to 4 min. Protein levels were assessed, and activity at 1 min was normalized to total protein using DC protein assay kit (Bio-Rad).

Real Time Quantitative Polymerase Chain Reaction

Polymerase Chain Reaction was utilized in order to amplify gene expression of α-CaMKII (Mm00437967_m1 Camk2a FAM-MGB / 20X), β-CaMKII (Mm00499266_m1 Camk2d FAM-MGB / 20X), γ-CaMKII (Mm00618054_m1 Camk2g FAM-MGB / 20X), δ-CaMKII (Mm00432284_m1 Camk2b FAM-MGB / 20X), and the housekeeping genes Hypoxanthine Guanine Phosphoribosyltransferase (HPRT) (Mm03024075_m1 HPRT FAM-MGB / 20X) and GAPDH (Mm99999915_g1 GAPDH FAM-MGB / 20X) in young and aged mouse hippocampal tissue samples. The Qiagen RNeasy Mini Kit was

used in order to isolate the RNA. And the ThermoFisher High-Capacity RNA to cDNA Kit was used to reverse transcribe the cDNA. Primers purchased from LifeTech Laboratories were used to amplify the desired segments of cDNA. Applied BioSystem TaqMan dye label for PCR per manufacturer's recommendation. The amplification was analyzed using a BioRad CFX Connect Real-Time PCR Detection System, and the Ct values were normalized to gene expression of HPRT using the ΔΔCT normalization method.

Data Analysis

Data collected from BioRad DC protein assays was quantitatively analyzed to establish a concentration curve using Microsoft Excel. Sigma Plot was utilized to formulate each of the graphs used in this research study. At multiple points in the study, unpaired t-tests were performed to determine the statistical significance of our collected data. When the absence of a discernable numerical pattern was evident in both preliminary data and Sigma Plot graphical analysis, the unpaired t-test was not utilized. However, the unpaired t-test was utilized in all experiments that were of central importance to the evaluation of our hypotheses. In this study, a p-value < 0.05 was considered statistically significant.

RESULTS

We first conducted *in vitro* phosphorylation assays in order to examine total CaMKII enzymatic activity in both young and aged mouse hippocampi. These in vitro

phosphorylation assays exhibited significantly decreased levels of CaMKII activity in the hippocampi of aged mice (Figure 2). Once we found CaMKII activity to be significantly diminished in the aged mouse hippocampus, we conducted other experiments in order to identify the cause of the enzyme's diminished activity. These experiments were immunoblots and Real-Time PCR. Immunoblotting was performed using various antibodies in order to enable us to attain a full picture of CaMKII protein expression in both the young and aged mouse hippocampus. The first western blot experiment performed used α-CaMKII (50 kDa) and GAPDH (40 kDA) antibodies as a means to examine relative α-CaMKII expression. Levels of relative alpha CaMKII expression were normalized to a value of one. The relative total CaMKII expression western blot yielded no discernable pattern linking total CaMKII protein expression to diminished CaMKII activity in the aged mouse hippocampus (Figure 3).

Like the first western blot, the second western blot also used an α-CaMKII antibody; however, the samples used in this blot included both supernatants and pellets in order to examine aggregation of total CaMKII. As mentioned previously, CaMKII is known to aggregate at the self-association domain under ischemic conditions (Dosemeci 2000; Tao-Cheng 2002; Hudmon 2005; Vest 2009). Aggregated CaMKII is unable to reactivate when needed (Hudmon 1996). In the laboratory, we can assess aggregation by means of differential centrifugation (Hudmon 1996). The α-CaMKII aggregation expression western blot yielded no discernable pattern linking total CaMKII aggregation to diminished CaMKII activity in the aged mouse hippocampus (Figure 4).

Next, we sought to examine the expression of the CaMKII inhibitor by way of western blot using a CaMKN antibody. Sadly, the experiment did not yield quantifiable

results. The gel image obtained from this western blot can be found in the supplemental data section (Supplemental Figure 5).

As stated previously the constitutive activity of CaMKII is thought to play a critical role in long-term potentiation. This constitutive activity occurs as a result of autophosphorylation of Thr-286, which effectively blocks CaMKII's autoregulatory domain from binding to the p-site. We measured the expression of phosphorylated Thr-286 CaMKII by means of the Cell Signaling Technology P-CaMKII (T286) rabbit monoclonal antibody, which recognizes CaMKII phosphorylated at Thr-286. Furthermore, we were able to quantify the relative expression of phosphorylated Thr-286 CaMKII by normalizing to total expression of α-CaMKII for each supernatant sample. Quantification of the relative expression of phospho-CaMKII exhibited that expression of phosphorylated Thr-286 CaMKII is significantly diminished in the aged mouse hippocampus (Figure 5). From this result, we were able to identify a likely link between diminished total CaMKII activity and diminished expression of phosphorylation of CaMKII at Thr-286.

