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The role of CaMKII binding NMDARs in synaptic plasticity and memory

Robert Francis Dallapiazza
University of Iowa

THE ROLE OF CAMKII BINDING NMDARS IN SYNAPTIC PLASTICITY AND MEMORY

by

Robert Francis Dallapiazza

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Pharmacology
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Thesis Supervisor: Professor Johannes Hell

Our memories are fundamental components of who we are as individuals. They influence almost every aspect of our lives such as our decisions, our personalities, our emotions, and our purpose in life. Diseases that affect memory have devastating impacts on the individuals who bear them. Imagine not being able to recall pleasant memories or even the faces of close family members. It's important to understand the biology of memory formation not only because it's an intriguing scientific question, but because of its consequences when these processes are lost. N-methyl-D-aspartate-type glutamate receptors (NMDARs) and calcium/calmodulin-dependent kinase II (CaMKII) are essential molecules involved in learning and its physiological correlate, synaptic plasticity. Calcium influx through NMDARs activates CaMKII, which translocates to the postsynaptic signaling sites through its interactions with the NMDAR subunits NR1 and NR2B. The significance of CaMKII's translocation is not fully known, however we hypothesize that it is an early molecular event that is necessary for the expression of synaptic plasticity and learning. Our laboratory has developed two strains of mice with targeted mutations to NR1 and NR2B (NR1KI and NR2BKI) that are deficient in their ability to bind to CaMKII to test the role of CaMKII binding to NMDARs in synaptic plasticity and learning. We found that CaMKII binding to NR2B is necessary for long-term potentiation (LTP), the most commonly studied form of synaptic plasticity. NR2BKI mice are able to learn spatial and cued tasks normally, however they are unable to consolidate spatial tasks for long-term memory storage. On the other hand, we found that CaMKII binding to NR1 is not necessary for LTP. Furthermore NR1KI mice do not show impairments in contextual or cued learning. We found that NR1 mutations resulted in an age-dependent truncation of the intracellular domains of NR1 that reduced its

activity leading to severe impairments in synaptic transmission, LTP, and learning. Our results suggest that CaMKII binding to NR2B is the more important for synaptic plasticity and memory formation than NR1. However, we found that the intracellular domains of NR1 are critical for NMDAR and synapse function. These results have broad implications for neuronal excitability and plasticity in the hippocampus and cortex. Most importantly we identified a key molecular step involved in the consolidation of spatial memories.

Abstract Approved:_____
Johannes Hell, Thesis Supervisor

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Title and Department

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Date

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Graduate College
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Iowa City, Iowa

CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

Robert Francis Dallapiazza

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in pharmacology at the May 2010 graduation.

Thesis Committee: _____
Johannes Hell, Thesis Supervisor

_____
Donna Hammond

_____
Stefan Strack

_____
Yuriy Usachev

_____
Michael Welsh

_____
John Wemmie

To my sisters, Mariana and Meggie, for all that my studies have led me to miss.

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ABSTRACT

Our memories are fundamental components of who we are as individuals. They influence almost every aspect of our lives such as our decisions, our personalities, our emotions, and our purpose in life. Diseases that affect memory have devastating impacts on the individuals who bear them. Imagine not being able to recall pleasant memories or even the faces of close family members. It's important to understand the biology of memory formation not only because it's an intriguing scientific question, but because of its consequences when these processes are lost. N-methyl-D-aspartate-type glutamate receptors (NMDARs) and calcium/calmodulin-dependent kinase II (CaMKII) are essential molecules involved in learning and its physiological correlate, synaptic plasticity. Calcium influx through NMDARs activates CaMKII, which translocates to the postsynaptic signaling sites through its interactions with the NMDAR subunits NR1 and NR2B. The significance of CaMKII's translocation is not fully known, however we hypothesize that it is an early molecular event that is necessary for the expression of synaptic plasticity and learning. Our laboratory has developed two strains of mice with targeted mutations to NR1 and NR2B (NR1KI and NR2BKI) that are deficient in their ability to bind to CaMKII to test the role of CaMKII binding to NMDARs in synaptic plasticity and learning. We found that CaMKII binding to NR2B is necessary for long-term potentiation (LTP), the most commonly studied form of synaptic plasticity. NR2BKI mice are able to learn spatial and cued tasks normally, however they are unable to consolidate spatial tasks for long-term memory storage. On the other hand, we found that CaMKII binding to NR1 is not necessary for LTP. Furthermore NR1KI mice do not show impairments in contextual or cued learning. We found that NR1 mutations resulted in an age-dependent truncation of the intracellular domains of NR1 that reduced its activity leading to severe impairments in synaptic transmission, LTP, and learning. Our results suggest that CaMKII binding to NR2B is the more important for synaptic

plasticity and memory formation than NR1. However, we found that the intracellular domains of NR1 are critical for NMDAR and synapse function.

TABLE OF CONTENTS

LIST OF FIGURES

Figure

CHAPTER 1: GENERAL INTRODUCTION

Historical Perspective

The first steps in understanding the cellular and molecular mechanisms of learning and memory took place at the turn of the Twentieth Century when Santiago Ramon y Cajal, a Spanish physician, realized that cells within the brain were discrete and not continuous as was previously thought. Ramon y Cajal was studying the histology of the brain using a newly developed staining method that stained relatively few cells. This allowed him to observe the intricate branching of the dendritic arborization and identify several different types of brain cells based on their architecture (Swanson, Grant et al. 2007).

Around the same time Sir Charles Sherrington, a British physician studying spinal reflexes, noted that brain cells -- which he termed neurons -- made contacts with each other. In his book *Integrative action of the central nervous system* he postulated that neurons transmit information at these points of contact, structures that he called synapses (Burke 2007). Although, at the time it was unclear how synapses transmitted their signals between neurons, it was quickly noted that modifications of synaptic structure could be the basis for learning and memory.

These two landmark observations led to a host of new questions regarding signaling between neurons. One of the prominent questions was how synapses mediate their signals. At the time, scientists knew that electrical stimulation could causes skeletal muscles to twitch, and peripheral nerves were viewed as wires that carried specific sensory or motor signals. Not surprisingly, some scientists thought that synaptic connections were electrically coupled. However, this model was unable to account for time delays in relatively simple circuits such as spinal reflexes.

In 1924, Otto von Loewi provided a key insight to this question while studying vagal innervation of the heart. Loewi isolated frog hearts with the vagus nerve still attached (Loewi 1949). He noted that stimulation of the vagus slowed the rate of contraction not only in the heart that was attached to the nerve, but also to other hearts that were incubating in the same chamber. This suggested that some diffusible substance was mediating the slowing of the heartbeat, which ultimately led to the discovery of the first neurotransmitter, acetylcholine (Brown 2006). Acetylcholine and other neurotransmitters are released from presynaptic vesicles into a space between the neurons known as the synaptic cleft where they diffuse and interact with receptors within the postsynaptic membrane. Postsynaptic receptors then propagate the signal to the next neuron or cell.

The next theoretical breakthrough came in 1949 with the publication of Donald Hebb's book, *The Organization of Behavior*. In it he postulated: "*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."* (Hebb 1950). More simply stated, neurons that are coordinately activated strengthen their connects. The inverse can be said about neurons with sporatic or weak coordinate activity. Their connections are weakened or lost.

This phenomenon is seen in the developing visual system where signals from the retina need to be relayed through the thalamus to the cortex in a topographical manner that maintains spatial information. During development, retinal ganglion cells project to several layers in the lateral geniculate nucleus, thus resulting in dual innervation of each layer by both eyes. Over time, these connections are pruned in an activity-dependent manner that results in an input-specific lamination of the LGN from each eye (Udin 1985). Similar processes have also been described for the motor unit, the auditory system,

and the somatosensory system (Bennett 1983; Scheich 1991; Killackey, Rhoades et al. 1995).

The previous examples demonstrate how activity-dependent synaptic pruning can shape neuronal development, however what about in the adult brain? Do mature synapses demonstrate Hebbian properties? The first experimental evidence for the activity dependent modification of mature synapses came in 1973 when Bliss and Lomo demonstrated that brief, high-frequency stimulation of the Schaffer collaterals in the rabbit hippocampus could produce long-lasting increases in synaptic field potentials. They termed this phenomenon long-term potentiation (LTP) (Bliss and Lomo 1973). Since its discovery LTP has been the dominant model for studying the molecular events underlying synaptic plasticity. Synapses are highly dynamic structures that are capable of not only enhancing their efficiency but also depressing it based on the pattern and frequency of their activation. In 1982, Ito and Kano first described long-term depression (LTD) in the cerebellum (Ito and Kano 1982). LTD appears to be the functional inverse of LTP. Since its discovery in the cerebellum, LTD has also been observed in the hippocampus thereby demonstrating bidirectional plasticity (Bramham and Srebro 1987). Synapses can be tuned up or down based on their activity. Synaptic plasticity appears to be a general property of neurons. It has been observed in almost every system from the hippocampus and cerebellum to the visual cortex, striatum, and spinal cord (Calabresi, Maj et al. 1992; Calabresi, Pisani et al. 1992; Randic, Jiang et al. 1993; Racine, Teskey et al. 1994; Racine, Wilson et al. 1994; Liu and Sandkuhler 1995).

Properties of LTP

What makes LTP such an attractive model for studying learning and memory (Bliss and Collingridge 1993)? First, the induction of LTP is rapid. Enhanced synaptic transmission is seen on the timeframe of seconds to minutes following stimulation (Malenka and Nicoll 1999). Second, LTP is long lasting. In acute hippocampal slice

preparations, LTP can last for hours and in awake, behaving animals LTP can last for days (Bliss and Lomo 1973). Other forms of enhanced synaptic transmission such as paired-pulse facilitation and post-tetanic potentiation last only seconds to minutes. Since memories can last a lifetime, the synaptic changes that are associated with memory should last for a long time. Third, LTP is associative and input specific. It requires coincident activation of both presynaptic and postsynaptic neurons (Andersen, Sundberg et al. 1977; Lynch, Dunwiddie et al. 1977). For example, in a dual input recording from a single postsynaptic field, LTP can be induced in one pathway without changing the properties of the second. These properties make LTP an attractive model for studying the underlying basis of learning and memory since they share so many fundamental characteristics.

Behavioral LTP

Since the properties of LTP were so compelling, next question to be addressed was whether synaptic enhancement is actually seen during learning (Rioult-Pedotti, Friedman et al. 1998). High frequency stimulation used to induce LTP in slices is unlikely to occur naturally. Searching for synapses that are potentiated during learning was a formidable task since there are a large number of synapses that are involved during behavior. Furthermore, learning would likely involve both mechanisms of synaptic tuning; possibly decreasing efficiency in some synapses while increasing it in others. This could lead to little or no net effect in field potential amplitudes (Stevens 1998). One group approached this problem in mice by studying auditory inputs to the lateral amygdala (LA) through the medial geniculate nucleus (MGN). LeDoux and colleagues evoked potentials in the LA by stimulating MGN fibers. High frequency stimulation of this pathway in intact animals results in LTP in the LA. Furthermore, this enhancement can also be elicited by natural auditory stimulation (Rogan, Staubli et al. 1997). This study demonstrated that normal auditory transmission could utilize enhancements in

synaptic plasticity elicited by artificial stimulation. Despite the promise of these findings, several subsequent studies found no change in synaptic enhancement after exhaustive training paradigms (Hargreaves, Cain et al. 1990). Therefore the question of whether LTP is a laboratory phenomenon or is actually occurring during learning remains an open question.

Memory Formation

The formation of stable, long-term memories is generally broken down into four phases: acquisition, consolidation, retrieval, and expression (McGaugh 2000). Here, we will focus on the principle cortical and subcortical structures involved in the first three phases of memory formation. The acquisition and short-term processing of explicative information requires the hippocampus. Most famously, patient HM had profound deficits in short term memory after bilateral surgical resection of his medial temporal lobes (including the hippocampus.) Although he was able to recall remote memories pre-dating his surgery, he was unable to remember recent events and those post-dating his surgery (Scoville and Milner 1957). Subsequent studies in primates and rodents demonstrated the role of the hippocampus in the acquisition of declarative memories. Animals with hippocampal resections were unable to learn new information at the same rate as animals with sham-operations, indicating that the hippocampus is a critical structure in the primary processing of new memories (Morris, Garrud et al. 1982; Squire 1992).

Consolidation refers to the process by which newly acquired memories are stored after a novel learning experience (Lechner, Squire et al. 1999; McGaugh 2000). During consolidation, information is transferred from structures that are involved in the initial, rapid processing of memories to areas that are involved in memory storage. For example, spatial memories are initially encoded by the hippocampus and are then transferred to the cortex for storage. This process may take days to years to complete depending on the

nature of the memory to be stored. Experiments in primates and rodents using post-training lesions of the hippocampus show that recent spatial memories are disrupted after hippocampal damage, while remote memories are recalled with equal reliability to sham-operated controls. One model suggests that the hippocampus acts as a rapidly encoding 'weigh-station' that has limited capacity for storage. The hippocampus is then able to transfer information to areas with slower processing but with higher storage capacities(McClelland, McNaughton et al. 1995). Why might the cortex have slower processing than the hippocampus? One speculation is that cortical storage requires organization of the newly acquired memory into existing theoretical frameworks, thus takes more time (Alvarez and Squire 1994; Alvarez, Zola-Morgan et al. 1994).

Memory retrieval refers to the process by which a previously encoded memory is brought to consciousness (Tronson and Taylor 2007). There is increasing evidence to suggest that retrieved memories are labile after recall, and that a set of distinct cellular processes must reestablish the memory trace. This is termed reconsolidation, and is functionally and molecularly distinct from consolidation (Tronson and Taylor 2007). Recent studies have shown that without the process of reconsolidation, memory traces can be lost once they are retrieved (Lee 2008; Rudy 2008). These studies show that once a memory has been consolidated, they are not permanent or rigid. Ongoing acquisition of new information allows memory traces to be shaped and modified.

Consolidated memories can be modified in other ways as well. Under the classical conditioning paradigm, pairing conditioned stimuli (CS) with unconditioned stimuli (US) results in the ability to elicit a conditioned response (CR) from the CS alone. Pavlov famously demonstrated this by ringing a bell before feeding his dogs. However, after repeated un-reinforced CS presentation (that is CS without US), the CR is reduced in a process called memory extinction. This also appears to be a distinct process behaviorally and molecularly (Barad, Gean et al. 2006; Ji and Maren 2007).

Memory acquisition, consolidation, reconsolidation, and extinction represent distinct behavioral and neuronal processes that have unique molecular characteristics. Working together and in opposition, these processes shape the information that we are able to store for future use and are fundamentally important to our ability to adapt and utilize our surrounding resources. Therefore, it is important to investigate the molecular mechanisms of these processes.

Excitatory Synaptic Transmission

Synaptic transmission in the central nervous system is supported by several neurotransmitter systems. The principal excitatory and inhibitory neurotransmitters are glutamate and GABA, respectively. In general, several other neurotransmitters like norepinephrine, dopamine, and serotonin play important roles in modulating excitatory and inhibitory synaptic transmission. Glutamate acts via binding to both metabotropic (Baskys 1992) and ionotropic receptors (Dingledine, Borges et al. 1999). Metabotropic receptors belong to the seven transmembrane spanning receptor family, while ionotropic receptors are ligand gated ion channels. There are three main types of ionotropic glutamate receptors: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate (KA) receptors. These designations were principally based on differences in their affinity for various pharmacological agonists. Ionotropic glutamate receptors are tetramers (Behe, Stern et al. 1995; Furukawa, Singh et al. 2005) comprised of 17 distinct yet highly homologous gene products (Suchanek, Seeburg et al. 1995). AMPARs consist of GluR1-4 (Boulter, Hollmann et al. 1990), NMDARs consist of NR1, NR2A-D, and NR3A-B, and KARs consist of GluR5-7 and KA1,2. All subunits within this family share the same membrane topology. Each contains an extracellular amino-terminus (Hollmann, Maron et al. 1994), four amphipathic helical domains (TMI-IV), and an intracellular carboxy-terminus. The second helical domain (TMII) does not span the membrane; rather it forms a reentrant

loop within the plasma membrane and constitutes the pore of the channel (Kuner, Wollmuth et al. 1996). The extracellular loop between TMII and TMIII along with the amino-terminus forms the ligand-binding domain that has the characteristic structure of a Venus fly trap (Stern-Bach, Bettler et al. 1994).

AMPAR receptors mediate "fast" excitatory transmission in neurons and constitute a majority of synaptic currents under basal conditions (Muller, Joly et al. 1988). They play a crucial role in activity dependent synaptic transmission including long-term potentiation (LTP) and long-term depression (LTD). AMPARs are expressed throughout the central nervous system, however, their subunit composition is developmentally, regionally, and temporally regulated (Monyer, Seeburg et al. 1991; Nakanishi 1992). In the CA1 region of the hippocampus, most AMPARs are composed of GluR1-GluR2 and GluR2-GluR3 subunits. AMPARs demonstrate dynamic trafficking to and from the synapse. These can be inserted or removed from the plasma membrane via intracellular postsynaptic vesicles at endocytic "hot spots" adjacent to the synapse, or they can be inserted directly into the synapse. In the case of the former, AMPARs can then laterally diffuse from extra-synaptic sites into the synapse. This process is regulated by postsynaptic scaffold proteins like PSD95 and the AMPAR auxillary subunit, stargazin (Bredt and Nicoll 2003; Stein, House et al. 2003; Tomita, Adesnik et al. 2005). Furthermore, the composition of AMPARs seems to determine how they are trafficked to the synapse. GluR2-GluR3 containing receptors are thought to cycle constitutively directly from the synapse, while GluR1-GluR2 containing receptors are thought to reach the synapse by lateral diffusion in an activity-dependent manner (Racz, Blanpied et al. 2004; Kennedy and Ehlers 2006; Lu, Helton et al. 2007; Wang, Edwards et al. 2008). AMPARs are also regulated at the mRNA level. GluR2 mRNA is post-transcriptionally modified to replace an encoded glutamine for arginine (607), which imparts ion selectivity on receptors containing GluR2. GluR2-lacking receptors are Ca2+ permeable and show inward rectification (Egebjerg, Kukekov et al. 1994; Puchalski, Louis et al.

1994). That is they conduct inward current more easily than they conduct outward current. Inward rectification of GluR2-lacking receptors is due to a block by endogenous polyamines on the cytoplasmic side of the ion channel (Washburn and Dingledine 1996).

NMDARs are widely expressed throughout the central nervous system (Monyer, Sprengel et al. 1992) and contain two NR1 and two NR2 subunits. NR2A and NR2B are the predominate subunits expressed in the hippocampus and cortex (Monyer, Burnashev et al. 1994; Petralia, Wang et al. 1994) while NR2C is predominate in the cerebellum (Kopke, Bonk et al. 1993). Consistent with the Hebbian hypothesis on synaptic plasticity, NMDARs act as coincidence detectors, requiring both the presence of coagonists, glutamate and glycine (Johnson and Ascher 1987), and postsynaptic depolarization to promote channel conductance. Both glutamate and glycine are required for full activation of the receptor, with glycine binding to NR1 (Kleckner and Dingledine 1988) and glutamate to NR2 subunits (Anson, Chen et al. 1998). Near resting potentials, the pore of the receptor is blocked by a Mg^{+} ion, which limits NMDAR contribution to basal synaptic transmission (Crunelli and Mayer 1984; Nowak, Bregestovski et al. 1984; Coan and Collingridge 1985; Sharma and Stevens 1996). However, upon postsynaptic depolarization the Mg^{+} ion is driven out of the channel. Therefore, greater current passes through NMDAR at depolarized membrane potentials than hyperpolarized potentials. NMDARs have characteristically slower activation and deactivation kinetics compared to AMPAR and kainite receptors (Erreger, Dravid et al. 2005; Erreger, Geballe et al. 2007). The ion-conducting pore of NMDAR allows influx of Na^{+} as well as Ca^{2+}, with Ca^{2+} serving as an important secondary messenger (Hume, Dingledine et al. 1991; Burnashev 1996). Whereas AMPARs are highly motile within synaptic and extrasynaptic compartments, NMDARs are relatively immobile. Recent studies using quantum dot analysis to measure receptor motility show that lateral diffusion rates for AMPARs are approximately 100 times greater than that of NMDARs (Triller and Choquet 2005). However, among NMDARs the NR2 subunits seem to be more mobility. NR2A-

containing NMDARs are more mobile than tightly anchored NR2B-containing receptors (Groc and Choquet 2006; Groc, Heine et al. 2006).

Postsynaptic Signaling Molecules

CaMKII is a multifunctional serine/threonine protein kinase composed largely of α- and β- subunits in neurons. Separate genes encode the α- and β- subunits, however they share a high degree of sequence homology (Braun and Schulman 1995). CaMKII is expressed in a variety tissues throughout the body, and it constitutes ~1% of the total protein in the brain (Bennett, Erondu et al. 1983; Kennedy, Bennett et al. 1983). It is involved in a number of neural functions including neurotransmitter synthesis, synaptic vesicle exocytosis, and postsynaptic signaling (Schulman and Hanson 1993).

CaMKII is functionally divided into a catalytic, regulatory, and association domains. The regulatory domain consists of an overlapping autoinhibitory and calmodulin-binding domain (Tobimatsu and Fujisawa 1989; Tombes and Krystal 1997). The autoinhibitory domain prevents kinase activity by binding to the ATP binding site in the catalytic domain (Colbran, Smith et al. 1989; Lisman, Schulman et al. 2002). The carboxy-terminal association domain mediates oligomerization of subunits into a functional dodecamer consisting of two six-member rings (Rosenberg, Deindl et al. 2005; Rosenberg, Deindl et al. 2006). The association domain forms the dodecameric core, while the catalytic and regulatory domains project outward in a spoke-like fashion. This structure allows each subunit to bind Ca^{2+}-calmodulin independently and it allows intersubunit autophosphorylation (Mukherji and Soderling 1994).

Binding of Ca^{2+}-calmodulin to the regulatory domain displaces the autoinhibitory domain from the active site, thereby allowing substrate binding (Meyer, Hanson et al. 1992). The autoinhibitory domain contains a conserved threonine residue (T286) that can be autophosphorylated in a Ca^{2+}-calmodulin-mediated manner (Colbran and Soderling 1990; Hanson, Meyer et al. 1994; Rich and Schulman 1998). T286 phosphorylation

renders CaMKII Ca^{2+}-independent by preventing re-assocation of the autoinhibitory domain with the catalytic domain. T286 phosphorylated CaMKII remains autonomously active until dephosphorylated by protein phosphatases (Hudmon and Schulman 2002). T286 autophosphorylation therefore acts as a molecular switch transducing transient elevations of postsynaptic Ca^{2+} into prolonged kinase activity (De Koninck and Schulman 1998).

Protein Kinase A (PKA), also known as cyclic AMP-dependent kinase, is an abundant kinase in neurons (Browning, Huganir et al. 1985). It's composed of two regulatory and two catalytic domains that form a dimer of dimers. The regulatory subunits are responsible for targeting of the enzyme and inhibiting the catalytic subunits. PKA is activated when cAMP binds to the regulatory domains thereby releasing the catalytic domains thus allowing them to phosphorylate downstream targets. cAMP is produced from ATP by the integral membrane protein adenylyl cyclase (AC) (Beam and Greengard 1976). AC has eight isoforms that are positively regulated by the G-protein, Gs, and negatively regulated Gi. Two isoforms of AC (AC1 and AC8) are stimulated by calcium (Ferguson and Storm 2004). Several seven membrane-spanning receptors (7TMR, also GPCR), such as the adrenergic receptor, can activate Gs, thereby activation AC and PKA. PKA has several target substrates at synapse including AMPARs (Esteban, Shi et al. 2003) and NMDARs (Leonard and Hell 1997), and it plays an important role in LTP and LTD (Nguyen and Woo 2003; Lu, Allen et al. 2007; Lu, Zhang et al. 2008). Activation of PKA can also affect gene transcription. It phosphorylates a cAMP response element binding protein (CREB) that translocated to the nucleus where it acts as a cofactor in transcription (Waltereit and Weller 2003).

Protein Kinase C is another important synaptic kinase. Like PKA, its activation is mediated through 7TMR and G-proteins, however, in the case of PKC the prototypical G-protein involved is Gq. Gq activates phospholipase, which cleaves membrane phospholipids like PIP2 into diacylglycerol (DAG) and inositol phosphates (IP3). IP3

raises intracellular calcium levels by releasing it from intracellular stores, and DAG directly activates PKC activity. Some isoforms of PKC also require calcium for full activation. PKC has several important substrates at the synapse (Pasinelli, Ramakers et al. 1995; Ramakers, Pasinelli et al. 1997). It can phosphorylate NMDARs directly as well as key components of NMDAR trafficking (Zheng, Zhang et al. 1997). Activation of PKC leads to increased NMDAR surface expression and peak currents. PKC also phosphorylates AMPARs (Boehm, Kang et al. 2006), which increase their channel conductance (Lee, Barbarosie et al. 2000).

Protein phosphatases are also important for synaptic function. They play a central role in the induction of LTD. One prominent phosphatase is PP2A. PP2A is a heterotrimer consisting of scaffolding, regulatory, and catalytic subunits (Xu, Xing et al. 2006). Multiple genes encode all three subunits, and alternative splicing confers further proteomic variability. PP2A is targeted to its substrates by its variable regulatory subunit (B subunit) (Strack, Ruediger et al. 2002). Bβ1 and Bβ2 are splice variants are expressed principally in neurons. Bβ2 targets PP2A to mitochondria where it regulates mitochondria fission and fusion (Dagda, Zaucha et al. 2003; Cribbs and Strack 2007), while Bβ1 has a diffuse, cytosolic localization. PP2A also targets NMDAR and calcium channel signaling complexes where it opposes the action of kinases (Davare, Horne et al. 2000; Hall, Davare et al. 2007).

Synaptic Scaffolding Proteins

Electron micrographs of synapses clearly demonstrate an electron-dense band in the postsynaptic cell adjacent to the vesicle release site of the presynaptic cell. This region is approximately 400 microns thick and is termed the postsynaptic density (PSD) (Harris and Stevens 1989). The PSD is enriched with ligand-gated ion channels, metabotropic receptors, adhesion molecules, signaling complexes, and scaffolding proteins. Two of the most important classes of scaffolding proteins are the membrane-

associated guanylate kinase (MAGUKs) (Kim and Sheng 2004) and A kinase anchoring proteins (AKAPs) (Colledge, Dean et al. 2000). These proteins have direct interactions with ionotropic glutamate receptors, modulators of synaptic transmission, and downstream signaling molecules (Leonard, Davare et al. 1998; Tavalin, Colledge et al. 2002; Lim, Merrill et al. 2003; Lu, Allen et al. 2007).

PSD95 is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins that includes PSD93, SAP97, and SAP102 (van Zundert, Yoshii et al. 2004). These proteins contain three amino-terminal PDZ domains, which bind to the D-S-X-L motif at the carboxy-terminus of several transmembrane proteins like AMPAR and NMDARs. An SH3 domain and an inactive guanylate kinase domain follow the three PDZ domains. These proteins are thought to be adaptors for signal transduction cascades, linking upstream receptors to their downstream signaling partners for efficient, localized signaling. For example, PSD95 links AMPARs to the β_2AR. The first PDZ domain of PSD95 binds to the GluR1 subunit of AMPARs, and the third PDZ domain binds to β_2AR. This allows for specific regulation of AMPAR surface expression by norepinephrine (Joiner and Hell, unpublished).

