ROLE OF REDOX STATE IN MEDIATING AGE-RELATED CHANGES IN HIPPOCAMPAL SYNAPTIC TRANSMISSION, PLASTICITY AND NEURONAL EXCITABILITY

By

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To my family, my friends, and Ramana

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LIST OF ABBREVIATIONS

°C	Degree Celsius (unit for expressing temperature)
x g	g-force (unit for expressing relative centrifugal force)
±	plus-minus sign (symbol for variability around a value)
AC	Alternating current
ACSF	Artificial Cerebro Spinal Fluid
ADP	Adenosine diphosphate
ad lib	ad libitum
AHP	Afterhyperpolarization
ANOVA	Analysis of Variance
AMPAR	Alpha-amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid Receptor
AP-5	2-Amino-5-Phosphonovaleric acid
AP_{amp}	Action Potential Amplitude (expressed in milli volts)
ATP	Adenosine-5'-triphosphate
BCA assay	Bicinchoninic acid assay (a method to determine total protein levels in a sample)
Ca^{2+}	Calcium (ionic form)
$[Ca_i]$	Intracellular Calcium concentration; usually expressed in nanomoles to micromoles
CA1	Cornu Ammonis Area 1
CA3	Cornu Ammonis Area 3
CaMKII	Ca^{2+}/Calmodulin-Dependent Protein Kinase II
CaN	Calcineurin

Cat	Catalase (Enzyme)
DC	Direct current
DG	Dentate Gyrus
DNA	Deoxyribonucleic acid (genetic material of living cells)
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)
DTT	Dithiothreitol
EC	Entorhinal Cortex
EPSP	Excitatory Post Synaptic Potential
F(x, y)	F-test statistic value of F-distribution
F344	Fischer 344 (strain of rat commonly used in aging studies)
fEPSP	Field Excitatory Post Synaptic Potential
Fisher's PLSD	Fisher's Protected Least Significant Difference
GABA	Gama Amino butyric acid
GSSG	Glutathione Disulfide (a dimer of two glutathione molecules)
H_2O_2	Hydrogen peroxide
HFS	High Frequency Stimulation
Hz	Hertz (unit for representing frequency of periodic events)
ICS	Intracellular Calcium Stores
kHz	Kilo Hertz (1000 Hertz)
L-GSH	L-Glutathione
L-type VGCC	L-type Voltage gated calcium channel
LTD	Long-Term Depression
LTP	Long-Term Potentiation

µm	Micro Meter (1/1000000 of a meter; unit of length)
µM	Micro Molar (unit for representing concentration of solutions)
µs	Microsecond (1/1000000 of a second; unit of time)
M	Molarity (unit for representing concentration of solutions)
MΩ	Mega Ohms (1000000 Ohms; unit of electrical resistance)
mg	Milli Grams (1/1000 of a gram; unit of mass)
Mg^{2+}	Magnesium (ionic form)
min	Minutes (unit of time)
mL	Milli Liters (1/1000 of a liter; unit of volume)
mm	Milli Meter (1/1000 of a meter; unit of length)
mM	Milli Molar (1/1000 of a Mol; unit for representing concentration)
mo	Months Old (Ex. 24 mo means 24 months old)
mV	Milli Volt (1/1000 of a Volt; unit of representing potential difference)
mU	Milli Units (1/1000 of a Unit of enzymatic activity)
myr-AIP	Myristoylated Autocamtide-2 Related Inhibitory Peptide
N	Normality (unit for representing concentration of solute in solution)
nA	Nano Ampere (1/1000000000 of an Ampere; unit for current)
Na^{+}	Sodium (ionic form)
nm	Nano meters (1/1000000000 of a meter; unit of length)
NMDAR	N-methyl D-aspartate Receptor
NMDAR-fEPSP	NMDAR Mediated Field Excitatory Post Synaptic Potential
O_2^{-}	Superoxide anion
OA	Okadaic Acid

OH^-	Hydroxyl Radical
PFV	Pre synaptic Fiber Volley
pH	Measure of acidity or basicity of solutions
PKC	Protein Kinase C
post hoc	Post Hoc Ergo Propter Hoc (Latin for “after this”)
PP1	Protein Phosphatase Type 1
PP2B	Protein Phosphatase Type 2B
R_{in}	Input Resistance
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RyR	Ryanodine Receptor
S.E.M	Standard Error of the Mean
s.or	Stratum Oriens
s.pyr	Stratum Pyramidale
s.rad	Stratum Radiatum
SC	Schaffer collateral
sec	Seconds (unit of time)
Ser/Thr	Serine/Threonine
SOD	Superoxide Dismutase (Enzyme)
V_m	Resting Membrane Potential (expressed in milli volts)
X/XO	Xanthine/Xanthine Oxidase

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The mechanisms that disrupt normal neuronal function during aging are poorly understood. Due to this fact we do not yet possess a reliable therapeutic strategy to treat age-related memory loss and cognitive dysfunction. Given the central role played by hippocampal CA1 pyramidal neurons in learning and memory, understanding the senescent changes to the biochemical and physiological properties of these neurons has become a necessary first-step in developing effective therapeutic strategies. The hypothesis that forms the basis for this dissertation is that increased oxidative stress or a more oxidative redox state mediates an age-related shift in Ca^{2+} homeostasis The experiments presented in this dissertation were designed to delineate the age-related changes to the N-methyl D-aspartate receptor (NMDAR) function of CA1 pyramidal neurons. We tested the hypothesis that the age-related decline in NMDAR function was linked to a more oxidative redox state of the neuron. We confirmed that the NMDAR function declines in the CA1 region of aged hippocampus. The results indicate that the intracellular redox state of the aged neurons shifts to a more oxidative environment. The oxidizing agent xanthine/xanthine oxidase (X/XO) decreased the NMDAR mediated

synaptic responses at hippocampal CA3-CA1 synapses, in slices from young (3-8 mo), but not aged (20-25 mo) F344 rats. Conversely, the reducing agent dithiothreitol (DTT) selectively enhanced the NMDAR mediated synaptic response in aged but not in young hippocampal slices. The age-dependent sensitivity of the NMDAR function to DTT was associated with facilitated induction of long term potentiation (LTP) in aged but not young animals. Moreover, experiments using membrane impermeable reducing agent L-glutathione (L-GSH) indicated that the NMDAR response was dependent on the intracellular redox state. The effect of DTT was not observed for the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (AMPAR).

The intracellular redox state dependent effects of DTT on NMDAR function indicated a role for various intracellular signaling cascades. We tested the hypothesis that the DTT-mediated increase in NMDAR function involved intracellular kinases and/or phosphatases. The blockade of DTT effect by H-7 indicated the involvement of Ser/Thr kinase(s) in mediating the increase in NMDAR function. The DTT-mediated increase in NMDAR function was not blocked by Bis-I (a protein kinase C inhibitor), but was blocked by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor - myristoylated autocamtide-2 related inhibitory peptide (myr-AIP), and the general CaM kinase inhibitor KN-62. Furthermore, the inhibition of the activity of protein phosphatases- PP1 and calcineurin had no effect on the DTT-mediated increase in NMDAR function. These results suggest a role for the CaMKII signaling cascade. Our results with CaMKII activity assays established that DTT increases CaMKII activity in CA1 cytosolic extracts from aged but not from young animals. The findings provide a link between intracellular redox state and CaMKII activity during aging, which causes

the decline in the NMDAR function, and subsequently impairs synaptic plasticity in the aged hippocampal neurons. Taken together the results provide a link between a hypothesized mechanism of aging (increased oxidative stress) and mechanisms of impaired memory (decreased NMDAR function and impaired synaptic plasticity).

We further tested the hypothesis that increased oxidative stress or a more oxidative redox state decreases neuronal excitability of aged neurons by increasing the post burst afterhyperpolarization (AHP). Application of DTT decreased the slow component of afterhyperpolarization (sAHP) in CA1 pyramidal neurons of aged but not young animals. The DTT-mediated decrease in aged-sAHP was blocked by the depletion of intracellular Ca^{2+} stores (ICS) using thapsigargin or blockade of ryanodine receptor (RyR) by ryanodine. Neither the inhibition of L-type voltage gated calcium channels (L-type VGCC) nor the inhibition of Ser/Thr kinases by H-7 had any effect on the DTT-mediated decrease in aged-sAHP. The results suggest that a more oxidative redox state during aging contributes to RyR oxidation, increases Ca^{2+} mobilization from the ICS, and increases the sAHP.

The results presented in this dissertation link oxidative redox state of aged neurons to decrease in the NMDAR function, and increase in sAHP. These two processes are very potent and functionally significant biomarkers of aging in the hippocampus. Hence the results of this study will have significant impact on the development of therapeutics that can offset senescent changes to the biochemical and physiological properties of hippocampal neurons and provide fundamental insights into the mechanisms that mediate age-related memory loss and neuronal dysfunction.

CHAPTER 1
INTRODUCTION

Learning and Memory

The mammalian brain is endowed with the amazing capacity for learning new information that can be stored as memories. It is fascinating that the brain possesses the unique capacity for learning and memory, in addition to being the seat of a wide variety of human faculties (Crick, 1995). Certain regions of the brain are designed to take part in distinct forms of learning and memory, for example, the hippocampus located in the medial temporal lobe of the brain is involved in the formation and retrieval of declarative/explicit memory. The two main classes of cells in the hippocampus that possess unique properties (Kupfermann et al., 2000), and enable the learning and memory function of the brain are the neurons and the glial cells. The neurons are the predominant type of signaling cells, which communicate through chemical neurotransmitters and receptors. The glial cells are the supporting cells, which provide nutrition and recycle neurotransmitters released by the neurons (Kandel, 1991, 2000a; Alberts et al., 2002). The learning and memory function of the hippocampus is accomplished by the utilization of a complex array of molecules and signaling mechanisms present in these cells.

The studies presented in this dissertation were designed to test the hypothesis that an increased oxidative stress or a more oxidative redox state mediates age-related shift in Ca^{2+} homeostasis and contributes to neuronal dysfunction. Neuronal function is defined by the neuron's synaptic transmission, plasticity and excitability (described in detail in the following sections). Our experiments were designed to test the effects of redox state modulators on important markers of neuronal function, namely the synaptic

transmission mediated by the N-methyl D-aspartate receptors (NMDARs), synaptic plasticity and neuronal excitability. Before describing the results in the following chapters, an overview of the key components of the hypothesis is provided in the following sections of this introduction.

Aging Effects on Hippocampus

The hippocampal function is particularly vulnerable to dysfunction during aging. The National Institute on Aging (NIA) has identified cognitive impairment due to memory dysfunction as a normal part of aging. In particular, the hippocampal function is impaired in aged animals such that they learn slower and forget easily (Barnes, 1979; Foster, 1999). In this context it is worthwhile to differentiate the effects of normal aging on hippocampus-dependent memory function and the effects arising from neurodegenerative processes. The pattern of changes to hippocampus during normal aging is different from that observed in individuals suffering from neurodegenerative disorders like Alzheimer's disease (AD). The primary difference seems to be the absence of major neuron loss in people with normal aging. Although initial data suggested neuronal loss during aging (Ball, 1977; Brizzee et al., 1980; Coleman and Flood, 1987), subsequent studies have conclusively proved that no significant neuron loss is observed (West et al., 1994; Rapp and Gallagher, 1996; Rasmussen et al., 1996; Gazzaley et al., 1997; Morrison and Hof, 1997; Pakkenberg and Gundersen, 1997; Merrill et al., 2001). Furthermore, the loss of memory function, due to neuron loss, is associated with neurodegenerative disorders but not normal aging (Rapp and Gallagher, 1996; Rasmussen et al., 1996). In other words, the basic elements required for hippocampus dependent learning and memory seems to be intact during normal aging; however these systems are progressively weakened in their function.

The absence of neuron loss indicates that the hippocampus does not exhibit an "anatomical lesion" during aging. Nevertheless, the memory systems dependent on hippocampus become dysfunctional during aging, such that aged animals learn slower and forget rapidly, as noted above (Barnes, 1979; Dunnett et al., 1990; Mabry et al., 1996; Oler and Markus, 1998; Foster, 1999; Norris and Foster, 1999). Based on the evidence presented in the following chapters, it is becoming increasingly clear that the neurobiological correlates of memory loss during normal aging are subtle physiological, biochemical and posttranslational changes accumulated in the hippocampal neurons. These lines of evidence support the idea of a "functional lesion" of hippocampus during aging characterized neuronal dysfunction.

Learning and Memory Systems Dependent on Hippocampus

In chapters 3, 4 and 5 we have presented results that suggest that increased oxidative stress or more oxidative redox state contributes to the physiological and biochemical changes in the hippocampal CA1 pyramidal neurons during aging. In order to better understand the functional significance of these results, a brief overview of the memory systems in the brain and a detailed overview of the role of CA1 pyramidal neurons in hippocampus dependent learning and memory are provided in the following section.

Although several memory systems are thought to utilize the neural networks of hippocampus (Riedel et al., 1999), the declarative/explicit memory system is particularly dependent on the intact functioning of the hippocampus (Eichenbaum, 1997; Mingaud et al., 2007). Declarative memory includes semantic and episodic memory (Squire and Zola, 1998). Semantic memory is the capacity to store general knowledge and recollect factual information (Squire and Zola, 1998; Kapur and Brooks, 1999; Holdstock et al.,

2002; Manns et al., 2003) and episodic memory is the capacity to store and recollect information about time, places and their context in a temporal order (Vargha-Khadem et al., 1997; Tulving and Markowitsch, 1998). Models of memory impairment in nonhuman primates (Mishkin, 1982; Squire et al., 2004), combined with extensive behavioral characterization (Zola-Morgan et al., 1994) has indicated that hippocampal pyramidal neurons are necessary for the acquisition and consolidation of declarative memory; while long-term storage occurs in the neocortical regions of the brain (McClelland et al., 1995; McClelland and Goddard, 1996; Eichenbaum, 2000; Fell et al., 2001; Kali and Dayan, 2004). The hippocampal CA1 pyramidal neurons mediate memory consolidation (Shimizu et al., 2000; Remondes and Schuman, 2004; Frankland and Bontempi, 2005; Ji and Wilson, 2007; Takehara-Nishiuchi and McNaughton, 2008), and the retrieval or recollection of recently formed memories (Gabrieli et al., 1997; Roozendaal et al., 2001; Smith and Squire, 2009). All these observations support the idea that the CA1 pyramidal neurons of the hippocampus play a crucial role in the formation, consolidation and retrieval of declarative/explicit memories in the mammalian brain. The hippocampal formation is also important for the acquisition of spatial memory, which denotes the capacity to store and retrieve information regarding the spatial location and relative orientation of objects (O'Keefe, 1993; O'Keefe and Burgess, 1996; Nakazawa et al., 2004; McNaughton et al., 2006). Spatial information is represented in the hippocampus through alterations in the firing properties of the CA1 pyramidal neurons in the hippocampus (O'Keefe and Speakman, 1987; Foster et al., 1989).

The intricate anatomy of the hippocampus enables its learning and memory function. One of the ideas used in constructing the hypotheses in chapters 3, 4, and 5

has been that age-related changes to the learning and memory function of the hippocampus arises, in part, from the physiological and biochemical changes to the CA1 pyramidal neurons. Since all studies presented in this dissertation concern the CA1 pyramidal neurons, a brief overview of the hippocampal neuroanatomy and the organization of the CA1 pyramidal neurons within the hippocampal CA1 subfield are provided below.

Neuroanatomy of Hippocampus

The hippocampus is a sea horse shaped structure located in the medial temporal lobe of the brain. It is part of the hippocampal formation in the medial temporal lobe, which includes the entorhinal cortex (EC), the subiculum, the presubiculum and the parasubiculum. The hippocampus is divided into three major subfields: the CA1 region, the CA3 region, and the dentate gyrus (DG). The abbreviation CA stands for *cornu ammonis*, due to its semblance to a ram's horn. The experiments described in the following chapters were all designed to study the synaptic transmission and plasticity in the CA3-CA1 synaptic contacts, which are part of the tri-synaptic pathway. In the tri-synaptic pathway, the first set of synaptic contacts occur between the axonal afferents from EC onto the DG pyramidal neurons, the second set of synaptic contacts between the afferents from DG pyramidal neurons onto the CA3 pyramidal neurons, and the third set of synaptic contacts between the afferents from the CA3 pyramidal neurons onto the CA1 pyramidal neurons (Kandel, 2000b; Amaral and Lavenex, 2007). In addition to the tri-synaptic connection between the principal pyramidal cells of the hippocampus, there are many recurrent and interneuronal connections in all the major subfields of the hippocampus thus providing a massive, yet organized, network of neurons.

The subfields of hippocampus are further distinguished into various layers which reflect the underlying laminar organization, orientation and location of the principal pyramidal cells. The CA1 subfield is distinguished into *stratum lacunosum molecular (s.l.m)*, *stratum radiatum (s.r)*, *stratum pyramidale* (*s.p* or the pyramidal cell layer), and *stratum oriens (s.o)* (Amaral and Lavenex, 2007). The dendrites of the CA1 pyramidal neurons are located in *s.l.m* and *s.r*; the cell body is located in *s.p*; and axon passes through *s.o*. In addition, the DG subfield is distinguished into the molecular layer, the granule cell layer and the polymorphic cell layer, and the CA3 subfield is distinguished into *stratum radiatum*, *stratum lucidum*, *stratum pyramidale* (or pyramidal cell layer) and *stratum oriens*.

The function of the CA1 pyramidal neurons is defined by the neuron's synaptic transmission, plasticity and excitability. In chapters 3, 4, and 5 we tested the hypothesis that a shift in the redox state to a more oxidative environment contributes to neuronal dysfunction by altering synaptic transmission, plasticity and excitability. In order to clarify the key components of this hypothesis, a brief description of the above mentioned parameters of CA1 pyramidal neurons is provided in the following sections.

Aging Effects on NMDAR Mediated Synaptic Transmission

The decline in NMDAR function is thought to be one of the critical biomarkers of aging in CA1 pyramidal neurons (Foster, 2006), which is also supported by previous reports (Barnes et al., 1997; Billard and Rouaud, 2007). In chapter 3, we tested the hypothesis that age-related decline in NMDAR function is caused by increased oxidative stress or a more oxidative redox state. In order to better understand the hypothesis and results, a brief description of the properties and function of NMDARs in the CA1 pyramidal neurons and their role in synaptic transmission and plasticity is provided in

the following sections. But first, an evaluation of the age-related changes to NMDAR function is discussed.

The NMDAR mediated synaptic transmission is critical for acquisition and consolidation of hippocampus dependent spatial learning and memory (Bannerman et al., 1995). As noted above, there is considerable evidence to indicate that aging is associated with a decline in NMDAR function within regions involved in processing and performing higher brain function including learning and memory (Gonzales et al., 1991; Pittaluga et al., 1993; Barnes et al., 1997; Magnusson, 1998; Eckles-Smith et al., 2000; Gore et al., 2002; Liu et al., 2008; Zhao et al., 2009). Perhaps the strongest evidence for a reduction in NMDAR function comes from physiological studies which indicate that the NMDAR mediated excitatory post synaptic potentials in the CA3-CA1 synapses of the hippocampus are reduced by approximately 50% in aged animals (Barnes et al., 1997; Eckles-Smith et al., 2000; Bodhinathan et al., 2010). However, age-related changes in the amplitude of NMDA-evoked responses were not observed in dissociated cortical neurons suggesting the possibility of regional specificity in the loss of NMDAR function (Kuehl-Kovarik et al., 2003). Several studies indicate a decrease in the level of NMDAR protein expression in the hippocampus during aging (Bonhaus et al., 1990; Kito et al., 1990; Miyoshi et al., 1991; Tamaru et al., 1991; Wenk et al., 1991; Magnusson, 1995; Magnusson et al., 2006; Billard and Rouaud, 2007; Das and Magnusson, 2008; Liu et al., 2008; Zhao et al., 2009). Moreover, the decrease has been localized to area CA1 of the hippocampus (Magnusson and Cotman, 1993; Gazzaley et al., 1996; Magnusson, 1998; Wenk and Barnes, 2000); wherein the studies report reduced binding of [^{3}H] glutamate (agonist site), [^{3}H] glycine (NR1 site), [^{3}H] CPP (a competitive

antagonist to the L-glutamate binding site), and [^{3}H] MK-801 (an open channel blocker) in the hippocampus of aged rats. However, others have reported no age-related change in antagonist binding (Kito et al., 1990; Miyoshi et al., 1991; Araki et al., 1997; Shimada et al., 1997), or an increased MK-801 binding in animals with learning and retention deficits (Ingram et al., 1992; Topic et al., 2007). It is important to note that MK-801 binds to the hydrophobic channel domain of NMDAR, exclusively labeling open channels. Thus, an apparent increase in NMDAR channel open time may act as a compensatory mechanism for the decrease in receptor number (Serra et al., 1994). However, the majority of reports, including our recent findings, indicate that the net function of the NMDARs decreases at CA3-CA1 hippocampal synaptic contacts during aging (Bodhinathan et al., 2010). Thus, our working hypothesis is based on the idea that the age-related changes in the NMDAR function are predominantly posttranslational, probably involving oxidation/reduction and/or changes in phosphorylation. Before describing the results which indicate a link between oxidative redox state and decrease in NMDAR function during aging, a brief overview of the general properties of synaptic transmission and plasticity involving the CA1 pyramidal neurons is provided in the following section.

Synaptic Transmission and Plasticity in CA1 Pyramidal Neurons

Information is transmitted between neurons through the synapses (a narrow cleft between two neurons). Neurotransmitters released by presynaptic neurons bind to and activate the receptors at the postsynaptic sites. This action constitutes synaptic transmission. The ability to modify the strength of synaptic transmission between the neurons is thought to underlie the learning and memory function of the brain, including hippocampal-dependent learning and memory (Bliss and Collingridge, 1993; Foster et

al., 1996; Malenka and Nicoll, 1999; Bailey et al., 2000; Martin et al., 2000; Dragoi et al., 2003). In particular, the neurons possess a vast array of signaling molecules that are responsive to various aspects of learning and memory (Lisman, 1994; Tsien et al., 1996; Benson et al., 2000; Abel and Lattal, 2001; Genoux et al., 2002; Koekkoek et al., 2003). Unfortunately the learning and memory functions of the hippocampus are weakened during aging and disease.

The role of hippocampus in learning and memory, described previously, depends on the complex synaptic properties of its neurons. The hypothesis presented in chapter 3 is that age-related increase in oxidative stress or oxidative redox state decreases the NMDAR mediated synaptic transmission. The focus of this hypothesis has been the CA3-CA1 synaptic contacts located in the CA1 subfield of the hippocampus. A part of the CA3 axons constitute the Schaffer collateral pathway that release L-glutamate, which binds to ionotropic and metabotropic glutamate receptors on the CA1 pyramidal neurons. Synaptic transmission is completed upon the activation of the receptors, leading to inflow of Na^+ and Ca^{2+} ions into the neuron, and outflow of K^+ ions from the neurons. The ability to modify the strength of synaptic transmission between neurons is termed synaptic plasticity. In this study we have investigated the influence of redox state on synaptic transmission and plasticity during aging. Specifically we tested whether the redox agents can modulate NMDAR mediated synaptic transmission and plasticity in an age-dependent manner.

Ionotropic Glutamatergic Transmission

The experiments described in chapters 3 and 4 were designed to analyze the effects of redox agents on NMDARs, and we also tested if they had any effect on AMPARs. The NMDARs and AMPARs are ionotropic glutamate receptors (iGluRs),

which are activated by the amino acid neurotransmitter L-glutamate (Collingridge et al., 1983) released from the axonal terminals of CA3 pyramidal neurons. The L-glutamate can also activate metabotropic glutamate receptors (mGluRs) on the CA1 pyramidal neurons; however all the studies presented in this dissertation involved the iGluRs.

The iGluRs mediate fast synaptic transmission and are classified into three major subtypes, named after the synthetic agonists that activate them. They are the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (abbreviated as AMPAR), the kainate receptor and the N-methyl D-aspartate receptor (abbreviated as NMDAR). The AMPARs and kainate receptors are also categorized as non-NMDARs. All the iGluRs are non-selective cationic channels that are permeable to both Na^+ and K^+ (Aidley, 1989; Johnston and Wu, 1995). However the NMDARs are relatively more permeable to Ca^{2+} ions (Garaschuk et al., 1996). The ionic fluxes mediated by the iGluRs in the CA1 pyramidal neurons are measured as the excitatory post synaptic potential (EPSP). The non-NMDARs generate the early phase of the EPSP (~ 0 to15 ms from stimulation) and the NMDARs contribute to the late phase of the EPSP (greater than 15 ms time window from stimulation). In this study we have analyzed both the AMPAR-mediated and NMDAR-mediated synaptic transmission.

Decreased NMDAR function is an important biomarker of aged CA1 pyramidal neurons, and is also the central aspect of hypotheses presented in chapters 3 and 4. We tested the hypothesis that increase in oxidative stress or oxidative redox state of aged neurons contributes to decrease in the NMDAR mediated synaptic transmission and plasticity. In order to better understand the results in support of this hypothesis, it is critical to understand the properties and function of NMDAR mediated synaptic

transmission and plasticity. The following section provides a brief overview of the ionic currents and synaptic mechanism that are associated with NMDAR function in neurons.

The NMDARs are hetero-tetrameric protein complexes composed of two classes of subunits, the ubiquitously expressed and essential subunit - NR1; and a modulatory subunit - NR2A/2B/2C/2D (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Cull-Candy et al., 2001). The activation of NMDAR requires binding of a ligand (glutamate), membrane depolarization (to remove the Mg^{2+} block on the channel), and binding of a co-agonist (glycine). These requirements for NMDAR activation makes it the ideal coincidence detector to integrate presynaptic and postsynaptic activity. Since NMDAR is a non-selective cation channel, its activation and opening leads to simultaneous conductance of Na^{+}, K^{+}, and Ca^{2+} ions (Chen et al., 2005). However the NMDARs are at least 19 times more permeable to Ca^{2+} ions than the AMPARs, which are the other major subtype of iGluR in the CA1 pyramidal neurons (Garaschuk et al., 1996), primarily because all the NMDAR subunits carry the polar but neutral residue arginine in the M2 region of their pore domain (Zarei and Dani, 1994; Ferrer-Montiel et al., 1996; Premkumar and Auerbach, 1996). The NMDARs also play a critical role in influencing the properties of AMPARs. For example the AMPARs are rapidly inserted into recently activated synapses (Hayashi et al., 2000; Zhu et al., 2000; Song and Huganir, 2002) where NMDARs have been activated (Petralia et al., 1999; Shi et al., 1999). This feature serves as the molecular basis for the induction and expression of synaptic plasticity at CA3-CA1 synapses (described in detail in the following section). Although AMPAR subunits carry an arginine, instead of glutamine in their pore domain, can generate significant Ca^{2+} influxes, akin to the NMDARs (Jonas et

al., 1994; Lomeli et al., 1994); the NMDARs are the primary source of iGluR derived Ca^{2+} in the CA1 pyramidal neurons.

NMDA Receptor Dependent Synaptic Plasticity: LTP and LTD

As described in more detail in the previous section, the NMDA receptor component of synaptic transmission is decreased in aged animals. This is important because, the influx of Ca^{2+} through the NMDARs is critical to the activation of signaling cascades in close proximity to the synapses, such that those synapses undergo a change in the strength of synaptic transmission. A change in the synaptic strength between the hippocampal neurons constitutes hippocampal synaptic plasticity which is thought to underlie the formation of memories in the mammalian brain (Morris et al., 2003). Age–related decline in memory function is associated with altered hippocampal synaptic plasticity (Foster, 1999). In chapter 4 we tested the hypothesis that the age-related increase in oxidative stress and decrease in NMDAR function contributes to the alteration in synaptic plasticity. In order to better understand the results, a brief overview of the relationship between NMDAR function, and synaptic plasticity is provided below.

One aspect of synaptic plasticity is long term potentiation (LTP), first reported in 1973 (Bliss and Lomo, 1973), as a long lasting enhancement in synaptic transmission between two neurons following brief high-frequency electrical stimulation. In contrast, long term depression (LTD) is a long lasting decrease in the strength of synaptic transmission between two neurons, which is observed after prolonged low-frequency stimulation. LTD was first reported in 1977 (Lynch et al., 1977), and an integrative model of the interplay between LTP and LTD has emerged since then. Although LTP and LTD are processes of synaptic plasticity with opposite outcomes, they are governed by changes in the Ca^{2+} dynamics of the postsynaptic neuron (Cummings et al., 1996;

Shouval et al., 2002), involving calcium influx predominantly through the NMDARs. In this dissertation we have investigated the effects of redox modulators on NMDAR function.

Experiments have shown that LTP is initiated upon a quick and large amplitude rise in the intracellular Ca^{2+} concentration in the postsynaptic neuron, while LTD induction requires a moderate rise in intracellular Ca^{2+} concentration over longer periods of time (Cho et al., 2001; Cormier et al., 2001). Other studies have shown that elevations in the cytosolic Ca^{2+} levels in the postsynaptic neuron is sufficient, by itself, to cause bidirectional changes in synaptic strength without presynaptic activity (Neveu and Zucker, 1996a, b; Yang et al., 1999). All these studies support the idea that *Ca^{2+} influx into the postsynaptic neuron through the NMDARs* is necessary for initiating LTP and LTD (Bliss and Collingridge, 1993; Bear and Malenka, 1994). The NMDAR-mediated Ca^{2+} influx into the neuron activates numerous Ca^{2+} sensitive signaling cascades that are involved in the induction and expression of LTP and LTD. The role of NMDARs in synaptic plasticity, learning and memory is supported by evidence showing that NMDAR antagonists (used at concentrations that block LTP *in vitro*) block acquisition of hippocampus dependent memory (Bolhuis and Reid, 1992; Davis et al., 1992). In addition, genetic models carrying a CA1 region specific knockout of the NMDAR subunit NR1 exhibit impaired hippocampal synaptic plasticity, and poor memory skills (McHugh et al., 1996; Tsien et al., 1996). Subsequent models connecting synaptic plasticity, learning and memory have proposed a sliding threshold for the direction of synaptic modification dependent on the frequency of neural activity (Shouval et al., 2002; Kumar and Foster, 2007).

One of the contributing factors for the NMDAR mediated Ca^{2+} influx during the induction of LTP and LTD is postsynaptic membrane depolarization. In the context of the current studies, neuronal excitability is closely linked to the membrane depolarization and determines synaptic transmission and plasticity. The depolarization of the postsynaptic membrane potential is necessary to remove the Mg^{2+} block on the NMDARs. Normally K^+ channels in the postsynaptic neurons gate the efflux of K^+, through the afterhyperpolarization (AHP) current that maintains the neurons in a hyperpolarized state. The synaptic inputs from the presynaptic neurons counteracts the effect of K^+ channels and shifts the postsynaptic membrane potential to more depolarized potentials, which ultimately aids in the removal of the Mg^{2+} block on the NMDARs. Thus a more hyperpolarized state makes it difficult for the neurons to depolarize and activate the NMDARs. The complex relationship that exists between processes that regulate membrane potential and processes that modify synaptic strength ultimately determines the expression of LTP and LTD. Increased AHP amplitude is a biomarker of aged CA1 pyramidal neurons, and is also the focus of a separate set of studies presented in this dissertation. We tested the hypothesis that the oxidative redox state of aged neurons contributes to increase in the amplitude of the slow component of AHP or sAHP. In order to better understand the results in support of this hypothesis, it is critical to understand the properties and function of AHP. The following section provides a brief overview of the ion channels, currents and signaling mechanisms that mediate the AHP in neurons.

Afterhyperpolarization in CA1 Pyramidal Neurons

The AHP is a post burst hyperpolarization in the membrane potential of the neurons which lasts over 1-2 seconds after the offset of the depolarizing pulse. The

AHP is divided into three broad phases based in time kinetics into the fast component (fAHP), the medium component (mAHP), and the slow component (sAHP) (Sah and Faber, 2002). Each component of AHP is mediated by distinct classes of ion channels that differ in their pharmacology and time kinetics. The fAHP, which lasts several tens of milliseconds, is mediated by the BK channels. The BK channels generate large K^+ currents with single channel conductance reaching 400 pS (Marty, 1981). The BK channels are dependent on Ca^{2+} binding and membrane depolarization for their activation (McManus, 1991; Cui et al., 1997). In the context of Ca^{2+} signaling in the CA1 pyramidal neurons, the BK channels contain a Ca^{2+} detection site on their intracellular domain (Wei et al., 1994; Schreiber and Salkoff, 1997). Although activated by cytosolic Ca^{2+}, the Ca^{2+}- sensitivity of BK channels is highly dependent on the membrane potential, which enables it to generate the hyperpolarizing K^+ currents within few milliseconds of the onset of the depolarizing pulse.

The mAHP, which lasts a few hundred milliseconds, is generated by the SK channels. The SK channels have small K^+ conductance ranging from 2-20 pS (Blatz and Magleby, 1986). In contrast to the BK channels, the SK channels are voltage insensitive (Hirschberg et al., 1998); however, their activation is dependent on rises in cytosolic Ca^{2+}. In the context of Ca^{2+} signaling, the SK channels possess unique Ca^{2+} binding properties. The SK channels do not directly bind to Ca^{2+} like the BK channels; however, reports indicate that they can bind to Ca^{2+} binding proteins like calmodulin (Xia et al., 1998; Keen et al., 1999; Schumacher et al., 2001), which leads to activation of the SK channel through a conformational change. All these properties of SK channels ideally suit them to generate the relatively late onset K^+ current which underlie the mAHP.