Having examined protein expression of CaMKII, we turned our attention to gene expression. Real-Time PCR was used in order to examine gene expression of the different CaMKII isoforms (α, β, γ, and δ) in young and aged mice. The results of the PCR were normalized to HPRT. The Real-Time PCR conducted in this study yielded no discernable pattern linking gene expression to diminished CaMKII activity in the aged mouse hippocampus (Figure 6).

Total CaMKII Activity

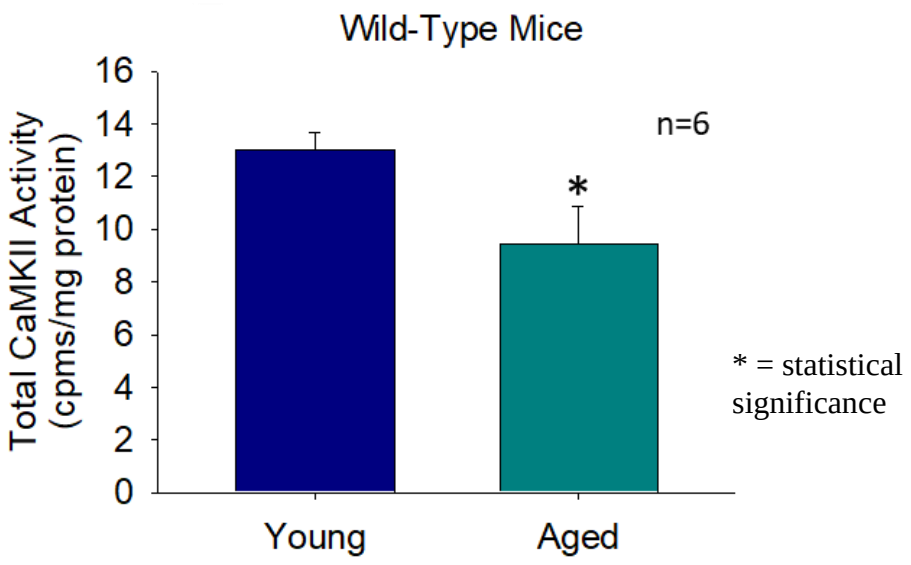


Figure 2: Total CaMKII Activity: Phosphorylation assays revealed that total CaMKII activity is diminished in the aged mouse hippocampus. Enzymatic activity was measured using counts per minute per milligram of protein (cpms/mg). The diminished activity of CaMKII in the aged mouse hippocampus was shown to be statistically significant (p-value < 0.05 vs. young mice). **Figure 2** provides a graphical quantification of the results of the total CaMKII activity assay.