A kinase anchoring proteins (AKAPs) bind to the regulatory subunits of PKA and target its catalytic activity to distinct subcellular regions. In addition to binding the regulatory subunits of PKA, these proteins also bind other signaling molecules such as phosphatases. Two AKAPs are prominent at synapses, AKAP150 and Yotiao. AKAP150 targets PKA to synapses via its final 36 residues (Dell'Acqua, Smith et al. 2006). We found that truncation of these residues in the mouse genome (D36) results in > 60% reduction in PKA targeting to the synapse. These mice demonstrate an age-dependent reduction in LTP, consistent with the developmental time frame that calcium-permeable AMPARs play a role in LTP. These findings led us to hypothesize that AKAP150-mediated anchoring of PKA facilitates the insertion of GluR2-lacking AMPARs shortly after LTP induction (Lu, Allen et al. 2007). Yotiao is another synaptic

AKAP. Whereas AKAP150 seems to modulate AMPARs, yotiao is implicated in regulating NMDAR signaling. Yotiao binds to the alternatively spliced C1 domain of the NR1 subunit of NMDARs (Lin, Wyszynski et al. 1998). Furthermore, PKA-mediated phosphorylation of NMDARs increases their permeability to calcium, thereby priming neurons for the induction of LTP (Skeberdis, Chevaleyre et al. 2006).

Synaptic CaMKII Accumulation

Activation of CaMKII changes in its sub-cellular localization. Inactive CaMKII primarily distributes throughout the cytosol, however upon Ca^{2+} influx through the NMDAR, CaMKII translocates and clusters at the postsynaptic density (PSD), a protein-dense structure adjacent to the postsynaptic plasma membrane. Activated CaMKII is recruited to the PSD presumably through high-affinity interactions with the NMDAR subunits NR2B and NR1 (Merrill, Chen et al. 2005). This places CaMKII in near proximity to additional calcium influx (through the NMDAR) and target substrates that are important mediators of synaptic transmission. CaMKII has several other synaptic binding partners such as densin180 (Strack, Robison et al. 2000; Dhavan, Greer et al. 2002; Krapivinsky, Medina et al. 2004; Okamoto, Narayanan et al. 2007), but NR1 and NR2B are its only identified partner that interact in activity-dependent manner (Merrill, Chen et al. 2005).

Interaction of CaMKII with NMDARs

CaMKII associates with the cytoplasmic tails of NR2B (residues 1290-1310, NR2B-C) (Strack and Colbran 1998; Strack, McNeill et al. 2000), NR1 (residues 847-858, NR1-C0) (Leonard, Lim et al. 1999; Bayer, De Koninck et al. 2001; Leonard, Bayer et al. 2002), and NR2A (Gardoni, Caputi et al. 1998; Gardoni, Schrama et al. 1999) in an activity-dependent manner (Figure 1). NR1 binding requires T286 phosphorylation, whereas binding to NR2B depends on either Ca^{2+}-calmodulin or T286 phosphorylation. NR2B binding locks CaMKII in its active conformation through its association with the

catalytic domain, thereby displacing the autoinhibitory domain even after removal of Ca^{2+}-calmodulin and independent of T286 phosphorylation (Bayer, LeBel et al. 2006). This region of NR2B is highly homologous to NR2A, however NR2A shows a much lower affinity for CaMKII than NR2B. Residue swapping between NR2A and NR2B demonstrated that L1298 and A1300 are necessary for CaMKII binding to NR2B (Strack, McNeill et al. 2000). Binding studies with extensive peptide libraries derived from NR1 C0 identified Q849 and N856 as critical residues for CaMKII binding (Leonard and Hell, unpubished).

The activity-dependent binding of CaMKII to the NMDAR stabilizes the kinase in its active form and promotes its clustering at postsynaptic signaling sites (Bayer, De Koninck et al. 2001; Barria and Malinow 2005). CaMKII has several other postsynaptic binding partners, however NR1 and NR2B are the only ones shown to strictly depend on CaMKII activation (see previous references). I hypothesize that CaMKII targeting to the NMDAR is a crucial event in the induction of synaptic plasticity where it places the kinase in proximity to its target substrates in active postsynaptic signaling sites. To test this hypothesis, we generated two strains of mice harboring mutations in NR1 and NR2B, which lack the necessary residues for activity-dependent binding of CaMKII to the NMDAR (Figure 2). Many studies suggest that NMDAR mediated targeting of CaMKII is important for synaptic plasticity (Merrill, Chen et al. 2005), however this has not been directly tested. These mice will allow us to assess the significance of activity-dependent CaMKII binding to the NMDAR in the induction of synaptic plasticity.

Mice with genetically modified NMDARs

Several groups have generated mice with genetically modified NMDARs (Sprengel and Single 1999). In the early 1990s, Forrest et al constructed mice that lacked the NR1 subunit ($NR1^{-/-}$). These mice died shortly after birth from an apparent respiratory failure. The gross neuroanatomical structures were intact, however there was

an impairment of whisker-related barrel formation in the somatosensory cortex, suggesting that while the projection of neurons to their target nuclei does not require NMDAR, fine-tuning of synaptic connections is NMDAR dependent (Forrest, Yuzaki et al. 1994; Li, Erzurumlu et al. 1994). Subsequent studies used the Cre-recombinase system to investigate the effects of region-specific knockout of NR1 within the hippocampal sub-fields (CA1, CA3, DG) in learning and memory. CA1-NR1 knockout mice have impairments in spatial representation and have difficulty solving nonspatial tasks. The authors conclude that CA1-NR1$^{-/-}$ mice adopt simple learning strategies that preclude the ability to solve complex tasks (McHugh, Blum et al. 1996; Tonegawa, Tsien et al. 1996; Tsien, Huerta et al. 1996). CA3-NR1 knockout mice show normal spatial learning in the Morris Water Maze (MWM) however; they show deficits in probe trials when a few of the spatial cues are removed from the maze, suggesting that they have impairments in spatial pattern completion (Nakazawa, Quirk et al. 2002; Nakazawa, Sun et al. 2003). DG-NR1 knockout mice also show normal acquisition of spatial memory, however they show selective impairments in spatial working memory (Niewoehner, Single et al. 2007). The results from these studies indicate that NMDARs within the hippocampus are necessary for proper short-term working memory.

Genetic knockout of NR2B (NR2B$^{-/-}$) also led to perinatal lethality, however these mice had deficits in the suckle response and likely died of starvation. These mice were viable to 7-8 days if they were hand fed. Like NR1$^{-/-}$ mice, NR2B$^{-/-}$ mice showed disruptions in whisker-related barrel formation in the somatosensory cortex, and synaptic long-term depression (LTD) was absent (Kutsuwada, Sakimura et al. 1996). NR2A$^{-/-}$ mice are viable and demonstrated deficits in hippocampal (CA1) LTP and had moderate deficits in spatial learning (Sakimura, Kutsuwada et al. 1995; Kiyama, Manabe et al. 1998).

Mice with genetically modified αCaMKII

Several genetic manipulations to CaMKII have also been investigated in mice (Elgersma, Sweatt et al. 2004). Complete genetic knockout of αCaMKII ($CaMKII^{-/-}$) results in impaired hippocampal LTP and spatial learning (Silva, Paylor et al. 1992; Silva, Stevens et al. 1992). Furthermore, disruption of αCaMKII autophosphorylation at T286 (T286D and T286A) also impairs hippocampal LTP and spatial learning (Mayford, Wang et al. 1995; Giese, Fedorov et al. 1998). These results indicate that persistent activation of CaMKII is necessary for the acquisition phase of memory formation.

Recently, Zhou et al investigated the role of CaMKII binding to NR2B in synaptic plasticity and learning by overexpressing the intracellular domains of NR2B (NR2B-CT) in an inducible manner. They found that overexpression of NR2B-CT reduced LTP and led to impairments in spatial learning in the MWM and radial arm maze (Zhou, Takahashi et al. 2007).

NMDARs in neurological and psychiatric diseases

Glutamatergic signaling is implicated in a wide array of neurological and psychiatric diseases including excitotoxicity during ischemia, neurodegenerative diseases, schizophrenia, and chronic pain. In this section, I will briefly discuss the role of NMDARs in these diseases. This will serve to highlight the importance of understanding the basic role of NMDARs in neurotransmission and synaptic plasticity.

Cardiovascular disease is highly prevalent in developed countries and in the U.S. it is the leading cause of death. One of the consequences of cardiovascular disease is cerebral ischemia, or stroke. During cerebral ischemia, glutamatergic neurons release excessive amounts of neurotransmitter resulting in the over-activation of NMDARs and excessive calcium influx. This leads to mitochondrial dysfunction, the activation of calcium dependent enzymes, and ultimately cell death (Lipton and Rosenberg 1994; Mattson 1997). NMDAR antagonists block excitotoxic cell death following ischemia

effectively in in vitro and whole animal models (Hajimohammadreza, Probert et al. 1995; Liu, Wong et al. 2007), however, the clinical utility of these drugs is diminished due to their highly adverse side effects (Muir and Lees 1995; Muir, Lees et al. 2004). One of the important questions researchers face concerning glutamate excitotoxicity is how to uncouple the pathological consequences of NMDAR over-excitation with the normal function of the receptor in unaffected neurons. This could lead to more efficient ways to treat patients suffering from stroke.

Alzheimer's Disease (AD) is characterized by progressive short-term memory loss that ultimately leads to dementia and death. Recent statistics suggest that 5.2 million Americans are currently living with AD, and this number will grow dramatically as the baby-boomers age into their 70s. The diagnosis of AD can be confirmed postmortem by the presence of neuritic plaques and neurofibrillary tangles, which are the remnants of dead neurons. Much of the research regarding AD has focused on the mechanisms of plaque and tangle formation. These processes seem to be the consequence of defective proteolytic processing of amyloid precursor protein by secretases. Recent clinical and basic research has demonstrated a role for NMDARs in the pathogenesis of AD (Small, Mok et al. 2001; Selkoe 2002). Clinically, patients that are treated with the partial NMDAR antagonist, memantine, show a slower decline in short-term memory loss (Scarpini, Scheltens et al. 2003). Recent studies have shown that Aβ-mediated spine loss in neurons requires NMDAR activity (Shankar, Bloodgood et al. 2007). These and other studies point to the important role of NMDARs in neurodegenerative diseases.

Schizophrenia is a chronic psychiatric disease characterized by hallucinations and delusions that affects approximately 1% of the world's population. It has a profound impact on thought processes and behavior, to the extent that many people with schizophrenia are unable to hold jobs and care for themselves. Despite the fact that acute psychosis and schizophrenia are principally treated with medications that target dopamine receptors (Levinson 1991), NMDAR dysfunction has been strongly implicated in these

disorders. One of the primary observations supporting this hypothesis is that acute intoxication of NMDAR antagonists, like ketamine, causes psychosis (Wachtel and Turski 1990). NMDARs and dopamine receptors are expressed in the same neurons within the striatum and they seem to be able to modulate each other's activity. Furthermore, two recently generated genetically modified strains of mice show behavioral abnormalities that are somewhat ameliorated by traditional antipsychotic therapy (Mohn, Gainetdinov et al. 1999; Duncan, Moy et al. 2004; Labrie, Lipina et al. 2008; Labrie, Clapcote et al. 2009). One strain expresses low amounts of NMDARs, and the other has a mutation in the ligand-binding domain that dramatically reduces agonist affinity. These studies not only offer further insight into the pathophysiology of psychosis and schizophrenia, but they also provide potential targets for more advanced therapeutics.

Chronic pain is a complex, debilitation disorder characterized by increased sensitivity of neurons in the nociceptive circuits. It can be caused by a variety of factors such as chronic inflammation or nerve injury. Nociceptive neurons in the periphery project to the spinal cord and synapse within the dorsal horn. These neurons are excitatory in nature, relying on glutamatergic signaling and ionotropic glutamate receptors (Bleakman, Alt et al. 2006; Qu, Cai et al. 2009; Zhuo 2009). Like neurons of the hippocampus, dorsal horn neurons can adjust their synaptic efficiency based on the pattern and frequency of their inputs as in LTP. In fact, several forms of sustained increases in synaptic transmission have been characterized. Like hippocampal LTP these forms of enhanced transmission are dependent on NMDARs (Woolf and Salter 2000). NMDAR antagonists block many forms of chronic pain in rodent models, highlighting their importance.

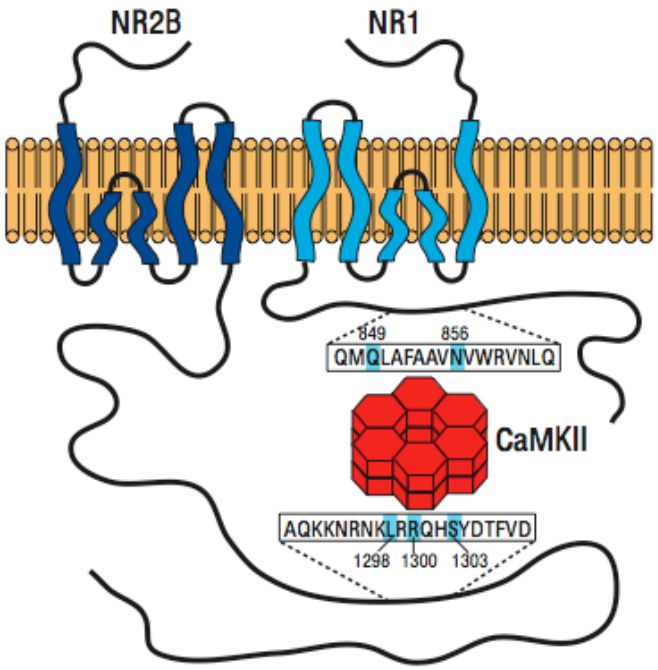

Figure 1: Schematic of CaMKII binding to NR1 and NR2B. The critical residues for NR1 and NR2B binding to CaMKII are shaded in blue. Figure contributed by Duane Hall.

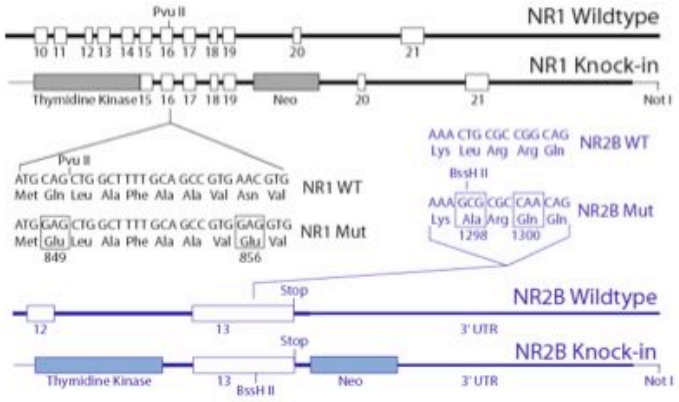

Figure 2: Schematic of NR1KI and NR2BKI mutations. NR1 targeting vector black: A Neomycin resistance gene flanked by two loxP sites was inserted in antisense orientation into the intron between exon 19 and 20 of the NR1 gene. This was flanked by a 2.2 kb short arm spanning exon 15-19 and a 6.1 kb long arm containing exon 20 and 21 (the first alternatively spliced exon). Two copies of the HSV thymidine kinase gene were attached 5' to the short arm for negative selection of randomly integrated vector. The short arm was generated by PCR using Sv129J mouse genomic DNA as template. Three point mutations were introduced to generate the Q849E and N856E mutations, simultaneously destroying a Pvu II site for diagnostic purposes. NR2B targeting vector (blue): a Neomycin resistance gene flanked by loxP sites was inserted in an antisense orientation 3' of the stop codon of the NR2B gene between a 2.1 kb short arm spanning exon 13 and a long arm containing 3.1 kb of the 3' untranslated region. Two HSV thymidine kinase genes were attached 5' to the short arm. The short arm was generated by PCR using Sv129J mouse genomic DNA. Three point mutations were introduced to generate the L1298A and R1300Q mutations, simultaneously creating a BssH II site for diagnostic purposes. Both targeting constructs were linearized by digestion with Not I and separately electroporated into E14 ES cells (Juntti, Brose, and Hell; unpublished data).

CHAPTER 2: THE ROLE OF CAMKII BINDING TO NR2B IN SYNAPTIC PLASTICITY AND MEMORY FORMATION

Introduction

The hippocampus is necessary for the acquisition and short-term processing of declarative memories (Morris, Garrud et al. 1982; Squire 1992). This was most famously demonstrated in Patient HM, who after bilateral resection of his medial temporal lobes, suffered from retrograde amnesia and was unable to acquire new declarative information. He was, however, able to accurately recount remote memories from his childhood (Scoville and Milner 1957). Further studies using experimental resection of the hippocampus in primates and rodents confirmed the role of the hippocampus in memory acquisition and in retrograde amnesia. Animals with hippocampal resections had a preferential loss of recently acquired memories but were able to remember and perform remotely trained tasks (Squire 1992). This led to the idea that initial processing of memories took place in the hippocampus, but long-term storage of declarative memories took place elsewhere and was independent of the hippocampus. Newly acquired memories must then be transferred from the hippocampus to areas of long-term storage such as the cortex, a process termed consolidation.

There is compelling evidence that N-methyl-D-aspartate receptors (NMDARs) are necessary for memory acquisition and for the induction of synaptic plasticity (Harris, Ganong et al. 1984; Muller, Joly et al. 1988; Murphy, Reid et al. 1997; Malenka and Nicoll 1999; Maren 1999). NMDARs are ionotropic glutamate receptors that have two unique properties. First, near resting membrane potentials, the pore of the receptor is blocked by a Mg^{+} ion, which limits NMDAR contribution to basal synaptic transmission. However, upon postsynaptic depolarization the Mg^{+} ion is driven out of the channel allowing current passes through NMDAR (Crunelli and Mayer 1984; Nowak,

Bregestovski et al. 1984; Coan and Collingridge 1985; Sharma and Stevens 1996). Second, NMDARs are highly permeable to calcium, a critical second messenger in neurons (Hume, Dingledine et al. 1991; Burnashev 1996).

During NMDAR activation, postsynaptic calcium entry triggers the activation of several signaling molecules including α calcium/calmodulin-dependent kinase II (αCaMKII) (Shifman, Choi et al. 2006). CaMKII also plays a central role in memory acquisition and in some forms of synaptic plasticity like hippocampal long-term potentiation (LTP). Disruption of CaMKII activity blocks LTP and impairs spatial learning (Malinow, Schulman et al. 1989; Tsien, Schulman et al. 1990). As a holoenzyme, CaMKII consists of 12 subunits forming a stacked hexameric ring structure. Each subunit contains three domains, catalytic, autoinhibitory, and association domains. CaMKII is initially activated by calcium-calmodulin displacement of the autoinhibitory domain from the catalytic core. However, with sufficient activation, the kinase is able to autophosphorylate adjacent subunits within the autoinhibitory domain, thus rendering CaMKII insensitive to drops in intracellular calcium and conferring persistent activity. These properties suggest that CaMKII functions as a molecular switch that can be activated for prolonged times without further rises in postsynaptic calcium (Hudmon and Schulman 2002). Along with the voltage-sensitivity of the NMDAR, these two molecules provide compelling molecular evidence for the input-specificity and longevity of LTP and memory.

Activated CaMKII is recruited to synapses through its interactions with the intracellular domains of the NMDAR subunits NR1 and NR2B (Strack and Colbran 1998; Leonard, Lim et al. 1999; Strack, McNeill et al. 2000; Bayer, De Koninck et al. 2001; Leonard, Bayer et al. 2002). This likely serves two important purposes. First, CaMKII binding to NR2B stabilizes the kinase in its active form. Residues 1290-1310 of NR2B bind to a pseudosubstrate site adjacent to the catalytic core of CaMKII, thereby preventing re-association of the autoinhibitory domain and further maintaining catalytic

activity of CaMKII (Bayer, LeBel et al. 2006). Second, CaMKII binding to NMDARs places the kinase in near proximity to further calcium influx and to its substrates involved in mediating LTP and learning. CaMKII phosphorylates several key proteins that are important for enhancing excitatory synaptic transmission including the AMPAR subunit, GluR1, and AMPAR auxillary proteins like stargazin (Hayashi, Shi et al. 2000). Given the importance of both NMDARs and CaMKII in LTP and learning, we hypothesized that CaMKII binding to NR2B is a crucial event in the induction of these processes.

To directly test this hypothesis and further characterize the importance of CaMKII recruitment to synapses, we generated a strain of knock-in mice with mutations in NR2B designed to block activity-dependent binding of CaMKII to the NMDAR (NR2BKI, see Figure 2). We exchanged residues L1298 and R1300 to A and Q, which have been shown to reduce CaMKII to NR2B by 90 percent in previous in vitro studies (Strack, McNeill et al. 2000). These mutations were designed to specifically block association of CaMKII with NR2B while having minimal impact on proteins associated with NMDARs like PSD95.

Results

Generation and Characterization of NR2BKI mice. We used homologous recombination to insert knock-in mutations within the NR2B gene. First, we first generated a plasmid containing a 2.1 kb short arm spanning exon 13 and a long arm containing 3.1 kb of the 3' untranslated region (Figure 2). Two HSV thymidine kinase genes were attached 5' to the short arm for negative selection of randomly inserted vector. For positive selection we inserted a neomycin resistance gene flanked by loxP sites in an antisense orientation 3' of the stop codon between the short and long arms. Three point mutations were introduced to generate the L1298A and R1300Q mutations, simultaneously creating a BssHII site for diagnostic purposes. The targeting plasmid was linearized by digestion with NotI and electroporated into E14 ES cells.

Mice were backcrossed into the C57/B6 for 7 generations before we began experiments. The neomycin cassette was removed in a backcross with mice expression Cre-recombinase.

NR2BKI mice bred normally and showed no obvious physical abnormalities. Nissl staining of the forebrain showed no gross morphological abnormalities within the hippocampus or layers of the cortex (Figure 4). Analysis of forebrain lysates and purified post-synaptic densities showed no change in the expression of NMDAR subunits in the KI mice (Halt, personal communication), and immunostaining of cultured hippocampal neurons showed proper localization of NR2B-containing receptors under both naïve and activity-dependent conditions (Halt, personal communication). We concluded that the KI mutations did not cause a gross developmental defect or affect expression or localization of NMDARs (Kutsuwada, Sakimura et al. 1996).

NR2B is necessary for activity-dependent CaMKII synaptic targeting (Barria and Malinow 2005). To test whether the KI mutations affected activity-dependent binding of CaMKII with NR2B, we co-immunoprecipitated (coIP) CaMKII from acute forebrain slices treated with vehicle or NMDA and probed for the NMDAR subunits NR2B and NR1. Consistent with previous findings, we saw an increase in NR2B and NR1 coIP with CaMKII after NMDA treatment in slices from WT mice. However we did not observe any increase in parallel experiments from KI mice (Figure 3). Importantly, we did not observe any change in the expression levels of NR1, NR2B, CaMKII, PSD95, or GluR1 in forebrain membranes; nor did we detect any change in PSD95 coIP with NR2B in KI mice compared to WT controls. These results demonstrate that residues L1298 and R1300 are necessary for activity-dependent binding of CaMKII to NMDARs, but do not affect the expression of NMDAR subunits or the association of NR2B with PSD95.

Previous work suggests that NR2B is important for activity-dependent synaptic recruitment of CaMKII (Barria and Malinow 2005). CaMKII associates with several postsynaptic proteins, including densin180, however NR2B and NR1 are the only ones

thus far shown to interact strictly in an activity-dependent manner (Merrill, Chen et al. 2005). We tested synaptic localization of CaMKII in cultured hippocampal neurons from WT and KI mice following treatment with vehicle or glutamate. We assessed colocalization of CaMKII and the presynaptic marker, synapsin. Following glutamate treatment, we saw punctate CaMKII immunostaining with increased colocalized with synapsin in WT neurons, whereas in KI neurons CaMKII only showed punctate staining but no increase in synapsin colocalization (Halt, personal communication). These results demonstrate that this motif is necessary for CaMKII recruitment to synapses during NMDAR activation.

CaMKII binding to NR2B is necessary for LTP. Previous studies have demonstrated that NMDARs and CaMKII are indispensable for the induction of LTP at hippocampal CA3-CA1 synapses. Pharmacological inhibition or genetic disruption of either NMDARs or CaMKII blocks LTP in these synapses (McHugh, Blum et al. 1996; Tonegawa, Tsien et al. 1996; Tsien, Huerta et al. 1996). Furthermore, recent work has shown that CaMKII binding to NR2B is important for LTP (Barria and Malinow 2005; Zhou, Takahashi et al. 2007). We tested LTP induction in acute slices and found that LTP was significantly reduced, albeit not absent, in slices from KI mice compared to wt controls with a 100Hz/1sec induction protocol (Figure 5). We also observed reduced LTP with two other stimulation protocols, 10Hz/15 sec and 10 theta bursts (Figure 6) (Thomas, Watabe et al. 1998). These results are consistent with previous reports and further confirm that CaMKII binding to NR2B is necessary for LTP (Barria and Malinow 2005). Several forms of synaptic plasticity at the CA3-CA1 synapse are not strictly dependent on CaMKII activation. Long-term depression (LTD) is the physiological opposite to LTP and results from the preferential activation of phosphatases during NMDAR activity. We found no difference in LTD in slices from KI mice compared to WT mice (Figure 5).

Differences in synaptic plasticity can be influenced by changes basal synaptic strength, therefore we sought to determine if there were changes in basal AMPAR or NMDAR synaptic function. We measured field potentials with increasing stimulation intensity and plotted fiber volley amplitude versus field potential slope. Our experiments were conducted in normal ACSF (principally AMPAR mediated) and in the presence of DNQX and 0 mM Mg+ (principally NMDAR mediated) and found no difference between KI and WT controls in either condition (Figure 8). We also measured spontaneous activity in CA1 pyramidal hippocampal neurons. Recordings were made in the presence of TTX, APV, and PTX to isolate AMPAR currents. We found no change in mEPSC amplitude or frequency (Figure 9). Together, these results indicate that CaMKII binding to NR2B is not necessary for basal synaptic function. Furthermore, we did not observe a cumulative effect on synaptic function.

Since LTP was not completely absent in slices from KI mice, we tested whether the remaining potentiation in the KI mice was NMDAR and CaMKII-dependent. We induced LTP in the presence of APV or KN93, respectively. Both treatments completely abolished LTP in both genotypes, suggesting that residual LTP in KI mice was NMDAR-dependent and not due to compensatory mechanisms (Figure 7). Furthermore, these results suggest that NMDARs and CaMKII play an important role in LTP independent of their association.