The sAHP is the primary focus of a separate set of studies presented in this dissertation. Unfortunately, the molecular identity of the ion channel underlying the sAHP is unknown. Nevertheless, numerous observations regarding the current that underlies the sAHP have given rise to interesting predictions. The current underlying sAHP has been observed to be voltage independent, but Ca^{2+} dependent (Sah, 1996), which is modulated by a range of neurotransmitters including glutamate, acetylcholine and serotonin (Nicoll, 1988). Although a class of SK channels could possibly mediate the sAHP current (Marrion and Tavalin, 1998; Bowden et al., 2001), experiments using clotrimazole analogs, which are highly selective synthetic inhibitors of sAHP current, have eliminated this possibility (Shah et al., 2001). Recent advances in understanding the molecular mechanisms underlying the sAHP current suggest that a diffusible molecule or second messenger system could be operating at the interface between cytosolic Ca^{2+} and the channels that mediate the sAHP current. For example, recent studies indicate that the neuronal Ca^{2+} sensing protein hippocalcin has been reported to activate channels that mediate the sAHP current in its membrane bound form (Tzingounis et al., 2007; Tzingounis and Nicoll, 2008). Another study suggests that the ionotropic kainate receptors could decrease sAHP currents through a unique metabotropic action involving protein kinase C (Melyan et al., 2002). A separate set of studies point to a phosphorylation mechanism for mediating the sAHP involving kinases like cAMP-dependent protein kinase A (Madison and Nicoll, 1986; Pedarzani and Storm, 1993), Ca^{2+}/Calmodulin dependent protein kinase II (Muller et al., 1992), and protein kinase C (Malenka et al., 1986). In summary, numerous slow-activating secondary systems have been implicated in mediating and modulating the sAHP

current; however the identity of the ion channel that actually conducts the K^+ ions underlying the sAHP current remains to be discovered. Nevertheless, the dependence of this unknown ion channel on cytosolic Ca^{2+} is a veritable starting point for investigations into the mechanisms that modulate sAHP, especially in the context of aging, because sAHP is reported to increase in aged hippocampal neurons (Landfield and Pitler, 1984; Moyer et al., 1992; Kumar and Foster, 2004; Matthews et al., 2009).

The cytosolic Ca^{2+} level, which determines the activation of K^+ channels underlying the sAHP current, is the outcome of processes involved in maintaining neuronal Ca^{2+} homeostasis. The Ca^{2+} homeostasis is maintained by the complex interaction between mechanisms that allow Ca^{2+} entry into the neuron and those mechanisms that remove Ca^{2+} from the cytosol and/or buffer them in internal stores. In addition to the sAHP, the intracellular Ca^{2+} concentration also modulates synaptic plasticity. Due to the fundamental role of intracellular Ca^{2+} homeostasis in the development of several hypotheses presented in this dissertation a brief discussion of the key components and regulators of Ca^{2+} homeostasis in CA1 pyramidal neurons is provided below.

Calcium Homeostasis in CA1 Neurons

There are three major sources for Ca^{2+} mobilization in neurons – the NMDARs, the L-VGCC's, and the intracellular Ca^{2+} stores (ICS). Cytosolic elevation in Ca^{2+} concentration, from these sources, activates signaling cascades involved in LTP/LTD, and mediates physiological processes like the AHP. Excessive levels of cytosolic Ca^{2+} can lead to excitotoxicity and neuronal death, which is offset by pumps and buffering mechanisms that remove Ca^{2+}. The interplay between the processes that control the elevation and decrease in cytosolic Ca^{2+} levels maintains the Ca^{2+} homeostasis in the

neurons (Fig. 1-1). Age-related neuronal dysfunction is thought to originate, in part, from the perturbation of Ca^{2+} homeostatic mechanisms. In aged neurons there is a shift in the relative contribution of Ca^{2+} by various sources; such that the NMDARs contribute less Ca^{2+}, and the L-VGCC's and ICS contribute more Ca^{2+} (Thibault and Landfield, 1996; Norris et al., 1998a; Thibault et al., 2001; Foster and Kumar, 2002; Kumar and Foster, 2004; Gant et al., 2006; Foster, 2007; Thibault et al., 2007; Kumar et al., 2009) for the maintenance of Ca^{2+} homeostasis. Poor regulation of intracellular Ca^{2+} concentration contributes to improper activation of the signaling cascades involved in synaptic plasticity, thus impairing LTP and LTD (Foster, 1999; Burke and Barnes, 2006, 2010). Age-related changes to Ca^{2+} homeostasis is highlighted in the "calcium hypothesis of brain aging", which states that the disruption of normal Ca^{2+} homeostasis underlies neuronal dysfunction during aging (Landfield and Pitler, 1984; Gibson and Peterson, 1987; Khachaturian, 1989, 1994). However, the mechanisms that "cause" the disruption in normal Ca^{2+} homeostasis are, as yet, poorly understood. The hypothesis that forms the basis for this dissertation is that increased oxidative stress or a more oxidative redox state mediates the age-related shift in Ca^{2+} homeostasis, and contributes to neuronal dysfunction. The results presented in chapter 3, 4, and 5 delineate the link between oxidative redox state and age-related changes to neuronal synaptic transmission, plasticity, and sAHP. In order to better understand the hypotheses and results, it is important to understand the cause and consequence of increased oxidative stress or an oxidative redox state. Hence an overview of the neuronal redox state, in the context of aging, is provided in the following section.

Redox State and Aging

Biological aging is thought to be the outcome of accumulation of changes in an organism over time (Hayflick, 1985, 2007). One school of thought considers aging as a biological process controlled by the expression pattern of genes (Kennedy et al., 1995; Martin et al., 1996; Kirkwood and Austad, 2000; Martin, 2007; Budovskaya et al., 2008) and/or alterations in the structure of chromosomes, specifically the events associated with the length and state of the ends of chromosomes called telomeres (Harley et al., 1990; Bodnar et al., 1998; Wright and Shay, 2002; Stewart et al., 2003; Blasco, 2005). However our current understanding is that accumulation of changes to the biological molecules (lipids, proteins, DNA, RNA etc) contributes to the process of aging by altering the structure and function of the biological molecules (Rattan et al., 1992; Butterfield et al., 1998; Finkel and Holbrook, 2000). In particular, protein oxidation is a commonly observed age-related change which alters the structure and function of proteins (Stadtman, 1988, 1992; Yin and Chen, 2005; Widmer et al., 2006; Riemer et al., 2009), including several neuronal proteins (Smith et al., 1992). Protein oxidation results in the formation of disulfide bonds on the thiol moieties of cysteine and/or methionine residues (Shacter, 2000; Davies, 2005). The formation of disulfide bonds modifies NMDAR function too, which is one of the major focus of this study (Choi and Lipton, 2000; Choi et al., 2001). Protein oxidation during aging arises from increased oxyradical production and/or weakened antioxidant capacity of the neurons (Foster, 2006; Poon et al., 2006), which shifts the redox state to a more oxidative environment and weakens the redox buffering capacity (Parihar et al., 2008). Thus progressive accumulation of oxidative damage due to increased oxidative stress and a more oxidative redox state leads to protein dysfunction and contributes, in part, to the age-

related alteration in the structure and function of proteins. One of the central tenets of all the hypotheses presented in the following chapters is that aging is associated with an increase in oxidative stress or an oxidative redox state. In order to better understand the results based on this idea, it is very critical to understand the production and removal of the free radicals that promote protein oxidation.

One of the widely accepted theories of aging is the Free Radical theory of aging proposed by Denham Harman in 1956. The theory hypothesized that biological aging is the consequence of free radical damage to the biological molecules (Harman, 1956, 1972). The free radicals commonly encountered in biological systems are the reactive oxygen species (ROS) and the reactive nitrogen species (RNS). Among its various functional roles, the free radicals participate in cellular signaling, immunological response, neurotransmission, and oxidative metabolism. However, due their toxic nature, cells have evolved an elaborate detoxification system to remove and neutralize them. Thus a balance exists between the production and removal free radicals. During aging there is an excessive production and improper removal of free radicals that leads to an accumulation of abnormal levels of ROS, and RNS, which leads to oxidative and nitrosative stress respectively. Although there is insufficient evidence to support the idea that free radicals determine life-span; there is nevertheless a consensus that increased oxidative stress has a significant role to play in age-related disorders (Beckman and Ames, 1998; Migliaccio et al., 1999; Finkel and Holbrook, 2000; Melov, 2000; Schriner et al., 2005; Muller et al., 2007).

Excessive levels of superoxide are one of the hallmarks of increased oxidative stress. The increase in the accumulation of free radicals shifts the redox environment of

the neurons to a more oxidative state, and contributes to the free-radical induced damage proposed by Harman. Normally at the end of the electron transport chain, the free electrons are absorbed by oxygen, which is reduced to water. However incomplete reduction of oxygen yields superoxide (denoted by $\cdot O_2^-$) that contains one free electron. The enzyme superoxide dismutase (SOD) catalyzes the dismutation of superoxide into hydrogen peroxide (H_2O_2). However H_2O_2 is a potent oxidizing agent, which can still cause oxidative damage to cellular components. The enzyme catalase then converts H_2O_2 into oxygen and water. In addition, cells utilize the enzyme glutathione peroxidase to convert H_2O_2 into water by simultaneous oxidation of reduced glutathione to oxidized glutathione. The age-related increase in the production of ROS overwhelms these antioxidant systems and ultimately leads to excessive accumulation of ROS. The increase in oxidative stress creates an oxidative redox state and weakens the redox buffering capacity of the aged neurons which is also indicated by lower resting levels of reduced nicotinamide adenine dinucleotide (phosphate), and reduced L-glutathione (L-GSH) (Parihar et al., 2008). The results presented in chapters 3, 4, and 5 indicate that oxidative redox state is one of the critical factors that paves the way for neuronal dysfunction during aging.

A key component of all the hypotheses presented so far has been neuronal dysfunction. Our experiments were designed to detect abnormal changes in neuronal function and understand the mechanisms that cause such dysfunction. As described in the previous sections, the neuronal dysfunction is closely linked to processes that regulate intracellular Ca^{2+} homeostasis. Increased oxidative stress or a more oxidative redox state during aging can modulate processes that maintain Ca^{2+} homeostasis by

decreasing NMDAR function (Bodhinathan et al., 2010), increasing the activity of L-type VGCC (Lu et al., 2002; Akaishi et al., 2004), and increasing Ca^{2+} mobilization from ICS (Hidalgo et al., 2004; Kumar and Foster, 2004). Redox state of the aged neuron can also affect synaptic plasticity in hippocampal slices (Serrano and Klann, 2004; Bodhinathan et al., 2010). The results presented in the following chapters enhance our understanding of the link between these diverse processes.

Summary

The overview presented above highlights the complex interaction between mechanisms that mediate normal functioning of hippocampal CA1 pyramidal neurons, and mechanism during aging (more oxidative redox state) that contribute to its dysfunction. At the outset normal aging has significant impact on NMDAR function, NMDAR-dependent synaptic plasticity, and neuronal excitability. One of the central themes highlighted in the following chapters is that the CA1 pyramidal neurons express a broad profile of changes in intracellular Ca^{2+} homeostasis during aging. This phenomenon is central to many mechanisms investigated in this dissertation including changes in synaptic transmission, plasticity, and neuronal excitability. Notably, the ideas presented in the preceding sections take a reductionist approach (in the context of aging) to describe age-related changes in learning and memory dependent on hippocampus, the oxidative redox state arising from increased oxidative stress, and the subtle age-related biochemical and physiological changes in the CA1 pyramidal neurons. The following chapters have built upon these ideas and provide novel results that enhance our understanding of neuronal dysfunction during aging.

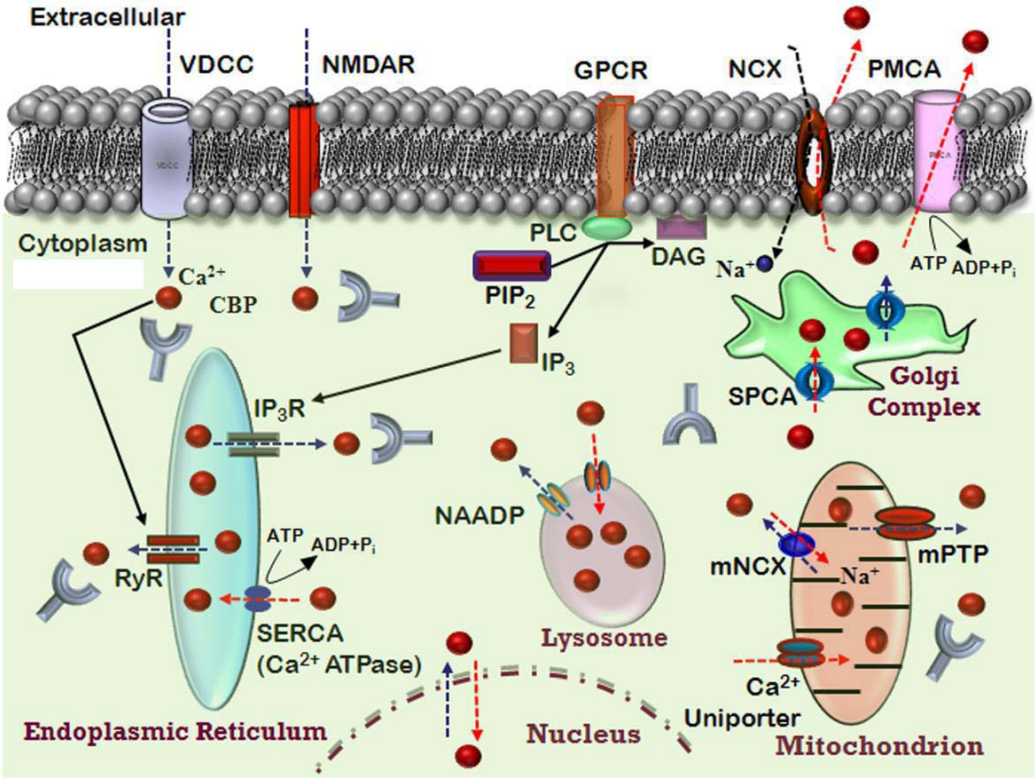

Figure 1-1. Calcium homeostasis in the neuron. Model depicting various Ca^{2+} sources, sequestrating, buffering mechanisms, and Ca^{2+} signaling events in a healthy neuron. Indicated are the voltage dependent Ca^{2+} channels (VDCC), n-methyl-d-aspartate receptor (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR), and g-protein coupled receptor (GPCR) involved in Ca^{2+} (*red balls*) influx into the cytosol (*blue dashed arrows*). The release of Ca^{2+} into the cytoplasm also occurs from the intracellular Ca^{2+} stores (ICS) through inositol (1, 4, 5)-triphosphate receptor (IP_3R) and ryanodine receptors (RyRs). Organelles, including the endoplasmic reticulum (ER), mitochondria, and lysosomes act as Ca^{2+} buffering systems, releasing and sequestering Ca^{2+}. Further, the model depicts Ca^{2+} buffering and extrusion pathways (*red dashed arrows*), involving Na^{+}/Ca^{2+} exchanger (NCX) and plasma membrane Ca^{2+}ATPase (PMCA), sarcoplasmic reticulum Ca^{2+} ATPases (SERCAs), nicotinic acid adenine dinucleotide phosphate (NAADP), various Ca^{2+} binding proteins (CBPs). Mitochondrial permeability transition pore (mPTP) and mitochondrial sodium-Ca^{2+} exchanger (mNCX) and secretory pathway Ca^{2+}-ATPases (SPCA) contribute to Ca^{2+} regulation (Adapted from Kumar A, Bodhinathan K, and Foster T C, Front Ag Neurosci 2009).

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CHAPTER 2
MATERIALS AND METHODS

Drugs, Solutions and Suppliers

All drugs were prepared according to the manufacturer's specifications and ultimately dissolved in ACSF prior to bath application on the slices. Appendix A provides a comprehensive list of all the drugs, solutions and their suppliers, used in this study. Drugs that need either DMSO or ethanol as the solvent were initially dissolved in DMSO or ethanol respectively and diluted in ACSF to a final DMSO concentration of less than 0.01% and final ethanol concentration of less than 0.0001%. Appendix B provides a list of the all the concentrations of various drugs used in this study. Commonly used laboratory chemicals were acquired from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animal Procedures

Procedures involving animals have been reviewed and approved by the Institutional Animal Care and Use Committee at the University of Florida. All procedures were in accordance with the guidelines established by the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals. Male Fischer 344 rats, young (3-8 mo) and aged (20–25 mo), were obtained from National Institute on Aging colony at Harlan Sprague Dawley Inc (Indianapolis, IA). All animals were group housed (2 per cage), and maintained on a 12:12 hr light schedule, and provided *ad libitum* access to food and water. Animal health was regularly monitored with the help of the Animal Care Services at the University of Florida.

Hippocampal Tissue Dissection for Electrophysiological Experiments

The protocol to prepare live hippocampal slices for electrophysiological experiments are derived from initial reports by Li and McIlwain (Li and McIlwain, 1957), which has been suitably modified and standardized in our lab (Kumar and Foster, 2004). Animals were deeply anaesthetized using isoflurane (Webster, Sterling, MA) and decapitated with a guillotine (MyNeurolab, St Louis, MO). The layer of skin covering the skull was pared open and the skull was removed using bone snips. The brains were rapidly removed and transferred to a beaker containing ice-cold artificial cerebrospinal fluid (ACSF) which was calcium free. The hippocampi were dissected out carefully for slicing. Hippocampal slices (~ 400μm) were cut parallel to the alvear fibers using a tissue chopper (Mickle Laboratory Engineering Co, Surrey, UK). The slices were incubated in a holding chamber (at room temperature) with ACSF containing (in mM): 124 sodium chloride (NaCl), 2 potassium chloride (KCl), 1.25 potassium phosphate monobasic (KH_2PO_4), 2 magnesium sulfate ($MgSO_4$), 2 calcium chloride dihydrate ($CaCl_2$), 26 sodium bicarbonate ($NaHCO_3$), and 10 D-glucose. At least 30 min before recording, slices were transferred to a standard interface recording chamber (Warner Instrument, Hamden, CT). The chamber was continuously perfused with oxygenated ACSF (95%-O_2 and 5%-CO_2) at the rate of 2 mL/min. The pH was maintained at 7.4 initially adjusted using 10N hydrochloric acid or 10M sodium hydroxide. The temperature was maintained at 30 ± 0.5°C using the automatic temperature controller TC-324B (Warner Instrument, Hamden, CT) (Fig. 2-1).

Electrophysiological Recordings: Extracellular Field Potentials

The extracellular *f*ield *e*xcitatory *p*ostsynaptic *p*otentials (fEPSP) represent the net influx of Na^+ and other positive ions like Ca^{2+} into the postsynaptic neuron. The net

movement of positive ions from the recording electrode is measured as a negative deflection on the oscilloscope. This negative deflection is called the fEPSP (Aidley, 1989; Johnston and Wu, 1995; Kandel, 2000b) and is indicated as total fEPSP (*black trace*) in Fig. 2-2A. The fEPSPs for studies described here were generated by stimulating the CA3 afferent fibers onto CA1 pyramidal neurons, known as the Schaffer-collateral pathway or the CA3-CA1 pathway. The fEPSPs were recorded using glass micropipette electrodes filled with artificial cerebrospinal fluid as the recording medium. The glass micropipette electrode resistances ranged from 4-6 MΩ. The glass micropipettes were pulled from thin-walled borosilicate capillary glass using a Flaming/Brown horizontal micropipette puller (Sutter Instruments, San Rafael, CA). The borosilicate capillary glass had an outer diameter of 1 mm, an inner diameter of 0.75 mm and was about 4 inches in length. Two concentric bipolar stimulating electrodes (FHC, Bowdoinham, ME) were localized to the middle of the stratum radiatum on either side of the recording electrode in order to stimulate the afferents onto CA1 pyramidal neurons. The outer pole of the bipolar electrode was made of stainless steel and was 200 μm in diameter. The inner pole was made of platinum/iridium alloy and was about 25 μm in diameter. Diphasic stimulus pulses of 100 μs duration were delivered by a stimulator (SD9 Stimulator; Grass Instrument, West Warwick, RI) and alternated between the two pathways such that each pathway was activated at 0.033 Hz.

Extracellular Field Potentials: Data Analysis

The signals corresponding to the extracellular field potentials were sampled at a frequency of 20-kHz; filtered and amplified between 1 Hz and 1 kHz using Axoclamp-2A (Molecular Devices, Sunnyvale, CA) and a differential AC amplifier (A-M Systems, Sequim, WA) and stored on a computer disk (Dell Inc, Texas) for off-line analysis. A

separate output from the differential AC amplifier was fed into an oscilloscope (Tektronix 2214, Tektronix Inc, Beaverton, OR) and audio monitor (AM8, Grass Technologies, West Warwick, RI) for real time visualization of the signals. Two cursors were placed to cover the initial descending phase of the waveform and the maximum negative slope (mV/ms) of the fEPSP was determined by a Sciworks computer algorithm (Datawave Technologies, Berthoud, CO) which determined the maximum change across a set of 20 consecutively recorded points between the cursors. To measure the amplitude of the fEPSP, the cursors were placed to cover the entire waveform of the fEPSP. Subsequently a Sciworks computer algorithm was used to compute the maximum amplitude (mV) of the fEPSP at the peak of the waveform (Fig. 2-2B).

Long-Term Potentiation and Paired-Pulse Ratio Recordings

For induction of LTP, the stimulation intensity was set to elicit 50% of the maximal fEPSP obtained by stimulating the CA3 afferents on CA1 pyramidal neurons. After stable baseline recording at 0.033 Hz for at least 20 min, high frequency stimulation (HFS) was delivered to the pathway at 100 Hz for 1 sec (100 pulses) at the baseline stimulation intensity, and recorded for at least 60 min post-HFS. A simultaneously recorded control (non-HFS) pathway received the test stimulation but not the HFS. In some cases the fEPSP was monitored in the presence of drugs to account for changes on baseline synaptic transmission, before delivering the HFS. The average fEPSP slope corresponding to the last 5 minutes from each pathway was used to compare changes in synaptic strength relative to the baseline.

For measuring the paired-pulse ratio, paired pulses were delivered through a single stimulating electrode at varying inter pulse intervals. The first pulse was set to elicit 50% of the maximal fEPSP. The various inter pulse intervals between successive

pulses were 50 ms, 100 ms, 150 ms, and 200 ms. The ratio of the maximum negative slope of the second pulse to the maximum negative slope of the first pulse was computed as the paired-pulse ratio.

Isolation of NMDAR Mediated Extracellular Synaptic Potentials

To obtain the NMDAR-mediated field excitatory postsynaptic potential (NMDAR-fEPSP) at the CA3-CA1 synapses, the slices were incubated in ACSF containing low extracellular Mg^{2+} (0.5 mM), 6, 7-dinitroquinoxaline-2, 3-dione (DNQX, 30 μM), and picrotoxin (PTX, 10 μM). Low extracellular Mg^{2+} was used to facilitate the removal of the "Mg^{2+} block" on the NMDAR; DNQX (AMPAR antagonist) was used to block the AMPAR component of the fEPSP; and PTX ($GABA_A$ antagonist) was used to minimize the GABA-ergic inhibition on the CA1 neurons. In each case the baseline response was collected for at least 10 min before experimental manipulations (drug application). The NMDAR-fEPSP is indicated in Fig. 2-2A (*blue trace*). Successful pharmacological isolation of the NMDAR-fEPSP was demonstrated by the application of the NMDAR antagonist AP-5 (100 μm), which is indicated by the *red trace* in Fig. 2-2A. Changes in the levels of synaptic transmission, induced by drug application, were calculated as percentage change from the averaged baseline responses.

Electrophysiological Recordings: Intracellular Sharp Microelectrode Recording

Intracellular excitatory post synaptic potentials were recorded from the CA1 pyramidal neurons using sharp microelectrodes. Sharp microelectrodes were pulled from thin walled borosilicate capillary glass (1 mm outer diameter; 0.75 mm inner diameter; 4 inches in length) using the Flaming/Brown horizontal micropipette puller (Sutter Instruments, San Rafael, CA). All microelectrode tips were filled with 3M potassium acetate and in some cases were filled with potassium acetate solution

containing the drug (for example L-GSH). The microelectrode resistances typically ranged from 39-55 MΩ. Microelectrodes were visually positioned in the CA1 pyramidal cell layer using a dissecting microscope (SZH10, Optical Elements Corp, Washington, DC) and a bipolar stimulating electrode was positioned to stimulate the CA3 afferents onto CA1 pyramidal neurons. On cell entry, positive or negative current was applied to clamp the neuronal membrane potential at -65 mV. Only neurons with a resting membrane potential (V_m) more hyperpolarized than -57 mV, and having an input resistance (R_{in}) >20 MΩ, and an action potential amplitude (AP_{amp}) rising ≥70 mV from the point of spike initiation were included in the analysis. The resting membrane potential and holding current was monitored through the entire course of the experiment. Variations in the resting membrane potential, holding current, input resistance, action potential amplitude or the microelectrode resistance was also monitored for those cases in which drugs were included in the pipette and eliminated accordingly. Diphasic stimulus pulses of 100 μs duration were delivered at 0.033 Hz and the stimulation intensity was adjusted to elicit an intracellular synaptic response, which was below the spike threshold. Baseline response recording began within 3 minutes after cell entry. To obtain the NMDAR-mediated intracellular synaptic potentials from CA1 pyramidal neurons, slices were incubated in ACSF containing low extracellular Mg^{2+}, DNQX and PTX as described above. An example of the NMDAR mediated intracellular synaptic potential is indicated in Fig. 2-2C.

Intracellular Synaptic Potentials: Data Analysis

The signals corresponding to the intracellular synaptic potentials were sampled at a frequency of 20 kHz; filtered and amplified between 1 Hz and 1 kHz using Axoclamp-2A (Molecular Devices, Sunnyvale, CA) and a differential AC amplifier (A-M Systems,

Sequim, WA) and stored on a computer disk (Dell Inc, Texas) for off-line analysis. A separate output from the differential AC amplifier was fed into an oscilloscope (Tektronix 2214, Tektronix Inc, Beaverton, OR) and audio monitor (AM8, Grass Technologies, West Warwick, RI) for real time visualization of the signals. Two cursors were placed to cover the entire waveform of the intracellular EPSP; from the pre-stimulus baseline to > 100 ms of the waveform. A Sciworks computer algorithm was used to compute the maximum amplitude (mV) of the intracellular EPSP at the peak of the waveform.

Intracellular Afterhyperpolarization: Data Analysis

The signals corresponding to the intracellular AHP were sampled at 20 kHz; filtered and amplified between 1 Hz and 1 kHz and processed as described previously for the intracellular synaptic potentials. The AHP was recorded from the neurons in the following manner. Depolarizing current pulses (duration: 100 ms; amplitude: 0.3 to 1.2 nA) were delivered every 20 seconds through the intracellular electrode in order to elicit a train of action potentials with 5 spikes. Since the AHP amplitude varies with the number of spikes in a train of action potentials, a train of 5 action potentials were maintained throughout the recording to study the effect of various treatment conditions on the AHP amplitude. The AHP recorded during the baseline and under the application of various drugs were elicited at the same membrane potential (-63 mV), which was achieved by manually clamping the membrane potential with DC current injection (-1 to +1 nA). The sAHP amplitude was measured as the difference between the average membrane potential during the 100 ms period immediately preceding the onset of the depolarizing current and the average membrane potential 400 to 500 ms after the offset of the depolarizing current. The sAHP amplitudes were computed in mV and changes under experimental conditions are assessed as percent change from the average

baseline value over 5 to 10 min period. A representative trace of the intracellular AHP is indicated in Fig. 2-2D.

Measurement of ROS in Hippocampal Slices

Hippocampal slices were incubated for 30 minutes in ACSF containing 10 μM of the ROS detection reagent 5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (c-H_2DCFDA; Molecular Probes Inc, Eugene, OR). Slices incubated for 30 minutes in absence of c-H_2DCFDA were used to detect background or auto fluorescence. Slices that were incubated with c-H_2DCFDA were imaged to quantify the levels of ROS. Fluorescent images were obtained with an Axiovert 40 CFL fluorescent microscope and Axiocam digital CCD camera (Carl Zeiss, Thornwood, NY). Fluorescence intensity was quantified as follows: fluorescent microscope was used to obtain images under uniform exposure time (100 ms) and intensity (150%). The images were then converted to grayscale images in Adobe Photoshop 5.5; the resulting images were quantified by densitometry analysis using Image J software (http://rsbweb.nih.gov/ij). An area of about 225 μm along the medial-lateral axis and 187.5 μm along the anterior-posterior axis, centered on the CA1 pyramidal neurons was selected for analysis of fluorescence intensity. The mean gray value intensities obtained from the aged and young animals are represented as the mean fluorescence intensities. The mean fluorescence intensity from the dye-exposed slices is normalized to the mean fluorescence intensity obtained from dye-unexposed slices (harvested from the same animal) using the following relationship:

Mean c-H_2DCFDA Fluorescence (% of control) = $[(F_e-F_u)/F_u] \times 100$

Where F_e and F_u were the mean fluorescence intensities obtained from dye-exposed and dye-unexposed slices respectively.

CaMKII Activity Assay

Hippocampi were isolated from aged F344 rats as described above. CA1 region was separated from the rest of the hippocampus, collected in an eppendorf tube, flash frozen in liquid nitrogen and stored at -80°C. The frozen CA1 tissue samples were placed in a dounce homogenizer containing 1 mL of the homogenization buffer (sucrose, 1M Tris pH 7.5, 1M KCl, protease inhibitor, protein phosphatase 1 inhibitor, protein phosphatase 2 inhibitor, 100 mg/mL sodium butyrate, 0.1 M phenyl methyl sulfonyl fluoride; all prepared in distilled H_2O) and homogenized using at least six strokes of the pestle. Homogenates were centrifuged at 7700 x g for 10 minutes at 4°C. The supernatant (containing the cytosolic fraction) was carefully isolated and stored at -80°C. Protein concentrations of the cytosolic fractions were determined using the BCA assay method (Pierce, Rockford, IL). CaMKII activity in the cytosolic fraction was measured using the CaMKII assay kit (CycLex Co., Ltd, Nagano, Japan). Briefly, uniform amount of cytosolic extracts (protein concentration = 2.0 µg per well) were loaded onto micro titer wells coated with a specific peptide substrate for CaMKII – Syntide-2, along with kinase reaction buffer with or without Ca^{2+}/calmodulin. Purified CaMKII (30 milli units per reaction; CycLex Co Ltd) was used as positive control and cytosolic extracts incubated with EGTA + myr-AIP (CaMKII specific peptide inhibitor) was used as negative control to obtain a measure of comparison for the CaMKII activity in the DTT treated and untreated cytosolic extracts. CaMKII activity is expressed as spectral absorbance units at 450 nm, normalized to the control.

Statistical Methods for Analysis of Data

Statistical analyses for purposes of hypothesis testing were performed using Stat View 5.0 (SAS Institute Inc, NC) and Excel 2003 (Microsoft Corp., Seattle, WA).

Student's *t*-tests (paired or unpaired as applicable) were used to examine for differences between data sets. The statistical level of significance was set at $p<0.05$. For tests involving more than one factor, analysis of variance (ANOVA) was employed. In general, ANOVA was followed by Fisher's protected least significant difference (PLSD) post hoc analysis in order to localize individual differences among various data sets. Unless otherwise stated, the effects of pharmacological treatments on the synaptic transmission are represented as percent of baseline (Mean ± Standard Error of Mean). Repeated measures ANOVA was used for statistical analysis of data sets obtained sequentially from the same experimental setup over time. For example, repeated measures ANOVA was used to compare the NMDAR-fEPSP obtained for consecutively higher stimulus intensities from a single hippocampal slice and recording setup. Where stated, n represents the number of slices used in each experiment, an indication of the statistical power of the analysis.

