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CHARACHTERIZATION OF CALCIUM/CALMODULIN DEPENDANT PROTEIN KINASE II AND ITS NECESSITY IN SYNAPTIC PLASTICITY

By

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ABSTRACT

Characterization of Calcium/Calmodulin Dependent Protein Kinase II and its Necessity in Synaptic Plasticity

By Dwight Higgin

Calcium-Calmodulin dependent protein kinase II (CaMKII) is an enzyme that is functionally involved in critical stages of synaptic plasticity and memory formation. CaMKII is involved in both the presynaptic and the postsynaptic strengthening and weakening of synapses. In the presence of CaMKII inhibitors, synaptic enhancements are impaired and cellular ionic balance is destabilized because of the reduction of sodium, calcium, and potassium ions due to the lack of CaMKII activity upon membrane receptors thus reducing ion influx leading to a reduction in synaptic plasticity.

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Chapter 1 - Introduction

Background

The brain is the most complex organ of the body. It is the maestro of the orchestra that controls an organism in every way. Our passions, feelings, desires, emotions, and fears are wrapped up into what amounts to an 8 pound mass of interconnecting cells. The central nervous system, CNS, is made up of the brain and the spinal cord. The CNS communicates with the peripheral nervous system, PNS, which sends signals to effector organs like the muscles. While we have a good idea of the structure of the nervous system, the intricate details of how it works is still somewhat a mystery. How we learn, and how we store memories are just a couple of the many questions that have plagued researchers for hundreds of years. An even more fundamental question is, who am I? It is quite interesting to note that for ages science has been leading us to answer these questions from a cellular standpoint and relate that to behavior. Research strategies have suggested the use of simpler organisms provides insights into the functions of more complex organisms. These kinds of studies have proved very beneficial for obtaining a better understanding of the brain, yet one still must look at the cell as an individual entity that globally governs higher function. For understanding behavior, the particular cells of interest are the neurons. Neurons are the fundamental signaling unit of the central nervous system. There are billions of neurons in the brain; each making connections to thousands of other neurons in the brain. The dendrites of a neuron receive information from neighboring

neurons and transmit the information down its own axon to continue the signal. The fast transmission characteristic of vertebrate neurons is made possible because of the protection by the myelin sheath on the axon. The myelin sheath insulates the axon and allows action potentials to propagate quickly. A synapse is a connection between two neurons. As a result of these connections, complex neural networks are formed. The neuron can undergo electrical, structural, and biochemical changes in response to changes in the environment. All of these changes can affect synaptic strength and can be accomplished, in some aspect, because of the role and the function of calcium Calmodulin dependent protein kinase II, CaMKII. The genetic composition and the structure are unique features that control the activation and function of this important enzyme. CaMKII is a necessary molecule in synaptic plasticity and plays a major role in many cellular processes. However, the protein does not act alone; rather it relies upon elevated calcium concentration activating Calmodulin. In addition, CaMKII belongs to a family of calcium Calmodulin protein kinases, all of which are involved in key cellular function involving phosphorylation. This paper will explore the function of CaMKII as the critical molecule in synaptic plasticity induced through three pathways that increase internal calcium in neurons; opening of voltage-gated calcium channels, activation of inositol 1,4,5-triphosphate receptors and release of calcium from internal stores, and opening of N-methyl-D-aspartate (NMDA) receptor channels.

Phosphorylation and dephosphorylation are the two most widely known and understood methods of covalent modification of proteins.

Interestingly, the pattern of protein phosphorylation leading to activity and dephosphorylation leading to inactivity is not standard. In some cases, as in the activation of hormone sensitive lipase, dephosphorylation activates the enzyme and the enzyme can liberate fatty acids from their triacylglyceride form and provide fatty acids to supply the body with needed energy. In other cases enzymes are activated by phosphorylation such as Signal Transducer and Activator of Transcription, STAT proteins. These proteins are responsible for regulating activation of transcription, as its name implies, and require phosphorylation to become activated.

The enzymes that phosphorylate proteins are kinases. The enzymes that dephosphorylate proteins are called phosphatases. A protein kinase is important in many cellular functions since it physically adds a phosphate to itself or other proteins and causes a change in function of the target protein. There are nearly 500 protein kinases in the human genome (Manning, 2002). Many of them are involved in signal transduction in which stimuli arrive in the periphery and are converted from mechanical, chemical, or photo energy into electrical energy. This conversion leads to gene regulation, receptor activation/deactivation, chemical/hormone release, and many other cellular activities. Kinases can communicate to a single protein or can generate a cascade of proteins involved in signal transmission. Therefore, kinases are very important, and can activate proteins in cascades to alter necessary cellular functions.

Synaptic plasticity

Before embarking on the functional necessity of CaMKII in synaptic plasticity, it is necessary to give a brief description of what synaptic plasticity is and provide background information on the topic of plasticity.

Synaptic plasticity refers to enhancement or depression of activity between two neurons. The changes can last from days to weeks and provide a basis for memory (Zucker, 1998). Presynaptic stimulation leads to plasticity. Two stimuli delivered within a short interval can lead to synaptic enhancement and two stimuli delivered after a long interval can lead to depression of a synapse (Zucker, 1998). This is referred to as paired pulse stimulation and has been a consistent research area across many different research backgrounds. One of the most recognized forms of synaptic plasticity is long term potentiation (LTP). LTP is marked by protein activations and phosphorylation. This phenomenon has been extensively studied in the Cornu Ammonis (CA) regions of the hippocampus. LTP is thought to lead to learning and memory because of synaptic strengthening of the CA regions of the hippocampus (Zola-Morgan, 1993). The opposing plasticity to LTP is long term depression (LTD). LTD is marked by protein dephosphorylation and deactivations and also more recently understood to be activated in the opposite manner of LTP and are primarily acting on the Cerebellum Purkinje cells leading to motor learning (Ito, 2001). In both forms of plasticity, there is a change in protein expression and signal transduction. The

induction of synaptic LTP requires activation of the NMDA subtype of glutamate receptors. Normally, glutamate released from the presynaptic neuron into the synapse, attaches to NMDA receptors, yet the receptor is not activated. A key feature of the NMDA receptor is the magnesium which blocks the pore. Only under circumstances of high glutamate release and depolarization of the post-synaptic cell is the magnesium block released and calcium and sodium are allowed to freely pass through the pore. The influx of Na and Ca cause a number of downstream cascades, one of which includes the up regulation of more AMPA receptors to the synapse (Hayashi, 2000). Over a prolonged period, this feature of NMDA activation can be sustained and alterations to the membrane can be long lasting. Activation of NMDA receptors in a spine result in growth of spines and filopodia which are precursors of synaptic spines (Engert, 1999). This process can also be reversed if the activating ions are removed or the receptor is inhibited (Engert, 1999).

Most studies of synaptic plasticity have implicated CaMKII as a modulator, and to a greater extent, LTD also involves CaMKII function. CaMKII is a necessary protein in synaptic plasticity. Synaptic enhancement and depression is discussed and is possible only by the function of changes in calcium concentration that underlie the activation of CaMKII.

Activators of CaMKII

Calmodulin

Calmodulin, abbreviated CAM, stands for calcium modulated protein; Calmodulin is a ubiquitous protein of approximately 16kD and is necessary for activation certain protein kinases. Calmodulin acts as a calcium sensor and a signal transducer to proteins that cannot themselves bind calcium (Hanson 1994). It mediates inflammation, metabolism, muscle contraction, short-term and long-term memory, and nerve growth. It is the predominant calcium binding protein in the cell nucleus (Bachs 1992). As noted before, calcium enters the cell or is released from internal stores of the endoplasmic reticulum. This calcium elevation results in the activation of effectors which use the calcium to produce changes within the cell. The action of Calmodulin becomes apparent when the calcium concentration is above 10^{-6} M. It can bind up to four calcium ions and can undergo post-translational modifications which include phosphorylation, acetylation, methylation, and proteolytic cleavage (Deisseroth 1998). Calmodulin levels can also be modulated. Stimulation of adrenal cortical cells with ACTH increased the CAM protein (Harper 1980). Also, treatment of cells with various calcium channel agonists also increased Calmodulin levels (Vendrell 1992). Figure 1 illustrates the factors required to activate CaMKII.

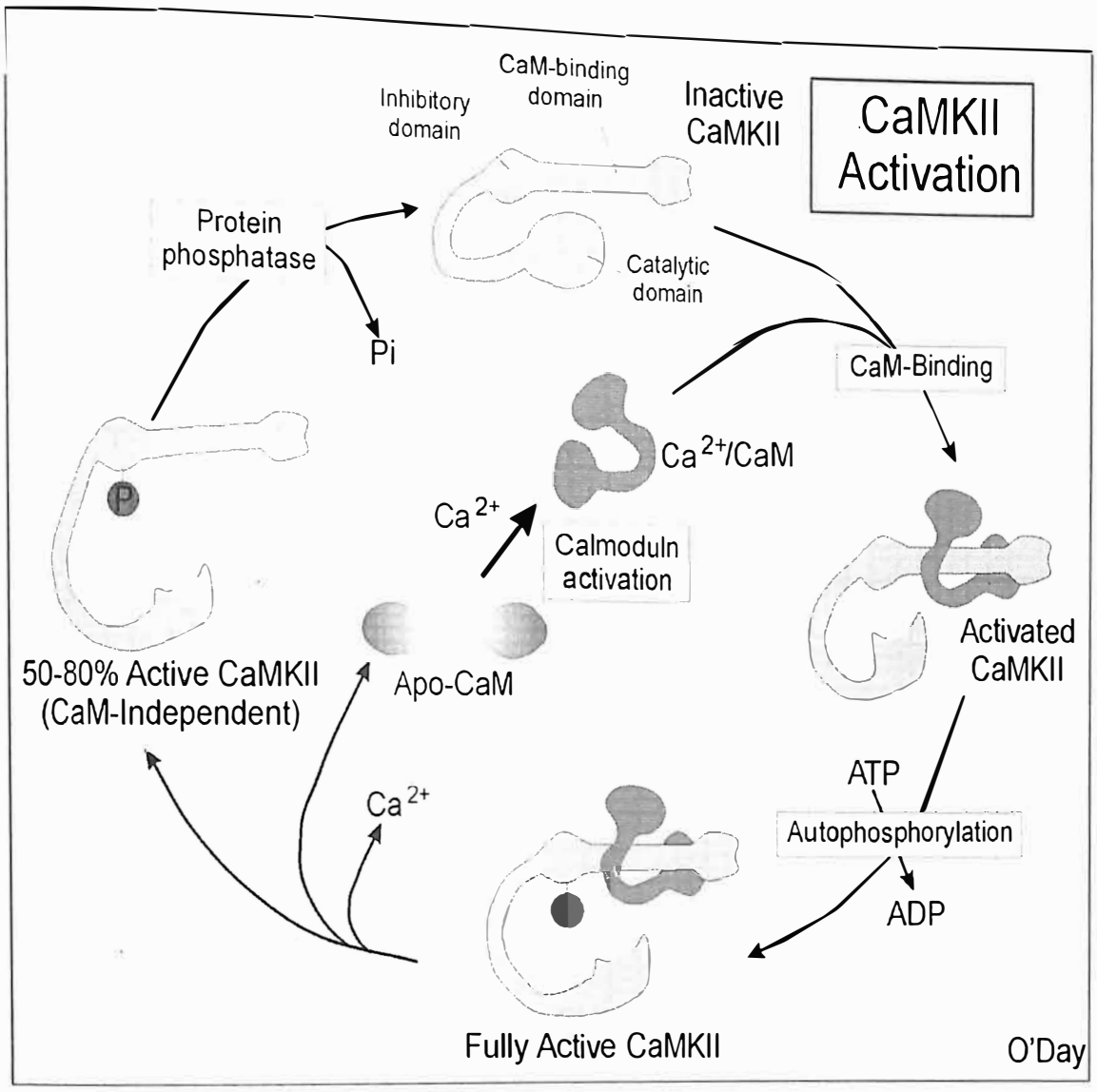

Figure 1 - Activation of CaMKII. University of Toronto, Dr. Danton O'Day

Another important feature of Calmodulin is its ability to translocate. In the presence of elevated calcium in smooth muscles, Calmodulin of the cytoplasm translocates to the nucleus (Luby-Phelps 1995). Stimulation of neurons and subsequent elevation of calcium is linked to the nuclear accumulation of Calmodulin (Deisseroth 1998). Bursts of Calmodulin migrating into the nucleus have been shown to be linked to the phosphorylation of cyclic AMP response element binding protein (CREB) and other proteins inside the nucleus giving

further evidence that the calcium bound Calmodulin is a means of communication to nuclear proteins and is the source of their activation. In particular, two ion channels are associated with this translocation, the L-type calcium channels and the NMDA receptors (Deisseroth 1998). The N and P/Q type calcium channels are not associated with NMDA translocation (K. Deisseroth 1998). The features of translocation involving these channels support the importance of CaMKII in synaptic plasticity. The activation of the Ca channels presynaptically results in the release of neurotransmitter at the synapse, thereby activating postsynaptic neurons. The stimulation state of the cell can be linked to the translocation and the concentration of Calmodulin within the nucleus and its downstream effector proteins.

