THE ANTITHETICAL EFFECTS OF MATRIX METALLOPROTEINASE INHIBITION ON HIPPOCAMPAL PLASTICITY BETWEEN YOUNG-ADULT AND AGED-ADULT RODENTS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of PETER CONKLIN MEIGHAN find it satisfactory and recommend that it be accepted.

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Abstract

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It is becoming increasingly evident that matrix metalloproteinases (MMPs), a family of zinc containing extracellular endopeptidases, participate in processes supporting hippocampal synaptic plasticity. Specifically, we have recently demonstrated that hippocampal MMPs are upregulated during acquisition of a spatial memory task, and inhibition of hippocampal MMPs is deleterious to both task acquisition and long-term potentiation (LTP). The purpose of this study was twofold: (1) to further our understanding of MMPs importance in hippocampal plasticity and (2) to gain a mechanistic insight as to the functional importance of MMP activity for hippocampal plasticity. Acute hippocampal slices were generated from 20-30 day old male Sprague Dawley rats. Following recovery, control and MMP inhibitor (FN-439 Calbiochem) treated slices were subjected to various stimulatory paradigms which produce either short term or long term modifications to synaptic efficacy. Slices exposed to FN-439 exhibited impairments in paired pulse facilitation and theta burst facilitation; furthermore, FN-439 treated slices failed to stabilize following induction for LTP and long-term depression (LTD). Given the capability of MMPs to alter the extracellular and pericellular environment by degradation of extracellular matrix molecules and cell adhesion molecules, we hypothesized that the effects of

FN-439 on LTP stabilization involves dysfunction to plasticity critical alterations to cell adhesion. To this end, we examined the temporal sensitivities of MMP inhibition by administering the inhibitor at various times following tetanization. Upon observing a similar temporal sensitivity pattern to that of integrin antagonist (GRGDSP Calbiochem), we predicted that FN-439 impacts LTP by interfering with integrin function. Consistent with this prediction, we observed that FN-439 competes for effect on LTP stabilization with the GRGDSP compound. Together, these data support a generalized role for MMPs for short term and long term hippocampal plasticity and that MMPs are a necessary facet of integrin mediated cell adhesion supporting LTP stabilization.

Results indicate that aged rats have markedly elevated levels of MMP-3 and that the ability to generate TBS induced LTP is limited. We hypothesized that excessive MMPs interferes with the normal remodeling processes that are requisite for the synaptic plasticity that accompanies learning and memory consolidation. To test this hypothesis, we first subjected hippocampal slices from aged-adult (20 months) Sprague-Dawley rats to a broad-spectrum MMP inhibitor prior to LTP testing. We observed that MMP inhibition enhanced LTP induction relative to untreated slices. This same treatment condition produced deficits in early-phase LTP maintenance in young-adult slices (3 months). Furthermore, we observed that exogenous application of catalytically active MMP-3 fragment to young-adult slices resulted in destabilization of LTP during early maintenance phase. These results suggest that age-related over-expression of hippocampal MMPs are deleterious to hippocampal plasticity.

TABLE OF CONTENTS

Page

LIST OF TABLES

APPENDIX C

LIST OF FIGURES

APPENDIX C

DEDICATION

I'd like to dedicate this work to my dad. Aside from being a wonderful dad, you are the smartest person I know. You are the one who taught me that asking questions and figuring things out can be fun. You are twice the scientist I'll ever be. Thanks for your help and support over the years. I love you dad.

GENERAL INTRODUCTION

Anatomical and Biochemical Substrates of Spatial Learning

In the laboratory, several distinct tasks can be used to assess spatial learning ability. The primary spatial used in these studies is the Morris water maze. The Morris water maze requires the animal to use primarily extra-maze cues to locate a small submerged pedestal within a large tank of water (Morris 1984). The water is sufficiently deep to force the animal to swim continuously until it locates the submerged pedestal. In this task the number of trials per day can vary from one to as many as 20 or 25 trials. The number of days of training can vary from 2 or 3 to a week or longer (McNamara and Skelton 1993). A significant advantage of using the Morris water maze, as opposed to other spatial tasks such as the radial mazes, is diminished confounding olfactory cues which are dissipated in the water in the Morris water maze, and does not rely on food deprivation to motivate the animals.

Readily retrievable explicit memories, such as those required for water maze navigation, require an intact hippocampus and medial temporal lobe (Cohen and Eichenbaum 1991; Squire 1992). The notion that the hippocampus plays an important role in spatial memory processing is supported by the observation that damage to the hippocampus results in an impaired ability to solve tasks that rely on spatial search strategies (Olton, Walker et al. 1978; Morris, Davis et al. 1990; Sutherland and McDonald 1990). Bilateral hippocampal lesions have also been shown to interfere with acquisition and/or retention of spatial memory in rats as measured by performance on the circular water maze task (Morris 1984; Rudy and Sutherland 1989; Stubley-Weatherly, Harding et al. 1996). Furthermore, hippocampal damage has been correlated with disruption of

spatial memory in a number of mammalian species including rat (Sutherland, Whishaw et al. 1982; Sutherland, Whishaw et al. 1983; Morris 1984; Nadel 1991; Jarrard 1993).

Although the precise mechanisms underlying long-term memory storage within the CNS are presently unknown, it is widely assumed that specific reconfigurations in the strength of synaptic connections are of paramount importance (Bailey and Kandel 1993; Bliss and Collingridge 1993; Doherty, Fazeli et al. 1995). One way in which synaptic efficacy is thought to be modulated is by alteration of dendritic spine morphology and density. Changes in spine density and shape are associated with many types of learning, including spatial (Moser, Trommald et al. 1997) and associative (Vozeh and Myslivecek 1996). In addition to demonstrating direct linkages between learning and synaptic remodeling, numerous additional studies have indirectly linked structural synaptic changes to learning by coupling changes to LTP. LTP, which is manifested as enhanced synaptic efficacy, is widely believed to represent a component of the cognitive process. With chemically induced LTP, the number of small spines increases (Doherty, Fazeli et al. 1995; Abel and Kandel 1998). Furthermore, the actual number of spine synapses per single axon was markedly increased following LTP (Toni, Buchs et al. 1999), thus, providing a plausible structural explanation for augmented synaptic efficiency. Spine density and shape can also be altered by application of glutamate receptor blockers again suggesting that the level of synaptic input is reflected by structural synaptic changes. Finally, it is noted that changes in spine morphology are accompanied by the redistribution of AMPA receptors which are required for sustained LTP and learning (Shi, Hayashi et al. 1999).

Proteinases and Plasticity

We hypothesize that MMPs effect pericellular proteolysis necessary for learning-based hippocampal remodeling to occur. The general idea that proteolysis plays a role in the plasticity of synapses was previously suggested. In 1984, evidence emerged that the *intracellular* protease calpain has an essential role in long-term synaptic changes (Lynch and Baudry 1984). Interestingly, the major substrates for calpains are elements of the dendritic cytoskeleton such as the spectrin-like molecule fodrin (Seubert, Baudry et al. 1987; Perlmutter, Siman et al. 1988) and cortactin (Huang, Tandon et al. 1997). Based on those observations it was hypothesized that proteolytic modification of neuronal morphology could contribute to synaptic plasticity. In subsequent years, interest turned toward calpain's role in neuropathological conditions (Lynch and Seubert 1989; Seubert, Nakagawa et al. 1989; Arai, Vanderklish et al. 1991; Lee, Frank et al. 1991). During this time, however, several reports emerged linking neurotransmitter receptor activity, LTP induction, NCAM modification to calpain-mediated proteolysis (Siman, Baudry et al. 1985; Seubert, Ivy et al. 1988; Staubli, Larson et al. 1988; Oliver, Baudry et al. 1989; Cerro, Larson et al. 1990; Cerro 1994; Vanderklish, Saido et al. 1995; Vanderklish, Bednarski et al. 1996). In recent experiments designed to investigate activity-dependant changes in adhesion molecules, Hoffman et al. demonstrated that activation of NMDA receptors within the hippocampus initiates a proteolytic degradation of adhesion molecules that is essential for the stabilization of LTP (Hoffman 1998). Hoffman et al., go on to demonstrate that NMDA receptor stimulation induces secretion of serine proteases, which suggests that activity-dependent proteolysis could be instrumental in architectural reconfiguration of active synapses.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of secreted and cell-surface endo-peptidases that are classified by their substrate specificity. MMPs degrade numerous pericellular molecules including other proteinases, latent growth factors, growth factor-binding proteins, cell surface receptors, cell-cell adhesion molecules, and nearly all extracellular matrix proteins (Sternlicht and Werb 2001). MMPs' catalytic activity is regulated on multiple levels, including: transcription, mRNA stability, secretion, activation, inhibition by endogenous inhibitors, and clearance. MMPs are secreted as an inactive zymogen which requires activation by proteolysis. The removal of the N-terminal "pro" domain of latent-MMP initiates MMPs' catalytic activity. Inactivation of MMPs is notably accomplished through sequestration by the endogenous tissue inhibitors of MMPs (TIMPs). To date, the TIMP family has only four TIMP molecules (TIMP1-4) – where each TIMP molecule is capable of inhibiting a distinct set of MMPs. MMP gene transcription is regulated by numerous promoting or suppressing factors. Stimuli which modulate MMP regulatory elements include integrin derived signals, specific ECM-adhesion molecule interactions, cell stress, and alterations of cellular morphology.

As their name implies, a major type of substrate for MMPs is the molecules comprising the extracellular matrix (ECM). The ECM is a specialized network of proteins and polysaccharides which forms an extracellular scaffolding system surrounding individual cells. ECM composition is the underlying basis for tissue formation, and is a determining factor for structural and morphological properties of tissue. Connections between the ECM and the cell surface are mediated through cell adhesion molecules. Many cell adhesion molecules possess signaling capabilities, which influence: actin cytoskeleton organization, ion flux, calcium signaling, gene transcription and other important cellular processes. Activation of the signaling

pathways that govern these responses, however, is typically dependent upon the solubility state of the ECM and the presence of particular ECM molecules. Therefore, MMPs are in a position to exert significant influence over a multitude of physiologic processes.

The importance of MMPs as key enzymes in both normal and abnormal neuronal development, structure, and remodeling, is increasingly evident. Expression of active MMP-9 is necessary for oligodendrocyte process extension and myelination (Uhm, Dooley et al. 1998; Oh, Larsen et al. 1999), and is elevated during postnatal cerebellar development (Vaillant, Didier-Bazáes et al. 1999). MMP-9 expression increases in the hippocampus following kainic acid induced seizures (Zhang and Gottschall 1997; Zhang, Deb et al. 1998; Jourquin, Tremblay et al. 2003), and is correlated with subsequent synapse formation. Following cerebral ischemia increased MMP-9 is associated with altered blood brain barrier function (Anthony, Ferguson et al. 1997; Romanic, White et al. 1998; Fujimura, Gasche et al. 1999; Gasche, Fujimura et al. 1999). Altered gelatinase regulation has also been implicated in cognitive impairments associated with several nervous system disorders. Both MMP-2 and -9 are elevated in multiple sclerosis and stroke patients (Anthony, Ferguson et al. 1997; Lim, Russell et al. 1997); and increased latent hippocampal MMP-9 in Alzheimer's patients has led to speculation that a decrease in active MMP-9 could be involved in the deposition of beta-amyloid (Backstrom, Lim et al. 1996; Lim, Russell et al. 1997; Asahina, Yoshiyama et al. 2001; Lorenzl, Albers et al. 2003; Lorenzl, Albers et al. 2003). In addition, MMP-9 is increased in the spinal fluid of HIV patients and is associated with HIV-related dementia (Conant, McArthur et al. 1999; Liuzzi, Mastroianni et al. 2000; Zhang, McQuibban et al. 2003). MMP-3 is a stromelysin that degrades ECM and also activates latent proMMPs, including MMP-9 (Ogata, Enghild et al. 1992). Alterations in MMP-3 have also been reported in patients with the same disease processes that

affect gelatinases (Anthony, Ferguson et al. 1997). Specifically MMP-3 is enriched in senile plaques of Alzheimer's disease patients (Yoshiyama, Asahina et al. 2000) and beta-amyloid peptide stimulates MMP-3 release from hippocampal cultures (Deb and Gottschall 1996).

Matrix Metalloproteinases and Synaptic Plasticity

Although the linkage of MMPs to neural development and axonal guidance has long been appreciated (see review (Ayoub, Cai et al. 2005; Luo 2005), linkage of MMPs to neural plasticity and especially that associated with cognition is recent. Initial indications that MMPs -3 and -9 might play a role in neuronal plasticity were derived from studies that examined the impact of physical or chemical trauma on synaptic plasticity (Zhang, Deb et al. 1998; Reeves, Prins et al. 2003; Kim, Fillmore et al. 2005) where the site of action seems to be the dendrite and its spines (Szklarczyk, Lapinska et al. 2002; Bilousova, Rusakov et al. 2006). Our recent study (Meighan, Meighan et al. 2006), was the first to demonstrate that MMPs are a critical component of efficient learning and synaptic plasticity. This conclusion is supported by a number of observations. First, MMP transcript and protein increases 4 hours subsequent to water maze training. These increases only occur during the acquisition phase of the task. Upon learning the task, additional maze training fails to promote MMP induction. Second, the observed induction to hippocampal MMPs is a result of NMDA receptor activity. This suggests that MMP increases are activity dependent, a critical feature of the theory of Hebbian based synaptic remodeling. Third, MMP inhibition interrupts LTP stabilization during early maintenance, without affecting the stability or intensity of baseline transmission (Meighan, Meighan et al. 2006) . Finally, both inhibition of MMP activity with FN-439 and reduction of MMP-3 and -9 protein with antisense oligo-nucleotides interfere with acquisition of the Morris water maze task.

Structural Plasticity of Dendritic Spines

Dendritic spines are microenvironments which serve as the functional units of excitatory post-synaptic neurotransmission in the CNS. Dendritic spine morphology has many implications for the nature and efficiency of neuronal synaptic connectivity. While the exact relationship or the coding between spatial learning, LTP and dendritic spine shape has yet to be deciphered, the literature does provide ample evidence that in mature animals, modification of the morphology of *existing* synapses and dendritic spines may account for long lasting changes to synaptic efficacy (Kasai, Matsuzaki et al. 2003). Neuronal cytoskeletal elements are concentrated within a specialized region of the cell membrane known as the post-synaptic density (PSD). The PSD is an area of the dendritic spine where the machinery of major signal transduction pathways are concentrated and organized to allow for integration of post-synaptic signaling events [for review see (Yamauchi 2002)]. The PSD receives synaptic transmission from neighboring cells, which is then transduced by components of the PSD to influence the local responsiveness of the spine or alteration of whole-cell behavior. Local level alterations to spine physiology usually involve regulatory changes in the activity state of proteins critical to signal transduction processes. These changes can in turn alter the responsiveness of the post-synapse to a given type or intensity of synaptic transmission stimuli. The signaling components of the PSD are physically organized by their connections to the actin cytoskeleton, mediated by scaffolding proteins with specialized binding sites. This puts the cytoskeleton in close proximity to other structures such as neurotransmitter receptors and signaling molecules that are also sequestered to PSDs. In fact, through various protein associations, excitatory post-synaptic receptors are linked physically to actin filaments allowing for the polymerization state of the actin cytoskeleton to influence receptor activity and signaling efficiency.

The structural stability of dendritic spines is thought to be reliant upon cell adhesion between dendritic spines and pre-synaptic structures (mediated by CAM-CAM interactions) and cell adhesion between dendritic spines and the ECM (mediated by CAM-ECM interactions) (Kasai, Matsuzaki et al. 2003). These interactions (i.e. CAM-CAM and CAM-ECM) are regulatable by MMP activity. Considering that dendritic spine structure is an important determinant of signaling efficacy within excitatory synapses, by extension, MMPs are strategically positioned to be critical mediators of hippocampal plasticity. Due to the relationships between MMPs, cell adhesion, and plasticity, the impact of MMPs might be inferred by examination of plasticity critical CAMs potentially influenced by MMPs. Specific plasticity critical CAMs likely affected by altered MMP activity are thus:

Cadherins. Localized to synaptic junctions, cadherins are adhesion molecules which are important mediators of direct cell-to-cell connections. Cadherins function by homophilically binding to cadherins on opposing cell membranes. The cytoplasmic segment of cadherins can bind and influence F-actin and cytoskeletal regulatory proteins via catenin binding protein. Disruption of the ecto-domains' homophilic interactions or cytoplasmic binding to catenin has a deleterious effect on dendritic spine morphogenesis and PSD-organization (Togashi, Abe et al. 2002). Interestingly, E-cadherin is a specific substrate for MMP-9 where knockdown of MMP-9 expression concomitantly results in increased surface expression of E-cadherin and the promotion of catenin mediated cytoskeletal and focal adhesion organization (Sanceau, Truchet et al. 2003). In a similar study, cadherin surface expression was increased simply by direct application of MMP inhibitors (Ho, Voura et al. 2001). Considering that cadherin function appears to be

intrinsically tied to cortactin translocation and regulation, cadherins are likely mediators of MMP-sensitive alterations to cortactin function).

Neural cell adhesion molecules (NCAMs). NCAM has three major isoforms (NCAM 120, NCAM 140, and NCAM 180). Research has focused on NCAM 180 because it is localized to synapses that are undergoing plastic changes (Peterson, Blankenship et al. 1996) and increases 90 minutes after long-term potentiation of the dentate gyrus (Fazeli, Breen et al. 1994). Similarly, NCAM knockout mice exhibit impaired spatial learning (Cremer, Chazal et al. 2000) and LTP (Cremer, Chazal et al. 1998). In addition to the particular NCAMs listed above, the Ig superfamily adhesion molecule, ICAM-5, is another potential effector of MMP activity. ICAM-5 (telencephalin) is the first dendritic-associated CAM to be identified. It appears ontogenetically coincident with dendritic development (Yoshihara, Oka et al. 1994) and is concentrated on the dendritic-soma membranes of hippocampal neurons where it stimulates neurite outgrowth (Yoshihara, Oka et al. 1994). Perhaps most relevant to neural plasticity is the observation that ICAM-5 antibodies suppress LTP at Schaffer collateral-CA1 synapses (Sakurai, Hashikawa et al. 1998). Although the data are not definitive, evidence is emerging that MMPs regulate the surface expression of NCAMs and ICAMs by enzymatic degradation (Maidment, Rucklidge et al. 1997; Fiore, Fusco et al. 2002).

Integrins. Hetero-dimeric transmembrane protein, a number of alpha and beta subunits give rise to the diverse physiological phenomena mediated by integrins. There exits a staggering number of ECM and non-ECM ligands which bind and modulate integrin

activity including: collagens, fibronectins, laminins, cadherins and ICAMs. It has also been suspected that some integrin variants form multimeric MMP integrin complexes and an activation mechanism for MMP-2 (Zigrino, Drescher et al. 2001) Furthermore, integrin-MMP docking is thought to help direct localization of MMP-mediated pericellular proteolysis (Brooks, Stromblad et al. 1996). Integrins are probably the most important mediators of cell-ECM junctions throughout the body. Among the most typical response to integrin-ECM engagement is the formation of focal adhesions and macromolecular signaling complexes on the cytoplasmic domains of alpha and beta integrin subunits (Loeser 2002). Uninhibited integrin function within the hippocampus is essential for both memory consolidation (Chan, Weeber et al. 2003) and LTP (Sakurai, Hashikawa et al. 1998). Similar to other adhesion molecules, MMPs influence integrin function by regulating the availability of specific integrin ligands and by affecting ECM stability.

The structural stability of dendritic spines is thought to be reliant upon cell adhesion between dendritic spines and pre-synaptic structures (mediated by CAM-CAM interactions) and cell adhesion between dendritic spines and the ECM (mediated by CAM-ECM interactions) (Kasai, Matsuzaki et al. 2003). In turn, the molecules which participate in these interactions have a large influence over a wide array of signaling processes (Dityatev and Schachner 2003). Perhaps the most critical of these processes, with respect to dendritic spine function, is the regulation of the actin cytoskeletal polymerization state. Our hypothesis' regarding the importance of MMPs in synaptic plasticity is largely based on the fact that MMPs are potent effectors of pericellular proteolysis. This property of MMPs is significant considering that

proteolytic degradation of ECM components and cell adhesion molecules sequentially affects several signal transduction pathways sensitive to cell adhesion. (Lochter, Taylor et al. 1995). This influence on signal transduction is typified by the presence or absence of focal complex formation at the cell-membrane. Focal complexes are hetero-multimeric protein signaling complexes, which are comprised of as many as several hundred signaling agents and structural constituents. Many of the signaling proteins comprising a focal complex are major regulators of the actin cytoskeleton, gene transcription, and ion conductance, among other vital cellular processes. Focal complex formation occurs on cytoplasmic portion of adhesion molecules, and their activity is heavily influenced by CAM-CAM and ECM-CAM interactions. Consequently, MMPs are thought to indirectly influence the signaling activity of focal complexes by effecting pericellular proteolysis. This supposition is supported by the observations of MMPs influencing the signaling capabilities of several classic constituents of focal complexes (e.g. FAK, paxillin, and integrins) (Takino, Watanabe et al. 2006) (Meriane, Duhamel et al. 2006). Of the many signaling molecules known to be influenced by MMP activity, several are known contributors to dendritic spine physiology and hippocampal plasticity. This makes it likely that MMPs influence synaptic plasticity is partially due to modulation of signaling pathways. Due to the relationships between MMPs, cell signaling, and plasticity, the impact of MMPs might be inferred by examination of plasticity critical signaling molecules potentially influenced by MMPs. Specific plasticity critical molecules likely affected by altered MMP activity are thus:

Focal Adhesion Kinase (FAK) FAKs are non-receptor tyrosine kinases which are heavily expressed in the CNS, most notably in the cerebral cortex, striatum and hippocampal formation. There are several isoforms of FAK which stem from alternative splicing of a single gene product. The FAK isoform most prevalent in the central

nervous system is FAK^{+} which differs from the classic FAK isoform, $p125^{FAK}$, by the inclusion of a short exon coding for 3 amino acids (Pro-Trp-Arg) on the c-terminal segment (Menegon, Burgaya et al. 1999). FAKs have an intrinsic focal adhesion targeting (FAT) sequence on the C-terminus that mediates the localization of FAKs to focal adhesions. Upon localization to cell adhesion associated signaling complexes, FAK undergoes autophosphorylation of Tyr397 which allows for SH2 domain mediated docking of src family kinases. Once associated with FAK, src kinases phosphorylate tyrosine residues in the catalytic and c-terminal domains which initiates FAKs signaling activity. Upon activation, FAK's main function is thought to be as a central scaffolding protein involved in the recruitment and activation of other signaling molecules, such as src family kinases, $p130^{cas}$ and paxillin (Girault, Costa et al. 1999). Downstream effectors of FAK are important modulators of gene transcription, cytoskeletal organization and NMDA receptor function. It has previously been demonstrated that integrin engagement is a prerequisite to FAK activation (Shattil, Haimovich et al. 1994; Slack 1998). An interesting consequence of this relationship, with respect to these studies, is the observation that FAKs activation state is sensitive to MMP pericellular proteolysis-- where FAK activity is potentiated by MMP inhibition (Ho, Voura et al. 2001). The fact that FAK activation is essential for LTP induction in the rat dentate gyrus (Yang, Ma et al. 2003) underscores its' physiologic importance.

Proline-Rich Tyrosine Kinase 2 (Pyk2). Also known as cell adhesion kinase β (CAKβ), FAK2, and related adhesion focal tyrosine kinase (RAFTK), Pyk2 is a non-receptor tyrosine kinase closely related to the FAK family. Pyk2 shares many of the regulatory

mechanisms and utilizes similar downstream effector systems as FAK. Pyk2 activation differs from that of FAK in that it appears to be dependent upon intracellular calcium, and presumably, protein kinase C activity (Siciliano, Toutant et al. 1996). Pyk2 associates with the NMDA receptor complex via PSD-95 and SAP102 scaffolding proteins where it is thought to mediate src-directed tyrosine phosphorylation of the NR2A and NR2B subunits resulting in augmented NMDA receptor function

Src family kinases. A major substrate of FAK and Pyk2, src kinases are non-receptor tyrosine kinases which modulates numerous cellular process including gene transcription, ion flux and cytoskeletal organization. With respect to cytoskeletal organization, src is particularly important for the targeting of cortactin to lamellapodia at the cell cortex, and down-regulating cortactin's ability to stabilize F-actin (Weed, Karginov et al. 2003). Src phosphorylates the NR2A and NR2B subunits resulting in an upregulation of NMDA receptor function (Suzuki and Okumura-Noji 1995; Yu, Askalan et al. 1997; Salter 1998; Hisatsune, Umemori et al. 1999). In regulating the actin-cytoskeleton, src kinases' functional role is to facilitate focal adhesion turnover and cytoskeletal remodeling (Dityatev and Schachner 2003); therefore src is thought to be critically involved in structural and morphological aspects of neural plasticity.

Cortactin. Cortactin, a substrate for the src family tyrosine kinase *fyn*, is a potent F-actin binding and cross-linking protein and is highly enriched in hippocampal tissue, notably in the post synaptic density (Husi, Ward et al. 2000). Cortactin binds Arp 2/3 and F-actin which results in the stabilization of F-actin branches (Weaver, Karginov et al. 2001). In

addition to promoting actin polymerization, cortactin also acts as a scaffolding protein to localize other proteins to actin polymerization sites (Naisbitt, Kim et al. 1999). Consistent with the idea that it's involved with activity-dependent synaptic remodeling, cortactin strategically associates with NMDA receptors complexes via the PDZ scaffolding protein, Shank (Naisbitt, Kim et al. 1999), and redistributes from dendritic spines to the dendritic shafts following NMDA receptor stimulation (Hering and Sheng 2003). Further support to the idea that cortactin is a critical mediator of structural plasticity, siRNA knockdown of cortactin expression results in decrease in dendritic spine density in hippocampal cultures (Hering and Sheng 2003). Conversely, cortactin over-expression induces dendritic spine elongation in hippocampal cultures and promotes the outgrowth of dendritic protrusions in aspiny hippocampal interneurons (Hering and Sheng 2003).

In addition to ECM proteins and CAMs, MMPs can activate growth factors (e.g. brain derived neurotrophic factor; BDNF) or release those that are sequestered in the ECM (e.g. TNFα). Factors, like BDNF and TNFα, can dramatically impact synaptic plasticity (Alonso, Vianna et al. 2002; Alonso, Vianna et al. 2002; Lu 2003; Golan, Levav et al. 2004).

Although the functional impact of MMP inhibition on hippocampal LTP has been identified, the means by which MMPs affects LTP has yet to be determined. Furthermore, it is unclear if MMP involvement is limited to LTP, or if MMPs have a more generalized role in promoting hippocampal plasticity. Chapter one is comprised of a series of experiments which address these points. First, we assessed the impact of MMP inhibition multiple forms of short-term and long-term hippocampal plasticity. Second, we examined the means by which MMPs affect LTP by varying the tetanization intensity, and by altering the timing of MMP inhibition

relative to tetanus. Finally, we attempted to determine if effects of MMP inhibition on hippocampal plasticity result from changes to cell adhesion. The results indicate that MMPs are important for multiple forms of hippocampal plasticity and that MMPs promote LTP by at least two distinct mechanisms.

Dysregulation of MMPs is implicated in tissue aging in a broad assortment of non-neuronal tissues [see Chapter 2: Introduction]. Considering that increased MMPs activity is implicated in pathological progression of several diseases and conditions which ultimately impact cognitive performance (e.g. Alzheimer's disease and stroke) we hypothesized that MMPs are involved in the cognitive deficits associated with aging. The experiments described in Chapter 2 were aimed at addressing this hypothesis. First, we assessed the levels of MMPs in aged hippocampi in comparison to those expressed by young adult rats. Second, we assessed the impact of inhibiting MMPs with broad-spectrum inhibitor FN-439 on hippocampal LTP from aged-adult rat slices. Third, we attempted to recapitulate the effects of aging on hippocampal plasticity and fEPSP waveform characteristics by artificially increasing MMP levels. The results indicate that increased hippocampal MMPs play an important role in the functional deficits associated with hippocampal aging.