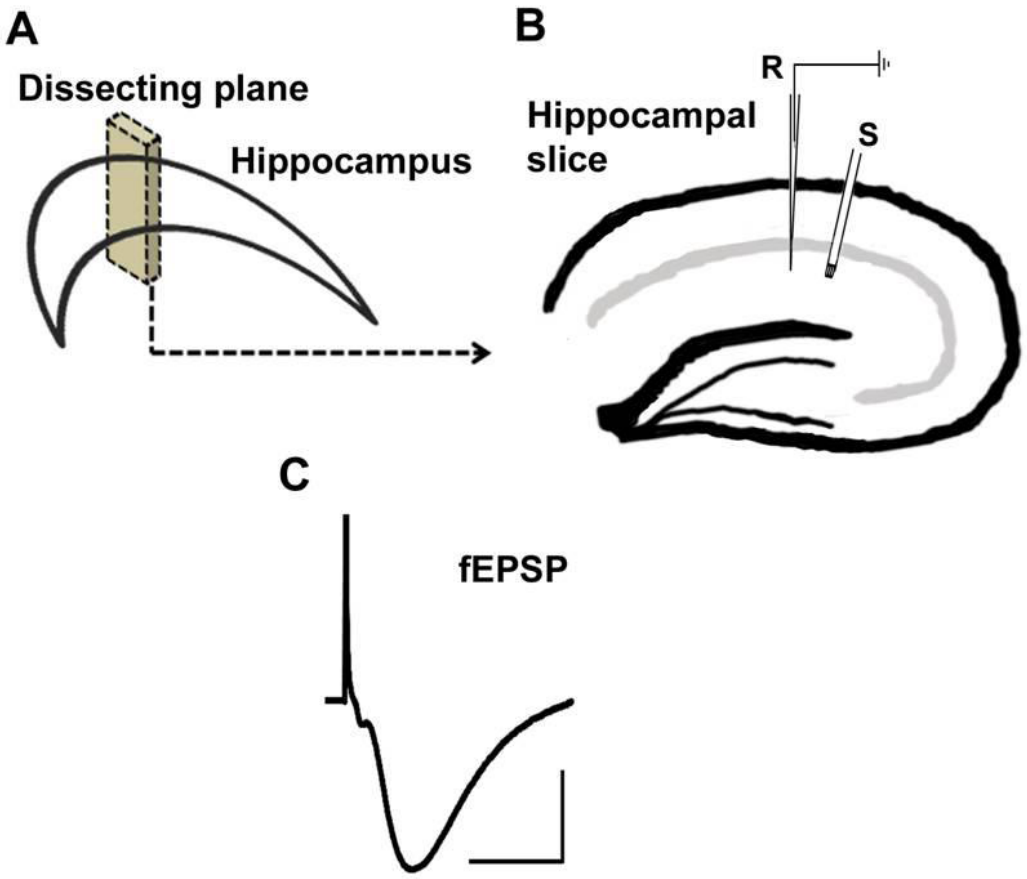

Figure 2-1. Hippocampal dissection and setup for electrophysiological recordings. A). Depiction of the dissecting plane in the hippocampus. The dissecting plane is parallel to the alvear fibers. B). Depiction of the hippocampal slice indicating the position of the recording (R) and stimulating (S) electrodes. The gray outline indicates the CA1 pyramidal cell layer. C). The fEPSP recorded from one such arrangement. Calibration Bars: 10 ms (horizontal) and 0.5 mV (vertical).

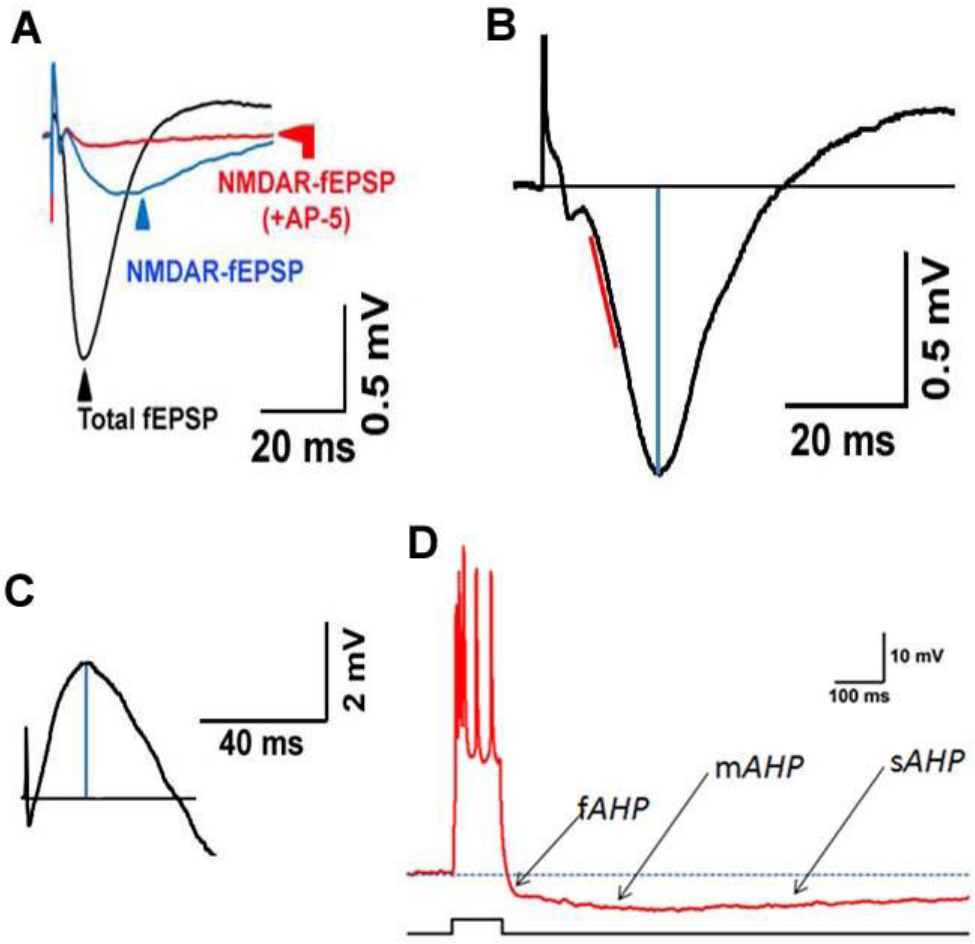

Figure 2-2. Analysis of electrophysiological signals from hippocampal slices. A) Representative traces demonstrating the total field potential (Total fEPSP, black trace), the pharmacologically isolated NMDA receptor mediated synaptic response (NMDAR-fEPSP, blue trace) and the NMDAR-fEPSP in the presence of the NMDAR antagonist AP-5 (NMDAR-fEPSP (+AP-5), red trace). B) Representation of the measurement of the fEPSP amplitude (blue) and the fEPSP slope (red) from a representative fEPSP (black trace). Calibration bars in A and B: 20 ms; 0.5 mV. C) Representation of the measurement of the amplitude (blue) of the intracellular NMDAR mediated synaptic potentials obtained from single CA1 pyramidal neurons in the hippocampus of aged animals. Calibration bars: 40 ms, 2 mV. D) Representative trace of the intracellular post-burst afterhyperpolarization (red trace), indicating the fast (fAHP), medium (mAHP), and slow (sAHP) components. The step current used to elicit a train of 5 action potentials is indicated beneath the AHP trace. Calibration bars: 100 ms, 10 mV.

CHAPTER 3
REDOX STATE DEPENDENT CHANGES IN NMDA RECEPTOR MEDIATED SYNAPTIC TRANSMISSION IN AGED HIPPOCAMPUS

Introduction

The NMDAR is a major source of Ca^{2+} influx into the postsynaptic neurons during the induction of LTP at hippocampal CA3-CA1 synapses (Bliss and Collingridge, 1993). The CA1 region specific knockout of the NR1 subunit of the NMDAR abolishes LTP and impairs spatial learning and memory (Tsien et al., 1996). Similar deficits in LTP and spatial memory are observed in aged, memory impaired animals. Most importantly, preliminary studies have indicated that the NMDAR component of the synaptic transmission at the CA3-CA1 synapses is decreased in aged animals (Barnes et al., 1997; Billard and Rouaud, 2007). These results have given rise to the "NMDAR hypofunction" hypothesis in the hippocampus during aging. The NMDAR hypofunction hypothesis suggests that age-related LTP and memory deficits are due to a decrease in the NMDAR mediated component of synaptic transmission (Foster, 1999; Rosenzweig and Barnes, 2003; Foster, 2007). This idea is further supported by reports indicating that the NMDARs contribute less Ca^{2+} to the induction of LTP in the CA1 region of aged hippocampus, when compared to the young hippocampus (Norris et al., 1998a; Shankar et al., 1998; Boric et al., 2008). However, it is still unclear what age-related mechanism underlies the NMDAR hypofunction.

Age-related alterations that may contribute to the NMDAR deficits include altered subunit expression, composition, and splice forms (Magnusson et al., 2005; Magnusson et al., 2006). However there is a debate concerning whether the NMDAR subunit expression decreases at the CA3-CA1 synaptic sites (Foster and Kumar, 2002; Kumar et al., 2009). It is thus possible that NMDAR hypofunction is related to posttranslational

modifications associated with oxidation/reduction and/or phosphorylation state rather than number and/or type of receptor subunits. Interestingly previous research examining the ability of reducing and oxidizing (redox) agents to modulate NMDAR activity in cell cultures and in tissue from neonates suggests that redox state is an important determinant of NMDAR function (Aizenman et al., 1989; Aizenman et al., 1990; Bernard et al., 1997; Choi and Lipton, 2000; Choi et al., 2001).

In this study we tested the hypothesis that the age-related NMDAR hypofunction is due to increased oxidative stress or a more oxidative redox state of the aged neuron. The current studies confirm that the NMDAR mediated synaptic potentials are decreased at CA3-CA1 synapses of the aged hippocampus. The NMDAR responses were modified by redox agents in an age-dependent manner; such that oxidizing agents decreased NMDAR responses to a greater extent in young than in aged animals, and reducing agents increased NMDAR responses to a greater extent in aged than in young animals. However, using a combination of extracellular and intracellular recordings with the relatively membrane impermeable reducing agent L-GSH, we found that intracellular redox state mediates that age-dependent shift in NMDAR responses. Moreover the intracellular redox state dependent increase in NMDAR function was independent of L-type VGCC activity. Thus, the results provide a link between oxidative redox state and decrease in NMDAR mediated synaptic transmission.

Results

NMDA Receptor Function Decreases in the Hippocampus of Aged Animals at Various Levels of Pre-synaptic Fiber Volley Amplitude

One of the potential mechanisms that might explain the loss of hippocampus dependent learning and memory function is decrease in the NMDAR function of the

CA1 pyramidal neurons. To study the alterations in the NMDAR function during aging, the NMDAR mediated field excitatory postsynaptic potentials (NMDAR-fEPSPs) were pharmacologically isolated (Kumar and Foster, 2004), recorded and analyzed from the hippocampus of young and aged F344 rats (Fig. 3-1A). The stimulation evoked presynaptic fiber volley (PFV) served as an indicator of the level of axon activation that gave rise to the NMDAR-fEPSP; thus enabling the comparison of NMDAR-fEPSP amplitudes across the age groups at increasingly higher PFV amplitudes (Table. 3-1). To examine the relationship between PFV and NMDAR-fEPSP across the two age groups, the PFV amplitude was separated into 0.4 mV bins and plotted against the corresponding NMDAR-fEPSP amplitude obtained from the aged and the young animals (Fig. 3-1B). An ANOVA revealed that the amplitude of the NMDAR-fEPSP was reduced in the aged animals (n = 6 animals) when compared to young animals (n = 5 animals) [$F(1, 47) = 27.47$, $p<0.0001$]. In fact, the maximum amplitude of the NMDAR-fEPSP was 0.73 ± 0.14 mV and 2.87 ± 0.9 mV in aged and young animals, respectively, approximately exhibiting a fourfold decrease during aging (Table 3-1).

Oxidizing Agents Decrease NMDAR Function in Young, but not in Aged, Hippocampal Slices

To test the hypothesis that the decrease in the NMDAR response was related to oxidizing conditions X/XO, an enzyme substrate combination which produces two types of ROS - superoxide anion and hydrogen peroxide, was applied to hippocampal slices of young and aged animals. X/XO has been previously used in an independent study that evaluated the effects of ROS on hippocampal synaptic transmission and plasticity (Knapp and Klann, 2002).

The stimulation intensity was set to evoke a response which was 30% to 50% of the maximal NMDAR-fEPSP attainable in that pathway, and the slope of the NMDAR-fEPSP was measured before and after pharmacological manipulations. Paired Student's *t*–test revealed that application of X/XO (20 µg/mL/ (0.25 units/mg of xanthine)) for 60 min, significantly decreased the slope of the NMDAR-fEPSP from the baseline levels in the young ($p<0.01$) but not in the aged animals. Furthermore, application of X/XO significantly [$F(1, 10) = 15.49$, $p<0.01$] decreased the NMDAR-fEPSP slope to a greater extent in the young animals (66.37 ± 7.04%, n = 5), when compared to the aged animals (96.41 ± 6.14%, n = 7) (Fig. 3-2). Paired *t*-test on the percent change in the PFV amplitudes (corresponding to the last 5 min of the 60 min recording) from the X/XO treated files indicated no effect ($p > 0.05$) of X/XO on the level of presynaptic axonal activation across the age groups (young: 95.16 $\pm$ 8.29%; aged: 108.23 $\pm$ 19.44%).

NMDAR Function in Young Animals Recovers From Exposure to Higher Concentrations of Oxidizing Agents

The effect of oxidizing agents on the NMDAR function in young animals was reversible, such that even in the presence of a higher concentration of xanthine oxidase (X/XO: (20 µg/mL) / (1 unit/mg of xanthine)), the NMDAR mediated synaptic response decreased to 54.21 ± 5.79%; however the response recovered to 88.11 ± 5.41% of the baseline (n = 5) following a 50 minute washout (Fig. 3-3). One possibility is that the redox state of the young hippocampal neurons is comparatively more reduced than oxidized, thus enabling a quick recovery of the NMDAR-fEPSP in the young animals upon washout of the oxidizing agent. Furthermore, the recovery of the NMDAR-fEPSP following washout indicates that young animals possess sufficient antioxidant and/or redox buffering capacity to absorb the excess oxyradicals due to application of X/XO.

Reducing Agents Increase NMDAR Function Selectively in Aged Hippocampus

The age-dependent sensitivity of NMDAR-fEPSP to oxidizing conditions suggests that the components of NMDAR signaling system are initially oxidized to a greater extent in aged animals. To test whether the decline in the NMDAR response in aged animals might be due to the age-dependent increase in the formation of disulfide linkages on the cysteine residues, the reducing agent DTT was applied to hippocampal slices from young and aged animals, and its effect on the NMDAR function in both the age groups was studied. DTT can reduce the disulfide bonds on cysteine residues of proteins into free thiols (Ciorba et al., 1997; Cai and Sesti, 2009; Long et al., 2009), thus partially reversing the effect of increased oxidative stress during aging. Paired *t*–test revealed that DTT significantly enhanced the slope of the NMDAR-fEPSP from the baseline levels in both the aged ($p<0.0001$) and young ($p<0.05$) animals. However, bath application of DTT (0.7 mM, 45 min) significantly [$F(1, 19) = 5.49$, $p<0.05$] increased the slope of the NMDAR-fEPSP to a greater extent in the aged animals (171.38 ± 13.26%, n = 16) when compared to young animals (114.55 ± 4.41%, n = 5) (Fig. 3-4A, 3-4B, 3-4C). Paired *t*-test on the PFV amplitude before and after application of DTT confirmed no change ($p > 0.05$) in the PFV amplitude for aged (102.81 $\pm$ 3.84) and young (97.08 $\pm$ 5.41) animals indicating that the effect of DTT on the NMDAR mediated response was not due to changes in the number of axons activated.

In a subset of these files, DTT was allowed to washout of the recording chamber, while its effect on the aged NMDAR-fEPSP was continuously monitored. Interestingly, the enhancement of the NMDAR-fEPSP in the aged animals was maintained (167.32 ± 20.91%, n = 5) following a 45 min washout of DTT (Fig. 3-4D). This finding indicates a persistent change associated with the NMDAR function upon application of the reducing

agent DTT. It raises the possibility that intracellular signaling cascades that are known to regulate the NMDAR function could underlie the DTT-mediated increase observed in aged animals.

Intracellular Location of Redox Sensitive Cysteines Revealed by Differential Application of Biologically Available Reducing Agent L-Glutathione

The results from the experiments using the membrane permeable reducing agent, DTT (Susankova et al., 2006), left open the question of the exact location of the redox sensitive cysteine residues. Sequence comparison with the structurally similar bacterial periplasmic binding protein predicts that the NMDARs must possess at least 7 cysteine residues on their extracellular surface (Choi and Lipton, 2000; Choi et al., 2001), which are redox sensitive and controls the function of NMDARs. In order to delineate the location of the cysteines that are responsive to the application of DTT, the biologically available and membrane impermeable reducing agent L-GSH was used to study its effect on the aged NMDAR-fEPSP. L-GSH is relatively membrane impermeable such that exogenous application of L-GSH is not effective in increasing intracellular free thiols when compared to DTT (Mazor et al., 1996; Zou et al., 2001; Susankova et al., 2006). Moreover previous findings suggest that L-GSH protects hippocampal neurons against damage due to oxidative stress (Shin et al., 2005; Shih et al., 2006; Yoneyama et al., 2008). Surprisingly, extracellular application of the reduced form of L-GSH (0.7 mM, 45 min) did not alter NMDAR-fEPSP in the aged animals (104.83 ± 8.39%, n = 6) (Fig. 3-5).

Since extracellular application of the membrane impermeable L-GSH failed to enhance NMDAR function, L-GSH was delivered into the intracellular compartment of the aged neurons using sharp microelectrodes. The NMDAR mediated intracellular EPSP was simultaneously recorded. Inclusion of the reduced form of L-GSH (0.7 mM to

1.4 mM) in the intracellular recording pipette significantly [$F(1,6) = 6.87$, $p<0.05$] enhanced the amplitude of the NMDAR mediated synaptic potentials (203.90 ± 31.38%, n = 5) in single hippocampal CA1 pyramidal neurons from aged hippocampus when compared to age matched control cells (91.55 ± 22.94%, n = 3), for which L-GSH was not included in the intracellular recording pipette (Fig. 3-6A). Application of 100 μM of NMDAR antagonist AP-5 abolished the intracellular EPSP (Fig. 3-6B, right), suggesting that the recorded component was specifically due to the activation of the NMDARs in the aged hippocampal neurons. The fact that intracellular delivery, but not extracellular application, of L-GSH enhanced the NMDAR response provides strong evidence for intracellular redox status as a mechanism for the age-associated modulation of NMDAR function.

One possibility was that intracellular delivery of the reduced form of L-GSH was increasing the activity of the L- type VGCCs. To test whether L-channel activity was modified, intracellular NMDAR EPSP was recorded in the presence of L-GSH and the L-type VGCC antagonist - nifedipine. Intracellular delivery of L-GSH in the presence of nifedipine (10 μM) continued to enhance the amplitude of the NMDAR mediated intracellular synaptic potentials (223.54 ± 46.56%, n = 3) suggesting that the DTT effect is not due to differences in L-channel activity (Fig. 3-7A, 3-7B).

Reducing Agent Mediated Recovery of NMDAR Function is Reversed by Oxidizing Agent, and Specific to NMDARs

To test whether the NMDAR-fEPSP in aged animals was indeed affected by the redox environment, the oxidizing agent 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) was applied subsequent to the DTT-mediated enhancement of the NMDAR response in aged animals. Bath application of DTNB (0.5 mM, 45 min) significantly decreased

($p<0.05$) the DTT-mediated increase in NMDAR-fEPSP in aged animals, such that the NMDAR-fEPSP slope was 86.49 ± 5.54% (n = 4) of the baseline (Fig. 3-8A). A repeated measures ANOVA comparing the mean NMDAR-fEPSP slope corresponding to the last 5 min under each condition indicated a significant [$F(2,9) = 6.24$, $p<0.05$] difference (Fig. 3-8 B). Post-hoc analysis revealed that the NMDAR-fEPSP slope was significantly ($p<0.05$) increased under DTT compared to baseline or DTNB ($p<0.05$), and no significant difference was observed between the NMDAR-fEPSP slopes in the baseline and under DTNB.

In order to delineate the specificity of the DTT effect on the NMDARs, the NMDAR-fEPSP was isolated and 100 μM AP-5 was applied prior to and during the application of DTT. With NMDARs blocked, there was no effect of DTT on any residual component of the field potential, such that the percent change in the residual fEPSP was 109.43 ± 7.15% (n = 6) of the baseline (Fig. 3-8C). Finally, the NMDAR-fEPSP was abolished by application of AP-5 (Fig. 3-8D) indicating that the response was generated by the activation of NMDARs.

To further examine the specificity of the DTT effects, the AMPAR component of synaptic transmission was studied before and after application of DTT in aged hippocampal slices. Application of DTT did not affect ($p>0.05$) the AMPAR component of the synaptic response such that responses were 101.63 ± 2.89% (n = 10) of the baseline after application of DTT for 45 minutes (Fig. 3-9A). In this case, the AMPAR component was recorded as the initial descending phase of the synaptic response (covering a 15 ms to 20 ms window) indicated in Fig. 3-9B. Finally, the AMPAR component of the fEPSP was isolated in the presence of 100 μM AP-5, and DTT was

applied to the non-NMDAR or predominantly AMPAR component of the synaptic transmission. Application of 0.7 mM DTT under these conditions did not significantly alter ($p>0.05$) the AMPAR-fEPSP from the baseline levels (94.27 ± 4.42%, n = 10) (Fig. 3-9C, Fig. 3-9D).

Together, the results indicate that oxidation of sulfhydryl groups can rapidly regulate responsiveness of NMDARs, and that an age-related reduction in the NMDAR response is linked to an oxidative redox state. Moreover this effect was exclusive to the NMDARs and had no discernable effect on the AMPAR component of the synaptic transmission.

Discussion

In this study we have used extracellular and intracellular recording techniques to understand the alterations in NMDAR responses during aging. The results presented in this chapter confirmed an age-related decrease in the NMDAR response (or "NMDAR hypofunction") and demonstrate age-dependent effects of redox modulators on the NMDAR response. First, the oxidizing agent X/XO selectively decreased NMDAR-fEPSP in young but not aged hippocampal neurons. The age dependent sensitivity of NMDAR function to oxidizing condition suggests that the NMDAR signaling system is oxidized to a greater extent in aged animals. Moreover, the recovery of NMDAR response under higher concentrations of X/XO in young neurons indicates sufficient redox buffering capacity in the young animals. Second, application of the reducing agent DTT increased NMDAR-fEPSP selectively in the aged but not in young hippocampal slices, suggesting that NMDAR signaling components are relatively less oxidized in young animals.

The redox state of the extracellular cysteine residues of the NMDARs have been implicated in regulating the NMDAR function in cell cultures and neonatal animals (Aizenman et al., 1989; Aizenman et al., 1990; Bernard et al., 1997). However extracellular application of the membrane impermeable biological reducing agent L-GSH failed to increase NMDAR-fEPSP. In contrast, intracellular application of L-GSH increased NMDAR mediated synaptic responses in single aged neurons. The use of NMDAR antagonist AP-5 confirmed that the intracellular response was mediated solely by NMDARs. The L-GSH mediated increase in intracellular NMDAR response was independent of L-type VGCC activity. Taken together, these results indicate that the age-related decrease in NMDAR function is due to a shift in the intracellular redox state favoring an oxidative state. This particular result is consistent with recent work in hippocampal cell cultures indicating a decrease in the intracellular redox ratio during aging, due in part to a deficit in the reduced form of GSH inside neurons (Parihar et al., 2008).

Furthermore, in accordance with previous reports (Gozlan et al., 1995; Abele et al., 1998), the reducing agent DTT had no effect on the AMPAR function of aged neurons. We have demonstrated this by studying DTT's effect on total fEPSP and AMPAR-mediated fEPSP, which was isolated by the application of NMDAR antagonist AP-5. With NMDARs blocked, there was no effect of DTT on any residual component of the field potential.

One of the consequences of NMDAR hypofunction is that aged neurons could engage a compensation mechanism in order to maintain Ca^{2+} homeostasis. Thus during aging the decreased Ca^{2+} contribution from the NMDARs is offset by the increased Ca^{2+}

contribution from the L-type VGCCs and ICS, as discussed previously. This situation poses a problem from a functional standpoint. The NMDARs are located at synaptic sites that are in close proximity to the Ca^{2+} sensitive kinases (Ex. CaMKII) and the postsynaptic density, which contain numerous signaling molecules that are critical for the induction and expression of synaptic plasticity. NMDAR hypofunction during aging would decrease the activation of these signaling cascades. Furthermore, L- type VGCCs are mostly clustered at the base of dendrites (Westenbroek et al., 1990), thus limiting their ability to activate the Ca^{2+} sensitive signaling cascades at the distant synaptic sites.

Our results suggest that age-related NMDAR hypofunction is the consequence of a shift in the intracellular redox state to a more oxidative state. The various alternative explanations for NMDAR hypofunction are discussed below. Excessive NMDAR activation is thought to contribute to excitotoxicity and neuronal death (Waxman and Lynch, 2005). Thus NMDAR hypofunction could be regarded as a cellular response to prevent excitotoxicity, and impaired NMDAR-dependent synaptic plasticity and memory decline may be the consequence of processes that mediate cell preservation (Foster, 1999). Alternatively it is possible that alterations in the NMDAR localization, through the insertion of receptors into the membrane or recruitment of extra-synaptic receptors into the synapse, may have important effects on NMDAR function during aging. However, it remains to be determined whether altered localization of the NMDARs (specifically extra-synaptic localization) is the mechanism by which the NMDAR function declines during senescence. Preliminary reports indicate that the NR2B subunit levels, but not the obligatory NR1 subunit levels of NMDARs, decrease in the synaptic sites (Zhao et

al., 2009). However these findings raise the possibility that the loss in NR2B at the synaptic sites could possibly be compensated by NR2A/2C/2D subunits that can combine with the unaltered NR1 subunits and form a normally functioning receptor. Another likely candidate mechanism extends the idea of posttranslational changes investigated in this chapter. The NMDAR function is regulated by phosphorylation state of its cytosolic tail, which is determined by the activity of neuronal kinases and phosphatases. In the next chapter we have demonstrated the role of one such kinase (CaMKII) in the DTT-mediated increase in the NMDAR function of aged animals.

In conclusion, the results presented in this chapter demonstrate a link between age-related decline in NMDAR function and increased oxidative stress or a more oxidative redox state of the aged neurons. Most importantly we have demonstrated that the NMDAR function can be recovered in the aged CA1 pyramidal neurons upon application of DTT. This is a veritable starting point for treatment of NMDAR hypofunction.

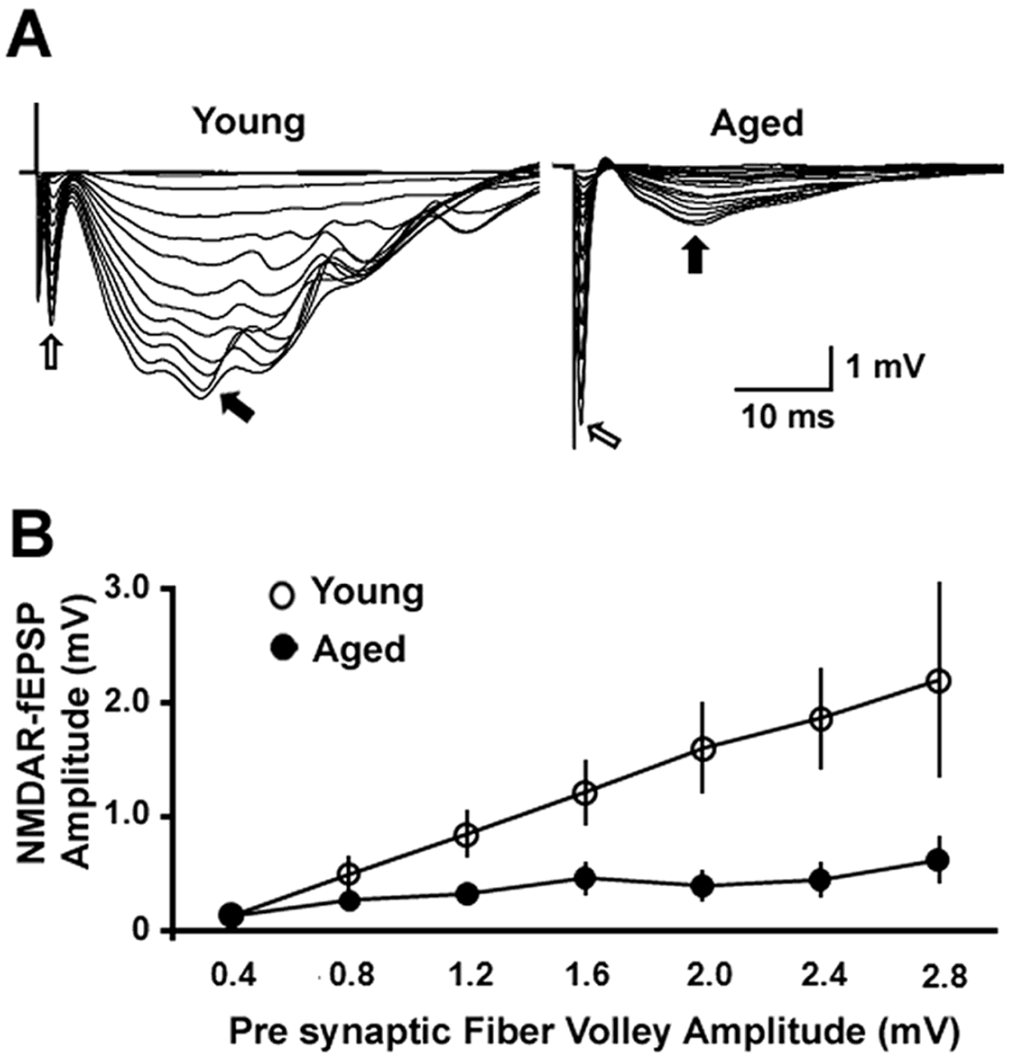

Figure 3-1. NMDAR mediated synaptic potentials (NMDAR-fEPSP) are reduced in area CA1 of the hippocampus during aging. A) Representative traces of NMDAR-fEPSPs obtained at consecutively higher stimulus intensities from the young (*left*) and aged animals (*right*). Open and filled arrows indicate the PFV and NMDAR-fEPSP, respectively. As observed in the traces, the aged animals exhibit a markedly reduced NMDAR mediated synaptic potential. Calibration bars: 10 ms, 1 mV. B) Plot of the mean NMDAR-fEPSP amplitude versus the PFV amplitude (at 0.4 mV binning width). The aged animals (*filled circles*) (n = 6) exhibited reduced NMDAR-fEPSP when compared to the young animals (*open circles*) (n = 5). In this and subsequent figures error bars represent S.E.M.

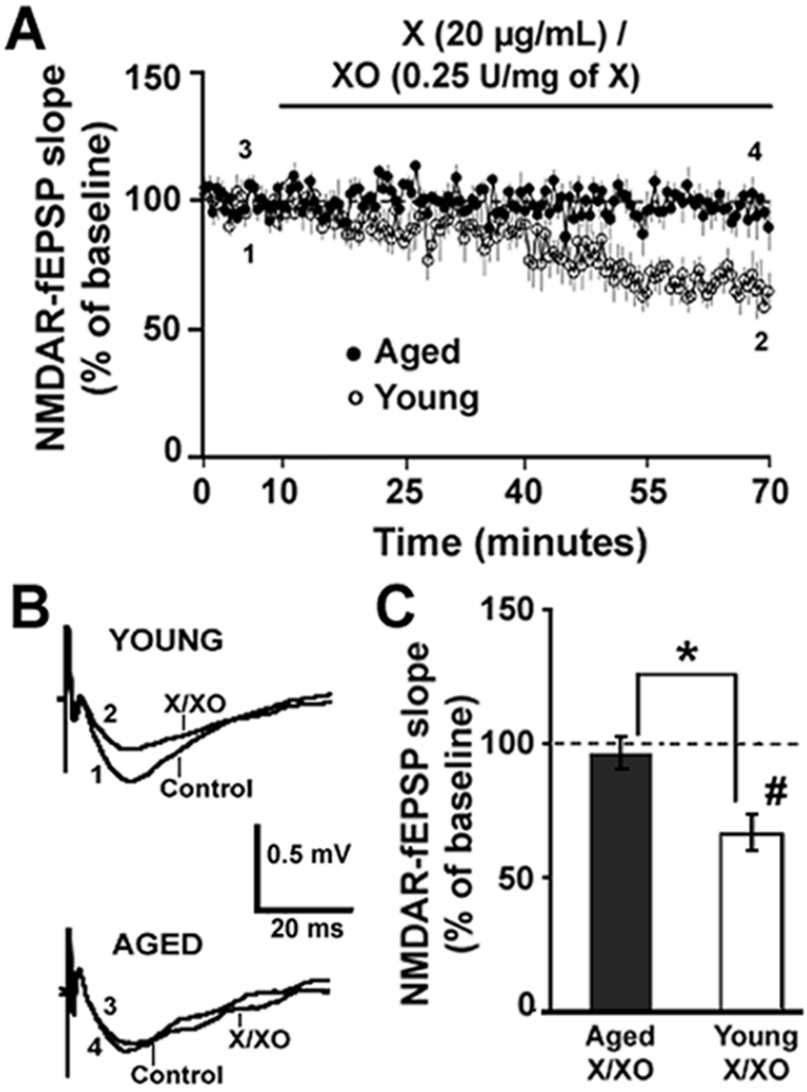

Figure 3-2. The oxidizing agent X/XO decreases NMDAR mediated synaptic potentials in young animals but not in aged animals. A) Time course of the change in the normalized NMDAR-fEPSP slope in the aged (*filled circles*) (n = 7) and in the young animals (*open circles*) (n = 5) following application of X/XO (20 µg/mL/ (0.25 unit/mg of xanthine)) for 60 minutes. B) Representative traces (average of 5 traces under each condition) illustrating the change in the NMDAR-fEPSP in the young (*top*) and aged animals (*bottom*) under *control* conditions and at the end of a 60 minute application of *X/XO*. Calibration bars: 20 ms, 0.5 mV. C) Quantification of mean percent change in the NMDAR-fEPSP slope from baseline (*dashed line*), corresponding to the last 5 minutes of a 60 minute application of X/XO in aged (*filled bar*) and young (*open bar*) animals.