Expression of Alpha CaMKII Protein

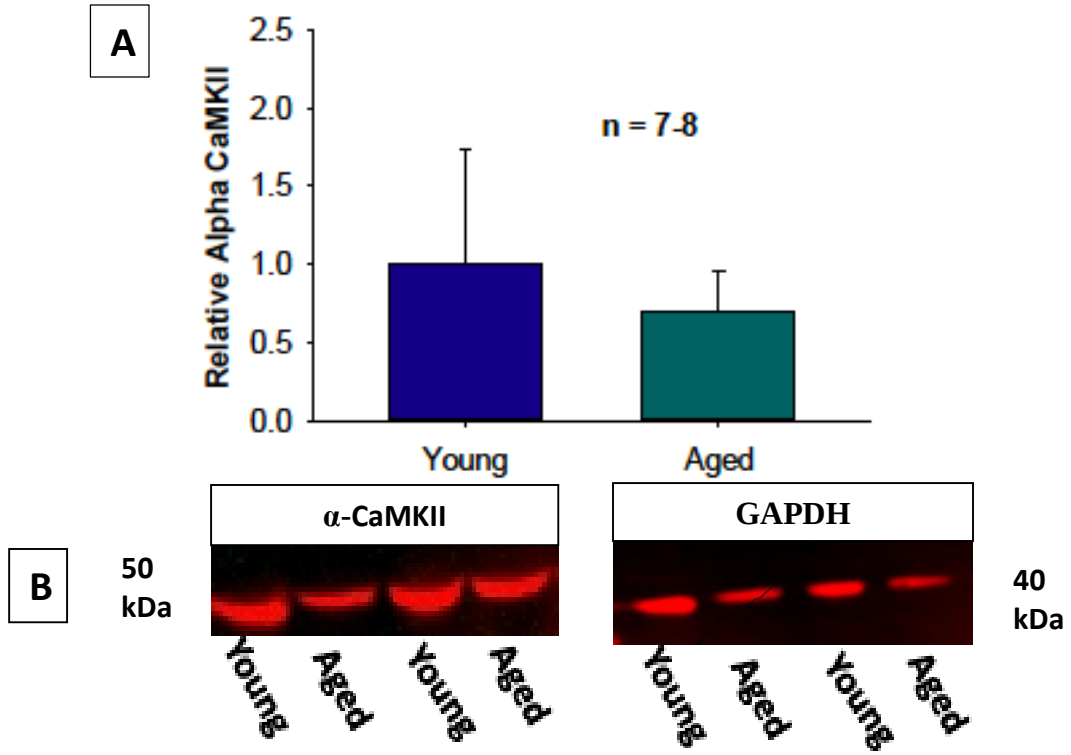


Figure 3: Relative Expression of the α-CaMKII Antibody: young and aged wild-type mouse supernatant hippocampal protein samples were blotted with an α-CaMKII antibody as well as an a GAPDH antibody. Levels of alpha CaMKII (α-CaMKII antibody) expression were normalized to levels of GAPDH expression. **A)** a graphical quantification of relative alpha CaMKII expression in young and aged wild-type mouse supernatant hippocampal tissue samples. **B)** a representative images of western blots used to quantify alpha-CaMKII expression in young and aged wild-type mouse supernatant hippocampal tissue samples. See supplemental data section for a full Western blot images (**Supplemental Figure 1**).

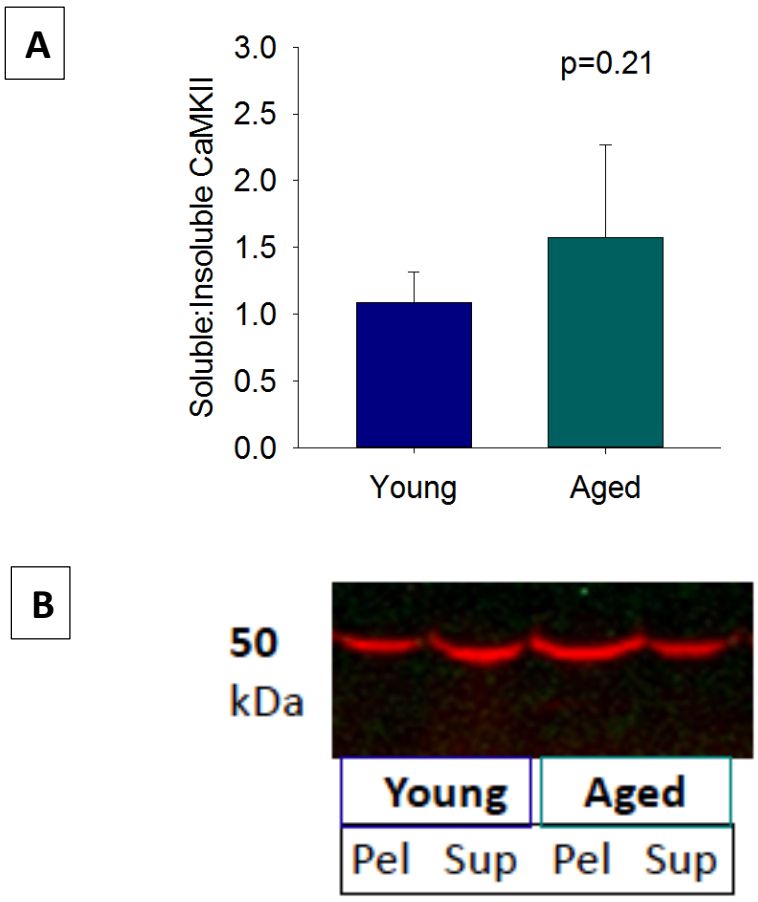


Figure 4: Expression of the α-CaMKII Antibody during Aggregation: young and aged wild-type mouse pellet and supernatant hippocampal tissue samples were blotted with a CaMKIIα antibody in order to quantify expression levels of CaMKIIα aggregation. **A)** a graphical analysis of quantified levels of total CaMKII aggregation expression in young and aged wild-type mouse pellet and supernatant hippocampal tissue samples. The ratio used in this analysis was soluble to insoluble CaMKII (supernatant to pellet). **B)** a representative image of a western blot gel used to quantify total CaMKII aggregation expression. See supplemental data section for a full Western blot image (**Supplemental Figure 2**).