CHAPTER I

EFFECTS OF MATRIX METALLOPROTEINASE INHIBITION ON SHORT AND LONG TERM PLASTICITY OF SCHAFFER COLLATERAL /CA1 SYNAPSES

ABSTRACT

It is increasingly evident that matrix metalloproteinases (MMPs), a family of zinc containing extracellular endopeptidases, participate in processes supporting hippocampal synaptic plasticity. Specifically, we have recently demonstrated that hippocampal MMPs are upregulated during acquisition of a spatial memory task, and inhibition of hippocampal MMPs is deleterious to both task acquisition and long term potentiation (LTP). The purpose of this study is twofold: (1) to further our understanding of MMPs importance in hippocampal plasticity and (2) to gain a mechanistic insight as to the functional importance of MMP activity for hippocampal plasticity. Acute hippocampal slices were generated from 20-30 day old male Sprague Dawley rats. Following recovery, control and MMP inhibitor (FN-439 Calbiochem) treated slices were subjected to various stimulatory paradigms which produce either short term or long term modifications to synaptic efficacy. Slices exposed to FN-439 exhibited impairments in paired pulse facilitation and theta burst facilitation; furthermore, FN-439 treated slices failed to stabilize following induction for LTP and long term depression (LTD). Given the capability of MMPs to alter the extracellular and pericellular environment by degradation of extracellular matrix molecules and cell adhesion molecules, we hypothesized that the effects of FN-439 on LTP stabilization involves dysfunction to plasticity critical alterations to cell adhesion. To this end, we examined the temporal sensitivities of MMP inhibition by administering the inhibitor at various

times following tetanization. Upon observing a similar temporal sensitivity pattern to that of integrin antagonist (GRGDSP Calbiochem), we predicted that FN-439 impacts LTP by interfering with integrin function. Consistent with this prediction, we observed that FN-439 competes for effect on LTP stabilization with the GRGDSP compound. Together, these data support a generalized role for MMPs for short term and long term hippocampal plasticity and that MMPs are a necessary facet of integrin mediated cell adhesion supporting LTP stabilization.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of secreted and cell-surface endo-peptidases that are classified by their substrate specificity. There are over 25 individually recognized MMPs, categorized by their distinct structural elements and their variable affinity for a variety of identified substrates. Secreted as an inactive zymogen, the proteolytic removal of the N-terminal "pro" domain of latent-MMP initiates MMPs' catalytic activity. Once active, MMPs are capable of degrading numerous pericellular molecules including other proteinases, latent growth factors, growth factor-binding proteins, cell surface receptors, cell-cell adhesion molecules, and nearly all extracellular matrix proteins [for review see (Sternlicht and Werb 2001)]. The sheer variety of substrates recognized by MMPs, and the influence that many of these substrates have on cell adhesion, enables MMPs to have a tremendous impact on a wide array of physiologic processes.

Although MMPs influence on cellular processes has been extensively described for a variety of tissues, their involvement in central nervous system physiology is only recently appreciated. Predominantly, the initial studies documenting MMPs participation in CNS physiology have focused on damage or disease associated CNS structural remodeling. For instance, MMPs have been described in the pathological progression of neuronal degeneration during stroke. Transient forebrain ischemia promotes the induction of MMP-3, resulting in gross architectural changes to the ECM. Furthermore, the neuronal degeneration following the ischemic insult is at least partially attributable to increased MMP-9 expression and activity. In addition to stroke, MMPs have been implicated in neuronal remodeling associated with kainic acid-induced epileptogenesis (Szklarczyk, Lapinska et al. 2002), recovery following traumatic brain injury (Falo, Fillmore et al. 2006) and deafferentation-induced collateral sprouting in the

dentate gyrus (Reeves, Prins et al. 2003). In each of the instances, MMPs impact tissue morphology or functional outcome by affecting structural remodeling.

Neurons within the hippocampus, a structure critical for memory acquisition, exhibit activity dependent modifications of signaling efficacy between pre- and postsynaptic elements. This process is thought to be a neurobiological basis for learning. Alterations to synaptic efficacy are thought to involve modification of synaptic morphology. Considering that morphological modifications necessitate restructuring of extracellular and pericellular architectures, a priori, MMPs are likely contributors to synaptic plasticity. This idea was initially tested by examination of hippocampal gelatinases of rats subjected to a spatial learning task (Wright, Kramar et al. 2002). Animals subjected to the spatial task displayed increased MMP-9 levels during the task acquisition phase. Since this initial observation, we have demonstrated that learning associated MMP increases are NMDA receptor dependent (Meighan, Meighan et al. 2006); this suggests that learning associated MMP increases are activity dependent. Furthermore, we have demonstrated that MMP activity is essential for both water maze learning and LTP.

Although the functional impact that MMP inhibition has on LTP has been identified, the means by which MMPs affects LTP has yet to be determined. Furthermore, it is unclear if MMP involvement is limited to LTP, or if MMPs have a general role in promoting multiple forms of hippocampal plasticity. To address these points, we first assessed the impact of MMP inhibition multiple forms of short-term and long-term hippocampal plasticity. Second, we examined the means by which MMPs affect LTP by varying the tetanization intensity, and by altering the timing of MMP inhibition relative to tetanus. Finally, we attempted to determine if effects of MMP inhibition on hippocampal plasticity result from changes to cell adhesion. The results

indicate that MMPs are important for multiple forms of hippocampal plasticity and that MMPs promote LTP by at least two distinct mechanisms.

MATERIALS AND METHODS

Generation of Hippocampal Slices

Rats were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ, USA), decapitated and the brain was rapidly removed. The brain was immediately placed into ice-chilled, oxygenated aCSF for approximately 30 s. The hemispheres were separated by a mid-sagittal cut and the hippocampus was removed from the right hemisphere using a custom-built wire loop instrument. Slices (400 um thick) from the middle third portion of the hippocampus were prepared using a McIlwain tissue chopper (Brinkmann, Gomshall, UK) and transferred to a gassed (95% O_2 / 5% CO_2) incubation chamber containing aCSF, where they were maintained for at least 1 h at 22–23_C. Single slices were then transferred to a perfusion–recording chamber and stabilized on the chamber floor (coated with Sylgard; Dow Corning, Midland, MI, USA) by platinum wires. Slices were continuously superfused with gassed aCSF (30–31_C) at a rate of 1–1.5 mL/min via a peristaltic pump (Rainen Rabbit-Plus, Woburn, MA, USA).

Electrophysiology

Extracellular recordings from the CA1b stratum radiatum layer were obtained using glass micropipettes filled with 0.15 M NaCl, yielding a resistance of 2–3 MΩ. Orthodromic activation of the Schaffer collaterals within the CA1c was accomplished using concentric bipolar stimulating electrodes (Rhodes Medical Instruments, Inc., Woodland Hills, CA, USA) positioned adjacent to the recording electrodes in the CA1c stratum radiatum layer. Test stimuli (0.1 ms, 0.1 Hz) were delivered using a stimulator (Model S88; Grass, Quincy, MA, USA) to elicit field excitatory postsynaptic potential (fEPSP) responses. The duration of stimulation was adjusted in

each case to produce a dendritic field potential that was 50–60% of the maximum spike-free response (.08–.12 ms).

LTP was induced by theta patterned high-frequency stimulation. For the *robust* tetanization, this was accomplished by the application of 4 trains of theta burst stimulations (TBSs) with an inter-train interval of 10 s. Each train was comprised of 6 bursts of four 100Hz , .35ms pulses, separated by 200ms. For the *attenuated* LTP tetanization, LTP was induced by a single train of six bursts and the pulse duration was reduced to .1ms. LTD was accomplished with low frequency stimulation (900 pulses at 1Hz) with pulse duration of .1ms. Paired pulse facilitation was generated by administering twin pulses with an inter-pulse interval of 50, 100 and 150 ms with constant pulse duration of .1ms. Slices were allowed to recover for 20 seconds between stimulations.

Extracellular signals were amplified (gain 1000x) and filtered (1 kHz) using an amplifier (Model 1800; A-M Systems, Newport, WA, USA). Data were digitized and analyzed using a computer interfaced PowerLab/400 (ADInstruments Inc., Dover, NH, USA).

Drug Application

For LTP and LTD, and input-output curves FN-439 (180 uM; Sigma Chemical) was either administered 10 minutes prior to tetanization, or administered at discrete intervals following tetanization (as indicated). For paired-pulse facilitation slices were subjected to a 20-minute pretreatment of FN-439 prior to testing

Data analysis

Three successive fEPSP amplitude measurements were averaged and recorded. LTP and LTD time-courses were expressed as the percent of the mean baseline response. Two-factor ANOVA, area under the curve, student T-tests, and regression analyses were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA). Specific analyses indicated within the figure legends. A test for interaction between factors was included for each two-factor ANOVA. In each case, the analysis failed to indicate a significant interaction between the tested factors. Because there were only two levels of treatment factor (FN-439 versus vehicle), a significant result was not followed by multiple comparisons testing. To test for coincidence of regression lines the fit of the data to the two regression lines was compared to a fit of the data with a single regression line. To accomplish this, an F ratio was generated by comparing the total sum-of-squares for the single regression to the combined total sum-of-squares from the two regression lines.

RESULTS

Inhibition of MMPs interferes with multiple forms of Schaffer collaterals-CA1 plasticity.

MMP inhibitor treated and control slices were subjected to a series of pulses with increasing stimulus duration (.04-.2 ms), at constant stimulus intensity and the amplitudes of the evoked responses were plotted as an input-output curve (I-O curve) **(Fig 1A)**. FN-439 had no effect on the input-output relationship. Failure of the MMP inhibitor to alter the I-O relationship indicates that the inhibitor does not alter tissue viability or basic electrical properties of the slice preparation. In contrast to the unchanging I-O relationship, MMP inhibitor treated slices evidenced a significant attenuation of paired-pulse facilitation (PPF) **(Fig 1B)**. The mechanisms underlying this form of short-term plasticity are predominantly confined to pre-synaptic elements [for review see (Thomson 2000)], therefore an attenuation of PPF indicates that inhibition of MMPs interfered with processes which underlie augmentation of neurotransmitter release. It should be noted that this interference does not extend to basal transmission, as it has been previously demonstrated that FN-439 treated slices fail to alter baseline synaptic responses (Meighan, Meighan et al. 2006).

To examine how MMP inhibition can affect different forms of long term plasticity, we determined FN-439's impact on the time-course and magnitude of LTP and LTD. To induce LTP, slices were subjected to a robust theta burst tetanization paradigm consisting of 4 trains with an inter-train interval of 10 seconds, where each train consisted of 6-bursts patterned after endogenous hippocampal theta rhythms (~5Hz). Consistent with our previously reported findings, FN-439 had minimal effect on the magnitude of LTP induction, but interfered with the stabilization of LTP **(Fig 1C)**. Furthermore, MMP inhibition diminished the magnitude and

stabilization of low frequency stimulated LTD (**Fig 1D)**. Taken together, these results support a generalized role for MMPs in modulating processes that underlie short-term and long-term hippocampal plasticity.

Temporal relationship between FN-439 administration and TBS differentially effects LTP stabilization.

Although initial studies employing LTP indicated that MMPs participate in processes governing LTP stabilization following theta burst stimulation, little is known about the mechanisms by which MMPs participate. In an initial attempt to elucidate these mechanisms, we examined the temporal relationship between inhibitor application and LTP stabilization. Previously, MMP-inhibitor was present during tetanization and throughout LTP time-course (see Figure 1c). From this experiment, it appears that the effects of MMP inhibition on LTP are largely associated with early-phase maintenance with a minor effect on the magnitude of LTP induction. To isolate the effects of MMP inhibition on LTP maintenance, FN-439 was administered immediately following tetanization (PT_0) and compared to slices that were subjected to pretreatment and non treated slices **(Fig 2A)**. Aside from the peak response immediately following tetanization, the rate of LTP decay was indistinguishable between FN-439 pretreated and PT_0 slices.

A priori, there are two possibilities regarding the timeframe by which MMPs promote stabilization of early-phase LTP: (1) MMPs participate in processes within a discrete temporal interval such that MMP inhibition outside of this interval has no effect on early-phase LTP maintenance or (2) MMP activity is required to promote early-phase LTP indefinitely such that MMP inhibition at any point in time following tetanization impacts LTP. To differentiate

between these two possibilities, LTP was induced by TBS and MMP inhibitor was administered 30 minutes post tetanus (PT_{30}). This time-point was selected as slices are well within early-phase LTP but safe from contamination by late-phase LTP. Application of MMP inhibitor 30 minutes post tetanus had no effect on rate of LTP decay **(Fig 2B)**. The divergence of effects between immediate FN-439 application and application 30 minutes following tetanization suggests that there exists a period, within the first 30 minutes following LTP induction, where early-phase LTP transitions from MMP dependent to MMP independent. To isolate this transition period, MMP inhibitor was delivered 15 minutes following tetanization (PT_{15}). Although MMP inhibition resulted in LTP destabilization on the outset of the treatment interval, LTP apparently stabilized and decayed at control levels towards conclusion of treatment interval **(Fig 2C)**. Regression analysis was used during these treatment intervals to quantify the relative efficacies of each treatment interval **(Fig 2D)**. For this analysis, the difference between the mean, time-specific, control values and the replicates of the FN-439 treatment time-courses were calculated. This transformation allows for easy assessment of rate of LTP decay, relative to control decay values, for the three temporal intervals studied. A positive slope indicates increased decay rate relative to mean control values and a zero slope denotes a rate of decay equal to that as mean control values. Slices administered MMP inhibitor immediately following tetanization displayed an enhanced rate of decay relative to control (slope: $p<.001$). This is in contrast to inhibitor delivery 30 minutes subsequent to tetanization, which did not significantly differ from control decay (slope: $p>.05$). Slices administered inhibitor beginning 15 minutes following tetanization (PT_{15}) expressed a significant non-linearity (Runs Test: $p<.05$) that was best fit to an exponential association function. Qualitatively the PT_{15} group showed an increased decay rate on the outset of the treatment interval which transitioned to control rates by the end of the interval. Taken

together, it appears that MMP-dependent events that promote early-phase LTP stabilization occur during the first 20-25 minutes following tetanization; subsequent to this interval, stabilization of early-phase LTP is no longer MMP-dependent.

Integrin antagonist, Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), occludes the effect of MMP inhibitor, FN-439, on LTP stability.

Although MMPs are recognized to participate in many processes which may ultimately affect cellular or neuronal physiology, MMPs are perhaps best known for their impact on cell adhesion. Considering the importance of cell adhesion in promoting LTP, it seems likely that at least part of MMPs influence on LTP is through modulation of cell adhesion. Multiple reports indicate that integrins are important for LTP stabilization during early maintenance phase (Staubli, Chun et al. 1998) (Chan, Weeber et al. 2003). To test the possibility that MMP inhibition ultimately impacts LTP stabilization by negatively affecting integrin function, we examined the combined effect of GRGDSP and FN-439 on LTP stabilization while looking for possible occlusion. If MMP inhibition affects plasticity critical processes independent of integrin function, then co-treatment with GRGDSP and FN-439 should have a significantly greater effect on LTP stabilization than GRGDSP alone. Consistent with previously published observations, slices treated with GRGDSP exhibited a similar effect profile (i.e. magnitude of induction, rate of decay) as FN-439 treated slices (**Fig 3**). Furthermore, treatment with GRGDSP abrogates the effect of MMP inhibition on LTP stability as slices subjected to co-treatment exhibited a decay rate of LTP nearly equal to that of GRGDSP treated slices. These results suggest that the effect of MMP inhibition on LTP stabilization was occluded by inhibition of integrins due to a competitive effect on a common process.

Effect of MMP inhibition on theta burst responses elicited during a *robust* theta burst paradigm. MMP inhibition significantly impaired short term plasticity in the form paired pulse facilitation (see **Fig 1B**). The implication of this observation is that rapid augmentation of neurotransmitter release is dependent upon MMP function. Considering that the theta burst stimulation paradigm employed in these experiments consists of bursts comprised by a series of 4 high frequency pulses (100 Hz), it is likely that within train facilitation during theta burst stimulation is supported by similar mechanisms. Based on this, one could predict that MMP inhibition would ultimately result in decreased facilitation during theta burst stimulation. To examine the possible effect of MMP inhibition on theta burst facilitation, evoked responses during theta burst stimulation were recorded and analyzed. Two burst response parameters were chosen for analysis for this experiment: t*otal burst area*, which corresponds to the total depolarization elicited by each burst in the theta train, and the magnitude of *burst after-positivity*, a persistent positive potential subsequent to a burst of activity. Evoked response bursts elicited by theta trains 1 and 4, for both FN-439 treated and control slices, were measured and calculated for total area **(Fig 4A)**. Area of burst response during theta stimulation was significantly lower for MMP inhibitor treated slices for both trains #1 ($p<.01$) and #4 ($p<.0001$). It is likely that this effect is not integrin-dependent, since application of GRGDSP had no effect on burst response during theta **(Fig 4B)**; a result that is consistent with previously reported findings (Staubli, Chun et al. 1998). Interestingly, the difference in total depolarization between MMPi treated and control slices was not reflected in the magnitude of after-positivity accompanying these burst responses **(Fig 4C)**. After-positivity is a result of calcium sensitive potassium conductance, primarily generated by small-conductance Ca^{2+}-activated K^{+} channels (SK channels). Under

normal conditions, after-positivity changes proportionally with the magnitude of the preceding theta burst response (Kramar, Lin et al. 2004) due to their shared relationship to calcium influx. One possibility for this observed discrepancy is that MMP inhibition either directly or indirectly altered functional characteristics of dendritic SK channels. If this were true, this alteration should manifest as a change in the relationship between total depolarization (i.e. area of burst response) and after-positivity. To examine this possibility, regression analysis was used to determine whether the relationship between burst-area and after-positivity was altered by treatment with MMPi **(Fig 4D)**. Because the regression lines for both FN-439 and control conditions were coincident (i.e. difference between slope and y-intercept for regression lines was not significant ($p>.05$)), it is unlikely that regulatory alterations to SK channels (e.g. phosphorylation, surface expression) were responsible for the lack of effect on after-positivity.

MMP inhibition impacts theta burst facilitation, afterpositivity and LTP induction when stimulated by an *attenuated* TBS protocol.

Although there was a difference in total depolarization between FN-439 and control slices, there appeared to be similar levels of SK channel activation (manifested as after-positivity). Because the relationship between area and after-positivity were nearly identical for both control and MMPi treated slices, and because SK channels are activated by increased cytosolic calcium, it is likely that similar levels of calcium entry occurred during theta for each treatment condition. Because the tetanization scheme employed for this study is a relatively robust protocol, this discrepancy could be due to a ceiling effect for calcium entry. To control for this possibility, LTP was elicited by an attenuated tetanization paradigm consisting of a single theta train of 6 bursts, with a reduction of pulse duration by 50%. Consistent with the robust tetanization scheme, slices

exposed to MMPi displayed a significant decrease in total depolarization during the burst response **(Fig 5A)** ($p<.001$). However, in contrast to the robust scheme, the difference in depolarization translated into a marked reduction of after-positivity amplitude **(Fig 5B)** ($p<.0001$). As with the robust scheme, the relationship between area and after-positivity for both control and MMPi treated slices expressed coincident regression lines (data not shown). Therefore, the decreased magnitude of after-positivity is likely due to diminished calcium entry during tetanization in MMPi treated slices.

Inhibition of MMPs had only a minor effect on induction when LTP was triggered by a robust tetanization protocol (see **Fig 1C**). Considering the importance of intracellular calcium for LTP induction, this finding is consistent with the after-positivity data (which suggests that similar levels of calcium entry occurred during tetanization for MMPi treated and control slices). Decreasing the intensity of tetanization produced a sizeable difference in the magnitude of after-positivity between MMPi treated and control slices. If the disparity in after-positivity was a result of decreased calcium entry for MMPi treated slices, it should manifest as reduced LTP induction of MMPi treated slices from this tetanization protocol. To examine this, MMPi and control slices were subjected to the attenuated TBS protocol and were subsequently monitored for induction and early maintenance of LTP **(Fig 6A)**. In contrast to control slices, this weak tetanization employed failed to induce LTP in MMPi treated slices. The timeframe of potentiation for the MMPi treated slices (a return to baseline values in approximately 10-15 minutes) is consistent with the time-course of short-term potentiation . Interestingly, the rate with which MMPi treated slices returned to baseline values appeared to be independent of previously observed effects of MMPi on LTP maintenance. This is evident in the difference between the two regression lines which quantify the rate of LTP decay relative to control values **(Fig 6B and 6C)**. Moreover, the

rate of decay for FN-439 treated slices is not significantly different than that of the controls (slope: $p>.05$); indicating a principal effect of MMPi on LTP induction with the weaker tetanization protocol.

DISCUSSION

Although it is becoming increasingly evident that MMPs participate in processes necessary for hippocampal synaptic plasticity, little is understood regarding the mechanisms by which they contribute. To further our understanding as to the scope of MMPs involvement in hippocampal plasticity, we have shown that MMP inhibition interferes with multiple forms of short term (e.g. PPF and theta burst facilitation) and long term (LTP and LTD) plasticity without altering basic electrical properties of hippocampal preparation. Furthermore, we have identified two independent roles by which MMPs support LTP: (1) MMPs promote the stability of early-phase LTP by facilitating integrin function and (2) MMPs augment TBS induced LTP induction; likely by enhancing the burst response during theta.

MMPs and stability of early-phase LTP

It's been recently demonstrated that theta burst stimulation of Schaffer collaterals, such that it is sufficient to induce LTP, promotes the formation of filamentous actin (F-actin) in CA1 dendrites (Kramar, Lin et al. 2006). This is a significant finding considering that augmentation of dendritic F-actin is antecedent to structural modifications of dendritic spines (i.e. post-synaptic elements of excitatory neurotransmission within the CNS) necessary for LTP consolidation during early maintenance phase (Krucker, Siggins et al. 2000). Integral to the process of dendritic actin polymerization, with the attendant effects on spine function, is cell adhesion. The cell adhesion state of dendritic spines and pre-synaptic elements, determined by both a direct interaction between pre- and post-synaptic cell adhesion molecules (CAM-CAM) and an indirect association between CAMs and the surrounding ECM (CAM-ECM), has been shown to influence plasticity critical regulators of the actin cytoskeleton. Our hypothesis regarding the importance of

MMPs involvement in synaptic plasticity is largely based on the fact that MMPs are potent effectors of extracellular and pericellular proteolysis. Considering that proteolytic degradation of ECM and CAMs are important regulatory aspects of cell adhesion, MMPs are strategically positioned to have a profound influence on spine dynamics underlying synaptic plasticity (Lochter, Taylor et al. 1995).

Advancing our hypothesis (regarding the means by which MMPs promote stability of early-phase LTP) necessitated that we identify the particular cell adhesion molecule(s) influenced by plasticity-associated MMP activity. Of the cell adhesion molecules that are demonstrably important for hippocampal plasticity, the three families which receive the most attention are NCAMs, cadherins and integrins. Although MMPs have been shown to influence the function of each of these families of cell adhesion molecules (Covington, Bayless et al. 2005) (Takino, Watanabe et al. 2006) (Fiore, Fusco et al. 2002), we ultimately focused our examination on integrins for a number of reasons. **First**, similar to MMP inhibition, inhibition of integrins has minimal affect on LTP induction. This is in contrast to inhibition of NCAM, which primarily affects LTP induction (Staubli, Chun et al. 1998). Moreover, inhibition of either N- or E-cadherin with an adhesion blocking antibody attenuates LTP induction (Tang, Hung et al. 1998). **Second**, as with MMP inhibition, interference of integrin function produces an enhanced decay of early-phase LTP that begins immediately following induction. Contrary to this, inhibition of either NCAMs or cadherins (either –N or –E) fails to affect LTP stability during early-phase maintenance (Tang, Hung et al. 1998) (Staubli, Chun et al. 1998). **Third**, integrin inhibition has a similar post-tetanus temporal sensitivity profile to that of MMPs (see Figure-2 and (Staubli, Chun et al. 1998)). Specifically, neither FN-439 nor RGD compounds are required to be present during tetanization to affect LTP stability, and loss of blockade efficacy progresses for each of

these inhibitors if LTP is allowed to develop for approximately 15 minutes prior to administration (with a complete loss of efficacy occurring after 30 minutes). This is in opposition to NCAM blockade as administration immediately following LTP induction is inconsequential to magnitude and stability of LTP. The inconsistencies between the MMP inhibition and the inhibition of cadherins and NCAMs make it unlikely that the effects of MMP inhibition on LTP stability during early-phase maintenance are related to impairment of NCAM or cadherin function.

The similarities between MMP and integrin inhibition on early-phase LTP stability, and the comparable temporal contingencies by which the inhibitors operate, suggests a functional association between MMP activity and integrin-mediated cell adhesion. We hypothesized that MMPs support LTP by promoting integrin function. As a corollary to this, we predict that inhibition of MMPs is deleterious to LTP by negatively impacting integrin operation. If true, the direct inhibition of integrin mediated adhesion with GRGDSP should circumvent the effects of MMP inhibition on LTP stability. Consistent with this prediction, the treatment of slices with GRGDSP abrogated the effect of FN-439 on LTP stability during early-phase. It is possible that the actual causal relationship between MMP and integrin inhibition is obverse (i.e. inhibition of integrins detrimental to MMP function). However, considering the functional relationship between MMPs, the ECM, and integrins, and the time-course by which these events occur, the most parsimonious interpretation is that MMP activity promotes early-phase LTP stability by facilitating integrin function. This hypothesized relationship between MMPs and integrins is strengthened by recent observations that exogenous application of catalytically active MMP-9 results in an integrin dependent potentiation (Nagy, Bozdagi et al. 2006); a finding which suggests that integrin activation is downstream from MMP activity.

MMPs, theta burst facilitation, and LTP induction

Theta burst stimulation is a tetanization protocol, modeled after endogenous hippocampal theta rhythms, that produces a NMDA dependent form of LTP. Under experimental conditions, the burst-response magnitude elicited by TBS directly translates to the level of NMDA receptor activity, and consequently, the degree of calcium influx during tetanization. Ultimately, increased intracellular calcium levels promote activation of effector signaling cascades resulting in the induction of LTP. Serving as a negative regulatory component of this processes are the function of SK channels. SK channels are potassium channels heavily expressed in CA1 dendrites. In the presence of increased intracellular calcium, SK channels produce hyperpolarizing conductances which ultimately suppress subsequent depolarization and resultant calcium entry thereby creating a feed-back loop with NMDA receptor activity. Therefore, suppression of SK channel conductances effectively eliminates this form of feedback regulation and can result in the enhancement of LTP induction (Ngo-Anh, Bloodgood et al. 2005). Furthermore, augmentation of SK channel conductances promotes dendritic hyperpolarization thusly limiting LTP induction (Ngo-Anh, Bloodgood et al. 2005).

Inhibition of MMPs by FN-439 caused a reduction of total area of the burst response during a relatively weak theta burst tetanization scheme; a situation that would likely result in comparably diminished calcium entry during tetanization. Consistent with this, the reduction of burst response with MMP inhibition was accompanied with a corresponding and proportional reduction of burst afterpositivity. The abatement of the burst response by MMP inhibition apparently impacted the induction of LTP. This was evidenced by the fact that a weak theta burst tetanization scheme was unable to produce LTP in MMPi treated slices, whereas a robust theta burst scheme was able to overcome these effects and generate LTP; presumably by effecting

sufficient depolarization so as to promote NMDA activity and calcium entry adequate for the induction of LTP.

Because the relationship between burst magnitude and afterpositivity was unaffected by MMP inhibition, the effect of MMP inhibition on LTP induction is likely unrelated to direct manipulation of SK channel conductances. Therefore, there are two main possibilities by which MMP inhibition could lead to a reduction of burst response. First, treatment with MMP inhibitor negatively affects AMPA receptor responsiveness. This seems doubtful considering that treatment with MMP inhibitor does not alter input-output relationships or baseline transmission. A second possibility is that MMP inhibition negatively affects burst associated neurotransmitter release. Implicit to this, MMP activity somehow promotes neurotransmitter release during the high-frequency bursts which comprise the theta train. Accordingly, inhibition of MMPs during theta burst would lead to a reduction of the burst response and a potential abatement of LTP induction. Although a plausible mechanism by which MMPs influence neurotransmitter release is unknown, some support for this possibility exists in the observation that FN-439 lead to a reduction of paired-pulse facilitation (PPF). It is generally accepted that PPF stems from increased release probability (i.e. probability that a competent neurotransmitter vesicle will be released by action potential) for a succeeding pulse. Under normal conditions, increased release probability is thought to be supported by residual presynaptic calcium that results in the priming of calcium sensitive neurotransmitter release machinery. Whether the effect of MMP inhibition on PPF relates to effects on theta burst facilitation (and by extension, LTP induction) will be determined by future inquiry.