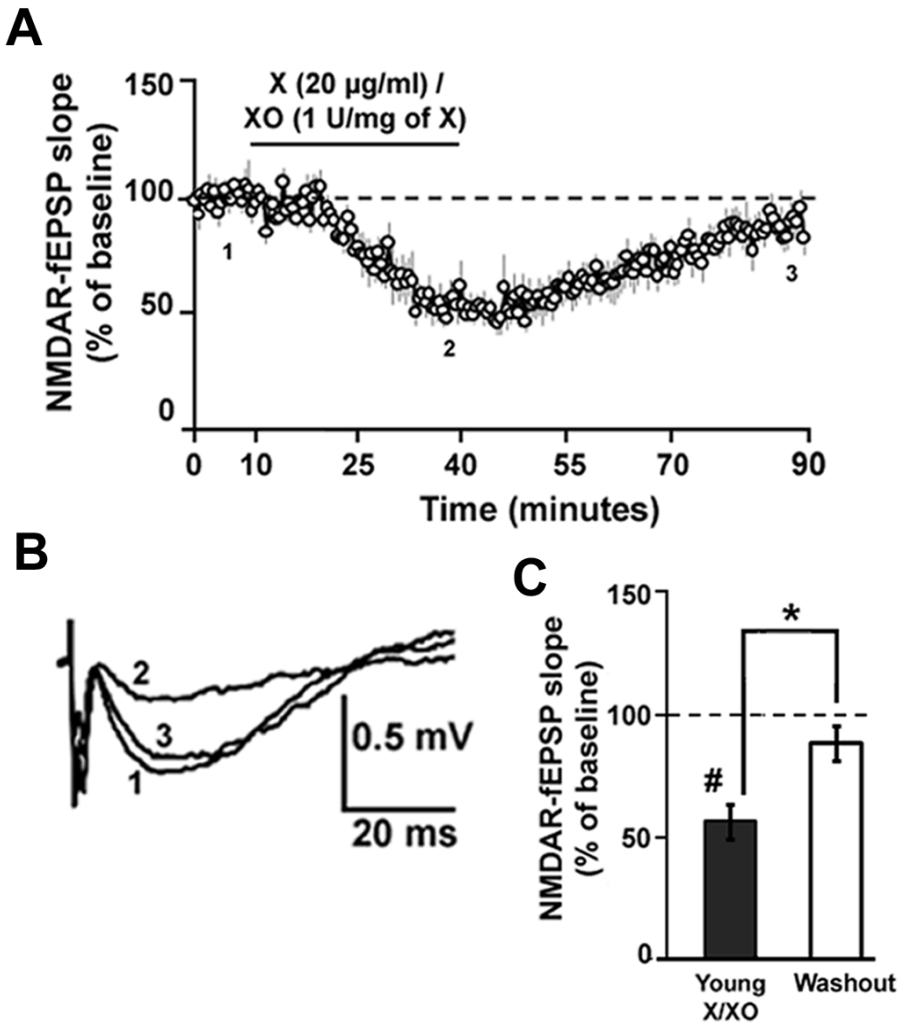

Figure 3-3. Effect of maximal concentrations of X/XO on NMDAR mediated synaptic potentials in young animals. A) Time course of the change in the normalized NMDAR-fEPSP slope in the young animals (n = 5) following application of X/XO (20 µg/mL/ (1 unit/mg of xanthine)) for 30 minutes and followed by a washout for 50 minutes. B) Representative traces (average of 5 consecutive traces) obtained during the indicated time points: baseline (1), under X/XO (2), and upon washout (3). Calibration bars: 20 ms, 0.5 mV. C) Quantification of the mean percent change in the NMDAR-fEPSP slope in young animals following the application of X/XO for 30 minutes (filled bar) and after washout for 50 minutes (open bar). In this and subsequent figures dashed lines represent the baseline level of 100%; pound signs indicate significant difference ($p<0.05$) from baseline level of 100%; asterisks indicate significant difference ($p<0.05$) between the indicated groups.

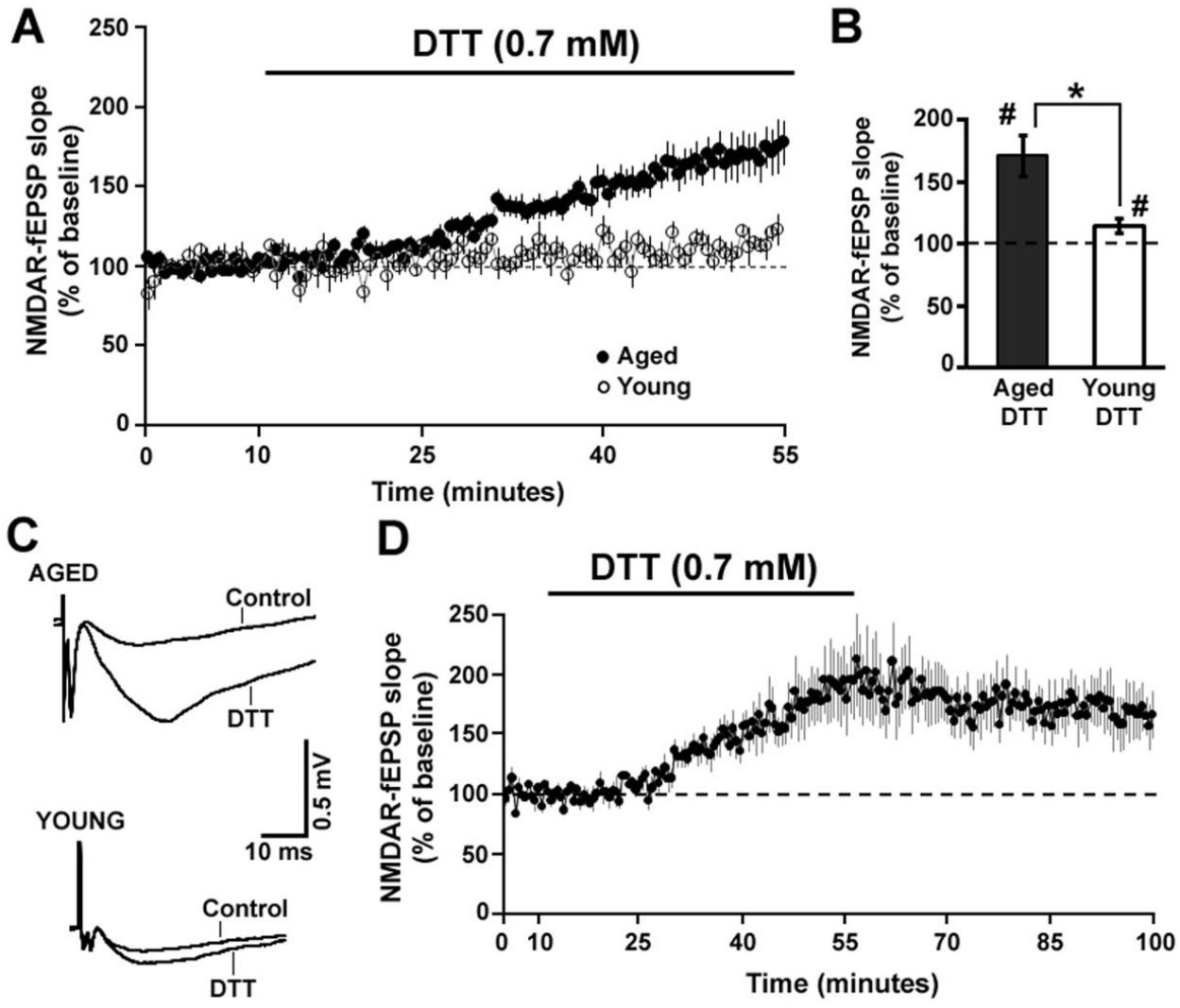

Figure 3-4. The reducing agent DTT increases NMDAR mediated synaptic responses to a greater extent in aged than in the young animals. A) Time course of the change in the normalized NMDAR-fEPSP slope in the aged (*filled circles*) (n = 16) and young animals (*open circles*) (n = 5) following application of DTT for 45 minutes. B) Quantification of the mean percent change in the NMDAR-fEPSP slope following application of DTT in aged (*filled bar*) and young (*open bar*) animals. C) Representative traces (average of 5 consecutive traces under each condition) illustrating the change in NMDAR-fEPSP in the presence of DTT in aged (*top*) and young animals (*bottom*). Calibration bars: 10 ms and 0.5 mV. D) Time course of the change in the normalized NMDAR-fEPSP slope in aged animals (*filled circles*) (n = 5) upon application of DTT for 45 minutes followed by a washout for 45 minutes.

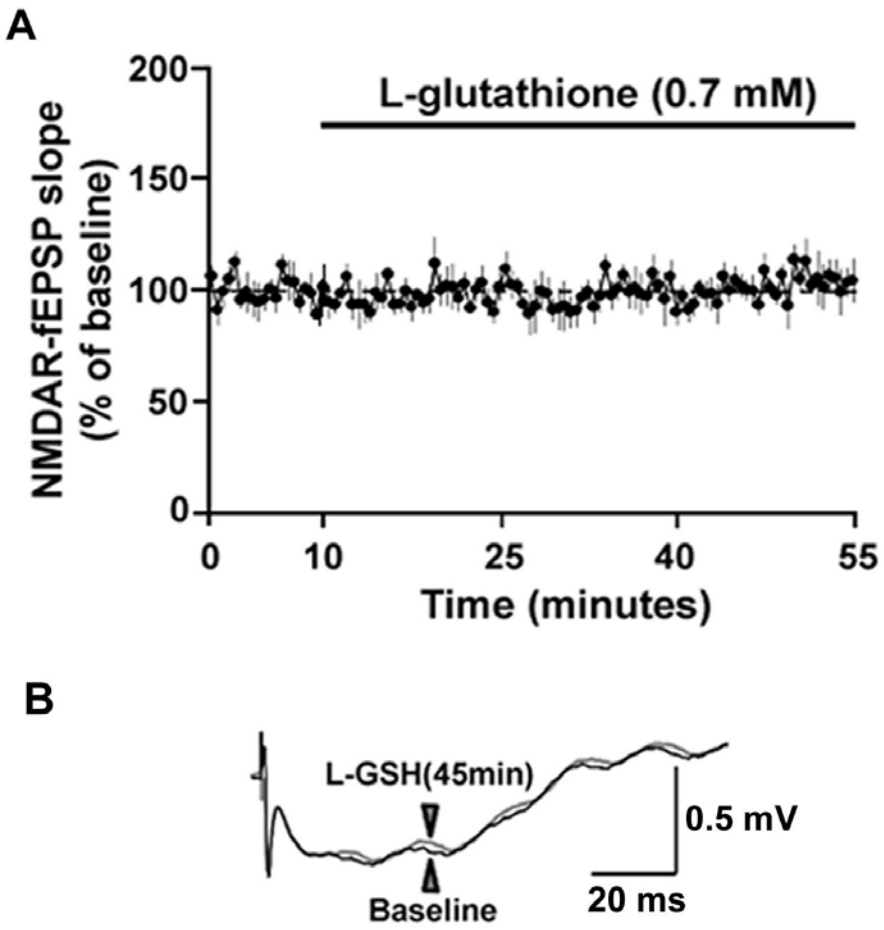

Figure 3-5. Extracellular application of reduced L-glutathione does not affect NMDAR function. A) Time course of the normalized NMDAR-fEPSP slope in the aged animals (n = 6) in response to extracellular application of the L-GSH. B) Overlay of the means of 5 consecutive responses during the baseline (black trace) and 45 minutes after application of L-GSH (gray trace). Calibration bars: 20 ms (horizontal) and 0.5 mV (vertical).

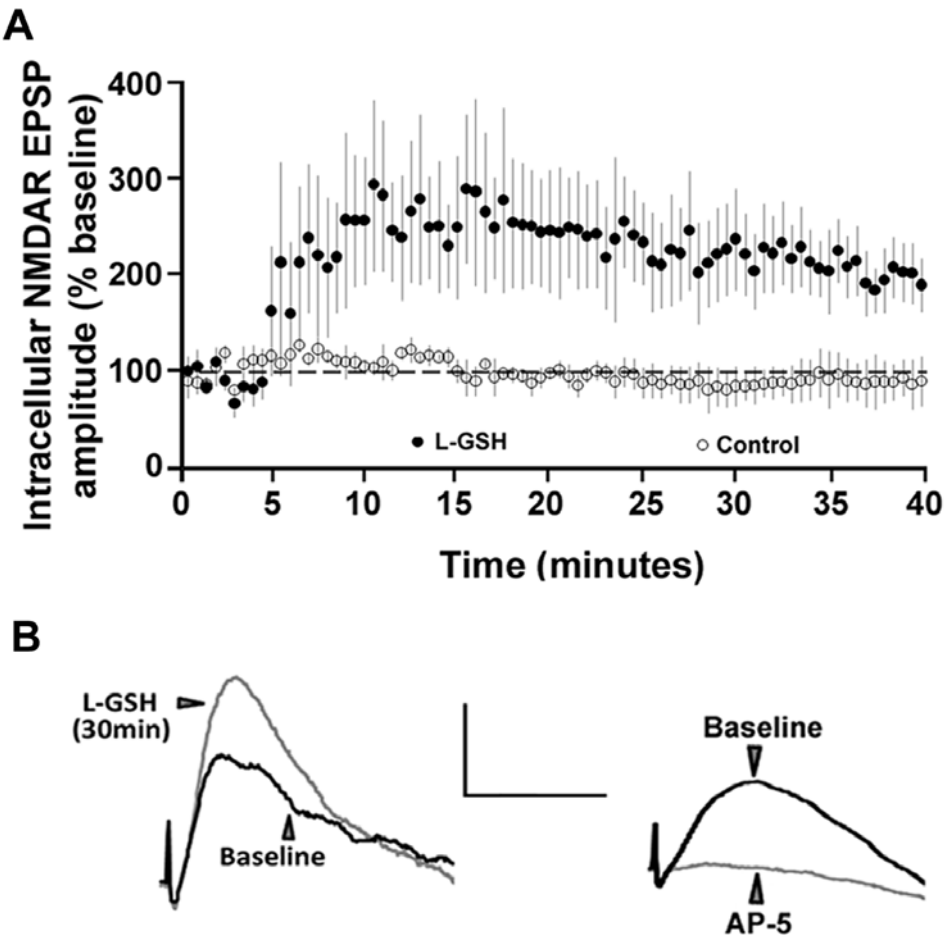

Figure 3-6. Intracellular application of reduced L-glutathione enhances intracellular NMDAR mediated synaptic potentials. A) Time course of the normalized NMDAR mediated intracellular EPSP amplitude in the aged animals obtained under control conditions (open circles, n = 3) or with L-GSH in the recording pipette (filled circles, n = 6). B) Left: Overlay of means from 5 consecutive responses obtained intracellularly during baseline (black trace) and 30 minutes after impalement (gray trace). Right: Overlay of means from 5 consecutive traces obtained intracellularly during baseline (black trace) and 25 minutes after application of AP-5 (gray trace). Calibration bars: 40 ms (horizontal) and 2 mV (vertical).

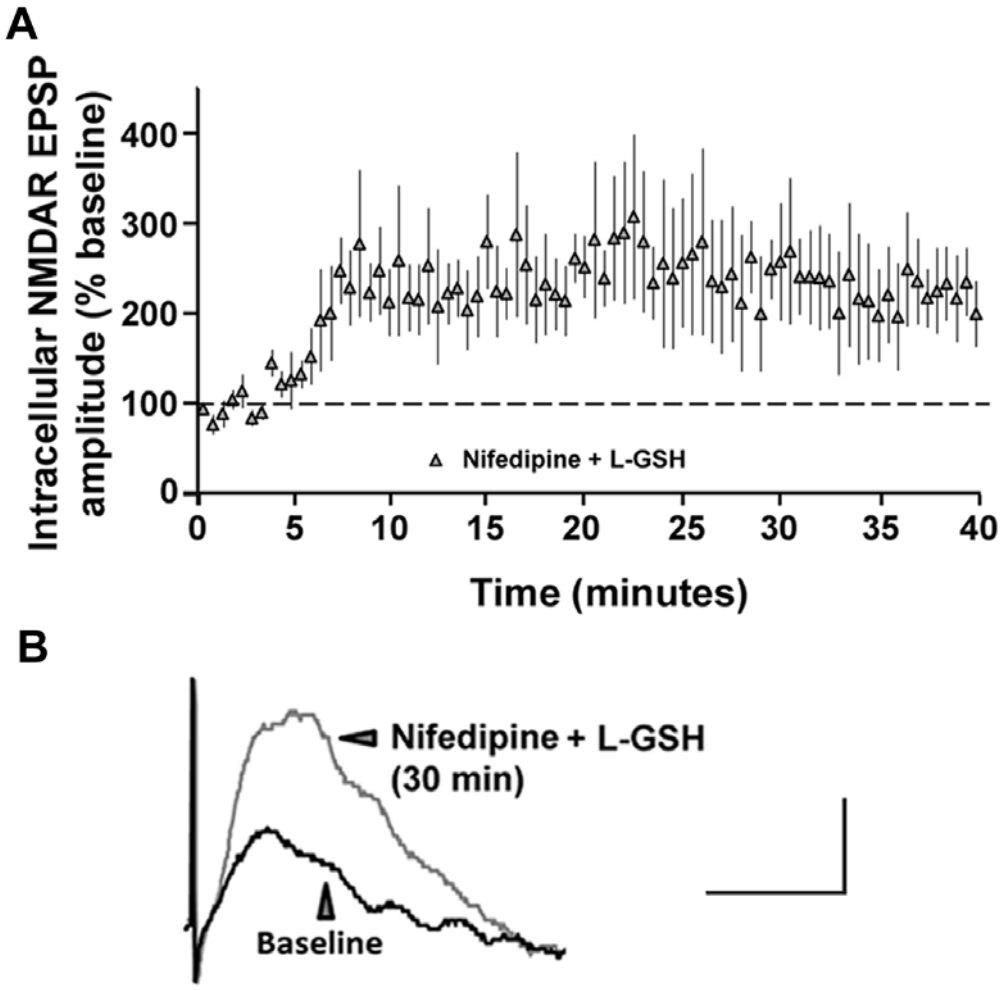

Figure 3-7. Glutathione mediated recovery of NMDAR function in aged animals does not involve L-type VGCC. A). Time course of normalized NMDAR mediated intracellular EPSP amplitude obtained with nifedipine along with ACSF and L-GSH in the recording pipette (filled triangles, n = 3). B) Overlay of means from 5 consecutive responses obtained intracellularly during baseline (*black trace*) and 30 minutes after impalement (*gray trace*). Calibration bars: 40 ms (horizontal) and 2 mV (vertical).

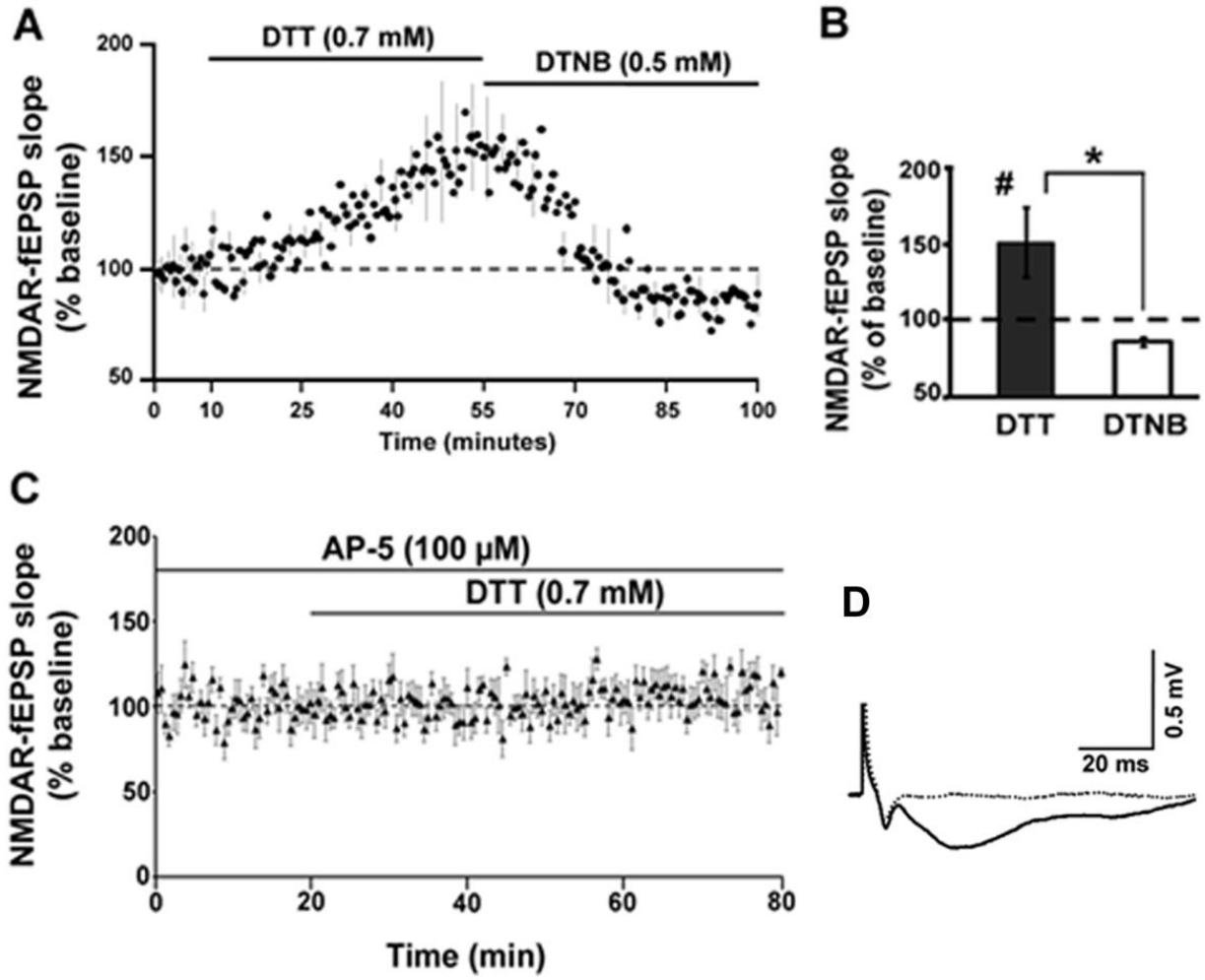

Figure 3-8. Redox modification of cysteine residues underlies NMDAR specific effect of DTT. A) Time course of the change in the normalized NMDAR-fEPSP slope in aged animals (n = 4) in response to the bath application of DTT followed by DTNB. The increase in NMDAR mediated synaptic responses by DTT was decreased by the oxidizing agent DTNB. Error bar in A is indicated for every fourth point, for purposes of clarity. B) Quantification of the mean percent change in NMDAR-fEPSP slope following application of DTT (filled bar) and DTNB (open bar) in aged animals. C) AP-5 (100 µM) was applied on the isolated NMDAR-fEPSP to block it, prior to DTT application. Time course of the change in any negative slope measured in a 20 ms window (n = 8) upon application of AP-5 followed by DTT. D) Representative traces (average of 5 consecutive traces) of the NMDAR-fEPSP recorded under control conditions (*solid black trace*), and upon application of 100 µM AP-5 (*dashed black trace*) for at least 30 min.

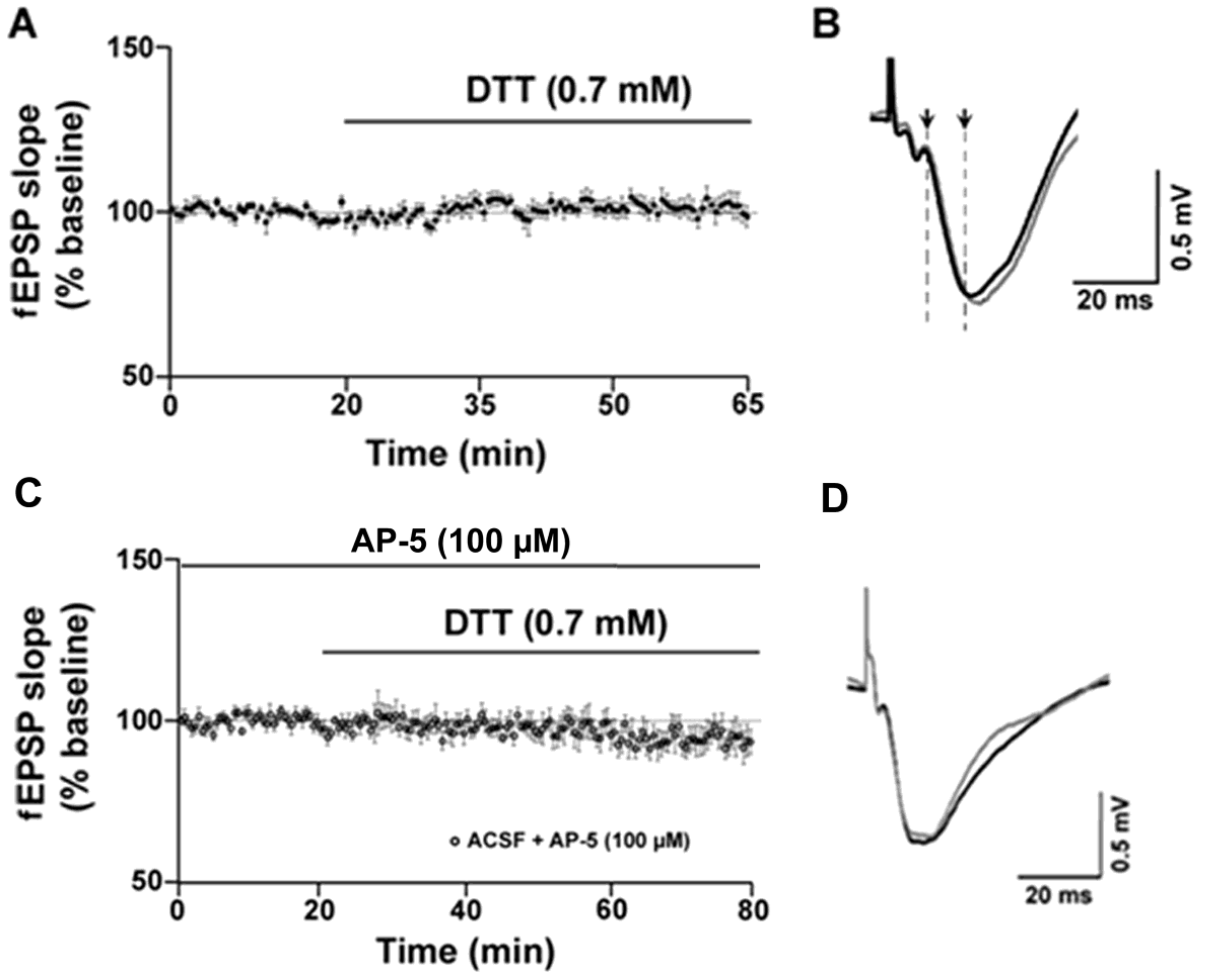

Figure 3-9. DTT does not affect the AMPAR function of aged animals. A) Time course of the change in fEPSP upon application of DTT for 45 minutes in aged hippocampal slices (n = 10). B) Representative traces (average of 5 consecutive traces) of the fEPSP recorded under control conditions (black trace) and after application of DTT for 45 min (gray trace). The downward pointing arrows and cursors indicate the 15 ms time window, where the initial descending phase of the fEPSP is predominantly mediated by AMPARs. Calibration bars: 20 ms and 0.5 mV. C) Time course of the change in the AMPAR mediated EPSP (isolated by the application of 100 µM AP-5 on the fEPSP) upon application of 0.7 mM DTT in the aged animals (n = 5). D) Representative traces (average of 5 consecutive traces) of the AMPAR mediated EPSP obtained under control conditions (*black trace*) and after application of DTT for 45 minutes (*gray trace*). Calibration bars: 20 ms, 0.5 mV.

Table 3-1. The NMDAR-fEPSPs from hippocampus of young and aged animals

PFV Bin Window (mV)	Young NMDAR-fEPSP (mean ± S.E.M)	Aged NMDAR-fEPSP (mean ± S.E.M)
0 – 0.4	0.13 ± 0.04	0.12 ± 0.04
0.4 – 0.8	0.49 ± 0.16	0.26 ± 0.05
0.8 – 1.2	0.85 ± 0.21	0.32 ± 0.09
1.2 – 1.6	1.21 ± 0.29	0.46 ± 0.15
1.6 – 2.0	1.61 ± 0.41	0.39 ± 0.14
2.0 – 2.4	1.86 ±0.46	0.44 ± 0.16
2.4 – 2.8	2.21 ± 0.87	0.62 ± 0.21
2.8 – 8.0	2.87 ± 0.91	0.73 ± 0.14

This table presents the NMDAR-fEPSP values recorded from the CA1 region of the hippocampus from young and aged animals. The NMDAR-fEPSP has been grouped under bin width of 0.4 mV of PFV amplitude.

CHAPTER 4
MOLECULAR MECHANISM UNDERLYING RECOVERY OF NMDAR FUNCTION AND HIPPOCAMPAL SYNAPTIC PLASTICITY IN AGED ANIMALS

Introduction

Age-related decrease in the NMDAR function of the hippocampal CA1 pyramidal neurons was reversed by the application of the reducing agent DTT or intracellular application of L-GSH (results from chapter 3). The strong links to intracellular redox state suggested a role for an intracellular signaling mechanism in causing the DTT-mediated increase in NMDAR function.

Extensive empirical evidence suggests that the NMDAR function is regulated by the phosphorylation state of the receptor. NMDAR function is increased upon phosphorylation by several intracellular kinases (Ben-Ari et al., 1992; Westphal et al., 1999; Li et al., 2001). Specifically the activation of tyrosine kinase (Wang and Salter, 1994; Heidinger et al., 2002), protein kinase C (PKC) (Ben-Ari et al., 1992; Chen and Huang, 1992), and protein kinase A (Raman et al., 1996) increases NMDAR mediated currents. In contrast, protein phosphatases, including calcineurin and protein phosphatase 1, decrease NMDAR currents (Lieberman and Mody, 1994; Wang et al., 1994; Raman et al., 1996). Moreover, phosphorylation state of NR2A and NR2B subunits can rapidly regulate surface expression and localization of the NMDARs (Gardoni et al., 2001; Chung et al., 2004; Hallett et al., 2006; Lin et al., 2006). For example, phosphorylation of serine residues within the alternatively spliced cassettes of the C-terminal tail of NR1 promotes receptor trafficking from the endoplasmic reticulum and insertion into the postsynaptic membrane (Scott et al., 2001; Carroll and Zukin, 2002). On the other hand, increased phosphatase activity has been linked to the internalization of NMDARs (Snyder et al., 2005). Hence, the kinases and phosphatases

act like molecular switches, which increase or decrease NMDAR function, respectively. Interestingly, aging is associated with a shift in the balance of kinase/phosphatase activity, favoring phosphatases (Norris et al., 1998b; Foster et al., 2001; Foster, 2007). Thus, alterations in the kinase/phosphatase activity in the postsynaptic neuron could underlie the decrease in the NMDAR function during aging. Moreover, the enzymatic activity of these kinases can be regulated by the reduction and oxidation of the cysteine residues located in their structure (Raynaud et al., 1997; Griendling et al., 2000; Knapp and Klann, 2000).

We tested whether the DTT-mediated increase in the NMDAR function was dependent on the activity of kinases and/or phosphatases that regulate the NMDAR function. Indeed we demonstrate that the mechanism for the age-dependent redox modulation of NMDARs involves CaMKII, but not PKC, PP1 or calcineurin/PP2B. CaMKII activity assays established that DTT increased CaMKII activity in CA1 cytosolic extracts in aged but not in young animals. Evidence is also provided to support the idea that the reducing agent DTT increases LTP in CA1 region of aged but not young hippocampal slices. The results presented in this chapter elucidate a molecular mechanism for the age-related NMDAR hypofunction and links oxidative redox state to impaired synaptic plasticity in aged CA1 pyrmaidal neurons.

Results

ROS Sensitive Dye Indicates Redox State of Live Hippocampal Neurons in *in vitro* Slices

The ROS sensitive dye 5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (c-H_2DCFDA) was used to detect ROS in live CA1 pyramidal neurons of the hippocampus from aged (23 month old) F344 rat. As described in the methods section,

the hippocampal slices were incubated for 30 minutes with c-H_2DCFDA. Images of the aged hippocampal CA1 pyramidal neurons were obtained under bright field illumination (Fig. 4-1A) and in the presence of a filter set to detect green fluorescence from the dye-exposed slices (Fig. 4-1B). Dye-unexposed slices obtained from the same aged F344 rat served as the control (Fig. 4-1C); which were used to detect auto-fluorescence. The green band pass filter (Excitation at 490 nm and Emission at 525 nm) was set to detect green fluorescence at uniform exposure time of 100 ms, and uniform exposure intensity set at 150%. The fluorescent signals from the dye-exposed slices were normalized to the dye-unexposed slices as described in the methods section.

The fluorescent signals originating from the hippocampal neurons incubated with c-H_2DCFDA are used as a direct measure of the levels of ROS; thus mandating a proper control for auto-fluorescent signals from the hippocampal tissue of aged animals. For all experiments described here, auto-fluorescence was assessed from dye-unexposed slices harvested from the same animal. No fluorescent signals were detected in the dye-unexposed slices, from aged (23 month old) and young (7 month old) hippocampi, when the imaging was performed with an exposure time of 100 ms (Fig. 4-2, middle panels). However, when the imaging was performed with an exposure time of 500 ms, clusters of fluorescent signals were detected in the aged but not in the young hippocampal slices (Fig. 4-2, right panels). The auto-fluorescent signals in the aged hippocampal slices could potentially arise from lipofuscin, an oxidized product known to accumulate during aging. Hence for the purposes of this study the imaging was performed at exposure times well below 500 ms, in order to eliminate auto-fluorescent signals and detect the fluorescence predominantly from ROS-oxidized c-

H_2DCFDA. For the results described in the following sections the imaging was performed at an exposure time of 100 ms, which successfully eliminated the auto-fluorescent signals.