Lineage of CaMKII

In 1977, a protein in the rat brain was reported to be activated by the depolarization of a neuron and the activation was mediated by calcium ion influx (Krueger 1977). Early researchers had no idea that the protein they had stumbled upon would be so valuable in understanding synaptic transmission and learning and memory. Notable researchers such as De Camilli, Navone, and Greengard began to expand upon the discovery of this protein and its involvement in neuronal maintenance. While CAMKII was known as synapsin kinase originally, it was later understood that CAMKII actually had more function than that of phosphorylating synapsin alone. It had many other substrates which included synapsin as well as upstream modulators of its activity (Menegon 2006).

CaMKII belongs to the multifunctional calcium/Calmodulin dependent protein kinase family (CAM kinase) and consists of three particular protein kinases; CAMKI, CAMKII, and CAMKIV. These proteins are activated as a result of the interaction with calcium bound Calmodulin and activated calcium Calmodulin kinase-kinase. Each type of CAMK is unique in the sense that their tissue distribution and substrate differs. They individually vary across eukaryotic systems and yet are recognizable across species of vertebrate and invertebrates. The major difference between the CAMK and other dedicated protein kinases is that the substrate pool they phosphorylate is wider. Dedicated kinases include phosphorylase kinase, myosin light chain kinase, and the eukaryotic elongation factor 2 kinase (EEF2). The phosphorylase kinase was the first protein kinase to be identified and is known to activate glycogen phosphorylase. The site of

importance for this kinase is in the muscle where glycogen breakdown is necessary to provide glucose as energy to produce muscle contractions. The dedicated kinase, myosin light chain kinase (MLCK), a serine/threonine kinase, whose function is to phosphorylate myosin II phosphorylates the regulatory light chain of myosin. The actions of MLCK are limited to particular tissues and a particular substrate; in contrast the CAMK family kinases are wide spread throughout the body and have many substrates (Gallagher 1997).

For example, EEF2 kinase (also known as CAM kinase III) is a very important kinase that phosphorylates EEF2. EEF2 is a GTPase and is responsible for the elongation step in protein translation (Taha, 2013). CAMKI is activated by calcium/Calmodulin kinase–kinase, CAMKK, after calcium bound Calmodulin is attached. This activity is regulated by cAMP dependent protein kinase A (PKA). PKA activates CAMKK, which in turn activates CAMKI (Matsushita 1999). CAMKK controls the activation of CAMKI and CAMKIV, exists in one of two isoforms and is enriched in the cytoplasm and the nucleus. CAMKIV is another member of the CAMK family that also requires phosphorylation for activation. This kinase is found in the brain and the thymus (Kitani 1994). CAMKI and CAMKIV are monomeric enzymes and share the same activation, and initiate cascades of phosphorylation for regulatory proteins (Soderling 1999). Both CAMK I and IV activate transcription factors and DNA binding proteins. CAMKIV is also referred to as CMKGr indicating its granular cell enriched feature. It phosphorylates synapsin I and was indicated to have association with chromatin as well (Jensen 1991). In addition, CAMGr also activates CREB and

associated CREB proteins. CREB is a transcription factor that has been shown to bind to DNA upon activation at certain sites called cAMP response elements and regulates transcription of genes (Purves, 2008). This indicates a role for CAMGr in calcium regulated gene transcription. CAMK must have access to the nucleus in order to have an effect on its substrate within. A nuclear localization signal is the means by which CAMK I and IV localize to the nucleus. Alternative splicing of the CAM kinase gene produces the signal and targets the kinase to the nucleus (Srinivasan 1994).

Composition of the CaMKII molecule

Genetics

Overall, CAMKII is encoded by four genes; alpha, beta, gamma, and delta. Each one of the genes can undergo alternative splicing within the association domain and gives rise to two or more isoforms of the protein. The alpha and the beta isoforms are found predominately in neuronal tissue, while the gamma and the delta forms are both have neuronal and non-neuronal tissue distribution. All isoforms of the protein contain four key domains. The domains are critical in the activation and the inactivation of the protein along with the overall function of the protein upon its substrate (for review see Cline et al 2002). The structure consists of an N-terminal catalytic domain, a regulatory domain that consists of a Calmodulin binding site, a regulatory autophosphorylation site, and a C- terminal association domain that initiates the formation of the entire holoenzyme (Stevens 2001). The C terminal domain forms the central core of each ring and the N terminal domain projects outward. The amino terminal domain is the site of ATP binding and the Calmodulin site is more towards the middle of the protein (Bennett and Kennedy 1987). In this structure, 80-90% of known CaMKII species have these domains in common (Meyer 1992).

The brain isoform of CAMKII is between 300 and 700 kD in size and consists of 6-12 subunits individually assembled and stacked as a hexameric ring (Braun and Schulman 1995). The protein exists as a holoenzyme which consists of two subunit types; alpha and beta. These two isoforms are restricted to the

neuron. The alpha subunit was found to be approximately 50 kD and the beta unit was found to be between 55 and 60 kD (Kennedy 1983). The doublet of alpha and beta subunits forming the brain isoform of CAMKII was originally stumbled upon by SDS-PAGE analysis of the protein and was thought to represent the degradation of the protein purification process (M. Bennett 1983).

Through biochemical and immunochemical analysis of purified CAMKII from various regions of the brain, it was shown that the composition of subunits varies among the brain regions (Miller and Kennedy 1985). Areas of interest include the forebrain, cerebellum, and the hippocampus. The forebrain CAMKII consists of 9 alpha and 3 beta subunits per holoenzyme. Cerebellar CAMKII is composed of 2 alpha and 8 beta subunits. In the forebrain, the composition of the alpha subunit is high in the post synaptic densities (Miller and Kennedy 1985). The alpha and beta isoforms make up nearly 2% of total protein in the hippocampus (Erondu and Kennedy 1985). Beta and alpha isoforms are of great importance, and have different roles in the neuron. Beta isoforms differ in the variable region for F-actin binding (Fink, 2002). Both isoforms have different affinities for Calmodulin. The half maximal level of Calmodulin binding for the alpha isoform is 130nM and is 15nM for the beta isoform (Fink, 2002). Phosphorylation of the different isoforms also results in varied translocation targets. Beta subunits translocate to the postsynaptic density when phosphorylated primarily due to the F-actin target function of beta isoforms (Abria, 2010). The alpha isoform translocates to the post synaptic density, but in response only to the NMDA receptor activation (Fink, 2002).

Anatomical distribution indicates function

The function of CAMKII is closely related to the brain area in which it is expressed and its subcellular location. The activity of the protein is not the same throughout the brain. The highest levels of activity are found in the hippocampus. The cerebral cortex also has a high level of activity in the hippocampus (Erondu and Kennedy 1985). CAMKII accounts for 2% of the total protein in the hippocampus (Dosemeci, 2000) and it stands to reason that the function in the hippocampus relates to learning and memory as we will see. Other brain regions that show substantial, but not high, levels of kinase activity include the cerebellum and the brain stem structures. The amygdala and the striatum also exhibit an intermediate level of CAMKII activity (Erondu and Kennedy 1985). The ratio of alpha to beta subunits also corresponds to the areas of high activity in the brain such as the hippocampus. As the alpha subunit ratio increases, the activity of CAMKII increases. The hippocampus not only exhibits the highest level of activity, but also the highest ratio of alpha subunit. In contrast, the cerebellum with low activity of CAMKII also had the lowest alpha to beta subunit ratio (Erondu and Kennedy 1985).

The subcellular location of the CAMKII also indicates a function specific localization of the protein. Originally, CAMKII was known as synapsin I kinase, which gave an indication for the presynaptic localization as well as involvement in the release of neurotransmitter. Further studies showed that was a limited description for the protein since it was also found at high levels in the post synaptic densities, PSD (Yoshimura 2002). CAMKII is found in 2% of the PSD

of the cerebellum and 40% in the forebrain, again indicates a site-specific localization for function in these different regions (Yoshimura 2002). The alpha subunit within the cerebellum acts as an anchor for the CAMKII in the PSD of the cerebellum.

Functions of activated CaMKII

The specific role of CAMKII in the vertebrate system differs from the other isoforms of the multifunctional CAMK family of proteins. CAMKII exists in neuronal and non-neuronal tissue and is dependent on gene splicing for its structurally distinct isozymes that lead to functional diversity and differential anatomical distribution (Griffith 2003). The ratio of the subunits of the neuronal CAMKII type alpha and beta is important because it determines the localization and function of the activated protein. Upon activation, CAMKII activates cell regulating proteins such as the Extracellular signal regulated kinase (ERK) / mitogen activated kinase (MAPK) (Lu 2005), gene transcription proteins such as CREB (cAMP response element binding protein) (Sun 1994), as well as ion channels for calcium (Zuhike 1999; Erikson 2001). These protein interactions modulate cell function and are crucial for the pathway for initiation of LTP leading to long term memory (Lledo 1995).

When not interacting with calcium bound Calmodulin, the CAMKII is held in an inactive state. The protein has an autoinhibitory domain (AID) in each subunit that prevents the enzyme from activation by blocking the binding of substrate and ATP. CAMKII differs from the other CAM kinases, CAMKI and CAMKIV because these require only phosphorylation at the activation loop, so their activation is one step (Ishida 2005). The activation of CAMKII is dependent on binding of calcium rich Calmodulin to a site overlapping the core of the AID which activates the protein by disrupting the interaction of the inhibition site and the active site (Rosenberg 2005).

The sensitivity of the activation to Calmodulin depends on the subunit composition of the enzyme. The alpha and the beta subunits bind Calmodulin in a calcium dependent manner, they bind ATP, and can also undergo auto-phosphorylation. Autophosphorylation is an important function of CaMKII; however it is must be regulated to prevent overactivity and cellular issues of overactivity (Umemura 2005). A pseudo substrate domain is located on the amino side of the Calmodulin binding site (Kwaitkowski 1987). The function of the pseudo substrate is to prevent autophosphorylation as Calmodulin binds and cause a conformational change which "pulls" the site away from the catalytic site (Hudmon 2002). The concept of the pseudo substrate has also been shown in myosin light chain kinase and protein kinase C (Kemp 1987). The activation of the CAMKII holoenzyme involves phosphorylation of specific resides on the protein. The kinase autophosphorylates at the threonine 286 residue that lies within the autoinhibitory domain after Calmodulin binds to CaMKII and changes its structural configuration.

The active kinase consists of at least two subunits and requires phosphorylation initiated by ATP. The distinct subunits differ in their phosphorylation sites. The subunit delta is phosphorylated at threonine 286, and gamma and beta are phosphorylated at threonine 287. Autophosphorylation of the threonine residue of each subunit is made possible when Calmodulin is bound to both subunits involved. There are six phosphorylation sites on the alpha subunit and eight sites on the beta subunit. The active protein phosphorylation sites are not all occupied in the active protein (Giese 1998). The active CaMKII protein

only requires three phosphates for function. Only after autophosphorylation does the protein lock into the active configuration. Calmodulin has two roles in activation: To first activate the subunit and then to present the autoinhibitory domain of a neighboring subunit for phosphorylation. First, upon activation of the subunit, the CAMKII is converted from a protein of low Calmodulin affinity to one with a high Calmodulin affinity. This conversion leads to prolonged binding of Calmodulin by CAMKII to nearly hundreds of seconds (Meyers 1992). Without the calcium enriched Calmodulin, CAMKII is inactive. After autophosphorylation, CAMKII has no "need" for the Calmodulin and can become self-functioning as a kinase (Miller and Kennedy 1986). With 12 subunits in the protein, activation is possible with 1/4 of the sites phosphorylated. The phosphorylation of the threonine residue 286 is the necessary step in the further activation of the non-Calmodulin bound protein function. Since CaMKII is a kinase, its inactivation is also an important feature to discuss. A protein phosphatase is an enzyme that removes a phosphate from a target protein. Its function is opposite to a protein kinase. A phosphatase removes a PO_4 that leads to target protein activation or deactivation, and can regulate signaling pathways of the cell. Phosphatase Types 1 and 2, found within the lipid bilayer of synaptic terminals are responsible for dephosphorylating CAMKII and returning it to a Calmodulin dependent state (Schworer 1986).

CAMK modulates the functionality of many proteins via binding at serine/threonine sites which exposes sites of phosphate binding. In general, kinases can both activate or inactivate a protein and this interaction can be

positive or negative. Modulators may bind to a particular substrate allosterically and modify the activity of the kinase and phosphatase. Allosteric activation is when one ligand binds to a protein and enhances the attraction of binding another protein or effector. Allosteric inhibition occurs when binding of a ligand decreases the attraction with the substrate active site. Allosteric binding must be reversible, non-covalent, and must be different from the catalytic site (Alberts 2002). The specificity of the substrate to the effector molecule is very important. The affinity of the regulatory enzyme to its substrate can result in allosteric activation or inhibition. Some notable modulators of CaMKII are KN-93, KN-62, and autocamtide-2-related inhibitory peptide (AIP) (Blatter 2005). KN93, N-[2-[[[3-(4-chlorophenyl)-2-propen-1-yl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxy- benzenesulfonamide, inhibits CaMKII and is associated with reduction of calcium currents in CaV1 and CaV2 (Anderson 1998). KN62 inhibits the phosphorylation of CaMKII and also changes the intracellular calcium concentration (Okazaki 1994). AIP completely inhibits CaMKII by non-competitively inhibiting the phosphorylation (Ishida, 1995). The three inhibitors act on CaMKII and modulate calcium concentration and result in inhibited calcium induced cellular function (Ademuyiwa 2005).