MMPs and plasticity

A recent article supports a role for MMP-9 in synaptic plasticity and learning (Nagy, Bozdagi et al. 2006). Although the overall conclusion precipitating from this paper are consistent with the findings presented here (i.e. an apparent role for MMPs in learning associated synaptic plasticity), there are a number of divergences as well. First of all, specific inhibition of MMP-9 failed to affect stabilization of early-phase LTP whereas stabilization of early phase LTP was clearly impacted by MMP inhibition with FN-439. Second, in contrast to specific inhibition of MMP-9, MMP inhibition by FN-439 resulted in an attenuation in the magnitude of LTP induction; an effect likely due to suppressed calcium entry during theta bursts. Third, FN-439 inhibited stabilization of LTD; on the contrary, inhibition of MMP-9 failed to affect LTD. A probable explanation for these divergent effects is that results presented here are due to inhibition of MMPs other than MMP-9. We have recently described a role for MMP-3 in synaptic plasticity underlying learning. MMP-3 protein and mRNA is up-regulated in CA3 and CA1 during acquisition of water maze spatial learning task. Learning associated MMP-3 increase is NMDA dependent and essential for water maze acquisition. Because MMP-3 participates in learning associated plasticity, we view MMP-3 as a prime candidate in future studies. Additionally, MMP-7 has been shown to induce morphological modifications to dendritic spines (Bilousova, Rusakov et al. 2006). Interestingly, these modifications were demonstrated to be NMDA dependent. Considering the importance of NMDA receptor activity and morphological dynamics of dendritic spines to learning associated plasticity, it is tempting to speculate that at least part of the effects elicited by FN-439 treatment are due to MMP-7 inhibition.

Conclusion

The work presented herein has demonstrated that MMPs are involved in multiple forms of short-term and long-term hippocampal plasticity. Although we have previously demonstrated that MMP activity is necessary for the stabilization of early-phase LTP, we now provide evidence MMPs promote LTP stabilization by actuating integrin function. In addition to LTP stability, we have also shown that MMP activity affects the threshold of LTP induction by affecting burst response during theta. Interestingly, the effect of MMP inhibition on burst response, with the consequential effect on LTP induction, does not appear to be mediated by integrin activity. Together, these findings argue for a complex involvement of MMPs in hippocampal plasticity. Future work should be directed at identifying the specific MMPs involved in these forms of hippocampal plasticity and teasing apart the specific mechanisms by which they operate.

FIGURE LEGENDS

Figure 1: Inhibition of MMPs interferes with multiple forms of Schaffer collaterals-CA1 plasticity. (A) Input-output curves generated from MMP inhibitor (FN-439) treated (filled circles) and control slices (open circles) with representative traces for each condition. **(B)** Paired pulse facilitation of amplitudes summary data expressed as percent change from fEPSP1 amplitude for a given interstimulus interval (ISI) (*$p<.05$; unpaired two tailed t-test). Representative traces for FN-439 treated (hashed line) and control (solid line) for 50ms ISI. Arrow highlights representative difference between control and FN-439 treated slices occurring for fEPSP2. **(C)** Effects of MMP inhibition on LTP induced by a robust TBS paradigm. LTP was induced at t=0 min. (indicated by arrowhead), by 4-TBS trains, and was subsequently monitored for 60 min. FN-439 administration began 10min. prior to tetanus and was present in bath throughout experiment. Representative traces provided for FN-439 treated and control slices for baseline (hashed arrow) and maintenance (solid arrow) phases. Data expressed as percent change from baseline of mean fEPSP amplitude (+/- S.E.M.). **(D)** Effects of MMP inhibition on long term depression (LTD). LTD induced by low frequency stimulation (LFS; 900 pulses at 1Hz frequency) in FN-439 treated or control slices. Administration of FN-439 began 10min prior to LFS and was present for duration of time-course. Representative traces provided for FN-439 and control slices for baseline (hashed arrow) and maintenance (solid arrow) phases.

Figure 2: Temporal relationship between FN-439 administration and TBS differentially effects LTP stabilization. Hippocampal slices were subjected to a 30 minute exposure of FN-439 in one of three temporal conditions: **(A)** immediately following TBS (PT_0), **(B)** 30 minutes

following TBS (PT_{30}), or **(C)** 15 minutes following TBS (PT_{15}) (filled circles: FN-439; open circles: control). Presence of FN-439 is represented by black bar adjacent to time-axis; tetanization indicated by arrowhead. Data expressed as percent change from baseline of mean fEPSP amplitude (+/- S.E.M.). **(D)** Comparison of the relative efficacy of FN-439 on LTP stabilization for the three administration schemes by regression analyses (black circles: PT_0, grey circles: PT_{15}, and open circles: PT_{30}). Amplitudes elicited during treatment interval were subtracted from temporally-matched mean control amplitudes. Regression analysis performed on replicates for each group (n=152/group) mean responses for each time-point shown for sake of clarity. PT_0: slope = 0.6816 ± 0.1779 ($p<.001$). PT_{30}: slope = 0.1251 ± 0.5438 ($p>.05$). PT_{15} exhibited a significant nonlinearity (Runs Test: $p<.05$) not present in PT_0 and PT_{30}, and was fit to a one-phase exponential association function (hashed line).

Figure 3: Integrin antagonist, Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), occludes the effect of MMP inhibitor, FN-439, on LTP stability. black circles), or vehicle (aCSF; open circles). Infusion of inhibitors began 10 minutes prior to tetanization and persisted for duration of experiment. Data expressed as percent change from mean baseline response (+/- S.E.M.).

Figure 4: MMP inhibition reduces theta burst facilitation elicited by a *robust* theta burst stimulation protocol. FN-439 treated and control slices were subjected to a theta burst paradigm consisting of 4-trains (consisting of 6 bursts per train at 5Hz frequency) with an interburst interval of 10 seconds. **(A)** Evoked responses for FN-439 treated (filled bars) (n=4) and control slices

(open bars) (n=6), elicited by TBS, were recorded and analyzed. Statistical analysis by two-factor ANOVA (treatment **x** burst #) indicates an independent effect of MMP inhibition on burst magnitude for both train-1 ($p<.01$) and train-4 ($p<.0001$). **(B)** In contrast to MMPi, treatment with integrin antagonist (GRGDSP) failed to produce a significant effect on burst response area for both train-1 and train-4 ($p>.05$). **(C)** Mean amplitudes of after-positivity (prolonged positive potential subsequent to negative "burst" potentials) for FN-439 treated and control slices. MMP inhibition failed to affect the magnitude of after-positivity elicited by robust TBS ($p>.05$). **(D)** Regression analyses of burst area versus resultant amplitude of after-positivity for both FN-439 treated (filled circles; hashed regression line) and control (open circles; solid regression line) slices. Difference between slopes and y-intercepts of two regression lines is not significant ($p>.05$).

Figure 5: MMP inhibition reduces theta burst facilitation and burst afterpositivity elicited by an *attenuated* theta burst stimulation protocol. FN-439 treated and control slices were subjected to a theta burst paradigm consisting of a single TBS train consisting of 6 bursts, with a 50% reduction of pulse duration. **(A)** Evoked responses for FN-439 treated (filled bars) (n=4) and control slices (open bars) (n=4), elicited by TBS, were recorded and analyzed. Statistical analysis by two-factor ANOVA (treatment **x** burst #) indicates an independent effect of MMP inhibition on area of burst response ($p<.001$). Sample waveforms of response for both MMPi and control conditions provided for both bursts #1 (solid line) and #4 (hashed line). **(B)** Mean amplitudes of after-positivity for FN-439 treated and control slices. Statistical analysis by two-factor ANOVA (treatment **x** burst #) indicates an independent effect of MMP inhibition on

amplitude of afterpositivity ($p<.0001$). Sample traces of afterpositivity (subsequent to burst #3) provided for both control (solid line) and MMPi treated (hashed line) conditions.

Figure 6: MMP inhibition disrupts induction, but not maintenance, when LTP is stimulated by an *attenuated* theta burst stimulation protocol. FN-439 treated and control slices were subjected to an attenuated theta burst paradigm and were subsequently monitored for LTP. **(A)** LTP timecourse for both FN-439 treated (closed circles) (n=4) and control (open circles) (n=4) conditions. Regression analysis was performed on replicates to assess rate of decay of MMPi treated slices, relative to control values, following robust tetanization and attenuated tetanization. **(B)** For robust tetanization, MMP inhibition produced a significantly greater rate of decay than controls (slope = 0.4002 ± 0.01734 ($p<.0001$) n = 71 replicates). **(C)** For attenuated tetanization, decay rate did not differ significantly from controls (slope = 0.1706 ± 0.1116 ($p>.05$) n = 29).

Figure 1

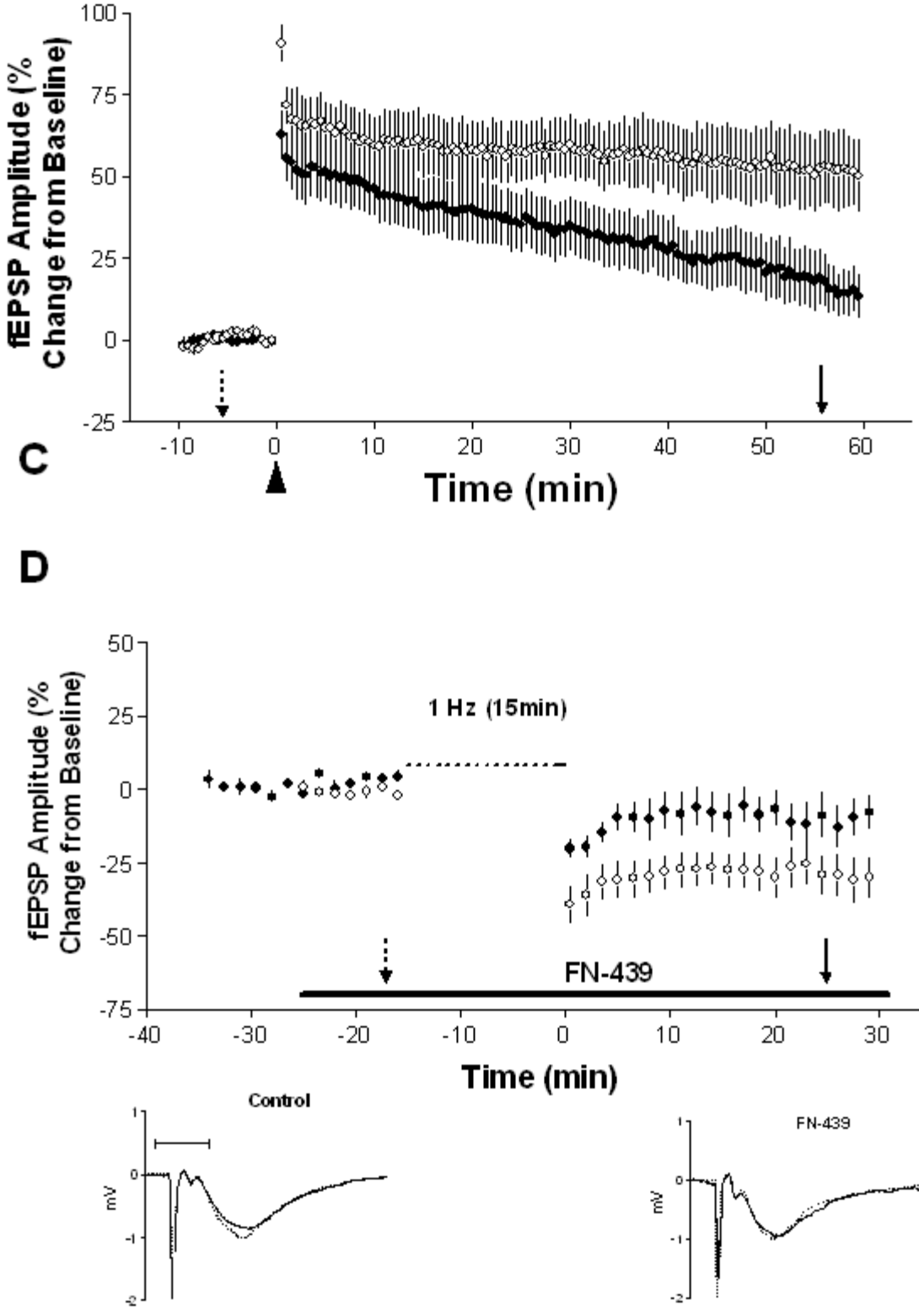
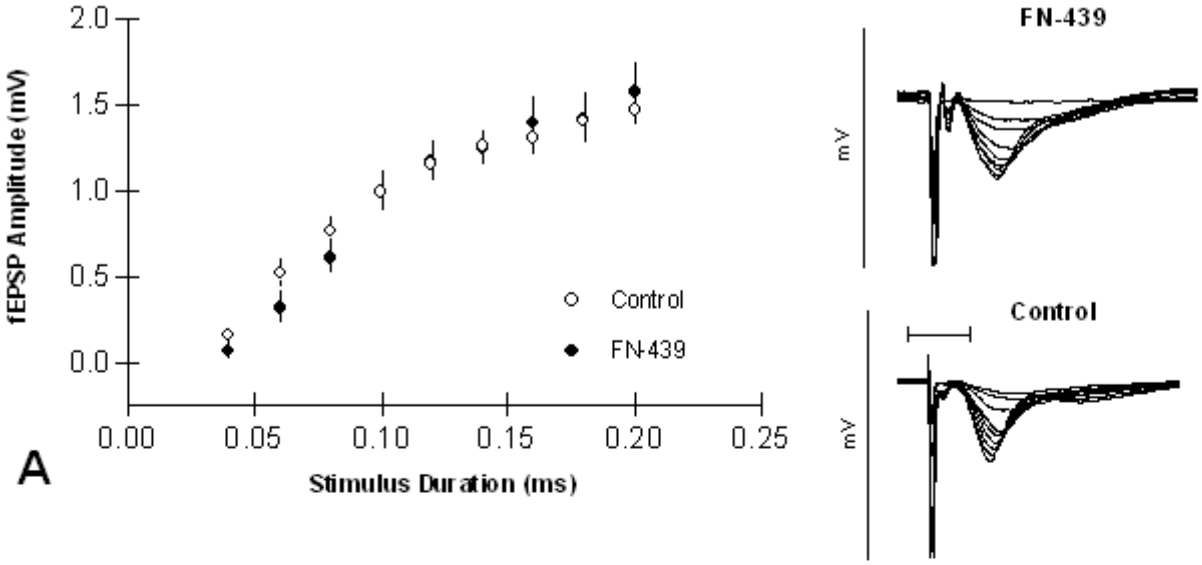

C
fEPSP Amplitude (% Change from Baseline)
100
75
50
25
0
-25
-10
0
10
20
30
40
50
60
Time (min)
D
fEPSP Amplitude (% Change from Baseline)
50
25
0
-25
-50
-75
1 Hz (15min)
FN-439
-40
-30
-20
-10
0
10
20
30
Time (min)
Control
FN-439
mV
1
0
-1
-2

Figure 1


2.0
1.5
1.0
0.5
0.0
fEPSP Amplitude (mV)
0.00
0.05
0.10
0.15
0.20
0.25
Stimulus Duration (ms)
Control
FN-439
A
FN-439
mV
Control
mV

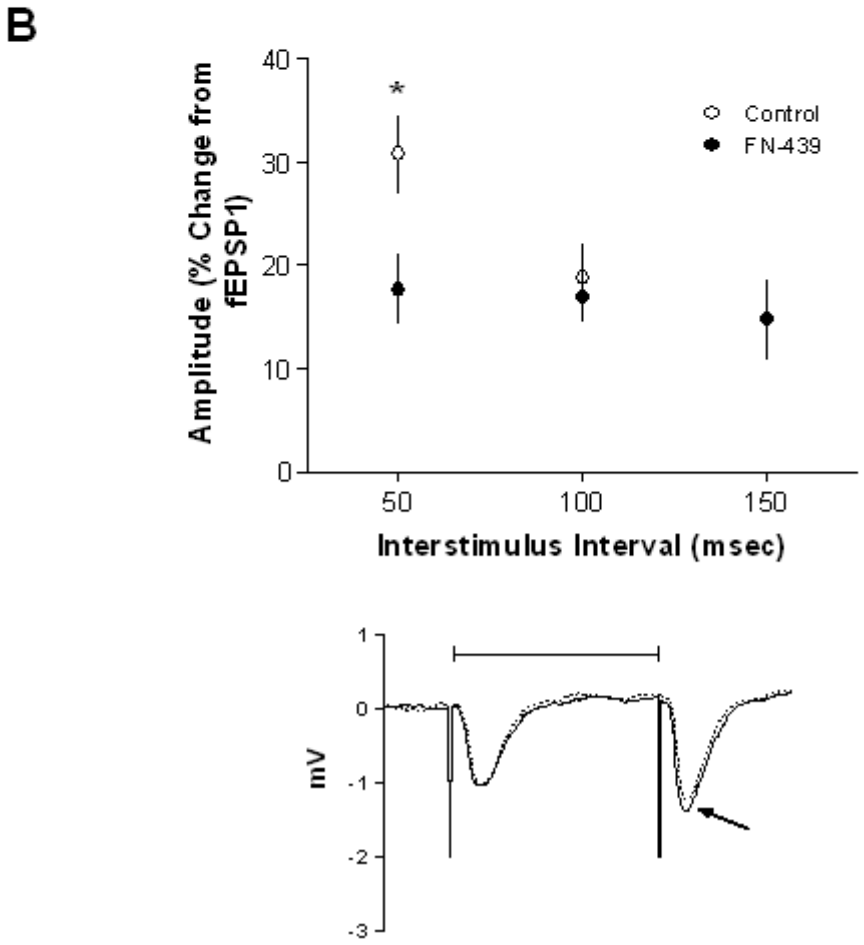

B
40
30
20
10
0
Amplitude (% Change from fEPSP1)
*
Control
FN-439
50
100
150
Interstimulus Interval (msec)
1
0
-1
-2
-3
mV

Figure 2

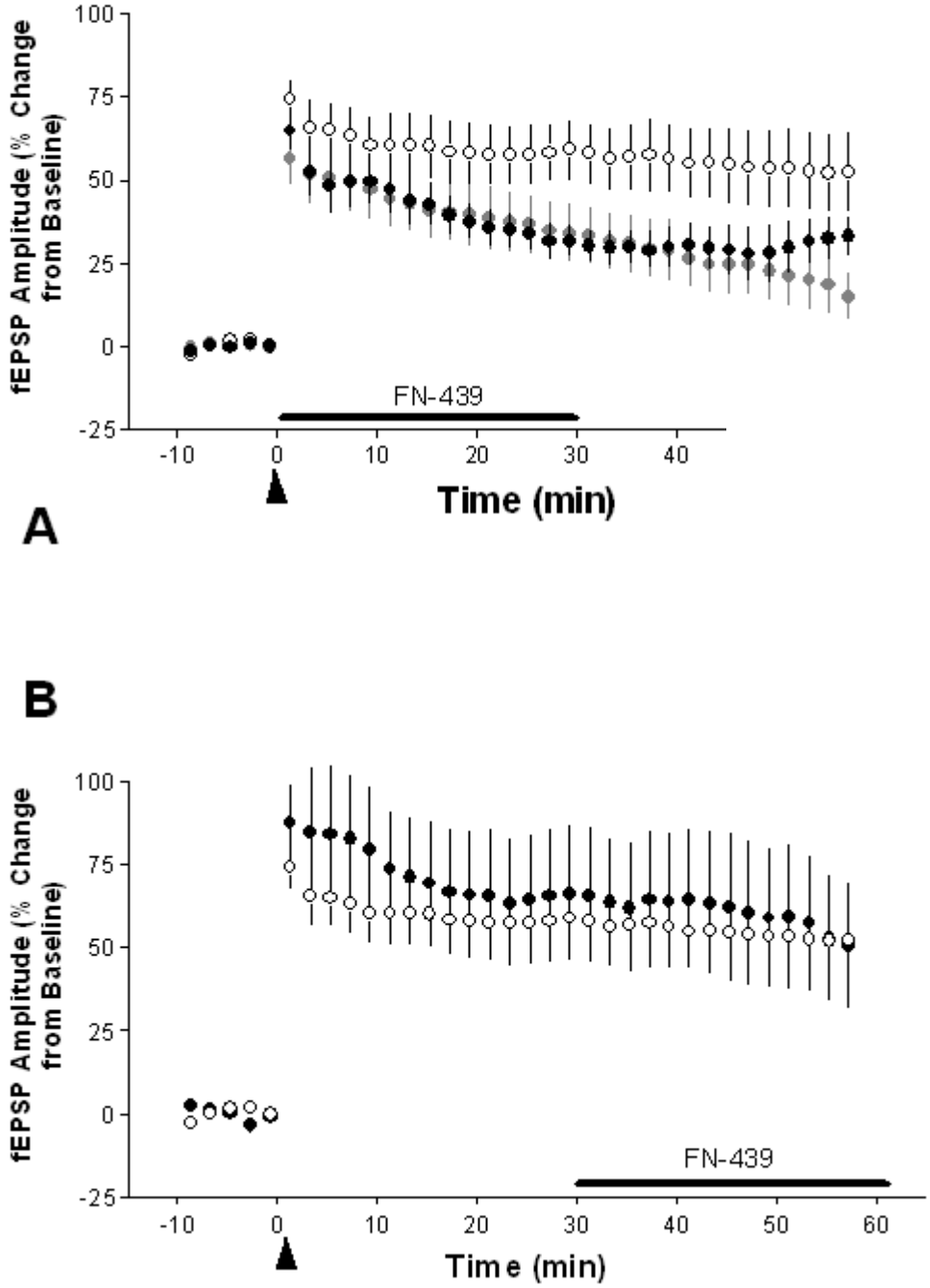
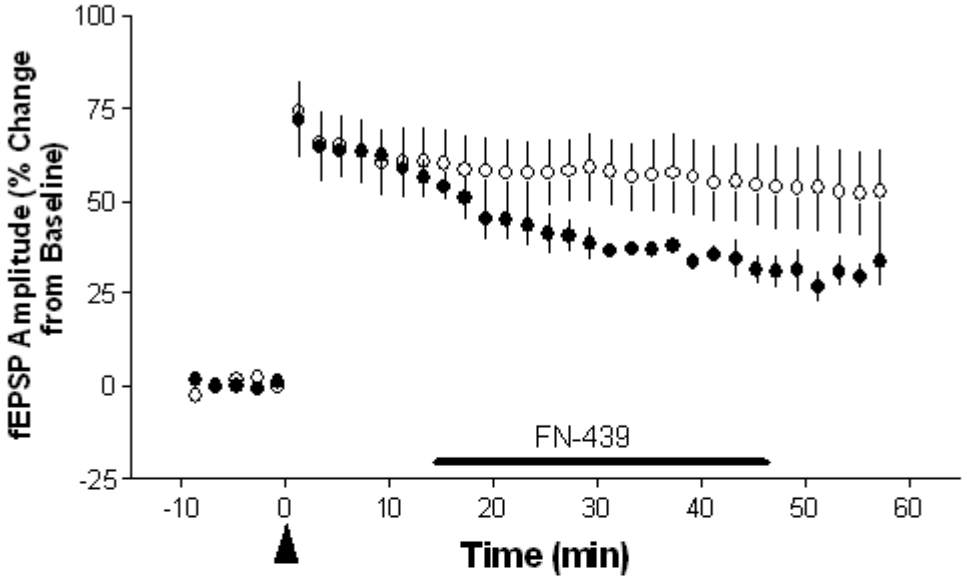

A
fEPSP Amplitude (% Change from Baseline)
100
75
50
25
0
-25
FN-439
-10
0
10
20
30
40
Time (min)
B
fEPSP Amplitude (% Change from Baseline)
100
75
50
25
0
-25
FN-439
-10
0
10
20
30
40
50
60
Time (min)

Figure 2


fEPSP Amplitude (% Change from Baseline)
100
75
50
25
0
-25
FN-439
-10
0
10
20
30
40
50
60
Time (min)

C

D

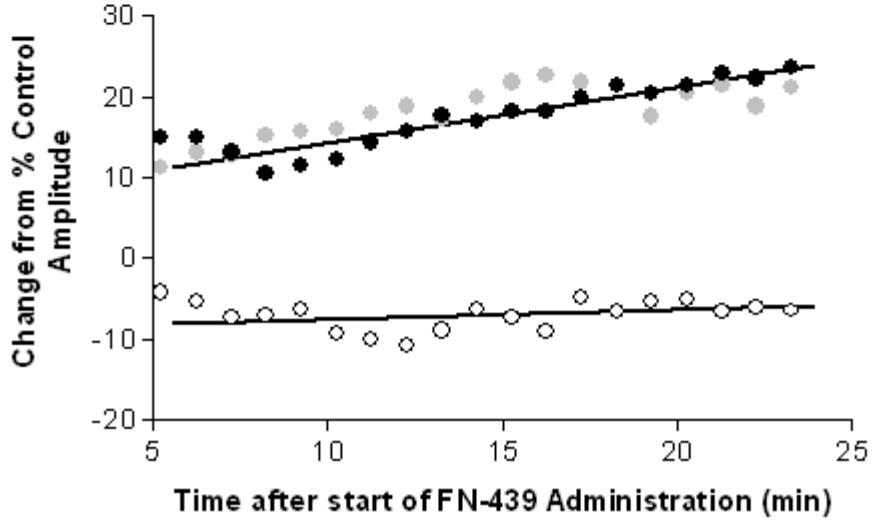

Change from % Control Amplitude
30
20
10
0
-10
-20
5
10
15
20
25
Time after start of FN-439 Administration (min)

Figure 3

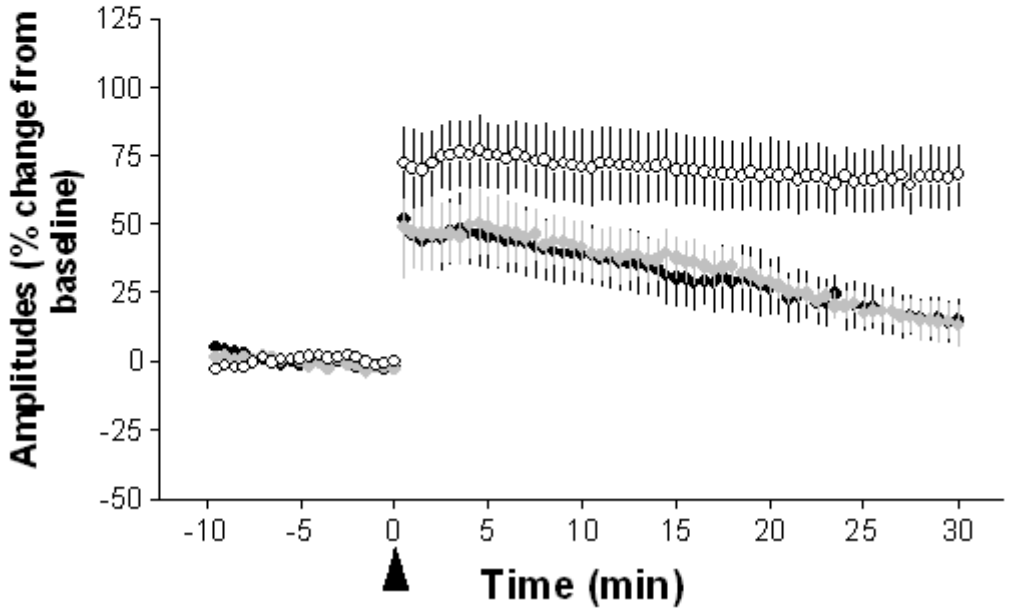
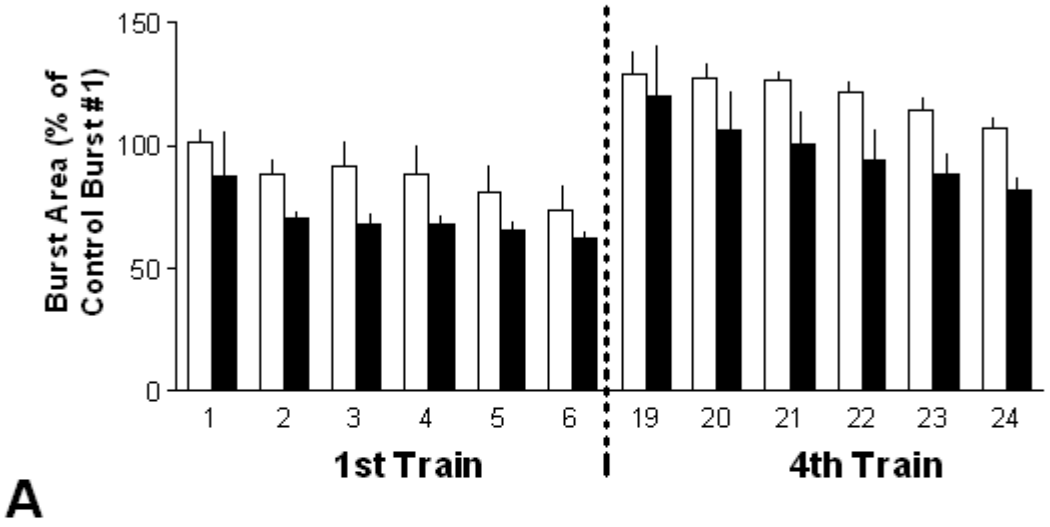