CaMKII binding to NR2B regulates its activity at the synapse. Since we saw deficits in hippocampal LTP we wanted to assess CaMKII activation by T286 phosphorylation and synaptic CaMKII accumulation in forebrain slices following NMDAR activation. For this we turned to the chemical LTP (cLTP) protocol developed by Makhinson et al. This protocol has been shown to both induce CaMKII T286 phosphorylation and persistently increase synaptic CaMKII accumulation in an NMDAR-dependent manner (Makhinson, Chotiner et al. 1999). We treated slices from WT and KI mice with the cLTP protocol and found that while CaMKII initially underwent

autophosphorylation at T286 after treatment, this increase did not persist after a 30 minute chase in slices from KI animals compared to WT controls (Figure 13). Furthermore, using crude PSD preparations we found that CaMKII did not accumulate in isolated PSDs from KI mice compared to WT controls. This data conclusively demonstrates the importance of CaMKII binding to NR2B in the synaptic recruitment of CaMKII and its prolonged activation, but not in its initial activation.

We next assessed the phosphorylation state of synaptic substrates for CaMKII. GluR1 S831 is a prominent substrate for CaMKII. Phosphorylation at this site increases AMPAR conductance and is thought to be important for LTP (Kauer, Malenka et al. 1988; Benke, Luthi et al. 1998). We treated slices with vehicle control or NMDA and assessed GluR1 S831 phosphorylation. We found NMDA treatment increased GluR1 S831 phosphorylation in slices from WT but not KI mice, consistent with the finding that S831 phosphorylation corresponds to activated CaMKII at the synapse (Figure 13). Another important CaMKII substrate in the hippocampus is γ-8, a member of the TARP family of proteins. TARPs are important mediators of synaptic trafficking of AMPARs. γ-8 has 9 phoshporylation sites within its intracellular domains, and phosphorylation regulates not only their association with synapse but also AMPARs (Hayashi, Shi et al. 2000). Again, we treated slices with the cLTP protocol and measured PSD association as a surrogate marker for phosphorylation (since commercial Abs are not available). Similar to our results with CaMKII autophosphorylation γ-8 association with the PSD initially increased upon treatment in both genotypes, but did not persist after a 30-minute chase period in KI samples compared to WT controls (Halt, personal communication). These results are the first to suggest that NR2B bound CaMKII is necessary for stabile synaptic localization of γ-8.

CaMKII binding to NR2B is necessary for memory consolidation. Activation of NMDARs and CaMKII are firmly established in the acquisition of spatial memory. Furthermore, CaMKII binding to NR2B has been suggested to be an important

component in learning spatial tasks (Barria and Malinow 2005; Zhou, Takahashi et al. 2007). To test the role of CaMKII binding to NR2B in learning and memory we turned to context fear conditioning. Mice were given a single foot-shock (0.5 mA, 2 sec) for five consecutive days over a 5 min trial. KI mice initially demonstrated equivalent freezing compared to WT mice on day 2; however on days 3 and 5, KI mice froze significantly less than WT mice (Figure 4F). Furthermore, on day 6, when no shock was delivered, KI mice froze only 33.2±3.7% of the time compared to 53.0±7.9% of the time for WT mice (Figure 11). These results suggest that CaMKII binding to NR2B is necessary for contextual fear learning. However, since trials were conducted on consecutive days, it was possible that memory recall was affected rather than memory acquisition.

To directly test this idea we trained mice using a contextual fear paradigm that avoided prolonged shock intervals. We delivered 5 footshocks (0.75 mA, 1 sec) during a 10 min trial (60 sec interval) (Wemmie, Askwith et al. 2003). With this training protocol, no difference was observed between WT and KI mice. This suggests that memory acquisition was not affected in KI mice. We tested half the mice 1 hour after training for immediate memory recall. Freezing times were not statistically different between genotype indicating that short-term memory was intact in the KI mice. We then tested the other group of mice 4 days after training and found that KI mice froze significantly less than WT mice (Figure 11). These results indicate that CaMKII binding to NR2B is not necessary for memory acquisition or immediate recall, but is necessary for the consolidation of contextual fear memories.

We also tested mice for cued fear learning and memory using five 20 sec tones co-terminating with a 0.75-mA/1-sec shock. Tone-shock pairings were delivered in a single 14-min trial (100 sec shock interval). Similar to our results with contextual fear conditioning, the acquisition of cued fear was not statistically different in KI mice compared to WT controls. One day or two weeks after initial training mice were placed in a novel context and were exposed only to the tone. WT and KI mice showed

equivalent freezing during tone presentation at both time points (Figure 12). These results suggest that CaMKII binding to NR2B is specifically necessary for the consolidation of context fear memories, but not cued fear memories. Context learning requires the hippocampus and the amygdala, whereas cued fear learning requires only the amygdala (LeDoux 2003).

Finally we tested spatial learning and memory in the Morris water maze (MWM). Mice were trained in two blocks for six days and probed without the hidden platform after day 5 training and 3-days after the completion of training. We found no difference in escape latency over the six-day training period (Figure 4A). Day 6 escape latencies were 16±3.5 and 10.6±2.6 sec for WT and KI, respectively. The fifth day probe without the platform showed no difference in time spent searching the target quadrant (TQ) or in the proximity of searching the target location (Figure 4B and 4C). WT mice spent 40.2±6.1% of time searching the TQ compared to 38.9±2.8% for KI mice, indicating that NR2BKI mice are able to learn and immediately recall spatial tasks normally. However, three days after training, the escape platform was again removed, and the mice were assessed by search time in the TQ and proximity to the target. WT mice searched the TQ 51.0±3.2% of time whereas KI mice spent only 35.9±4.1% of time in the TQ (Figure 4D). WT mice searched much closer to the target than KI mice (45.5±3.3 cm versus 35.7±2.1 cm, Zhou and Silva, personal communication). These results are consistent with our findings from contextual fear conditioning. KI mice are able to learn and immediately recall context and spatial tasks normally, however they are unable to properly consolidate these memories. This suggests that CaMKII binding to NR2B is important for the consolidation of spatial memories but not during the acquisition and short-term processing of these memories.

Discussion

Synaptic accumulation of CaMKII. CaMKII associates with several components of the postsynaptic density, and it has multiple synaptic substrates (Strack, Robison et al. 2000; Merrill, Chen et al. 2005). However, CaMKII's association with NR1 and NR2B are the only interactions that have been shown to be specifically activity-dependent thus far (Merrill, Chen et al. 2005). Our findings in cultured hippocampal neurons indicate that CaMKII binding to NR2B is critical for activity-dependent accumulation of CaMKII in the postsynaptic density (Barria and Malinow 2005). Further, in acute slice preparations from NR2KI mice, stimulation of NMDARs leads to robust activation and autophosphorylation of CaMKII. However CaMKII activity is not persistent, and autophosphorylation levels return to baseline within 30 minutes, while autophosphorylation remains high in slices from WT mice. This suggests that NR2B is critical for postsynaptic accumulation of CaMKII, and that this interaction is necessary for persistent CaMKII activity.

Synaptic accumulation of CaMKII has been shown to leads to increased phosphorylation of GluR1 S831, a site that has been proposed to be important for increased conductance of AMPARs and recruitment of AMPARs into the synapse. Considering our findings that CaMKII did not accumulate at synapses in NR2BKI mice, it was not surprising to find that GluR1 S831 phosphorylation levels were reduced upon NMDAR stimulation in slices from NR2BKI mice (Kauer, Malenka et al. 1988; Benke, Luthi et al. 1998). However, we were surprised to find that levels of synaptic TARP γ-8 were reduced in crude PSD preparations from NR2BKI mice, suggesting that CaMKII binding to NR2B was important in the stabilization of TARP γ-8 at the synapse (Hayashi, Shi et al. 2000).

CaMKII binding to NR2B in LTP. Consistent with previous studies, we found that CaMKII binding to NR2B is necessary for hippocampal LTP, however disruption of the CaMKII-NR2B interaction does not completely abolish LTP (Barria and Malinow

2005; Zhou, Takahashi et al. 2007). Our finding that initial CaMKII activation is equivalent in slices from KI mice but does not persist compared to WT mice correlates well with our LTP studies where post-tetanic potentiation is normal but LTP is clearly reduced by 30 minutes in NR2BKI mice. These results strongly suggest that activity-dependent binding of CaMKII to NR2B is necessary for persistent enhancements of activity in hippocampal neurons, and that synaptic accumulation of activated CaMKII is necessary for orchestrating the molecular events underlying LTP.

CaMKII in contextual memory consolidation. Our results suggest that CaMKII binding to NR2B is necessary for the consolidation of spatial and contextual fear memories, but not for the consolidation of cued fear memories. In contrast to previous studies, we find that this interaction is not necessary for the acquisition phase of learning or in immediate memory recall (Barria and Malinow 2005; Zhou, Takahashi et al. 2007). Previous studies have strongly implicated NMDAR and CaMKII activation in memory acquisition. Pharmacological blockade of either NMDARs or CaMKII causes deficits in many forms of learning (Malinow, Schulman et al. 1989; Tsien, Schulman et al. 1990). Our studies show that specifically blocking the interaction of CaMKII with NR2B leads to deficits in memory consolidation. This suggests that NMDARs and CaMKII have other effects independent of their association during memory acquisition. CaMKII activation by NMDARs may be important for the acquisition of memories, however, persistent CaMKII activity and synaptic accumulation seem to be important in hippocampal dependent memory consolidation.

Our model is limited by the fact that all neurons in the CNS express a form of NR2B that has reduced affinity for activated CaMKII. This system does not allow a clear interpretation for the role of CaMKII binding to NR2B in any particular region involved in the consolidation process. The observed deficits in contextual memory consolidation may be due to aberrations in any number of regions involved in consolidation, or may be the result of cumulative deficiencies in multiple brain regions.

However, one key finding was that the deficits we observed in NR2BKI mice seemed to be specific for the consolidation of hippocampal-dependent memories. Cued fear conditioning relies on the amygdala and is largely independent of hippocampal function. This has two important implications. First, our results suggest that transference of memories from the amygdala to the cortex remains intact, implying that cortical neurons are able to undergo activity-dependent changes that are required for long-term memory. However, consolidation of hippocampal dependent memories is impaired when CaMKII cannot associate with NR2B. This suggests that CaMKII-dependent modulation of hippocampal excitability and processing are the primary deficits in NR2BKI mice.

Second, these results suggest that the molecular mechanisms that underlie consolidation of hippocampal-dependent memories are fundamentally different than the consolidation of amygdala-dependent memories. The acquisition of cued fear memories is dependent on NR2B-containing NMDARs in the amygdala (Rodrigues, Farb et al. 2004), however our data suggest that CaMKII binding to NR2B is dispensable for this process. Furthermore, our data suggest that this interaction is not required for sustained activity of amygdaloid neurons during the consolidation phase. On the other hand, CaMKII binding to NR2B is necessary for the consolidation of classically known hippocampal memories.

Model for memory consolidation. The molecular mechanisms of memory consolidation are poorly understood. Recent studies have focused NMDAR activity and the role of protein synthesis during the consolidation phase of memory. Cui et al recently described an inducible, reversible transgenic model for inhibiting NMDAR activity. They found that inhibiting NMDARs after fear training blocked consolidation of both contextual and cued fear memories (Cui, Wang et al. 2004). From these experiments they proposed a model were several rounds of NMDAR-dependent synaptic adjustments are required for memory consolidation, a process they termed synaptic reentry reinforcement (SRR) (Wang, Hu et al. 2006). They suggest that this process serves as a

cellular mechanism for the hippocampus to transfer newly acquired memories to the cortex for long-term storage. This model is logical since most forms of cortical plasticity are dependent on NMDARs. One conceivable notion is that enhanced activity in hippocampal neurons drives to excitability of downstream neurons in the entorhinal cortex and association cortices during memory consolidation. Our results fit this model and further delineate the role of CaMKII binding to NMDARs in this process. From the results of this study we conclude that activity-dependent association and synaptic accumulation of CaMKII by NR2B is necessary for the consolidation of hippocampal-dependent memories, however this mechanism does not seem to be conserved for hippocampal-independent forms of memory consolidation.

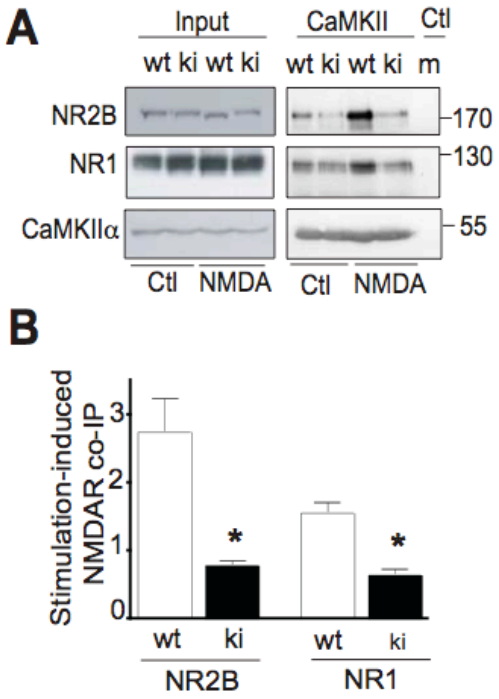

Figure 3: Activity-dependent CaMKII association with NMDARs is reduced in NR2BKI mice. (A) Right immunoblots: CaMKII was immunoprecipitated from deoxycholate-solubilized membranes prepared from acute forebrain slices. Slices were treated with vehicle control or 100 uM. CoIP of NR2B and NR1 increased with NMDA treatment in slices from WT mice, however there was no increase in coIP in slices from NR2BKI mice. Left immunoblots: Relative amounts of CaMKII, NR1, and NR2B were equal in forebrain lysates used for coIPs. **(B)** Quantification of NMDAR coIP with CaMKII. NR1 and NR2B coIPed significantly more in slices from WT mice compared to KI ($p < 0.05$). Respective contributions: Amy Halt performed these experiments and analyzed the data with the assistance of Robert Dallapiazza.

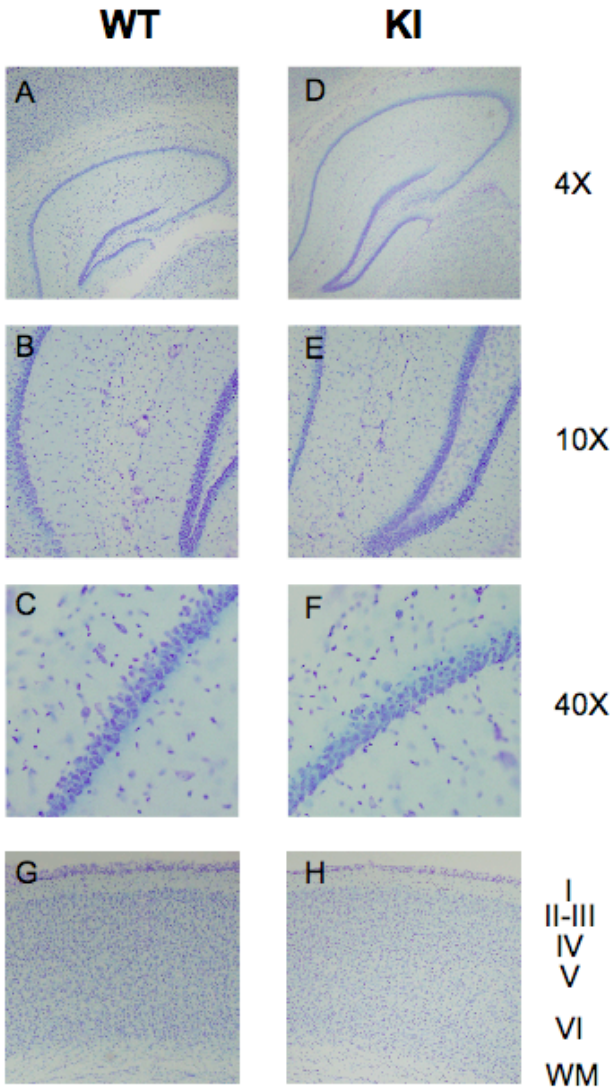

Figure 4: Gross morphology of the hippocampus and cortex is normal in NR2BKI mice. (A-C) Nissel stained sections (60 microns) from the hippocampus of WT mice (Magnification: 4X, 10X, 40X). **(D-F)** Nissel stained sections from the hippocampus of NR2BKI mice show no gross morphological abnormalities (Magnification: 4X, 10X, 40X). Respective contributions: Robert Dallapiazza prepared and stained the hippocampal and cortical sections with the help of Ronald Merrill.

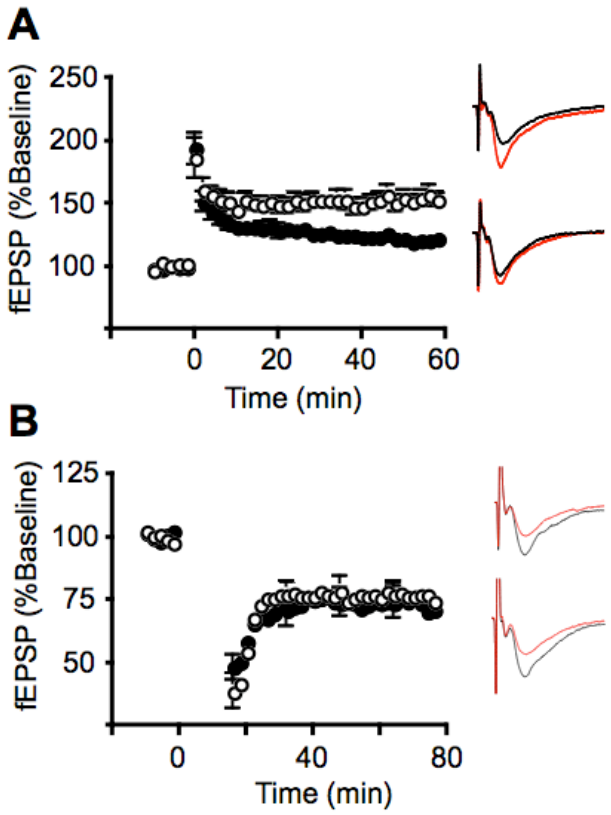

Figure 5: Hippocampal LTP is reduced in NR2BKI mice. (A) LTP induced by two trains of 100-Hz stimulation showed a significant difference (Students t-test, $p < 0.05$) between slices from WT (N = 10) and NR2B KI (N = 13) mice. Baseline representative traces are in black and traces 60 min after LTP induction are shown in green. Scale bars are 5 ms and 1 mV. **(B)** Long term depression (LTD) induced by 1-Hz stimulation for 15 min showed stabilization at 76% and 72% of the original fEPSP amplitude in slices from WT (N = 9) and NR2BKI (N = 10) animals (Student's t-test, $p > 0.05$). Baseline representative traces are in black and traces 60 min after LTD induction are shown in green. Scale bars are 5 ms and 1 mV. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

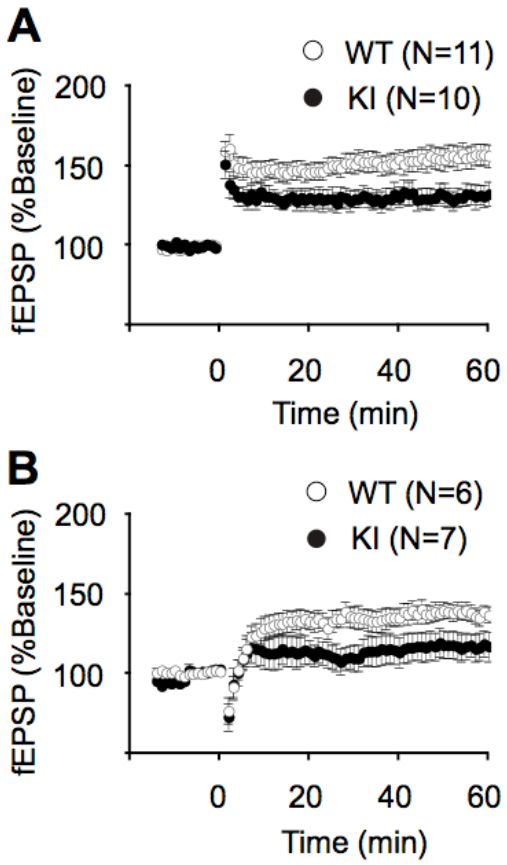

Figure 6: Hippocampal LTP is reduced with multiple induction protocols. (A) LTP induced by ten bursts of theta stimulation showed a significant difference (Student's t-test, $p<0.05$) between slices from WT (N = 11) and NR2BKI (N = 9) mice. **(B)** LTP induced by 10-Hz stimulation for 15 seconds showed a 151% increase in fEPSP left slope in slices from WT mice (N = 7) and an 135% increase in slices from NR2BKI mice (N = 7, Student's t-test p = 0.27). Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

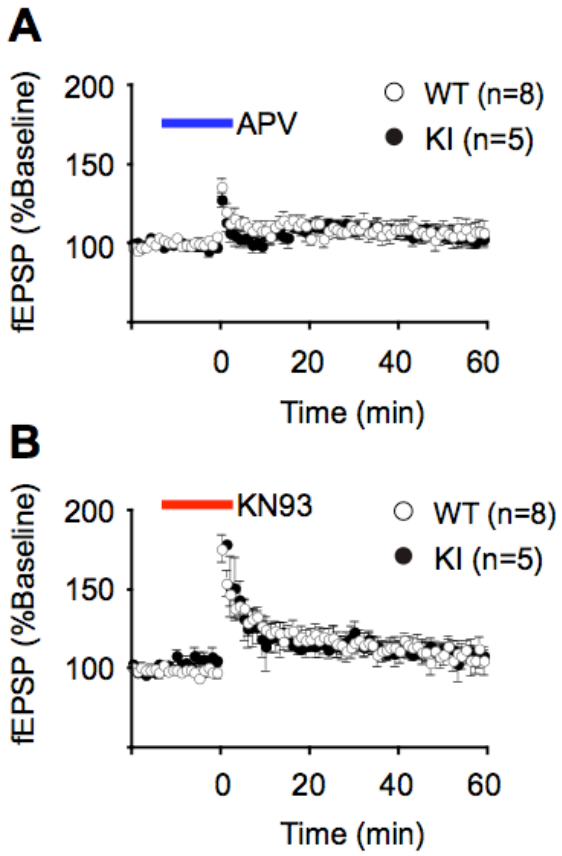

Figure 7: Hippocampal LTP is abolished with APV and KN93 in both WT and NR2BKI mice. (A) LTP induced by 2-trains 100-Hz is blocked by 100 uM APV in slices from both WT and KI mice. **(B)** LTP induced by 2-trains 100-Hz is blocked by KN93 in slices from both WT and KI mice. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

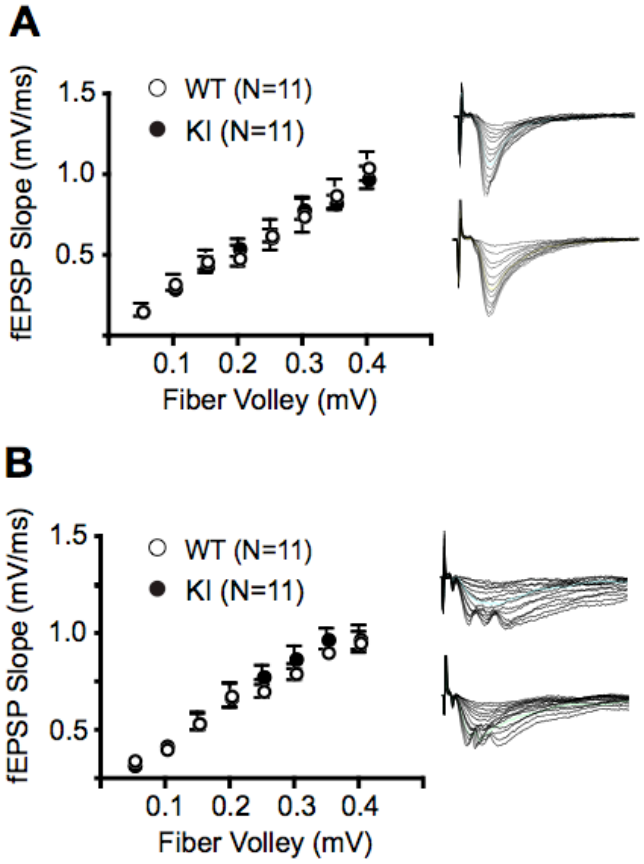

Figure 8: Basal synaptic transmission is normal in NR2BKI mice. (A) Stimulus-response curves plotted presynaptic fiber volley amplitude versus postsynaptic fEPSP left slope show no difference between WT (N = 9) and NR2BKI mice (N = 9). Representative traces are shown for the range of stimulus intensities. Scale bars are 5 ms and 2 mV. **(B)** Recordings were performed in the presence of 8 μM CNQX and 0 mM Mg to isolate NMDARs. Stimulus response curves show no difference in recordings from slices obtained from WT (N = 11) and NR2B KI (N = 11) mice across increasing fiber volley amplitude. Scale bars are 20 ms and 1 mV. Robert Dallapiazza performed these experiments and analyzed the data. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

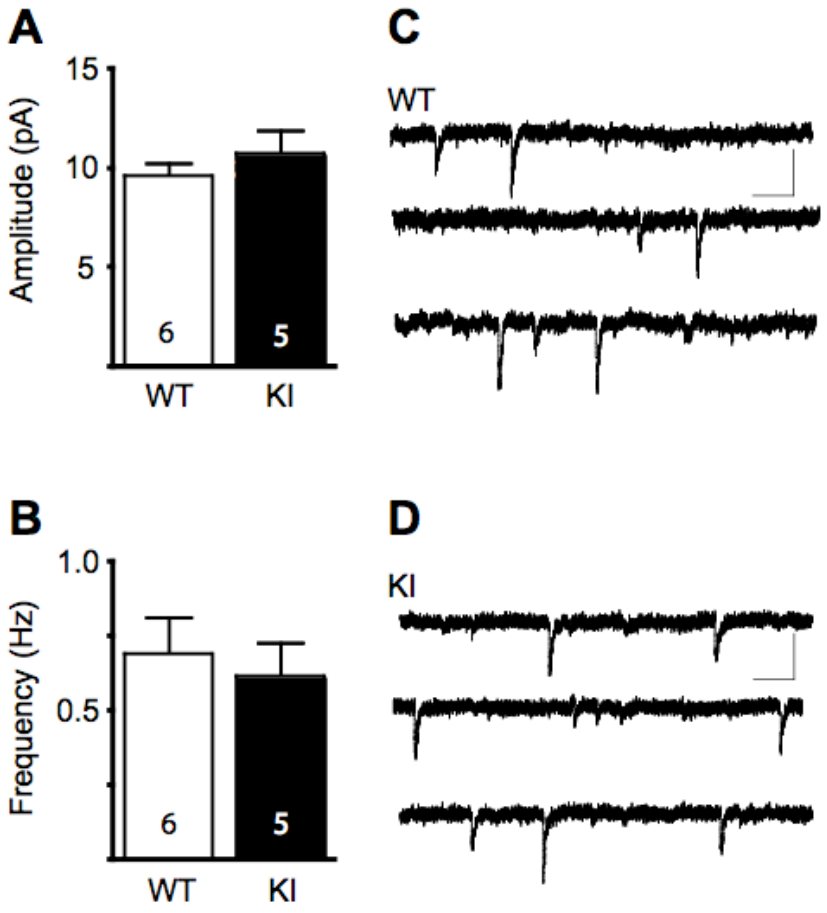

Figure 9: mEPSC analysis is normal in NR2BKI mice. (A) Amplitude of miniature EPSC is not statistically different in neurons from WT (N = 6) and NR2BKI (N = 5) mice. **(C)** Frequency of hippocampal mEPSC is normal in NR2BKI mice compared to WT controls. **(B, D)** Representative traces from WT and KI mice. Scale bar is 10 pA, 20 msec. Robert Dallapiazza performed these experiments and analyzed the data.