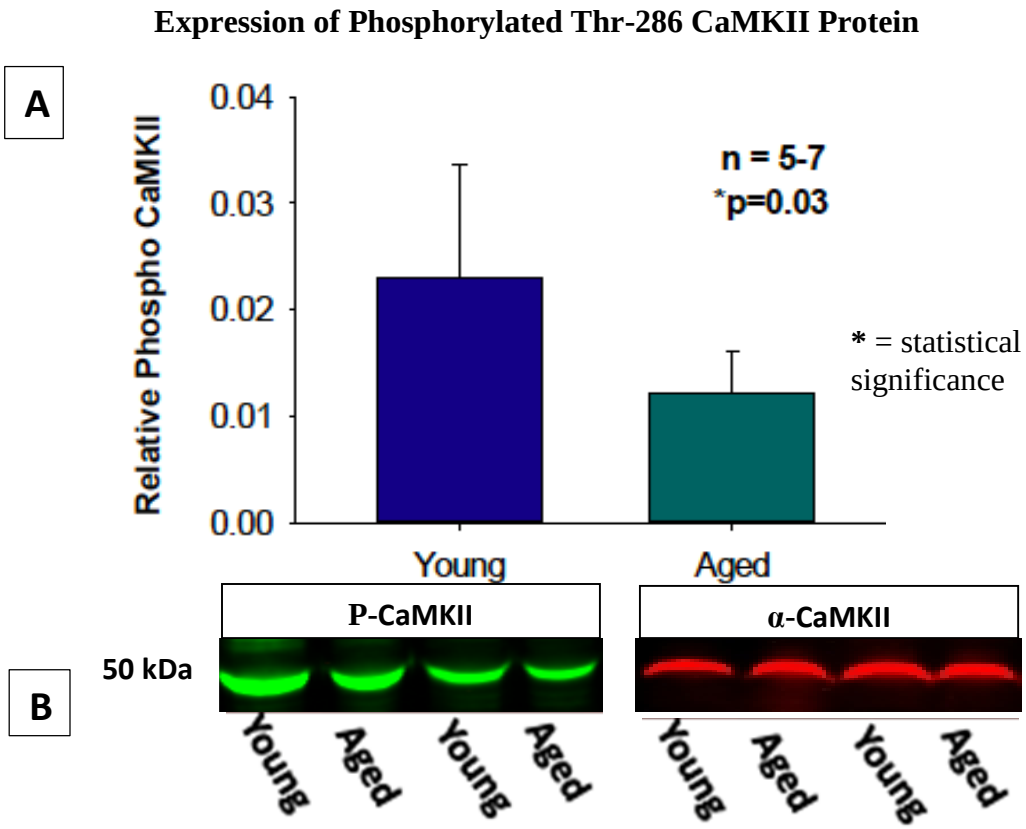


Figure 5: Relative Expression of the P-CaMKII Antibody: young and aged wild-type mouse supernatant hippocampal tissue samples were blotted with a p-CaMKII antibody and an α-CaMKII antibody. Levels of phospho-CaMKII expression (p-CaMKII antibody) were normalized to levels of total CaMKII expression (α-CaMKII antibody). **A)** a graphical quantification of relative phospho-CaMKII expression in young and aged wild-type mouse supernatant hippocampal tissue samples (p-value = 0.03). **B)** representative images of the western blot gel used to quantify relative phospho-CaMKII expression in young and aged mouse supernatant samples. See supplemental data section for full western blot images (**Supplemental Figure 3**).