Amplitudes (% change from baseline)
125
100
75
50
25
0
-25
-50
-10
-5
0
5
10
15
20
25
30
Time (min)

Figure 4


Burst Area (% of Control Burst #1)
150
100
50
0
1
2
3
4
5
6
19
20
21
22
23
24
1st Train
4th Train

A

B

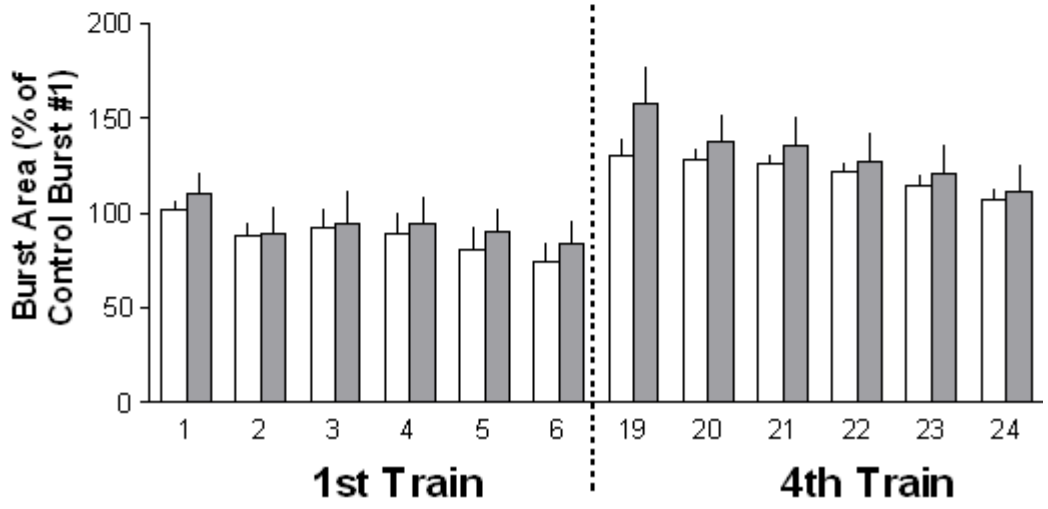
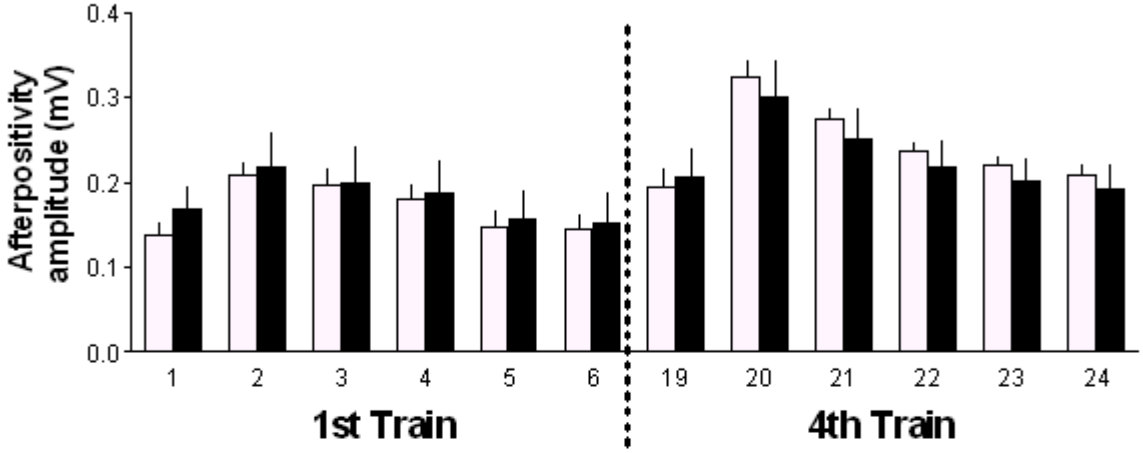

Burst Area (% of Control Burst #1)
200
150
100
50
0
1
2
3
4
5
6
19
20
21
22
23
24
1st Train
4th Train

Figure 4


Afterpositivity amplitude (mV)
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
19
20
21
22
23
24
1st Train
4th Train

C

D

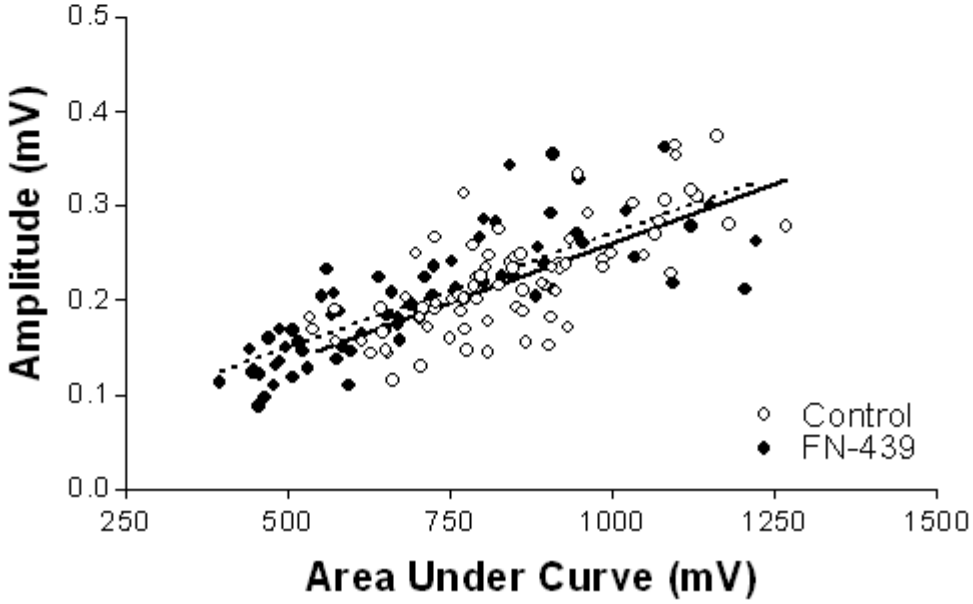

Amplitude (mV)
0.5
0.4
0.3
0.2
0.1
0.0
250
500
750
1000
1250
1500
Area Under Curve (mV)
Control
FN-439

Figure 5

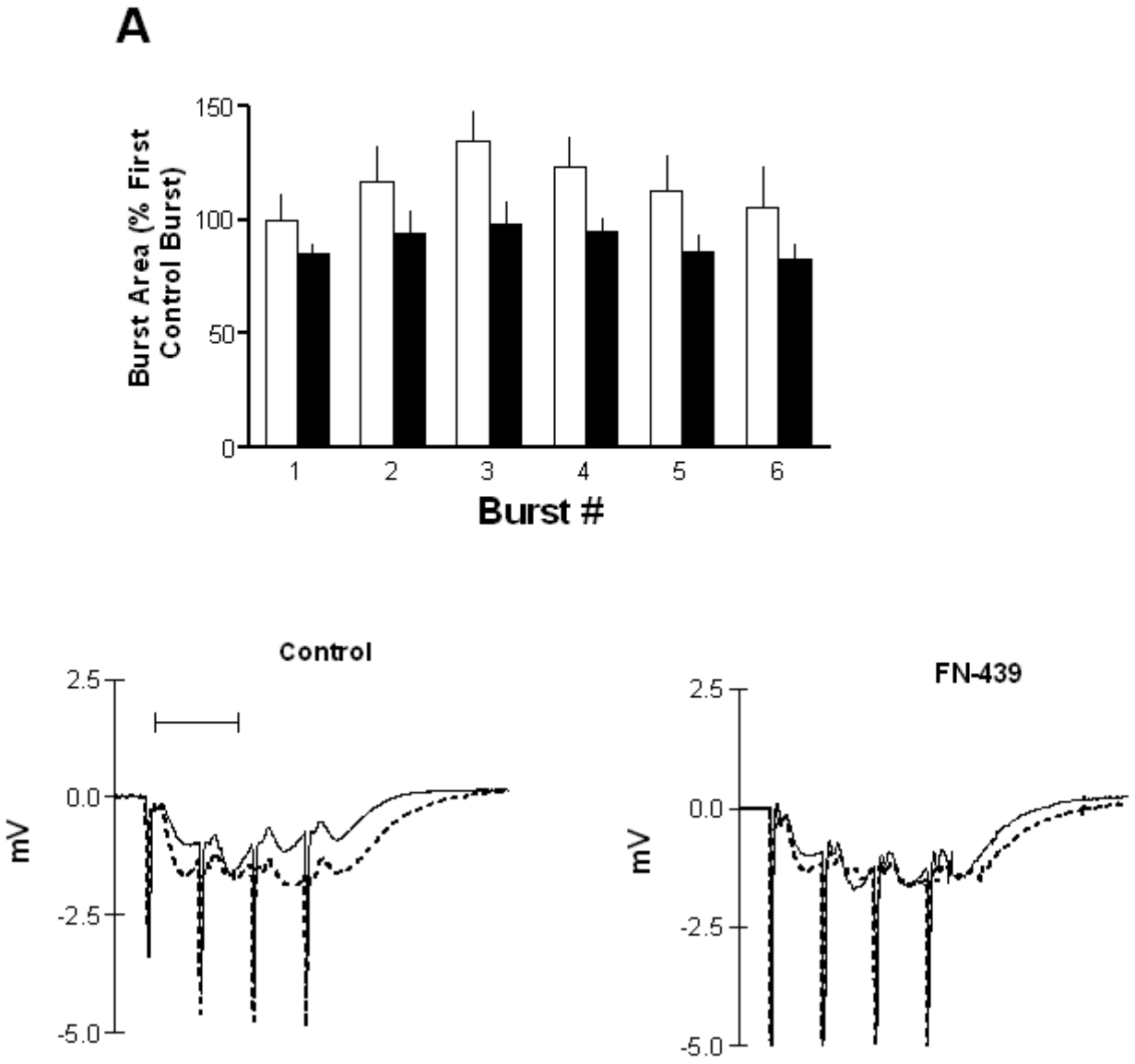

A
Burst Area (% First Control Burst)
150
100
50
0
1
2
3
4
5
6
Burst #
Control
2.5
0.0
-2.5
-5.0
mV
FN-439
2.5
0.0
-2.5
-5.0
mV

Figure 5

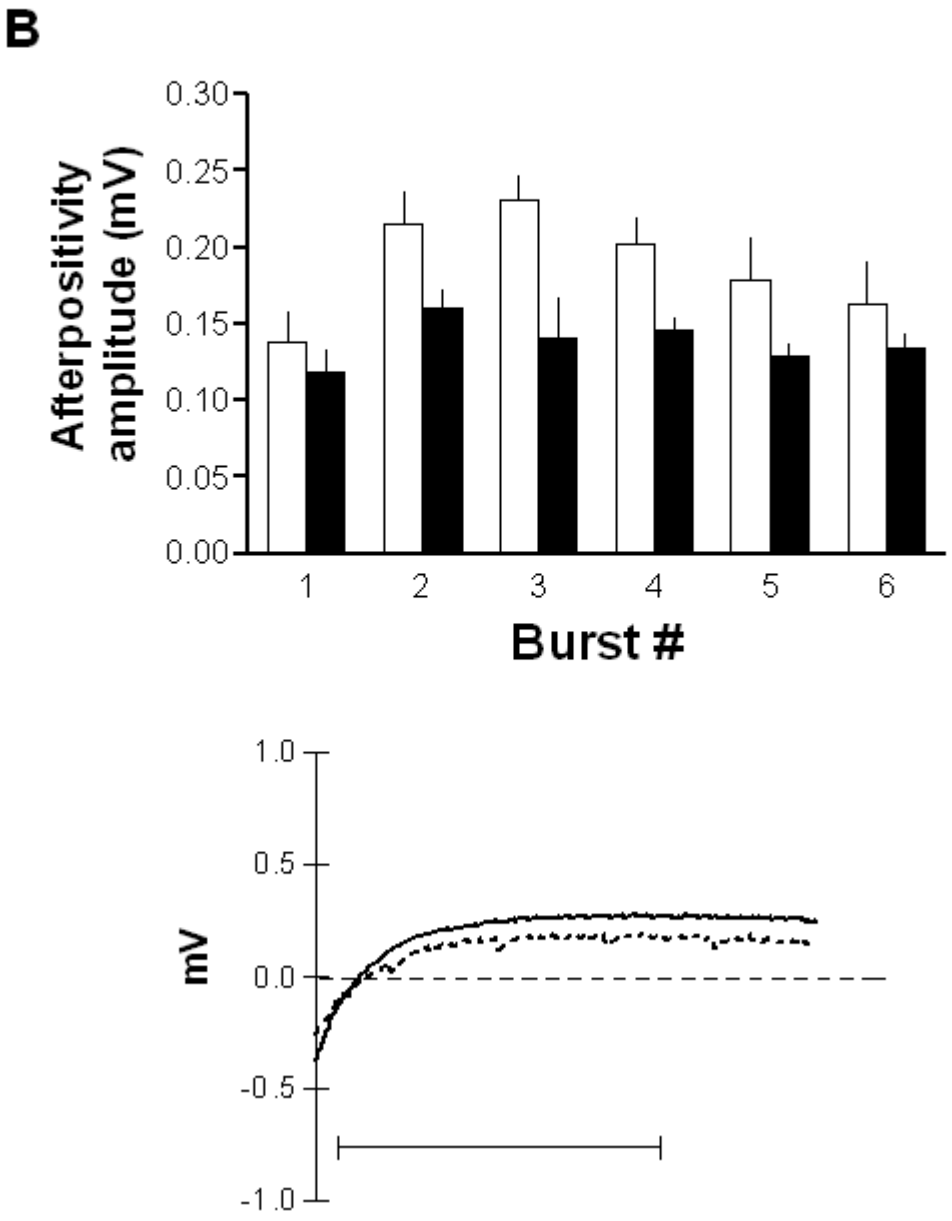

B
Afterpositivity amplitude (mV)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1
2
3
4
5
6
Burst #
1.0
0.5
0.0
-0.5
-1.0
mV

Figure 6

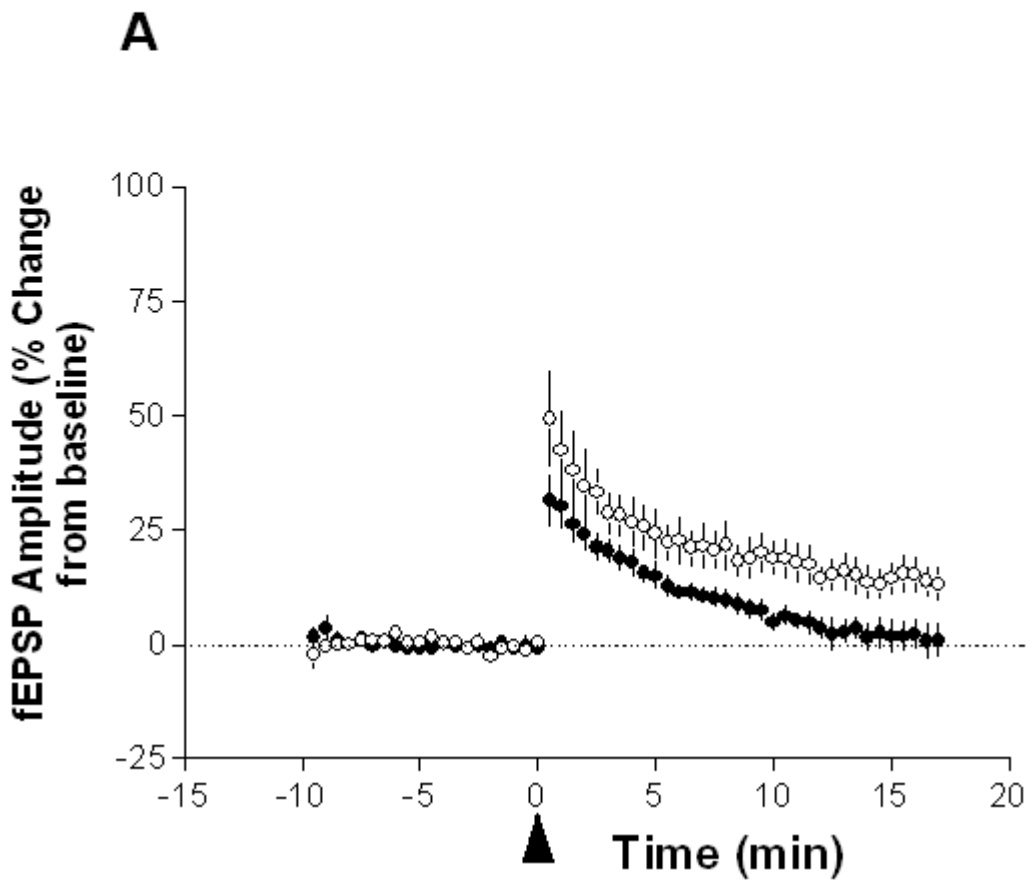
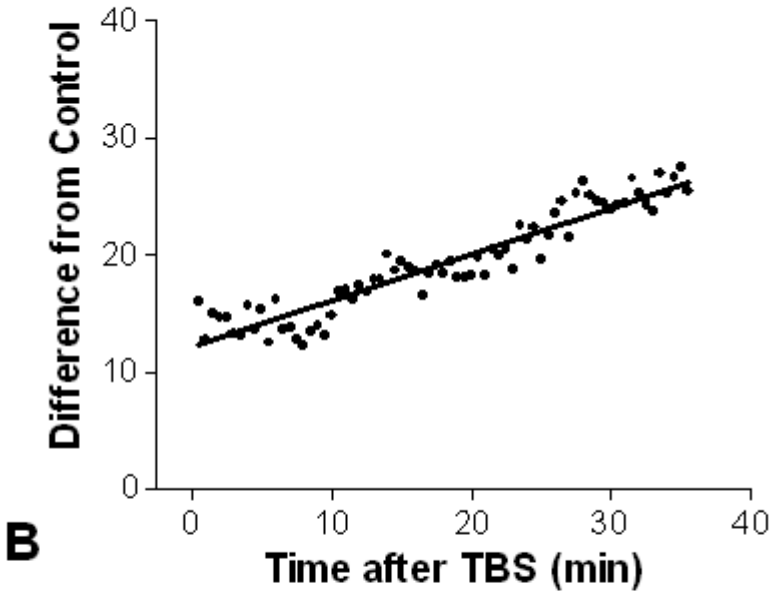
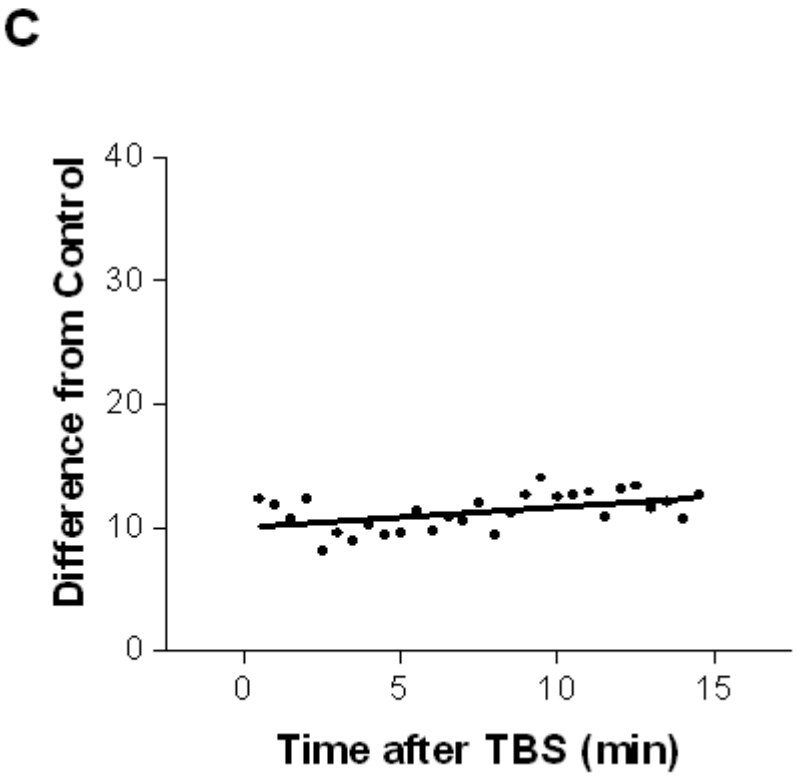

A
fEPSP Amplitude (% Change from baseline)
100
75
50
25
0
-25
-15
-10
-5
0
5
10
15
20
Time (min)

Figure 6


B
Difference from Control
40
30
20
10
0
0
10
20
30
40
Time after TBS (min)


C
Difference from Control
40
30
20
10
0
0
5
10
15
Time after TBS (min)

CHAPTER II

ELEVATED MATRIX METALLOPROTEINASE -3 ASSOCIATED WITH AGING IS DETRIMENTAL TO HIPPOCAMPAL PLASTICITY

ABSTRACT

Matrix metalloproteinases (MMPs) are a well recognized family of proteases that are actively involved in the regulation of tissue remodeling. While most past studies have focused on this action of MMPs outside of the central nervous system, recent investigations have begun to probe the potential role of these proteins in synaptic remodeling. Ongoing studies in our laboratory have indicated that transient expression of MMPs is essential for spatial learning and hippocampal LTP. Results indicate that aged rats have markedly elevated levels of MMP-3 and that the ability to generate TBS induced LTP is limited. We hypothesized that excessive MMPs interferes with the normal remodeling processes that are requisite for the synaptic plasticity that accompanies learning and memory consolidation. To test this hypothesis, we first subjected hippocampal slices from aged-adult (20 months) Sprague Dawley rats to a broad-spectrum MMP inhibitor prior to LTP testing. We observed that MMP inhibition enhanced LTP induction relative to untreated slices. This same treatment condition produced deficits in early-phase LTP maintenance in young-adult slices (3 months). Furthermore, we observed that exogenous application of catalytically active MMP-3 fragment to young-adult slices resulted in destabilization of LTP during early maintenance phase. These results suggest that age-related over-expression of hippocampal MMPs are deleterious to hippocampal plasticity.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of secreted and cell-surface endopeptidases found in nearly every tissue-type where they typically participate in tissue remodeling. They are classified by their substrate specificity and degrade numerous pericellular molecules including other proteinases, latent growth factors, growth factor-binding proteins, cell surface receptors, cell-cell adhesion molecules, and the majority of extracellular matrix proteins (ECM) (Sternlicht and Werb 2001) . MMP catalytic activity is regulated on multiple levels, including: transcription, mRNA stability, secretion, activation, inhibition by endogenous inhibitors, and clearance. MMPs are secreted as an inactive zymogen which requires activation by proteolysis. The removal of the N-terminal "pro" domain of latent-MMP initiates MMPs' catalytic activity. Inactivation of MMPs is notably accomplished through sequestration by the endogenous tissue inhibitors of MMPs (TIMPs). To date, the TIMP family is composed of four TIMP proteins (TIMP1-4) – where each TIMP molecule is capable of inhibiting a distinct set of MMPs.

MMPs have been identified as key enzymes in both normal and abnormal neuronal development and remodeling and disease states associated with neurodegeneration. Expression of active MMP-9 is necessary for oligodendrocyte process extension and myelination (Uhm, Dooley et al. 1998; Oh, Larsen et al. 1999), and is elevated during postnatal cerebellar development (Vaillant, Didier-Bazáes et al. 1999). MMP-9 expression increases in the hippocampus following kainic acid induced seizures (Zhang and Gottschall 1997; Zhang, Deb et al. 1998; Jourquin, Tremblay et al. 2003) and is correlated with subsequent synapse formation. Following cerebral ischemia increased MMP-9 is associated with altered blood brain barrier function (Anthony, Ferguson et al. 1997; Romanic, White et al. 1998; Fujimura, Gasche et al. 1999; Gasche, Fujimura

et al. 1999). Altered gelatinase regulation has also been implicated in cognitive impairments associated with several nervous system disorders. Both MMP-2 and -9 are elevated in multiple sclerosis and stroke patients (Anthony, Ferguson et al. 1997; Lim, Russell et al. 1997); and increased latent hippocampal MMP-9 in Alzheimer's patients has led to speculation that a decrease in active MMP-9 could be involved in the deposition of beta-amyloid (Backstrom, Lim et al. 1996; Lim, Russell et al. 1997; Asahina, Yoshiyama et al. 2001; Lorenzl, Albers et al. 2003; Lorenzl, Albers et al. 2003). In addition, MMP-9 is increased in the spinal fluid of HIV patients and is associated with HIV-related dementia (Conant, McArthur et al. 1999; Liuzzi, Mastroianni et al. 2000; Zhang, McQuibban et al. 2003). MMP-3 is a stromelysin that degrades ECM and also activates latent proMMPs, including MMP-9 (Ogata, Enghild et al. 1992). Alterations in MMP-3 have also been reported in patients with the same disease processes that affect gelatinases (Anthony, Ferguson et al. 1997). Specifically MMP-3 is decreased in senile plaques of Alzheimer's disease patients (Yoshiyama, Asahina et al. 2000) and beta-amyloid peptide stimulates MMP-3 release from hippocampal cultures (Deb and Gottschall 1996). Several of these early studies have provided subtle portent to the possible role of MMPs in age-associated disease states.

Although the linkage of MMPs to neural development and axonal guidance has long been appreciated (see review (Ayoub, Cai et al. 2005; Luo 2005), linkage of MMPs to neural plasticity and especially that associated with cognition is recent. Initial indications that MMPs -3 and -9 might play a role in neuronal plasticity were derived from studies that examined the impact of physical or chemical trauma on synaptic plasticity (Zhang, Deb et al. 1998; Reeves, Prins et al. 2003; Kim, Fillmore et al. 2005) where the site of action seems to be the dendrite and its spines (Szklarczyk, Lapinska et al. 2002; Bilousova, Rusakov et al. 2006). Our recent study (Meighan,

Meighan et al. 2006), along with a nearly concurrent study by Nagy et al. (Nagy, Bozdagi et al. 2006) were the first to demonstrate that MMP modulation is necessary for efficient learning and synaptic plasticity.

Alterations to MMP function appear to be a common feature of aging for a variety of non neuronal tissues. When serial passage of human dermal fibroblasts was used to simulate aging, later passage ("older") cultures were found to have higher MMP expression than early passage cultures (Millis, McCue et al. 1992; Khorramizadeh, Tredget et al. 1999). Fibroblasts cultured from individuals with Werner syndrome, a disease characterized by premature senescence, expressed MMP levels similar to late passage normal fibroblasts (Millis, Hoyle et al. 1992). Senescent fibroblasts have also been observed to release large amounts of MMP-3, which appears to trigger the differentiation of surrounding cells to a malignant phenotype, a finding that has broad implications for our understanding of age-associated pathologies (Parrinello, Coppe et al. 2005). It has been hypothesized that delayed wound healing in aged individuals, which is heavily dependent on fibroblast function, could be a result of MMP over-activity (Ballas and Davidson 2001). It also appears that extrinsic (i.e. UV radiation photoaging) and intrinsic (chronological) factors associated with skin aging both have a common underlying component, over-induction of MMPs. These studies have led to the use of topical MMP inhibitors, Melanocin A and eicosapentaenoic acid, as treatments for damage-associated skin aging (Park, Lee et al. 2005; Kim, Cho et al. 2006). Furthermore, aging has also been shown to be associated with increased MMP levels in: vascular tissue (Jacob 2003; Wang, Takagi et al. 2003; McNulty, Spiers et al. 2005), the myocardium (Lindsey, Goshorn et al. 2005) and chondrocytes (Forsyth, Cole et al. 2005). Increased MMP levels have also been implicated in lens cataract formation (Descamps, Martens et al. 2005), a pathology commonly associated with aging.