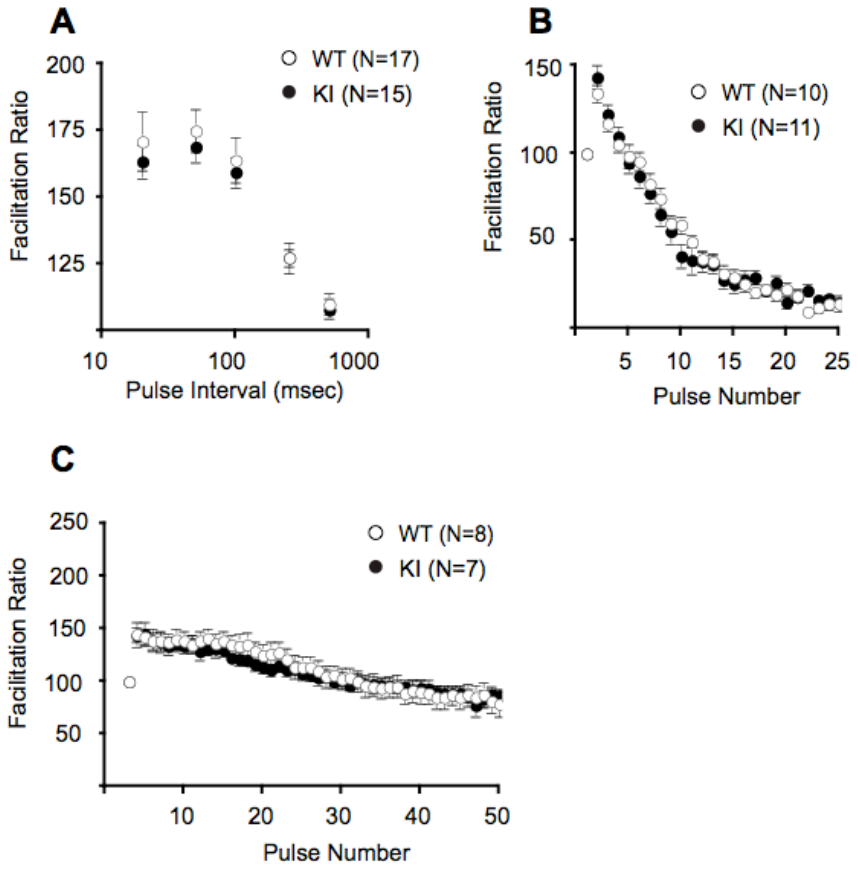

Figure 10: Presynaptic function is normal in NR2BKI mice. A) Facilitation was assessed by paired stimulation ranging from 20-500 msec. No difference in facilitation was observed in recordings in slices from WT (N = 9) and NR2BKI mice (N = 10). Peak facilitation was measured at 50 msec in both groups. RRV and RV were assessed by recording repetitive stimulation at frequencies of **(B)** 100 and **(C)** 10 Hz, respectively. Repetitive stimulation of slices from WT and NR2B KI mice show nearly identical responses. Robert Dallapiazza performed these experiments and analyzed the data.

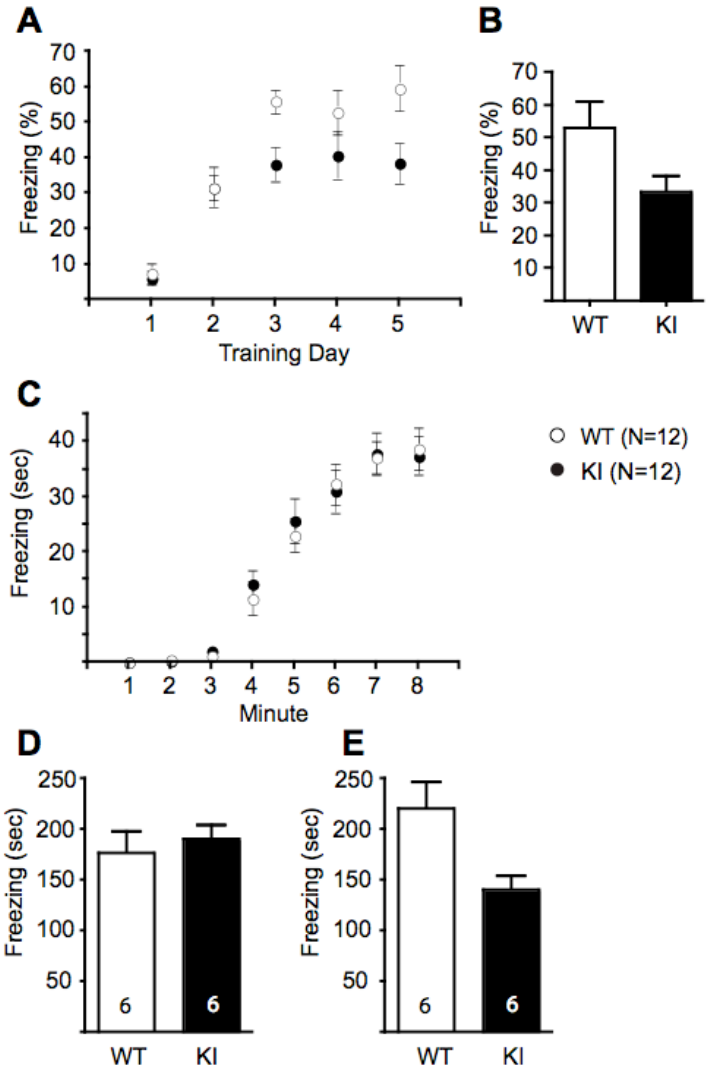

Figure 11: Consolidation of context fear conditioning is impaired in NR2BKI mice. **(A)** Fear training with one shock each day for 5 days. NR2BKI mice showed statistically less freezing after day 2 compared to WT controls. **(B)** Contextual recall on day 6 after fear training. **(C)** Fear training with five shocks delivered in 10 minutes showed normal context fear acquisition in NR2BKI mice. **(D)** Immediate recall 1-hour after training is normal in NR2BKI mice. **(E)** Recall 4-days after training is impaired in NR2BKI mice ($p<0.05$). Respective contributions: Yu Zhou, Uche Maduka, and Robert Dallapiazza performed these experiments and analyzed the data.

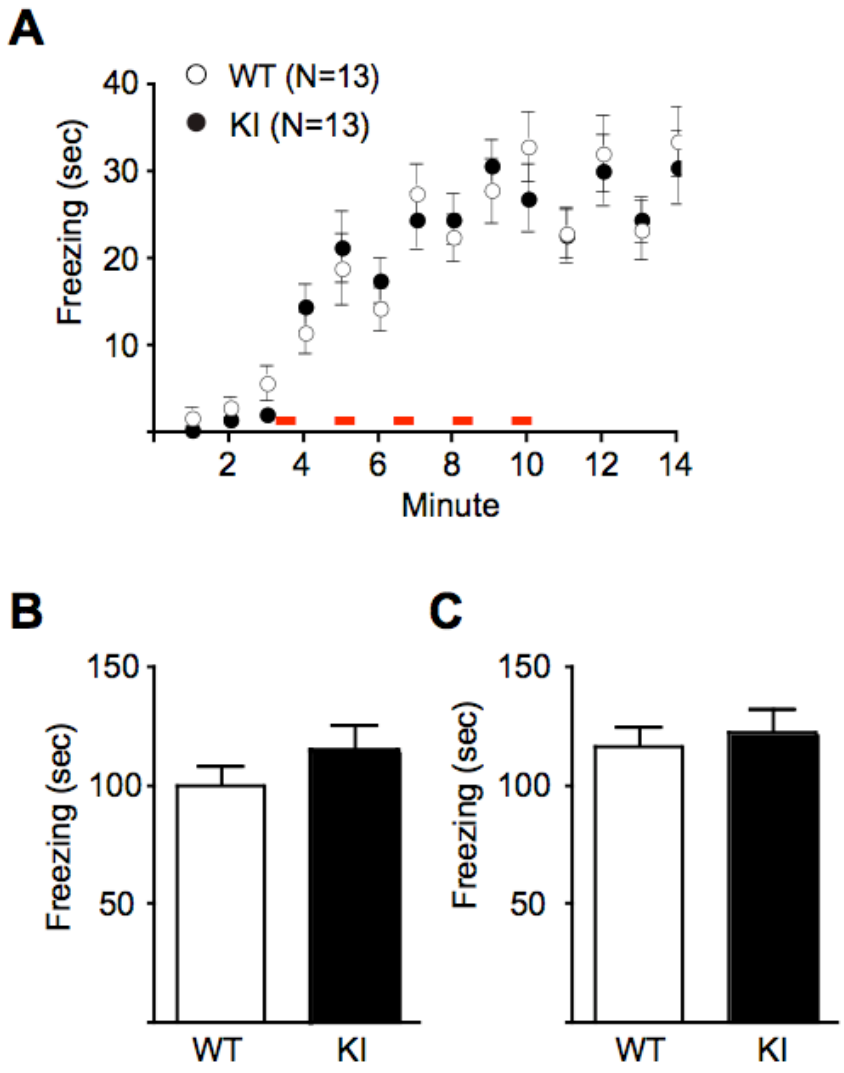

Figure 12: Consolidation of cued fear conditioning is normal in NR2BKI mice. (A) Fear training with five tone-shock pairings during a 14 minute training period showed normal cued fear acquisition. **(B)** 24-hour cued recall is normal in NR2BKI mice. **(C)** Cued fear recall is normal two weeks after training in NR2BKI mice. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

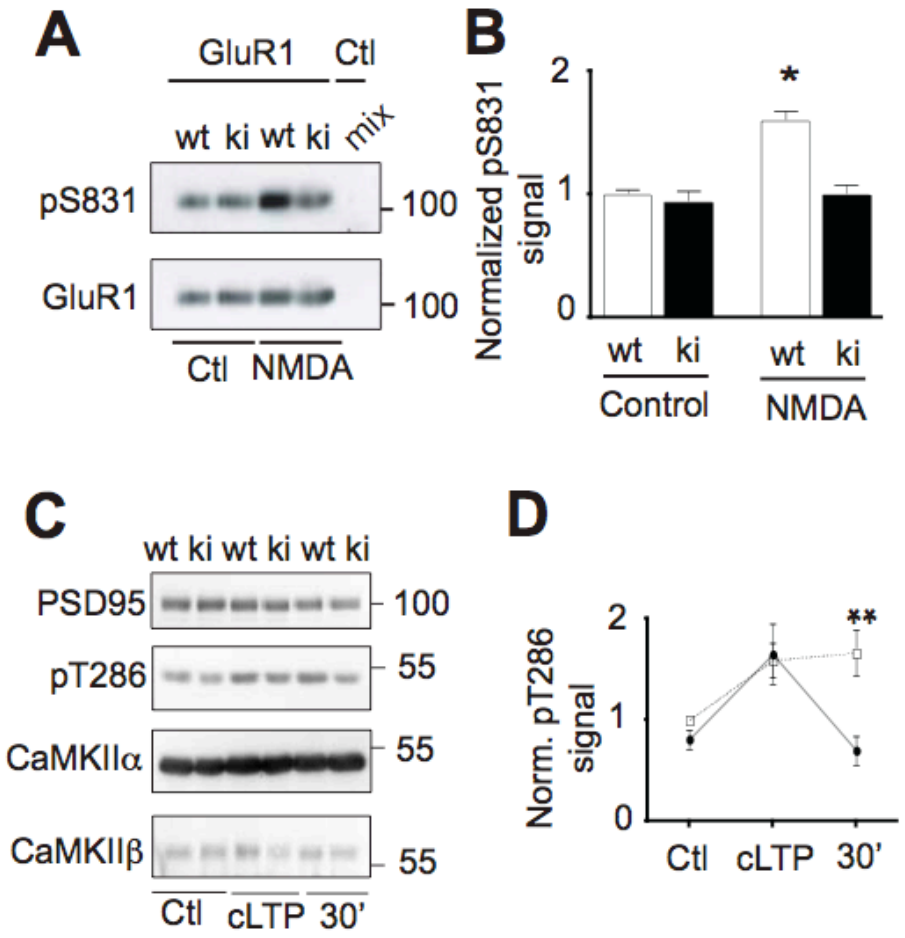

Figure 13: Activity dependent CaMKII phosphorylation of GluR1 and T286 are reduced in NR2BKI mice. (A) Immunoblot showing increase in GluR1 S831 phosphorylation following treatment of NMDA in forebrain membranes of WT mice but not in KI mice. **(B)** Quantification of results from experiments (A). GluR1 S831 was statistically increased after NMDA treatment in WT slices but not KI slices. **(C)** Immunoblot showing CaMKII autophosphorylation increased following cLTP and persisted for 30' in WT slices. CaMKII autophosphorylation initially increased in KI slices following cLTP treatment, but returned to baseline within 30'. **(D)** Quantification of CaMKII autophosphorylation following cLTP treatment.

CHAPTER 3: CAMKII BINDING TO NR1 IS DISPENSABLE FOR LTP AND LEARNING

Introduction:

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors that are critical for several forms of synaptic plasticity and learning. Several studies have shown that disruption of NMDAR function leads to deficits in these processes (Harris, Ganong et al. 1984; Muller, Joly et al. 1988; Murphy, Reid et al. 1997; Malenka and Nicoll 1999; Maren 1999). Calcium flux through NMDARs leads to the activation of Ca^{2+}/calmodulin dependent kinase II (CaMKII). CaMKII is also critical for several forms of synaptic plasticity and learning (Malinow, Schulman et al. 1989; Tsien, Schulman et al. 1990). The structure of CaMKII is unique among kinases. The holoenzyme consists of 12 subunits that associate in stacked, six-member rings. Each monomer of CaMKII is functionally divided into a catalytic, regulatory, and association domains. The regulatory domain consists of an overlapping autoinhibitory domain and calmodulin-binding site. The autoinhibitory domain blocks kinase activity by binding to the ATP binding pocket in the catalytic domain. The carboxy-terminal association domain mediates oligomerization mediating the formation the dodecameric core, while the catalytic and regulatory domains project outward in a spoke-like fashion (Hudmon and Schulman 2002). This structure allows each subunit to bind Ca^{2+}/calmodulin independently, and it allows intersubunit autophosphorylation (Mukherji and Soderling 1994).

The autoinhibitory domain contains a conserved threonine residue (T286) that can be autophosphorylated in a Ca^{2+}/calmodulin-mediated manner. T286 phosphorylation renders CaMKII Ca^{2+}-independent by preventing reassociation of the autoinhibitory and catalytic domains. T286 phosphorylated CaMKII remains autonomously active until dephosphorylated by protein phosphatases (Colbran and Soderling 1990; Hanson, Meyer et al. 1994; Rich and Schulman 1998). T286 autophosphorylation therefore acts as a

molecular switch transducing transient elevations of postsynaptic Ca^{2+} into prolonged kinase activity (De Koninck and Schulman 1998). Mutation of T286 to Ala blocks persistent activation and leads to deficits in LTP and learning (Mayford, Wang et al. 1995; Giese, Fedorov et al. 1998).

Activated CaMKII associates with the NMDAR though its interactions with intracellular domains of NR1 and NR2B (Strack and Colbran 1998; Leonard, Lim et al. 1999; Strack, McNeill et al. 2000; Bayer, De Koninck et al. 2001; Leonard, Bayer et al. 2002). CaMKII association with each of the NMDAR subunits has distinct requirements of activation. NR1 binding to CaMKII requires autophosphorylation at T286, whereas binding to NR2B requires only activation by Ca^{2+}/calmodulin. Furthermore, NR2B binding locks CaMKII in its active conformation through its association with the catalytic domain, thereby displacing the autoinhibitory domain even after removal of Ca^{2+}/calmodulin and independent of T286 phosphorylation (Bayer, LeBel et al. 2006). However, autophosphorylation of CaMKII is not sufficient for CaMKII recruitment to the synapse by NMDARs. Expression of constitutively activated CaMKII T286D that mimics autophosphorylation has the same diffuse cytosolic expression pattern as WT CaMKII. Like WT CaMKII, CaMKII T286D is only recruited to synapses following increases in intracellular calcium (Shen and Meyer 1999). In addition to activating CaMKII, calcium has another important function in recruiting the kinase to synapses (Thalhammer, Rudhard et al. 2006). We previously found that α-actinin-2 and CaMKII compete for binding on NR1 within the C0 domain and that this process is dependent on calcium/calmodulin displacement of α-actinin (Merrill, Malik et al. 2007). We hypothesized that increased postsynaptic calcium not only activates CaMKII but also unmasks a synaptic CaMKII binding site that allows for stabilization of the enzyme near its synaptic targets, perhaps by binding to NR1.

We previously found that CaMKII binding to NR2B is required for hippocampal LTP and consolidation of spatial memory (Halt et al), however the significance of CaMKII binding to NR1 remains untested. In this study we further identified the residues within NR1 necessary for CaMKII binding and generated a second strain of mice with mutations in the mouse *grin1* gene designed to specifically block the association of CaMKII with NR1.

Results:

Identification of CaMKII binding site on NR1. We previously identified residues 848-860 as the core-binding site of CaMKII on NR1 (corresponding to the distal portion of C0). These residues overlap with the core residues necessary for calmodulin and α-actinin-2 binding, but are shifted three amino acids toward the amino terminus (Leonard, Lim et al. 1999). This region of NR1 likely adopts an a-helical conformation suggesting that CaMKII binds on the opposite face of an α-helix from calmodulin and α-actinin-2. We modeled the side chain orientation in a putative a-helix for residues 845-860. Residues 849-857 are essential for effective CaMKII binding. Within this core sequence, Q849 and N856 are the only two hydrophilic residues. Their side chains are predicted to point in the same direction. We substituted glutamate at various positions since this segment of C0 does not contain negatively charged residues and introducing such residues would change the character of each side of the C0 helix.

To test whether exchanging endogenous sequences for glutamate would affect CaMKII association, we determined the EC50 values for each mutant peptide for displacement of a fluorescein-labeled peptide that covered residues NR1 838-865. The fluorescein-labeled peptide showed saturating binding curves in fluorescence polarization assay with Ca^{2+}/calmodulin, CaMKII, and α-actinin-2. The apparent Kd value for Ca^{2+}/calmodulin was 80 nM and for CaMKII ~1 μM, in good agreement with earlier studies in which similar values were obtained by different methods. The apparent Kd for

a-actinin-2 was 300 nM. An unlabeled peptide containing the WT sequence of NR1 from residues 848 to 859 effectively displaced the fluorescent peptide from CaMKII, Ca^{2+}/calmodulin, and α-actinin-2. Displacement curves were sigmoidal in semi-logarithmic plots and indicated EC50 values of 2.62+.193 for CaMKII, 0.5.+.096 for Ca^{2+}/calmodulin, and 2.61+.072 for α-actinin-2. Substituting Q849 and N856 with glutamate ("QN peptide") increased the EC50 for displacement by nearly an order of magnitude for CaMKII, but not for Ca^{2+}/calmodulin or a-actinin-2. These results suggest that CaMKII association with NR1 is inhibited by Q849E and N856E mutations while calmodulin and α-actinin-2 remain intact (Leonard and Hell, personal communication).

Generation of NR1KI mice. We used homologous recombination to insert knock-in mutations within the grin1 gene (NR1, Figure 2 and 14). First, we first generated a plasmid containing a 2.2 kb short arm spanning exon 15-19 and a 6.1 kb long arm containing exon 20 and 21 (the first alternatively spliced exon). Two HSV thymidine kinase genes were attached 5' to the short arm for negative selection of randomly integrated vector. For positive selection we inserted a neomycin resistance gene flanked by loxP sites in an antisense orientation into the intron between exon 19 and 20 of the NR1 gene. Three point mutations were introduced to generate the Q849E and N856E mutations, simultaneously destroying a PvuII site for diagnostic purposes. The targeting plasmid was linearized by digestion with NotI and electroporated into E14 ES cells. Mice were backcrossed into the C57/B6 strain for 7 generations before we began experiments. The neomycin cassette was removed in a backcross with mice expressing Cre-recombinase.

CaMKII association with NMDARs is unaffected in NR1KI mice. To test whether the KI mutations affected activity-dependent binding of CaMKII with NR1, we co-immunoprecipitated (coIP) NMDARs from the deoxycholate-solubilized membranes from the forebrains of NR1KI or WT mice and probed for CaMKII. Interestingly, despite our *in vitro* studies that demonstrated reduced affinity with Q849E/N856E peptides, we

found no change in CaMKII coIP with NMDARs in KI mice compared to WT (Dallapiazza and Hell, unpublished). We also performed the reverse coIP with CaMKII, however, this yielded similar results. These results suggest that CaMKII binding to NR2B is sufficient for association and that the NR1 binding site is dispensable for CaMKII binding to the NMDAR complex under basal conditions.

CaMKII is normally recruited to synapses in NR1KI mice. Although coIP of CaMKII with NMDARs was unaffected in membrane-solubilized forebrains, we next sought to test whether CaMKII association with NMDARs was affected after NMDAR activation in NR1KI mice. Association of CaMKII with NR1 requires CaMKII autophosphorylation, and under basal conditions the interaction with NR2B may be more important since it only requires calcium-calmodulin-activation for its interaction (Strack and Colbran 1998). Furthermore, postsynaptic calcium may be necessary to displace a-actinin from NR1C0 in order for CaMKII to associate with NR1 (Shen and Meyer 1999). To assess activity-dependent CaMKII accumulation at the synapse, we measured synaptic localization of CaMKII in cultured hippocampal neurons from WT and NR1KI mice following treatment with glutamate. Our previous studies showed that CaMKII binding to NR2B was necessary for synaptic localization of CaMKII. We assessed colocalization of CaMKII and the presynaptic marker, synapsin following vehicle or glutamate treatment. In contrast to our studies with NR2B, we found no difference in the distribution of CaMKII upon glutamate treatment in NR1KI mice immediately following stimulation or with a 20 min chase (Figure 15). These results suggest that CaMKII recruitment to synapses does not rely on binding to NR1, and that NR2B is sufficient for synaptic accumulation of CaMKII.

Hippocampal LTP is normal in NR1KI mice. Since we previously observed that CaMKII binding to NR2B is important for LTP (Halt and Dallapiazza, unpublished), we tested LTP induction in acute slices from NR1KI mice. In contrast to our results from NR2BKI mice, we found that LTP was unchanged in slices from KI mice compared to

WT controls with a 100Hz/1sec induction protocol. In addition, we did not observe changes in AMPAR-mediated stimulus response curves or in paired pulse facilitation (Figure 16). These results suggest that CaMKII binding to NR1 is dispensable for activity-dependent synaptic transmission in the hippocampus.

Contextual and cued fear conditioning is normal in NR1KI mice. Next, we tested cued and contextual fear conditioning using five 20 sec tones co-terminating with a 0.75-mA/1-sec shock. Tone-shock pairings were delivered in a single 14-min trial (100 sec shock interval) (Wemmie, Askwith et al. 2003). Similar to our results with NR2BKI mice, the acquisition of fear conditioning was not statistically different in NR1KI mice compared to WT controls. One day after initial training mice were placed in a novel context and were exposed only to the tone. WT and KI mice showed equivalent freezing during tone presentation. The second day after training mice were placed back into the original training context. Similar to our results with cued fear retention, there was no significant difference in the amount of time KI mice froze compared to WT controls (Figure 17).

Discussion

We previously identified the critical residues for activity-dependent CaMKII binding to NR1. The core site spans residues 848-860 of C0 domain (Leonard, Lim et al. 1999). This site is shifted approximately one helical turn from the calmodulin and α-actinin-2 binding sites suggesting that they associate on opposite sides of the predicted helical NR1C0. We exchanged residues Q849 and N856, which from our modeling are predicted to project from the same side of the helix, to glutamate. FP studies using peptides with these substitutions did not compete with CaMKII binding to NR1, suggesting that these residues are necessary for CaMKII association with NR1. Importantly, peptide Q849E/N856E competitively inhibited both calmodulin and α-

actinin-2 binding, indicating that while CaMKII required residues Q849 and N856, calmodulin and α-actinin-2 did not.

Activity-dependent synaptic recruitment of CaMKII. CaMKII binds to several synaptic proteins, however NR1 and NR2B are the only ones shown to interact in an activity-dependent manner. Upon NMDAR-dependent activation by calcium/calmodulin, CaMKII accumulates at the synapse and is stabilely localized for prolonged periods of time. Activation of CaMKII alone does not result in synaptic localization, however since the overexpression of constitutively active CaMKII T286D does not cluster at synapses without calcium flux through NMDARs (Shen and Meyer 1999). This suggests that NMDAR-mediated calcium influx, in addition to activating CaMKII, has a second role in the recruitment of the kinase to the synapse. We hypothesized that this second role might be the calcium/calmodulin displacement of α-actinin-2 from NR1C0. Our previous work demonstrated that CaMKII and α-actinin-2 compete for binding of NR1 in a calcium-dependent manner (Merrill, Malik et al. 2007). However, the data from our present study suggests that dissociation of α-actinin from NR1C0 is not critical for activity-dependent recruitment of CaMKII to the synapse. Based on our FP studies, we designed a strain of mice with targeted mutations in NR1 that specifically block the association of CaMKII with NR1 while not affecting calmodulin or α-actinin-2 binding (NR1KI mice). In hippocampal cultures from these mice, activity-dependent recruitment of CaMKII to the synapse is unaffected. We stimulated neurons with glutamate and measured the synaptic localization of CaMKII over time. We observed no difference in CaMKII localization in cultured neurons from NR1KI mice compared to WT over the course of 60 min. This data suggests that calcium/calmodulin displacement of α-actinin-2 from NR1C0 is not critical for the activity-dependent recruitment of CaMKII to the synapse.

CaMKII binding to NR1 is dispensable for LTP and fear conditioning. CaMKII binds to NR2B at two distinct sites, termed NR2B-P and NR2B-C. The critical residues within NR2B-C have been identified, however the exact binding site within NR2B-P

remains undefined (Strack and Colbran 1998; Leonard, Lim et al. 1999; Strack, McNeill et al. 2000; Bayer, De Koninck et al. 2001; Leonard, Bayer et al. 2002). Like NR1, binding of CaMKII to NR2B-P requires autophosphorylation, whereas the binding to NR2B-C requires only activation by calcium/calmodulin. We previously demonstrated that NR2B-C is critical for sustained CaMKII activity and synaptic accumulation (Halt et al., Chapter 2). Although our previous results suggested that CaMKII association with NR1 was not critical for synaptic localization of the kinase, we hypothesized that CaMKII binding to NR1 may further stabilize the interaction with the receptor complex thereby facilitating LTP. However, when we induced LTP in slices from NR1KI mice we found that it was unaffected as well. Furthermore, cued and context fear conditioning were not affected in NR1KI mice. These results strongly suggest that CaMKII binding to NR1 is dispensible for synaptic plasticitiy and in learning and memory. Instead, CaMKII binding to NR2B seems to be most important for mediating sustained synaptic recruitment of CaMKII, the expression of LTP, and is involved in the consolidation of spatial memories.