Enhanced ROS Production in the CA1 Region of the Hippocampus of Aged Animals

The ROS detection technique (standardized as described above) was used to evaluate differential rates of ROS production in hippocampal slices from young and aged animals. The slices were incubated with c-H_2DCFDA for 30 minutes prior to imaging (Fig. 4-3A). Dye-unexposed slices (aged control: 36.14 ± 4.42%, n = 3 animals; young control: 31.97 ± 3.16%, n = 3 animals) showed no significant difference in fluorescence across the age groups. Thus, fluorescence intensity from c-H_2DCFDA - unexposed slices was used to normalize the fluorescence intensity obtained from c-H_2DCFDA-exposed slices. Incubation of the hippocampal slices from young and aged animals with c-H_2DCFDA for 30 min resulted in significantly (unpaired *t*-test; $p<0.05$) enhanced fluorescence in aged animals (242.19 ± 20.96%, n = 3 animals) when compared to young animals (141.61 ± 11.78%, n = 3 animals) (Fig. 4-3B).

The dye was designed to detect ROS produced in the intracellular space of the neurons. However, to further eliminate the possibility of signal contribution from ROS outside the cells, aged hippocampal slices were incubated with superoxide dismutase (SOD; 121 units/mL) and catalase (260 units/mL) along with the dye for 30 min prior to imaging. There was no significant difference ($p>0.05$) in the ROS-oxidized c-H_2DCFDA fluorescence observed between the SOD/catalase exposed (292.388± 28.04%, n = 3) and unexposed aged hippocampal slices.

Broad Spectrum Ser/Thr Kinase Inhibitor Blocks DTT-Mediated Recovery of NMDAR Function in Aged Hippocampal Neurons

To test if serine/threonine (Ser/Thr) kinases were involved in the DTT-mediated increase of the NMDAR response, the broad-spectrum and membrane-permeable Ser/Thr kinase inhibitor H-7 was bath applied prior to and during the application of DTT. In the presence of H-7 (10 μM, 45 min), DTT application failed to produce the robust increase (one-group *t*-test; $p>0.05$) in the NMDAR-fEPSP slope (111.86 ± 6.92%, n = 7) from the baseline levels (Fig. 4-4A).

In order to narrow down the identity of the Ser/Thr kinase that could potentially underlie the DTT-mediated increase in NMDAR-fEPSP several specific inhibitors were used. It was likely that Protein Kinase C (PKC) could underlie the DTT-mediated increase in NMDAR-fEPSP, due to the fact that PKC increases NMDAR function through phosphorylation mechanisms and PKC's activity could be regulated by redox mechanisms. To test whether PKC was responsible for the DTT-mediated increase in NMDAR-fEPSP, the membrane permeable PKC inhibitor, Bis-I (Knapp and Klann, 2002), was applied prior to, and during, the application of DTT on aged hippocampal slices. Application of Bis-I (500 nM, 45 min) failed to block the DTT-mediated increase in the NMDAR-fEPSP (142.58 ± 13.06%, n = 6) (Fig. 4-4B). Finally, *t*-tests indicated no effect of 0.01% DMSO (96.38 ± 7.64, n = 5) or kinase inhibition per se (H-7: 103.01 ± 4.21%, n = 7; Bis-I: 110.72 ± 11.44%, n = 6) on the baseline NMDAR-fEPSP slope in aged animals. Thus, DTT-mediated increase in NMDAR function was independent of PKC activity.

CaM Kinase II specific Inhibitors Block DTT-Mediated Recovery of NMDAR Function in Aged Hippocampal Neurons

One of the intracellular kinases that can enhance NMDAR function, and whose activity is regulated by redox agents like DTT, is Ca2+/Calmodulin dependent Protein Kinase II (CaMKII). To test if CaMKII underlies the DTT-mediated increase in NMDAR function in aged neurons, the CaMK inhibitor, KN-62, was bath applied prior to and during the application of DTT. In the presence of KN-62 (10 μM, 45 min) (Tokumitsu et al., 1990), DTT application failed to produce the robust increase in NMDAR-fEPSP in aged animals (97.9 ± 7.98%, n = 5) (Fig. 4-5A). Furthermore, the specific peptide inhibitor of CaMKII, myr-AIP (5 μM, 60 min), which was bath applied prior to and during the application of DTT, effectively blocked (104.48 ± 4.29%, n = 4) the DTT-mediated increase in NMDAR-fEPSP in aged animals (Fig. 4-5B). Finally, *t*-tests indicated no effect of 0.01% DMSO (96.38 ± 7.64, n = 5) or CaM kinase inhibition per se (KN-62: 96.23 ± 8.3%, n = 5; myr-AIP: 100.52 ± 4.42%, n = 4) on the baseline NMDAR-fEPSP slope in aged animals.

An ANOVA comparison of the effect of DTT in the presence and absence of the all the pharmacological kinase inhibitors, indicated a significant effect of kinase inhibition on the DTT effect in aged animals [$F(4, 33) = 5.85$, $p<0.01$]. Post hoc comparisons (Fisher's PLSD) indicated that the DTT-mediated increase in the NMDAR response was blocked by H-7, myr-AIP, and KN-62 but not Bis-I (Fig. 4-5C). This conclusively proves that CaMKII underlies the DTT-mediated increase in the NMDAR-fEPSP slope in aged CA1 hippocampal neurons.

DTT-Mediated Recovery of NMDAR Function in Aged Animals is Independent of Neuronal Protein Phosphatases

It has been previously reported that neuronal kinases and phosphatases act in tandem to regulate the function of the NMDARs. While kinases are known to increase NMDAR function, phosphatases decrease NMDAR function. The activity of Ser/Thr phosphatases like calcineurin (CaN) and protein phosphatase 1 (PP1), is thought to contribute to altered synaptic plasticity during aging (Foster et al., 2001). Moreover, increase in phosphatase activity decreases NMDAR function through dephosphorylation of the cytosolic tails of the NMDARs (Lieberman and Mody, 1994; Wang et al., 1994). To test if DTT effects were mediated by CaN, the CaN inhibitor FK-506 (10 μM) (Norris et al., 2008) was bath applied for 45 minutes prior to and during the application of DTT on aged hippocampal slices, while simultaneously recording the NMDAR-fEPSP. Application of FK-506 per se did not affect the NMDAR-fEPSP slope (109.61 ± 9.16%, n = 5). Importantly, FK-506 failed to block the DTT-mediated increase in the NMDAR response in aged animals such that the NMDAR-fEPSP slope increased to 148.61 ± 16.42% (n = 5) (Fig. 4-6A).

To examine the role of PP1, in mediating the DTT effect on aged NMDAR function, the PP1 inhibitor okadaic acid (OA) (1 μM, 30 min) (Schnabel et al., 2001) was (Schnabel et al., 2001) applied prior to and during application of DTT on aged hippocampal slices. Application of OA significantly increased the NMDAR-fEPSP slope during the baseline recording period itself (121.46 ± 9.19%, n = 5, $p<0.05$) (Fig. 4-6B). Therefore, a new stable baseline was recorded prior to the application of DTT. Relative to the new baseline, DTT increased the NMDAR-fEPSP slope (137.89 ± 3.99%, n = 5) in the presence of OA (Fig. 4-6C). An ANOVA comparing the effect of DTT in the

presence and absence of the phosphatase inhibitors, indicated no significant effect of phosphatase inhibition on DTT's effect [F (2, 15) = 1.37, p>0.05]. A summary of the results obtained with phosphatase inhibitors is presented in Fig. 4-6D.

Long-Term Potentiation is Enhanced in Aged Hippocampal Slices Exposed to DTT

For studies on Long-Term Potentiation (LTP) and paired-pulse ratio, slices were bathed in normal ACSF, in order to record the AMPAR and NMDAR component of the synaptic response. LTP was induced by a single episode of high frequency stimulation (HFS) of 100Hz (1second). Hippocampal slices from aged animals were either incubated in normal ACSF (Aged Control) or incubated in ACSF containing 0.7 mM DTT (Aged DTT) for at least 45 min prior to the delivery of HFS. In addition, a second pathway in the same slice that did not receive HFS (but received the baseline test pulses at 0.033 Hz, at baseline stimulation intensity) was used as the control pathway. The control pathway was used to monitor changes in slice health and to ensure stability of recording. In each case the fEPSP was recorded for 20 min prior to, and 60 min after, delivery of HFS. The magnitude of LTP was greater in aged hippocampal slices pre-incubated with DTT for 45 min (136.53 ± 2.77%; n = 10) (Fig. 4-7A), when compared to the aged controls not exposed to DTT (118.15 ± 4.63%; n = 9) (Fig. 4-7B). Although the levels of LTP showed considerable variation (Fig. 4-7C), the LTP was significantly [F (1, 17) = 12.14, p<0.01] greater in Aged-DTT group than the Aged-Control group.

In order to evaluate the role of presynaptic transmitter release on the DTT mediated enhancement in aged LTP, the paired pulse ratio was computed. Examination of paired-pulses delivered at varying inter-pulse intervals (Δt = 50 ms, 100 ms, 150 ms, 200 ms) (Fig. 4-7D), under control conditions and 45 min after the bath application of

DTT, indicated no effect of treatment across the four inter-pulse intervals (Table. 4-1). This suggests that the increase in aged LTP under DTT recruits postsynaptic mechanisms, involving the NMDARs in the postsynaptic CA1 pyramidal neurons.

Reducing Agent does not Alter Long-Term Potentiation in Young Hippocampal Slices

DTT had selectively enhanced NMDAR function in aged, but not in young, hippocampal slices. In order to test whether DTT had any age-dependent effects in enhancing LTP, young hippocampal slices were incubated with either normal ACSF (Young Control) or ACSF containing 0.7 mM DTT (Young DTT), before delivering LTP inducing HFS. In contrast to the effect observed in the aged animals, there was no difference ($p>0.05$) in the levels of HFS-induced LTP between young controls (130.17 ± 8.64%; n = 6) (Fig. 4-8A) and the young slices exposed to DTT (117.09 ± 12.24%; n = 5) (Fig. 4-8B). Furthermore, the fEPSP observed in the HFS pathway was significantly higher than the fEPSP in the control pathway (Fig. 4-8C).

CaMKII Activity is Enhanced in Aged Hippocampal CA1 Cytosolic Extracts Treated with DTT

To determine whether DTT was directly influencing CaMKII activity, cytosolic extracts from CA1 region of the hippocampus from aged animals were assayed for CaMKII activity by examining the phosphorylation of the synthetic peptide, syntide-2, in the presence and absence of DTT. Relative to baseline control levels, cytosolic CaMKII activity was significantly enhanced ($p<0.05$) in the presence of 0.7 mM (113.11 ± 3.47%, n = 3) and 1.4 mM DTT (120.46 ± 3.14%, n=3) in the aged CA1 cytosolic extracts (Fig. 4-9A). Higher levels of DTT (2.8 mM) resulted in a decrease in CaMKII activity, presumably due to denaturation of the enzyme. In addition, the CaMKII activity was

significantly ($p<0.05$) blocked (Aged: 5.36 ± 2.85%, n = 3) in the presence of Ca^{2+} chelator EGTA (2 mM) and CaMKII-specific peptide inhibitor myr-AIP (10 μM).

However DTT's effect on CaMKII activity from aged CA1 cytosolic extracts raised the possibility that DTT was acting on the CaMKII activity regulator calmodulin (CaM), and not exclusively on CaMKII. In this case the effect of DTT on CaMKII activity will be reduced by the addition of exogenous and un-oxidized CaM. To test this idea the assay was repeated in the absence of exogenously added CaM. The CaMKII activity from aged CA1 cytosolic extracts, in the presence of 0.7 mM DTT, and in the absence of exogenous CaM, (112.11 ± 3.91%, n = 3) was not enhanced beyond that observed following addition of exogenous CaM, suggesting that DTT effects were not mediated by reducing effect on CaM (Fig. 4-9B).

DTT does not Alter CaMKII Activity in Young Hippocampal CA1 Cytosolic Extracts

It was possible that DTT's effect were specific to the oxidized CaMKII present in the aged CA1 cytosolic extracts, and not the relatively un-oxidized CaMKII present in the young CA1 cytosolic extracts. To test this idea, the CaMKII activity was measured from the young CA1 cytosolic extracts, in the presence and absence of DTT. In contrast to the effect observed in aged animals, DTT had either no effect or decreased CaMKII activity in CA1 cytosolic extracts from young animals (Fig. 4-10A). However, as observed in aged animals, the CaMKII activity was inhibited ($p<0.05$) by the addition of Ca^{2+} chelator EGTA (2 mM) and CaMKII-specific peptide inhibitor myr-AIP (10 μM) (Young: 19.99 ± 9.01%, n = 3). Finally, addition of DTT to purified CaMKII (CycLex Co Ltd) decreased CaMKII activity ($p<0.05$) (0.7 mM DTT: 86.62 ± 6.04%, n = 3; 1.4 mM DTT: 70.72 ± 18.58%, n = 3) (Fig. 4-10B), indicating that the DTT effects were specific for CaMKII present in the aged CA1 cytosolic extracts.

Discussion

The DTT-mediated enhancement of NMDAR responses was specific to CaMKII activity because CaMKII inhibitors, myr-AIP and KN-62, blocked the DTT-mediated increase in NMDAR-fEPSP in aged animals. The DTT effects were not blocked by inhibition of PKC, PP1, or CaN/PP2B. The results point to CaMKII as a critical link between the intracellular redox state and NMDAR hypofunction. The role of CaMKII was further confirmed by enzyme activity assays which established that DTT increased CaMKII activity only in CA1 cytosolic extracts from aged animals. In contrast, DTT did not increase CaMKII activity in CA1 cytosolic extracts from young animals. In fact, DTT decreased the CaMKII activity in a purified sample of CaMKII, presumably due to enzyme denaturation by the reducing action of DTT.

Upon comparative analysis of all results, an interesting observation arises based on one of our previous results in chapter 3. In Chapter 3, we demonstrated that the DTT-mediated increase in NMDAR-fEPSP persisted for more than 45 min after aborting DTT application (Fig. 3-4D). The results presented in this chapter complement the previous findings and suggest that the lasting increase in NMDAR function was sustained by DTT's effect on CaMKII. In fact DTT's effect on NMDARs expressed in heterologous and non-neuronal systems has a quick onset and immediate washout (Tang and Aizenman, 1993; Kohr et al., 1994; Choi et al., 2001). The persistent increase in NMDAR function of aged neurons upon DTT application can be explained only when we invoke the neuronal CaMKII signaling mechanism, as proved by the results presented in here.

Oxidation of methionine residues on CaM has been reported to decrease the ability of CaM to activate CaMKII (Robison et al., 2007). Our results are not likely due to

CaM methionine oxidation since DTT possesses higher selectivity to reduce cysteine residues over methionine residues (Ciorba et al., 1997; Cai and Sesti, 2009; Long et al., 2009). In addition, DTT had equivalent effects in activating CaMKII, regardless of whether exogenous CaM was added to the CaMKII activity assay. The results indicate that oxidation of CaMKII, rather than CaM, underlies the reduction in kinase activity and are consistent with a recent report demonstrating that oxidative stress induced by ischemia results in disulfide linkages on the cysteine residues of CaMKII which decrease kinase activity (Shetty et al., 2008). While the data provide a link between age-related changes in intracellular redox state, CaMKII activity, and NMDAR function, the exact mechanism through which CaMKII regulates the NMDAR response remains to be determined. In addition to regulating phosphorylation state of proteins, including AMPARs, synaptic CaMKII participates in protein-protein interactions with several proteins localized to the dendritic spine which could ultimately alter NMDAR location and function (Lisman et al., 2002; Robison et al., 2005). In this context an independent report suggests that reduced CaMKII activity is associated with a specific decrease in synaptic NMDARs and decreased LTP (Gardoni et al., 2009).

In addition to a role for CaMKII, we observed that PP1 inhibition resulted in a modest increase in the NMDAR-fEPSP in aged hippocampal neurons. Age differences in the NMDAR response, which depend on kinase/phosphatase activity, are reminiscent of the age-dependent effects of kinase and phosphatase inhibitors on the rapid component of synaptic transmission mediated by AMPARs (Norris et al., 1998b; Hsu et al., 2002; Foster, 2007) and suggest that PP1 activity contributes to a reduction in AMPAR and NMDAR components of synaptic transmission (Foster et al., 2001;

Morishita et al., 2005), a characteristic specific to senescent CA1 synapses (Rosenzweig and Barnes, 2003). Our results indicate that a shift in the intracellular redox state towards oxidizing conditions during aging may cause or magnify the imbalance in the kinase/phosphatase activity, favoring phosphatases (Foster, 2007).

Our results using the ROS-sensitive dye provided us a direct and real-time indication of the intracellular redox state of the hippocampal neurons. Several points indicate that the readout of our ROS detection experiment was an accurate indication of the intracellular redox state. First the ROS sensitive dye c-H_2DCFDA, is preferentially cleaved by intracellular esterases to yield a non-fluorescent product; a pre-requisite for subsequent oxidation by ROS. Second, upon oxidation by ROS, the dye is converted into a fluorescent product which is membrane impermeable; thus the fluorescent readout is primarily due to intracellular signals. Finally, extracellular application of SOD and catalase did not affect c-H_2DCFDA fluorescence, simply due to the fact that these proteins are relatively membrane impermeable, and also because only intracellular events gave rise to c-H_2DCFDA fluorescence. While quantifying ROS-derived fluorescence from aged neurons, a significantly large auto-fluorescent signal was detected in dye-unexposed slices, mainly from lipofuscin. Lipofuscin, a breakdown product of lipid oxidation, is reported to accumulate in the aged hippocampal neurons (Landfield et al., 1981; Oenzil et al., 1994) and is capable of emitting auto-fluorescent signals overlapping the upper end of green, and the lower end of the yellow emission spectra (Haralampus-Grynaviski et al., 2003). Hence the imaging for detection and quantification of c-H_2DCFDA fluorescence was performed at 100 ms time-window in

order to eliminate the auto-fluorescent signals arising from lipofuscin accumulation in aged neurons.

Finally, NMDAR function is critical to the induction of LTP and we observed that DTT improved LTP in the CA3-CA1 synapses of aged animals. The interaction of NMDARs with CaMKII has been proposed as a model of memory (Lisman et al., 2002) and recent work indicates that disruption of the interaction between CaMKII and NMDAR impairs the induction of LTP and spatial learning (Zhou et al., 2007). We have provided evidence to indicate that a more oxidized redox state is a biological mechanism that can progressively inhibit NMDAR function in the hippocampus during senescence. Together, the results suggest that age-related changes in the redox state contributes to a decline in CaMKII activity, which ultimately leads to a decline in the NMDAR response. The outcome of such senescent mechanisms is an alteration in the synaptic plasticity at the CA3-CA1 synapses which contributes to age-related cognitive decline.

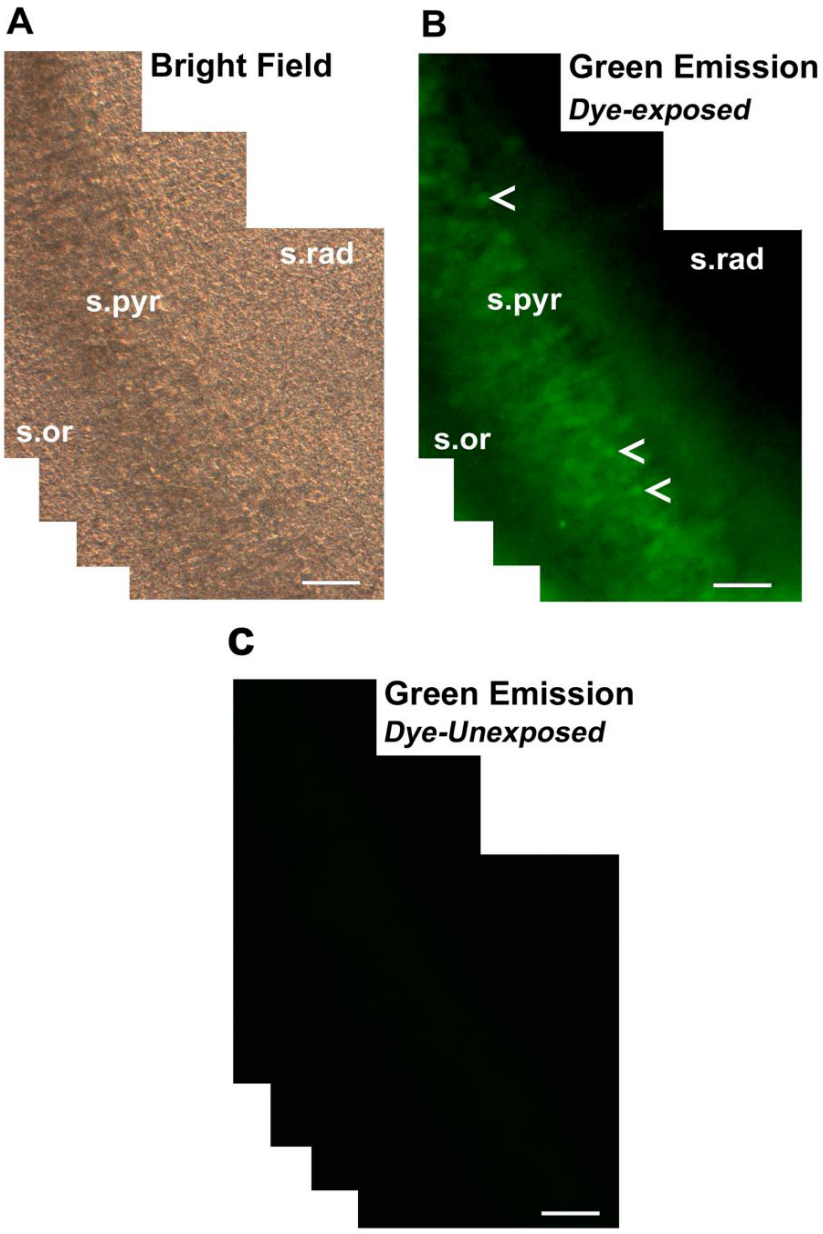

Figure 4-1. Detection of ROS in live hippocampal slices. A) Bright field image of the CA1 region of the hippocampus from an aged F344 rat. Indicated are the various layers of hippocampal area CA1 – stratum radiatum (s.rad), stratum pyramidale (s.pyr) and stratum oriens (s.or). B) The same image as in (A) was obtained with a green filter. White arrow heads indicate few of the many CA1 pyramidal neurons that have oxidized the ROS detection dye into a green fluorescent product. C) Image obtained from one of the dye-unexposed slices from the same rat. Scale bars = 50 μm.

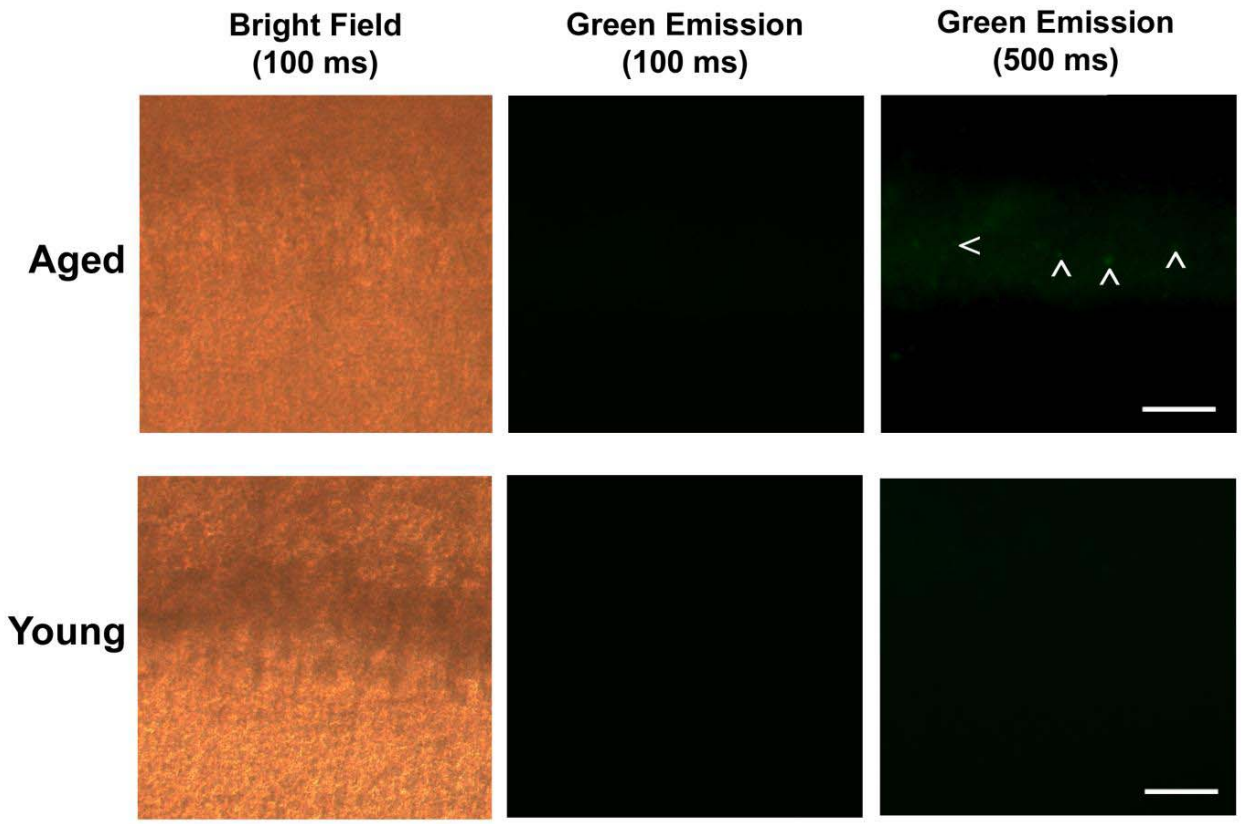

Figure 4-2. Detection of auto-fluorescence from dye-unexposed hippocampal slices. Shown are the images of the CA1 region of the hippocampus from aged (*top panel*) and young (*bottom panel*) animals. Dye-unexposed slices were used to obtain the bright field image (*left*), and the images with a filter designed to detect green fluorescence with exposure time set at 100 ms (*middle*) and 500 ms (*right*). Auto-fluorescent signals were detected, for a 500 ms exposure time, in the stratum radiatum of aged hippocampal slices (*white arrowheads*) but not in the young hippocampal slices. Scale Bar = 50 μm.

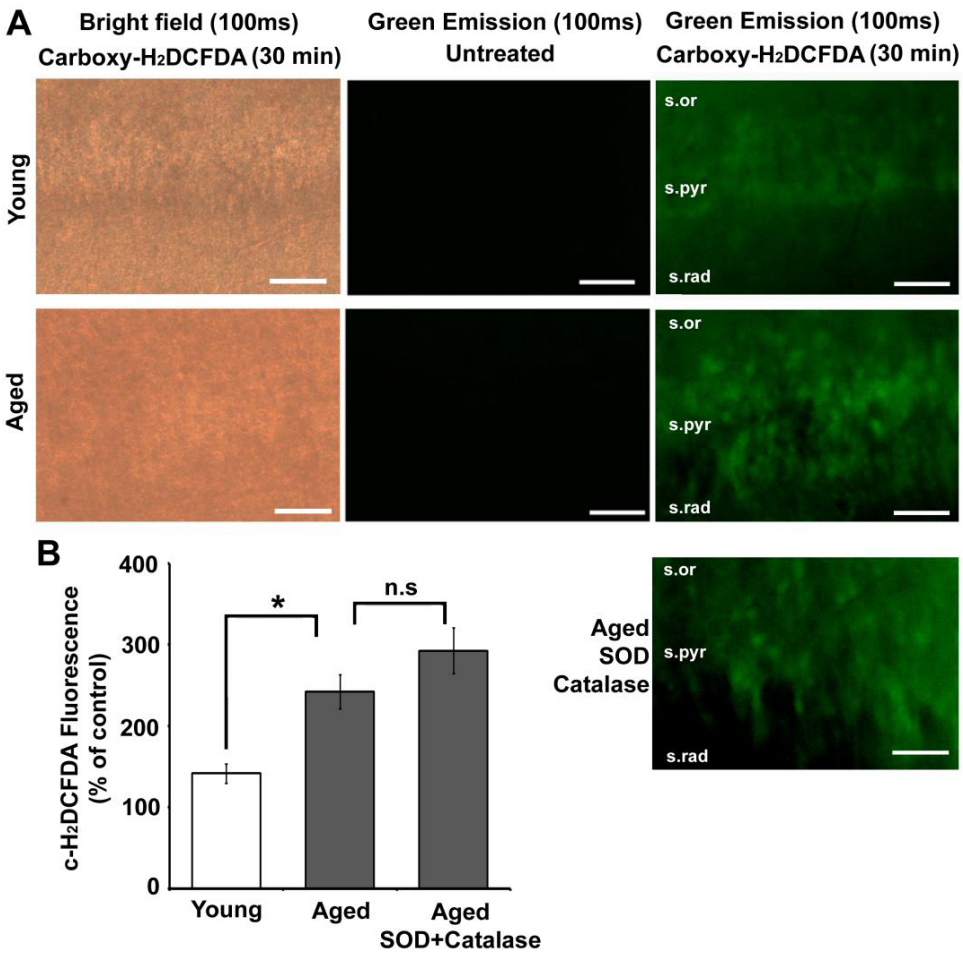

Figure 4-3. Enhanced ROS production is observed in hippocampal tissue from aged rats. A) Indicated above each image column are the imaging conditions (*bright field* or *green emission)* and the exposure time (*100 ms*) for hippocampal slices that were either *untreated* or treated *(Carboxy-H_2DCFDA)* with the dye. The rows are images of the CA1 region of young (*top row*) and aged (*second row*) hippocampal slices. The lowermost image is a *Carboxy-H_2DCFDA* treated slice from an aged animal, which was incubated with SOD + catalase. The various layers of hippocampal area CA1- stratum radiatum (*s.rad*), stratum pyramidale (*s.pyr*) and stratum oriens (*s.or*) are indicated in the last set of images. Scale bars = 50 μm. B) Quantification of the mean fluorescence intensity generated by the oxidation of c-H_2DCFDA (*c-H_2DCFDA Fluorescence*) from young (n = 3) (*open bar*), aged (n = 3), and SOD + catalase exposed aged hippocampal slices (n = 3) (*gray bars*) expressed as percent of fluorescence in untreated (control) slices from the same animal. In this and subsequent figures *error bars* represent standard error of mean (S.E.M), *asterisks* indicate significant difference ($p<0.05$) between the groups indicated, and *n.s* indicates no significant difference.

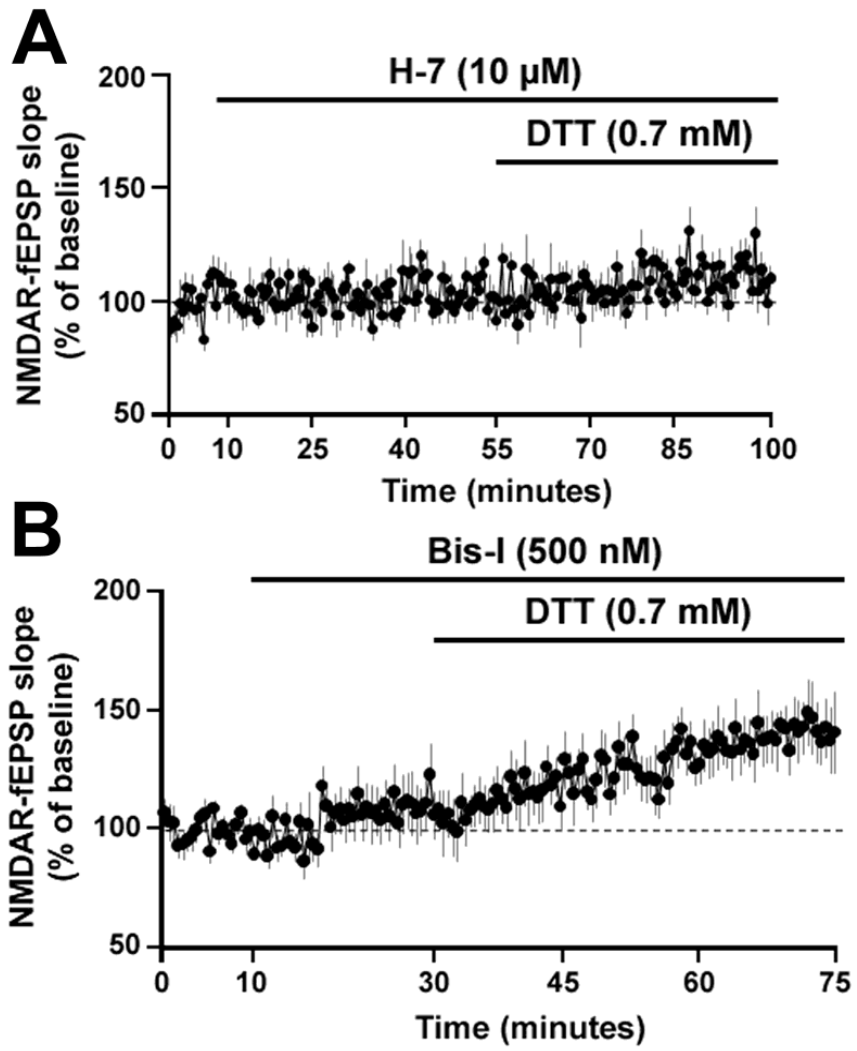

Figure 4-4. A Serine/Threonine (Ser/Thr) kinase, but not protein kinase C, mediates DTT mediated increase in NMDAR function in aged hippocampus. A) Time course of the change in the normalized NMDAR-fEPSP slope in the aged animals that were incubated with the broad spectrum Ser/Thr kinase inhibitor H-7 dihydrochloride (10 μM, n = 7) prior to and during DTT application. B) Time course of the change in the normalized NMDAR-fEPSP slope in the aged animals that were incubated with the PKC inhibitor Bis-I (500 nM, n = 6) prior to and during DTT application.