The dysregulation of MMPs in a broad assortment of aged tissues and organs coupled with apparent dysregulation of MMPs in age-associated nervous system diseases, such as Alzheimer's disease, led us to hypothesize that MMP dysregulation may also be a property of brain aging. Considering that MMPs have an important role in hippocampal plasticity, we focused our examination on the effects of aging on hippocampal MMPs. We hypothesized that if hippocampal MMPs were altered as a result of senescence, then this dysregulation would translate into functional deficits. To address these related hypotheses, we first assessed the levels of MMPs in aged hippocampi in comparison to those expressed by young adult rats. Second, we assessed the impact of inhibiting MMPs with broad-spectrum inhibitor FN-439 on hippocampal LTP from aged-adult rat slices. Third, we attempted to recapitulate the effects of aging on hippocampal plasticity and fEPSP waveform characteristics by artificially increasing MMP levels. The results indicate that increased hippocampal MMPs play an important role in the functional deficits associated with hippocampal aging.

MATERIALS AND METHODS

Tissue preparation

Rats were sacrificed by decapitation, and the hippocampus from each hemisphere was quickly dissected on ice, immediately frozen in liquid nitrogen and stored at -80^0C until all sample groups were collected. One hippocampus from each animal was analyzed for MMP protein levels and the other for MMP mRNA expression (see *RT-PCR analysis* methods).

Tissues for protein analysis were weighed and immediately homogenized on ice in a volume of homogenization buffer (50mM Tris HCl pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij 35, 0.02% NaN_3) to give a final sample concentration of 1mg/ml. Homogenates were centrifuged at 12,000 x g for 5 min, 4^{o}C and the supernatant fraction was recovered for analysis by immunoblotting.

Western immunoblotting

Supernatants were mixed 1:1 with 2x Laemelli sample buffer plus -mercaptoethanol. Samples were subjected to SDS-PAGE and subsequently transferred onto a nitrocellulose membrane. Following transfer, membranes were pre-blocked in 4% milk/ TBS prior to the addition of primary antibody. Membranes were incubated in primary antibody overnight at 4^o C [1:2000, MMP-9 (Abcam, Cambridge, MA); 1:2000, MMP-3 (RDI, Flanders, NJ); 1:1000, MMP-2 (Chemicon, Temecula, CA); 1:2000 cortactin (Upstate, Charlottesville, VA)]. After rinsing, blots were incubated for 2 hours with a 1:10,000 dilution of HRP-conjugated secondary antibody and rinsed again in TBS/TTBS. Visualization was achieved with Pierce SuperSignal and subsequent exposure

to Kodak X-Omat Blue film. Signal intensity per volume was quantitated using TotalLab Image Analysis software.

Generation of Hippocampal Slices

Rats were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ, USA), decapitated and the brain was rapidly removed. The brain was immediately placed into ice-chilled, oxygenated aCSF for approximately 30 s. The hemispheres were separated by a mid-sagittal cut and the hippocampus was removed from the right hemisphere using a custom-built wire loop instrument. Slices (400 um thick) from the middle third portion of the hippocampus were prepared using a McIlwain tissue chopper (Brinkmann, Gomshall, UK) and transferred to a gassed (95% O_2 / 5% CO_2) incubation chamber containing aCSF, where they were maintained for at least 1 h at 22–23_C. Single slices were then transferred to a perfusion–recording chamber and stabilized on the chamber floor (coated with Sylgard; Dow Corning, Midland, MI, USA) by platinum wires. Slices were continuously superfused with gassed aCSF (30–31_C) at a rate of 1–1.5 mL/min via a peristaltic pump (Rainen Rabbit-Plus, Woburn, MA, USA).

Electrophysiology

Extracellular recordings from the CA1b stratum radiatum layer were obtained using glass micropipettes filled with 0.15 M NaCl, yielding a resistance of 2–3 MΩ. Orthodromic activation of the Schaffer collaterals within the CA1c was accomplished using concentric bipolar stimulating electrodes (Rhodes Medical Instruments, Inc., Woodland Hills, CA, USA) positioned adjacent to the recording electrodes in the CA1c stratum radiatum layer. Test stimuli (0.1 ms, 0.1 Hz) were delivered using a stimulator (Model S88; Grass, Quincy, MA, USA) to elicit field excitatory

postsynaptic potential (fEPSP) responses. The duration of stimulation was adjusted in each case to produce a dendritic field potential that was 50–60% of the maximum spike-free response (.08–.12 ms).

LTP was induced by theta patterned high-frequency stimulation. This was accomplished by the application of 4 trains of theta burst stimulations (TBSs) with an inter-train interval of 10s. Each train was comprised of 6 bursts of four 100Hz , .35ms pulses, separated by 200ms. Extracellular signals were amplified (gain 1000x) and filtered (1 kHz) using an amplifier (Model 1800; A-M Systems, Newport, WA, USA). Data were digitized and analyzed using a computer interfaced PowerLab/400 (ADInstruments Inc., Dover, NH, USA).

Drug Application

For LTP FN-439 (180 uM; Sigma Chemical) was administered for 3 hours prior to transfer to recording chamber. Once positioned within the recording chamber, slices were allowed to recover for 1 hour prior to testing.

Data analysis

Three successive fEPSP amplitude measurements were averaged and recorded. LTP and time-courses were expressed as the percent of the mean baseline response. Two-factor ANOVA, area under the curve, student T-tests, and regression analyses were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA). Specific analyses indicated within the figure legends. A test for interaction between factors was performed for each two-factor ANOVA. In each case, the analysis failed to indicate a significant interaction between the tested factors. Because there were only two levels of treatment factor (FN-439 versus

vehicle), a significant result was not followed by multiple comparisons testing. Tests for normality and equal variance were performed by NCSS 2004 (Kaysville UT USA). Data which failed tests for normality or equal variance were analyzed with non-parametric methods (as indicated in figure legends).

RESULTS

Effects of age on and LTP and fEPSP characteristics from Schaffer collaterials/CA1 field recordings .

Hippocampal slices generated from 3-month (young) and 22-month (aged) Sprague Dawley rats were analyzed for differences in evoked field responses during baseline and potentiated conditions. In accordance with previously published findings (Tombaugh, Rowe et al. 2002), slices generated from aged rats had a significant deficit in magnitude of theta burst stimulated LTP during induction and early maintenance phases compared to younger animals (two factor ANOVA; $p<.01$) **(Figure 1A,B)**.

Situations where an augmented synaptic response is elicited, by either increased magnitude of stimulation or by synaptic potentiation, it's typical for the fEPSP peak to experience a leftward shift (i.e. peak values are achieved more rapidly than basal level responses). This phenomenon represents a change in the temporal efficiency in the interval of time between presynaptic depolarization (with concomitant neurotransmitter release) and maximum post-synaptic responsiveness. Quantification of this relationship between pre and post synaptic elements can by performed by measuring the latency from the peak of the presynaptic fiber volley to the peak of fEPSP (peak-to-peak latency). In accordance with this phenomenon, slices generated from young rats experience a significant reduction of peak-to-peak latency (i.e. increased temporal efficiency) upon tetanization which persists through early maintenance (t=25min -30min post tetanus). Conversely, tetanus induced modifications to peak-to-peak latency is relatively diminished in slices generated from aged rats (Aspin-Welch test; $p<.001$). This is particularly true during early

maintenance where peak-to-peak latency is not significantly greater than pre-tetanus levels (one sample t-test; $p>.05$). Interestingly, however, a comparison of baseline peak-to-peak latencies from both young and aged rats reveals a significantly reduced peak-to-peak latency in evoked field potientials from aged rats as compared to younger counterparts. The divergence in the temporal efficiency between young and aged animals was not due to difference in fEPSP amplitudes as there was no statistical difference in baseline amplitudes between the two groups.

Aged rats have chronic elevations of hippocampal MMPs.

Rats learning a hippocampal dependent task experience significant increases in hippocampal MMP-3 levels during task acquisition (Meighan, Meighan et al. 2006). It was demonstrated that these MMP alterations are necessary for water maze learning and synaptic plasticity. Classically, MMP expression/activity within the CNS has been linked to pathological situations, such as ischemia (Lee, Tsuji et al. 2004), and tissue trauma (Phillips and Reeves 2001). A feature of MMP regulation during these conditions is that MMPs experience a persistent increase for days after initial insult. These seemingly divergent roles for MMPs led us to examine if there is evidence for a functional distinction between pathologically associated increases and the increases observed during learning. An observation which may distinguish between these differences is that learning associated MMP-3 increases return to basal levels within 24 hours following training (**Figure 2A**). Therefore a potentially important characteristic of learning associated MMP increases is that MMP upregulation during these conditions are *transient*; contrasting chronic elevations seen in pathologically associated conditions. This suggests that learning

associated MMP elevations are perhaps only appropriate during a discrete phase of learning associated synaptic remodeling.

There exists extensive literature which documents MMP dysregulation in tissue aging (particularly in skin, and cardiac). Considering the importance of MMPs to hippocampal plasticity, that aged rats exhibit deficits to long term potentiation, and that MMPs are dysregulated in multiple tissues during aging, we explored the possibility that abnormalities in the expression pattern of MMPs hippocampal tissue in aged rats are present. In contrast to the low levels found in young-adult rats, our assessment of hippocampal tissue from aged-adult rats uncovered chronic basal elevations in MMP-3 levels (**Figure 2B**). The increased MMP-3 levels are also significantly present in TIMP-sequestered form (**Figure 2C**).

MMP inhibitors facilitate LTP in hippocampal slices from aged rats.

Following our initial observations, there were two competing possibilities which could potentially explain the functional consequences of increased hippocampal MMP expression in aged rodents. One possibility is that age-linked MMP increases attend to compensatory function; this possibility seems reasonable considering MMPs importance in synaptic plasticity. A logical consequent to this premise would be that increased MMP activity is *beneficial* in helping preserve plasticity of hippocampal networks. A second contending possibility is that increased MMPs are a significant source of burden placed on plasticity critical mechanisms. A consequent to this premise is that increased MMP activity is *detrimental* to processes underlying hippocampal plasticity. We predicted that if the *former* premise were true (i.e. MMPs are serving compensatory function in

hippocampal aging) inhibition of MMPs should be a detrimental to hippocampal plasticity. Conversely, if the *latter* premise were true (i.e. MMPs are a burden to processes underlying hippocampal plasticity), MMP inhibition would be predicted to enhance plasticity dependent processes. We sought to test these contending hypotheses by observing the impact of MMP inhibition on long term potentiation. Hippocampal slices from aged rats were pretreated with a relatively dilute concentration of FN-439 (18 uM) for 4 hours prior to being introduced to the recording chamber. Once transferred to the recording chamber, slices were allowed a 1 hour wash-out phase prior to testing. Slices pretreated with MMP inhibitors experienced augmented LTP induction which persisted for at least 30min following tetanization (**Figure 3A**). This enhancement was also reflected in the total evoked dendritic depolarization during the recorded time (**Figure 3B**). In addition to promoting LTP, MMP inhibitor treated slices displayed an interesting but curious alteration to the development of LTP. Regression analysis indicates that the amplitude of evoked responses continued to gradually increase for up to 15 minutes following tetanization, before the onset of LTP decay (**Figure 3C**). This is in contrast to non-treated slices which exhibited a linear decay following tetanization (**Figure 3D**).

MMP inhibition promotes young-like fEPSP characteristics in slices from aged rats.

In addition to LTP deficits, slices derived from aged-rats also exhibited abnormalities in the temporal characteristics in the evoked field potentials. Specifically, aged field potentials reached maximum at a much quicker rate than younger animals, and failed to shift during potentiated conditions (see **Figure 1**). If these temporal features are associated with age related MMP elevations they would likely be sensitive to MMP

inhibition. In support of this idea, MMP inhibitor treated slices experienced a significantly greater peak-to-peak latency during baseline stimulatory conditions (i.e. a reduction in the temporal efficiency of the post-synaptic response)(two sample t-test; $p<.0001$) without a corresponding effect on amplitude (**Figure 4A**). This rightward shift represents a transformation of the fEPSP into a more young-like conformation. From this observation, we hypothesized that the decreased temporal efficiency during baseline conditions would provide the latitude for a dynamic leftward shift (i.e. increased temporal efficiency) upon potentiation; a form of responsiveness which is more typical of young slices rather than old. Consistent with this hypothesis, MMP inhibitor treated slices experienced a significantly greater decrease in peak-to-peak latency, for induction and early maintenance phases (**Figure 4B**), compared to non treated controls (two-factor ANOVA; $p<.0001$). In light of these results, it seems likely that age-associated MMP escalation is a source of burden on plasticity critical processes. Moreover, these data provide initial evidence that the potentially detrimental effects of age-related MMPs increases on hippocampal function may yet be at least partially reversed by MMP inhibition.

MMP inhibition fails to enhance LTP and alter fEPSP characteristics in slices from young rats.

Although unlikely, it's possible that beneficial effects of FN-439 pretreatment could be unrelated to MMP inhibition and due to some unknown, nonspecific effects on neuronal function. To control for this possibility, hippocampal slices generated from young animals were subjected to MMP inhibitor pretreatment and assessed for LTP and fEPSP characteristics. Due to the relatively low basal MMP levels present in hippocampal

tissue we predicted that either the treatment would not have an effect on LTP and fEPSP characteristics or if the treatment did have an effect it would be detrimental to synaptic plasticity. Consistent with our prediction, the MMP inhibitor treatment conditions which produced LTP enhancement in aged slices produced slight deficits in LTP induction and early maintenance in young slices (**Figure 5A, B**). Furthermore, MMP inhibition did not affect peak-to-peak latency for baseline and post-tetanus conditions (**Figure 5 C-E**). These findings provide validation to our hypotheses regarding the impact of elevated hippocampal MMP levels and further support our interpretation of how FN-439 administration affects hippocampal function in slices derived from aged rats.

Exogenous application of active MMPs impairs hippocampal LTP

To further test the hypothesis that an increased MMP activity is antecedent to deficits to synaptic plasticity, we administered a 1-hour pre-incubation of an active MMP-3 fragment (bath concentration of 1nM) to a hippocampal slice preparation and monitored the effects on LTP. MMP-3 treated slices experienced a moderate reduction in LTP induction magnitude and increased maintenance decay were (**Figure 6A**). The impact of exogenous MMP-3 treatment on LTP induction and early maintenance was particularly reflected in total area of fEPSP, a measure of evoked dendritic depolarization, 30 minutes post tetanus (**Figure 6B**).

In addition to the effects on LTP, pretreatment with MMP-3 produced a leftward shift (i.e. decreased peak-to-peak latency) of the evoked field potentials without a corresponding effect on fEPSP amplitude (**Figure 6C**). Although post-tetanus modifications to peak-to-peak latency were unaffected (data not shown) the changes to

plasticity and baseline peak-to-peak latency are coherent with the MMP associated effects during aging.

DISCUSSION

MMPs, aging and plasticity

We have previously demonstrated that MMPs are essential for synaptic plasticity underlying long term potentiation and hippocampal dependent learning (Meighan, Meighan et al. 2006). This conclusion is based on a number of observations. First, MMP transcript and protein increases 4 hours subsequent to water maze training. These increases only occur during the acquisition phase of the task. Upon learning the task, additional maze training fails to promote MMP induction. Second, MMP inhibition interrupts LTP stabilization during early maintenance, and can interfere with LTP induction if a peri-threshold tetanization protocol is used (manuscript in preparation?). Finally, both inhibition of MMP activity with FN-439 and reduction of MMP-3 and -9 protein with antisense oligo-nucleotides interfere with acquisition of the Morris water maze task. On the surface, these results seems paradoxical to the conclusion that increased MMP levels are detrimental to synaptic plasticity. This apparent paradox may be reconciled by considering the temporal characteristics of the expression patterns of MMP-3 increases during normal learning versus aberrant condition which promote MMP increases. For instance, the temporal pattern of MMP modulation is vastly different for models of injury induced plasticity as compared to learning associated plasticity. It's been recently described that MMP-3 is upregulated during reactive synaptogenesis following traumatic brain injury (TBI) from a period of 2-15 days post insult (Reeves, Prins et al. 2003). Interestingly, a maladaptive injury model which combines TBI with a bilateral entorhinal cortical lesion (TBI + BEC) produces an enhancement in the expression and duration of MMP-3 levels compared to TBI alone. Furthermore, inhibition of TBI + BEC induced MMP expression promotes functional recovery (Falo, Fillmore et al. 2006). Another

example of augmented MMPs deleterious to neuronal physiology is the induction of gelatinases following an ischemic insult. Hippocampal MMPs -2 and -9 exhibit a persistent increase for at least 72 hours following transient occlusion of common carotid arteries. These increases were concomitant with enhanced neuronal damage to hippocampal pyramidal neurons. Inhibition of MMP activity reduced the neuronal damage attributable to the ischemic insult. In addition to injury induced plasticity models, MMPs have been implicated in the pathogenesis of Alzheimer's disease (Yoshiyama, Asahina et al. 2000).

In contrast with these persistent, and potentially deleterious, augmented MMP levels, the learning associated elevations of MMPs is relatively short (i.e. a cycle which peaks and returns to baseline levels within 24 hours). Given the persistence of aged-associated MMP-3 increases and the apparent negative impact of these increases to plasticity and learning, we contend that age-associated elevation of MMP-3 is contiguous with injury and pathology associated MMP induction.

Effects of aging on temporal fEPSP characteristics

The LTP induction deficits exhibited by aged slices are consistent with previous reports (Tombaugh, Rowe et al. 2002). However, the differences in temporal-characteristics of waveforms between young and aged derived slices have not been reported. Young slices demonstrated a persistent decrease of peak-to-peak latency of post-tetanus evoked responses. In contrast to this, waveforms from aged slices failed to exhibit this pattern (i.e. decreased peak-to-peak latencies following tetanization). This result is likely due to the fact that evoked fEPSPs from aged slices are predisposed to relatively low peak-to-peak latencies during baseline stimulation. Ultimately, these data lead to one of two logical conclusions regarding the fEPSP

characteristics in aged derived slices. Either the mechanisms or features which promote increased temporal efficiency are omnipresent in aged rats, or mechanisms or features which promote decreased temporal efficiency are absent in aged rats.

Peak-to-peak latency is a frequently ignored aspect of fEPSP characteristics. This is perhaps due to the fact that under normal conditions, peak-to-peak latency appears to be inextricably linked to the magnitude of the evoked potential. However, we have demonstrated that changes to fEPSP amplitude can exist without a proportional and corresponding change to peak-to-peak latency. An implication of this finding is that peak-to-peak latency, rather than being intrinsic to fEPSP magnitude, might correspond to a partially independent aspect of synaptic communication. We speculate that peak-to-peak latency reflects the temporal efficiency of synaptic transmission. As such, a change to peak-to-peak latency has the potential to influence aspects of synaptic integration (e.g. temporal summation). Because the determinants of peak-to-peak latency are partially distinct from the determinants of synaptic response magnitude, activity dependent changes to temporal efficiency may represent a distinct form of plasticity (i.e. temporal plasticity). Regardless, it is clear that the temporal characteristics of fEPSPs from aged slices are different than those derived from young. These observations raise the possibility that temporal aspects of synaptic responsiveness, in addition to aspects of magnitude, underlie age related impairments to synaptic plasticity.

MMPs and peak-to-peak latency

MMP inhibition caused temporal fEPSP characteristics of aged-slices to adopt features similar to young-slices (i.e. increased peak-to-peak latency during baseline conditions and decreased latency during potentiated conditions). Moreover, treatment of young-slices with a

catalytically active MMP-3 fragment produced temporal fEPSP characteristics similar to those observed from aged-slices (i.e. reduced peak-to-peak latency during baseline conditions). From this, we conclude that MMP activity influences peak-to-peak latency. This raises an obvious question: how might MMP activity accomplish this? Although there are a number of possibilities which may account for the relationship between MMP activity and peak-to-peak latency, the most plausible is that MMPs affect neurotransmitter diffusion. Movement of neurotransmitter across the synaptic cleft and through extracellular space (ECS) is affected by the ECM. One way the ECM affects neurotransmitter diffusion is by influencing the volume of extracellular space (ECS). The ECM increases ECS volume by electrostatic repulsion via negatively charged moieties, and by retention of osmotically active cations resulting in increased ECS turgor. Increased electrostatic repulsion and turgor within the ECS counters compressive forces ultimately resulting in a healthy expansion and stabilization of the ECS. Specific ECM molecules thought to contribute to theses forces include tenascins, condroiten sulfate proteoglycans, and hyaluronic acid. Loss of these specific ECM proteins results in diminished ECS volume. Speculatively, a reduction of ECS volume would result in a decreased neurotransmitter diffusion time between signaling neurons. It has been shown that, reduced ECS volume is detrimental to extrasynaptic transmission important for plasticity. Interestingly, aged rats exhibit reduced hippocampal ECS volume (Sykovâa, Mazel et al. 1998). Furthermore, this reduced ECS volume appears to be associated with reduction of volume critical ECM proteins and correlates to poor performance in hippocampal dependent tasks (Sykovâa, Mazel et al. 2002).

Another means by which ECM affects neurotransmitter diffusion is by generating extracellular tortuosity. Tortuosity is defined by the ratio between the free diffusion coefficient of a substance and the apparent diffusion coefficient in the brain (Sykovâa 2004). The ECM

influences tortuosity by imposing steric hindrance on neurotransmitter diffusion paths. This imposition is important for generating neurotransmitter anisotropy (i.e. directed diffusion to extrasynaptic receptors) underlying synaptic plasticity. Similar to altered ECS volume, aged rats exhibit reduced hippocampal tortuosity. Again, this is partially attributable to reduced levels of particular ECM proteins. Age associated alterations to tortuosity and ECS volume would be predicted to decrease neurotransmitter transit time between associated neurons (both synaptically and extrasynaptically). This could potentially explain the reduced peak-to-peak latency in aged slices. We speculate that the age associated alterations to the ECM, resulting in reduced ECS volume and tortuosity, is attributable to heightened MMP activity. Support for this lies in the fact that (a) MMPs are increased within the hippocampus in aged rats and (b) many of the ECM molecules which influence ECS volume and tortuosity are established MMP substrates. Consequently, MMP inhibition would thereby promote increased peak-to-peak latency by enabling the restoration of neurotransmitter diffusion barriers and increased ECS volume.

How might MMPs be increased?

The observation that MMP-3 is dramatically increased in aged hippocampal tissue begs the following question: what are the physiological conditions responsible for this increase? One possibility is that this MMP-3 induction is a result of oxidative stress. Oxidative stress has been implicated as a causative agent for neuronal aging, particularly in the hippocampus. Interestingly, reactive oxygen species (ROS) promote both the induction and the activation of MMPs. This sensitivity to ROS manifests as a pathological induction of MMPs in non-neuronal tissues. Considering the relationship between aging, ROS, and MMPs, it reasonable to suppose that oxidative damage is responsible for aged associated increase of hippocampal MMP-3. Another

possibility which may account for these changes is age related calcium dysregulation. Previously, we have demonstrated that learning associated MMP-3 induction downstream from NMDA receptor activity (Meighan, Meighan et al. 2006). This suggests that increased intracellular calcium promotes MMP-3 gene transcription. Evidence suggests neurons in aged animals exhibit deficiency of intracellular calcium homeostasis. These deficits result in a persistent increase of intracellular calcium. Persistent increase of intracellular calcium is likely detrimental to plasticity as intracellular injection of calcium chealator rescues LTP in aged slices.

General conclusions regarding MMP-3 and plasticity

The explicit objective of this study was to assess the possibility that MMP-3 is involved in aged associated impairments to synaptic plasticity and learning. From these observations, however, we may derive some aspects of MMP's role in general plasticity. First**,** basal hippocampal MMP-3 levels are relatively low in young-adult rats. From this we may conclude that during quiescent conditions, only low-levels of MMP activity are necessary to promote neuronal function. Moreover, keeping basal MMP levels relatively low presumably provides the system with adequate latitude to promote increased MMP levels on demand. Second, although increased MMP activity is essential for synaptic plasticity underlying LTP and learning, MMP changes supporting plasticity are transient. We speculate that transient increase of MMP activity serves to temporarily alter the balance between ECM deposition and degradation. It is hypothesized that temporarily disrupting ECM / adhesive constituents is necessary for the reconfiguration of structural elements. This idea is supported by the fact that MMP inhibition with FN-439 loses efficacy if applied 30 minutes following tetanization. This suggests that MMPs are

involved in plasticity processes during a discrete temporal window. Beyond this window, MMP activity is no longer necessary to support plasticity.

In summary, we have demonstrated the hippocampal MMP-3 reaches abnormally high basal levels as a result of aging. Inhibition of MMPs with a broad-spectrum inhibitor enhances LTP induction and promotes young-like fEPSP characteristics. Additionally, exogenous application of the MMP-3 catalytic fragment resulted in the destabilization of LTP and fEPSP characteristics which mimicked those generated by aged slices. Considering that there are over 25 MMP subtypes, it is unlikely that the data presented here represents the extent of MMP involvement on hippocampal plasticity during aging. Future examination will focus on examining other MMPs and determining the mechanisms by which they impact hippocampal plasticity.

FIGURE LEGENDS

Figure 1: Effects of age on fEPSP characteristics and LTP. Acute hippocampal slices were generated from 3-month old (young) and 22-month old (aged) Sprague-Dawley rats. **(A)** Time-courses of theta burst stimulated LTP from slices of both young (open circle) and aged (grey circle) rats; tetanus indicated by arrowhead. Data expressed as mean +/- S.E.M. **(B)** Comparison of the magnitude of fEPSP amplitude, for young (open bars) and aged (grey bars) during LTP induction and early maintenance phases. Induction was calculated as the average amplitude from t=4.5 to t=5.5 min following tetanization; early maintenance was calculated as the average amplitude from t=27.5 to t=28.5 following tetanization. Statistical analysis by two factor ANOVA (age x phase) indicates an independent effect of age on fEPSP amplitude ($p<.01$). **(C)** Dynamic shift of peak-to-peak latency following tetanization during LTP induction (t= 3-6min post tetanization) and early maintenance (t= 25-30min post tetanization). Data expressed as mean +/- S.D. Due to violation of equal variance assumption, data analyzed with Holmes corrected Aspin-Welch tests (young-adults versus aged-adults: ***$p<.0001$). Sample waveforms provided from slices of both young (top waveforms) and aged (bottom waveforms), for both baseline (solid lines) and potentiated (hashed lines) conditions. **(D)** Baseline latency from peak-presynaptic fiber volley to peak fEPSP (peak-to-peak latency) and baseline amplitudes for slices from both aged and young rats (data expressed as percent young +/- SEM). Due to violation of equal variance assumption for peak-to-peak latency, data were analyzed with Aspin-Welch test (***$p<.0001$). Baseline amplitudes were analyzed with two sample t-test ($p>.05$). Sample waveforms provided from both young (solid line) and aged (hashed line).

Figure 2: Aged rats exhibit greater basal hippocampal MMP-3 levels and MMP-3 / TIMP-2 complex formation than younger rats. (A) Western blot analysis of hippocampal MMP-3 protein from 3-month old Sprague Dawley rats. Tissue was collected either 5minutes, 4 hours or 24 hours subsequent to first day training in Morris Watermaze. (B) Western blot analysis of hippocampal MMP-3 levels from 6 month old and 24 month old Sprague-Dawley rats. **(B)** Tissues were also examined for MMP-3 / TIMP complex formation. Identity of MMP-3 / TIMP-2 complex was verified by immunoprecipitating with anti MMP-3 and subsequently probing with anti TIMP-2 **(C)**.

Figure 3: MMP inhibition enhances hippocampal LTP in slices from aged rats. Acute hippocampal slices were generated from 22month Sprague-Dawley rats. Following initial recovery from dissection, slices were incubated in the presence or absence of FN-439 for four hours. Subsequent to a one hour washout of FN-439 in the recording chamber, LTP was induced by TBS. **(A)** Timecourse of fEPSP amplitudes for FN-439 treated (closed circle) or control (open circle). Data expressed as mean values +/- S.E.M. **(B)** Area under curve was calculated from randomly selected fEPSP waveforms, for both FN-439 treated (filled bars) and control slices (open bars), between 3-5min post tetanus, 13-16 min post tetanus, and 25-30 min post tetanus. Data expressed as mean values +/- S.E.M. Analysis by two factor ANOVA (LTP phase x treatment) revealed an independent effect of FN-439 on area under curve ($p<.001$) n=16 waveforms per group. **(C)** Regression analysis was performed to examine rate of LTP decay of fEPSP amplitude replicates for FN-439 treated slices. Fit of regression line was significantly improved by fitting data with a nonlinear model (second order polynomial) ($p<.0001$). Statistical peak of function

occurs at t=14 minutes post tetanus. Means (+/- S.E.M.) of replicates are presented for clarity. **(D)** Post tetanus nonlinearity was absent in timecourse from untreated slices.