CaMKII binding to NR1 in synaptic regulation. Since we found that CaMKII binding to NR1 is not required for synaptic recruitment of CaMKII and anchoring the kinase during LTP, the physiological significance of this interaction remains unknown. However, we have two leading ideas. First, NR1 association with CaMKII might be required for the specific recruitment of the kinase to NR2A-containing receptors and may play an important role in synaptic plasticity in brain regions where NR2B expression is low (Kopke, Bonk et al. 1993).. NR2A and NR2B are both abundantly expressed in the hippocampus and cortex, however in the cerebellum NR2A is the predominant NR2 species (Monyer, Burnashev et al. 1994; Petralia, Wang et al. 1994). NR2A has also been reported to bind to CaMKII, however its affinity for CaMKII is roughly ten-fold lower than NR1 or NR2B (Gardoni, Caputi et al. 1998; Gardoni, Schrama et al. 1999). Second, CaMKII binding to NR1 may be an important mechanism of negative feedback

by preventing over-excitation of the receptor. Under resting conditions α-actinin binds to NR1C0 through its spectrin repeat domain thus linking the receptor to the actin cytoskeleton and maintaining it in a high open probability state. However, after calcium enters postsynaptic spines, complexing with calmodulin and the EF-hand domains of α-actinin, calcium-calmodulin displaces α-actinin from NR1C0 thereby reducing peak currents by calcium-dependent inactivation. CaMKII binding to NR1 could then block the association of α-actinin (Legendre, Rosenmund et al. 1993; Rosenmund, Feltz et al. 1995), thus limiting the activation of NMDARs by changing their biophysical properties.

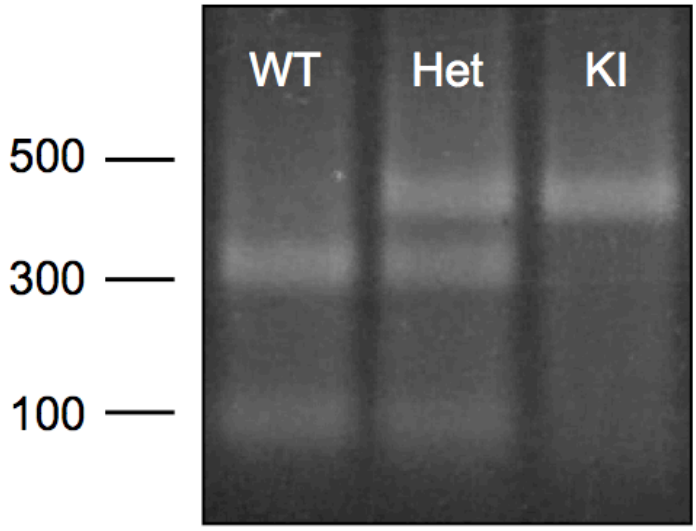

Figure 14: Genotyping of NR1KI mice. A Neomycin resistance gene flanked by two loxP sites was inserted in antisense orientation into the intron between exon 19 and 20 of the NR1 gene. This was flanked by a 2.2 kb short arm spanning exon 15-19 and a 6.1 kb long arm containing exon 20 and 21 (the first alternatively spliced exon). 2 copies of the HSV thymidine kinase gene were attached 5' to the short arm for negative selection of randomly integrated vector. The short arm was generated by PCR using Sv129J mouse genomic DNA as template. Three point mutations were introduced to generate the Q849E and N856E mutations, simultaneously destroying a PvuII site for diagnostic purposes (see figure 2). Representative gel showing the PvuII digestion of the genotyping amplicon. WTs have two bands (350, 96 bp), heterozygous have three bands (450, 350, and 96 bp), and NR1KIs have one band (450 bp). Respective contributions: Scott Juntti made the targeting vectors and Robert Dallapiazza performed the genotyping experiments.

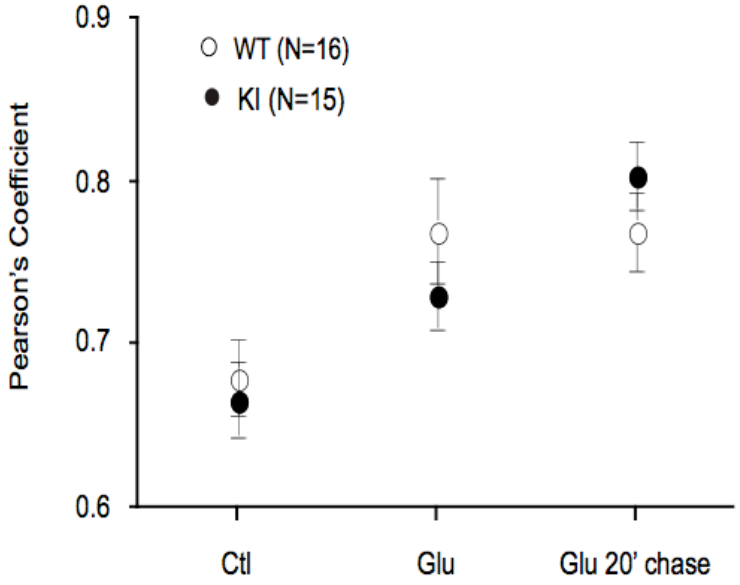

Figure 15: CaMKII is normally recruited to synapses in hippocampal NR1KI neurons. Quantification of synaptic localization of CaMKII and synapsin before, after 5' glutamate treatment, and 20' after glutamate treatment showed no difference in CaMKII synaptic clustering in NR1KI neurons compared to WT controls. Respective contributions: Ivar Stein performed the experiments and analyzed the data.

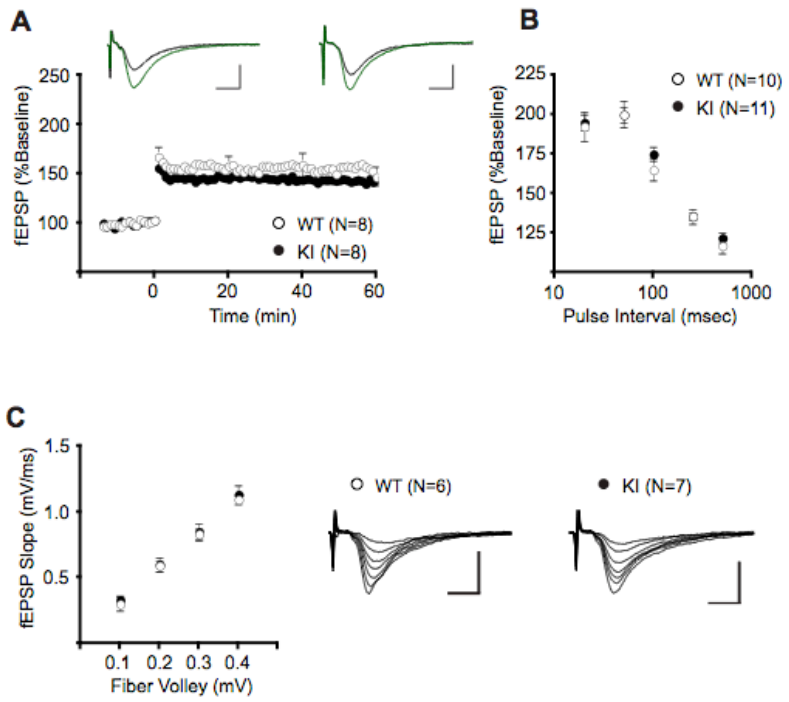

Figure 16: LTP and synaptic transmission are normal in NR1KI mice. (A) LTP induced by two trains of 100-Hz stimulation showed no difference in slices from WT (N = 8) and NR2B KI (N = 8) mice. Baseline representative traces are in black and traces 60 min after LTP induction are shown in green. Scale bars are 5 ms and 1 mV. **(B)** No difference in facilitation was observed in recordings in slices from WT (N = 10) and NR1KI mice (N = 11). **(C)** Stimulus-response curves plotted presynaptic fiber volley amplitude versus postsynaptic fEPSP left slope show no difference between WT (N = 6) and NR2BKI mice (N = 7). Representative traces are shown for the range of stimulus intensities. Scale bars are 5 ms and 2 mV. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

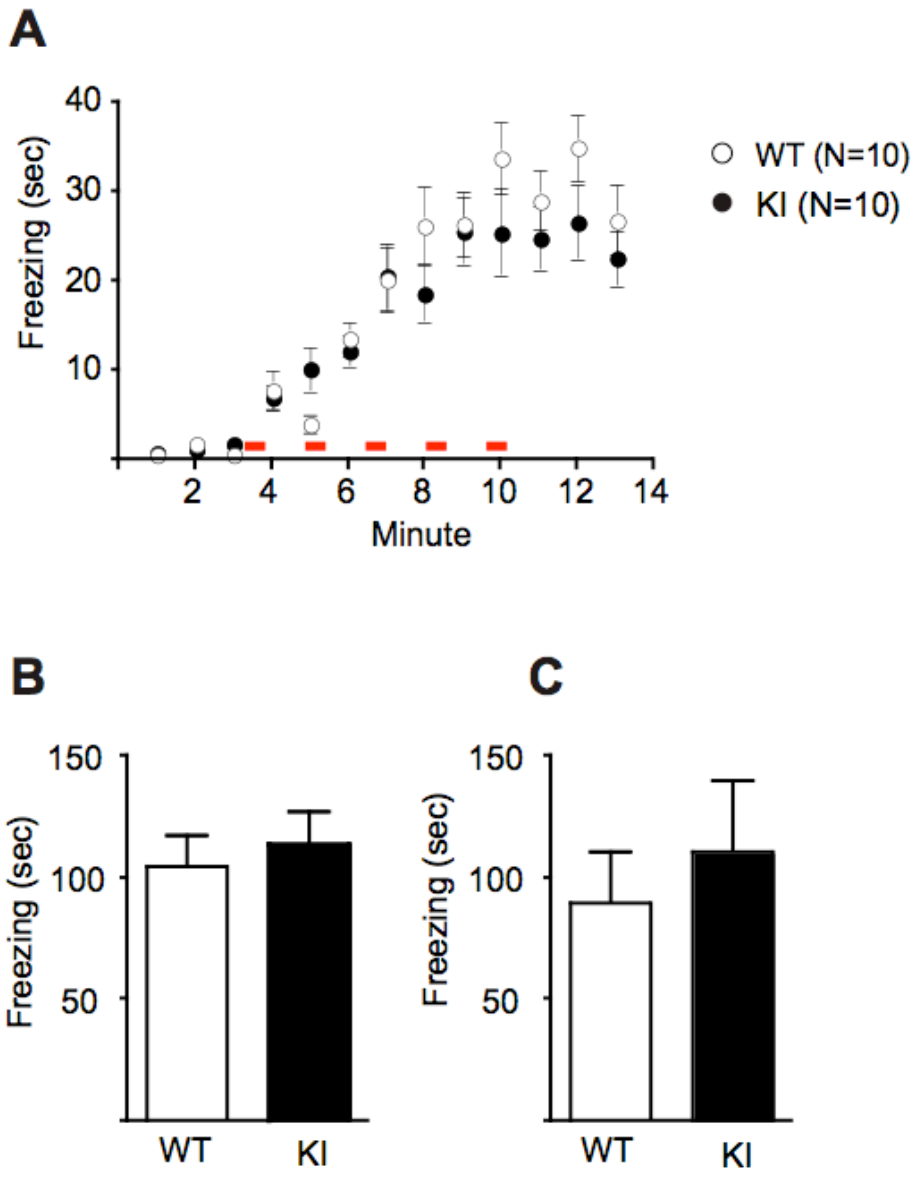

Figure 17: Cued and contextual fear conditioning are normal in NR1KI mice. (A) Fear training with five tone-shock pairings during a 14 minute training period showed normal cued fear acquisition. **(B)** 24-hour cued recall is normal in NR1KI mice. **(C)** Context fear recall is normal two weeks after training in NR1KI mice. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

CHAPTER 4: INTRACELLULAR DOMAINS OF NR1 ARE NECESSARY FOR NMDAR ACTIVITY AND LEARNING, BUT NOT REMOTE MEMORY RECALL

Introduction

N-methyl-D-aspartate receptors (NMDARs) are important for many neuronal processes including synapse formation and plasticity at mature synapses. NMDARs also play a key role in several pathological states (Lau and Zukin 2007). Much of the focus on NMDARs in disease has been in their over-excitation and contribution to excitotoxic cell death following ischemia and epilepsy (Lipton and Rosenberg 1994; Mattson 1997). However, recent studies have also focused on the role of attenuated NMDAR function in disease. Loss of NMDAR function is suggested as an inciting factor in the pathogenesis of Alzheimer's disease (Small, Mok et al. 2001; Selkoe 2002), and synaptic dysfunction is present long before cognitive manifestations in some mouse models of familial AD. Conditional double knockout of presenilins –1 and –2 in mice shows reduced NMDAR activity during development, which precedes behavioral deficits in learning and memory (Saura, Choi et al. 2004). Additionally, two mouse models with decreased NMDAR function show symptoms consistent with schizophrenia (Mohn, Gainetdinov et al. 1999; Labrie, Lipina et al. 2008). Therefore, mechanisms of attenuated NMDAR function have important clinical implications.

NMDARs are tetramers typically composed of two NR1 and two NR2 subunits. NR2 subunits are encoded by four separate genes designated A-D (Monyer, Sprengel et al. 1992). In the cortex NR2A and NR2B are predominant. NR1 subunits are encoded by one gene that is differentially spliced at both amino- and carboxy-termini. The carboxy-terminus of NR1 (residues 838-938) is encoded on three separate exons that are alternatively spliced (Laurie and Seeburg 1994). The membrane-proximal region (C0) is encoded by exon 19 and spans 30 residues. This sequence is common to all NR1

subunits. Exon 20 encodes a 37-residue domain (NR1C1) that can be alternatively spliced. The final coding exon contains two alternate splice acceptor sites resulting in either a 38- or 22- residue tail (designated C2 and C2', respectively). Along with an amino-terminal splice site, this allows for eight distinct NR1 subunits.

The intracellular domains of NR1 interact with a number of proteins that regulate several important aspects of NMDAR signaling. These domains play an important role receptor trafficking (Horak, Chang et al. 2008; Horak and Wenthold 2009), they act as scaffolds for downstream signal transduction cascades (Lin, Wyszynski et al. 1998) and they regulate channel properties (Ehlers, Zhang et al. 1996). NR1C0 associates with three calcium-sensing proteins, calmodulin, α-actinin, and CaMKII (Merrill, Malik et al. 2007). Both a-actinin and calmodulin are important for regulating channel properties, and α-actinin can anchor NMDARs to the actin cytoskeleton (Legendre, Rosenmund et al. 1993; Rosenmund, Feltz et al. 1995). The role of CaMKII binding to NR1C0 is unknown, however our previous work suggests that, in contrast to CaMKII binding to NR2B, this interaction is not necessary for LTP or learning (Chapter 3). NR1C1 binds to calmodulin as well as the A-kinase anchoring protein (AKAP) yotiao that recruits PKA to NMDAR signaling complexes (Lin, Wyszynski et al. 1998). The C1 domain can be phosphorylated at several serine and threonine residues by PKC and PKA (Leonard and Hell 1997), which regulate calcium permeability. NR1C1 also has an ER retention motif that can be suppressed by association with PDZ domain-containing proteins or association with NR2 subunits. Recent finding suggest that NR1C1 has a bipartite nuclear localization sequence (Holmes, Mattar et al. 2002).

We recently described NR1KI mice that were initially designed to study the role of CaMKII association with NR1 in synaptic recruitment of CaMKII, LTP, and learning. We hypothesized that under basal calcium levels the CaMKII binding site was masked by the association of α-actinin. During NMDAR-mediated calcium influx, however, α-actinin is displaced by calcium/calmodulin allowing CaMKII association. We found that

synaptic CaMKII recruitment, LTP, and learning were normal in NR1KI mice, however, during the course of our characterization of NR1KI mice, we noticed some discrepancies in our results that seem to be focused on the age at which the mice tested.

Results:

Carboxy-terminal truncation of NR1 in NR1KI mice. We first tested NMDA receptor expression in deoxycholate-solubilized membranes from WT, het, and KI forebrains. Western blot analysis of NR2A, NR2B, and NR1 proteins demonstrated equivalent expression levels across genotypes (Figure 18). However, we noticed that NR1 consistently ran 5-10 kDa lower in lysates from KI mice compared to WT controls using an antibody directed at the ligand binding domain (mouse anti-NR1 54.2). We also observed a slightly lower band in the WT lane, which we assumed could be due to alternative slicing of the C1 domain or exon 5 in the amino-terminus. To test whether the size shift we observed in NR1 was due to alternative slicing, we isolated postsynaptic densities from WT and KI mice and probed for the intracellular domains, C0, C1, C2, and C2'. Surprisingly, these domains were completely absent in samples from KI animals despite robust signals in WT controls (Figure 18). Probings for NR2B and NR1 showed that NMDA receptors were normally enriched in the KI PSDs. Both NR1 and NR2B signals showed a greater than 2-fold increase in the PSD fraction compared to the syanptosomal fraction suggesting that, despite the lack of the NR1 carboxy-terminus, receptors were still trafficked and maintained at the synapse. Similar PSD enrichments were seen for the AMPA receptor subunit GluR1, PSD95, and CaMKII. Synaptophysin, used as a control for PSD purity, was present in the synaptosomal fraction but not in the PSD (Data not shown). These results indicated that the carboxy-terminal domains of NR1 were truncated in KI mice.

NR1 mRNA is intact in NR1KI mice. We next isolated total forebrain RNA to assess the integrity of *Grin1* mRNA in NR1KI mice. We designed one forward primer in

the 5' region of C0 and four reverse primers spanning the rest of the mRNA. Two reverse primers were chosen in C0, one before the KI mutations and one following them. The third and fourth primers were at the end of C1 and C2, respectively. We did not see any change in size of amplicons in KI samples compared to WT controls (Figure 19). All bands were the expected sizes. We subsequently sequenced the C1 amplicon from KI and WT samples and found only our intended point mutations, ruling out a frameshift. We next took a quantitative approach to assess the relative levels of *Grin1* mRNA splicing. We designed two sets of primers that covered splice junctions of C1-C2 and C1-C2'. A third set was used as an internal control. It was designed for a splice site within the ligand-binding domain, which takes place in every *Grin1* mRNA. Quantitative comparison of C1-C2 and C1-C2' splice sites showed no difference between KI and WT, suggesting that the truncation of NR1 in KI mice was due to proteolytic processing or that translation was terminated early (Figure 19).

Truncation of NR1 is age-dependent in NR1KI mice. To further characterize the carboxy-terminal truncation of NR1 in KI mice, we prepared forebrain lysates from mice of varying ages. Our previous experiments were performed only using aged animals (>4 months). Interestingly, we did not observe the size-shift in NR1 in lysates from 1-month KI mice, and corresponding probings for C0 and C2 showed equivalent expression at this age. However, beginning at 2-months and continuing at 3-months of age we observed the development of the size-shift in NR1 in KI mice, and the corresponding probing for C0 and C2 showed decreases in signal intensity at these ages. NR2B and CaMKII were used as loading controls. These results indicate that at young ages NR1 is normally expressed in the KI but undergoes progressive truncation of the carboxy-terminus (Figure 18).

NMDAR-dependent and NMDAR-independent LTP is inhibited in NR1KI mice. Next, we tested long term potentiation (LTP) at the Schaffer collateral-CA1 synapse with 2x 100-Hz, 1 sec tetani. Recordings from WT mice showed stable 150% potentiation following LTP induction, but both post-tetanic potentiation (PTP) and LTP were

completely absent in slices from KI mice (Figure 20). These results are consistent with region-specific knockout of the NR1 allele and NR1 hypomorphic mice where the synaptic expression of NMDA receptors is severely attenuated (McHugh, Blum et al. 1996; Tonegawa, Tsien et al. 1996; Tsien, Huerta et al. 1996). We demonstrated that NR1KI mice have normal synaptic expression of NR1. These results indicate that the carboxy-terminal domains of NR1 are necessary for hippocampal LTP.

Since the carboxy-terminal domains of NR1 are involved in both regulating channel properties and organizing downstream signaling we sought to delineate these functions by inducing LTP in the presence of elevated extracellular calcium (5 mM). By increasing Ca^{2+} we sought to elevate the Ca^{2+} charge transfer during LTP induction with the thought that this would be sufficient to activate downstream Ca^{2+}-dependent signaling that is necessary for LTP. Previous work has shown that increasing extracellular calcium can overcome deficits in NMDA receptor function (Skeberdis, Chevaleyre et al. 2006). We perfused slices with 5 mM Ca^{2+} and saw a 50% increase in fEPSPs from WT slices whereas fEPSPs from KI slices only increased 25%. Following LTP induction, slices from WT mice showed robust PTP and LTP, however, even in the presence of elevated extracellular Ca^{2+} both PTP and LTP were dramatically reduced in slices from KI mice (Figure 20). One interpretation of these results is that truncated NMDA receptors have a lower open probability and are unable to conduct sufficient Ca^{2+} despite elevation of extracellular levels. Another interpretation is that carboxy-terminal domains of NR1 are necessary for proper postsynaptic scaffolding of downstream signaling.

Since NR1KI mice were deficient in NMDA receptor-dependent LTP, we also assessed NMDA receptor-independent LTP. We used 10 bursts of 200-Hz (200 msec burst interval) in the presence of 100 uM APV to measure calcium channel-dependent LTP. Surprisingly, we found that LTP induced with this protocol also showed reduced levels in slices from KI mice compared to WT controls (Figure 20). Like NMDA receptor-dependent LTP, calcium channel-dependent LTP is dependent on Ca^{2+} influx,

but the latter it is dependent on the activation of calcineurin rather than kinases (Onuma, Lu et al. 1998).

NMDAR but not AMPAR potentials are reduced in NR1KI mice. We next sought to determine if truncation of the carboxy-terminus of NR1 affects synaptic transmission. We measured field potentials (fEPSPs) in the stratum radiatum of the CA1 subfield using increasing stimulation intensity. We conducted our initial experiments in normal ACSF (principally AMPAR mediated) and plotting fiber volley amplitude versus field potential slope. We found no difference KI and WT synaptic responses under these conditions. However, in subsequent experiments we performed our recordings in the presence of the AMPA receptor antagonist, DNQX, and 0 mM Mg+ to isolate NMDAR-mediated field potentials. When we plotted fiber volley amplitude versus field potential slope we saw dramatic reductions in responses from KI compared to WT controls (Figure 21). In addition, we recorded typical fEPSPs and perfused ACSF containing DNQX and 0 Mg+ to monitor AMPA:NMDA receptor ratios (Figure 21). Ratios from KI mice were significantly higher compared to WT controls. These results indicate that the carboxy-terminus of NR1 is necessary for NMDA receptor potentials, however AMPA receptor responses remain intact.

Short-term facilitation is decreased in NR1KI mice. Next, we tested facilitation at the Schaffer collateral-CA1 synapse with paired and repetitive stimulations. First, we applied paired stimulations over a wide range of intervals and found that facilitation was significantly reduced in slices from KI mice compared to WT controls at intervals < 100 msec (Figure 22). We also tested whether acute inhibition of NMDA receptors would affect facilitation, however application of APV did not alter facilitation in either genotype (Figure 22). Since facilitation is thought to reflect presynaptic calcium cycling and vesicle release probability we lowered the extracellular calcium concentration to 0.8 mM (from 2.2 mM) (Hojjati, van Woerden et al. 2007). This reduced the amplitude of the field potentials, however under these conditions facilitation at 50 msec was equivalent in

slices from WT and KI mice (Figure 22). Next, we applied repetitive stimulations at 100- and 10-Hz. Consistent with our observations with paired stimulations, we found that responses in slices from KI mice were more quickly attenuated that those from WT controls (Figure 22). Collectively, these results suggest that synaptic vesicle release probability is lower in NR1KI mice.

NR1KI mice have reduced body mass and show impairments in breeding. At birth and at the time of weaning NR1KI mice are physically indistinguishable from their littermates. However, by 6 months of age NR1KI mice are visibly smaller than their WT littermates. At 7 months, the average weight for male WT mice was significantly higher than for male NR1KI mice (Figure 23: 41.6 +/- 1.6 g, N = 11 compared to 32.5 +/- 1.7 g N = 11, $p = 0.0007$). NR1KI mice also showed impairments in breeding. We set up 4 WT and 4 KI breeder pairs for 5 months. The WT mice bred regularly and had viable pups every 23 days. In contrast, over the 5-month breeding period NR1KI pairs had only 2 viable litters total.

NR1KI mice demonstrate impaired fear conditioning. NMDARs are necessary for the acquisition of spatial and cued memory(Kim, DeCola et al. 1991; Fanselow and Kim 1994; Fanselow, Kim et al. 1994; Young, Bohenek et al. 1994). We tested aged NR1KI mice in a fear-conditioning paradigm to assess whether the intracellular domains of NR1 are necessary for spatial and cued fear learning. Mice were presented with five 20 sec tones (conditioned stimulus, CS) that co-terminated with a 0.75 mA footshock (unconditioned stimulus, US). Shocks were delivered in one trial with 100 sec intervals. During the training phase, KI mice froze significantly less that WT mice, indicating that they were unable to learn spatial or auditory cues to adverse stimuli (Figure 24). The subsequent day, animals were placed in a new context and presented with the CS. During CS presentation, KI mice froze significantly less than WT. Similarly, when mice were placed back into the original training context KI mice froze less the WT (Figure 24).

These results clearly demonstrate that the intracellular domains of NR1 are necessary for the acquisition of contextual and cued fear.

Since fear conditioning can be influenced by an animal's motivation to explore and basal anxiety level, we tested WT and KI mice in the open field and light-dark box test. KI mice explored the open field to the same extent as WT, and they spent the same percentage of time in the dark chamber (Figure 25). These indicate that our behavioral deficits in fear conditioning were not due to differences in motivation or basal anxiety. Since fear conditioning can also be influenced by differences in nociception, we also tested vocalization to electric shock in WT and KI mice and found that they had equivalent thresholds (Figure 25).

Remote fear memory recall is not affected in NR1KI mice. Several recent studies have found that during remote memory recall the same neurons are activated in sequence as those that were activated during the initial acquisition of the memory trace. Furthermore, NMDARs have been suggested to play an important role not only in the acquisition of memory but also in memory recall, suggesting that NMDAR activity is necessary for maintenance or updates in memory (Cui, Wang et al. 2004). Cui et al used a reversible, inducible knockout of NR1 and showed that prolonged inactivation of NMDARs disrupted remote memory recall. We tested whether the intracellular tail of NR1 is necessary for memory recall by taking advantage of the fact that in young NR1KI mice NR1 is fully intact. Fear conditioning did not significantly differ between WT and KI mice at one month of age, nor did one-day cued or contextual recall (Figure 26). Three months after initial training, when the intracellular domains of NR1 are fully truncated in KI mice, we tested cued and contextual fear responses. Surprisingly, we found no difference in these tests comparing WT to KI mice. At four months, KI mice were able to recall both cued and contextual fear memories with equal efficiency at WT controls (Figure 26). These results suggest that full NMDAR activity is not necessary during the recall phase of memory.