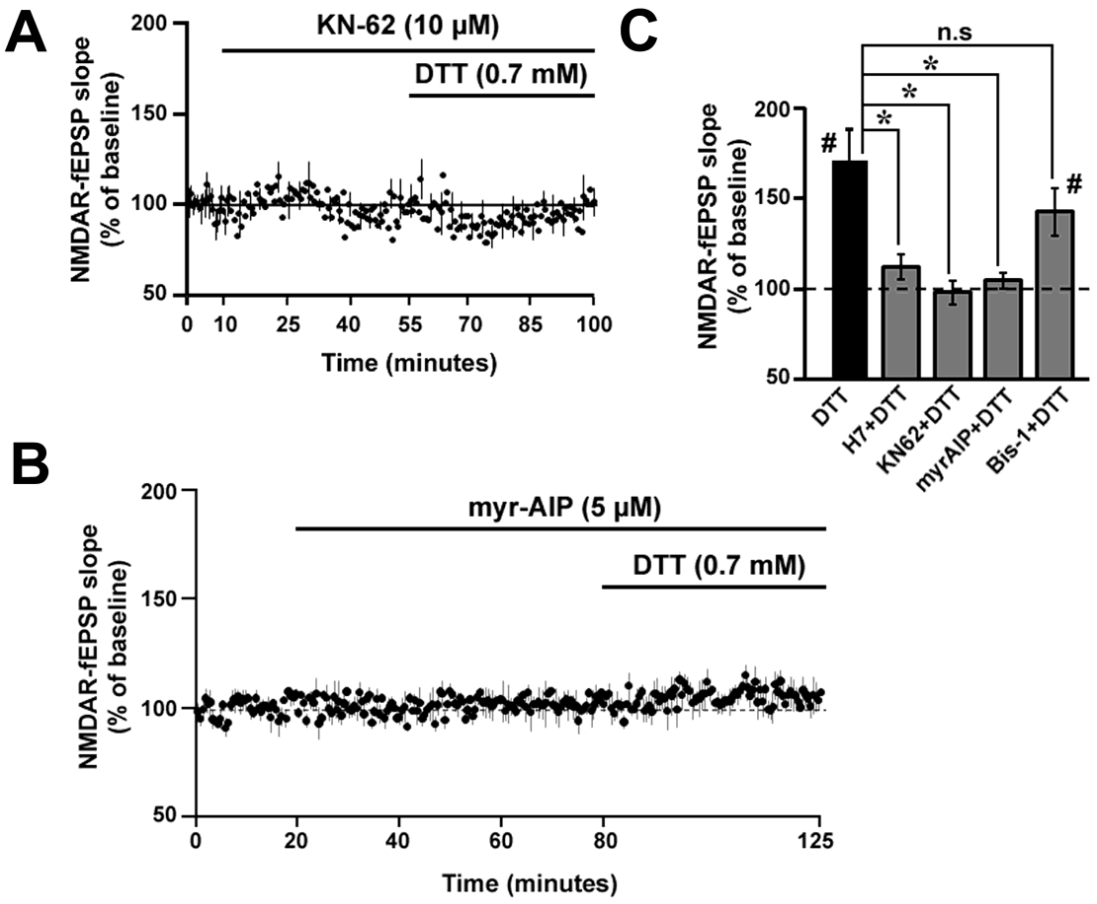

Figure 4-5. CaMKII involvement in the DTT mediated enhancement of NMDAR synaptic responses in aged animals. A) Time course of the change in the normalized NMDAR-fEPSP slope in the aged animals that were incubated with the CaMK inhibitor KN-62 (10 μM, n = 5). B) Time course of the change in the normalized NMDAR-fEPSP slope in the aged animals that were incubated with the specific CaMKII inhibitor myr-AIP (5 μM, n = 4). C) Quantification of the mean percent change in the NMDAR-fEPSP slope for aged animals under DTT alone (*filled bar*), and DTT applied in the presence of H-7, KN-62, myr-AIP and Bis-I (*gray bars*). Asterisk indicates a significant difference ($p<0.05$) between the increases observed in presence of DTT alone relative to DTT applied in the presence of H-7, KN-62, and myr-AIP; n.s indicates no significant difference between the increases observed in presence of DTT alone and DTT applied in presence of Bis-I. Pound sign indicates a significant ($p<0.05$) increase in the response relative to baseline level of 100%, following DTT application.

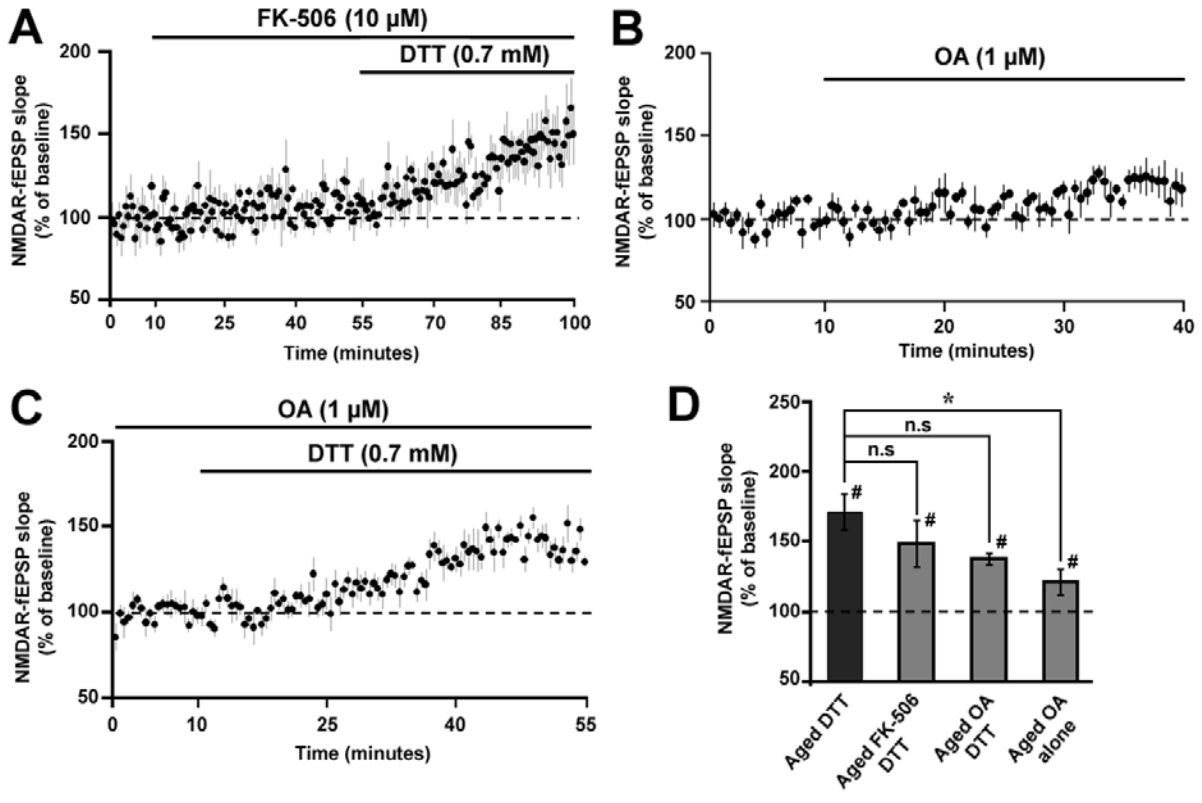

Figure 4-6. Calcineurin/PP2B and PP1 are not involved in the DTT mediated enhancement of NMDAR synaptic responses in aged animals. A) Time course of the increase in the NMDAR-fEPSP slope in slices from aged animals that were incubated with FK-506 (10 μM), 45 minutes prior to and during the application of DTT (n = 5). B) The NMDAR-fEPSP slope exhibited a modest increase (121.46 ± 9.19%) following a 30 minute incubation with OA (1 μM) (n = 5). C) Following stabilization of the response in OA, the baseline was recalculated. The figure illustrates the time course for the increase in the re-normalized NMDAR-fEPSP slope following application of DTT (n = 5). D) Quantification of the mean percent change in the NMDAR-fEPSP slope for aged animals under DTT alone (*filled bar*), and in the presence of FK-506 + DTT, OA + DTT and OA alone (*gray bars*).

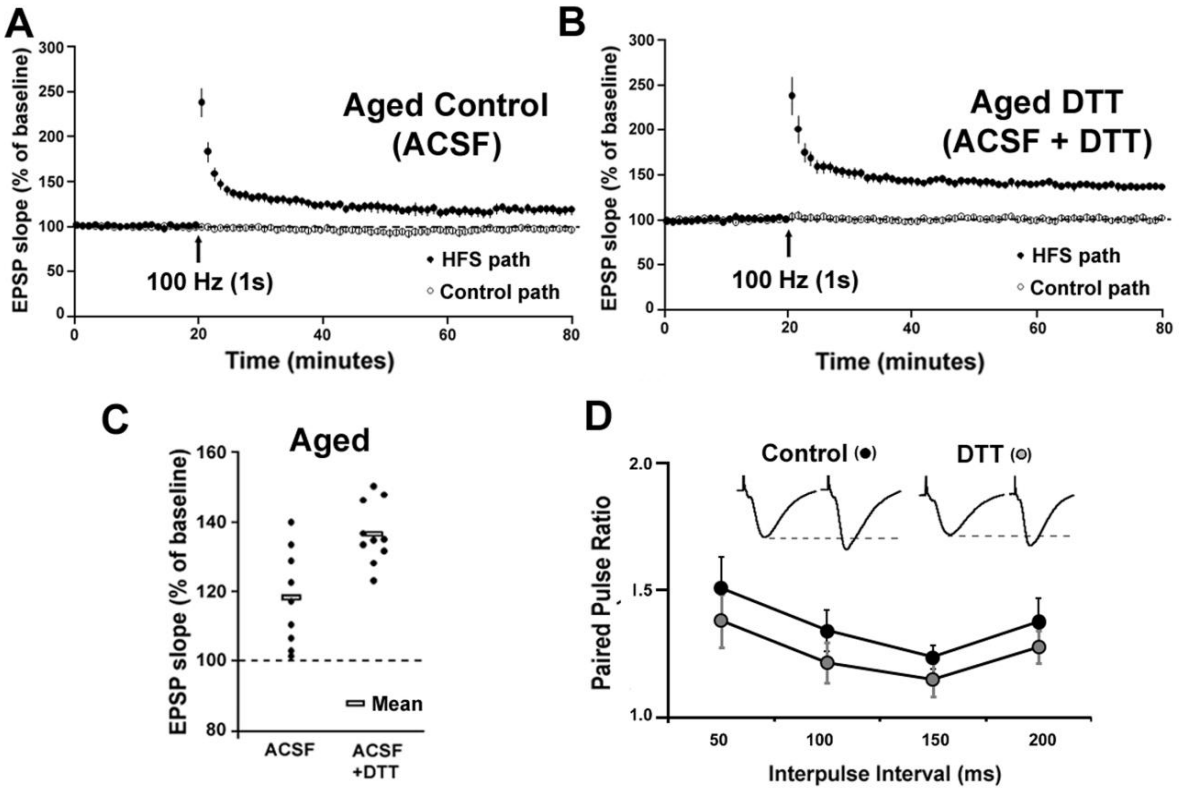

Figure 4-7. DTT enhances LTP in hippocampal area CA1 of aged animals. A) Time course for the expression of LTP recorded in aged hippocampal slices bathed in control ACSF (n = 9) for at least 45 minutes prior to HFS. Baseline stimulation was applied to a control pathway (*open circles*) and to a second pathway that received HFS (100 Hz, 1s) (*filled circles*). B) Time course for the expression of LTP recorded in aged hippocampal slices bathed in ACSF containing DTT (n = 10) for at least 45 minutes prior to HFS. Baseline stimulation was applied to a control pathway (*open circles*) and to a second pathway that received HFS (100 Hz, 1s) (*filled circles*). Arrows in A and B denote HFS delivery. For purpose of clarity, each point represents the mean of two consecutive responses. C) Distribution of the LTP magnitude for individual slices from aged animals bathed in control ACSF and ACSF+DTT. The rectangular boxes indicate the mean of each group. D) Quantification of the mean percent change in the fEPSP slope recorded from the control (*Cont*) and HFS (*HFS*) pathways from young slices bathed in ACSF or ACSF+DTT. E) Plot of the paired-pulse ratio obtained under control conditions (*black circles*) and after 45 minute bath application of DTT (*gray circles*) for four inter-pulse intervals (50, 100, 150, 200 ms). *Inset*: Responses obtained upon paired pulse stimulation (average of 5 consecutive traces; 50 ms inter-pulse interval) under control conditions and under DTT application.

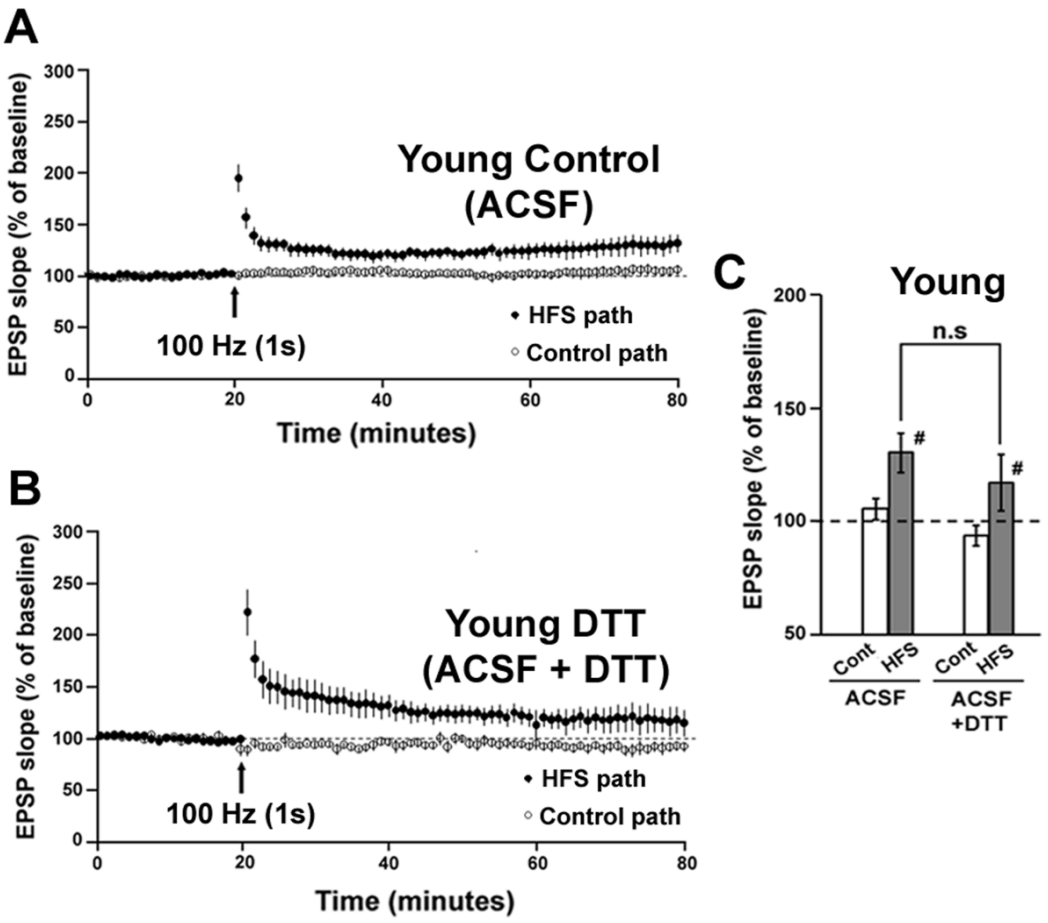

Figure 4-8. DTT does not alter the LTP in hippocampal area CA1 of young animals. A) Time course for the expression of LTP recorded in young hippocampal slices bathed in control ACSF (n = 6) for at least 45 min prior to HFS. Baseline stimulation was applied to a control pathway (*open circles*) and to a second pathway that received HFS (100Hz, 1s) (*filled circles*). B) Time course for the expression of LTP recorded in young hippocampal slices bathed in ACSF containing DTT (n = 5) for at least 45 min prior to HFS. Arrows in A and B denote HFS delivery. For purpose of clarity, each point represents the mean of two consecutive responses. C) Quantification of the mean percent change in the fEPSP slope recorded from the control (*Cont*) and HFS (*HFS*) pathways of the young slices bathed in ACSF or ACSF+DTT.

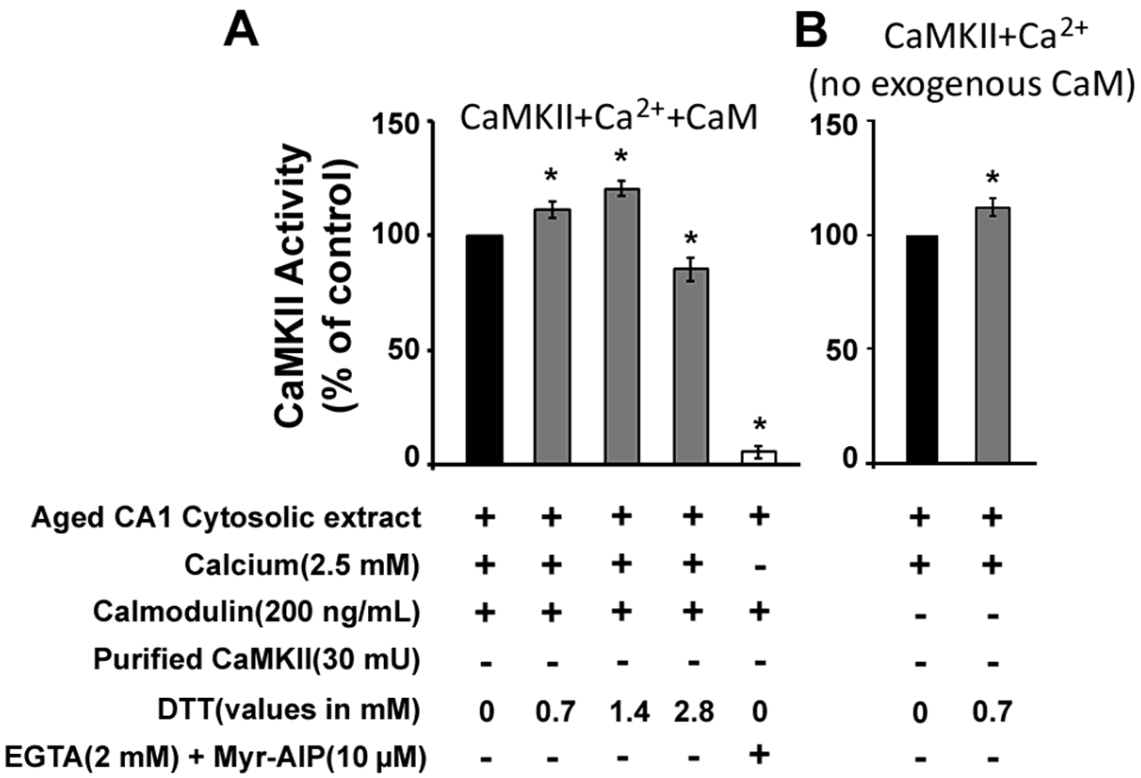

Figure 4-9. DTT enhances CaMKII activity in aged hippocampal CA1 cytosolic extracts. A) CaMKII activity measured from the hippocampal CA1 cytosolic extracts of aged F344 rats. CaMKII activity is represented as percent of control activity (*black bars*) in the presence of exogenous calmodulin. CaMKII activity was significantly enhanced in the presence of 0.7 mM and 1.4 mM DTT (*gray bars*), and was blocked by the addition of EGTA (2 mM) + myr-AIP (10 μM) (*white bars*). B) Removal of exogenous calmodulin did not further enhance the DTT (0.7 mM) effect on CaMKII activity suggesting that DTT is not acting through oxidized calmodulin in aged animals. *Asterisk* indicates a significant difference ($p<0.05$) from respective controls. *Plus* and *minus* represent the presence and absence (respectively) of the indicated component in the reaction mix.

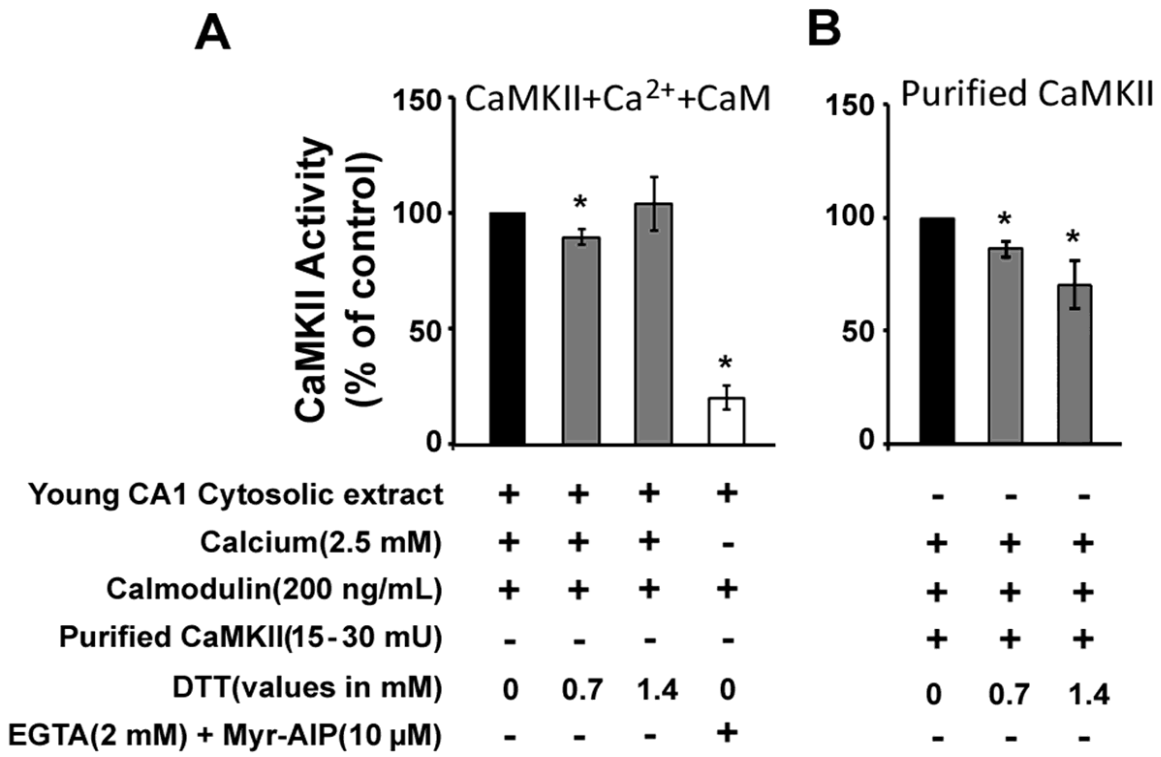

Figure 4-10. DTT does not enhance CaMKII activity in young hippocampal CA1 cytosolic extracts. A) CaMKII activity measured from the hippocampal CA1 cytosolic extracts of young F344 rats. CaMKII activity is represented as percent of control activity (*black bars*) in the presence of exogenous calmodulin. Addition of 0.7 mM and 1.4 mM DTT did not increase CaMKII activity in hippocampal CA1 cytosolic extracts of young F344 rats. B) Addition of 0.7 and 1.4 mM DTT decreased the activity of purified CaMKII. *Asterisk* indicates a significant difference ($p<0.05$) from respective controls. *Plus* and *minus* represent the presence and absence (respectively) of the indicated component in the reaction mix.

Table 4-1. Paired-pulse ratios from aged animals

Inter pulse Interval (ms)	Paired pulse ratio Control (mean ± S.E.M)	Paired pulse ratio DTT (mean ± S.E.M)
50	1.51 ± 0.12	1.38 ± 0.11
100	1.34 ± 0.08	1.21 ± 0.08
150	1.24 ± 0.05	1.15 ± 0.07
200	1.38 ± 0.09	1.28 ± 0.06

The table represents the paired pulse ratio obtained from control, aged hippocampal slices and aged hippocampal slices that were incubated with DTT for at least 45 minutes. The paired pulses ratio is the ratio of the slopes of two consecutive fEPSPs elicited apart by a time interval indicated by the inter pulse interval.

CHAPTER 5
REDOX MODULATION MEDIATES REDUCTION IN NEURONAL AFTERHYPERPOLARIZATION OF AGED HIPPOCAMPAL NEURONS

Introduction

An age-related decline in hippocampus-dependent memory is thought to result from dysregulation of Ca^{2+}-dependent processes in CA1 pyramidal neurons including synaptic plasticity and neuronal excitability (Foster, 1999, 2007; Kumar et al., 2009; Burke and Barnes, 2010; Magnusson et al., 2010; Oh et al., 2010). The results presented in chapters 3 and 4 dealt with NMDAR hypofunction, a significant biomarker of aging in CA1 pyramidal neurons. One of the other well characterized markers of aging in CA1 pyramidal neurons is an increase in the slow component of the Ca^{2+} activated, K^+- mediated afterhyperpolarization (sAHP) (Landfield and Pitler, 1984; Moyer et al., 1992; Kumar and Foster, 2004; Thibault et al., 2007; Matthews et al., 2009)

The exact mechanism that underlies the age-related increase in sAHP is unknown. The increase in the sAHP may be due to altered Ca^{2+} regulation, including an increase in L-type voltage gated Ca^{2+} channels (L-type VGCC) (Thibault and Landfield, 1996; Veng and Browning, 2002) or increased release of Ca^{2+} from intracellular Ca^{2+} stores (ICS) (Kumar and Foster, 2004; Gant et al., 2006) or an increase in the function or density of K^+ channels that mediate the sAHP (Power et al., 2001; Power et al., 2002). Importantly, aging is associated with increased oxidative stress that could influence the highly redox sensitive RyRs, which mediate Ca^{2+} release from ICS (Eager and Dulhunty, 1998; Hidalgo et al., 2004; Bull et al., 2008; Huddleston et al., 2008). Moreover, aged neurons are characterized by a decrease in their redox buffering capacity (Parihar et al., 2008; Bodhinathan et al., 2010) and recent work from our lab

demonstrates that the shift in redox state contributes to altered Ca^{2+} regulation in CA1 neurons from aged animals (Bodhinathan et al., 2010). Based on these observations we tested the hypothesis that the redox state of the aged neuron contributes to the increase in sAHP (Foster, 2007; Kumar et al., 2009).

The results reveal that the sAHP is decreased by the reducing agent dithiothreitol (DTT) in an age-dependent manner. Application of ryanodine, to block RyRs, prevented the DTT-mediated decrease of sAHP in the aged neurons. Depletion of ICS by the application of thapsigargin also blocked the DTT effect on aged-sAHP. The DTT-mediated decrease in aged-sAHP was independent of the activity of L-type VGCC or Ser/Thr kinase activity. Finally inhibition of the big conductance potassium (BK) channel activity did not influence DTT-mediated decrease in aged-sAHP. The results point to an ICS-dependent and RyR-mediated mechanism that links oxidative redox state during aging and the enhanced sAHP in CA1 pyramidal neurons. Reversal of the redox state of aged hippocampal CA1 pyramidal neurons is a potential therapeutic strategy to ameliorate Ca^{2+} dysregulation, decrease sAHP and restore normal functionality in aged neurons.

Results

Age Dependent Decrease in the sAHP Following DTT Application

To study the effects of oxidative redox state on the aged-sAHP, the reducing agent DTT was applied to aged and young hippocampal CA1 pyramidal neurons while continuously recording the sAHP. In confirmation of previous studies, the sAHP was significantly ($p<0.05$) increased in aged (6.44 ± 0.32 mV, n = 40) relative to young CA1 pyramidal neurons (4.23 ± 0.17 mV, n = 12). The properties of the CA1 pyramidal neurons recorded from the young and aged animals are indicated in Table. 5-1. In a

subset of these neurons, after a stable baseline recording for 10 min, DTT was applied for 40 min. Application of DTT significantly ($p<0.05$) decreased the sAHP amplitude form the baseline levels in the aged (48 ± 14% of baseline, n = 5), but not in the young animals (105 ± 10%, n = 3) (Fig. 5-1A, 5-1B). The DTT-mediated reduction in sAHP of aged neurons does not appear to be due to altered membrane properties, since the holding current required to maintain the membrane potential at -63 mV did not differ ($p>0.05$) between baseline and 45 minutes after DTT application. The DTT-mediated decrease, specific to the aged-sAHP, suggests a link between oxidative redox state and the increased sAHP amplitude in aged neurons.

DTT Mediated Decrease in Aged-sAHP Involves Intracellular Calcium Stores and Ryanodine Receptors

To test the hypothesis that the DTT-mediated decrease in the aged-sAHP was due to decrease Ca^{2+} mobilization from ICS, ICS were depleted by the application of thapsigargin prior to and during the application of DTT to aged hippocampal slices. Application of thapsigargin for 30 min significantly decreased ($p<0.05$) the amplitude of aged-sAHP to 58 ± 8% (n = 7) of the baseline levels (Fig. 5-2A). In a subset of these cells (n = 4), a new baseline was established and DTT was applied. Application of DTT for 50 min failed to decrease the aged-sAHP amplitude (104 ± 23%) (Fig. 5-2B, 5-2C). The results suggest that ICS provide a redox sensitive Ca^{2+} source that contributes to the age-related increase in the sAHP.

RyRs mobilize Ca^{2+} from the ICS and are highly redox sensitive. To test whether RyRs were involved in the DTT-mediated decrease in aged-sAHP, RyRs were block by ryanodine prior to and during the application of DTT. Application of ryanodine for 40 min, significantly ($p<0.05$) decreased the aged-sAHP amplitude to 47 ± 10% (n = 4).

Application of DTT for 50 min failed to further decrease the aged-sAHP amplitude such that it was 54 ± 7% (n = 4) of the original baseline (Fig. 5-3A, 5-3B).

Both DTT (Fig. 5-1) and ryanodine (Fig. 5-3) decreased the aged-sAHP to ~50% of baseline. The similar magnitude effect raises the possibility of a "floor effect" of ryanodine, which may have masked DTT influences on the sAHP. To address this issue, the sAHP of aged neurons was enhanced by increasing the extracellular Ca^{2+} concentration from 2 mM to 4 mM. Increasing the extracellular Ca^{2+} to 4 mM increased the sAHP almost two fold, from 6.71 ± 0.79 mV (n=11) to 11.06 ± 1.09 mV (n = 6) (Fig. 5-4A, 5-4B). In five cells a baseline was recorded in 4 mM Ca^{2+}, followed by application of ryanodine for 40 min, which was then followed by the application of DTT for 50 min (Fig. 5-4C). Application of ryanodine decreased ($p<0.05$) the aged-sAHP amplitude to 53 ± 6% (5.31 ± 0.86 mV) and application of DTT for 50 min failed to further decrease the aged-sAHP amplitude (51 ± 7%, 5.06 ± 1.05 mV) of the original baseline (Fig. 5-4B, 5-4C, 5-4D). Thus, DTT failed to reduce the sAHP amplitude under high Ca^{2+} and ryanodine application, despite the fact that sAHP amplitude was similar to the baseline under normal 2 mM Ca^{2+} conditions (Fig. 5-4B). The results indicate that the ryanodine blockade of the DTT-mediated decrease in the sAHP was not due to a floor effect of the sAHP during ryanodine application. Rather, these data suggest that the DTT effect on sAHP in aged animals is mediated by RyRs.

DTT Mediated Reduction in the Aged-sAHP is Independent of L-VGCC

L-type VGCCs are another major source of Ca^{2+} for the sAHP. To test the hypothesis the DTT-mediated decrease in the aged-sAHP involves the L-type VGCC, nifedipine was applied prior to and during the application of DTT to aged hippocampal slices (Fig. 5-5A). Application of nifedipine for 20 min decreased the sAHP to 68 ± 4%

(n = 5) of the baseline. Subsequent application of DTT for 30 min further decreased the amplitude of aged-sAHP to 34 ± 4% (n = 5) of the original baseline. The results suggest that the effects of nifedipine and DTT may be independent. In fact, using the sAHP responses recorded in nifedipine (bath application for at least 20 min) as the baseline, application of DTT decreased the amplitude of the aged-sAHP to 48 ± 6 % ($p<0.05$; n = 6) (Fig. 5-5B), a decrease comparable to that observed following DTT application in the absence of nifedipine (Fig. 5-7B).