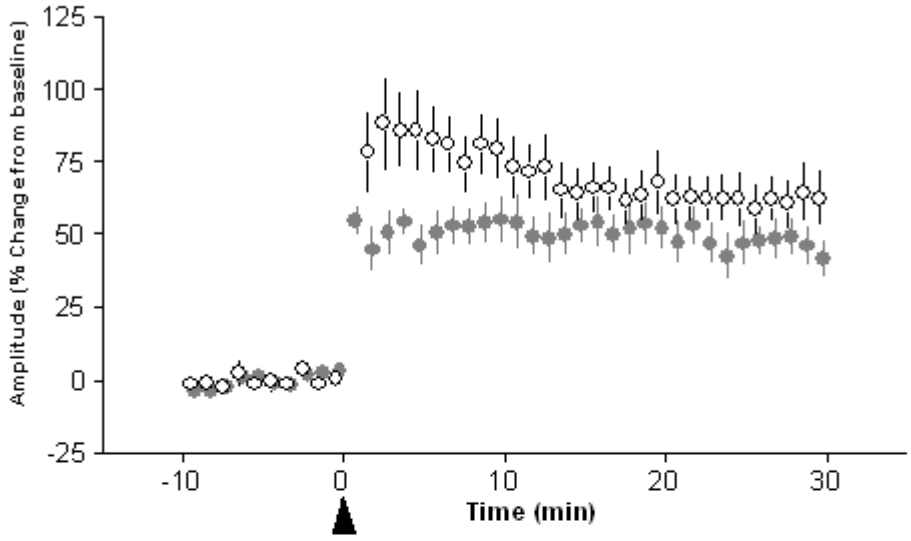
Figure 4: MMP inhibition promotes young-like fEPSP characteristics in slices from aged rats. Slices generated from aged rats were pretreated with FN-439 were assessed for alterations to fEPSP characteristics. **(A)** Peak-to-peak latency (open circles) and mean response amplitude (filled circles) during baseline transmission for FN-439 treated and untreated conditions. Data expressed as % change from control (untreated) conditions (mean +/- S.E.M.). Each pair of data analyzed with two-sample T-Test. MMP inhibitor treated slices experienced a significant increase in baseline peak-to-peak latency (***$p<.0001$); this difference was not reflected in baseline response amplitudes ($p>.05$). Provided are sample waveforms for both MMPi treated (hashed line) and control (solid line) slices generated from an individual aged animal. Arrowhead denotes presynaptic fiber volley (1^{st} peak) for both treated and control slices, solid arrow approximates peak from untreated slice, hashed arrow approximates peak from treated slice. **(B** Dynamic shift of peak-to-peak latency during induction (3-5 min post tetanization) and early maintenance (25-30 post tetanization) phases. Data expressed as mean +/- S.D. Analysis with two-factor ANOVA (treatment x time) indicates an independent effect of FN-439 on latency shift ($p<.0001$). Included are sample waveforms for both control (top) and MMPi treated (bottom) conditions during baseline (solid line) and early maintenance (hashed line) phases.

Figure 5: MMP inhibition fails to enhance LTP or alter fEPSP temporal-characteristic in slices from young rats. MMPi pre-treated slices generated from young rats were assessed for LTP. **(A)** LTP time course of amplitudes for MMPi treated (filled circle) and untreated (open circle) conditions. Data expressed as % change from baseline (mean +/- S.E.M.). **(B)** Pooled amplitudes for both control (open

bars) and MMPi treated (closed bars) during induction (3-5 min post tetanus) and early maintenance (25-30 min post tetanus). Data expressed as % change from baseline (mean +/- S.E.M.). **(C)** Area under curve during induction and early maintenance phases. Data expressed as % change from baseline (mean +/- S.E.M.). **(D)** Baseline peak-to-peak latency for MMPi treated and control slices generated from young rats. Data expressed as % change from control (untreated) conditions. MMP inhibition failed to alter baseline peak-to-peak latency (two sample T-test; $p>.05$). **(E)** Dynamic latency shifts for both control and MMPi treated conditions in slices generated from young rats during induction and early maintenance. Data expressed as mean +/- S.D. MMP inhibition failed to impact tetanus induced latency shift (two factor ANOVA (treatment x phase); $p>.05$).

Figure 6: Exogenous MMP-3 application impairs hippocampal LTP maintenance. Acute hippocampal slices were generated from 3-month old Sprague-Dawley rats. Thirty minutes prior to tetanization, slices were exposed to catalytically active, MMP-3 (which was present during duration of timecourse). LTP was induced by TBS (indicated by arrowhead). **(A)** Timecourse of fEPSP amplitudes for MMP-3 treated (closed circle) or control (open circle). **(B)** Area under curve was calculated from randomly selected fEPSP waveforms, for both MMP-3 treated and control slices, between 3-5min post tetanus (induction) and 25-30 min post tetanus (maintenance). Data expressed as mean +/- S.E.M.

Figure 1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50
25
0
-25
-10
0
10
20
30
Time (min)

A

B

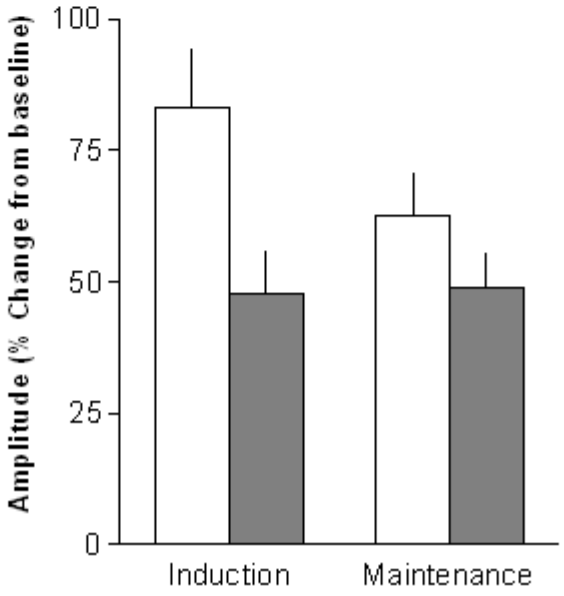
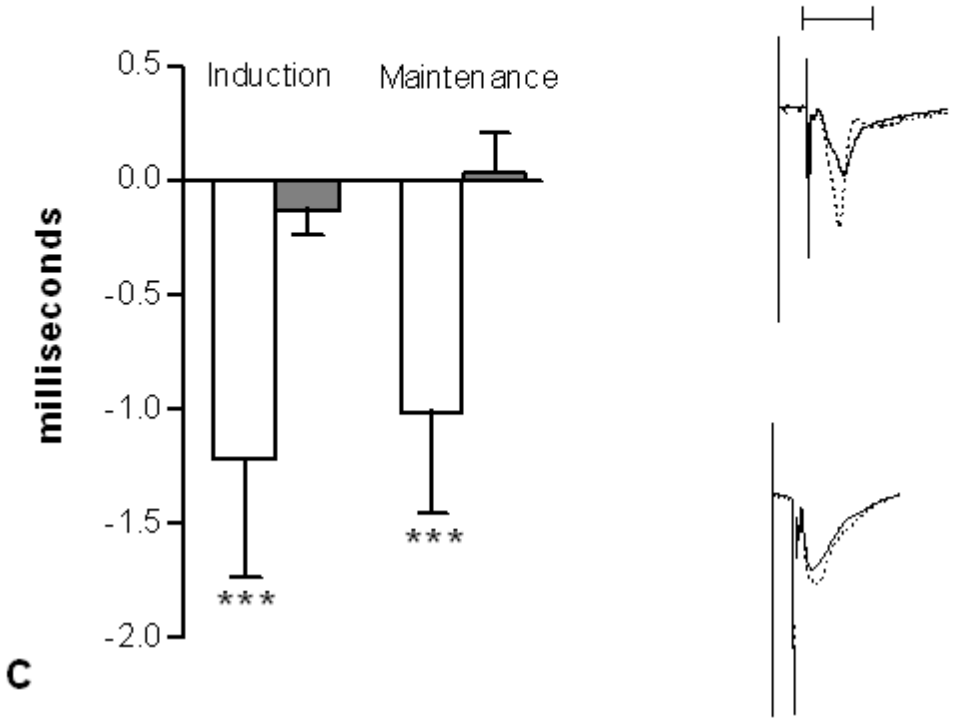

Amplitude (% Change from baseline)
100
75
50
25
0
Induction
Maintenance

Figure 1


0.5
0.0
-0.5
-1.0
-1.5
-2.0
milliseconds
Induction
Maintenance
***
***
C

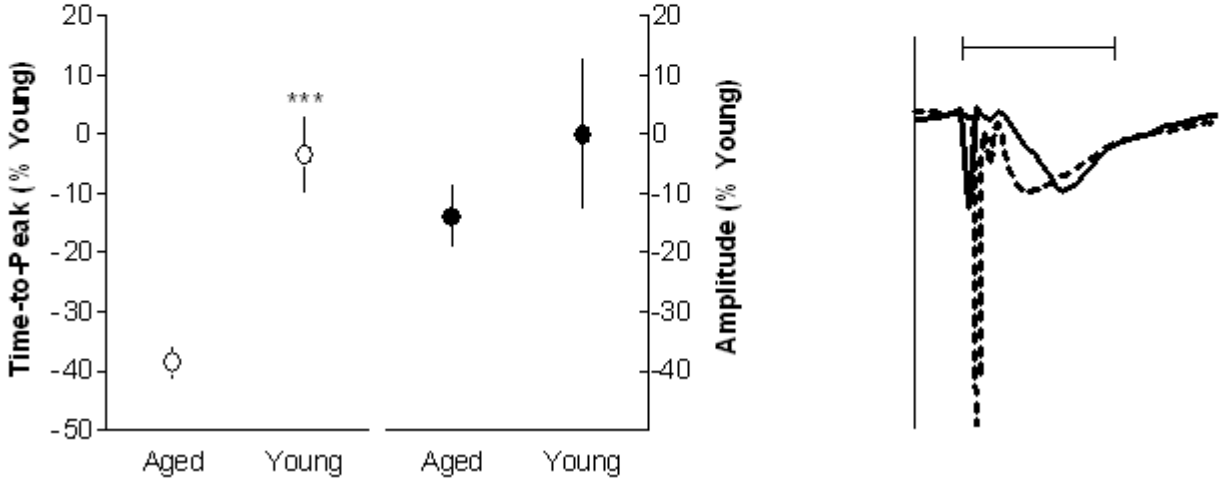

D
20
10
0
-10
-20
-30
-40
-50
Time-to-Peak (% Young)
Amplitude (% Young)
***
Aged
Young
Aged
Young

Figure 2

A

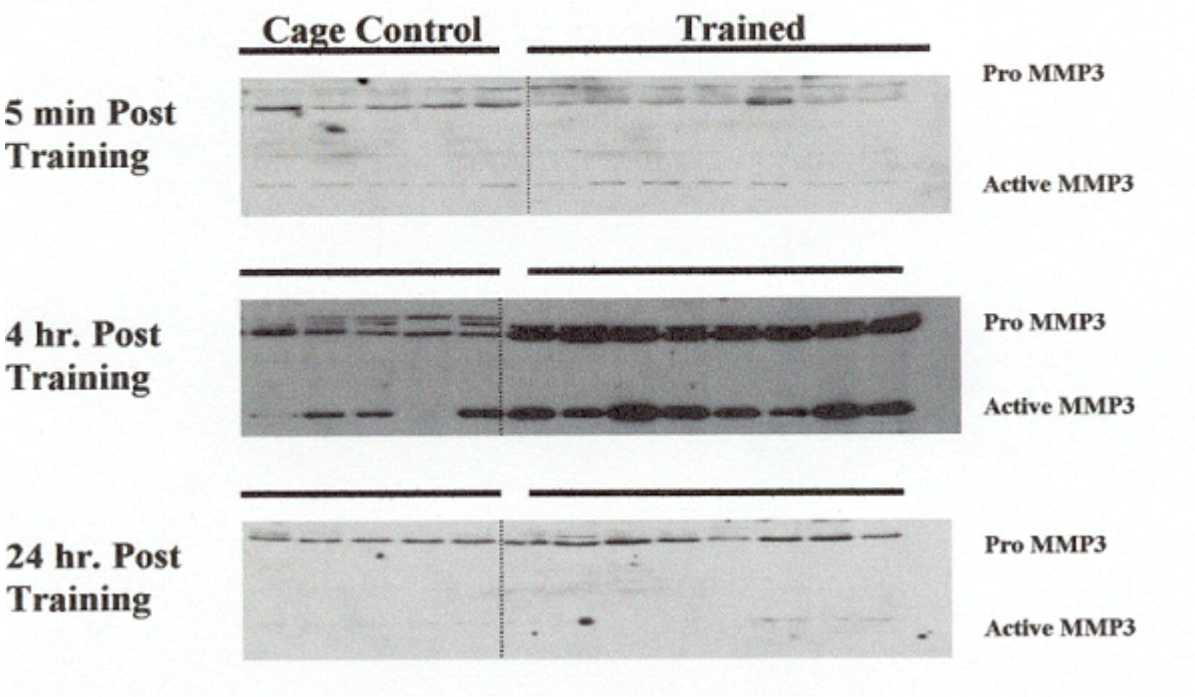
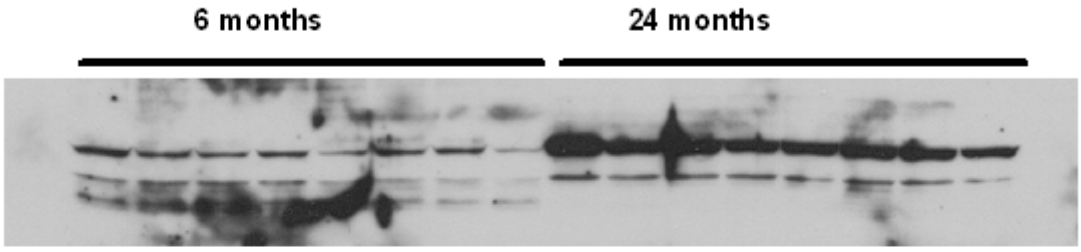
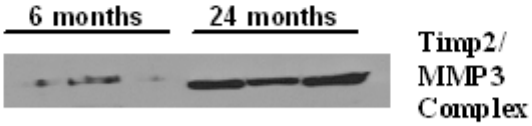

Cage Control
Trained
5 min Post Training
4 hr. Post Training
24 hr. Post Training
Pro MMP3
Active MMP3
Pro MMP3
Active MMP3
Pro MMP3
Active MMP3

Figure 2

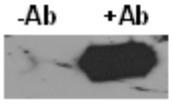


B

C



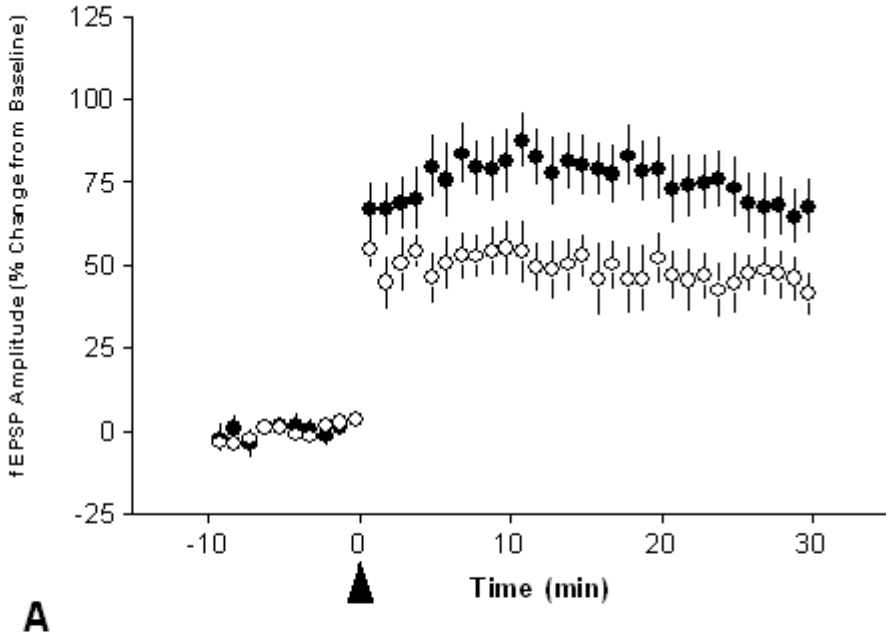
Timp2/
MMP3
Complex



MMP3/TIMP2
Complex

IP: MMP-3
IB: TIMP-2

Figure 3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100
75
50
25
0
-25
-10
0
10
20
30
Time (min)

A

B

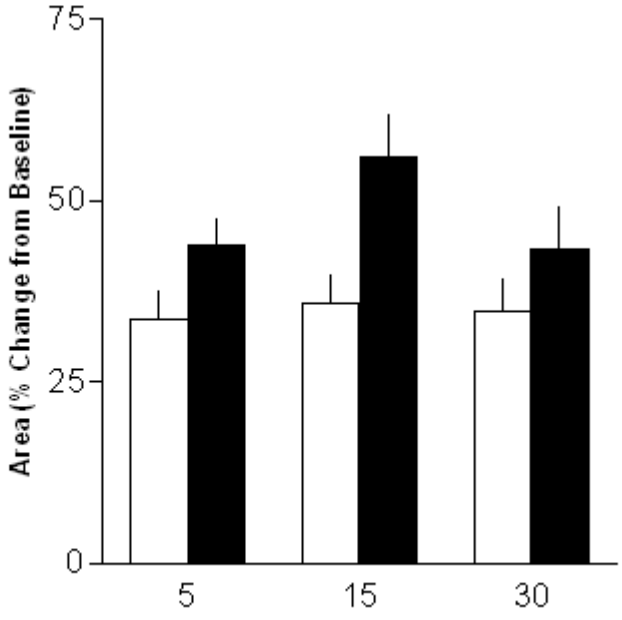
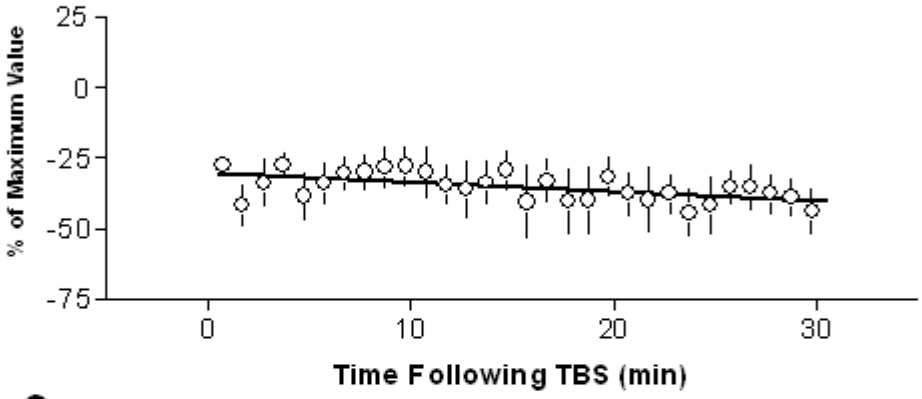

Area (% Change from Baseline)
75
50
25
0
5
15
30

Figure 3


25
0
-25
-50
-75
% of Maximum Value
0
10
20
30
Time Following TBS (min)

C

D

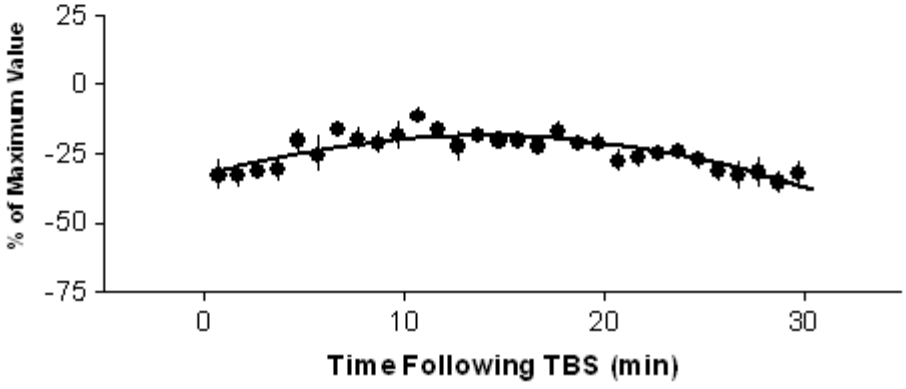

25
0
-25
-50
-75
% of Maximum Value
0
10
20
30
Time Following TBS (min)

Figure 4

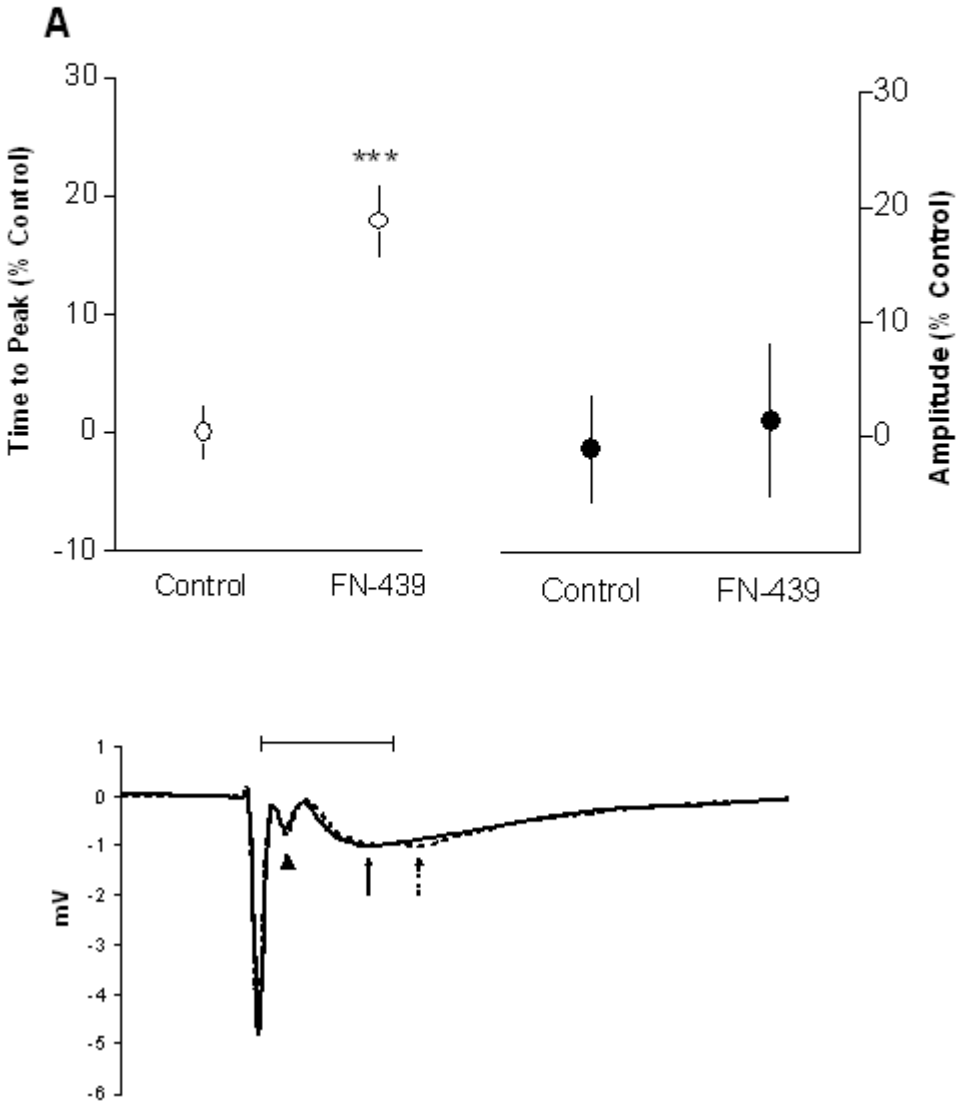

A
Time to Peak (% Control)
30
20
10
0
-10
***
Control
FN-439
Amplitude (% Control)
30
20
10
0
Control
FN-439
mV
1
0
-1
-2
-3
-4
-5
-6

Figure 4

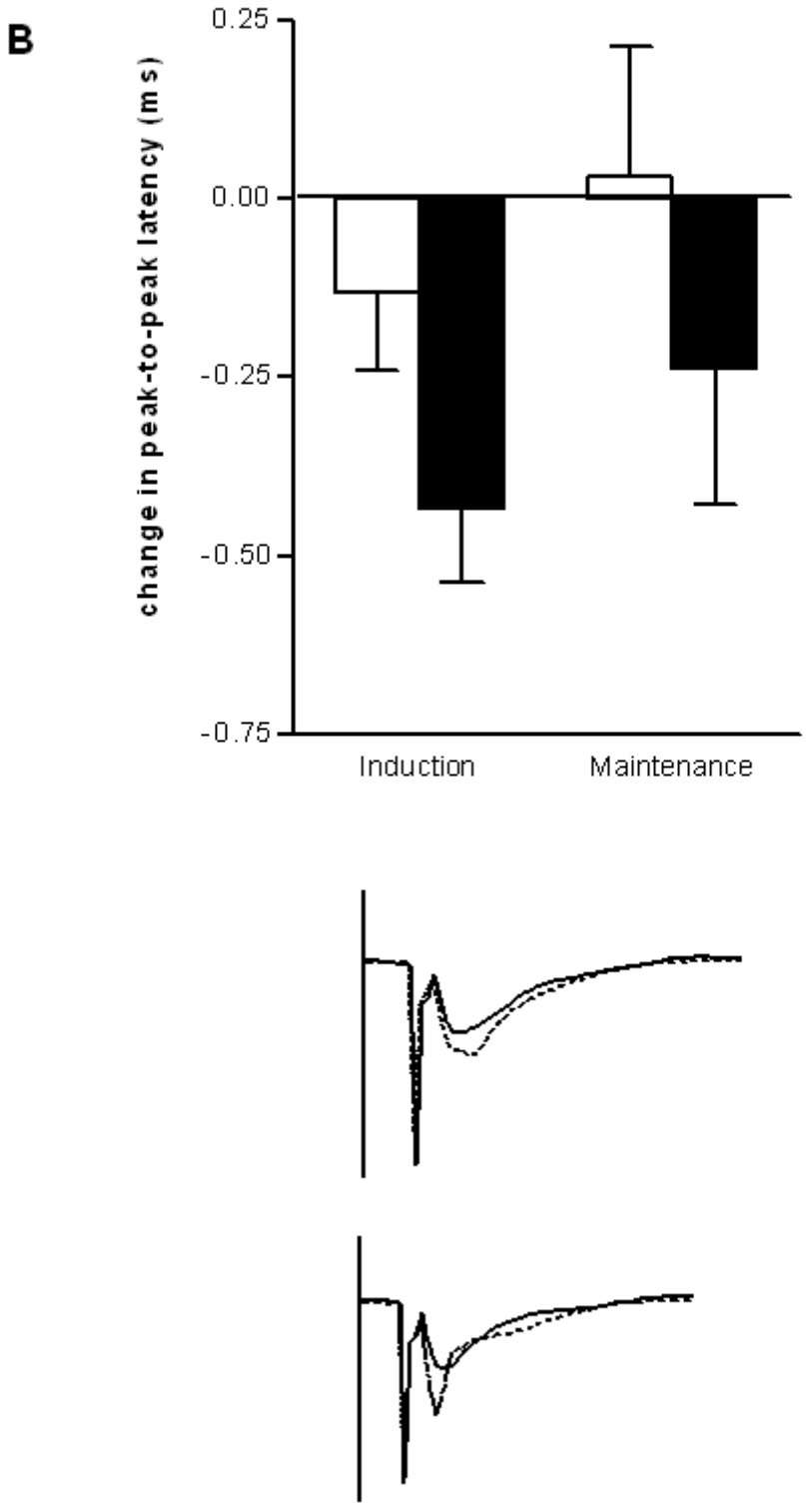
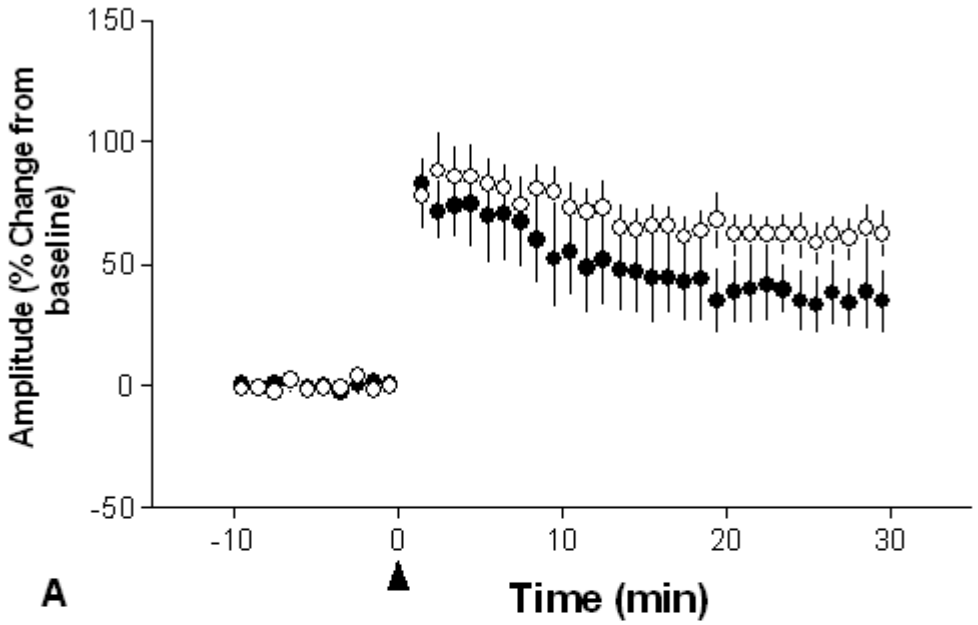