CAMKII is known to modify many ion channels and proteins leading to gene regulation and critical cellular function. Among the notable substrates are AMPA receptors (Tan 1994), CREB (Wu 2001), the NMDA receptor (Omkumar 1996), and tyrosine hydroxylase (Itagaki 1999). There are more than 60 substrate targets of CAMKII as shown in table 1. CAMKII has been shown to be abundant

in the synaptosome (Soderling et al, 2000). These areas are isolated terminals of a neuron that are formed from the phospholipid layer of a cell membrane and synaptic proteins. Synaptosomes contain the important components for the uptake, storage, and release of neurotransmitters. Also associated with synaptosomes are tyrosine and tryptophan hydroxylase, enzymes that are the rate limiting enzymes in the synthesis of monoamine neurotransmitters. Tyrosine hydroxylase catalyzes the hydroxylation of tyrosine to form DOPA which is then converted dopamine. Production of norepinephrine and epinephrine (Griffith 1988) relies upon CAMKII function to activate necessary hydroxylase for their formation. Therefore, CaMKII is known to affect the rate of catecholamine synthesis and the presynaptic neurotransmitter release. Synapsin (synapsin I), a vesicular membrane protein, regulates the release of transmitter vesicles following activation and is phosphorylated by CAMKII. CaMKII also phosphorylates synapsin along with other key regulatory proteins. The C-terminus auto regulatory region of CaMKII attaches to synapsin (Valtora 1992). This binding reduces the interaction of synapsin to the vesicle, thereby allowing docking to occur. The fact that CAMKII activates proteins associated with the synthesis of neurotransmitters and regulation of cellular vesicle docking is evidence that CAMKII is involved in synaptic transmission and cellular communication.

The cellular basis for learning focuses on the presynaptic and post synaptic modulation of chemical synaptic transmission (Malenka 1999). Nobel Prize winner Eric Kandel showed that the presynaptic facilitation of synaptic transmission that causes the learning phenomenon of sensitization is due to an

enhancement of neurotransmitter release from sensory neurons (Kandel 1976) and provided a link between CaMKII and synaptic plasticity. CAMKII increases pattern activity and synaptic strength and contributes to learning (Micheau 1999). CAMKII regulates firing patterns of neurons and as such, is a very important functioning protein involved in synaptic function (Peretz 1998).

Chapter 2 - CaMKII is necessary for synaptic plasticity

Calcium – a crucial cell signaling molecule

In order to understand the role that activation of CAMKII plays in cell signaling, it is necessary to understand the role that changes in calcium concentration and phosphorylation play in the modulation of cell activity. Calcium is a cell signaling molecule that plays a functional role in cellular motility, enzyme activity, and cytoskeleton maintenance. Calcium can be found both intra- and extra-cellularly. Because of its tendency to react with physiological concentrations of phosphate to form insoluble calcium phosphate intracellularly, its concentration in the cell must be tightly regulated. Calcium concentration within the cell is between 10 and 100 nM. Excess calcium is pumped into the endoplasmic reticulum. The intracellular stores of calcium are stored within the endoplasmic reticulum and when necessary, provide calcium to the cell. Calcium within the nucleus ranges from approximately 100-300 nM in the basal state. Following stimulation, the calcium concentration ranges from 350 to 1200 nM in the cell (Badminton 1996). Levels and timing of intracellular calcium increase determines synaptic plasticity as shown in figure 2.

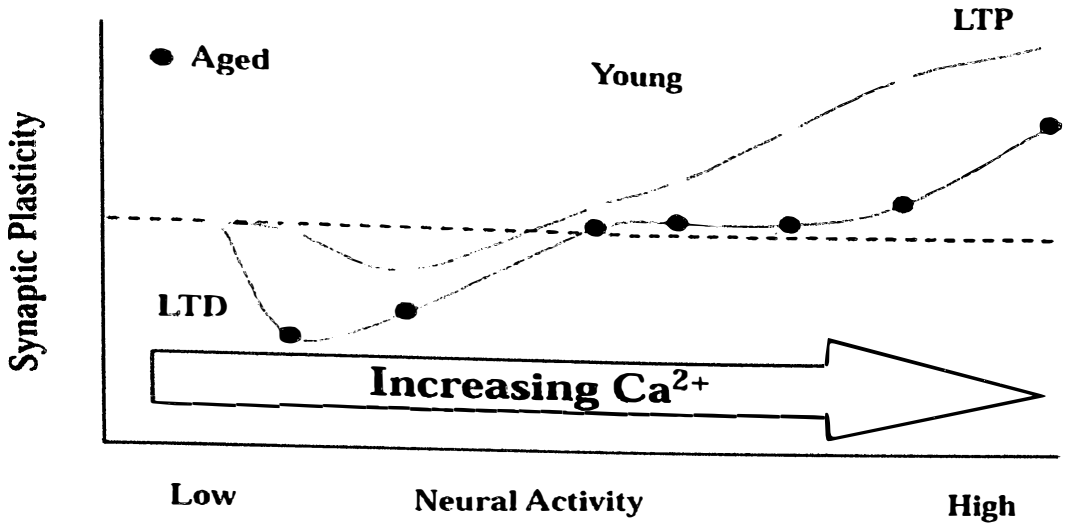

Figure 2- Calcium concentration determines synaptic plasticity enhancement or depression (Kumar & Foster, 2007)

The calcium concentration is directly correlated to increased receptor density in the postsynaptic neuron. In addition, calcium mediated synaptic plasticity can be initiated by stimulation. Low frequency synaptic stimulation of approximately 5 Hz produces LTD and high frequency synaptic stimulation of between 50 and 100 Hz produces LTP (Bi, 1998). Together, these findings indicate a prominent role of calcium in synaptic remodeling.

The importance of calcium has been established in many cell types. Of particular interest is the importance of calcium in neurons, and to an even greater extent, calcium within the nucleus. Evidence has shown that increases in cytosolic calcium levels are accompanied by elevation of calcium within the nucleus. In 1985, Roger Tsien developed a dye molecule that binds molecules of calcium and provided a basis for understanding the role of calcium in cellular

processes. Detection of intracellular calcium transients is accomplished with the fluorescent dye FURA-2 and shows better performance than previous methods. An excitation spectra measures the calcium concentration with FURA-2 dye in a wavelength specific range and focuses on calcium binding, affinity, and selectivity and sheds light on particulaly important plasticity processes (Grynkiewicz, 1985). Activation of the IP_3 ligand gated calcium channel, NMDA receptor, and voltage gated calcium channels in the membrane generate precise calcium signals within the cytosol. This provides a basis for activation of calcium dependent proteins within the cytosol and nucleus, namely Calmodulin, and the necessary plasticity induction protein CaMKII.

Learning and Memory requires CaMKII

Aristotle said in his text Metaphysics, *"By nature animals are born with the faculty of sensation and from sensation, memory is produced in some of them, though not in others. And therefore, the former are more intelligent and apt at learning than those which cannot remember: those which are incapable of hearing sounds are intelligent, though they cannot be taught, e.g. the bee, and other races of animals that may be like it: and those which besides memory have this sense of hearing can be taught."* (Aristotal 350 B.C.). As far back as Aristotle, we as humans have been intrigued with memory. This phenomenon is unrivaled in its complexity and aspects of brain integration. As humans, we rely upon it each moment, and daily we attempt to strengthen and add to it, and some of the time, we fail. Memory involves two components; spatial and temporal. Spatial components of memory refer to the brain structure processing of the information into a retention form and its location. Temporal aspects of learning involve stages of learning which are acquisition and integration of perceived information, storage and retention of information, and retrieval and recollection of stored information (Squire 1987; Eichenbaum 2001). In many types of learning, the crucial area of brain involvement is the hippocampus (Abel 2001). Learning is not an exact process, but is affected by the situation of the presentation and the timing of the cue. Studies have shown that if aversive stimuli are administered during crucial stages of the learning, the process will be disrupted (Bohbot 2001). Memories are created through both long term and short term cellular changes. Long term memory involves long term changes to cellular structure that exists

even after months and years. Prior to this stage of memory, the initial learning, undergoes consolidation where the short term cellular changes are converted into a more stable form of cellular remodeling that leads to long term memory. The study of an amnesiac patient named Henry Gustav Molaison, HM, opened the door for understanding the nature of memory consolidation (Scoville 1957). HM was unable to convert short term memory into long term memory as a result of his doctor's treatment to relieve his epilepsy by removing part of his temporal lobe on both sides which resulted in bilateral lesions in the medial temporal lobe and most of his hippocampus. Other patients that had similar more specific lesions to the hippocampus also experienced anterograde amnesia, or deficiencies in the conversion of short to long term memory as did HM. Since then, studies of lesions to the hippocampus in rodents showed distinct similarities to human characteristics of hippocampal damage (Broadbent 2005).

Subcellular areas of the hippocampus are responsible for spatial aspects of memory as well as processing and retention of information (temporal). The entorhinal cortex is a major site of input and output for the hippocampus. There are three excitatory pathways of the hippocampus: the entorhinal cortex to the perforant path, the dentate gyrus to mossy fiber path, and the CA3 pyramidal cell layer to the Schaffer collateral to the CA1 pyramidal cell layer path (Buzsaki 1990). The level of the excitatory synapses within the hippocampus results in the formation and consolidation of memory. Therefore, the synthesis of proteins and the strengthening of the postsynaptic neuron within the hippocampus are critical

for consolidation leading to long-term memory and are accomplished by the functional role of CAMKII within the neuronal PSD (Buzsaki, 1990).

CAMKII is one of most characterized proteins involved in learning and memory and is vital for synaptic plasticity (Soderling 2000). Synaptic plasticity refers to the process of modulating the strength of synaptic connections between neurons and changes the amount of neurotransmitter released from the presynaptic cell and the responsiveness of the post-synaptic cell (Gaiarsa 2002). According to the Hebbian theory, an increase in synaptic efficacy is a result of repeated and persistent stimulation of the post-synaptic cell (Hebb 1949). Hebb states, *When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.* Changes in synaptic strength are very important in the development of the nervous system. Ramon y Cajal gave neuroscience the first look into the cellular exploration of memory storage in the brain. He hypothesized that the cellular process that underlies a memory may be due to the structural modifications made on the individual signaling unit, which is the neuron (Jones 1994). This study laid the foundation for synaptic plasticity.

CAMKII has been shown to play an essential role in long term potentiation (LTP), a synaptic model of memory. The LTP phenomenon was coined during animal research studies of learning and memory in the early 70's (Bliss and Lomo 1973). LTP is initiated by activation of the NMDA receptors on postsynaptic neurons and it is very important that calcium concentrations rise due

to calcium entering the neuron from the extracellular or from internal stores (Cooke 2006). The calcium concentration increases within the post-synaptic density, and activates Calmodulin, which then activates CAMKII. CaMKII is known to play a role in induction of LTP and more recently, maintenance of LTP (Aslam 2009). The role of CAMKII in LTP has been studied extensively in the hippocampus which is known to be the foundation for memory and has been studied experimentally in rat and mouse brain for synaptic changes in CA1 and CA3 neurons through the Schaffer collateral pathway (Schuman 1994). The long term changes in the CA1 area occurs over hours and weeks, and increasingly strengthens the synapse. CaMKII plays a role in both presynaptic and postsynaptic induction of LTP (Bliss 1993). Studies of the effects of CaMKII in both the presynaptic and the post-synaptic neuron have been a major component of understanding its necessity. In the presynaptic axon, CaMKII acts to increase neurotransmitter release, and also to activate other kinases. Postsynaptically, CaMKII phosphorylates the AMPA receptor, glutamate receptor GluR1 and in so doing, creates an increase of glutamate receptors in the postsynaptic density as well as an increase calcium influx through up regulation of NMDA receptors. The function of CaMKII in these examples will be discussed below and provide the basis for understanding why CaMKII is the necessary molecule for synaptic plasticity. CAMKII is one of the most important proteins that act as a molecular switch to maintain cellular changes (Miller 2005). CaMKII is considered to be a molecular switch because the interaction of the autophosphorylation site acts as a switch in turning the enzyme on and as the calcium concentration dissipates; the

enzyme turns off (Miller 1986). An active and stable conformation that controls its synapse strengthening properties is the basis for the molecular switch classification. The state of the switch is based on the autophosphorylation. An up-state is autophosphorylated and highly phosphorylated with a high concentration of calcium. This is the ideal scenario for LTP induction. When the LTP is induced, CAMKII is converted from a down-state to a persistent up state (Miller 2005)

Mechanisms of calcium concentration increase leading to CaMKII dependent synaptic plasticity

Calcium, as discussed earlier, is an activator of CaMKII dependent Calmodulin and the underlying effects of CaMKII dependent plasticity, involves the increase of calcium via one of three key mechanisms; through NMDA receptors, Voltage gated calcium channels, or activation of the IP_3 receptor. Calcium can also enter via the calcium permeable AMPA receptors or the kainite receptors, yet these don't account for the bulk increase of calcium associated with synaptic plasticity.