The activity of BK channels is sensitive to oxidation (DiChiara and Reinhart, 1997). Moreover, an increase in BK channel activity can reduce the sAHP amplitude by decreasing the action potential spike width (Giese et al., 1998; Shao et al., 1999; Murphy et al., 2004). To test the hypothesis that the DTT-mediated decrease in aged-sAHP involves the BK channels, paxilline first applied to inhibit BK channel activity (Sanchez and McManus, 1996). Aged hippocampal slices were incubated in paxilline for at least 60 min prior to recording the sAHP and applying DTT. Paxilline failed to block the DTT-mediated decrease in aged-sAHP, such that DTT application was still able to decrease the amplitude of the aged-sAHP to 28 ± 11 % (n = 3) of the baseline (Fig. 5-6A, 5-6B). Furthermore, the DTT-mediated decrease in the presence of paxilline was not significantly ($p>0.05$) different from the decrease observed in the presence of DTT alone.

Serine/threonine kinases provide another potential mechanism for regulating RyRs and the K^+ channels that mediate the sAHP. Protein kinase A increases the activity of cardiac RyRs (RyR subtype 2) (Yoshida et al., 1992; Danila and Hamilton, 2004; Xiao et al., 2007; Morimoto et al., 2009), and kinase activity inhibits the sAHP (Madison and

Nicoll, 1986; Malenka et al., 1986; Muller et al., 1992; Pedarzani and Storm, 1993; Melyan et al., 2002). In order to test whether the changes in kinase activity underlies the decrease in sAHP of aged neurons upon application of DTT, the broad spectrum serine/threonine kinases inhibitor H-7 was applied prior to and during the application of DTT. Aged hippocampal slices were incubated with H-7 for at least 60 minutes before recording the sAHP. In the presence of H-7, application of DTT significantly ($p<0.05$) decreased the aged-sAHP to 53 ± 14 % (n = 3) of the baseline (Fig. 5-7A). The results suggest that DTT is not altering the sAHP through modulation of kinase activity.

Fig. 5-7B summarizes the change in the sAHP amplitude following DTT application under various conditions. In each case, the response was normalized to the pre-DTT application baseline. In addition, the percent change for application of nifedipine-alone or ryanodine-alone relative to the pre-drug baseline is illustrated for comparison. Treatments that blocked Ca^{2+} release from ICS (thapsigargin, ryanodine) blocked the DTT-mediated reduction in the sAHP. In all cases in which DTT reduced the sAHP; including in the presence of nifedipine, the reduction was ~50%. A similar reduction was observed following treatment with ryanodine-alone, consistent with previous reports in aged animals (Kumar and Foster, 2004; Gant et al., 2006). Application of nifedipine-alone decreased the sAHP by ~30%, consistent with previous reports in young and aged animals (Power et al., 2002; Disterhoft et al., 2004).

Discussion

The results demonstrate a link between the age-related increase in the sAHP and redox state, through the release of Ca^{2+} from ICS. A shift in Ca^{2+} regulation and altered Ca^{2+} channel function is a characteristic of certain aging neurons (Foster, 1999, 2007; Kumar et al., 2009; Burke and Barnes, 2010; Magnusson et al., 2010; Oh et al., 2010).

Recently we demonstrated that DTT could reverse an age-related decrease in NMDA receptor function in region CA1 (Bodhinathan et al., 2010). In the current study, the reducing agent, DTT, decreased the sAHP in aged, but not in young CA1 neurons. The data are consistent with the weakened redox buffering in aged animals as a mechanism contributing to Ca^{2+} dysregulation and electrophysiological changes observed in aged neurons. Redox modulation has been observed for several ion channels including K^+ and Ca^{2+} channels (Ruppersberg et al., 1991; Chiamvimonvat et al., 1995; Stephens et al., 1996; DiChiara and Reinhart, 1997; Hidalgo et al., 2004), which could contribute to the sAHP. The identity of the K^+ channel that underlies the sAHP is unknown (Furuichi et al., 1994; Sah and Faber, 2002); however, the amplitude to the sAHP is reduced by activation of Ser/Thr kinases, including PKA (Madison and Nicoll, 1986; Pedarzani and Storm, 1993), CaMKII (Muller et al., 1992), and PKC (Malenka et al., 1986). In the current study, the broad spectrum Ser/Thr kinase inhibitor H-7 had no influence on the DTT-mediated decrease in aged-sAHP indicating that the reduction was not mediated through kinase activity.

In the case of K^+ channels, previous reports indicate cysteine specific oxidation decreases BK channel activity (Tang et al., 2001; Tang et al., 2004), and that the reducing agent DTT increases BK channel activity (DiChiara and Reinhart, 1997). The BK channel is involved in repolarization of action potential, and an increase in BK channel activity will reduce the width of the action potential (Shao et al., 1999). Moreover, a decrease in the spike width can decrease the sAHP, by limiting the duration of depolarization-induced Ca^{2+} entry through L-type VGCCs (Giese et al., 1998; Murphy et al., 2004). Thus, DTT could be acting on the BK channels to decrease

L-type VGCC activity and the sAHP amplitude. However several pieces of evidence suggest that this might not be the case. First, blockade of BK channels with paxilline did not block the DTT-mediated decrease in aged-sAHP. Second, blockade of VGCC's with nifedipine did not influence the DTT-mediated decrease in aged-sAHP. Finally, the fast AHP, which is mediated by the BK channel, is not altered with age (Matthews et al., 2009)

The amplitude of the sAHP is dependent on the level of cytosolic Ca^{2+}. L-type VGCCs play a role in determining the amplitude of the sAHP (Landfield and Pitler, 1984; Moyer et al., 1992; Norris et al., 1998a) and contribute to the increase in the sAHP during aging (Thibault and Landfield, 1996; Veng and Browning, 2002). However, it does not appear that the DTT-mediated reduction in the aged-sAHP is acting through L-channels. The DTT-mediated reduction in the sAHP was larger than that observed for L-channel blockade, and was specific to aged animals. Previous research indicates that the decrease in the AHP following blockade of L-channels is quantitatively larger in aged animals; however, the percent decrease (~30%) is similar across ages, suggesting other mechanisms contribute to the age-related increase in the AHP animals (Power et al., 2002; Disterhoft et al., 2004). In the current study, blockade of the L-channel reduced the aged-sAHP ~30%, consistent with previous reports (Power et al., 2002). Regardless of L-channel function, DTT reduced the aged-sAHP by ~50% and the effect of DTT was specific to aged animals. The results suggest that DTT is acting on mechanisms other than the L-channel, which may mediate the age-related increase in the sAHP.

Release of Ca^{2+} from ICS, through RyR activation, plays a role in determining the sAHP amplitude (Sah and McLachlan, 1991; Usachev et al., 1993; Davies et al., 1996; Borde et al., 2000; van de Vrede et al., 2007). Ca^{2+} from ICS contributes to altered physiology during aging (Kumar and Foster, 2004, 2005; Gant et al., 2006). In addition, the RyRs are highly redox sensitive (Bull et al., 2008), such that oxidation of the cysteine residues increases the Ca^{2+} sensitivity and activity of RyR (Eager and Dulhunty, 1998; Hidalgo et al., 2004; Huddleston et al., 2008). In the current study, the DTT-mediated decrease in the sAHP was blocked upon depletion of ICS by thapsigargin or blockade of RyRs by ryanodine indicating the involvement of ICS and RyRs in the decreased Ca^{2+} mobilization by DTT application. Together the results suggest that the increase in the sAHP in aged neurons is related to redox sensitive Ca^{2+} mobilization from the ICS through the RyRs. Interestingly, decreased sAHP is observed in aged memory-unimpaired and young rats but not in aged memory-impaired rats (Moyer et al., 2000; Tombaugh et al., 2005; Murphy et al., 2006; Matthews et al., 2009). It is interesting to speculate that the decrease in the sAHP of memory unimpaired animals may result from a shift in redox state associated with learning (Shvets-Teneta-Gurii et al., 2007). Alternatively, treatments that modify intracellular redox state may provide a novel therapeutic strategy to restore Ca^{2+} homeostasis in the aged neurons.

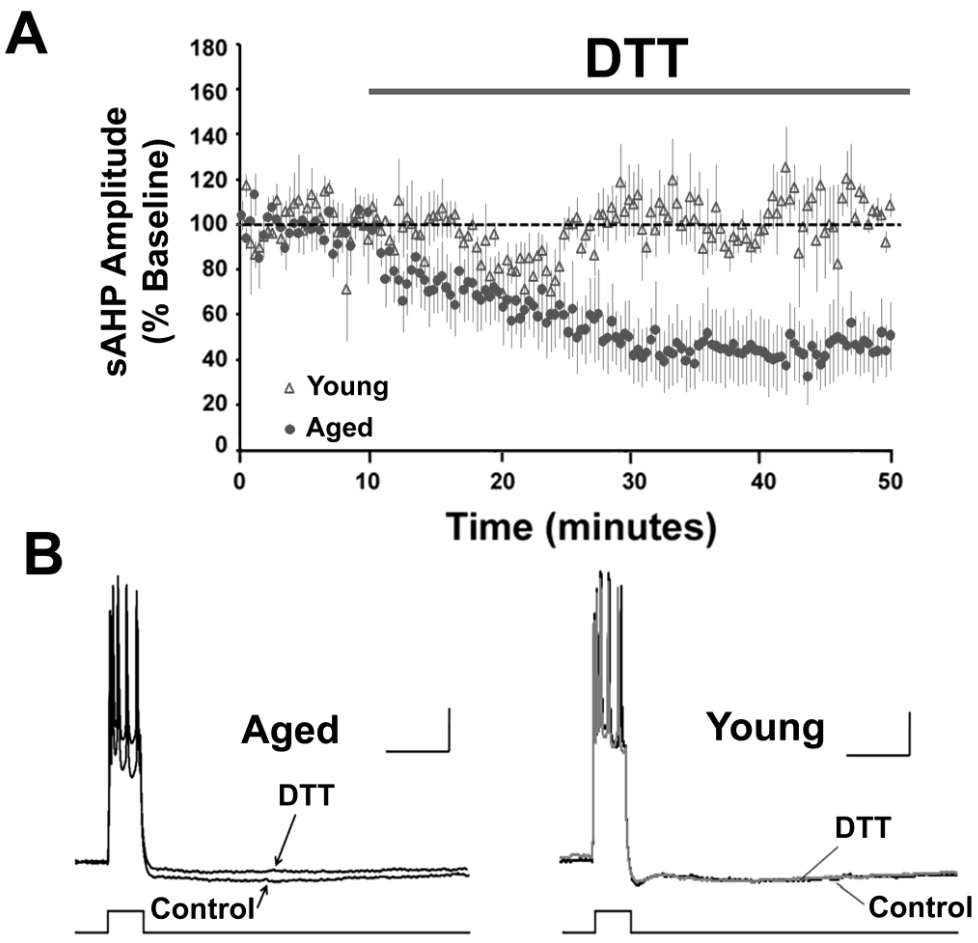

Figure 5-1. Age-dependent reduction in the sAHP by DTT. (A) Time course of the change in the normalized sAHP amplitude in the aged (filled circles) (n = 5) and young (open triangles) animals (n=3), following application of DTT for 40 minutes. (B) Representative traces illustrating the change in the AHP of aged (left) and young (right) animals under control conditions and at the end of a 40 minute application of DTT. The line beneath the traces indicates the onset and offset of the step current used to elicit a train of 5 action potentials. Calibration bars: 200 ms, 10 mV.

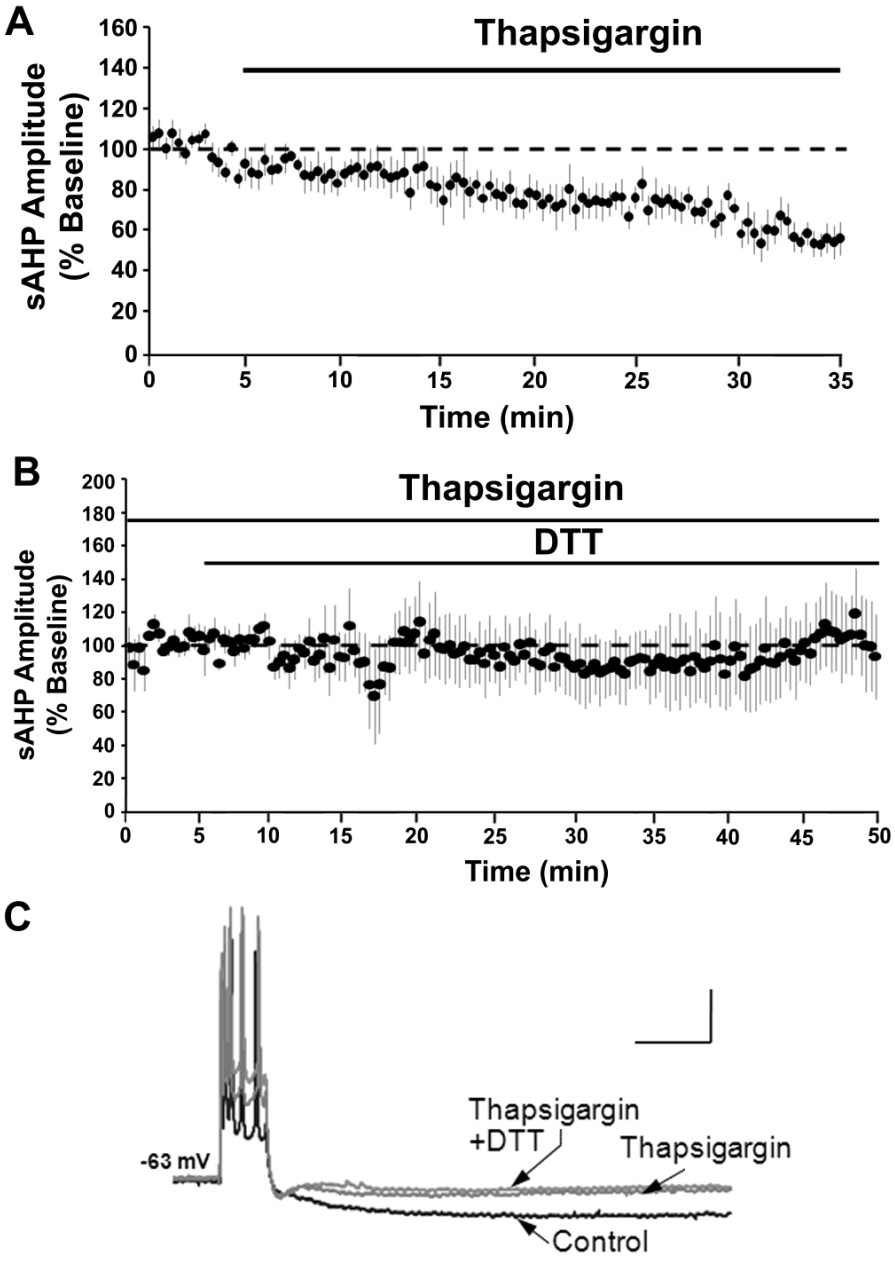

Figure 5-2. Intracellular calcium stores underlie DTT-mediated decrease in aged-sAHP. (A) Time course of the change in the normalized sAHP amplitude in the aged animals that were incubated with thapsigargin (n = 7). (B) Time course of change in the normalized sAHP amplitude in cells (n = 4) incubated with thapsigargin prior to and during DTT application. (C) Representative traces illustrating the AHP of aged animals under control condition (black trace), and at the end of a 40 minute application of thapsigargin (gray trace) and at the end of 50 min application of thapsigargin+DTT (gray trace). Calibration bars: 200 ms, 10 mV.

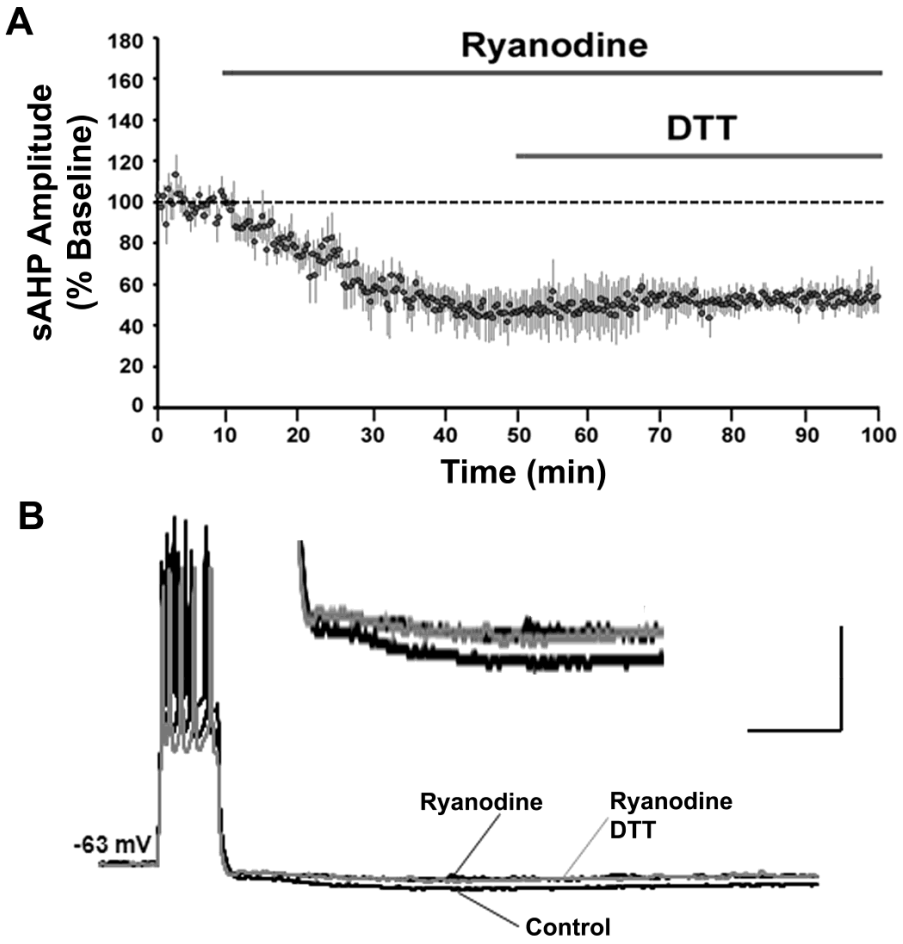

Figure 5-3. RyR blockade inhibits DTT mediated decrease in aged-sAHP. (A) Time course of the change in the normalized sAHP amplitude recorded from aged animals that were incubated with the ryanodine receptor antagonist ryanodine (n = 4) prior to and during application of DTT application. (B) Representative traces illustrating the change in the AHP of aged animals under control condition (black trace), at the end of a 40 minute application of ryanodine (black trace), and at the end of 50 min application of ryanodine+DTT (gray trace). Calibration bars: 200 ms, 20 mV. Inset: Magnified representation of change in the aged AHP under control condition, ryanodine, and ryanodine + DTT.

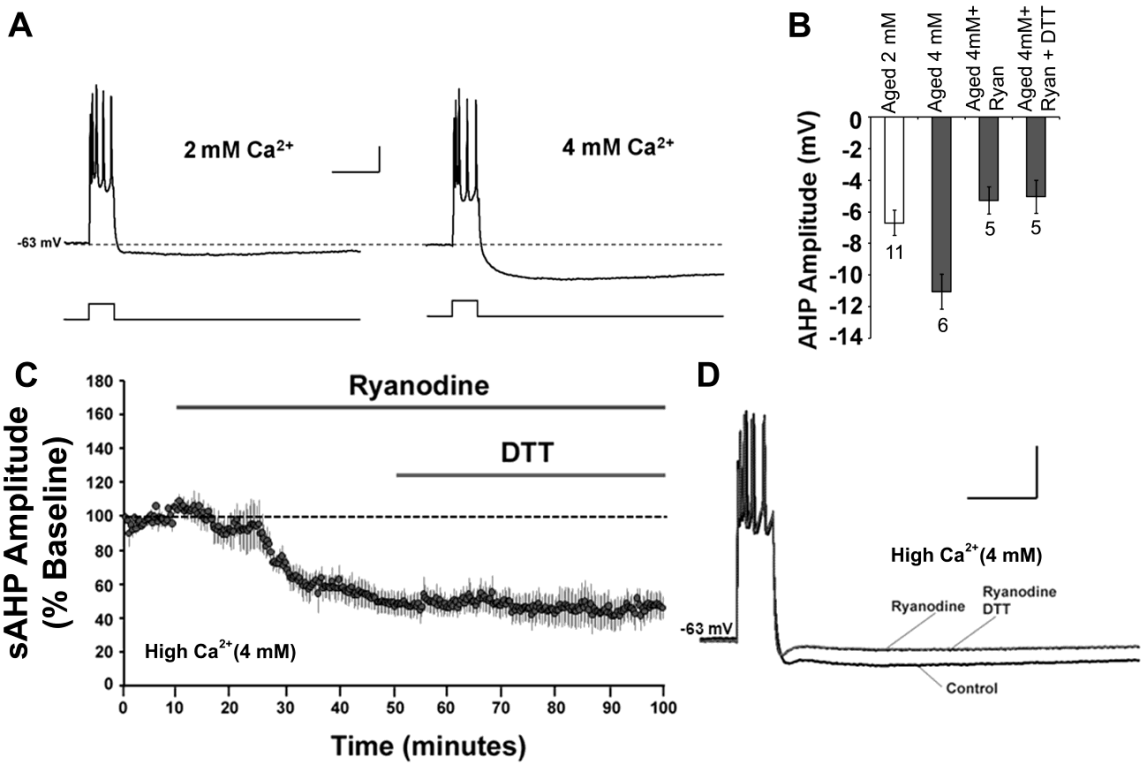

Figure 5-4. RyR blockade inhibits the DTT-mediated decrease in aged-sAHP when the AHP is increased by increasing calcium in the recording medium. (A) Representative traces illustrating the AHP from aged neurons recorded under conditions of 2 mM or 4 mM Ca^{2+}in the ACSF. Calibration bars: 200 ms, 10 mV. (B) Quantification of the mean sAHP amplitude in aged neurons recorded under 2 mM Ca^{2+} (n = 11, open bar), under 4 mM Ca^{2+} (n = 6), under 4 mM Ca^{2+} with ryanodine (n = 5), and under 4 mM Ca^{2+} with ryanodine + DTT (n = 5); all values under 4 mM Ca^{2+} are represented as filled bars. (C) Time course of the change in the normalized sAHP amplitude recorded in 4 mM ACSF from aged animals and incubated with ryanodine (n = 5) prior to and during DTT application. (D) Representative traces illustrating the AHP of aged animals under control condition, and at the end of a 40 minute application of ryanodine and at the end of 50 min application of ryanodine + DTT. Calibration bars: 200 ms, 20 mV.

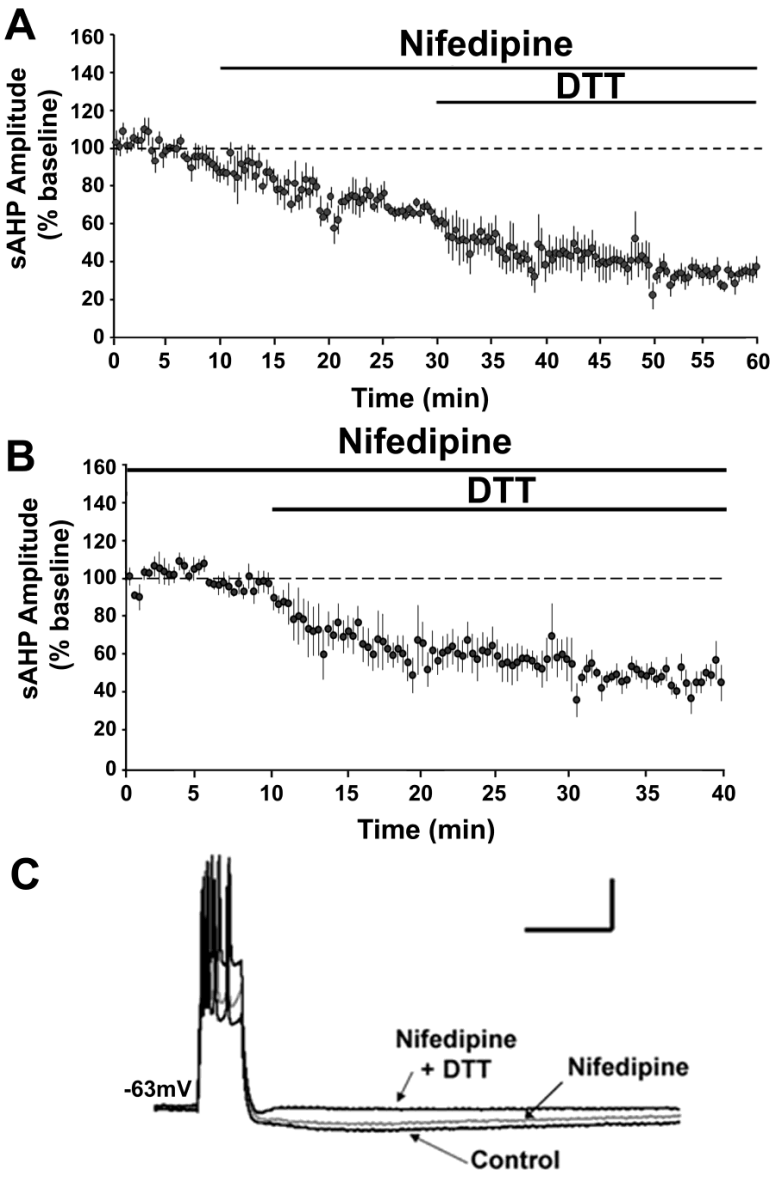

Figure 5-5. DTT mediated decrease is independent of L-type calcium channel function. A) Time course of the change in the normalized sAHP amplitude in the aged animals (filled circles) that were incubated with nifedipine (n = 4). B) Time course of the change in the normalized sAHP amplitude in the aged animals (filled circles) that were incubated with nifedipine (n = 6) for at least 45 min prior to the application of DTT for 30 min. Calibration bars: 200 ms, 10 mV. C) Representative traces illustrating the change in the AHP of aged animals under control condition (black trace), and at the end of a 20 min application of Nifedipine (gray trace) and at the end of 30 min application of Nifedipine + DTT (black trace).

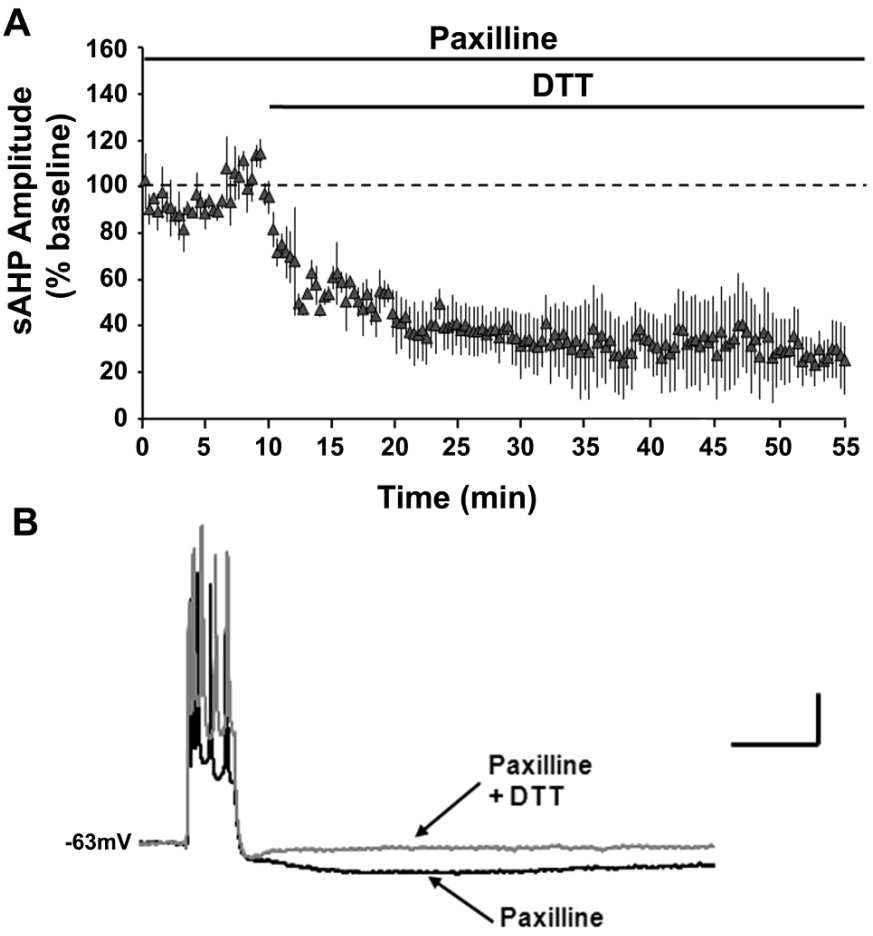

Figure 5-6. DTT effects on aged-sAHP are independent of BK channel function (A) Time course of the change in the normalized sAHP amplitude in the aged animals (filled triangles) that were incubated with paxilline (n = 3) for at least 60 min prior to DTT application. (B) Representative traces illustrating the change in the AHP of aged animals under paxilline (black trace), and at the end of 45 min under paxilline+DTT (gray trace). Calibration bars: 200 ms, 10 mV.

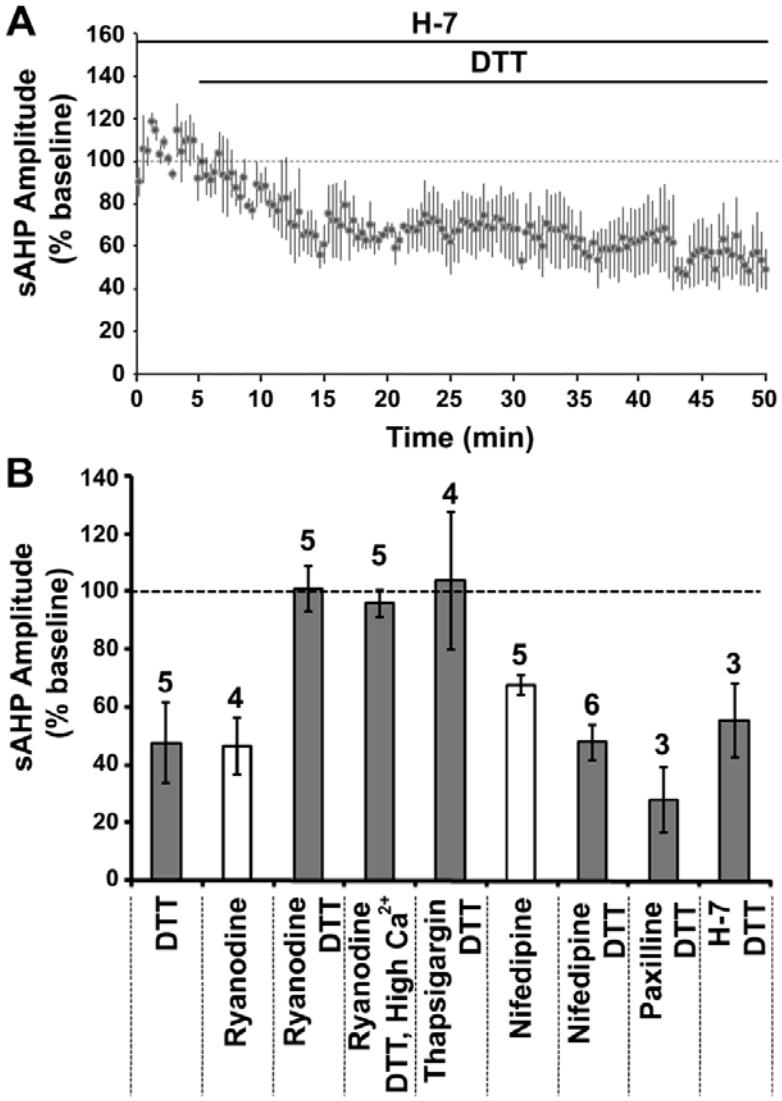

Figure 5-7. Ser/Thr kinase activity does not mediate DTT effects on aged-sAHP. (A) Time course of the change in the normalized sAHP amplitude in the aged animals that were incubated with the broad spectrum Ser/Thr kinase inhibitor H-7 (n = 3) for at least 60 min prior to DTT application. (B) Summary diagram representing the mean percent change in the sAHP amplitude of aged neurons following DTT application under various conditions. In each case, the response represents the percent change relative to the pre-DTT application baseline (dashed line). The effect of nifedipine or ryanodine (open bars) on the pre-drug baseline is presented for comparison. The numbers above each bar represents the number of neurons recorded in each condition

Table 5-1. Physiological properties of CA1 neurons from young and aged animals

	IR (MΩ)	RMP (mV)	Sp Amp (mV)
Young (n = 12)	37.0 ± 2.5	-62.8 ± 1.5	80.6 ± 1.9
Aged (n= 40)	37.8 ± 1.1	-61.9 ± 0.6	82.8 ± 0.7

The values for input resistance (IR), resting membrane potential (RMP), and spike amplitude (Sp Amp) are indicated as mean ± S.E.M. The values of holding current (HC) is presented as a range. The number in parentheses indicates the number of cells from the young and aged animals.