Gene Expression of the CaMKII Isoforms

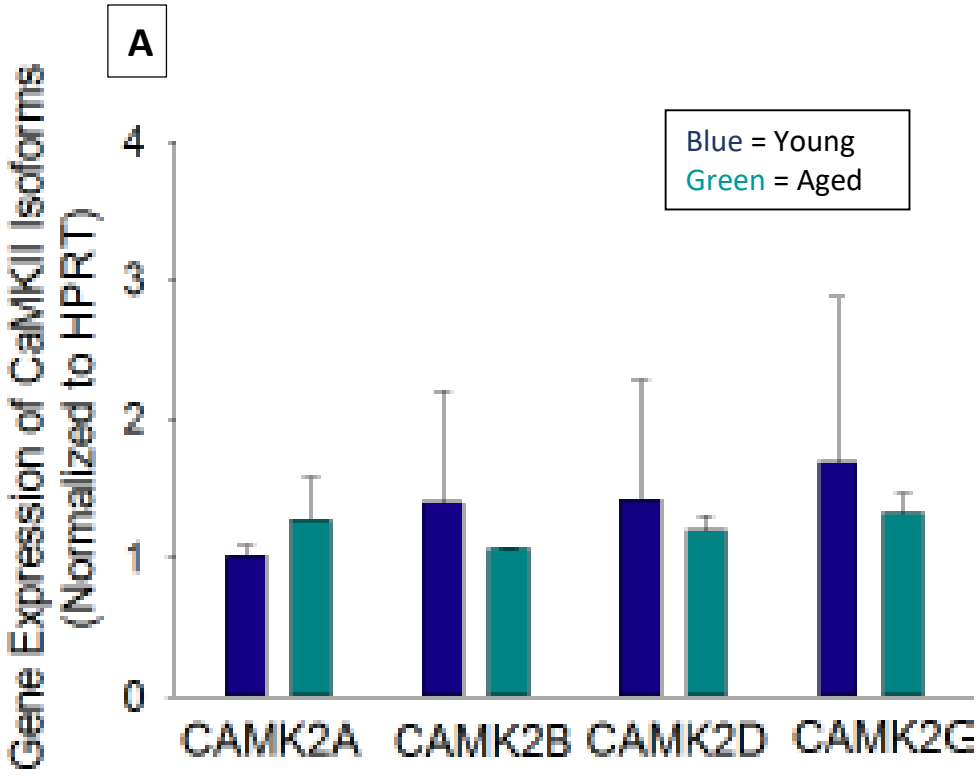


Figure 6: Real-Time PCR of the CaMKII Isoforms: mouse hippocampal tissue samples underwent real-time qPCR in order to analyze gene expression of the different CaMKII isoforms. The major CaMKII isoforms are α, β, γ, and δ. The CT values were normalized to gene expression of HPRT using the ΔΔCT normalization method. **A)** a graphical analysis of quantified gene expression levels of the CaMKII isoforms (α, β, γ, and δ) in young and aged mouse hippocampal tissue samples.

DISCUSSION

We hypothesized that when compared to young wild-type mouse hippocampi, aged wild-type mouse hippocampi would have lower levels of total CaMKII activity, lower levels of phosphorylated CaMKII expression, and higher levels of CaMKII inhibitor expression. Activity assays, qPCR, protein quantification, and western blots were conducted in order to test our hypotheses and to explore alternate explanations. The results of the activity assay supported our hypothesis that total CaMKII phosphorylation activity levels would be lower in aged mouse hippocampi than in young hippocampi. Likewise, the results of our western blot analysis of relative phospho-CaMKII expression supported our hypothesis that phospho-CaMKII expression would be diminished in the aged wild-type mouse hippocampi. It is worth noting that the results of the relative phospho-CaMKII western blot were significant having a p-value of 0.03 (p-value<0.05). Our finding that phospho-CaMKII expression is significantly diminished in the aged hippocampus suggests that dysfunction within the process of CaMKII phosphorylation at Thr-286 contributes to the decline in total CaMKII activity. Furthermore, because of the importance of Thr-286 autophosphorylation in the enzyme's mediation of LTP induction, it is plausible that diminished phosphorylation of CaMKII at Thr-286 contributed to the learning and memory impairments exhibited by the aged mice in behavioral studies. As was mentioned earlier, we were unsuccessful in our attempt to quantify expression of the CaMKII inhibitor; it is unclear as to whether this result was due to human error or a faulty antibody. The results of the qPCR did not exhibit that changes in gene expression of the CaMKII isoforms occur in aged hippocampi. Similarly, the results of the relative

α-CaMKII western blot did not exhibit diminished expression of α-CaMKII in aged hippocampi. Also, the results of the CaMKIIα aggregation western blot did not exhibit that aggregation of α-CaMKII causes the decline in CaMKII activity in aged hippocampi.

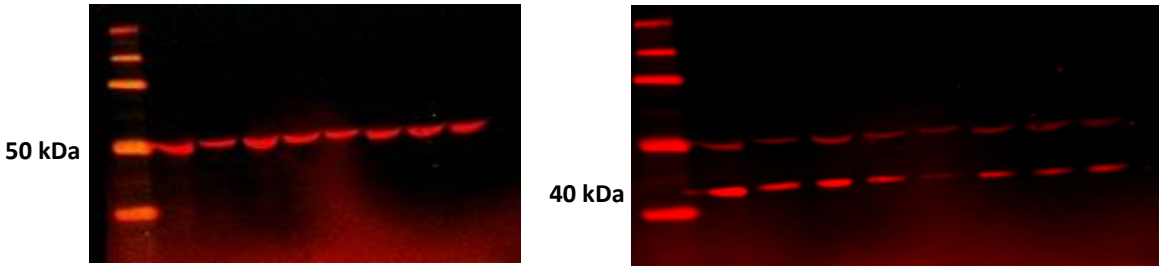
FUTURE STUDIES

The impact of our findings lies in the illumination of the mechanisms causing CaMKII activity to decline in the aged wild-type mouse hippocampus. In the future, researchers should seek to build upon the link between decreased phosphorylated CaMKII expression and decreased CaMKII activity. Likewise, researchers should formulate strategies that increase CaMKII phosphorylation in order to alleviate learning and memory problems in the aging brain. In terms of building upon the methods used in this study, it would be beneficial to increase the number of animal subjects and to widen the range of subject ages at time of euthanasia. Because of the fact that our CaMKII inhibitor western blot was unsuccessful, this experiment should be repeated. Also, the expression of the other CaMKII isoforms in advanced age should be evaluated. Lastly, the effects of aging on other kinase proteins should be evaluated.