N-methyl D-aspartate receptor and LTP

One of the most important aspects of synaptic plasticity involves the activation of the NMDA receptor. The receptor acts as a mediator for the enhancement of synaptic plasticity and hippocampal dependent learning and has been shown to play a role in synaptic modification and specifically associative, episodic, and spatial memory (Morris 1986; Rampon 2000). The receptor itself is an ionotropic glutamate receptor that when activated allows influx of calcium into the postsynaptic neuron. One of the vital steps in the initiation of LTP, plasticity, and many other CAMKII induced cellular interactions, is calcium influx. Presynaptically, the neurotransmitter glutamate is released and binds to the N-Methyl-D-Aspartic acid receptor, NMDAR, and opens the channel. Additionally, there is a depolarization of the postsynaptic neuron that causes magnesium in the channel pore to be removed. In any event of disruption of the calcium concentration by chelators, or calcium influx by NMDAR antagonists, LTP is not initiated (Collingridge 1983; Lynch 1983). Figure 3 illustrates the cascade of calcium influx leading to LTP in an NMDA receptor.

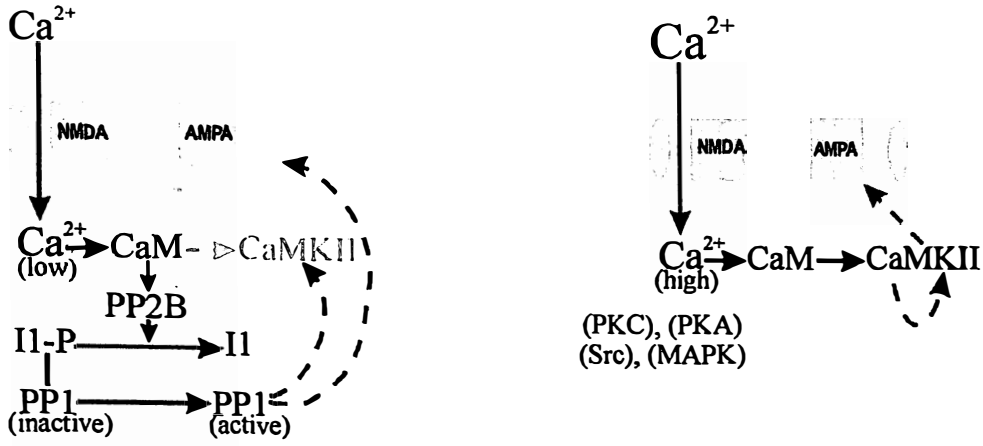

Figure 3 – Model for signal cascades in LTD and LTP in NMDA receptor cells (Malenka, 2002).

LTP begins with the release of glutamate from the presynaptic neuron to bind to the post synaptic AMPAR and NMDAR. AMPAR activation by glutamate results in the influx of sodium leading to post synaptic depolarization. This depolarization activates L-Type calcium channels resulting in the influx of calcium and also, dislodging of magnesium from the glutamate bound NMDAR resulting in calcium influx through those channels as well. At rest, the CAMKII is located at the base of the synaptic spine. Following the activation of NMDAR, it translocates to the PSD and interacts with the NR2B subunit of the NMDAR as shown in figure 4 (Bayer 2001; Shen and Leonard 2002; Pradeep 2009).

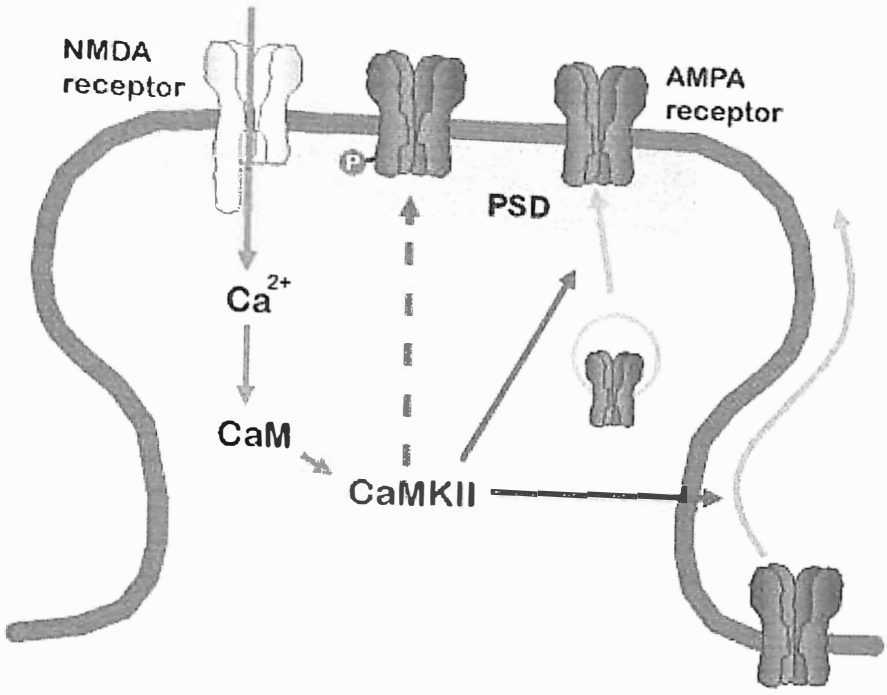

Figure 4 – Description of NMDA channel activation of CaMKII and the subsequent translocation of AMPAR to the PSD.

A structural note of the CAMKII holoenzyme is its ability to bind to F-actin (Shen 1998). Also the translocation of the inactivated CAMKII holoenzyme from the f-actin correlates with the increase activity of CREB (K. Deisseroth 1998) resulting in increased protein synthesis. Increases in calcium concentration activate a number of proteins within the PSD that converts the chemical signal into cellular modifications. CAM, binds calcium, and activates CAMKII. The CAMKII subunits then autophosphorylate and are activated to phosphorylate associated substrate molecules. MAP2, a microtubule associated protein that is responsible for microtubule assembly, is a substrate for CAMKII and is activated during calcium/Calmodulin activation of CAMKII amongst other cellular function

and structural maintaining substrate. As a result of the increase activity in the PSD, AMPAR and NMDAR are synthesized and inserted into the membrane, further strengthening the activity (Shen, 1999; Strack 2000). CAMKII continues its activity until the protein phosphatase type 1 dephosphorylates it.

The role of the NMDA receptor in learning was confirmed by selective gene knockout experiments where the deletion of the protein was achieved at the genetic level in mice (Capecchi 1989). The deletion of the NMDA receptor type-1 subunit of the NMDA receptor, NMDAR, disrupted spatial memories and resulted in failure to induce NMDAR-dependent LTP (Tsien 1996). Deletion of the receptor also diminishes performance in learning experiment paradigms (Shimizu, 2000). Interestingly, enhancement of the NMDAR function by increasing the expression of NMDARs results in a gain of function in performance in associated learning tasks (Tang 1999). CAMKII plays a critical role in activation of the NMDAR, and CAMKII is crucial for plasticity and memory. The NMDAR is capable of acting as a coincidence detector by becoming more active in the presence of increased calcium which leads to increased neurotransmitter release (Nakazawa 2004).

The role of the NMDAR in hippocampal learning has been investigated for more than twenty years. Throughout this time, the importance of the subunit NR1 has been well established. Within the CA1 region of the hippocampus, the deletion of the NR1 subunit of NMDAR resulted in memory deficit when tested by the Morris water maze (Tsien 1996). The NMDAR is necessary to form new memory. A demonstration of this point was illustrated when an NMDAR

antagonist known as 2-amino-5-phosphopropionic acid (AP5) was infused into the hippocampus of an intact rat performing a spatial learning task (Morris 1986). The rat's learning was impaired and the spatial performance was negatively affected. This demonstrated that function of the NMDAR was crucial for memory formation in the brain. Calcium entry through NMDA receptors is adequate to induce synaptic plasticity on a short term basis, but long term synaptic changes of plasticity requires both NMDAR and voltage-gated calcium channels to initiate molecular processes leading to long term plasticity changes (Hugh, 2001). CaMKII is required for NMDA control of synaptic plasticity. According to Barria et al (2005), CaMKII is necessary for its interaction with the NMDAR in synaptic plasticity.

Figure 5 shows the initial response that blocking CaMKII has on LTP. In the slices, there is a lack of LTP when KN93 is introduced to the slices. It is well understood that KN93 inhibits CaMKII function and as such inhibits LTP in this case.

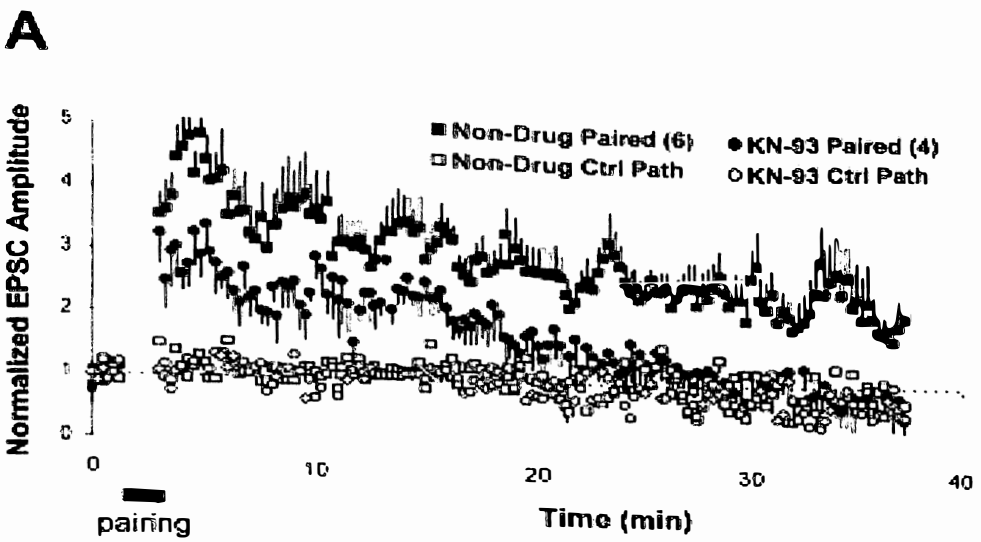

Figure 5 – Inhibition of LTP by blocking CaMKII. Hippocampal slices perfused with KN93 before whole cell recording from a cell in CA1 region. Control slices show no potentiation before pairing. Non drug pairing shows potentiation while KN93 shows no potentiation (Barria, 2005).

Further experiments, shown in figure 6 on the next page, demonstrate the need for both NMDAR and CaMKII for mEPSC frequency in CA1 pyramidal neurons. These results contribute to the fact that NMDAR, specifically NR2B, is necessary for EPSC. By either increasing magnesium or by introducing ifenprodil, an inhibitor of NMDAR, mEPSC's are reduced. Binding of CaMKII to NR2B is necessary and a simple mutation to the NR2B catalytic site that interacts with CaMKII results in a reduced synaptic plasticity.

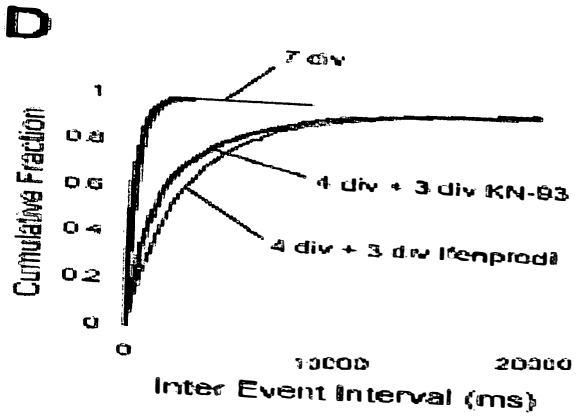

Figure 6 – Blockage of NR2B and CaMKII by KN93 and ifenprodil shows a reduction on mEPSC frequency in CA1 pyramidal neurons (Barria, 2005)

Data in Figures 7 and 8 indicate the need for CaMKII to associate effectively with NMDAR NR2B to induce potentiation leading to synaptic plasticity. Mutations to the NR2B receptor lead to prevention of LTP due to the lack of CaMKII association. To an even further extent, CaMKII must interact with NR2B in order to modulate synaptic plasticity *in vivo* and this leads to behavior dependent learning (Zhou, 2007).

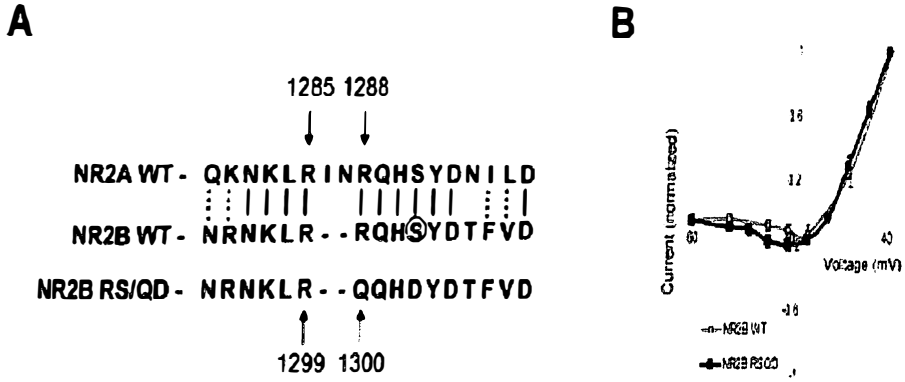

Figure 7 – Mutation of NR2B inhibits CaMKII binding, but not I/V of NMDAR currents. The NR2B sequence compared to NR2A and the consequent mutation creating NR2B RS/QD (A). Current voltage relationship of wt NR2B and the mutated NR2B RS/QD showing no difference in relationship (B) (Barria, 2005).