Discussion:

Truncation of the NR1 subunit. We found that by introducing glutamate residues at Q849 and N856, the carboxy-terminal domains of NR1 were truncated in an age-dependent manner such that the entire intracellular domains of NR1 were missing by the 4-months of age. We were unable to detect any abnormalities in NR1 mRNA qualitatively or quantitatively suggesting that the truncation was due to proteolysis rather than dysfunctional mRNA processing.

NMDARs are modulated by proteolytic cleavage by intracellular and extracellular proteases. Most notably, NR2 subunits are substrates for the calcium-activated protease, calpain (Guttmann, Sokol et al. 2002). Sustained activation of NMDARs leads to elevations in calcium that activates calpain, which is able to cleave the intracellular domains of NR2B. Interestingly, calpain cleavage of NR2B is dependent on the protein phosphatase, calcineurin, and PSD95 is able to protect NR2B from calpain cleavage (Yuen, Ren et al. 2008). This has important functional consequences. Calpain-mediated cleavage of NR2B reduces NMDAR activity and is thought to be an important negative feedback mechanism during over-excitation of NMDARs. NR1 also seems to be a substrate for calpain. One study found that treatment of synaptic membranes with calpain I resulted in the cleavage of NR1 and NR2 subunits, however further investigation in cultured forebrain slices with kainate did not demonstrate NR1 proteolysis but showed cleavage products of NR2 subunits (Bi, Rong et al. 1998). Therefore, while NR1 may be a substrate for calpain, it's unclear which conditions promote NR1 cleavage or whether it's physiologically significant.

Several recent studies have characterized proteolytic cleavage of NR1 by some unexpected serine proteases – thrombin and tPA. These proteins are well known for their role in the coagulation cascade, but they may also play an important role in synaptic function by modulating NMDARs (Gingrich, Junge et al. 2000). Treatment of forebrain membranes with thrombin results in specific cleavage of NR1 that bears striking

resemblance to our observations in NR1KI mice (NR2A or NR2B do not seem to be affected).

The protease responsible for NR1 cleavage remains to be identified, although calpain is our prime candidate. Several other proteases may be involved, however. NMDARs associate with secretase complexes that are involved in familial AD (Saura, Choi et al. 2004). One question that will also need to be addressed is whether NR1 Q849E/N856E is simply a better substrate for proteolytic cleavage or whether the processing of NR1 Q849E/N856E is a consequence of dysregulated signaling that activates a protease responsible for NR1 truncation.

Intracellular domains of NR1 in NMDAR assembly and trafficking. Another remaining question regarding the truncation of NR1 is the subcellular compartment in which the cleavage takes place. Since nearly all of NR1 subunits are cleaved in total forebrain membranes by 4-months of age, we think that this process is taking place extrasynaptically before NMDARs arrive at the synapse. Regardless of the site of proteolysis, our immunoblots from purified PSDs suggest that despite loss of the intracellular domains of NR1, NMDARs are targeted normally to the PSD. This strongly suggests that synaptic targeting of NMDARs is independent of the intracellular domains of NR1 (Horak, Chang et al. 2008; Horak and Wenthold 2009). Whether or not the intracellular domains of NR1 are important for receptor mobility in the PSD remains untested, although NMDARs are relatively immobile compared to AMPARs. Not surprisingly, there was no difference in the coimmunoprecipitation of NR1 with NR2 subunits when the intracellular domains were truncated, since this interaction is mediated by the association of the membrane-spanning regions.

Impaired NMDAR function after NR1 truncation. We found no change in AMPAR-mediated responses in the hippocampus of aged NR1KI mice, however there was a dramatic reduction of NMDAR-mediated responses, and NMDAR-dependent LTP was completely absent in these mice. Furthermore, impairments in LTP could not be

corrected by increasing extracellular calcium during LTP induction. These results suggest that the intracellular domains of NR1, while not necessary for synaptic targeting, are important for channel activity. Several groups have reported the association of NR1C0 with α-actinin-2 (Rosenmund, Feltz et al. 1995; Ehlers, Zhang et al. 1996; Merrill, Malik et al. 2007). This interaction is important for several reasons. First, α-actinin links NMDARs to the actin cytoskeleton and may be important for anchoring (but not targeting) the receptor in the synapse. Second, α-actinin binding to NR1 promotes an open conformation. Single channel NMDAR recordings show that association of a-actinin increase channel open probability but not conductance (Rycroft and Gibb 2004). Finally, dissociation of α-actinin from NR1 is important for calcium-dependent channel inactivation (CDI) (Krupp, Vissel et al. 1999). Our results fit the model that NR1C0 association with α-actinin is required for full NMDAR channel activity, and that truncation of this domain results in decreased activation.

Remote memory recall is intact in NR1KI mice. Considering our findings that NMDAR activity is reduced after truncation of the intracellular domains of NR1 and that hippocampal LTP is completely abolished, it was not surprising to find that acquisition and recall of conditioned fear was impaired. NMDAR activity is firmly established in these processes. However, since young NR1KI mice display normal hippocampal LTP and fear conditioning, we asked whether NMDAR activity is necessary for remote fear memory recall. Current models suggest that NMDARs are necessary to maintain consolidated memories within the cortical networks. One study used inducible, reversible knockout of NR1 to assess remote memory recall and found that remote memories were impaired when NMDARs were knocked out for a one-month interval, but not for a one-week time period (Cui, Wang et al. 2004).

We tested cued and contextual fear memory recall in NR1KI mice three months after training These mice were trained at one-month of age when LTP and fear conditioning is normal and the secondary tests were conducted at a time when NMDARs

are truncated. We found no difference in the recall of remote cued or contextual fear memories in NR1KI mice compared to WT controls. These results imply that full NMDAR activity or LTP-like processes are not required for memory maintenance or recall. There are key differences between our study and Cui et al. that might explain the difference in our results. NR1KI mice have NMDARs that are still largely intact. NR1 subunits are able to complex with NR2 subunits, and these are synaptically targeted. However, these receptors have reduced activity that precludes the induction of LTP. The method utilized by Cui et al. completely depletes NMDARs from synapses. This approach likely has dramatic consequences for synaptic function and stability (Cui, Wang et al. 2004). So while Cui et al find that NMDARs are important for the preservation of remote memories, we find that impairing NMDAR activity does not affect remote memory recall. One interesting follow up experiment would be to simply train animals and treat them for a prolonged period of time with an NMDAR antagonist after consolidation. We expect that this treatment would not affect remote memory recall if treatment was discontinued prior to recall testing.

The results from this study make two important contributions to the understanding of NMDAR function. First, we find that the intracellular domains of NR1 are necessary for activation of NMDARs, but not for targeting them to the synapse. We suspect that this is mainly mediated through the interaction of NR1 with α-actinin. Second, our studies suggest that NMDAR activity or LTP-like processes are not necessary for the maintenance of remote memories as previously thought.

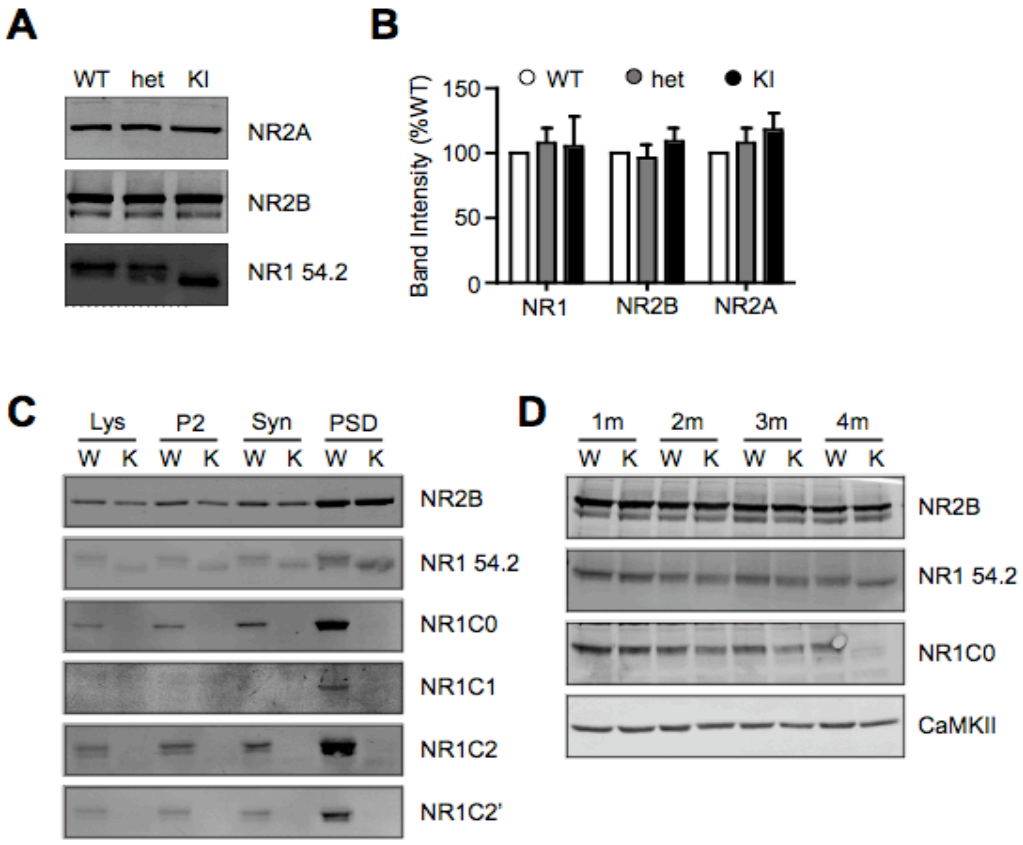

Figure 18: Age-dependent truncation of the intracellular domains of NR1 in KI mice. (A) Western blot analysis of the deoxycholate-soluble fraction of forebrain lysates from WT, het, and KI mice shows an apparent shift in the size of NR1 QN849/856EE. **(B)** Quantitative analysis of forebrain lysates shows equal protein levels of NR1, NR2B, and NR2A across genotypes. **(C)** Isolation and purification of forebrain postsynaptic densities (PSD) demonstrates an enrichment of NR1 QN849/856EE equal to WT NR1. Antibodies against the intracellular domains, C0, C1, C2, and C2' are unable to detect these domains in NR1 QN849/856EE. **(D)** Forebrain lysates from mice ages 1-4 months shows that the intracellular domains are truncated within 4 months of age but are intact in young animals. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

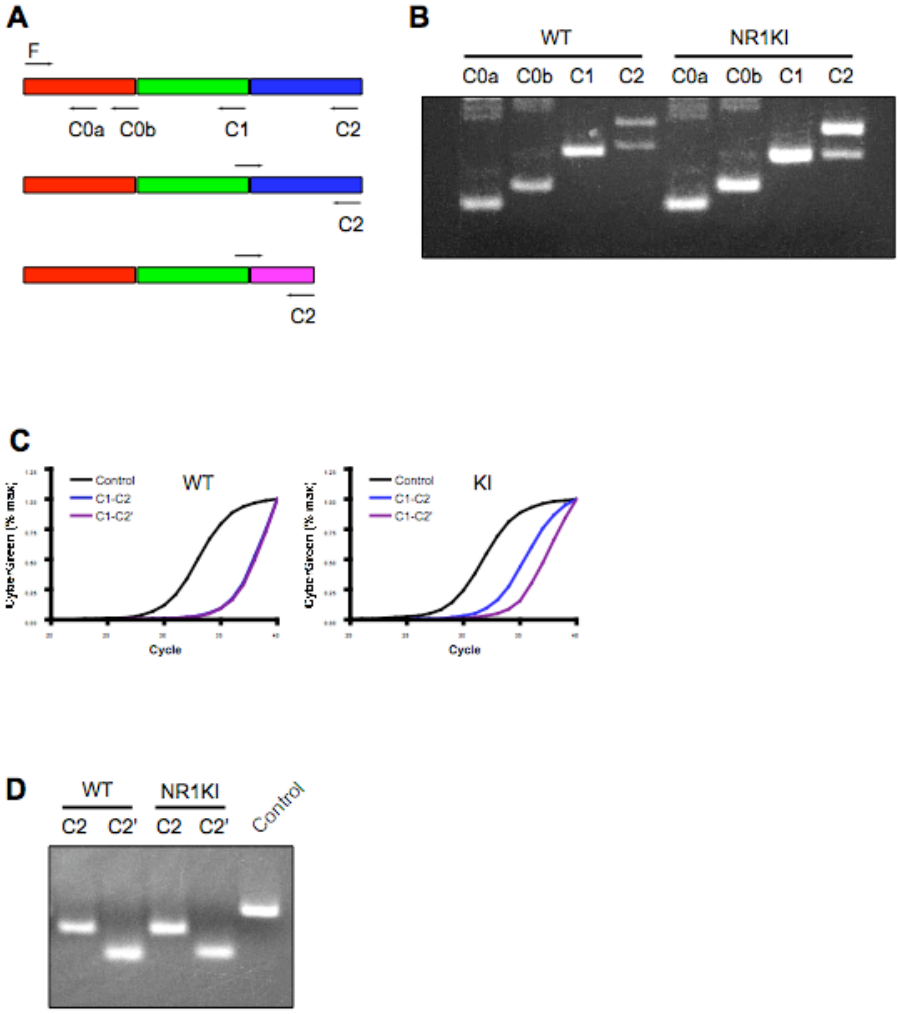

Figure 19: *Grin1* mRNA is intact. **(A)** Schematic representation of final three exons of mature *Grin1* mRNA and the primer hybridization sites. **(B)** Representative gel showing reverse transcription of the *Grin1* mRNA from total forebrain RNA with the primers from the top schematic. There were no differences in amplification between RNA samples from WT and KI forebrains. **(C)** Quantitative rtPCR showed that splicing between the C1-C2 and C1-C2' exons were not different in samples from WT and KI mice. An upstream splice site in the ligand-binding domain was chosen as a control and should be spliced in all mature *Grin1* mRNA. **(D)** Representative gel showing the quantified rtPCR amplicons. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

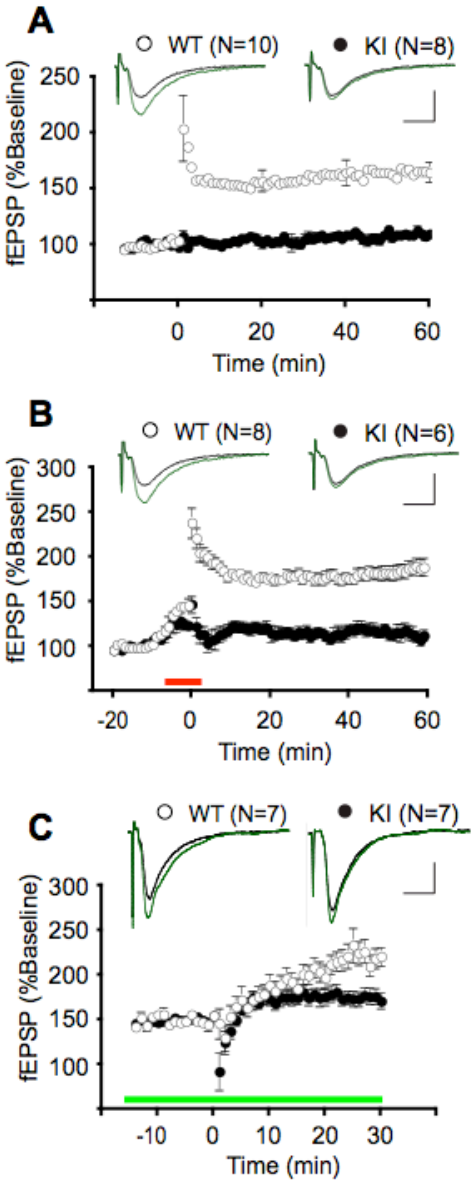

Figure 20: LTP is absent in aged NR1KI mice. (A) Long term potentiation (LTP) induced by 2 tetani of 100-Hz is absent in NR1KI mice (N = 8) compared to WT controls (N = 10, Student's t-test $p < 0.05$). **(B)** LTP induced with 2 tetani of 100-Hz in the presence of 5 uM calcium (red line) is impaired in aged NR1KI mice (N = 6) compared to WT (N = 8, Student's t-test $p < 0.05$). **(C)** LTP induced by 10 bursts of 200-Hz input in the presence of 50-100 uM APV (green line) is reduced in slices from NR1KI (N = 7) compared to WT controls (N = 7). Representative baseline traces are in black, and 50 min post LTP induction are in green. Scale bars are 5 ms and 1 mV. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

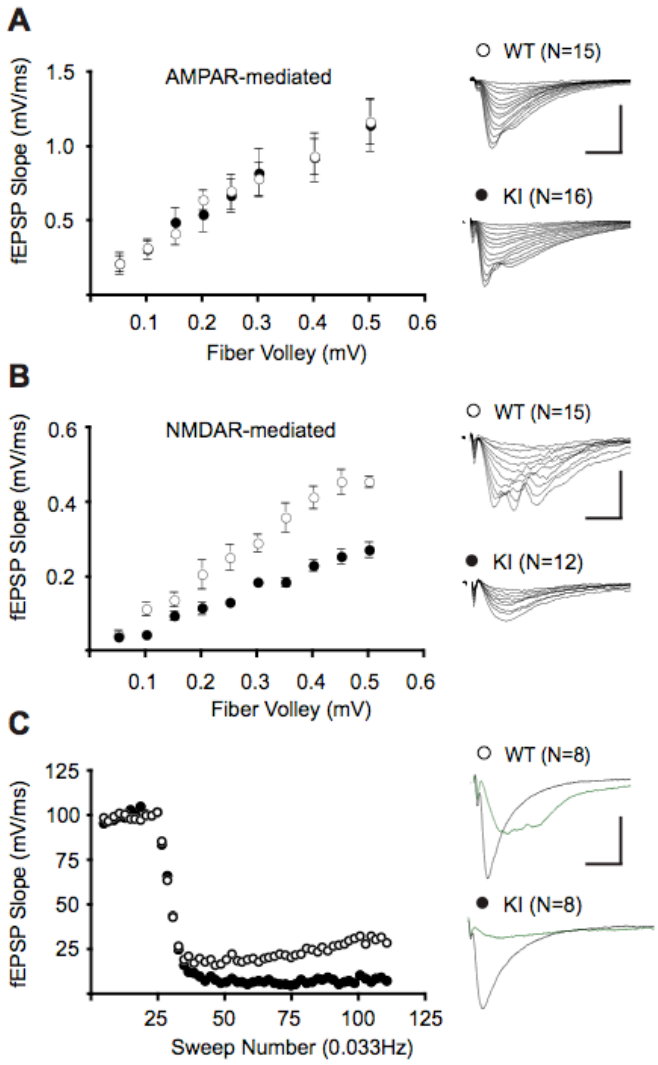

Figure 21: Normal AMPA Receptor but impaired NMDA Receptor function in NR1KI mice. (A) Field recordings from the CA1 stratum radiatum with normal ACSF using increasing stimulation intensity. The plot of fiber volley amplitude versus fEPSP left slope shows normal input-output for AMPARs in NR1KI mice (N = 16) compared to WT (N = 15). Representative traces to the right. **(B)** NMDAR-mediated fEPSPs from the CA1 stratum radiatum were recorded with ACSF containing 10 uM DNQX and 0 mM Mg+. Plotting fiber volley amplitude versus fEPSP left slope shows decreased NMDAR activity in slices from NR1KI mice (N = 12) compared to WT (N = 15). Representative traces to the right. **(C)** Application of 10 uM DNQX and 0 Mg+ demonstrates the isolation of NMDAR-mediated potentials. AMPAR:NMDAR ratios are increased in recordings from NR1KI animals compared to WT controls. Black traces are AMPAR fEPSPs, and green traces are NMDAR fEPSPs. Scale bars are 5 ms and 1 mV. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

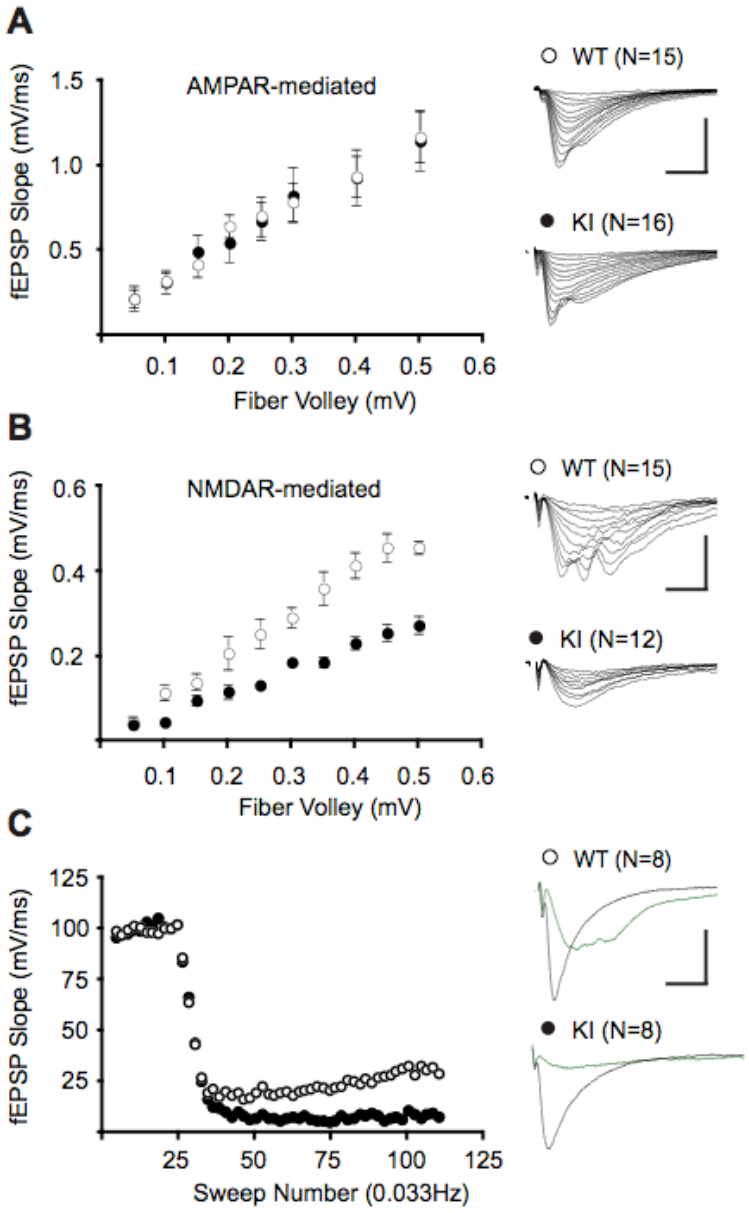

Figure 22: Short-term synaptic facilitation is reduced in aged NR1KI mice. (A) Paired pulse facilitation is significantly reduced over short interpulse intervals (IPIs) in slices from NR1KI mice (N = 15) compared to WT controls (N = 17, Student's t-test $p < 0.05$). **(B)** Peak facilitation at 50 msec IPI is not changed by acute inhibition of NMDARs by application of 100 uM APV (WT, N = 10; KI, N = 9). **(C)** Peak facilitation is corrected in low (0.8 mM) Ca2+ recording conditions (WT, N = 10; KI, N = 10). Repetitive stimulation at frequencies of **(D)** 100 and **(E)** 10 Hz of slices from WT and NR1KI mice show decreased responses in NR1KI mice. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

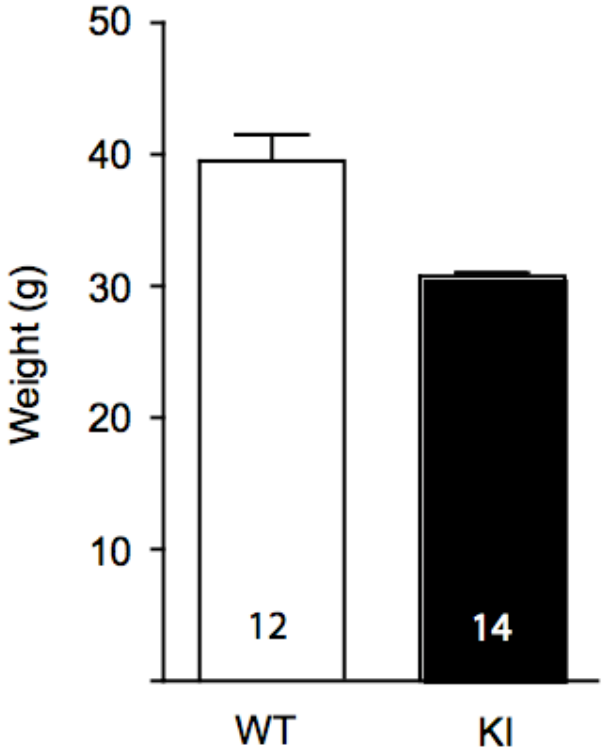

Figure 23: Aged NR1KI mice have reduced body mass. Mice were weighed at approximately 7-months of age. NR1KI mice (N = 14) weighed significantly less than WT litter-matched controls (N = 12, Student's t-test $p < 0.05$). Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

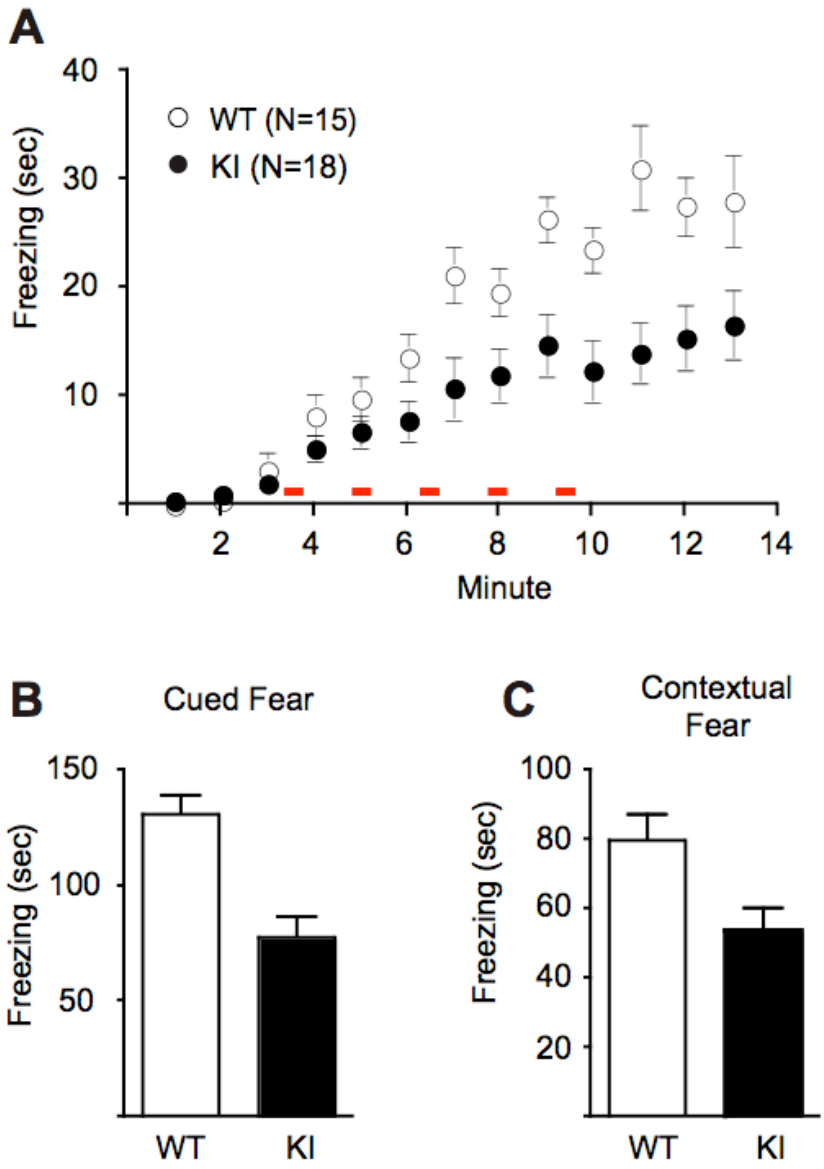

Figure 24: Impaired learning in aged NR1KI mice. (A) Mice were trained at ages 4-7 months with five tone-shock pairings (20 sec, 5 dB; 0.75 mA) in context A. NR1KI mice (N = 18) showed impaired learning over the course of the training compared to WT controls (N = 15). **(B)** Cued fear responses were measured 24 h after training with the same tone (180 sec) in context B. NR1KI mice demonstrated significantly less freezing during the tone compared to WT controls (Student's t-test $p<0.05$). **(C)** Mice were placed in context A for five minutes 1 d after cued fear responses were assessed. NR1KI mice demonstrated significantly less freezing during the trial compared to WT controls(Student's t-test $p<0.05$). Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