SUPPLEMENTAL DATA

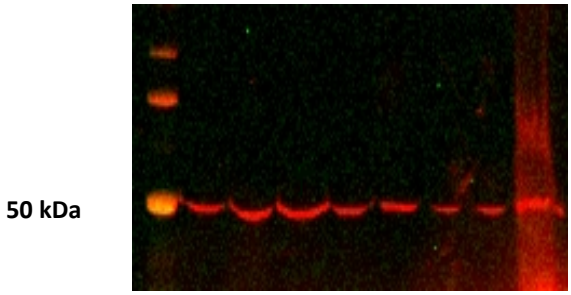
LIST OF SUPPLEMENTAL FIGURES

Supplemental Figure 1



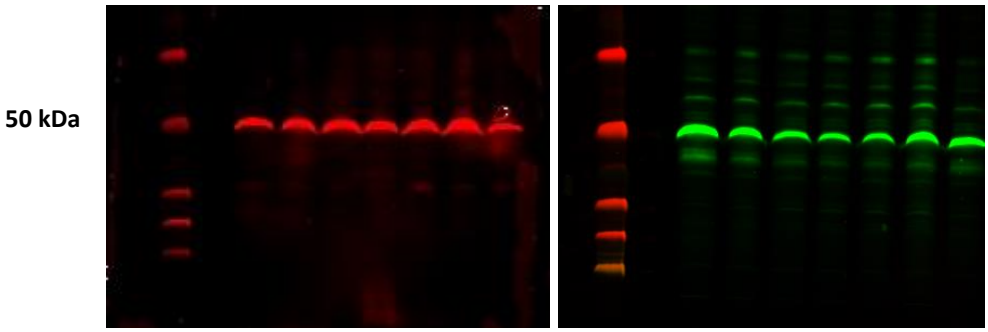
Supplemental Figure 1: complete images of a western blot used to quantify relative α-CaMKII expression in young and aged wild-type mouse supernatant hippocampal tissue samples. Expression of α-CaMKII (50 kDa) and GAPDH (40 kDa) were quantified then used to determine relative α-CaMKII expression. In the first blot image, the blot was incubated with an α-CaMKII primary antibody. In the second blot image, the blot was incubated with a GAPDH primary antibody. Levels of relative alpha CaMKII expression were normalized to a value of one. The ordering of samples of each image from left to right is young-aged-young-aged etc.

Supplemental Figure 2



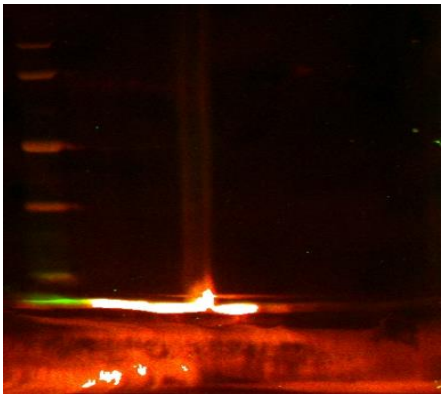
Supplemental Figure 2: A complete image of a western blot used to quantify the expression of total CaMKII (CaMKIIα) aggregation in both young and aged pellet and supernatant hippocampal tissue samples. The ordering of samples in the image from left to right is young-aged-young-aged etc.

Supplemental Figure 3



Supplemental Figure 3: complete images of western blots used to quantify the relative expression of phospho-CaMKII in both young and aged supernatant hippocampal tissue samples. The expression of p-CaMKII was normalized to α-CaMKII. In the first blot image, the blot was incubated with an α-CaMKII primary antibody. In the second blot image, the blot was incubated with a P-CaMKII primary antibody. The ordering of samples of each image from left to right is young-aged-young-aged etc.

Supplemental Figure 4



Supplemental Figure 4: A complete image of a western blot gel used to quantify the expression of CaMKII inhibitor (CaMK-Nβ) (6.5 kDa) in both young and aged supernatant hippocampal tissue samples. The ordering of samples in the image from left to right is young-aged-young-aged etc.

LIST OF REFERENCES

Ashpole, N. M., & Hudmon, A. (2011). Excitotoxic neuroprotection and vulnerability with CaMKII inhibition. *Molecular and Cellular Neuroscience, 46*(4), 720-730. 10.1016/j.mcn.2011.02.003

Ashpole, N. M., Johnson, D. E., & Hudmon, A. (2011). Molecular mechanism of alpha-CaMKII self-association. *Biophysical Journal, 100*(3), 85a-86a. 10.1016/j.bpj.2010.12.672

Ashpole, N. M., Song, W., Brustovetsky, T., Engleman, E. A., Brustovetsky, N., Cummins, T. R., & Hudmon, A. (2012). Calcium/calmodulin-dependent protein kinase II (CaMKII) inhibition induces neurotoxicity via dysregulation of glutamate/calcium signaling and hyperexcitability. Journal of Biological Chemistry, 287(11), 8495-8506. 10.1074/jbc.M111.323915

Bayer KU, Harbers K, Schulman H. alphaKAP is an anchoring protein for a novel CaM kinase II isoform in skeletal muscle. EMBO J 1998; 17: 5598–605.