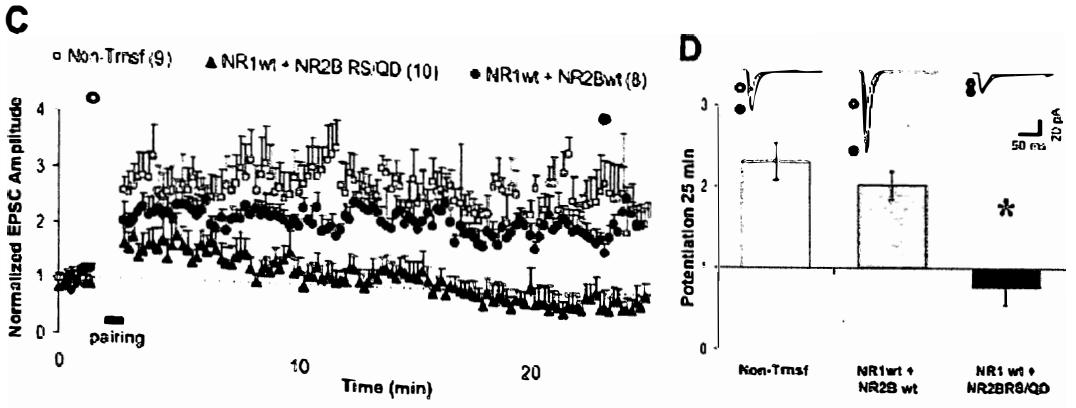

Figure 8 – NR2B mutant NR2B RS/QD reduces synaptic plasticity. NR2B wt resembles the non-transfected cells level of EPSC amplitude. Mutant NR2B RS/QD does not show potentiation (C). Quantification of potentiation shows potentiation of transfected NR2B and not in mutant NR2B RS/QD (Barria, 2005).

Construction of a transgenic mouse to explore the interactions between CaMKII and NR2B has been important for demonstrating their role in memory. One such study illustrates the importance of the interaction between CaMKII and NR2B and how this interaction results in behavior changes. The transgenic mouse expresses a ligand-activated NR2B fragment called cNR2B. Fusing this to a tamoxifen-dependent mutant of the estrogen receptor ligand domain, allows activation of the protein with the introduction of tamoxifen. The cNR2B fragment binds to endogenous CaMKII in neurons and interferes with the interaction of CaMKII and NMDAR at the synapses. This leads to decreased AMPAR phosphorylation and synaptic plasticity in the form of LTP. Figure 9 on the next page shows successful generation of the LBD^{G512}cNR2B mouse. In vivo, TAM activated LBD^{G512}cNR2B binds to CaMKII and interferes with CaMKII/NR2B interaction.

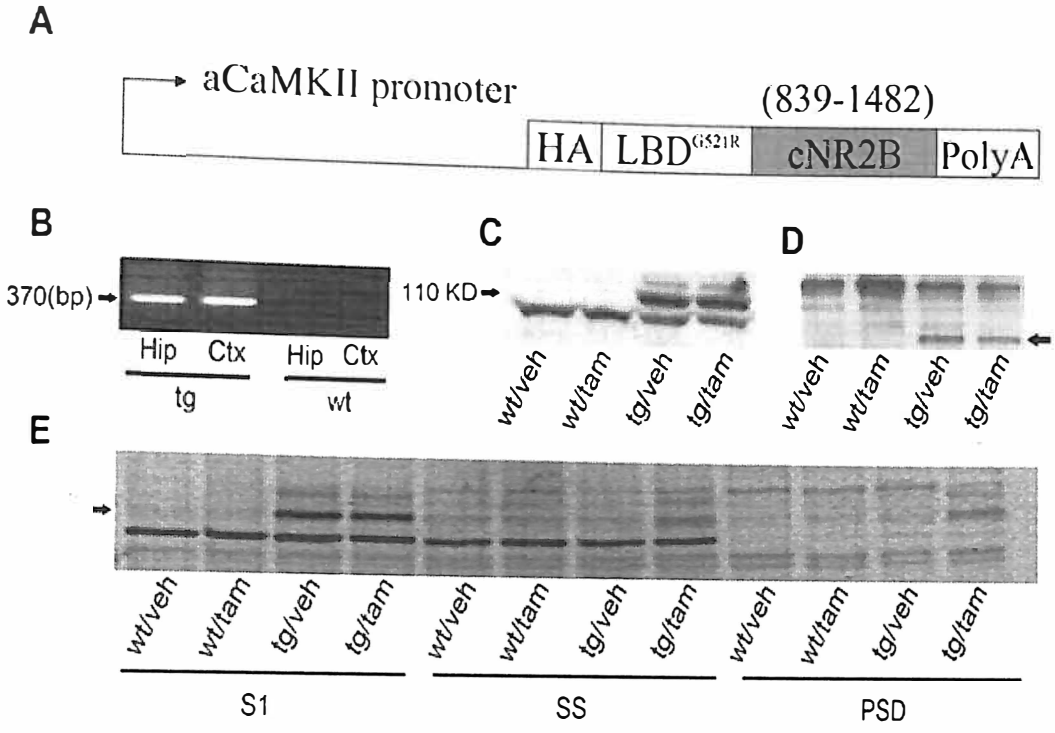

Figure 9 – Generation of LBD^{G512}cNR2B transgenic mice. Schematic diagram of the construct used for transgenic mice (A). RT-PCR showing transgenic LBD^{G512}cNR2B mRNA in hippocampus (Hip) and cortex (ctx) (B). Immunoblots for transgenic protein showing the protein exists only in transgenic (tg) mice (C). Immunoblots of forebrain homogenates against endogenous NR2BC and showing the presence of LBD^{G512}cNR2B protein in the forebrain samples of tg mice (D). Immunoblots of homogenates S1, synaptosomes SS, and PSD enriched fractions PSD showing the presence of the protein in the synaptosome and PSD (E) (Zhou, 2007).

NR2B binds to PSD95 (Kornau, 1995). To rule out the association of the PSD95 interaction with the mutant protein Co-IP is done along with immunoblots and figure 10 shows CaMKII is associated with the LBD^{G512}cNR2B protein and that the protein is not precipitated with PSD95.

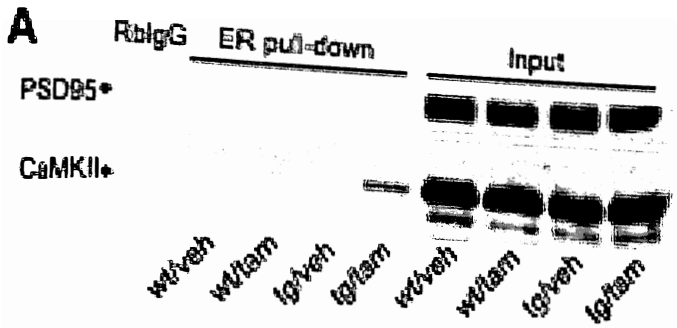

Figure 10 - LBD^{G512}cNR2B binds to CaMKII not PSD95 (Zhou, 2007).

The figures 10, and 11, illustrate the result of the activation of the LBD^{G512}cNR2B protein on the effects of LTP. This proves that when tamoxofen is used against CaMKII, LTP is reduced providing solid evidence that CaMKII interaction with the NR2B subunit is necessary for synaptic plasticity.

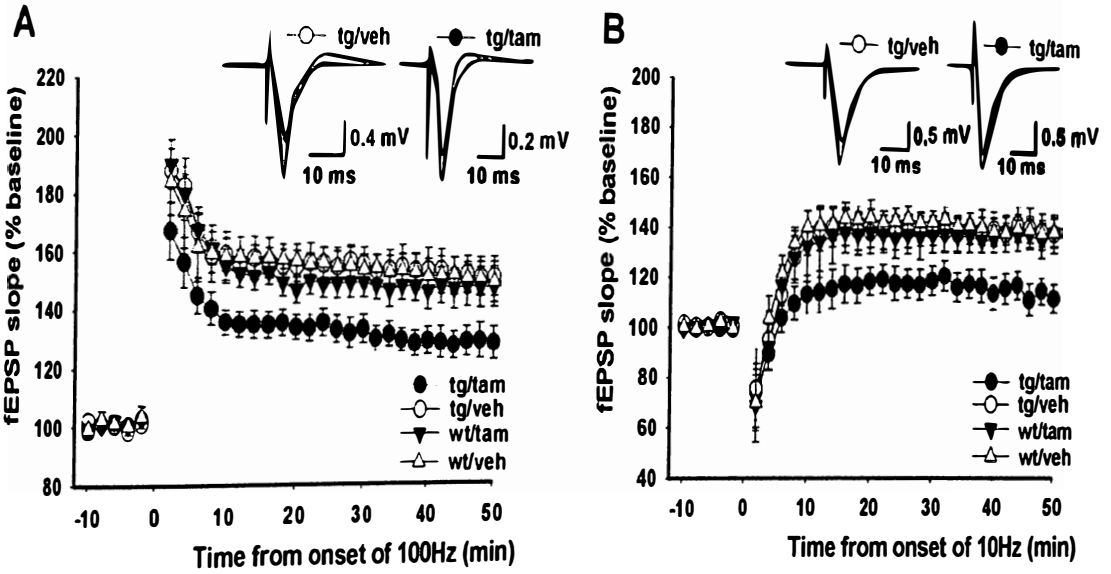

Figure 11 – Reduction in LTP in cNR2B transgenic mice. A reduction in LTP following 100 Hz stimulation is evident in transgenic tamoxofen administered mice. The other groups displayed no significant difference (A). 10 Hz stimulation induced LTP which is reduced again in the transgenic tamoxofen mice (B) (Zhou, 2007).

The relationship between CaMKII and the NR2B subunit is a necessary interaction for synaptic plasticity and it is evident that CaMKII must bind in order

to have synaptic plasticity in the form of LTP. Mutation of the binding sites and inhibiting CaMKII in some manner either by KN93 in vitro or by LBD^{G512}cNR2B protein *in vivo* leads to reduced synaptic plasticity.

Voltage gated calcium channels and presynaptic facilitation

The voltage gated calcium channels (VGCC's) are a family of proteins that are widely expressed throughout the body in a variety of cell types including neurons, cardiac and skeletal tissue where they control action potential and firing (Dolphin, 2006). The Cav2 family, associated with neurons, consists of three members; 2.1 (conducts p/q currents), 2.2 (conducts n currents, and 2.3 (conducts r currents) (Alberts, 2002). Global and local rises in calcium are detected by calcium binding EF domains in the N terminal and the C terminal respectively. (Alberts, 2002). Prolonged depolarization results in a global rise in calcium. The Cav2.1 channels consists of an IQ motif and is the site of CaMKII binding intracellularly (Guerter, 2008).

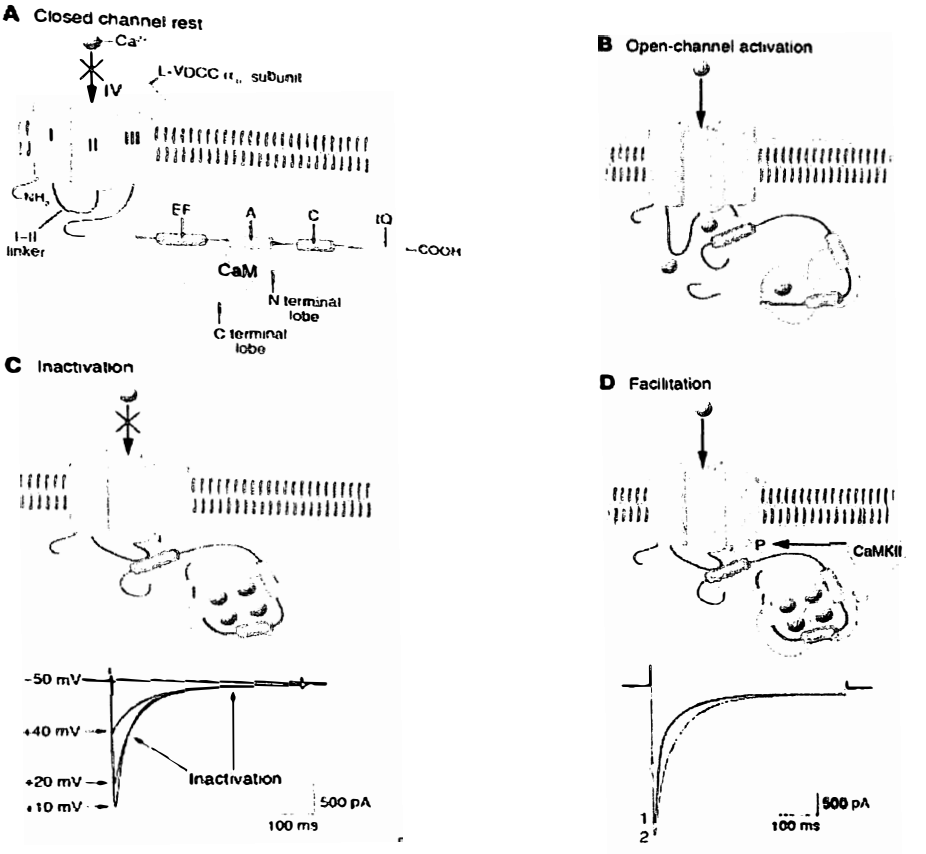

Figure 12 – L type calcium channel (A) at rest no calcium influx. Calmodulin binds to peptide A. (B) Depolarizing stimulus, calcium binds to Calmodulin and structural change. EF hand prevents I-II linker from blocking calcium influx. Depolarization leading to increased calcium causes conformational change and EF hand permits I-II to rapidly inactivate channel (C). CaMKII enhances the facilitation of calcium current by phosphorylation of channel (Ilona, 2005).