CHAPTER 6
CONCLUSION AND FUTURE DIRECTIONS

Conclusion

While numerous mechanisms could contribute to dysfunctional hippocampal synaptic transmission during aging, we were primarily interested in the role of increased oxidative stress and oxidative redox state in mediating NMDAR hypofunction, altered synaptic plasticity, increased sAHP, and altered Ca^{2+} homeostasis. The results presented in Chapter 3 describe the age-related changes in the baseline NMDAR mediated synaptic transmission, and indicate how these changes are linked to the redox state of the aged neurons. Briefly, measurement of the NMDAR-fEPSP amplitude in the young and aged neurons, and comparison across 0.4 mV bins of PFV amplitude, indicated a significant age-related decrease in the NMDAR function. The use of oxidizing agent X/XO decreased NMDAR function in the young but not in the aged neurons; and the use of reducing agent DTT, increased the NMDAR function in aged but not in the young animals. The effect of X/XO was washed out in young animals, even under higher concentrations of X/XO, indicating a transient effect on young NMDAR function. This effect could possibly be due to a robust antioxidant capacity or better redox buffering capacity in the young neurons. In contrast, the DTT effect in aged animals could not be washed out even 45 minutes after switching-off DTT application. This observation predicted secondary signaling mechanisms underlying the DTT-mediated increase in NMDAR function. Quite remarkably, this prediction was accurate and the data presented in Chapter 4 delineates the signaling mechanism underlying DTT-mediated increase in NMDAR function of aged animals.

Results presented in Chapter 4 describe the age-related increase in the rate of ROS production in the hippocampal slices. The use of dye-based ROS detection technique in live hippocampal neurons suggested that the rate of oxyradical production is higher in the aged neurons, when compared to young neurons. Moreover the use of membrane impermeable antioxidant enzymes (SOD and catalase) in the extracellular solution did not affect the dye-based fluorescence, suggesting that the source of oxyradical production is intracellular. Furthermore, in Chapter 4, the use of kinase and phosphatase inhibitors localized the specific effects of DTT to CaMKII, a Ca^{2+} sensitive Ser/Thr kinase that participates in regulating the NMDAR function and is also redox sensitive. First, physiological studies indicated that the DTT effect on aged NMDAR function was blocked by CaMKII inhibitors KN-62 and myr-AIP. Second, biochemical assays suggested that DTT increases CaMKII activity in aged but not in young CA1 cytosolic extracts of hippocampal neurons. In contrast no effect of phosphatase inhibition was observed on the DTT-mediated increase in NMDAR function. Neither the inhibition of CaN with FK-506 nor the inhibition of PP1 with OA had any effect on the DTT-mediated increase in aged NMDAR function. Finally, DTT treatment increased LTP in aged neurons following a single episode of 100 Hz stimulation. Taken together the data presented in Chapter 4 is indicative of the link between oxidative redox state and oxidized CaMKII signaling pathways in the aged neurons, which ultimately contributes to the decreased NMDAR function observed in Chapter 3, and weakened hippocampal function during aging. This complex relationship is summarized by the schematic in Fig. 6-1.

NMDARs in aged neurons can be subject to numerous post-translational changes that could mediate the observed deficit in activation. Results provided in Chapters 3 and 4 suggest a role for oxidative redox state. However numerous alternative mechanisms could mediate deficit in NMDAR function. Altered mRNA and protein expression of specific NMDAR subunits is proposed as a potential mechanism for the observed decrease in NMDAR function (Magnusson, 2000). Significant decreases have been observed in the expression of NR1 protein (Eckles-Smith et al., 2000; Mesches et al., 2004; Liu et al., 2008) and NR1 mRNA (Adams et al., 2001) in aged hippocampus. In contrast, other studies report no age-related decrease in NR1 protein expression in the whole hippocampus (Sonntag et al., 2000; Zhao et al., 2009). These studies point to a lack of congruent changes in hippocampal NR1 subunit expression. Some studies indicate age-related changes in the modulatory NR2 subunits. A decrease in the NR2A protein expression has been observed in the hippocampus (Sonntag et al., 2000; Liu et al., 2008), which is not observed in the frontal cortex (Sonntag et al., 2000). Furthermore, NR2A mRNA expression was reported to decline in the ventral hippocampus (Adams et al., 2001). In contrast, other studies report no significant change in the NR2A protein expression levels in the hippocampus and cortex (Sonntag et al., 2000; Martinez Villayandre et al., 2004). Age-related changes have also been reported for NR2B subunit of the NMDAR; in particular the expression of NR2B protein (Mesches et al., 2004; Zhao et al., 2009) and NR2B mRNA (Adams et al., 2001; Magnusson, 2001) declines in the aged hippocampus. This effect may be region specific since a decline in NR2B protein is not observed in the frontal cortex (Sonntag et

al., 2000). In contrast, NR2B mRNA decreases in the frontal cortices of aging macaque monkeys, but not in the hippocampus (Bai et al., 2004).

In conclusion, a lack of a clear model describing mRNA and protein changes of various NMDAR subunits in aged hippocampus gives rise to another alternative mechanism for reduced NMDAR activation in these neurons. The other possible mechanism is that alterations in the NMDAR localization, through the insertion of receptors into the membrane or recruitment of extra-synaptic receptors into the synapse, may have important effects on NMDAR function during aging. It has been suggested that NR2B containing receptors may be more prevalent at extra-synaptic sites (Massey et al., 2004), before being internalized into the cytoplasm (Blanpied et al., 2002; Lau and Zukin, 2007). A decrease in the NR2B protein expression in the synaptic membrane fraction, but not in whole homogenates (Zhao et al., 2009) suggests an age-related sequestration of NR2B in the extra-synaptic sites. Recent work indicates that extra-synaptic NMDARs couple to different signaling cascades, and initiate mechanisms that oppose synaptic potentiation, by shutting off the activity of cAMP response element binding protein and decreasing expression of brain-derived neurotropic factor (Hardingham et al., 2002; Vanhoutte and Bading, 2003). However, it remains to be determined whether altered localization of the NMDARs (specifically extra-synaptic localization) is the mechanism by which the NMDAR function declines during senescence.

The other likely candidate mechanism for regulating NMDAR function during aging is posttranslational modification of the receptor and/or its associated signaling cascades (investigated in Chapters 3 and 4). NMDAR function can be altered by the

oxidation/reduction of sulfhydryl moieties on their structure. Previous research demonstrates that oxidizing agents like 5,5′-dithiobis(2-nitrobenzoic acid) (Aizenman et al., 1989), hydroxyl radicals generated by xanthine / xanthine oxidase (Aizenman, 1995) and oxidized glutathione (Sucher and Lipton, 1991) decrease NMDAR function in the neuronal cell cultures. The decrease in NMDAR function under oxidizing conditions is thought to result from the formation of disulfide bonds on the sulfhydryl group containing amino acid residues in NMDARs (Aizenman et al., 1990; Sullivan et al., 1994; Choi et al., 2001); specifically the cysteine residues are more susceptible to oxidation over the methionine residues (Shacter, 2000). The aging brain is associated with an increase in the levels of oxidative stress and/or a decrease in redox buffering capacity, which contributes to a shift in the redox state favoring an oxidative state (Foster, 2006; Poon et al., 2006; Parihar et al., 2008). Thus conditions during aging should promote a decrease in NMDAR function. Another candidate mechanism associated with posttranslational modification is altered phosphorylation state of the receptor. In particular, the function of the NMDAR is influenced by its phosphorylation state. Activation of the tyrosine kinase (Wang et al., 1994; Heidinger et al., 2002), protein kinase C (Ben-Ari et al., 1992; Chen and Huang, 1992) and protein kinase A (Raman et al., 1996) increases NMDAR mediated currents. In contrast, protein phosphatases, including calcineurin and protein phosphatase 1, decrease NMDAR currents (Lieberman and Mody, 1994; Wang et al., 1994; Raman et al., 1996). Interestingly, Ser/Thr kinases promote NMDAR trafficking from endoplasmic reticulum and insertion into the postsynaptic membrane (Scott et al., 2001; Carroll and Zukin, 2002), while phosphatases promote internalization of NMDARs into the cytoplasm (Snyder et al.,

2005). Thus, the kinases and phosphatases act like molecular switches which increase or decrease NMDAR function, respectively. During aging there is a shift in the balance of kinase/phosphatase activity, favoring an increase in the phosphatase activity (Norris et al., 1998b; Foster et al., 2001; Foster, 2007). Thus alterations in the phosphorylation state of the NMDARs could mediate the decreased NMDAR activation in aged neurons (Coultrap et al., 2008).

In this study we have presented evidence that suggests that age-related increase in oxidative stress or oxidative redox state contributes to the decrease in NMDAR function. Alternatively it is also possible that increased nitrosative stress affects NMDAR function. In the hippocampal neurons, nitric oxide is produced by neuronal nitric oxide synthase (nNOS), which is activated upon stimulation of NMDARs. In memory impaired, aged F344 rats there is no loss of NOS containing neurons, rather a decreased production of NO (Meyer et al., 1998), probably due to decreased activation of NMDARs themselves (Barnes et al., 1997; Billard and Rouaud, 2007; Bodhinathan et al., 2010). Subsequently, nitric oxide reacts with superoxide to produce peroxynitrite, a potent oxidant and nitrating agent (Squadrito and Pryor, 1998), which has been reported to decrease NMDAR function (Lipton and Stamler, 1994; Lipton et al., 1998); and proposed to inhibit NMDAR-dependent LTP (Wang et al., 2004). The nitrosative stress mediated decrease in NMDAR function is thought to occur by S-nitrosylation of the receptor (Lipton and Stamler, 1994; Choi et al., 2000; Takahashi et al., 2007). Thus, excess amounts of superoxide plays an important role in mediating the effects of nitrosative stress, by contributing to the production of the peroxynitrite, which leads to S-nitrosylation of the NMDARs. Although we cannot completely discount the effect of

nitrosative stress on age-related decrease in NMDAR function, it is likely that the DTT mediated increase in NMDAR function does not involve the removal of S-nitrosyl groups on NMDARs.

The results presented in Chapter 5 describe the link between a more oxidized redox state and increased sAHP during aging. Increased sAHP is a physiological marker of aging, which makes it harder for the CA1 pyramidal neurons to reach the threshold for action potential firing. Our results indicate that application of the reducing agent DTT significantly decreases the sAHP in aged but not in young CA1 pyramidal neurons. Moreover, the DTT-meditated decrease in sAHP amplitude involves decreased Ca^{2+} mobilization from the ICS through a decrease in RyR function. Although the L-type VGCC's and the BK channels contain redox sensitive cysteine residues, they do not contribute to the DTT-mediated decrease in aged-sAHP. Furthermore, DTT's effects are mediated by a direct reducing action on the cysteine residues of RyR, and not due to redox modulation of Ser/Thr kinases which are known to regulate the function of RyRs. In summary, our results suggest that during aging there is enhanced Ca^{2+} mobilization from the ICS through the RyRs, which leads to increased activation of the Ca^{2+} dependent K^{+} current that underlies sAHP. The age-related increase in RyR function does not appear to be due to increased RyR expression (Martini et al., 1994), rather an oxidative stress induced shift in the intracellular redox state may enhance the responsiveness of RyRs during aging (Hidalgo et al., 2004; Bull et al., 2007; Gokulrangan et al., 2007). Hence posttranslational changes (primarily redox modification) of the RyRs are thought to underlie age-related increase in sAHP. Previous reports indicate that RyR contributes negatively to the induction of LTP and

interferes with normal spatial learning (Futatsugi et al., 1999). In fact blockade of RyRs using ryanodine ameliorates hippocampal markers of aging (Gant et al., 2006). Thus our model indicates that decreasing RyR function in aged hippocampal neurons, by shifting the redox environment to a more reductive state would enhance LTP and memory (as indicated in chapter 3 and 4), and decrease the sAHP amplitude (as demonstrated in Chapter 5).

Therapeutic Potential of the Current Study

The fact that "functional lesion" of the hippocampus during normal aging can be reversed to a certain extent, is an exciting starting point for the development of therapeutic strategies aimed to treat memory loss and cognitive dysfunction. The therapeutic potential of the current study is highlighted in the following points-

1). normal memory function could be restored in aging brains by reversing the subtle physiological, biochemical and posttranslational changes to the neurons.

2). the ease of using antioxidant or pill-based therapeutics far outweighs the complexity of the therapeutics based on cell replacement strategies. Therapeutics based on stem cell would be more suited to treat neurodegenerative disorders that are characterized by neuron loss; as opposed to treating memory dysfunction during normal aging that is characterized by posttranslational changes like oxidation/reduction. In fact, antioxidants (Socci et al., 1995; Cotman et al., 2002; Zhang et al., 2007; Li et al., 2009) and antioxidant mimetics (Stoll et al., 1993; Liu et al., 2003) have been reported to ameliorate age-related learning and memory deficits by reducing oxidative damage. Antioxidants are also effective in improving spatial memory in rats following brain injury (Long et al., 1996; Koda et al., 2008) pointing at a general protective role for antioxidants in learning and memory.

3). the possibility of a non-genetic approach for the treatment of memory loss during aging eschews the complexities of gene based therapeutics.

The results presented here indicate that a majority of neurons lose their function during aging; however it is still possible that some neurons retain normal functionality in the aged brain. If we assume that the normal functionality of these aged neurons derives from proper functioning of certain mechanisms, then the identification of such mechanisms is critical to treating cognitive impairment and memory dysfunction. The strategy to prevent a normal neuron from becoming dysfunctional can be achieved by creating suitable “therapeutic barriers” (Fig. 6-2). In this context, therapeutic barriers could be strategies that keep intracellular Ca^{2+} concentrations within tolerable levels or strategies that decrease oxidative damage to neurons like spin trap agents that scavenge ROS, nutritional supplements, regular exercise, or caloric restriction. In the provided conceptual framework (Fig. 6-2) integrated Ca^{2+} levels is considered as the biomarker upon which a hypothetical therapeutic barrier could be applied. As an extension of this idea, potentially any age-related biomarker, that marks the transition of the neurons from a functional to a dysfunctional stage, could be subjected to a tailor made therapeutic barrier. Based on the findings of the studies presented here, the function of the NMDARs and/or the amplitude of sAHP of the CA1 pyramidal neurons could be a biomarker for cognitive impairment and memory decline; and the therapeutic barrier could be strategies that prevent an oxidative redox state or increased oxidative stress.

Future Directions

Understanding the age-related changes in the Ca^{2+} handling mechanisms of the neurons is critical to the development of therapeutics aimed to ameliorate cognitive

dysfunction and memory loss. Amongst the key players that maintain Ca^{2+} homeostasis in the neurons (Fig. 6-3), NMDARs stand out for their significant role in synaptic plasticity, learning and memory. The findings presented in this dissertation delineate the biochemical and physiological changes to the NMDARs during aging. Debate surrounding NMDAR function during aging and neurodegeneration are at the heart of developing suitable therapeutics that reverse the biochemical and physiological changes and reinstate normal function to dysfunctional aged neurons. One of the important goals for future research is to distinguish between situations that demand an increase in NMDAR function (like improving neuronal function) versus situations that demand a decrease in NMDAR function (like preventing neuron death). The decrease in the NMDAR function of aged neurons might represent a compensatory neuroprotective mechanism associated with inappropriate receptor activity. It is well documented that NMDAR associated Ca^{2+} influx triggers neurotoxicity and activates cell death programs in neurons (Chen et al., 1992; Lei et al., 1992; Pivovarova et al., 2004). Thus aged neurons could progressively down regulate neurotoxicity-associated NMDAR activation for purposes of cell preservation (Foster, 1999). However one of the consequences of down regulating NMDAR function is impaired NMDAR-dependent synaptic plasticity and memory. Thus treatment strategies that deal with NMDARs in aged neurons have to reconcile the opposing features of “functional rescue” by NMDAR activation and over expression, with “neuroprotection” by NMDAR blockade and down regulation.

Interestingly, over expression of NR2B subunit improves synaptic plasticity and memory in aged mice (Cao et al., 2007). However, NMDAR blockade by memantine improves cognition and synaptic plasticity (Barnes et al., 1996; Norris and Foster, 1999;

Pieta Dias et al., 2007), possibly by blocking inappropriate NMDAR activation (Rosi et al., 2006; Matute, 2007; Chang and Gold, 2008); thus indicating a “functional rescue”. In the case of neurodegenerative disease, decreased expression of NR1 mRNA has been observed in brain regions that are most at risk for cell death, including Huntington's disease, wherein a decrease in NR1 mRNA expression is observed in the neostriatum (Arzberger et al., 1997). Furthermore, there is evidence for decreased NMDAR expression in the hippocampus during the early stages of Alzheimer’s disease (Mishizen-Eberz et al., 2004; Jacob et al., 2007), hinting at “neuroprotection” mechanisms employed by those neurons. Thus, it will be important for future research to determine whether enhancing or inhibiting NMDAR function will be beneficial in preserving hippocampus dependent learning and memory function during normal aging and in the face of neurodegenerative disease. A sound strategy would be to find a balance between the degree of functional rescue and the extent of neuroprotection needed for successful aging and preserved neuronal function.

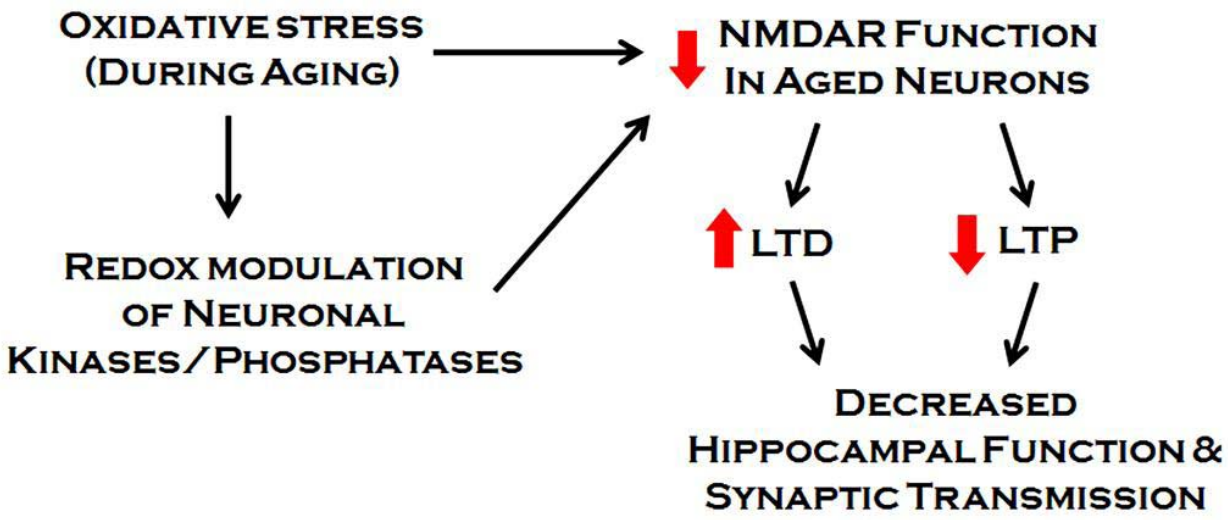

Figure 6-1. The biochemical model of brain aging and hippocampal dysfunction. The proposed model linking increased oxidative stress and decreased NMDA receptor activity during normal aging [either directly or indirectly through kinases and phosphatases]. The outcome would be enhanced LTD and impaired LTP for neural activity occurring at the modification threshold. If the proposed model turns out to be true, it could explain the age related weakening of synaptic connections in the hippocampus

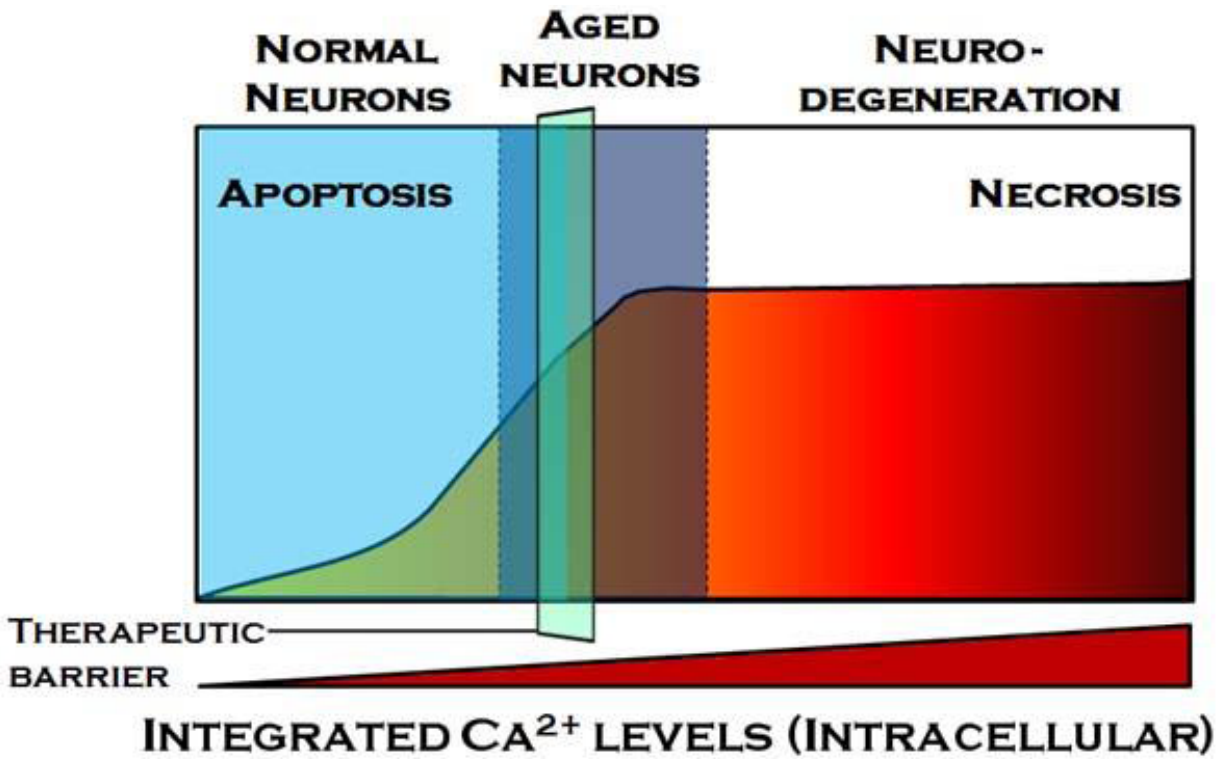

Figure 6-2. Conceptual framework for age-related neuronal dysfunction based on intracellular calcium levels. Integrated Ca^{2+} levels within the neurons can be used as an effective marker for differentiating the events associated with normal aging and neurodegeneration. The integrated Ca^{2+} levels follow a sigmoid pattern of increase as neurons transition from normal (light blue) to aged category (dark blue). Apoptosis can be observed at these stages. Sustained increase in integrated Ca^{2+} levels activates neurotoxic pathways and leads to necrosis or neuron death observed in neurodegeneration (darker shades of red). From a therapeutic standpoint, a “therapeutic barrier” could be erected, in the form of nutritional or therapeutic intervention, which will potentially prevent the normal neurons from exhibiting the intracellular Ca^{2+} profile of aged neurons. This crossover is proposed to precede neuronal dysfunction and ultimately memory dysfunction.

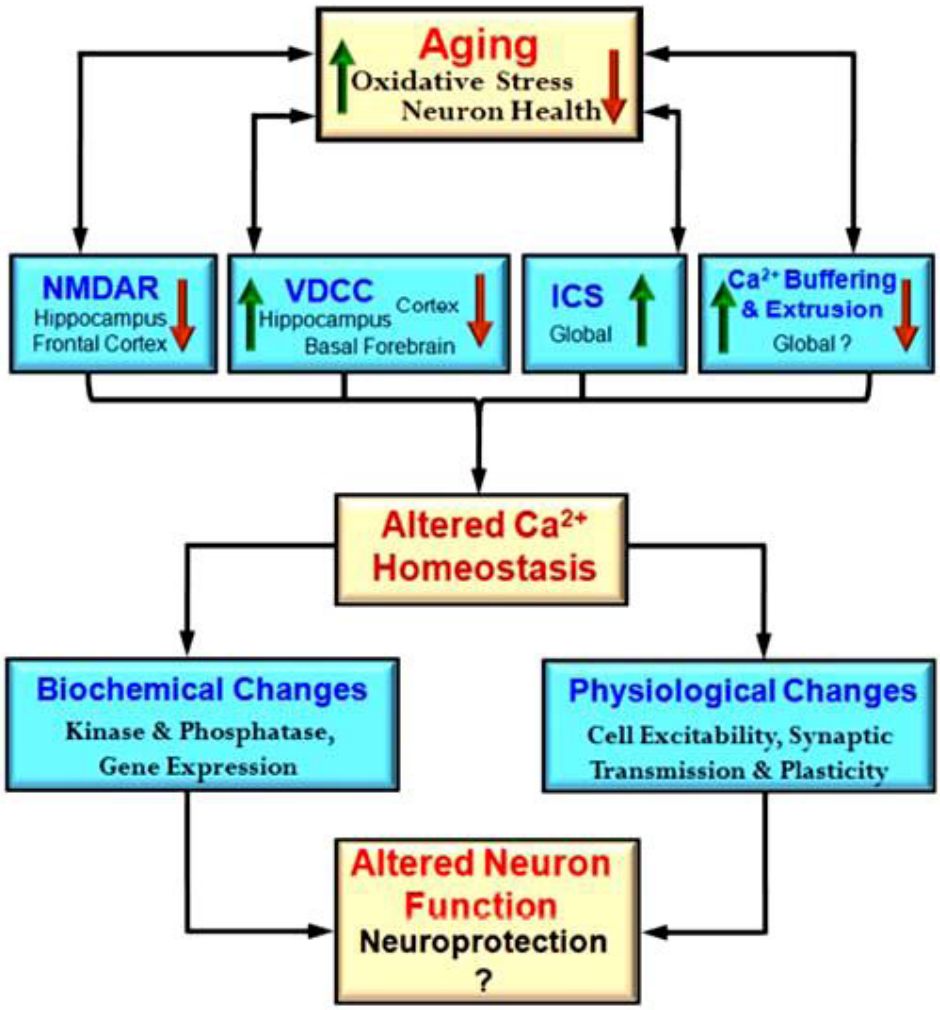

Figure 6-3. Integrative model of the impact of aging on the calcium handling mechanisms and physiological processes. During aging there is an interaction between increased oxidative stress and decreased neuron health with mechanisms for Ca^{2+} regulation that includes the NMDA receptors (NMDAR), voltage-dependent Ca^{2+} channels (VDCC), intracellular Ca^{2+} stores (ICS), and Ca^{2+} buffering and extrusion mechanisms. An indication of regional specificity (hippocampus, frontal cortex, cortex, basal forebrain) and the direction of change (increase – red arrow and decrease – green arrow) for each mechanism are also provided. The shift in Ca2+ homeostatic mechanisms may represent neuroprotective mechanisms to decrease further rise in intracellular Ca^{2+} by decreasing neuron activity. These changes impair the function of the neuron (Adapted from Kumar A, Bodhinathan K, and Foster T C, Front Ag Neurosci 2009)

APPENDIX A
DRUGS, SOLUTUIONS, AND SUPPLIERS

AP-5 (DL-2-Amino-5-phosphonovaleric acid); NMDAR antagonist; Sigma, St. Louis, MO

ATP-Na^+ lyophilized salt (Adenosine-5'-triphosphate); phosphate donor in kinase reactions; CycLex Co Ltd, Nagano, Japan

Bis-I (bisindolylmaleimide-I); specific inhibitor of protein kinase C; Calbiochem, San Diego, CA

c-H_2DCFDA (5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate); fluorescent dye used to detect reactive oxygen species; Molecular Probes Inc, Eugene, OR

CaM (Calmodulin, purified from bovine brain); Ca^{2+} binding protein and co activator of CaM-Kinase II; CycLex Co Ltd, Nagano, Japan

CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II); Ser/Thr kinase; CycLex Co Ltd, Nagano, Japan

Catalase (purified from human erythrocytes); antioxidant enzyme - converts hydrogen peroxide to water; Sigma, St. Louis, MO

DMSO (dimethyl sulfoxide); non-aqueous solvent for various drugs; Sigma, St. Louis, MO

DNQX (6, 7-Dinitroquinoxaline-2, 3(1H, 4H)-dione); AMPAR antagonist; Sigma, St. Louis, MO

DTNB (5, 5′-Dithiobis (2-nitrobenzoic acid); oxidizing agent; Sigma, St. Louis, MO

DTT (Dithiothreitol); reducing agent; Sigma, St. Louis, MO

EGTA (Ethylene glycol-bis-(2-aminoethyl)-N, N, N', N'-tetraacetic acid); chelating agent; CycLex Co Ltd, Nagano, Japan

Ethanol; non-aqueous solvent for various drugs; Fisher Scientific, Pittsburgh, PA

FK-506; Calcineurin/Protein Phosphates 2B inhibitor; LC Laboratories, Woburn, MA

H-7 ((±)-1-(5-Isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride); broad spectrum Serine/Threonine kinase inhibitor; Tocris Bioscience, Ellisville, MO

HCl (Hydrochloric acid); general acid; Fisher Scientific, Pittsburgh, PA

HRP conjugated anti-phospho-Syntide-2 antibody; CycLex Co Ltd, Nagano, Japan

KN-62 (4-[(2S)-2-[(5-isoquinolinylsulfonyl) methylamino]-3-oxo- 3-(4-phenyl-1-piperazinyl) propyl] phenyl Isoquinolinesulfonic acid ester); specific CaMKII inhibitor; Tocris Bioscience, Ellisville, MO

L-Glutathione reduced form (L-GSH); biologically available reducing agent; Sigma, St. Louis, MO

Myr-AIP (myristoylated autocamtide-2 related inhibitory peptide); specific peptide inhibitor of CaMKII with the following sequence [Myr-N-Lys-Lys-Ala-Leu-Arg-Arg-Gln-Glu-Ala-Val-Asp-Ala-Leu-OH]; Calbiochem, San Diego, CA

Nifedipine; L-type Voltage-gated Ca^{2+} Channel Antagonist; Tocris Bioscience, Ellisville, MO

NaOH (Sodium Hydroxide); used to adjust the pH of solutions; Sigma, St. Louis, MO

OA (Okadaic acid); Protein Phosphatase 1 inhibitor; Tocris Bioscience, Ellisville, MO

Picrotoxin (PTX); $GABA_A$ receptor antagonist; Tocris Bioscience, Ellisville, MO

Ryanodine (RyR); Ryanodine receptor antagonist; Calbiochem, San Diego, CA

Superoxide Dismutase (SOD, from human erythrocytes); antioxidant enzyme – converts superoxide anion to hydrogen peroxide or oxygen; Sigma, St. Louis, MO

TMB (Tetra methyl-benzidine); chromogenic substrate for horseradish peroxidase; CycLex Co Ltd, Nagano, Japan

Xanthine (X); substrate for Xanthine Oxidase which react together to produce superoxide radicals; Calbiochem, San Diego, CA

Xanthine Oxidase (XO); an enzyme which reacts with Xanthine to produce superoxide radicals; Roche Diagnostics, Indianapolis, IN

APPENDIX B
DRUG CONCENTRATIONS USED IN THE EXPERIMENTS

AP-5 (100 μM)

ATP-Na^+ lyophilized salt (62.5 μM)

Bis-I (500 nM)

c-H_2DCFDA (10 μM)

Ca^{2+}/Calmodulin-dependent protein kinase II (15 to 30 mU)

Calmodulin (200 ng/mL)

Catalase (260 units/mL)

DMSO (final solvent concentration of < 0.01%)

DNQX (30 μM)

DTNB (500 μM)

DTT (700 μM)

EGTA (2 mM)

Ethanol (final solvent concentration of < 0.0001%)

FK-506 (10 μM)

H-7 (10 μM)

KN-62 (10 μM)

L-Glutathione reduced form (700 μM)

Myr-AIP (5 μM)

Nifedipine (10 μM)

Okadaic acid (1 μM)

Paxilline (10 μM)

Picrotoxin (10 μM)

Ryanodine (20 μM)

Superoxide Dismutase (121 units/mL)

Thapsigargin (1 μM)

Xanthine (20 μg/mL)

Xanthine Oxidase (0.25 to 1.00 units/mg of Xanthine)

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BIOGRAPHICAL SKETCH

Karthik was born in Madurai, India, to Rajeswari Bodhinathan and Bodhinathan Sundarapandian. Karthik's primary schooling was at the Air Force School, Bangalore, situated in the midst of India's scientific complex on Sir C. V. Raman road (named after the late Nobel Laureate Sir C. V. Raman). Karthik graduated from T.V.S. Lakshmi Matriculation Higher Secondary School in 2001. He then graduated "first-class honors" in 2005 with a Bachelor of Technology (Major: Biotechnology) from P.S.G. College of Technology in Coimbatore, affiliated with Anna University, one of India's eminent engineering universities. His initial scientific pursuits were shaped by a Summer Research Fellowship (2003 and 2004) awarded by The Jawaharlal Nehru Center for Advanced Scientific Research in Bangalore, India. Consequently, the fellowship helped him pursue his undergraduate research work in the lab of Dr. Saumitra Das at the Indian Institute of Science in the spring of 2005. Karthik was recruited by the Interdisciplinary Program for Biomedical Research at the University of Florida, in the fall of 2005, with the Alumni Fellowship. The exciting and unanswered questions of brain function and dysfunction led him, in the summer of 2006, to join the lab of Dr. Thomas C. Foster, the McKnight Chair for Research on Aging and Memory in the Department of Neuroscience at the University of Florida.