Bezprozvanny I, Mattson MP. Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. Trends Neurosci. 2008;31(9):454–63.

Cammarota, M., Bevilaqua, L. R. M., Viola, H., Kerr, D. S., Reichmann, B., Teixeira, V., . . . Medina, J. H. (2002). Participation of CaMKII in neuronal plasticity and memory formation.*Cellular and Molecular Neurobiology, 22*(3), 259-267. 10.1023/A:1020763716886

Steven J Coultrap, Rebekah S VEST, Nicole M Ashpole, Andy Hudmon K Ulrich Bayef. (2011). CaMKII in cerebral ischemia. *32*(7), 861-872. 10.1038/aps.2011.68

Coultrap, S. J., & Bayer, K. U. (2012). CaMKII regulation in information processing and storage. Trends in Neurosciences, 35(10), 607-618. 10.1016/j.tins.2012.05.003

Dosemeci, A., Reese, T.S., Petersen, J., Tao-Cheng, J.H., 2000. A novel particulate form of Ca(2+)/calmodulin-dependent [correction of Ca(2+)/CaMKII-dependent] protein kinase II in neurons. J. Neurosci. 20, 3076–3084.

Elgersma, Y., Sweatt, J. D., & Giese, K. P. (2004). Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. The Journal of Neuroscience, 24(39), 8410-8415. 10.1523/JNEUROSCI.3622-04.2004

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ. 1998. Autophosphorylation at Thr286 of the α calcium-calmodulin kinase II in LTP and learning. Science 279: 870–873

Giese, K. P., & Mizuno, K. (2013). The roles of protein kinases in learning and memory. Learning & Memory, 20(10), 540-552. 10.1101/lm.028449.112

Ghosh, A., & Giese, K. P. (2015). Calcium/calmodulin-dependent kinase II and alzheimer's disease. Molecular Brain, 8(1), 78. 10.1186/s13041-015-0166-2

Glutamatergic neuron markers. (2018, March 29). Retrieved March 29, 2018, from http://www.abcam.com/neuroscience/glutamatergic-neuron-markers-and-their-functions

Hinds, H., Tonegawa, S., and Malinow, R. (1998). CA1 long-term potentiation is diminished but present in hippocampal slices from αCaMKII mutant mice. Learn. Mem. 5:344–354

Hudmon A, Aronowski J, Kolb SJ, Waxham MN. Inactivation and self-association of Ca2+/calmodulin-dependent protein kinase II during autophosphorylation. J Biol Chem 1996; 271: 8800–8.

Hudmon, A., & Schulman, H. (2002). Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. The Biochemical Journal, 364(Pt 3), 593-611. doi:10.1042/BJ20020228

Hudmon, A., & Schulman, H. (2002). Neuronal CA2+/calmodulin-dependent protein kinase II: The role of structure and autoregulation in cellular function. Annual Review of Biochemistry, 71(1), 473-510. doi:10.1146/annurev.biochem.71.110601.135410

Hudmon, A., Lebel, E., Roy, H., Sik, A., Schulman, H., Waxham, M.N., De Koninck, P., 2005. A mechanism for Ca2+/calmodulin-dependent protein kinase II clustering at synaptic and nonsynaptic sites based on self-association. J. Neurosci. 25, 6971–6983.

Institute of Medicine (US) Forum on Neuroscience and Nervous System Disorders. (1970, January 01). Overview of the Glutamatergic System. Retrieved March 29, 2018, from https://www.ncbi.nlm.nih.gov/books/NBK62187/

Irvine EE, Vernon J, Giese KP. 2005. αCaMKII autophosphorylation contributes to rapid learning but is not necessary for memory. Nat Neurosci 8: 411–412.

Irvine, E. E., von Hertzen, L. S. J., Plattner, F., & Giese, K. P. (2006). αCaMKII autophosphorylation: A fast track to memory. Trends in Neurosciences, 29(8), 459-465. 10.1016/j.tins.2006.06.009

Irvine EE, Danhiez A, Radwanska K, Nassim C, Lucchesi W, Godaux E, Ris L, Giese KP. 2011. Properties of contextual memory formed in the absence of αCaMKII autophosphorylation. Mol Brain 4: 8.

Kimura T, Yamashita S, Nakao S, Park JM, Murayama M, Mizoroki T, Yoshiike Y, Sahara N, Takashima A. 2008b. GSK-3β is required for memory reconsolidation in adult brain. PLoS One 3: e3540.

Lacor PN, Buniel MC, Chang L, Fernandez SJ, Gong Y, Viola KL, et al. Synaptic targeting by Alzheimer's-related amyloid beta oligomers. J Neurosci. 2004;24(45):10191–200. doi: 10.1523/JNEUROSCI.3432-04.2004.

LaFerla FM. Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. Nat Rev Neurosci. 2002;3(11):862–72

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, et al. Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A. 1998;95(11):6448–53. doi: 10.1073/pnas.95.11.6448.