VGCC's, are found in the synaptic terminal. During the voltage change induced by an action potential, these channels are triggered to open allowing extracellular calcium to flow inward. The vesicles filled with neurotransmitters fuse to the pre-synaptic membrane as a result of this influx of calcium and eventually release neurotransmitter into the synapse towards the post synaptic neuron. The accumulation of the protein receptors and enzymes in the postsynaptic cell as a response of the release of the neurotransmitter from the

presynaptic cell is a precursor of synaptic plasticity where the number of receptors indicates a change in plasticity (Liao, 1995). Among the notable receptors activated during VGCC activation are the NMDA and AMPA receptors which will be discussed later. In 1973, Bliss and Lomo showed the effect of lasting changes in hippocampal cells was result of interaction between pre and post synaptic cells (Bliss and Lomo, 1973). Later studies of the synaptic plasticity phenomenon were shown in experiments pairing the presynaptic stimulation with the postsynaptic depolarization.

Presynaptic calcium channels, Cav2.1 and Cav2.2, are associated with calcium release and are regulated by CaMKII in an activity dependent manner (Abria, 2010). CaMKII binds to the Cav1.2 L type calcium channel at the beta 1b or the beta 2a subunits of the channel resulting in phosphorylation of the channel at threonine 498 leading to enhanced transmission (Greuter, 2006). CaMKII binds to the IQ motif of the Cav2.1 channel leading to synaptic facilitation which provides a mechanism for synaptic plasticity (Greuter, 2006). Cav2.1 type channels are the primary calcium channels in the glutamatergic nerve terminal and are associated with activation of NMDA receptors (Pietrobon, 2005). The critical role of activated Cav2.1 in synaptic plasticity has been identified through experiments using a giant central synapse called the "calyx of Held" within the past decade. It is found in the auditory nucleus of the brainstem and has been valuable for discovery of the channels critical role in synaptic plasticity (Neher, 2007; Haustein, 2008). Projections from this nucleus project to the lateral

superior olive, involved in sound processing, a function which necessitates fast transmission.

An important note on VGCC activation is that its initial activation is dependent on the local calcium concentrations. While CaMKII is not required to activate the channel at low calcium local levels, and to some extent global calcium levels, CaMKII is required to induce VGCC facilitation which leads to increase rapid intracellular calcium concentration, increase channel activity, and leads to increased vesicle fusion resulting in neurotransmitter release (Yuan W 1994; Dzhura, 2000; Guerter, 2008; Abria, 2010). VGCC's are localized in areas that that contain CaMKII. CaMKII dependent VGCC facilitation depends on calcium activation of the Calmodulin/CaMKII complex as illustrated in figure 12. The facilitation is blocked by a fast calcium chelator BAPTA (Abria, 2010). CaMKII associates with an important alpha 1 subunit of a VGCC. Mutation of the motif completely prevents binding and modulation of Cav2.1 type channels and facilitation. To determine the impact of CaMKII on the regulation of Cav2.1 channels, ion currents can be analyzed in transfected tsA-201 cells using whole cell patch clamp with intracellular recording solution containing a high concentration of EGTA, a highly effective chelating agent of calcium, and using barium as the permeant ion of the extracellular saline (Jiang, 2008). CaMKII is necessary for facilitation of VGCC's and its binding to the channel site of CaV2.1 substantially increases the channel activity (Jiang, 2008).

CaMKII is necessary for the modulation of CaV2.1 channels. The activated CaMKII enhances activation of the L type currents. CaMKII binds to an

alpha1 site at the C terminal domain of the VGCC. Coimmunoprecipitation of CaMKII with the C terminal expressed alpha1 domain indicates CaMKII binds specifically to the channel. The addition of a competing peptide (Cav2.1 1897-1912) for the same sequence blocked coimmunoprecipitation (Alberts, 2002). To determine if CaMKII binding to the site specified in the alpha1 domain of the C terminal is necessary for channel regulation, dialysis of competing peptide into tsA201cells and patch clamp can be used. With the addition of the peptide, inactivation was enhanced.

CaMKII relies for its activation on the calcium Calmodulin complex. Inhibition of this activator system leaves endogenous CaMKII relatively inactive. Figure 13 illustrates the inactivation of the VGCC in the presence of CaMKII inhibitors. CaMKII can bind to Cav2.1 to enhance calcium modulation. In the presence of CaMKII inhibitors KN-93 and CaMKIIN, channel inactivation is not prolonged. While not effective at preventing modulation of Cav2.1 channels, these inhibitors may play a role in preventing conformational change in the CaMKII complex which would normally lead to binding to Cav2.1 channels. The results show the necessity for activated CaMKII to prolong channel openings and slow the inactivation of the VGCC. Inhibitors or regulators of CaMKII enhance inactivation of VGCC.

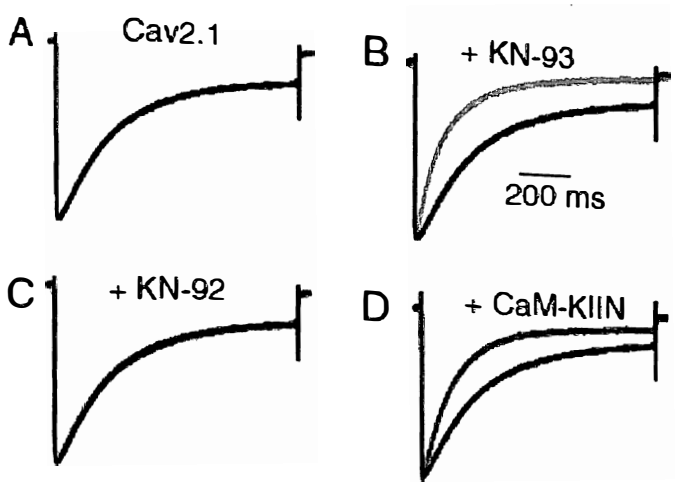

Figure 13 - Inhibition of Cav2.1 channels by CaMKII inhibitors KN93 (B), KN92 (C), and CaMKIIN (D).

The results in figure 13 show that CaMKII binding to the channel is necessary for modulation. KN93 and the competing enzyme function through the same mechanism. IgG used as a negative control because of its lack of specific antigen binding. Figure 14 illustrates the need for CaMKII to bind to the specific alpha1 site for inhibition of inactivation and modulation of the VGCC. Results from experiments performed with Cav2.1 myc tagged c terminal construct showed that in the presence of CamKII inhibitors KN-93 and CaM-KIIN, CaMKII does not bind to the c terminal sequence of the Cav2.1 channel. Also in the presence of the competitive inhibitor Cav2.1(1897-1912), CaMKII does not bind to the c terminal site of Cav2.1. These results indicate CaMKII associates with the VGCC and modulates the activity of the channel by preventing inactivation, which leads to facilitation of the synapse.

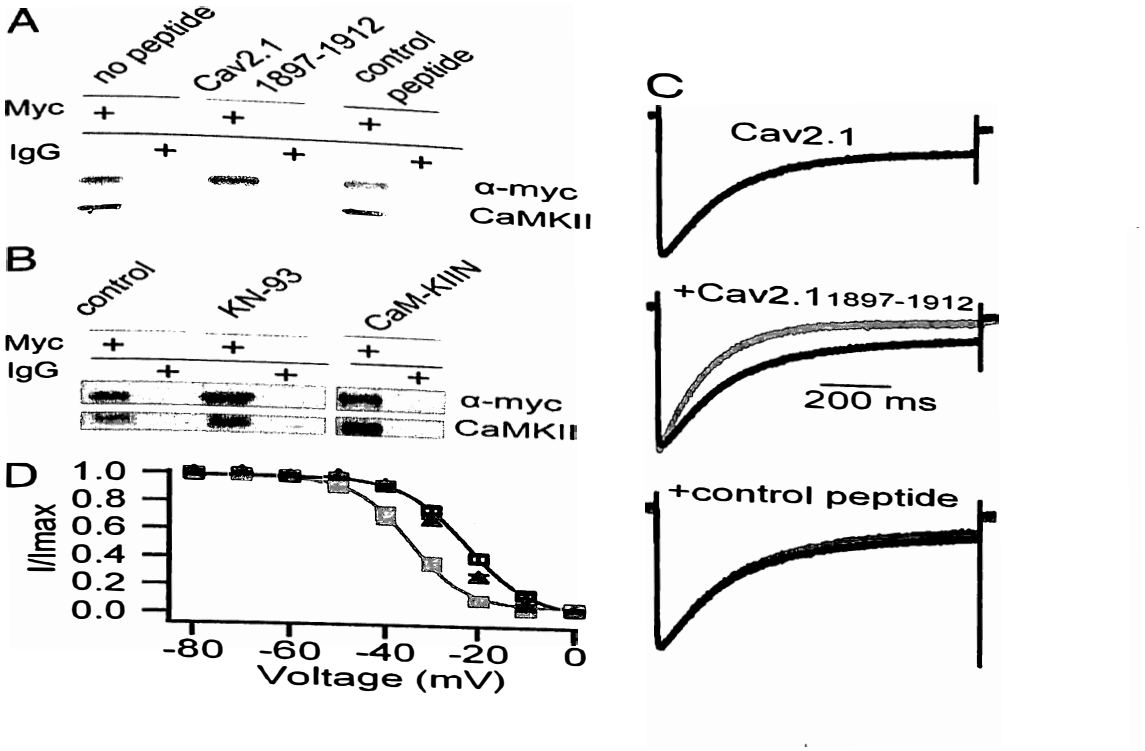

Figure 14 – CaMKII binding to alpha1 domain of C terminal is required for modulation of the VGCC. Addition of competing peptide CaV2.11897-1912 (A). Addition of CaMKII inhibitor KN93 (B). Inactivation with the addition of CaV2.1 competing peptide (C). Effect of competing peptide on voltage dependence of inactivation (D).

Figure 15 shows results of an experiment in which rat brain samples were solubilized and analyzed by immunoprecipitation using antibodies against the alpha1 domain and CaMKII. To narrow down the effects of CaMKII in the function of specific currents, toxins can be used to remove non-associated currents. Incubation of pyramidal neurons with nimodipine and conotoxin omega removes L and N type currents. Agatoxin removes nearly 85% of the remaining current shown in Figure 15 (B). The remaining currents are P/Q currents. By applying KN93, CaMKII's activity is inhibited and inactivation occurs.

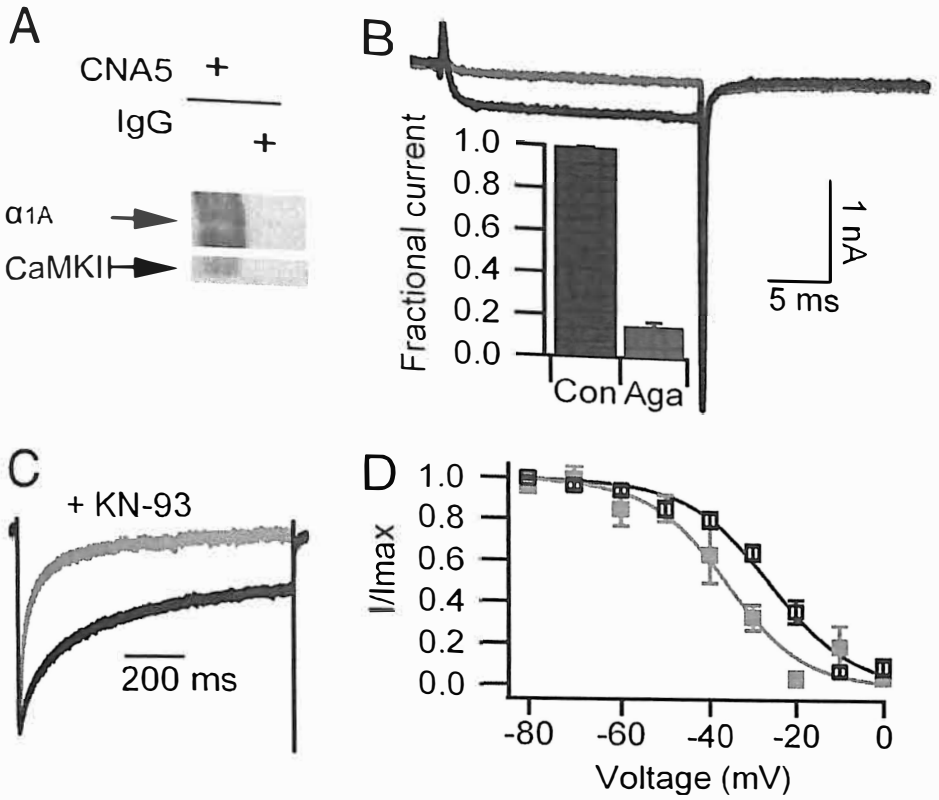

Figure 15 – Binding and modulation of calcium channels in adult rat hippocampal pyramidal neurons by CaMKII. Anti-alpha1 and CaMKII antibodies (A). Inhibition of nonspecific calcium channels using toxins, and subsequent removal of P/Q currents with agatoxin (B). Specific inhibition of CaMKII with KN93 causes a steep inactivation of VGCC (C). KN93 causes a shift in the voltage dependent inactivation (D).