B
change in peak-to-peak latency (ms)
0.25
0.00
-0.25
-0.50
-0.75
Induction
Maintenance

Figure 5


150
100
50
0
-50
Amplitude (% Change from baseline)
-10
0
10
20
30
Time (min)
A

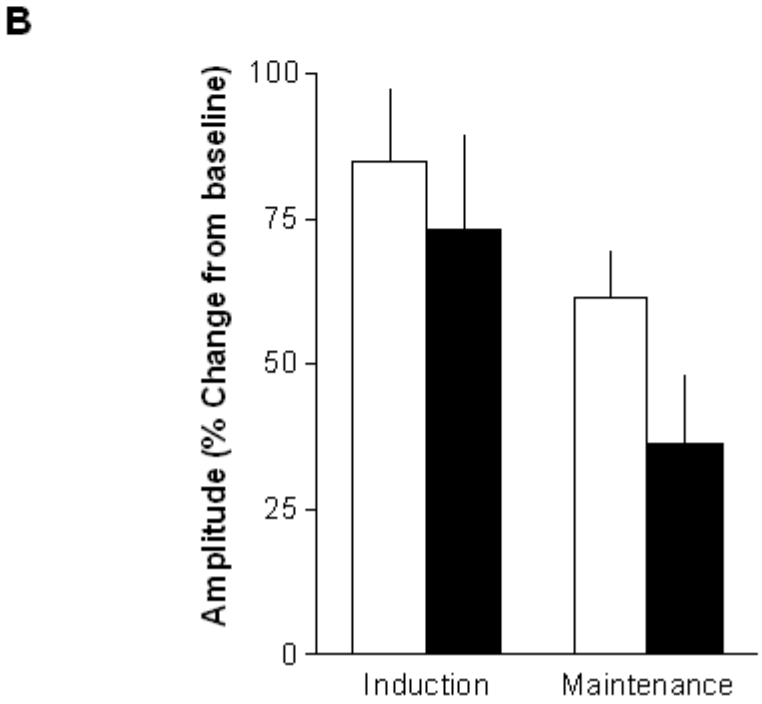
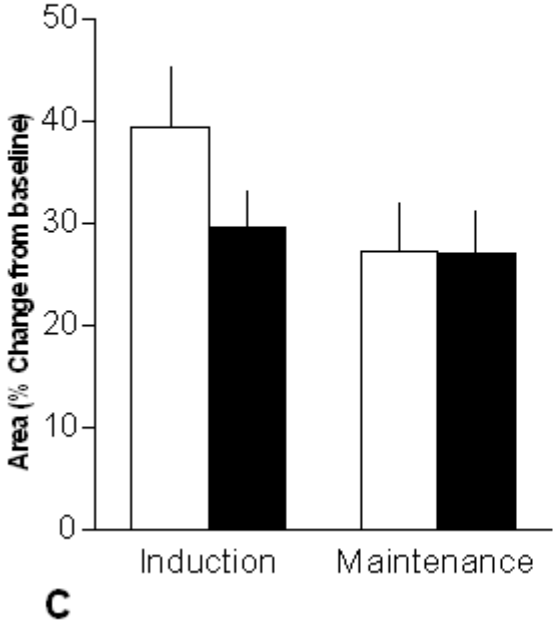
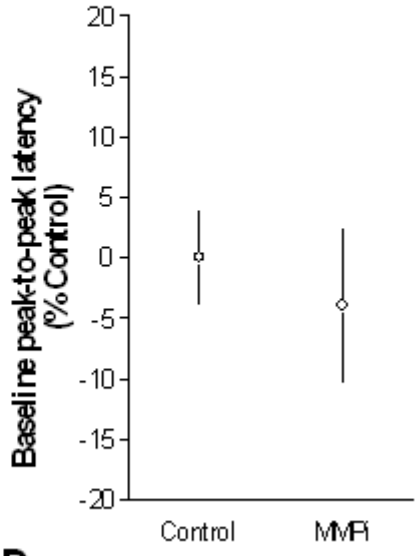

B
100
75
50
25
0
Amplitude (% Change from baseline)
Induction
Maintenance

Figure 5


50
40
30
20
10
0
Area (% Change from baseline)
Induction
Maintenance

C


20
15
10
5
0
-5
-10
-15
-20
Baseline peak-to-peak latency (% Control)
Control
MMPI

D

E

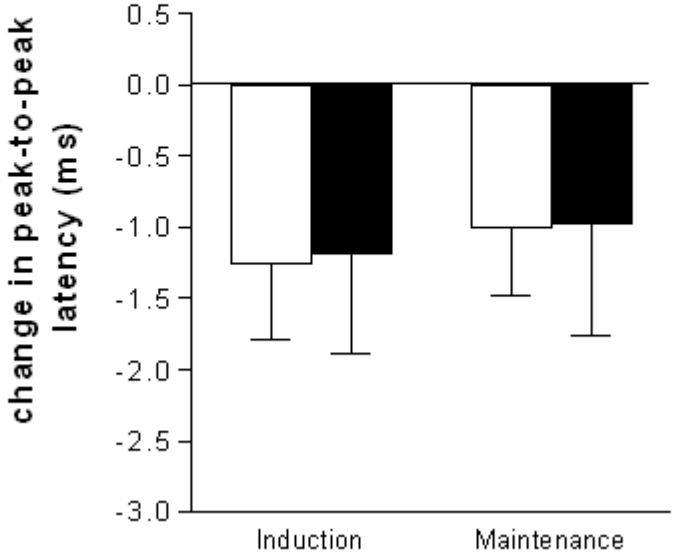
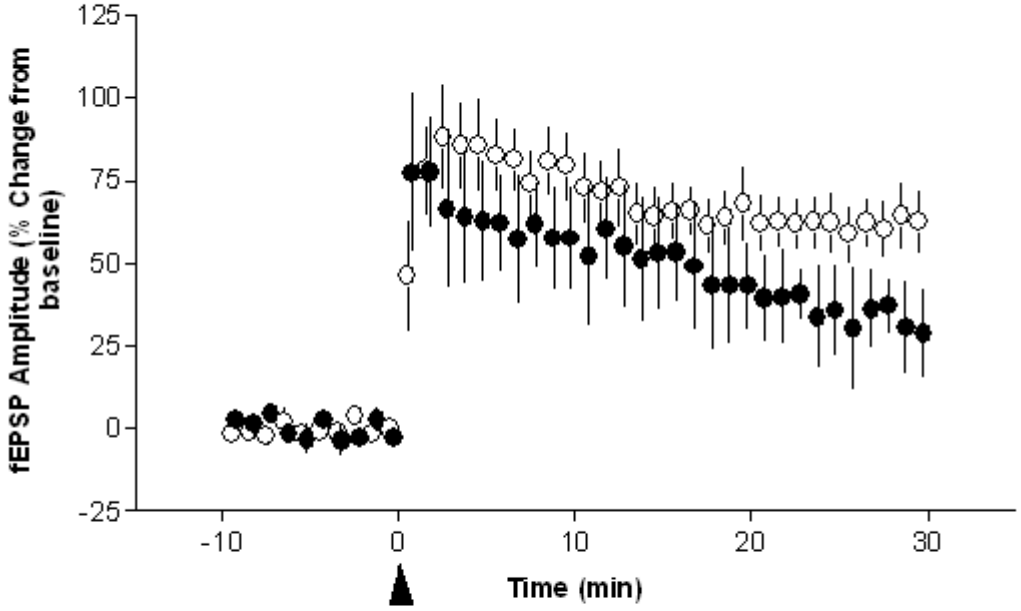

0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
change in peak-to-peak latency (ms)
Induction
Maintenance

Figure 6


fEPSP Amplitude (% Change from baseline)
125
100
75
50
25
0
-25
-10
0
10
20
30
Time (min)

A

B

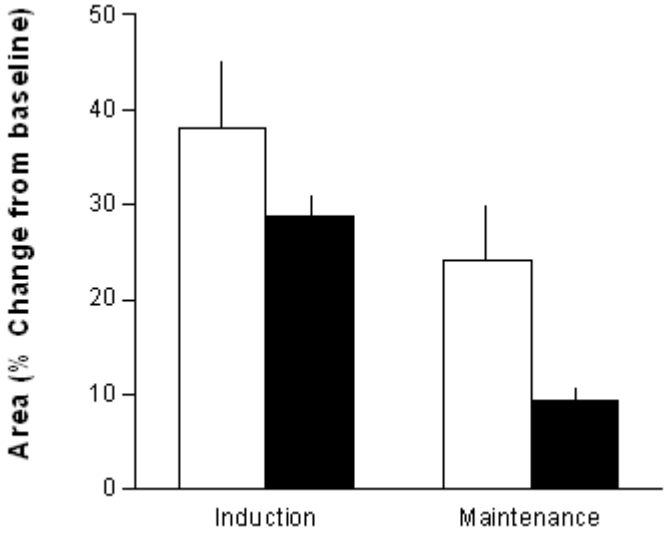
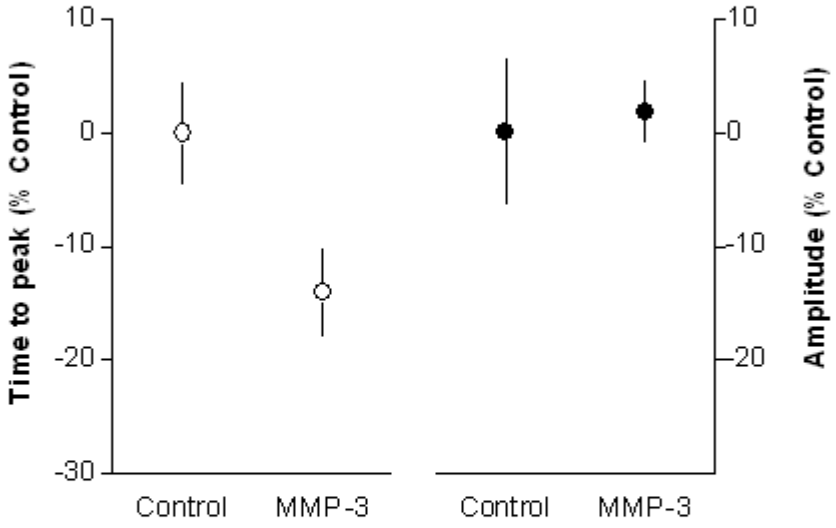
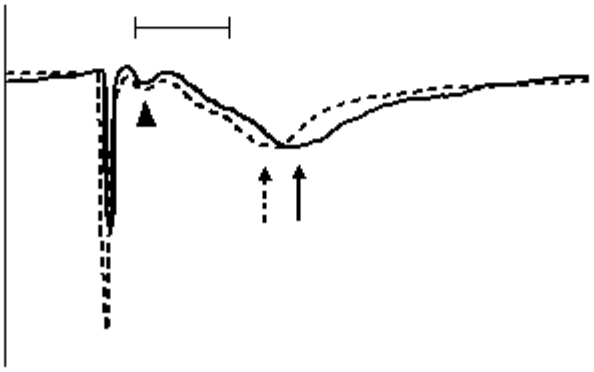

Area (% Change from baseline)
50
40
30
20
10
0
Induction
Maintenance

Figure 6

C


10
0
-10
-20
-30
Time to peak (% Control)
Control
MMP-3
10
0
-10
-20
Amplitude (% Control)
Control
MMP-3



GENERAL CONCLUSIONS

MMPs and Plasticity: A New Picture Emerges

We have determined that a crucial aspect of MMP involvement in the remodeling process is the transient nature of the elevations that are required. Our data indicate that the levels of MMPs-3 and -9 reach a maximum three to four hours after an initial exposure to the Morris water maze returning to basal levels by 24 hours. This pattern is repeated on subsequent training days until the task is fully learned and performance can no longer be improved with additional training. The MMP induction pattern is modified by a continual diminution of the peak response over four days until MMPs remain stable at baseline levels.

Therefore, two aspects of the MMP change seem essential if learning and memory consolidation are to occur-1) there must be an increase in MMP levels and 2) this increase must be transient. In support of these contentions are the observations that either inhibition of MMP activity or continual application of excess MMPs prevents learning and/or LTP. Prior to a learning experience young rats exhibit very low hippocampal MMP levels. During active learning, however, they exhibit the transient pattern of MMP elevation described above. Aged rats, on the other hand, possess basal levels of MMP-3 and MMP-9 that surpass the peak levels that are attainable by young rats during learning. These data are fascinating and suggestive; however, considering the size of the family of MMPs, they are incomplete in detail. Studies on brain MMP function, including our own published work and our preliminary studies, have focused primarily on MMP-9 and most recently MMP-3. This is not due to a lack of significant findings with other MMPs, but instead reflects initial limitations in technology for the assessment of the variety of MMPs and the

rapidly expanding lst of known MMPs. Historically, gelatinases (which include MMP-9) have

been a primary focus in research due to the widespread use of the easily adapted technique gelatin zymography in MMP research. The nature of this technique limits its use to primarily the gelatinase subfamily. With the recent availability of MMP antibodies suitable for rodent MMP assessment, there has been an expansion of research to include a wider variety of MMPs. More than likely our preliminary results represent the "tip of the iceberg" in terms of the extent of regulation and involvement of MMPs in brain aging.

In order to make our descriptive observations concerning MMPs, learning and aging more mechanistically meaningful we must ask the critical question: Why are low basal levels of hippocampal MMPs and the ability to support transient elevations in MMPs essential for learning? To try to address this question we

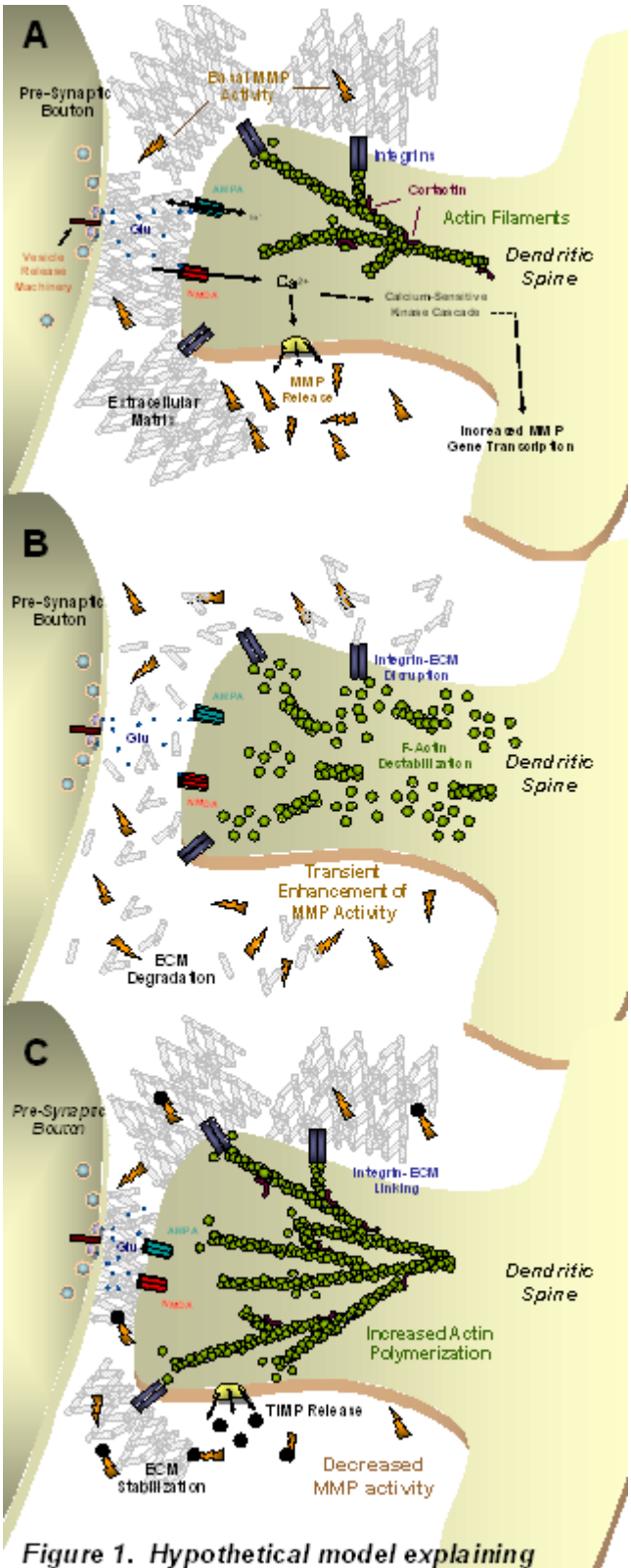


Figure 1. Hypothetical model explaining relationship between MMPs and spine dynamics before (A), during (B), and after (C) transient increase of MMP activity.

have developed a putative model to help us conceptualize the involvement of MMPs in learning-associated synaptic plasticity **[Figure 1]**. Synaptic plasticity involves changes in the efficiency of synaptic communication that reflects the historical level of action potential traffic at a given synapse. This change in efficiency is in large part a result of structural alterations in synaptic elements (e.g., dendritic spines) that impact the neurotransmitter release process and/or the responsiveness of postsynaptic cells. The importance of past performance on current synaptic effectiveness necessitates that plastic processes must be activity-dependent. This, of course, must be true for MMPs if they serve a role in this plastic process. Several facts suggest that a likely activity-dependent signal for MMP regulation could be an increase in postsynaptic intracellular calcium **[illustrated in Figure 1A]**. First, changes in intracellular calcium are already known to be critically involved in several postsynaptic processes that are entral to synaptic plasticity (e.g., modulation of AMPA receptor function via CAM kinase II). Second NMDA receptors, which are glutamate dependent calcium channels, are essential for most forms of hippocampal synaptic plasticity. And third and most important, inhibition of NMDA function with MK-801 completely eliminates both spatial learning and learning-associated MMP increases (REF).

Once in the synaptic cleft, MMPs can have several actions all of which could influence the structure and thus function of synaptic elements **[Figure 1B]**. MMPs most likely alter synaptic morphology by degradation of extracellular substrates that are critical for synaptic stability. The classic function of MMPs is the degradation of ECM proteins. Since several ECM proteins are essential activators of cell adhesion molecules (e.g., fibronectin activating integrins) that dictate actin cytoskeleton polymerization and, therefore, dendritic structure one would predict that their destruction might impact dendritic function and synaptic communication. This prediction has been recently verified for MMP-7 (Bilousova, Rusakov et al. 2006).

Implicit in this model is the notion that at a normally functioning synapse this morphological restructuring leads to a "new and optimal" functional orientation of synaptic elements. A corollary to this idea is that stable orientations must be destabilized to enable the necessary reorganization of synaptic elements to occur during the plastic event. A second corollary is that once synaptic rearrangements have occurred the new orientation must be re-stabilized to preserve the "improved" synaptic architecture. Thus, the necessity of MMP elevations being transient becomes apparent. The transient elevation of MMPs may serve to temporarily destabilize the synaptic structure while a subsequent downregulation of MMP activity could allow for re-stabilization **[Figure 1C]**. It is likely that MMP downregulation occurs by at least two mechanisms: proteolytic degradation and sequestration by TIMPs.

MMPs and Aging: Too much of a good thing?

Cognitive decline with aging has been shown in laboratory rodents, non-human primates, as well as, humans. Among this variety of species, there exist common themes and mechanisms. Most notably, in the absence of neurodegenerative disease status (i.e. Alzheimer's), there appears to be little actual neuronal loss with aging even with diminishing cognitive ability (Rapp and Gallagher 1996; von Bohlen und Halbach and Unsicker 2002). Instead, it appears there is detrimental loss of synaptic connectivity and/or unfavorable molecular changes at intact synapses (von Bohlen und Halbach, Zacher et al. 2006)rev.(Hof and Morrison 2004), both of which, as we have discussed, could conceivably result from MMP dysregulation. Although to our current understanding the gradual decay of cognitive function remains a complex event, the lack of significant neuron loss makes it a highly approachable problem.

Age-associated cognitive decline is a nearly universal consequence of aging, ranging from mild to severely debilitating. At the heart of this reality is the recognition that as medical research strives to increase the quantity of life it must also endeavor to maintain the quality of life in an increasing population of elderly individuals. Although we possess the medical tools to frequently keep the body relatively healthy well into the seventh and eighth decades of life, we now search for ways to effectively preserve the health of the mind.

Two of the major concepts at the forefront in understanding the mechanisms of brain aging are 1) age effects on molecules which effect synaptic plasticity and the dendritic morphology of the neuron and 2) neuronal damage and dysfunction as a result of age-associated reactive oxygen species accumulation. We present evidence that a group of enzymes known as matrix metalloproteinases (MMPs) are a major regulator of the negative alterations in synapse and dendritic morphology, synaptic plasticity and cognitive function which accompany aging. When MMPs are chronically over-expressed or artificially elevated, the ability of the ability to initiate and/or preserve new optimal synaptic arrangements during the learning process is flawed. These observations spawn an obvious question: how do elevated levels of MMPs affect plasticity? To address this question, we present a model **[Figure 2]** which synthesizes our understanding of how MMPs participate in normal learning-associated synaptic remodeling, and how MMPs influence aged-hippocampal plasticity. We hypothesize that persistently elevated MMP levels results in perpetual interruption of ECM-cell adhesion contacts **[Figure 2A]**. Consequently, this situation would result in destabilized actin cytoskeleton and altered spine morphology. Furthermore, we predict that heightened MMP activity results in altered intracellular signaling capabilities of adhesion-sensitive pathways (not shown). In addition to the proposed physiologic effects on dendritic spines, increased ECM degradation potentially results in diminished neurotransmitter

diffusion barriers (evidenced by reduced peak-to-peak latencies during baseline stimulation). Speculatively, treatment with MMP inhibitors promotes hippocampal plasticity by restoring cell adhesion and by facilitating the reestablishment of appropriate neurotransmitter diffusion barriers **[Figure 2B]**. This model will serve as a focal point for the testing of future hypotheses regarding the impact of MMPs during aging.

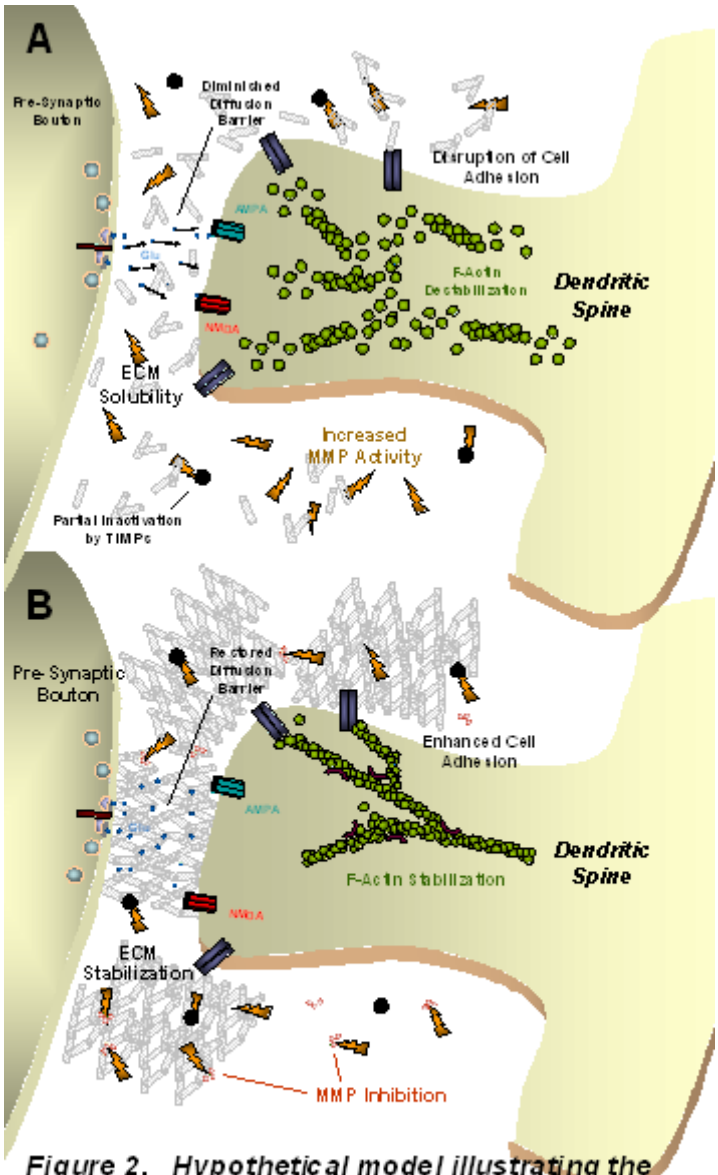


Figure 2. Hypothetical model illustrating the effects of perpetually elevated MMP levels on spine plasticity (A) and the theoretical effects of MMP inhibition on restoring normal plasticity (B).

The findings incorporated in this thesis have the potential to impact the fields of learning and memory, brain aging, and pharmacological approach to treating cognitive decline. Furthermore, these findings will expand our understanding of the relatively new and rapidly developing field of brain MMPs. This knowledge should provide a strong intellectual basis for the development of pharmacological and dietary based therapies for the prevention or reversal of age-associated cognitive decline. In addition, these studies are likely the first to directly explore the utility of MMP inhibitors as cognitive enhancers

in aged animals. Particularly exciting is that the impact of these studies and their underlying hypotheses could have immediate and profound clinical implications. Several large pharmaceutical companies have developed extensive libraries of MMP inhibitors, ostensibly for oncology applications. Although, the utility of these inhibitors as anti-cancer treatments has been almost universally disappointing, the companies, nevertheless, possess a vast selection of MMP inhibitors, many of which are FDA approved. Most germane to the effective treatment of age-related cognitive decline is that many of these inhibitors are permeable to the blood-brain-barrier and thus could be easily administered to suppress the exaggerated MMP activity that we have described in aged individuals. Taken together, these studies have immense potential for integrating the fields of brain aging, MMPs and oxidative stress and for providing a foundation for new and viable therapeutics.

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APPENDIX A: MMPs, Aging, and Reactive Oxygen Species

The Generation of Reactive Oxygen Species (ROS)

Most of the chemical energy provided to meet cellular requirements is produced by oxidative energy metabolism occurring within the mitochondrial matrix. Pyruvate and fatty acid molecules enter the mitochondrial matrix and are subsequently broken down to acetyl CoA. Acetyl CoA molecules participate in the TCA cycle by ultimately causing the reduction of NAD^+ to NADH. NADH acts as a soluble electron carrier molecule which shuttles electrons from the TCA cycle to the matrix membrane. NADH donates electrons to a series of transmembrane electron acceptor complexes- which use the energy from the redox reaction to create a proton gradient between the internal and external mitochondrial structures. The electrons donated to the electron acceptor complexes are ultimately delivered to the highly electronegative dioxygen molecule (O_2) acting as a thermodynamic sink for the free electrons. In most instances, the net result of electron donation to molecular oxygen is a reaction with free hydrogen ions to produce water molecules ($2H^+ + ½\ O_2 + 2e^- = H_2O$), where a single dioxygen molecule requires a 4-electron reduction to generate water. However, roughly 2-5% of O_2 consumed for energy metabolism participates in a single or double electron reduction and is released from the respiratory chain as a free reactive oxygen species (ROS) (Hensley, Pye et al. 1998). A majority of endogenously generated ROS molecules exist as super-oxide anions ($O_2^{\bullet -}$), or hydrogen peroxide (H_2O_2) from single and double electron reductions respectively (Simonian and Coyle 1996). Due to the highly reactive nature of these molecular species, most cellular systems have intricate mechanism to prevent ROS accumulation. ROS scavenger enzymes such as super oxide dismutase (SOD), catalase and glutathione peroxidase, along with the non-enzymatic molecules such as glutathione, Vitamins A, C, E, and flavenoids

eliminate ROS from the mitochondria, cytosolic space and the extracellular milieu. The generation of ROS by oxidative metabolism is typically balanced with the elimination of ROS by the antioxidants listed above. A situation where the rate of ROS generation significantly exceeds ROS elimination results in a condition termed oxidative stress.