Li S, Hong S, Shepardson NE, Walsh DM, Shankar GM, Selkoe D. Soluble oligomers of amyloid Beta protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. Neuron. 2009;62(6):788–801. doi: 10.1016/j.neuron.2009.05.012.

Lisman, J., Schulman, H., & Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. Nature Reviews Neuroscience, 3(3), 175-190. 10.1038/nrn753

Lisman, J. (2012). Memory erasure by very high concentrations of ZIP may not be due to PKM-zeta. Hippocampus, 22(3), 648-649. 10.1002/hipo.20980

Lucchesi, W., Mizuno, K., & Giese, K. P. (2010;2011;). Novel insights into CaMKII function and regulation during memory formation. Brain Research Bulletin, 85(1), 2-8. 10.1016/j.brainresbull.2010.10.009

Marcello E, Epis R, Di Luca M. Amyloid flirting with synaptic failure: towards a comprehensive view of Alzheimer's disease pathogenesis. Eur J Pharmacol. 2008;585(1):109–118. doi: 10.1016/j.ejphar.2007.11.083.

Moolman DL, Vitolo OV, Vonsattel JP, Shelanski ML. Dendrite and dendritic spine alterations in Alzheimer models. J Neurocytol. 2004;33(3):377–87.

Need AC, Giese KP. 2003. Handling and environmental enrichment do not rescue learning and memory impairments in αCamKII(T286A) mutant mice. Genes Brain Behav 2: 132–139.

Ohno, M., Sametsky, E. A., Silva, A. J., & Disterhoft, J. F. (2006). Differential effects of αCaMKII mutation on hippocampal learning and changes in intrinsic neuronal excitability. European Journal of Neuroscience, 23(8), 2235-2240. doi:10.1111/j.1460-9568.2006.04746.x

Otmakhov, N., Griffith, L., and Lisman, J. (1997). Postsynaptic inhibitors of Cap2C/calmodulin-dependent protein kinase type II block induction but not maintenance of pairing induced long-term potentiation. J. Neurosci. 17:5357–5365.

Reese LC, Laezza F, Woltjer R, Taglialatela G. Dysregulated phosphorylation of Ca(2+) /calmodulin-dependent protein kinase II-alpha in the hippocampus of subjects with mild cognitive impairment and Alzheimer's disease. J Neurochem. 2011;119(4):791–804

Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, et al. Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med. 2008;14(8):837–42. doi: 10.1038/nm1782

Silva, A., Paylor, R., Wehner, J., and Tonegawa, S. (1992). Impaired spatial learning in alpha-calciumcalmodulin kinase II mutant mice. Science 257:206–211.
Schulman H, Greengard P. Stimulation of brain membrane protein phosphorylation by calcium and an endogenous heat-stable protein. Nature 1978; 271: 478–9.

Takeuchi Y, Yamamoto H, Fukunaga K, Miyakawa T, Miyamoto E. Identification of the isoforms of Ca(2+)/Calmodulin-dependent protein kinase II in rat astrocytes and their subcellular localization. J Neurochem. 2000;74:2557–2567.

Tao-Cheng, J.H., Vinade, L., Pozzo-Miller, L.D., Reese, T.S., Dosemeci, A., 2002. Calcium/calmodulin-dependent protein kinase II clusters in adult rat hippocampal slices. Neuroscience 115, 435–440.

Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol. 1991;30(4):572–80

Tobimatsu T, Fujisawa H. Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J Biol Chem. 1989;264:17907–17912.

Townsend M, Mehta T, Selkoe DJ. Soluble Abeta inhibits specific signal transduction cascades common to the insulin receptor pathway. J Biol Chem. 2007;282(46):33305–33312.

Vest, R. S., O'Leary, H., & Bayer, K. U. (2009). Differential regulation by ATP versus ADP further links CaMKII aggregation to ischemic conditions. FEBS Letters, 583(22), 3577-3581. doi:10.1016/j.febslet.2009.10.028

Wang DM, Yang YJ, Zhang L, Zhang X, Guan FF, Zhang LF, et al. The alterations of Ca2+/calmodulin/CaMKII/CaV1.2 signaling in experimental models of Alzheimer's disease and vascular dementia. Neurosci Lett. 2013;538:60–5.

Wayman, G. A., Lee, Y., Tokumitsu, H., Silva, A., & Soderling, T. R. (2008). Calmodulin-kinases: Modulators of neuronal development and plasticity. Neuron, 59(6), 914-931. 10.1016/j.neuron.2008.08.021

Yamasaki N, Maekawa M, Kobayashi K, Kajii Y, Maeda J, Soma M, Takao K, Tanda K, Ohira K, Toyama K, et al. 2008. α-CaMKII deficiency causes immature dentate gyrus, a novel candidate endophenotype of psychiatric disorders. Mol Brain 1: 6.