These data indicate CaMKII is necessary for VGCC channels function in the facilitation plasticity of CaV2.1 type channels by slowing inactivation of the channel when activated CaMKII is bound. CaMKII binds to a particular sequence on the CaV2.1 channel. If the binding site on CaMKII is occupied by a competing peptide so that it can no longer interact with the channel, depolarization will lead to rapid inactivation of the channel. Despite the fact that CaMKII binds to the alpha1 site of the channel and can be inhibited by KN93, it must also be noted that CaMKII must be in its activated form to have an effect on the channel. When

CaMKII binds to the channel in its activated form, it enhances the channel activity by slowing inactivation. Constitutive modulation of the VGCC depends on CaMKII activation by Calmodulin and calcium concentration.

Inositol 1,4,5-triphosphate induced calcium release, CAMK and their roles in LTD

Liberation of internal stores of calcium requires two steps; first metabotropic receptors on the plasma membrane must be activated; then opening of ionotropic neurotransmitter receptor channels on internal membrane compartments. Activation of plasma membrane receptors activates phospholipase C which then hydrolyzes the phospholipid PIP2 into two products; IP3 and DAG. IP_3 is a substrate for the IP3 receptor. When bound to ionotropic receptors on the endoplasmic reticulum, IP3 causes the release of calcium from internal stores Calcium within the neuron, acts as a second messenger. In addition to the IP3, calcium, itself can also act as a substrate for the IP3 receptor and cause the release of calcium. The most effective activation of the IP3 receptor release of calcium is when calcium and IP3 are presented together. Ryanodine receptors are activated by intracellular calcium along with cyclic ADP-ribose. Both the Ip3 and ryanodine receptors are located in the membrane of the ER. These receptor types are found in neurons of the hippocampus, dentate gyrus, and the neocortex (Mori et al, 2005). The activation of either Ryanodine the receptor or IP_3 receptor (or both in cells that have both) results in a calcium-induced-calcium-release (CICR)

phenomenon, a positive feedback cycle where released calcium further activates receptors, further releasing calcium (Berridge 1998). Ryanodine receptors of the brain are the RYR2 and RYR3. Type 1 RYR exists in muscle and at a low concentration in the hippocampus, and types 2 and 3 are found in a high concentration in the cortex, hippocampus, and dentate gyrus (Kuwajima et al, 1992, Galeotti et al, 2008). Figure 16 illustrates how CICR works.

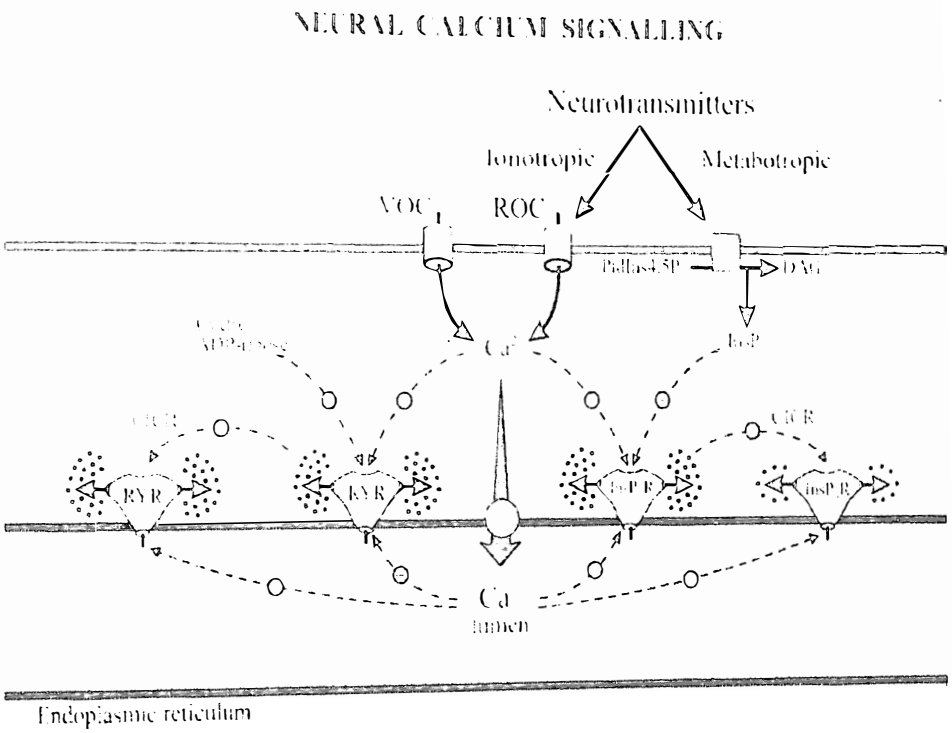

Figure 16 – Signal activation by neurotransmitters activates cascades that release internal calcium into the cytoplasm further activating protein cascades which depend upon calcium.

The interaction of the inositol triphosphate and ryanodine receptors upon the endoplasmic reticulum is responsible for releasing calcium from the internal store. Since it is understood that IP_3 receptors are intracellular calcium release channels, the regulation of opening is of high importance. Increases in calcium

by the IP3 pathway results in an increase in CaMKII clustering at the synapse, and inhibition of the IP3 pathway failed to show a synaptic clustering indicating the importance of IP3 elevation of calcium to plastic changes in CaMKII (Gu 2004). Very interestingly, increased CaMKII activity has been shown in disease models such as schizophrenia, and also in use of addictive drugs such as amphetamine. It is well understood that drug use leads to addictive plasticity and therefore provides a link between the function of CaMKII and cell processes that increase calcium concentration (Wang 2003). Another key aspect of IP3 induced plasticity through calcium concentration involves the activation of the ryanodine receptor by CaMKII leading to further calcium concentration increase and CaMKII activity (Macmillan 2005). The IP3 receptor initiates the CICR increase in calcium, which is a key component of synaptic plasticity (Emptage 2001; Nagarkatti 2008).

The IP3 receptor is regulated by CaMKII and CaMKII plays a role in IP3 induced calcium release (IICR). CaMKII's regulation of the IP3 receptor was reported to be a necessary component of calcium release from internal stores leading to cellular homeostasis (Berridge, 1998; Ferris, 1991). Results similar to these contribute to the understanding that the modulatory role of CaMKII on the internal calcium receptor is a necessary part of synaptic plasticity. Initial studies of the interaction of CaMKII with the IP3 receptor showed there were no interactions between the two (Supattapone, 1988), but this was later found to be incorrect. The detergents used to isolate IP3 were inhibiting the function of not only CaMKII but also other proteins and because of this mishap; CaMKII was not

reported to be associated with the IP3 receptor (Ferris, 1991). To overcome the weakness in the original experiment, reconstitution of the receptor in vesicles without the inhibiting detergents was a construct used to reexamine the functional relationship between IP3 and CaMKII. Results of experiments with purified and reconstituted IP3 receptors demonstrated that they are phosphorylated by CaMKII. Figures 17 - 19 illustrate the results of experiments showing that CaMKII phosphorylates the IP3 receptor and it is also noted that the relationship between CaMKII and the IP3R is more extensive than for other kinases (figure 16). CaMKII has a more rapid phosphorylation rate than the other enzymes since it has a half maximal phosphorylation at around 10 minutes.

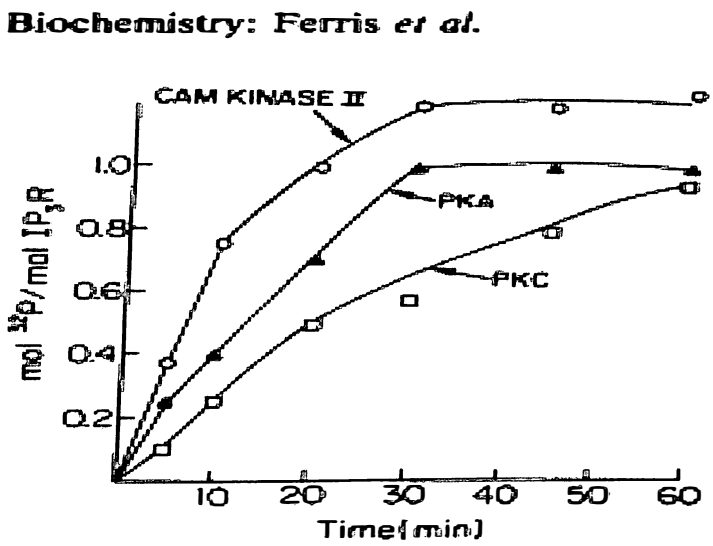

Figure 17 – Phosphorylation of purified IP3 receptors by CaMKII (Ferris, 1991). CaMKII at a greater extent activates the IP3 receptor, over a 1 hour time period, more than the activation of both PKA and PKC as illustrated in figure 17.

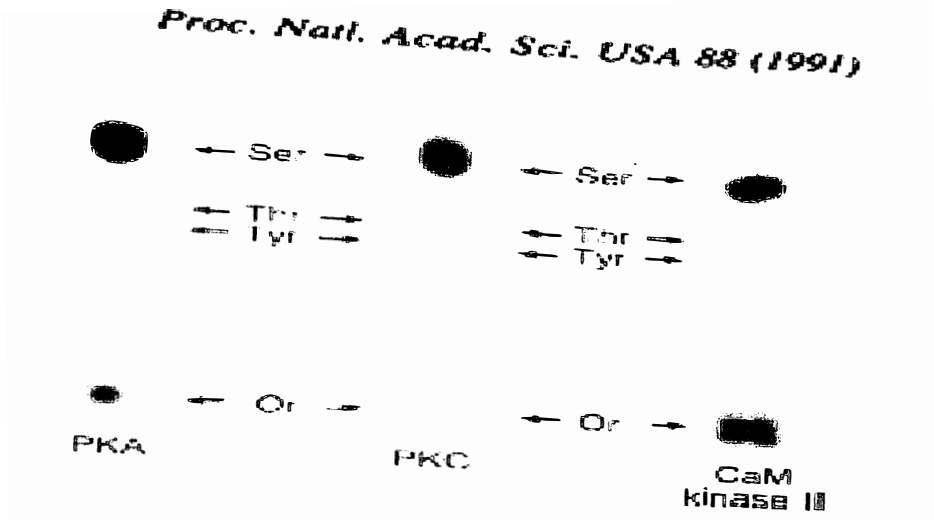

Figure 18 – CaMKII phosphorylates IP3R to a greater extent than PKA and PKC and all at either serine or threonine phosphorylation sites (Ferris, 1991).

The IP3 receptor also is reduced in the presence of an inhibitor of both PKA and PKC, but to an even greater extent, removal of calcium and Calmodulin. Because CaMKII relies upon these factors, removal results in a tenfold reduction in phosphorylated IP3 receptor concentration as shown in figure 19.

Table 1. Additive phosphorylation of IP_3 receptor by PKA, PKC, and CaM kinase II

Phosphorylating condition	$^{32}P/IP_3$ receptor, mol/mol
PKA	1.0
+ Walsh inhibitor	<0.1
PKC	0.9
− Ca^{2+}	<0.1
CaM kinase II	1.25
− Ca^{2+}	<0.1
− Calmodulin	<0.1
PKA + PKC + CaM kinase II	2.7

Specific and stoichiometric phosphorylation of purified and reconstituted IP_3 receptor by PKA (catalytic subunit), PKC, and CaM kinase II. Phosphorylation was performed as described. The incubation was for 60 min at 30°C. This experiment was replicated with <10% variation.

Figure 19 – Table of phosphorylation of IP3 receptor with inhibiting agents and reducing conditions (Ferris, 1991).

Furthermore, two dimensional phosphopeptide mapping of CaMKII phosphorylated IP3R produces a single highly acidic phosphopeptide. With the understanding that CaMKII phosphorylates IP3R, The localization becomes important. An established study demonstrated that CaMKII is localized in Purkinje cells of the cerebellum, the site where IP3R is highly concentrated as noted in figure 20 (Ferris, 1991).

Figure 20 – Two dimensional phosphopeptide map of CaMKII phosphorylated IP3R. (Ferris, 1991).

To date, the actual direct function of CaMKII activation of IP3R has not been fully elucidated, but there have been recent experiments that explore the components needed for IP3R activation needed for calcium concentration increase leading to synaptic plasticity. LTD, long term depression, is a form of synaptic plasticity that can result from weak synaptic stimulation or strong synaptic stimulation (Massey, 2007). In the case of the hippocampus, LTD is a result of

weak stimulation and in the case of the cerebellum; LTD is the result of strong stimulation and provides the basis for motor learning (Ito, 2001). In the case of the cerebellum, postsynaptic calcium elevation is necessary (Ito, 2001).