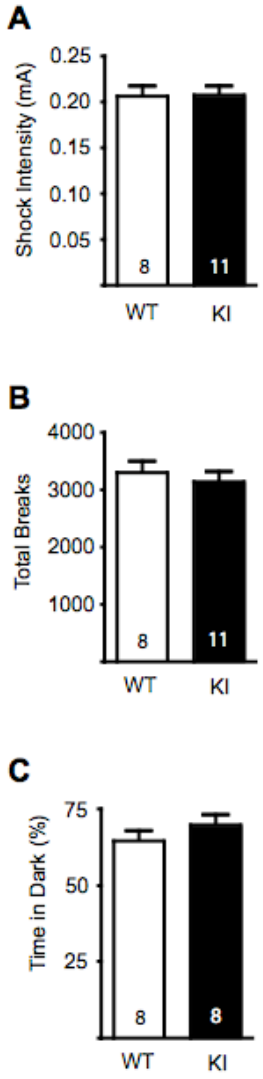

Figure 25: Shock nociception, locomotion, and basal anxiety are normal in NR1KI mice. **(A)** Shock vocalization threshholds were equivalent in aged NR1KI mice (N = 11) compared to WT controls (N = 8). **(B)** Locomotor activity in the open field test demonstrated that NR1KI mice (N = 11) explore and ambulate to the same degree as WT controls (N = 8). Total number of breaks was not statistically different over the 30 min trial. **(C)** There is no difference in the amount of time that NR1KI (N = 8) and WT (N = 8) mice spend in the dark in a light-dark box assay. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

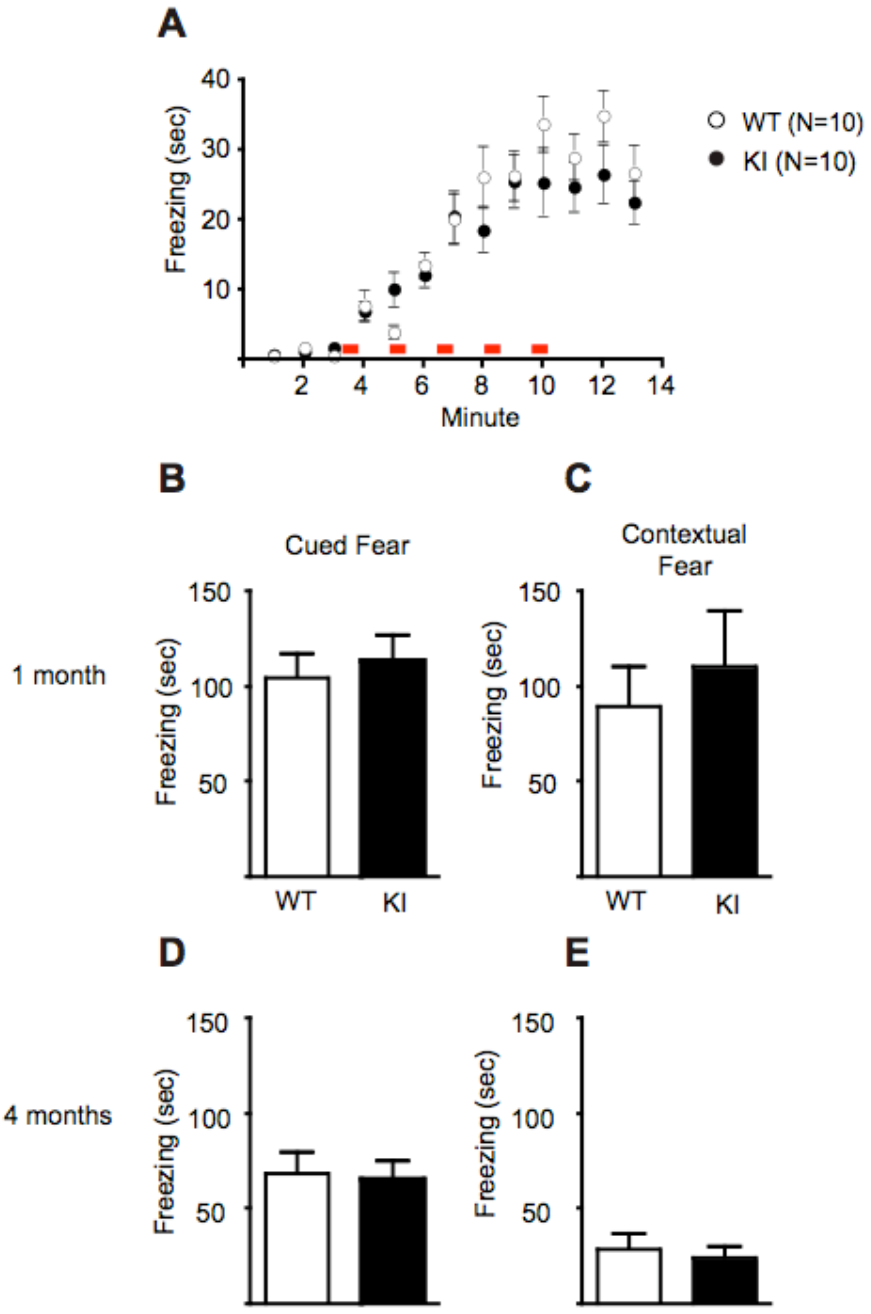

Figure 26: Remote memory recall is normal aged in NR1KI mice. **(A)** Young mice (1 m) were trained in the same paradigm as above. Freezing in NR1KI mice was indistinguishable from WT. **(B)** Cued and **(C)** contextual fear responses were normal after training. **(D)** Three months after training cued and **(E)** contextual fear recall were not statistically different (Two way ANOVA, P = 0.71). Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

CHAPTER 5: GENERAL DISCUSSION

CaMKII binding to NMDARs in LTP

NMDARs and CaMKII both have central roles in hippocampal LTP (McHugh, Blum et al. 1996; Tonegawa, Tsien et al. 1996; Tsien, Huerta et al. 1996), and the discovery of their activity-dependent biochemical interaction implied that this association was crucial for LTP. There are at least 4 interactions between CaMKII and NMDARs: two within the carboxy terminus of NR2B (Strack and Colbran 1998; Strack, McNeill et al. 2000), one in the C0 region of NR1(Leonard, Lim et al. 1999; Bayer, De Koninck et al. 2001; Leonard, Bayer et al. 2002), and one within the carboxy terminus of NR2A (Gardoni, Caputi et al. 1998; Gardoni, Schrama et al. 1999). Although CaMKII's affinity for NR2A seems to be an order of magnitude lower than for NR2B and NR1.

The role of CaMKII binding to NR2B in LTP has been previously tested using other experimental approaches. Barria et al transfected NR2B subunits with the mutations NR2B R1300Q/S1303D into cultured slices and measured LTP using a pairing protocol. These mutations were previously found to reduce CaMKII association with NR2B. However it disrupts S1303, which is a putative phosphorylation site for CaMKII on NR2B. They found that overexpression of NR2B QD did not alter NMDAR current-voltage (IV) curves or change synaptic localization of NR2B. However, they found that overexpression of NR2B QD decreased the amplitude of synaptic AMPAR- and NMDAR-mediated currents that was prevented by blockade of spontaneous activity by high magnesium. Under high magnesium conditions, where synaptic transmission seems to be normal Barria et al found that expression of NR2B QD completely abolished pairing-induced LTP in transfected hippocampal neurons compared to non-transfected neighboring neurons. This study was the first to demonstrate that CaMKII binding to NR2B is important for LTP (Barria and Malinow 2005), however there are several drawbacks to their study design. This study used overexpression of both NR1 and NR2

subunits in cultured slices. While there was no difference in synaptic transmission or LTP in untransfected neurons and neurons transfected with WT NR1 and NR2B, this does not exclude the possibility that there is no subtle differences in receptor numbers or densities. Furthermore, expression of NR2B QD changed the amplitude of both AMPAR- and NMDAR-mediated responses in culture conditions that permitted spontaneous activity.

The role of CaMKII binding to NR2B was also tested in transgenic mice. Zhou et al used the α-CaMKII promoter to overexpress a fusion protein containing the carboxy-terminus of NR2B fused to the estrogen receptor ligand-binding domain in the forebrain of mice (Zhou, Takahashi et al. 2007). This construct was designed to unfold upon treatment with tamoxifen, thereby allowing the NR2B-C to interact with CaMKII. Using this approach Zhou et al found that expression of TAM-NR2BC reduced CaMKII association with native NR2B by roughly 25%. There was no observable change in basal synaptic transmission in these mice. In acute forebrains slices from TAM-NR2BC mice treatment with 1 μM 4-hydroxytomaxifen prior to experiments reduced LTP by roughly 50% compared to WT mice and untreated TAM-NR2BC slices. These results further supported the findings of Barria et al that CaMKII binding to NR2B is necessary for LTP.

Both of the previous studies used overexpression systems to study the role of CaMKII binding to NR2B in LTP, however we addressed this question without altering the expression levels of NMDAR subunits or expressing large fragments of NR2B. We specifically knocked in two residues that were previously shown to decrease activity-dependent association of CaMKII with NR2B. Furthermore, we chose mutations in NR2B that left S1303, a putative CaMKII substrate, intact. These mutations should not affect other postsynaptic associations that NR2B has, such as PSD95 and α-actinin. Consistent with the findings of Zhou et al we found that inhibiting CaMKII binding to NR2B reduced LTP by 50% without having an affect on basal AMPAR- or NMDAR-mediated synaptic transmission. These results strongly suggest that CaMKII binding to

NR2B is necessary for the full expression of LTP in the hippocampus. Blocking CaMKII association with NMDARs does not fully block LTP though. One hypothesis is that CaMKII binding to either NR1 or the proximal portion of NR2B is also important for LTP. The role of CaMKII binding to NR1 is discussed below, but CaMKII may have other important targets in LTP that are independent of its binding to NMDARs. Furthermore, we found that inhibition of CaMKs by KN93 completely blocked LTP in slices from both WT and NR2BKI mice.

While several studies have examined the role of CaMKII binding to NR2B in LTP, no study thus far has examined the role of CaMKII binding to NR1 in LTP. Association of αCaMKII with NR2B and NR1 requires different levels of kinase activation (Strack and Colbran 1998; Leonard, Lim et al. 1999). Calcium/calmodulin are sufficient for association with NR2B, but T286 autophosphorylation is required for association with NR1. Furthermore, in neurons expression constitutively active αCaMKII (T286D) the kinase does not accumulate at synapses unless calcium is allowed into the synapse (Shen and Meyer 1999). This suggests that calcium plays two roles in activity-dependent recruitment of CaMKII to synapses. First, it activates the kinase resulting in autophosphorylation. Second, it's possible that calcium influx unmasks a CaMKII binding site. We hypothesized that this site might be NR1C0 since under basal conditions the receptor is associated with calmodulin and α-actinin and that α-actinin and CaMKII compete for binding of NR1C0 under calcium conditions. We tested this hypothesis by knocking in mutations to the C0 region of NR1 that were designed to specifically block the association of CaMKII with NR1, while leaving calmodulin and α-actinin binding unaffected. However, LTP is unaffected in NR1KI mice. In addition, we found that CaMKII association with NMDARs is unchanged in NR1KI mice and that CaMKII is normally recruited to synapses following NMDAR activation in hippocampal neurons from NR1KI mice. These results strongly suggest that CaMKII binding to NR1 is not necessary for LTP.

Collectively our results suggest that CaMKII binding to NR2B is important for hippocampal LTP but CaMKII anchoring by NR1 is dispensable. However, simply disrupting CaMKII association with NR2B does not completely abolish LTP. In NR2BKI mice the remaining LTP is unlikely to be due to CaMKII binding to NR1 subunits. One explanation for this observation is that since the mutations in NR2B do not completely block the association of CaMKII with NR2B, LTP is not fully inhibited. A second explanation is that CaMKII has synaptic targets that are important for LTP that are independent of its association with NR2B. Identifying these targets would be would be of much interest since CaMKII regulates a number synaptic processes.

CaMKII binding to NMDARs in memory acquisition

NMDARs and CaMKII activation are firmly established as central molecules involved in the acquisition of memory. Pharmacological blockade of either of NMDARs or CaMKII impairs memory acquisition, as does genetic deletion of either αCaMKII or hippocampal NMDARs (McHugh, Blum et al. 1996; Tonegawa, Tsien et al. 1996; Tsien, Huerta et al. 1996). There is further evidence that suggests CaMKII binding to NR2B is necessary for the acquisition phase of memory (Zhou, Takahashi et al. 2007). Zhou et al report their tamoxifene-inducible expression of NR2B-C strain of mice demonstrate deficits in the ability to learn spatial tasks like the Morris water and radial arm mazes. However, we find that memory acquisition in three different types of learning are unimpaired in NR2BKI mice, suggesting that CaMKII binding to NR2B is not necessary during the first phase of memory. One major difference between our study and that of Zhou et al is that expression of the entire carboxy-terminus of NR2B likely affects initial CaMKII activation. They find that CaMKII autophosphorylation is decreased with tamoxyfene treatment in the TAM NR2BC mice compared to WT and untreated transgenic mice. On the contrary, we find that CaMKII autophosphorylation is unchanged in isolated PSDs and activated normally following cLTP treatment. However,

we do not see persistent CaMKII autophosphorylation in NR2BKI mice. This suggests that the initial activation of CaMKII is important for memory acquisition, but the binding of CaMKII to NR2B is not necessary for this process.

We also found that memory acquisition was unaffected in NR1KI mice suggesting that CaMKII binding to NR1 is not necessary for this phase of memory formation. This result is not suprising considering we did not see any change in synaptic localization of CaMKII in hippocampal neurons during NMDAR stimulation and there was no change in hippocampal LTP in these mice.

CaMKII binding to NR2B in hippocampal-dependent memory consolidation

Although memory acquisition was normal in NR2BKI mice we found that these memories did persist. During the contextual fear training of NR2BKI mice using the 1 shock/day protocol we noted that the deficiency we observed after day 2 could be due to deficits in memory recall or in consolidation. To directly test whether NR2BKI mice had impairments in consolidation we trained a group of mice for context fear and either tested their memory recall after 1 hour or 4 days. After training we randomly split the mice into two groups to test immediate recall 1 hour after training or to test consolidation 4 days after training. This was done to avoid extinction during the 1-hour immediate recall. We found no difference between WT and NR2BKI mice in immediate memory recall, however NR2BKI mice showed significantly less freezing that WT mice in the 4-day consolidation probe trial. We observed similar results in the Morris water maze. NR2BKI mice are able to acquire spatial information and immediately recall it normally, however after three days they are unable to normally perform the spatial task.

From these data we conclude that while CaMKII activation by NMDARs may be important for the acquisition of memories, persistent CaMKII activity and synaptic accumulation seem to be important in hippocampal dependent memory consolidation.

This applies to both spatial and contextual memories. We suggest that persistent synaptic accumulation of CaMKII by NR2B is required for prolonged excitability of hippocampal neurons. Furthermore, we speculate that this increased excitability within the hippocampus drives further CaMKII-dependent processes in the entorhinal cortex and beyond. This idea is supported by the fact that LTP can last for days in freely behaving animals (Bliss and Lomo 1973), and this is a timeframe consistent with the process of consolidation.

CaMKII binding to NR2B in hippocampal-independent memory consolidation

One of the more interesting findings from this study was that CaMKII binding to NR2B did not seem to be necessary for all forms of consolidation. NR2BKI mice were also able to acquire cued fear memories normally, but in contrast to the water maze and context conditioning, NR2BKI mice were also able to recall these memories for as long as two weeks. Although memory acquisition in the hippocampus and amygdala both require the activation of NMDARs and CaMKII, the same may not be true during the consolidation phase. Our data suggests that the mechanisms of memory consolidation in the amygdala are different than that of the hippocampus. Whereas binding of CaMKII to NR2B is necessary for consolidation of hippocampal dependent memories, it is not required for consolidation of amygdala dependent memories. Previous studies have made similar conclusions while investigated the role of CaMKII in consolidation in both the amydala and hippocampus (Rodrigues, Farb et al. 2004). Memory consolidation is blocked when CaMKII inhibitors are injected into the hippocampus or amydala following fear training. However, administration of dopamine agonists or norepinephrine can restore consolidation after post-training infusion of CaMKII antagonist in amydala, however this is not true for the hippocampus (Barros, Izquierdo et al. 1999). Therefore consolidation of amygdala-dependent memories may be more heavily modulated by

dopaminergic and adrenergic inputs into the limbic system than hippocampal-dependent memory. This could reflect one of the fundamental differences between the functions of these two brain regions.

Age-dependent truncation of NR1 in NR1KI mice

We were surprised to find that NR1 ran at a lower molecular weight in forebrain lysates from NR1KI mice compared to WT. Since the carboxy-terminus is differentially spliced in NR1 (Laurie and Seeburg 1994), we first set out to test whether the difference in size we observed was due to alternative splicing. When we probed with specific antibodies to each of the carboxy-terminal domains of NR1 (C0, C1, C2, and C2'), we were even more surprised to find that although we were able to detect robust signals in lysates from WT mice we detected virtually no signal in lysates from KI mice. Especially concerning for us was the fact that we did not detect and C2 or C2', which contain the stop codons for NR1, nor did we detect C0, the domain that contained the KI mutations. We were concerned that our targeting construct had caused a frameshift mutation of the 3' end NR1 gene. We used rtPCR and sequencing to confirm that the grin1 mRNA was intact and that the sequence was as designed. Furthermore, we used quantitative rtPCR to measure the relative levels of 3' splicing. We essentially saw no difference in 3' splicing of the C2 and C2' exons in RNA from WT versus KI mice. The exon 17-18 junction was used as a control since this splice takes place in every NR1 mRNA. These results suggested that the truncation of NR1 was due to proteolytic processing and not due to a problem with mRNA.

This suspicion was further supported by the fact that when we prepared forebrain lysates from various aged animals, we found that there was no size shift or loss of C0, C1, C2, or C2' in lysates from young animals compared to older animals. Furthermore, lysates prepared from intermediate aged animals showed the development of low

molecular weight band and the progressive loss of C0 and C2. These results suggest that the truncation of NR1 is due to proteolytic cleavage.

Intracellular domains of NR1 in synaptic transmission

Although we found no change in AMPAR-mediated responses in the hippocampus of aged NR1KI mice, there was a dramatic reduction of NMDAR-mediated responses suggesting that the intracellular domains of NR1 important for channel activity (since NMDARs are appropriately targeted to the PSD). We think that reduced NMDAR activity seen in NR1KI mice is principally due to the disruption of the interaction with α-actinin-2. α-actinin binding to NR1 promotes an open conformation in NMDARs. Single channel recordings show that association of α-actinin increases NMDAR channel open probability but not conductance (Rycroft and Gibb 2004). Furthermore, dissociation of α-actinin from NR1 is important for calcium-dependent channel inactivation (CDI). Our results fit the model that NR1C0 association with α-actinin is required for full NMDAR channel activity, and that truncation of this domain results in decreased activation.

We also observed decreased responses in paired pulse facilitation and 10- and 100-Hz repetitive stimulations in the aged NR1KI mice. These findings suggest that hippocampal neurons form NR1KI mice have reduced presynaptic vesicle release probability. One explanation for this observation is that synaptic vesicles are spontaneously released more frequently, thus generating a hyperexcitable state and depleting synaptic vesicle pools. This is seen in several transgenic mouse models and frequently occurs when NMDARs are inhibited for prolonged periods of time. For example in CaMKII KO mice, which die prematurely due to seizures, have decreased PPF.

Intracellular domains of NR1 in remote memory recall

We found that acquisition and recall of conditioned fear was impaired in aged NR1KI mice. This is consistent with previous work that overwhelmingly implicates

NMDAR activity in these processes. However, since young NR1KI mice display normal hippocampal LTP and fear conditioning, we were able to ask whether NMDAR activity is necessary for remote fear memory recall. The current thinking is that NMDARs are necessary to maintain consolidated memories within the cortical networks. This idea is mainly based on the use of inducible, reversible knockout of NR1. Cui et al conditioned mice in cued and context fear and assessed remote memory recall nine months later. They found that remote memories were impaired when NMDARs were knocked out for a one-month interval, but not for a one-week time period. With their inducible, reversible system they were able to retest their mice after re-establishing NMDAR expression (driven from a transgene).

We used a similar approach but instead relied on the age-dependent truncation and reduced NMDAR function in NR1KI mice. We tested cued and contextual fear memory recall in NR1KI mice three months after training. These mice were trained at one-month of age when LTP and fear conditioning is normal and the secondary tests were conducted at a time when NMDARs were truncated. We found no difference in the recall of remote cued or contextual fear memories in NR1KI mice compared to WT controls. These results imply that full NMDAR activity or LTP-like processes are not required for memory maintenance or recall. There are key differences between our study and Cui et al. that might explain the difference in our results. NR1KI mice have NMDARs that are still largely intact. NR1 subunits are able to complex with NR2 subunits, and these are synaptically targeted. However, these receptors have reduced activity that precludes the induction of LTP. The method utilized by Cui et al. completely depletes NMDARs from synapses. This approach likely has dramatic consequences for synaptic function and stability. So while Cui et al find that NMDARs are important for the preservation of remote memories, we find that impairing NMDAR activity does not affect remote memory recall. One interesting follow up experiment would be to simply train animals and treat them for a prolonged period of time with an NMDAR antagonist after

consolidation. We expect that this treatment would not affect remote memory recall if treatment was discontinued prior to recall testing.

CHAPTER 6: FUTURE DIRECTIONS

The role of CaMKII binding to NMDARs in fear extinction

NMDARs are not only important for the acquisition of fear memories, but they're also necessary for their extinction (Sotres-Bayon, Bush et al. 2007). During extinction training, repeated presentation of an unreinforced conditioning stimuli leads to a decrease in conditioned responses. Fear extinction is not simply forgetting; it's an active learning process that requires NMDARs in the amygdala and hippocampus. Studying the role of NMDARs in the amygdala has proven to be difficult since traditional NMDAR antagonists like APV and CPP can block basal synaptic transmission and can affect acquired fear expression (Bauer, Schafe et al. 2002). However, the NR2B-selective antagonist ifenprodil blocks fear acquisition but does not interfere with conditioned fear expression or basal synaptic transmission in the amygdala(Rodrigues SM 2001). Administration of ifenprodil systemically or into the lateral amygdala blocks extinction of fear memories. CaMKII is important for extinction of single trial avoidance tasks, a process that is hippocampal-dependent (Szapiro, Vianna et al. 2003). CaMKII also plays a central role in the acquisition of cued fear memories(Rodrigues, Farb et al. 2004), however it's role in fear extinction in the amygdala has not been tested.

We have preliminary evidence to suggest that CaMKII binding to NR2B is necessary for the extinction of both cued and contextual fear memories. We trained WT and NR2BKI mice with a 5-shock paradigm (discussed in Chapter 2). We subsequently exposed these animals to the training context for the next 4 days in 5 min trials to assess freezing. During extinction training the mice did not receive any additional foot shocks. WT mice showed a rapid decrease in the time spent freezing during the extinction training, while NR2BKI mice froze significantly more during the first 3 days of training. These results suggest that in addition to its role in memory consolidation, CaMKII association with NR2B is also important for fear extinction. It's interesting to consider

that CaMKII binding to NR2B is necessary for both the consolidation and extinction of memories. Some studies suggest that consolidation of new memories share the same molecular mechanisms as memory extinction. Therefore, it will be interesting to further explore the role of CaMKII in both of these processes. One explanation for these results is that the neurons that were initially activated during fear training are distinct from those that are involved in fear extinction. Both acquisition and extinction of fear context-specific memories involve the hippocampus and amygdala the neuron populations that are involved may be distinct.

NMDARs and CaMKII in drug addiction

NMDARs are also important in the development of cocaine abuse, where excitatory synapses within the limbic system undergo activity-dependent changes that are similar to hippocampal LTP. A single administration of cocaine can increase AMPAR responses in dopaminergic neurons of the ventral tegmental area (VTA) for sustained periods of time (Ungless, Whistler et al. 2001). Mice administered with cocaine show both short and long-term behavioral changes (Sanchis-Segura and Spanagel 2006). Shortly after an intra-peritoneal dose of cocaine mice increase their locomotor activity that can further increase after repeated, fixed doses. This process is termed behavioral sensitization. Mice can also be conditioned to spend more time in chambers that they associate with the administration of cocaine, termed conditioned place preference (CPP). Like fear conditioning, CPP can be extinguished after several un-reinforced trails and can be reinstated. These behaviors serve as models for drug seeking and relapse. Acute injection of NMDAR antagonists into the VTA block behavioral sensitization and CPP (Carlezon and Nestler 2002), however recent studies using floxed NR1 alleles showed that specifically deleting NMDARs in dopaminergic neurons of the VTA did not attenuate CPP but had an effect on reinstatement of drug seeking (Engblom, Bilbao et al.

2008). These studies suggest that non-DA neurons in the VTA are playing a crucial role in NMDAR-dependent drug sensitization and reinforcement.