Reactive Oxygen Species (ROS) and Oxidative Stress

Oxidative stress is a condition caused by critical accumulations of ROS and is typically associated with oxidative damage to three macromolecular components: proteins, lipids and nucleic acids. The amount of oxidative stress to a tissue can be inferred by analysis of specific molecular markers. Presence of these molecular markers is indicative of oxidative damage to a particular cellular component**.** For proteins, oxidative damage can be measured by amino acid carbonylation. Peroxidation of lipids generates measurable aldehyde and isoprostane compounds. Damaged DNA bases are usually removed and replaced by cellular reparation mechanisms. Presence of free damaged-bases can then be analyzed by HPLC-electrochemical detection (HPLC-ECD). A prominently studied base-lesion indicative oxidative damage to DNA is 8-OHdG (Floyd, West et al. 1990). Other markers used as an index for oxidative damage are activation of the stress-activated protein kinases (SAPKs), IL-1 expression, and heme oxygenase-1(HO-1) immunoreactivity (Nicolle, Gonzalez et al. 2001).

ROS accumulations during an oxidative insult can also influence cellular physiology by direct activation of particular kinase cascades. Many of the signaling cascades initiated by a ROS-insult modulate cell proliferation, morphology and apoptosis. Although the exact physiologic effect of oxidative insult is dependent upon type, concentration, and duration of ROS exposure, several signaling pathways are preferentially activated by ROS. Signaling pathways sensitive to

ROS exposure include the MAPKs, JAK-STATs, PLC, Src family kinases, and the PI-3K/Akt cascades (Wright, Fischer et al. 1996; Abe and Berk 1999; Bae, Seo et al. 1999; Kwon, Pimentel et al. 2003). Many of the signaling pathways induced by ROS regulate gene-transcription, suggesting that a mere transient ROS-insult can exert long-term changes to cellular physiology and tissue function.

Hippocampal Aging and the Oxidative Stress Hypothesis

The idea that oxidative stress is a contributing factor of aging was introduced in 1956 by Harmon (Harman 1956). It was proposed that aging is a result of cumulative damage to cellular macromolecules by ROS generated during normal metabolic processes. Since then, this relationship between ROS, oxidative stress and aging has been further supported by experiments detailing the rate of $O_2\bullet^-/H_2O_2$ production and increased levels of oxidative damage to protein, DNA and lipids during aging. Oxidative stress, by the overproduction or accumulation of ROS, is thought to be a mechanism for both aging and the progression of age-related chronic diseases.

A neural structure of increasing interest with regards to aging and oxidative stress is the hippocampus. The hippocampus is amendable to the study of neurodegeneration and cognitive impairment for several reasons: First- The hippocampus is a prominent neural structure which is critical for learning and memory. Second- The hippocampus is an early target for age related morphological and physiological alterations. Third- The hippocampus is particularly susceptible to oxidative stress due to its relatively high aerobic metabolic rate. Fourth- Experimental oxidative damage to hippocampal subfields produce cognitive impairments similar to age-related cognitive impairment. Fifth- LTP, an experimental analog of memory formation is best characterized in hippocampal tissue. For these reasons, hippocampal aging is emerging as an

important model for the oxidative stress hypothesis. Consistent with hippocampal aging and the oxidative stress hypothesis, the hippocampus of aged rats express many features indicative of oxidative stress. Nicolle *et al.*, performed an extensive examination of molecular markers associated with oxidative stress in the hippocampus of aged rats. They reported multiple signs of oxidative stress, including mitochondrial DNA damage, lipid peroxidation, protein carbonylation and heme oxygenase-1 (HO-1) expression. Interestingly, they found a significant correlation between hippocampal HO-1 immunoreactivity and impairment in hippocampal dependent spatial learning tasks (Nicolle, Gonzalez et al. 2001). Similar to age related oxidative stress, intermittent hypoxia (IH) induced ROS generation caused lipid peroxidation, isoprostane production and resulted in deficits to spatial task acquisition. However, evidence of oxidative stress and spatial deficits could be attenuated by antioxidant administration (Row, Liu et al. 2003). Several other studies have demonstrated the ability of antioxidants to attenuate age-relate LTP deficits in the rat hippocampus (Murray and Lynch 1998).

Aging—Matrix Metalloproteinases and Reactive Oxygen Species

The vast literature detailing the regulation of MMPs by ROS within a multitude of cells and tissues provides strong evidence that ROS accumulation during aging contributes to the MMP dysregulation that we have observed in the hippocampus [for detailed review see (Nelson and Melendez 2004)]. This regulation is typically accomplished through the convergence of signaling pathways downstream of ROS and upstream of MMP regulation. For instance, Ras signaling is highly sensitive to ROS, and Ras expression has been shown to increase expression of collagenase-, stromelysin- and gelatinase-type MMPs (Thomas, Khokha et al. 2000). Most of the ROS-modulated signaling cascades lead to the activation of transcription factors that are critical

for the regulating the expression of MMPs. Elevated ROS, for example, typically lead to increased levels of AP-1 and nuclear translocation of NF-κB, both of which directly modulate MMP gene transcription (Nelson and Melendez 2004). Despite the overwhelming evidence that ROS modulate MMP levels in a variety of tissues, the effect of ROS on brain MMP regulation remains to be investigated.

APPENDIX B: Hippocampal Electrophysiology

Below is a brief description of some of the electrophysiological techniques employed in this thesis:

Theta Burst Facilitation

For these studies, long term potentiation (LTP) tetanization was accomplished by theta burst stimulation (TBS). Theta burst stimulation consists of a series of brief high frequency bursts; each burst is usually comprised of four .1ms pulses at 100 Hz frequency. Bursts are delivered at a frequency of 5 Hz. This induction protocol is thought to be a physiologically relevant tetanization scheme as the burst frequency is patterned to mimic the endogenous theta rhythms of the hippocampus. Short term facilitation resulting from theta burst stimulation produces a robust depolarization in post-synaptic elements sufficient for the activation NMDA receptors. Sequentially, NMDA receptor activation permits calcium entry requisite for LTP stabilization. Due to resultant activation of NMDA receptors, burst response facilitation is thought to be a critical determinant of theta-induced LTP induction (Kramar, Lin et al. 2004). Given this relationship, the effects of MMP inhibition on relevant burst response parameters was assessed. The following features of theta burst facilitation include: within-burst fEPSP (comparison of fEPSP amplitudes within each burst), inter-burst facilitation (comparison of the total post-synaptic depolarization occurring with each burst within the theta train) and magnitude of after-hyperpolarizations (AHPs) (hyperpolarizations which typically manifest between each high frequency burst within a theta train).

Input-Output Curves

Input-output curves (I-O curves) were generated by increasing stimulus duration with constant stimulus intensity. Stimulus duration during I-O curve generation will be at discrete levels between .04 ms to .3ms. To control for quality of slice preparation and handling, I-O Curves can be used to pre-screen slices for a suitable level of basic electrical sensitivity. Maximum population-spike free amplitudes achieved during generation of I-O curve will be used to determine test-pulse duration (the duration that will be used will be that which yields a response that is 50% of the maximal response). Additionally, I-O curves can be used to ascertain the effects of pharmacological treatments on slice physiology. This assessment helps determine if treatment affects electrical properties of slices or if treatment effects are restricted to plasticity-relevant mechanisms (therefore having little impact on I-O relationships).

Paired-Pulse Facilitation

When twin stimuli are administered with an inter-stimulus interval (ISI) between 50 and a few hundred milliseconds, an augmented evoked response is induced from the second pulse (as compared to the initial evoked response). This form of short term plasticity is termed paired pulse facilitation (PPF). The cellular basis for PPF is thought to involve the presence of residual presynaptic calcium, stemming from the initial pulse, effectively priming neurotransmitter release during the subsequent pulse. Because mechanisms supporting PPF are thought to be predominantly confined to pre-synaptic elements governing neurotransmitter release, treatment conditions affecting paired pulse are thought to impact elements responsible for neurotransmitter release (possibly affecting vesicle docking, vesicular release probability, etc... for review see (Thomson 2000)). For diagnostic purposes, positive and negative PPF results are useful in helping determine the mechanistic impact of MMP inhibition on hippocampal plasticity in aged slices.

Long Term Depression

Long term depression (LTD) is a NMDA-dependent process characterized by an enduring decrease in synaptic efficacy resulting from persistent low-frequency stimulation. Low frequency stimulation accomplishes LTD by causing a modest increase of post-synaptic intracellular calcium, preferentially promoting the activation of phosphatases. Phosphatase activation ultimately results in AMPA receptor downregulation and synaptic depression. Although there are a variety of LTD tetanizing protocols, LTD is commonly initiated with low frequency stimulation (LFS); stimulating for 15 minutes at 1Hz frequency. In addition to the direct effects on AMPA, the long-term expression of LTD is thought to involve many of the same features as LTP (e.g. gene transcription, structural remodeling of synapses). It should be noted that the physiological significance of LTD, and its relationship to depotentiation (i.e. the reversal of LTP), are controversial.

APPENDIX C: Additional Observations

- **Evidence for the regulation of hippocampal MMPs by acute exposure to hydrogen peroxide.**

MMPs are induced by ROS under oxidative stress conditions for a variety of tissue types. However, this relationship between ROS and MMP modulation has not been reported in hippocampal tissue. To determine if MMPs are regulated by ROS or modulated in experimental conditions used to induce oxidative stress in the hippocampus, we utilized a hippocampal slice preparation from 6-month-old animals. Hippocampal slices were treated with an acute (6 hours) exposure of the ROS hydrogen peroxide (H_2O_2) of concentrations typical for experimental oxidative stress test conditions. Upon H_2O_2 treatment, we observed an increase in MMP-3/TIMP-2 complex formation **[Figure 1]**, but no change of total MMP-3 and MMP-9 protein levels (data not shown). The fact that the H_2O_2 treatment failed to induce expressional changes for either MMP-3 or MMP-9 is not surprising considering that other studies reported ROS induction of MMPs required long term ROS exposure.

- **The cytoskeleton-regulatory protein *cortactin* is regulated by MMP activity**

Plasticity within the hippocampal synaptic environment depends on subtle architectural remodeling as a result of activity-driven input. This architecture is constantly stabilized and destabilized by the interactions among a multitude of plasticity-associated molecules involved in modulating cell surface interactions, cell signaling and cytoskeletal stability. Previously, we found that the hippocampal levels of the actin cytoskeleton-regulatory molecule, *cortactin*, are regulated by MMP activity [**Figure 2; also (Meighan, Meighan et al. 2006)**]. Specifically, during learning when MMP levels are elevated, hippocampal cortactin levels are low. Furthermore, if rats are

administered MMP inhibitor during this time, cortactin levels are dramatically elevated indicating an inverse relationship between MMPs and cortactin. This inverse relationship between MMP activity and cortactin has been observed in aged-hippocampus as well. In hippocampal tissue from aged rats, where MMP levels are dramatically elevated, cortactin levels are attenuated [**Figure 3**]. Cortactin functions to stabilize the actin cytoskeleton and thus stabilizes dendritic spine structure. It is likely that the excessive cortactin loss associated with chronic MMP elevation could be facilitating a destabilization of dendritic cytoskeletal elements and retraction of dendritic spines. This could lead to an overall loss of functional synapses. In fact, underscoring this relationship, a recent manuscript was published describing a disruption in F-actin and the loss of dendritic spines in hippocampal neurons as a direct result of MMP-7 upregulation (Bilousova, Rusakov et al. 2006). It's likely that additional plasticity critical proteins are also affected by heightened MMP activity.

- **Increased MMP levels correlate to decreased weight of the aged hippocampus**

It is likely that hippocampal atrophy plays a significant role in the development of cognitive aging. Current evidence suggests that age-associated hippocampal atrophy is linked to dendritic field density reduction rather than cellular loss. Consistent with this, age associated increased MMP activity is concomitant to cortactin downregulation, a situation likely to result in destabilization of structural elements reliant on actin filaments. Due to plausible connections between aging, MMP hyper-induction, cortactin downregulation and hippocampal atrophy, we choose to see if evidence exists that increased MMPs linked to neurodegeneration. To make a preliminary assessment of this possible association, the mass of dissected hippocampi from 24

month old Sprague Dawley rats were measured and related to their corresponding active MMP-3 levels by regression analysis [**Figure 3**]. The relationship between active MMP-3 and hippocampal mass is such that increasing presence of MMP-3 is associated with decreasing hippocampal mass. This relationship is nonexistent in 3-month old control animals (data not shown).

Effects of MMP inhibition on Morris water maze learning for aged-adult rats

MMPs are known to be critical for normal neuronal functioning during learning in young adult rats; however, excessive MMP levels may have serious consequences for hippocampal function. We previously described that MMP inhibition improves the electrophysiological characteristics of the aged hippocampus. We hypothesize that these inhibitors will also ameliorate age-associated cognitive decline. The primary goal of this study is to determine if MMP inhibitors are effectual at improving age-associated learning deficits in aged rats performing a spatial learning task, the Morris water maze. To test this idea, the MMP inhibitor FN439 was administered, intraperitoneally, to 22 month old F344 rats and their learning ability assessed in the water maze. Aged-adult rats treated with FN-439 were more efficient in the navigation of the water maze task than untreated rats (Figures 4,5). Furthermore, FN-439 treated rats were more successful at locating hidden pedestal, within the first three days, than the untreated controls. These data suggest that administration of the MMP inhibitor FN-439 two hours prior to water maze acquisition training improved the acquisition rate of the task in these animals. This result is in contrast to behavioral data demonstrating inhibition of task acquisition for younger rats (Meighan, Meighan et al. 2006).

Figure 1: Effects of H_2O_2 exposure on MMP-3 activity regulation in acute hippocampal slices. Twelve 200 micron Hippocampal slices from 6 month old rats were randomized and pre-incubated in ACSF for 2 hours prior to treatment. Six slices were treated with .6mM H_2O_2 in ACSF for 6 hours and compared to six slices from the same hippocampus, incubated in ACSF + vehicle forhours. (A) H_2O_2 treated slices (lane 2) observed an increased MMP-3 / TIMP-2 complex formation as compared to control slices (lane 1). (B) Data expressed as mean values (+/- SEM) n = 3.

Figure 2: Hippocampal cortactin levels are regulated by MMP activity. Training in the Morris water maze for one day significantly reduced hippocampal cortactin levels as compared to home cage controls (n=8, *p=0.035). MMP inhibition with FN-439 reversed water maze associated cortactin down-regulation and increased cortactin levels well above baseline. (n=6 per group). (*p<0.05, ***p<0.001, significantly different than vehicle control) Accompanying blot images demonstrate the typical cortactin doublet immunoreactivity at approximately 80 and 85 kDa.

Figure 3: Cortactin levels are depressed in aged hippocampal tissue. Hippocampal tissue from 3, 12 and 24 month old rats was assessed for cortactin levels by Western blotting. 24 month old animals have severely attenuated hippocampal cortactin levels during a time in which they have chronic elevations in MMPs. This corresponds with our previous data (Figure 4) demonstrating an inverse relationship between MMP activity and cortactin.

Figure 4: Active MMP-3 protein inversely correlates with hippocampal mass. Hippocampi from 24 month old Sprague-Dawley rats were dissected and weighed prior to assessment of MMP levels by Western blot analysis. Mass and MMP-3 levels were subjected to analysis byt linear regression. Rats demonstrated an inverse relationship between hippocampal MMP-3 levels and hippocampal mass (p=.011).

Figure 5: Effects of MMP inhibition on the distribution of daily water maze latencies for aged-adult rats. Fisher 344 (F344) rats were subjected to five days of Morris water maze training (4 maze trials per day). MMP inhibitor treated rats were given a daily IP injection of FN-439 2-hours prior to water maze training. Distribution of mean latencies represented as box plots; the box represents the interquartile range (IQR; 25^{th} to 75^{th} percentiles), the median value, and the adjacent values [defined as (1.5) x (IQR)]. Green and red filled circles represent moderate outliers [(1.5-3.0) x (IQR)] and severe outliers [(> 3.0) x (IQR)] respectively. C1-C5 corresponds to control animal latencies on days 1-5 and FN1-FN5 corresponds to MMP inhibitor treated animal latency for days 1-5 respectively.

Figure 6: Effects of MMP inhibition on mean water maze latencies. F344 rats were treated as previously described (Figure 5). Mean values (+/- SEM) are expressed for both control (open circles) and MMPi treated (filled circles). Data fit with exponential decay functions for both treated and control groups. Analysis with two-factor ANOVA (treatment vs. day) reveals an independent treatment effect on daily mean latencies (p=.0063).

Table 1: Contingency table describing the effect of MMP inhibition on successful completion of water maze task for days 1-3. Animals are allotted 120s, for each trial, to successfully complete water maze task. Successful completions (the ability to locate pedestal in allotted time) is scored as a "hit" and unsuccessful completion (inability to locate pedestal in allotted time) is scored as a "miss". A contingency table was constructed using pooled data from experiments where MMP inhibitor is administered ICV (n=3 animals) and IP (n=9 animals for treated and n=11 for control). *P-values are Holmes corrected.

Figure 1

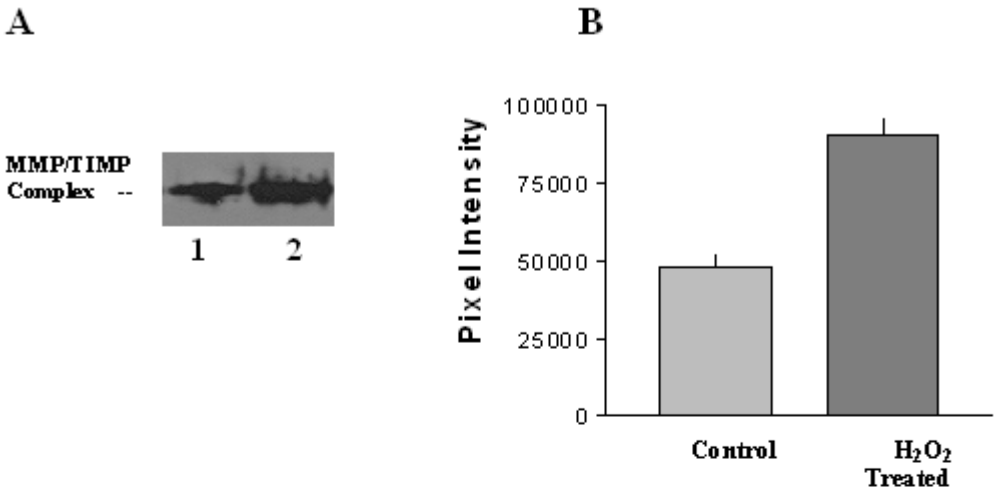

Figure 2

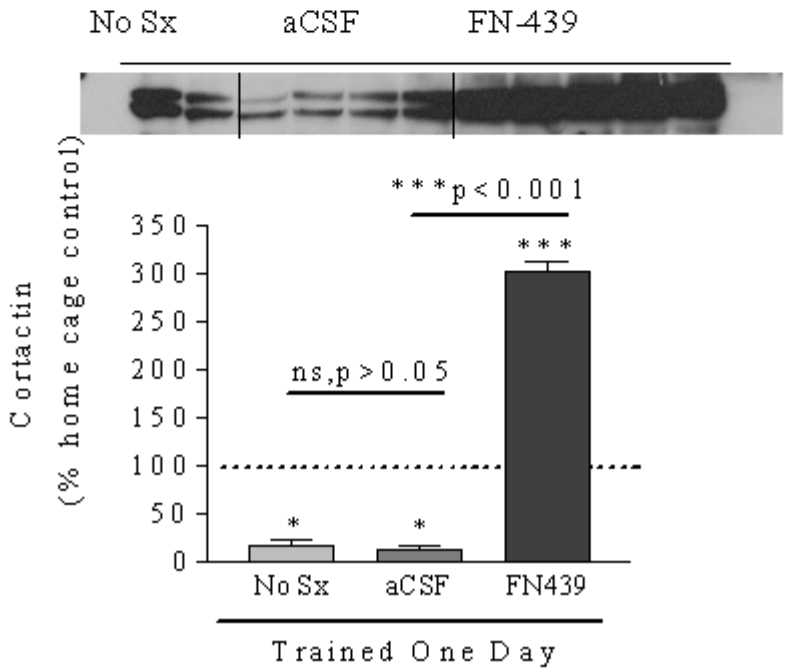

Figure 3

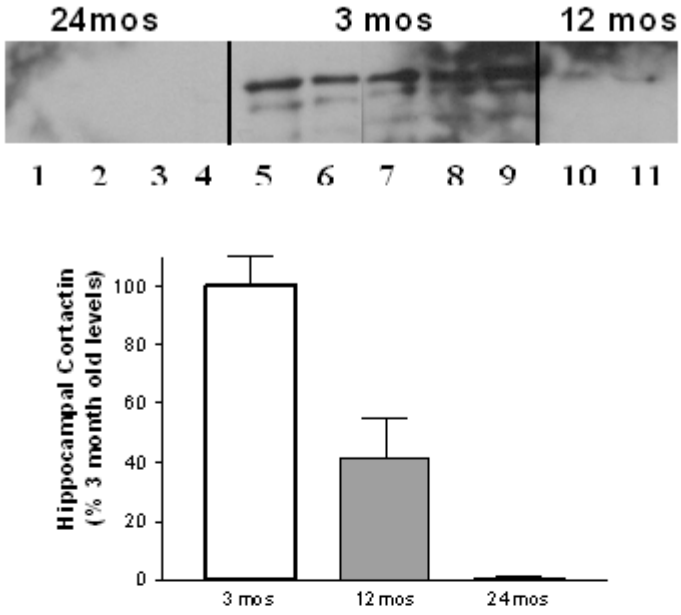

Figure 4

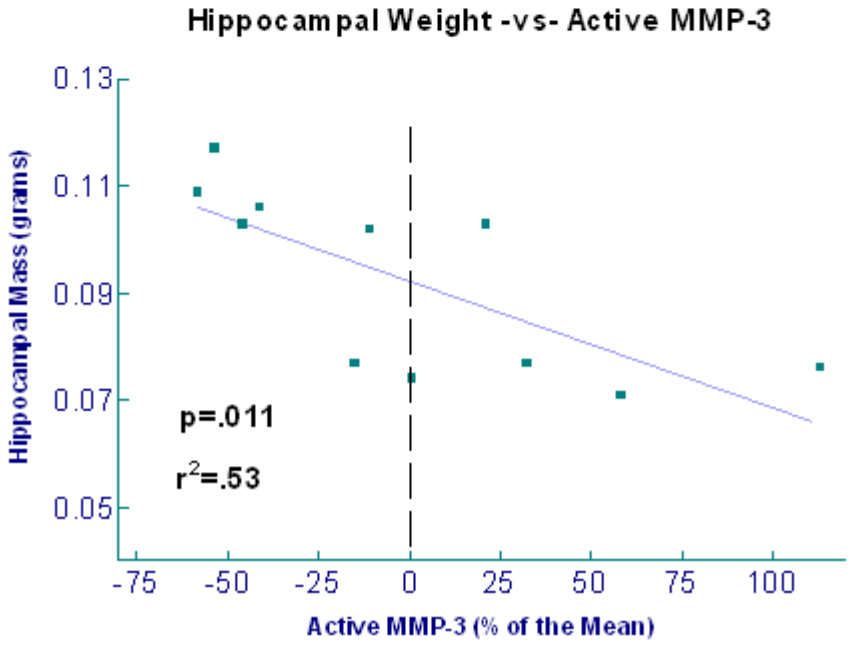

Figure 5

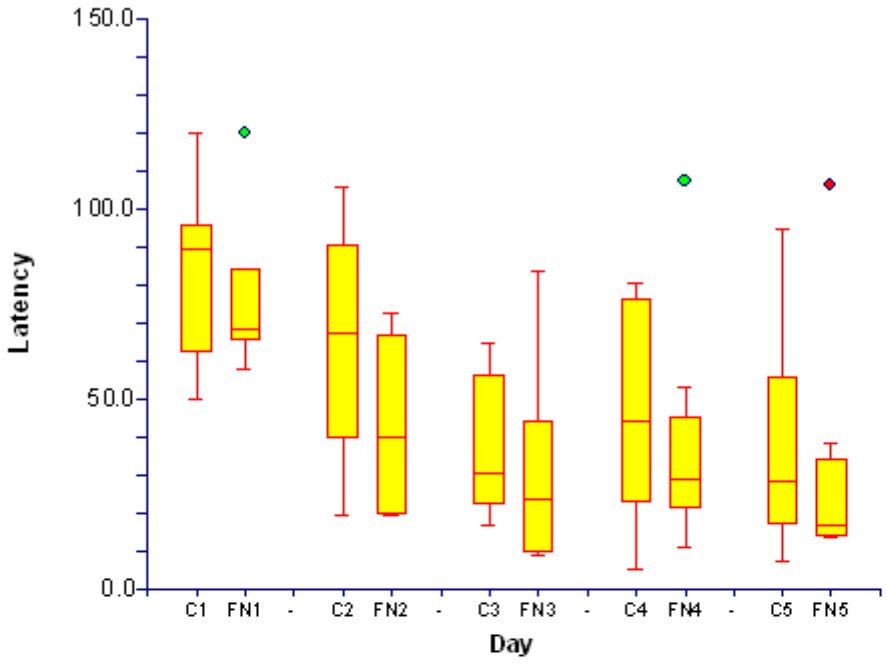

Figure 6

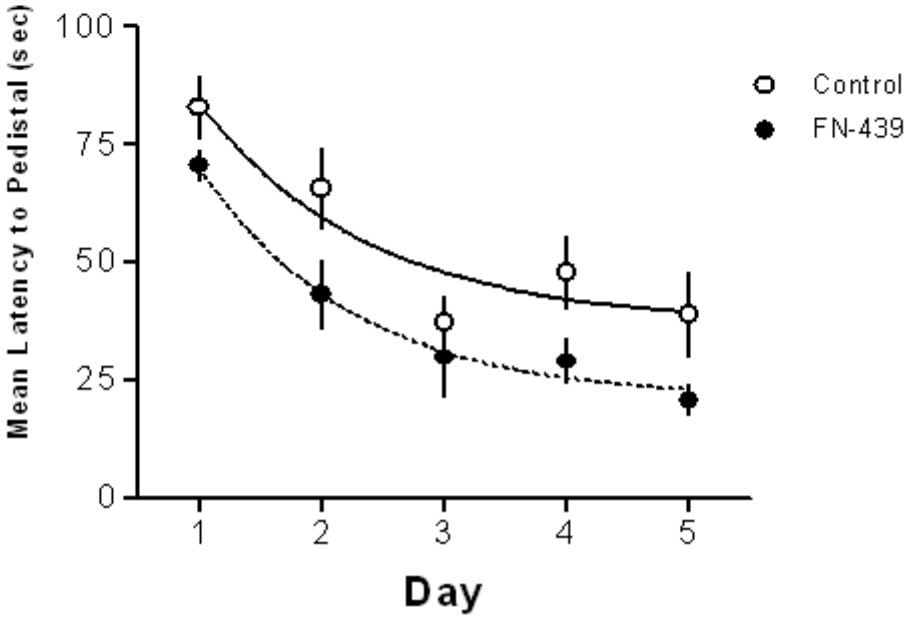

Table 1

		Hit	Miss	Total	Odds Ratio	p-value
Day 1	Control	28	28	56	1.97	p=.1280*
	MMPi	32	16	48		
Day 2	Control	37	19	56	4.21	p=.0134*
	MMPi	44	5	48		
Day3	Control	48	8	56	-	p=.0192*
	MMPi	48	0	48		
Total	Control	113	55	168	2.87	p=.0002
	MMPi	124	21	145		