A protein of note, Homer 3, is a member of the metabotrophic glutamate receptor family that interacts with the ryanodine receptor type 1. Homer 3 is localized to the Purkinje cells and more specifically in the dendritic spines (Shiraishi, 2007). Homer3 is a substrate for CaMKII that activates IP3R and contributes to synaptic depression necessary for motor learning. This provides further evidence that CaMKII is a required protein for synaptic plasticity and does not only lead to enhancement of a synapse, but is functionally important in depression of a synapse especially in the cerebellar Purkinje cells.

Homer 3 is a scaffolding protein that couples mGluR1 to IP3R thereby mediating intracellular calcium signaling. Its functions also include interactions with transcription factors that regulate nuclear function. Homer 3 is activated by CaMKII and leads to synaptic plasticity (Mizutani, 2008).

Sites of CaMKII phosphorylation in Homer3 include three sites containing serine residues as shown in figure 21. Mutation of the serine sites prevents CaMKII phosphorylation via the inhibition of group 1 mGluRs (Tu et al, 1998, Kammermeier et al, 2000). This reduction in calcium signaling decreases activation of CamKII. The sites of phosphorylation are conserved across species and can provide a link between species. Figure 21 illustrates the specific binding of Homer 3 over Homer 1 and 2 in the cerebellar Purkinje cells (Tu, 1998).

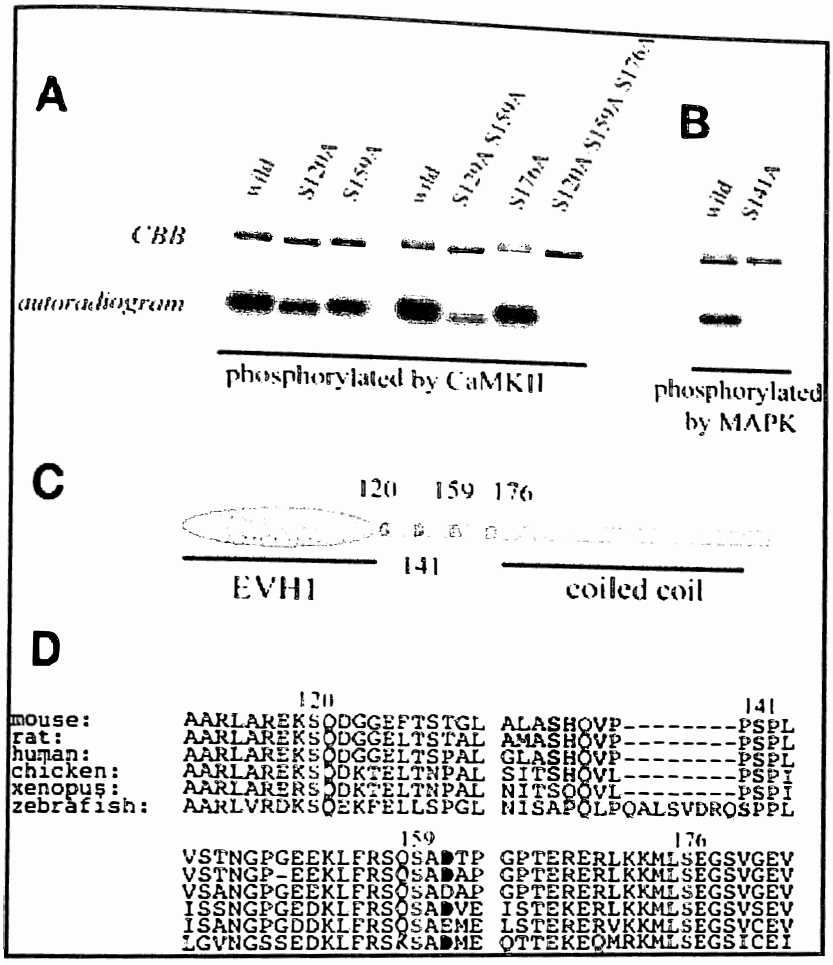

Figure 21 – In vitro CaMKII phosphorylation of Homer 3 at serines 120, 159, and 176. Wild type and mutated phosphorylation sites presented to CaMKII (A). Illustration of the Homer 3 protein (C). Alignment of amino acid sequence indicates conserved regions across species (D) (Mizutani, 2008).

Homer 3 coprecipitates with IP3 as shown in figure 22. The importance of this is the link between CaMKII and the purkinje cells responsible for LTD. As is evident in the blots, IP3 co precipitates with Homer 3 to a greater extent than the type 1 and 2 homologs and this leads to an understanding of the importance of CaMKII in the cerebellum.

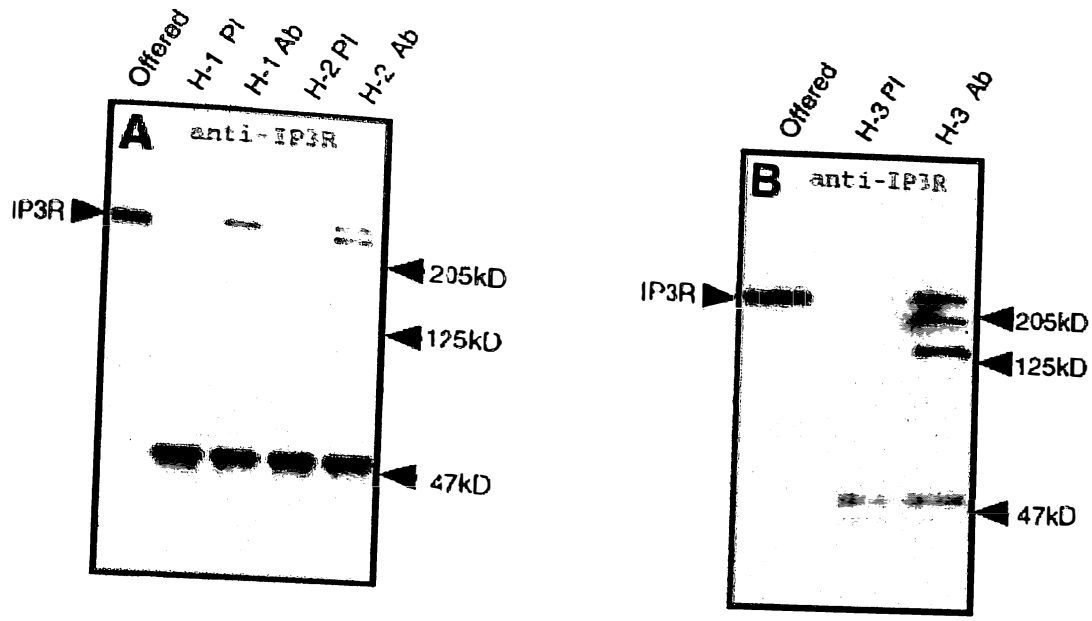

Figure 22 – IP3R coprecipitates with Homer 3. Homer 1 and 2 (A). Homer 3 (B) showing a greater level of IP3R binding (Tu, 1998).

Further evidence for the importance of CaMKII activation of Homer 3 in the initiation of LTD is the reduction of phosphorylated sites in the presence of CaMKII inhibitors. The current understanding is that Homer 3 facilitates the function of ryanodine receptor. The ryanodine receptor release of calcium in the purkinje cells leads to long term depression. When a CaMKII inhibitor is introduced to a purkinje cell, phosphorylated sites on Homer 3 are reduced over a 10 minute period and thus a reduction in Homer 3 activity is shown, as in figure 23.

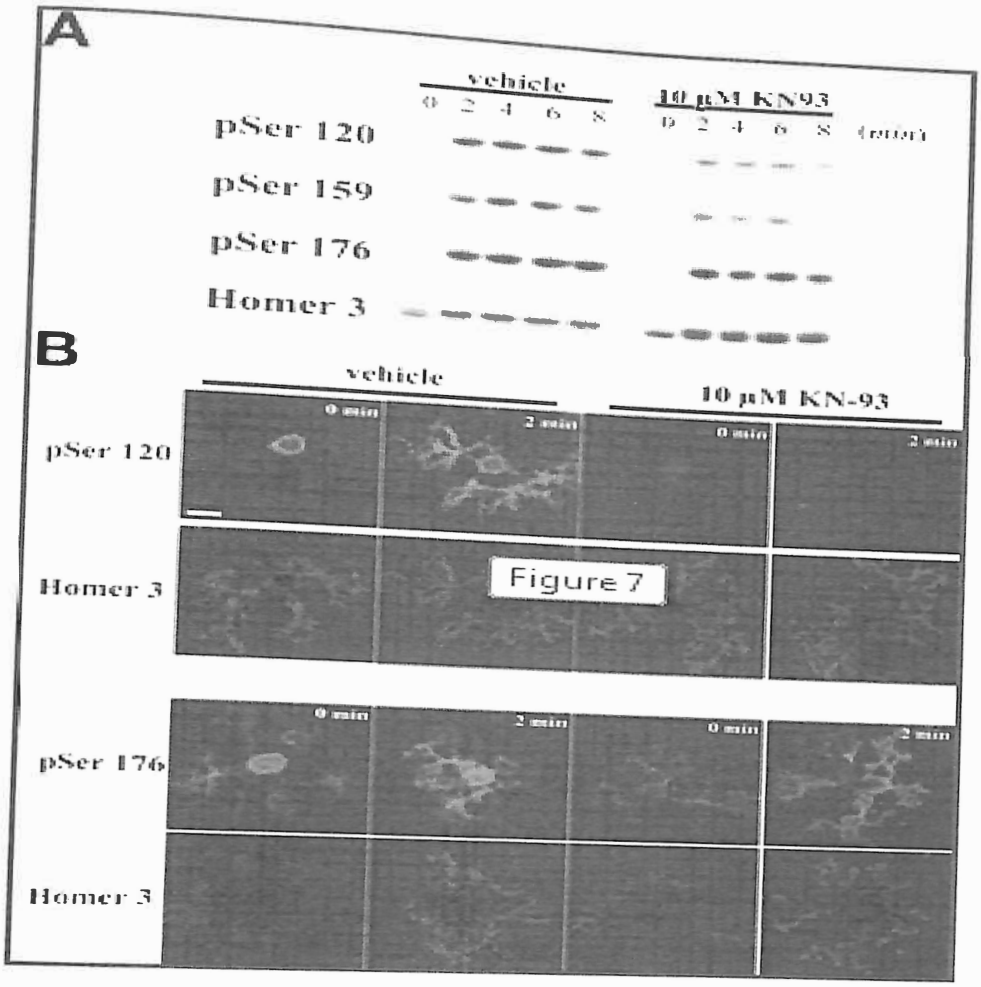

Figure 23 – Phosphorylation of Homer 3 at the serine sites 120, 159, and 176 by CaMKII induced by depolarization in primary cultured Purkinje cells. Time course shows that after depolarization, serine sites are phosphorylated and the reduction of phosphorylation following treatment with CaMKII inhibitory KN93 (A). Immunocytochemical analysis of primary cultured Purkinje cells with specific treatment with and without KN93 (B) (Mizutani, 2008).

Current studies of the CaMKII and IP3R relationship leading to synaptic plasticity examine the effect of their interactions and function during drug addiction and disease models. Repeated exposure to amphetamine increases PKA induced sensitization of IP3 receptors leading to an increase in NMDAR activation of LTP facilitated by CaMKII (Ahn 2010). Amphetamine also

increased CaMKII induced cell protein phosphorylation including the extracellular signaling kinase ERK (Choe 2002). Inhibition of the CaMKII by KN62 reduced the number of similar protein phosphorylation (Choe 2002). In schizophrenia CaMKII has been shown to be upregulated (Frankland 2008). In both of these conditions, the effects of the CaMKII function is magnified lending further evidence to the necessity of CaMKII in synaptic plasticity under conditions of increased calcium.

CONCLUSION

CAMKII has been studied *in vitro* extensively. CAMKII can regulate its activity through autophosphorylation, but its activity depends on the presence of Calmodulin. The protein is essential for functions of many cellular processes. CaMKII is necessary for synaptic plasticity and works through calcium concentration increase by voltage gated calcium channels, IP3 receptor activation, or NMDA receptor activation. Experimentally, CaMKII has been shown to be a key mediator for plasticity using gene knockout models, calcium cheaters, CaMKII inhibitors, and CaMKII mimic proteins to name a few of the tools. The work of elucidating CaMKII function completely is still ongoing. A greater understanding of CaMKII would prove valuable in the field of spinal cord injuries. Since we now understand that the protein is an essential component of synaptic plasticity, bridging the gap of information on its role in the spinal cord and neuromuscular junction, may shed light on the function of spinal cord processes lost during injury or disease of peripheral nerves. A central topic of biomechanics and motor control involves the exploration of the nature, function, and role of the rhythmic networks surrounding the concept of the Central Generator (CPG). A CPG is an endogenous neural network that, without sensory or central input, can produce rhythmic motor output. The CPG underlie the rhythmic motor patterns that has been studied as specific activated neural networks and include behavior such as walking, scratching, and breathing. The basis of the network in each behavior requires a threshold necessary to trigger

activation of the network. These behaviors all involve a level of complex patterning and are not subjected to voluntary control. Therefore, finding a link of CaMKII in such processes, and knowing the key activators of the protein, one could make an argument that the process of locomotion that is a result of the activated pattern generator can be initiated by manipulation of the CaMKII cascade.

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