CaMKII has also recently enjoyed the spotlight in the substance abuse research field. Infusion of CaMKII inhibitors into the shell of the nucleus accumbens (NAc, another limbic structure involved in drug sensitization and reinforcement) attenuated cocaine seeking in a dose-dependent manner (Anderson, Famous et al. 2008). Furthermore, in rats that self-administered cocaine, levels of autophosphorylated CaMKII and GluR1 were increased in the NAc compared to control rats. This increase in CaMKII activity during cocaine administration seems to be mediated by L-type calcium channels through the activation of D1 dopamine receptors. Interestingly, D1Rs interact with NR1 subunits of NMDARs through their intracellular domains (Kruse, Premont et al. 2009). CaMKII is also able to bind and phosphorylate some dopamine receptors subtypes in the striatum (Hell 2009). These findings suggest that CaMKII may link dopaminergic and glutamatergic signaling in the striatum. Since we developed mice that are unable to form activity-dependent CaMKII-NMDAR complexes it would be interesting to test these mice for behavioral changes that accompany cocaine administration to test the hypothesis that CaMKII binding to NMDARs is important for cocaine seeking and reinforcement. Furthermore, since aged NR1KI mice do not express the domains necessary for associated with D1Rs, it would be interesting to test this strain of mice for drug addiction as well.

CaMKII association with NR1 in epilepsy

One of our first observations in the NR1KI mice was that the character of their post decapitation motor activity was abnormal. We typically sacrifice our mice by rapid decapitation without anesthesia since some anesthetics interfere with hippocampal field recordings. After decapitation, WT mice have a 5-6 second delay before decerebrate motor activity ensues, a process best termed opisthotonus, which is characterized by

repetitive limb extension. In WT mice this typically lasts for 10 seconds and is quite reproducible. However, in NR1KI mice opsithotonus begins immediately and lasts for 15 seconds (Figure 27). These findings led us to believe that neurons in NR1KI mice are hyperexcitable, which may lead to differences in seizure threshold, duration, or delayed termination. In acute hippocampal slice preparations we observed a decreased response in paired pulse facilitation and 10- and 100-Hz repetitive stimulations in the aged NR1KI mice. These findings suggest that hippocampal neurons form NR1KI mice have reduced presynaptic vesicle release probability. One explanation for this observation is that synaptic vesicles are spontaneously released more frequently, thus generating a hyperexcitable state. Increased spontaneously release of vesicles is seen in several transgenic mouse models and frequently occurs when NMDARs are inhibited for prolonged periods of time (Ultanir, Kim et al. 2007; Adesnik, Li et al. 2008; Zweifel, Argilli et al. 2008). For example in CaMKII KO mice, which die prematurely due to seizures, have decreased PPF (Silva, Paylor et al. 1992; Silva, Stevens et al. 1992).

There is considerable evidence that synaptic glutamate is the trigger for spreading cortical activation during the onset of epileptiform activity in neurons (Rogawski and Loscher 2004). Epileptiform activity is blocked by competitive and noncompetitive inhibitors of NMDARs including magnesium, APV, and MK-801 (Kleinrok, Turski et al. 1995). However the use of these drugs has been limited by their negative side effect profiles (except magnesium which is administered during eclampsia and status epilepticus). CaMKII may also play an important role in epilepsy; CaMKII KO mice and CaMKII SS305/306DD mice (these mice have mutations in the inhibitory phosphorylation sites) display increased susceptibility to seizures (Elgersma, Sweatt et al. 2004). However, little is known about how CaMKII binding to NMDARs regulates their activity or whether this association is important in the development of epilepsy. One possibility is that CaMKII docking on NMDARs through its association with NR2B or

NR1 regulates further NMDAR activity. This hypothesis remains untested and the role of CaMKII in regulating NMDARs activity is an open question.

One interesting experiment would be to simply test seizure severity after intraperitoneal injection of either kainate or pilocarpine to induce seizures in WT, NR2BKI, and NR1KI mice. Considering our observations with NR1KI mice with regards to their exaggerated opisthotonus response, these experiments might yield interesting results that have implications for theraputic targets in epilepsy.

The role of NMDARs in innate behavior

NMDARs have classically been studied in synaptic plasticity and learning. However, these receptors may play a role in some innate behaviors. Innate behaviors are thought to be processes that do not require previous experience for their performance. Therefore, it seems counterintuitive that a receptor so profoundly implicated in learning should be involved in a process that doesn't require learning, per se.

We found that in addition to impairments in conditioned fear acquisition and memory recall, NR1KI mice also show deficits in freezing when exposed to the predator odor, trimethyl-thiazoline (TMT), a component of fox urine (Figure 28). This is thought to be an innate defense response in rodents that does not require previous exposure to elicit fear behaviors. In NR2BKI mice the response to TMT is indistinguishable from WT mice. Our results could be due to abnormalities in amygdala function and fear expression. It's possible that NR1KI mice have alternate fear responses that preclude freezing. This is unlikely, however, since aged NR1KI mice are able to recall and express remote fear memories normally (Chapter 3). These results suggest that NMDARs are important for innate responses.

This observation is not unprecedented. NR2BKI mice die shortly after birth because they have an abnormal suckle response and they starve. These mice are able to survive 7-8 days if they are hand-fed (Kutsuwada, Sakimura et al. 1996). The suckle

response is also an innate behavior. Furthermore, NR1KO mice died shortly after birth, but they expire due to reduced respiratory drive.

Acute inhibition of NMDARs in the amygdala blocks the expression of conditioned fear, and synaptic responses from amydaloid neurons are sensitive to antagonists of NMDARs (Farb and LeDoux 1997). This suggests that basal excitatory processing in the amygdala requires NMDARs whereas in the hippocampus NMDARs contribute little to basal synaptic transmission. One key experiment would be to inject the basolateral amygdala with an NMDAR antagonist and measure TMT induced freezing. Based on our previous results and the results of others we would expect that acute inhibition of NMDARs impairs TMT induced freezing further underscoring the role of NMDARs in innate behavioral responses.

CaMKII association with NR1 in cerebellar plasticity

Delay eyeblink conditioning is one of the most studied forms of associative learning and is a model of declarative memory that involves the circuitry of the cerebellum as well as several other brain regions involved in acquisition and storage of memories. In delayed eyeblink conditioning, subjects are presented with a conditioning stimulus (CS) such as a tone or flash of light that coincides with an unconditioned stimulus (US), a puff of air to the cornea leading to a conditioned response (CR, eyeblink). Trace conditioning, on the other hand, involves a brief delay between the CS and US (typically 500 msec), during which time the subject has to retain the memory 'trace'. Although these training paradigms seem similar they engage slightly different brain regions during the acquisition phase of memory formation. Whereas subjects with damage to the hippocampus and associated medial temporal lobes are able to acquire delay eyeblink conditioning, these subjects demonstrate impairments in trace eyeblink conditioning suggesting that the hippocampus plays an essential role in the trace period (Christian and Thompson 2003). Both of these forms of conditioning require the

circuitry of the cerebellum including the sensory nuclei, pontine nuclei, middle and superior cerebellar peduncles, interpositus nucleus (deep cerebellar nuclei), and the rubrospinal tract (Christian and Thompson 2003).

Systemic infusion or cerebellar microinjection of NMDAR antagonists impairs acquisition of delayed eyeblink responses and blocks trace eyeblink conditioning (Thompson and Disterhoft 1997). Moreover, analysis of heterozygous NR1KO and NR2BKO mice, which express lower levels of NMDARs, have similar deficits in delay and trace eyeblink conditioning (Elgersma, Sweatt et al. 2004). CaMKII is abundantly expressed in Purkinje cells, which are the sole output of the cerebellar cortex. Furthermore, α-CaMKII KO mice display deficits in cerebellar LTD and in cerebellar-dependent forms of learning (optokinetic reflex and vestibulo-ocular reflex) (Hansel, de Jeu et al. 2006). These forms of adaptation allow animals to adjust eye movements to reflect head movements allowing for stable visual perception. Since both NMDARs and CaMKII are involved in cerebellar plasticity and learning, we hypothesize that CaMKII binding to NMDARs is important for this form of learning as well.

Interestingly, NMDARs in the mature cerebellum largely consist of NR1 and NR2A subunits, and NR2B expression is low (Monyer, Burnashev et al. 1994; Petralia, Wang et al. 1994). Since NR2B association with CaMKII seems to be the most critical interaction for NMDARs in the hippocampus and cortex, it would be important to test the role of CaMKII binding to NR1 in brain regions where NR2B expression is low such as the cerebellum. The NR1C0 binding site may be important for CaMKII docking to NR2A containing receptors, which could contribute to cerebellar plasticity in Purkinje cells and cerebellar learning. We are currently testing the role of CaMKII binding to NR1 in delayed and trace trace eyeblink conditioning.

CaMKII association with NR2B is not necessary for consolidation of cued fear memories

Our results suggest that CaMKII binding to NR2B is necessary for consolidation of hippocampal dependent memories but not for hippocampal independent memories. NR2BKI mice were able to acquire spatial and contextual memories in both the Morris water maze and in contextual fear conditioning, however several days after training they were unable to recall these memories. NR2BKI mice were also able to acquire cued fear memories normally, but in contrast to the water maze and context conditioning, NR2BKI mice were able to recall these memories for as long as two weeks. Although memory acquisition in the hippocampus and amygdala both require the activation of NMDARs and CaMKII, the same may not be true during the consolidation phase (Rodrigues, Farb et al. 2004). Our data suggests that the mechanisms of memory consolidation in the amygdala are different than that of the hippocampus.

Previous studies have made similar conclusions while investigated the role of CaMKII in consolidation in both the amydala and hippocampus. Memory consolidation is impaired following injection of CaMKII antagonists into the hippocampus or amydala after. Administration of dopamine agonists or norepinephrine can restore consolidation after post-training infusion of CaMKII antagonist in amydala, however the same is not true for the hippocampus (Barros, Izquierdo et al. 1999).

Therefore, NR2BKI mice could be useful tools to investigate the differences in hippocampal-dependent versus hippocampal-independent memory consolidation. Since cued fear acquisition and memory consolidation is not affected in NR2BKI mice it would be interesting to test whether LTP is affected in the amydala of these mice. Also, the amydala expresses different types of AMPAR auxillary proteins (TARPs). These may impart distinct activity-dependent AMPAR trafficking during LTP or fear learning. Furthermore, enhanced synaptic transmission in the amygala may require trafficking and insertion of GluR2-lacking receptors.

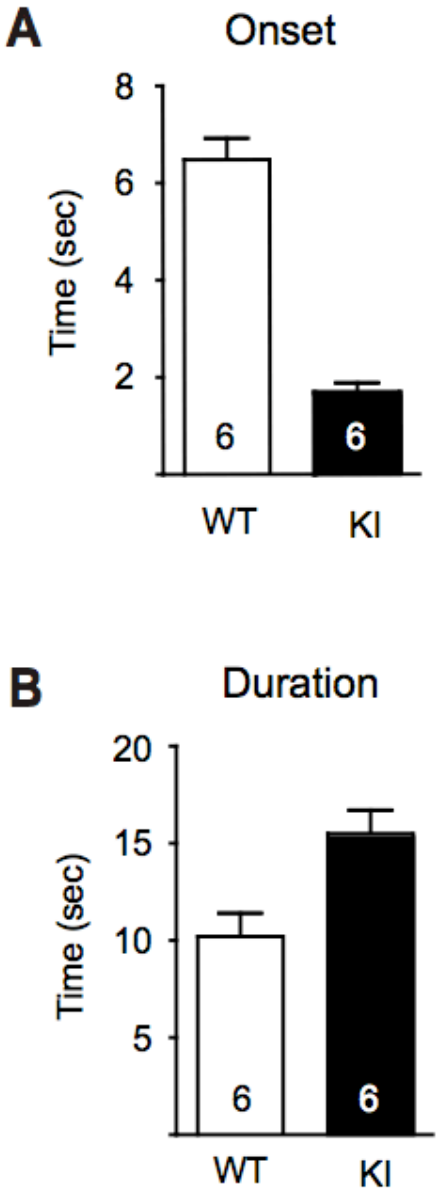

Figure 27: Onset and duration of opisthotonus are altered in NR1KI mice. (A) Onset of opisthotonus is statistically earlier and **(B)** lasts longer in NR1KI mice (N = 6) compared to WT mice (N = 6, Student's t-test, $p < 0.05$). Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data with the help of Jason Ulrich.

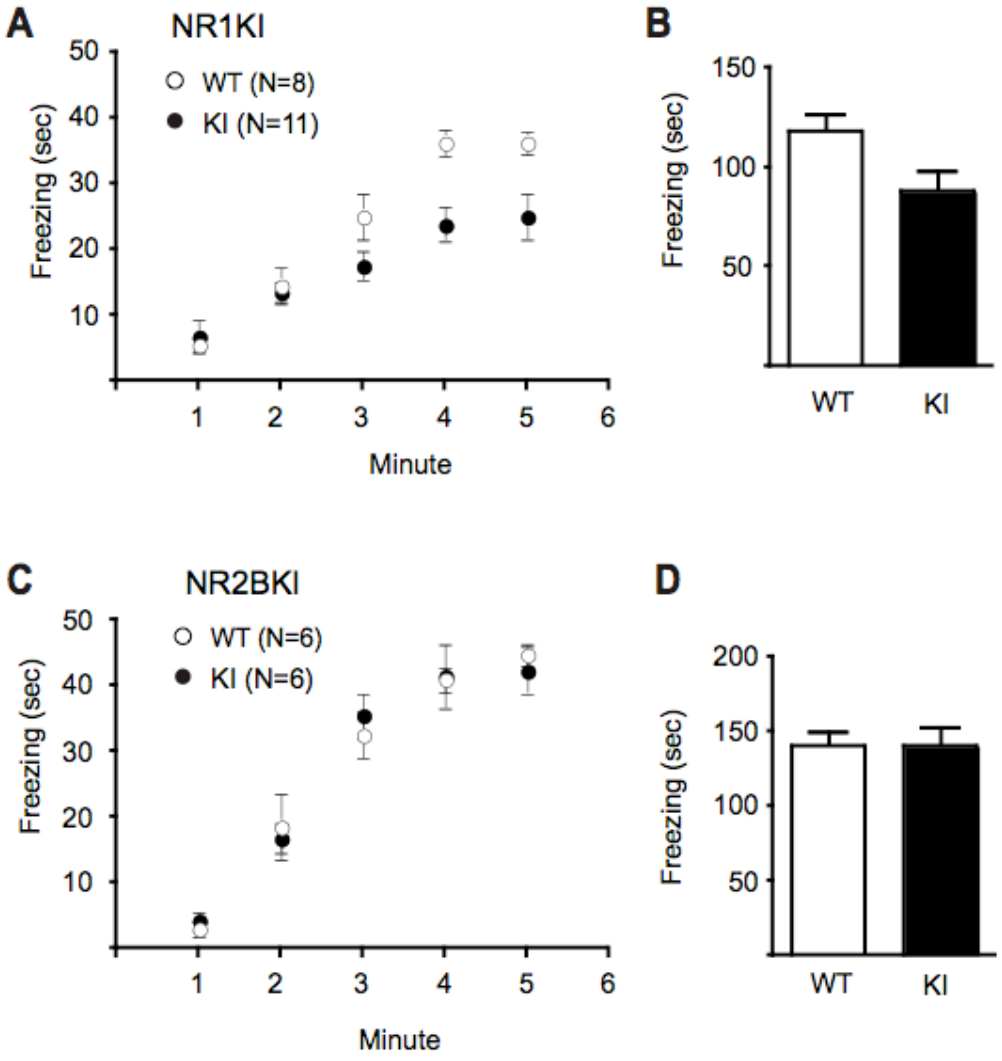

Figure 28: TMT-induced freezing is impaired in NR1KI mice. (A) NR1KI mice (N = 11) show significantly less freezing (Student's t-test, $p < 0.05$) when exposed to TMT compared to WT controls (N = 8). **(B)** There is no difference between WT (N = 6) and NR2BKI (N = 6) in TMT-induced freezing. Respective contributions: Robert Dallapiazza performed these experiments and analyzed the data.

CHAPTER 7: METHODS

Animal Husbandry and Breeding:

All procedures were in accordance with the University of Iowa Institutional Review Board and conducted under ACURF #0606028 and #0704069 (JWH). Animals were housed in a pathogen-free facility and had free access to food and water. The F1 generation of NR1 knock-in mice was from the 129/SvJ strain. These animals were backcrossed seven times into the C57/B6 strain prior to experimentation.

Genotyping of NR1KI mice:

Tail clips were solubilized in an SDS digest buffer (containing: 100 mM TrisHCl, pH 8.5, 400 mM NaCl, 0.2% SDS, 20 ug/mL RNase A, 500 ug/mL Proteinase K) overnight at 37 C. EDTA (5 mM) was added to the lysate, and insoluble material was pelleted by centrifugation. Genomic DNA was precipitated with 100% ethanol, pelleted, dried, and resuspended in 300-500 uL of sterile, DNase-free water.

To distinguish genotypes, PCR was performed using a complimentary 5' primer (CTGGTGGCTGGAGGCATCGTAG) upstream of the mutations and a 3' primer (GGCTTCCCTTGGCCCAGCTTG) downstream resulting in a 354 bp amplicon. Following PCR, the amplicon was digested overnight with PvuII and run on a 2% agarose gel. The PvuII restriction site was disrupted in the NR1KI transgene. Digested amplicons from wild-type animals had two fragments with sizes of 262 and 92 bp; heterozygous animals had three fragments with sizes of 354, 262, and 92; and KI animals had only one band 354 bp band consistent with the presence or absence of the PvuII site.

Acute Slice Preparation:

Both male and female C57/B6 mice were used in experiments (age indicated in results). Mice were sacrificed by decapitation and forebrains were rapidly dissected from the cranial vault through a mid-line incision. The tissue block containing the

hippocampal formation was removed, mounted in a slicing chamber, and bathed in ice-cold ACSF (containing in mM: 127 NaCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.9 KCl, 1.1 $CaCl_2$, 2 $MgSO_4$, 10 glucose) saturated with 95% O_2 and 5% CO_2. Transverse cortical slices (350 μM) were prepared using a Leica VT1000S vibrating microtome. Slices were hemisected and immediately placed in a chamber to recover at 30°C for 30 minutes and at room temperature thereafter for at least 1 hour prior to experiments.

Extracellular Recording

Slices were transferred to a submersion-type recording chamber perfused with oxygenated ACSF (containing in mM: 127 NaCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.9 KCl, 2.2 $CaCl_2$, 1 $MgSO_4$, 10 glucose) at a rate of 2 ml/min at 32°C. Slices were allowed to equilibrate in the chamber for 10 minutes prior to recording. An ACSF filled glass electrode was positioned in the stratum radiatum of the CA1 region of the hippocampus, and a bipolar tungsten electrode (World Precision Instruments) was adjacently positioned. Field excitatory postsynaptic potentials (fEPSP) were evoked by 0.1 msec stimulation of the Schaffer collaterals at 0.067 Hz. Field potentials were amplified with an AxoClamp 2B amplifier, filtered at 1 kHz, digitized at 10 kHz with Axon Digidata 1200 (Axon Instruments), and stored on a PC hard drive. Field EPSP rising slopes were calculated using Clampfit 9 software (Axon Instruments). The average slope of the baseline recordings were used to normalize the response. Field potentials with absolute amplitudes <0.5 mV or unstable fiber volley amplitudes were excluded from data analysis.

Stimulation intensity was adjusted to evoke a synaptic response that was 40-60% of the maximum. Baseline responses were recorded for 10-15 minutes prior to conditioning stimuli. LTP or LTD were induced using one of the following stimulation protocols: 10 Hz/15 sec, 100 Hz/1 sec, theta burst stimulation, or 1 Hz/900 sec.

Whole-cell Recording:

Pyramidal neurons in the CA1 are visualized under IR with an inverted microscope. Neurons are whole-cell patch-clamped with a borosilicate glass electrode (2-4 MΩ) filled with (in mM): 125 K-Gluconate, 10 KCl, 3 Mg-ATP, 1 $MgCl_2$, 10 HEPES, pH 7.25, 290 mOsm. Holding potentials (V_{hold}) are –70 mV. Slices are superfused with ACSF with the modifications noted. AMPA-R mediated mEPSC are recorded in the presence of 200 nM TTX to block action potentials, 10 μM bicucilline to block $GABA_A$ receptors, and 50 μM AP5 to block NMDA-R. Criteria for data inclusion in the analysis are: (1) stable series resistance <25MΩ and (2) stable input resistance > 100MΩ. Series resistance is monitored by a 50 msec depolarization from –60 to –55 mV before and after recording. AMPA-R mediated mEPSC frequency, amplitude, and decay constant are calculated (pClamp 9, Axon Instruments) and expressed as mean values using SEM for variance. We find that mEPSC frequency and amplitude are normally distributed. Student's T-test is used to calculate statistical significance between the groups.

Antibodies, Western blotting, and immunoprecipitations:

Immunoblots were performed using the following antibodies: NR1 mouse monoclonal antibody 54.2 (ascites), NR2B rabbit polyclonal C-term antibody (serum), and NR2A rabbit polyclonal C-term antibody (serum). In order to address the issue of the NR1 size shift in NR1KI mice, we probed our blots with antibodies to the intracellular domains of NR1. We used the following antibodies: NR1C0 rabbit polyclonal antibody, AA 834-864 (Millipore, Billerica, MA), NR1C1 mouse monoclonal antibody (Phosphosolutions, Aurora, CO), NR1C2 mouse monoclonal antibody (Phosphosolutions, Aurora, CO), NR1C2' mouse monoclonal antibody (Phosphosolutions, Aurora, CO). All other antibodies were previously described and available commercially.

Mouse forebrains were homogenized in sucrose buffer and centrifuged at low speed to remove cell debris. Membrane fractions were isolated by high-speed centrifugation and solubilized in 1% deoxycholate buffer. Protein concentrations were determined by BCA (Pierce). Lysates were resolved by SDS-PAGE and transferred to PVDF for immunoblotting. For coimmunoprecipitations, 500-1000 ug of total protein were incubated with 1-2 ul of antibody and 30 ul of protein A conjugated beads for at least 4 hours at 4°Celsius. Beads were washed 4-5 times in TBS, and remaining proteins were solubilized in sample buffer, electrophoresed, transferred to PVDF, immunoblotted, and imaged with an Odyssey Imaging System (Licor).

PSD were isolated as previously described. In summary 3-4 forebrains were pooled and homogenized in 15 mL 0.32 M sucrose, 1 mM $MgCl_2$, 1 mM HEPES, pH 7.0, plus protease inhibitors and centrifuged at low speed (2,500 rpm, 10 min) and high speed (20,000 rpm, 20 min; P2). P2 fraction was resuspended in 0.32 M sucrose/1 mM HEPES for sucrose step gradient centrifugation (0.85/1/1.25 M; 24,000 rpm, 2 h) [Lu, 2007 #1468]. The synaptosome fraction (Syn) at the 1/1.25 M interface was treated with 0.5% Triton X-100 and centrifuged (24,000 rpm, 30 min). The pellet (Tx-1) was resuspended in 0.32 M sucrose/1 mM HEPES for sucrose step gradient centrifugation (1/1.5/2.0 M; 35,000 rpm, 2 h) to obtain the PSD fraction at the 1.5/2.0 interface. Protein concentrations were determined with BCA assay.

Reverse transcription PCR:

To detect NR1 mRNA transcripts, we designed a series of DNA primers targeting the last three exons of NR1, which correspond to the carboxy-terminal domains. Two primers were chosen within the C0 domain coding sequence, one 5' and the other 3' of the mutations. Primer sequences were C0a: TACAAGCGACACAAGGATGC, C0b: CTGCAGGTTCTTCCTCCACAC, C1: CGTGTCTTTGGAGGACCTACG, C2: GTCTCAGCTCTCCCATGACG). A complimentary 5' primer was chosen within the

upstream M4-domain sequence (NR1F1: CTTCCGCTCCGGCTTTGGCATCG). Qiagen OneStep RT-PCR kit was used following manufacturer's instruction to amplify NR1 mRNA segments with the various primer sets. Expected amplicon sizes were C0a – 271 bp, C0b – 331 bp, C1 – 442 bp, and C2 – 559 bp. Following RT-PCR, the 331 bp C1 fragment was purified and sent for sequencing at the University of Iowa DNA Facility to confirm that the mutations were present.

For quantitative rtPCR (qPCR) forward primers were designed to target the C1-C2 and C1-C2' exon junctions, and reverse primers were designed to target the 3' ends of exons C2 and C2'. Together, these two splice events should be present in all NR1 transcripts. Forward and reverse control primers were designed to cover the splice junctions of exon 16, which should be present in all mature *Grin1* mRNA transcripts. Qiagen OneStep RT-PCR was used as described above to amplify the intended mRNA segments with the addition of Cyber Green for detection in a BioRad QPCR machine. Reactions were electrophoresed post quantification to assure only one band was amplified. Primer sequences were as follows: C1-C2 F TCCTCCAAAGACACGAGCACCG, C2 R CTCAGCTCTCCCTATGACGGG, C1-C2' F CAAAGACACGCAGTACCATCCC, C2' R ACACCACGGTGCTGACCGAGG, Ex 16 F ATGACCCCAGGCTCAGAAACC, Ex 16 R GGAGCTTGTTGTCCCGCACAGC

Fear Conditioning:

Experimentally naive mice were placed in a well-lit conditioning chamber made of aluminum walls, a plexiglass door, and rod flooring. After a three-minute habituation period, the mice were presented with five tones (80 dB, 20 sec) that each co-terminated with an electric foot-shock (0.75 mA, 1 sec, intershock interval 100 sec). After fear training the mice were returned to their home cage. The next day the mice were placed into a contextually distinct chamber: the lights were dimmed, the chamber geometry was altered from square to triangular, the rod-flooring was covered with white linoleum, and

peppermint was wiped throughout the chamber. After three minutes of habituation, mice were presented with the CS tone for 180 sec. After the tone presentation, mice were returned to their home cages. The following day mice were placed in the same context as day one for five minutes. Freezing behavior was defined as an absence of motion except that which was necessary for respiration and was measured using VideoFreeze Program (Location).

Foot Shock Vocalization Threshhold:

Foot shock vocalization thresholds were tested in a chamber similar to those used in fear conditioning. Five foot shocks were delivered for 1 sec starting at 0.08 mA, and subsequent shocks were increased by steps of 0.02 mA until vocalization occurred on consecutive shocks or 3/5 shocks at one intensity.

TMT responses:

Mice were placed in a 10X10X10 cm Plexiglas chamber containing a beaker with trimethyl-thiazoline (TMT, 30 ul, 1M), an active component of fox urine. Mice were video recorded and subsequently scored for time spent freezing. Freezing behavior was defined as an absence of motion except that which was necessary for respiration and was measured by a trained observer who was blinded to the genotype of the mice.

Open Field Test:

Experimentally naïve mice were placed in a 42X42X20 cm Plexiglas box with gridded, infrared beams spaced approximately 2.5 cm apart. Mice were allowed to explore the space for 30 min and the number of infrared beam crossing was recorded by BeamID protocol (San Diego Instruments). Total beam crossings were used to assess locomotive activity and exploratory behavior.

Light-Dark Assay:

Mice were placed in a 60X60X45 cm chamber divided into two compartments: one open-topped, painted white and brightly lit; the other covered, unlit, and painted black. The two compartments were connected by a 7X7 cm hole. Each mouse was placed in the apparatus for 10 min and its movements were monitored by a video tracking system (View Point Life Sciences) to quantify the total time that mice spent in the light versus the dark compartment.

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