# THE INVOLVEMENT OF MATRIX METALLOPROTEINASES IN NICOTINE CONDITIONED PLACE PREFERENCE IN ADOLESCENT FEMALE RATS

By

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## Abstract

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The process of learning new information or modifying existing information requires synaptic plasticity in the brain. In order for these plasticity events to occur, the extracellular matrix (ECM) is reconfigured and reorganized by their primary modulators – the matrix metalloproteinases (MMP). MMPs are a class of proteinases that are known to be involved in the learning and memory process by modulating synaptic plasticity. Our hypothesis is that MMP dependent synaptic restructuring takes place during the acquisition of drug dependent learning. We used a nicotine conditioned place preference (CPP) model to understand the underlying synaptic events that drives drug preference. There has been some debate regarding the ability of nicotine to elicit CPP in rats. Therefore the goal of the studies described in chapter 2 was to first establish that nicotine induced CPP. Our results show that nicotine produces robust, reproducible CPP at 0.03mg/kg dose using a 5 day drug administration protocol. We also observed that higher doses of nicotine produced conditioned place aversion. In chapter 3 we noted changes in the activity of MMPs – 2, 3, and 9 in the hippocampus and prefrontal cortex after conditioning. Inhibition of MMPs during nicotine conditioning interfered with the development of CPP. Elevation in MMP-3, but not MMP-2 and MMP-9 expression, accompanied re-activation of

previously learnt drug related memory in both the hippocampus and the prefrontal cortex. Changes in the activity of cortactin, an actin bound cytoskeletal marker protein, were also observed during the acquisition of CPP but not following re-exposure to the drug context. These results suggest that MMPs – 2, 3, and 9 are involved in facilitating intracellular and extracellular events required for synaptic plasticity and memory consolidation while MMP-3 is uniquely involved in the reconsolidation process.

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## DEDICATION

I dedicate this dissertation to my parents and my grandmother, Ranganayagi, who taught me the value of an inquisitive mind.

# Chapter 1

General Introduction

## Introduction

Tobacco use is the second leading cause of death in the world, accounting for nearly 5 million mortalities each year. Cigarette smoking is the most common type of tobacco consumption. There are more than 1.1 billion smokers worldwide, and on an average one in 10 adults throughout the world are smokers. Almost every first time use of tobacco occurs in adolescents and teenagers, and the addictive effects of smoking are believed to be greater during this period than during adulthood (Chambers et al., 2003; Merikangas, 2004; Adriani et al. 2002).

Nicotine is the primary addictive component of tobacco, and dependence on nicotine leads to persistent behavioral, psychological, and physiological changes in a person. These changes manifest as dependence, craving, tolerance and withdrawal. All of these phases results from alterations in normal brain activity through reconfiguring existing neural synapses. The difference with drug-induced learning and normal learning is that the drug subverts the normal learning and memory (L&M) mechanisms and uses it to produce aberrant reward functions in the brain.

With repeated drug exposure the body's normal homeostatic parameters reconfigure to a setting that is highly biased towards additional drug acquiring behavior. Normal reward value is overridden so that food or sex no longer brings as much pleasure to the animal as the drug does, and eventually the entire functioning of the animal becomes geared towards obtaining more drugs. Initially the drug taking is rewarding, but when a person or animal develops a dependence on the drug, the absence of the drug begins to produce strong negative sensations. This eventually results in the addict taking drugs not because they favor it, but because the absence of

the drug has highly undesirable physical consequences (Hyman and Malenka, 2001; Hyman 2005; Koob and Le Moal, 2001).

Recently, there has been much interest in understanding the molecular mechanisms by which neuronal plasticity occurs during the consolidation and reconsolidation of drug memory. The research presented in the subsequent chapters of this dissertation focuses on changes in extracellular matrix (ECM) related molecules that trigger synaptic restructuring in areas of the brain implicated in learning and memory formation. The following review summarizes the background of the hypothesis investigated within this dissertation.

**Animal models of addiction**

Conditioned place preference (CPP) and self-administration (SA) are the most widely used models to study the rewarding effects of many different psychoactive drugs.

In general, conditioned place preference experiments have 3 phases – preconditioning, conditioning and postconditioning. During preconditioning the initial preference for each chamber of a two or three compartment box is determined. During conditioning, animals are given drug injections in one compartment and saline injections in the other over a course of few days. At this time the animal forms associations between the types of drug received (unconditioned stimulus) and the compartment (conditioned stimulus) in which it receives it. At the end of this phase the animals are once again tested for chamber preference in the absence of the drug. Increase in preference for the drug-paired side indicates a greater reward value for the drug compared to saline. Most drugs of abuse like cocaine, morphine and amphetamine have been shown to readily induce CPP.

In the SA model, the animals are initially trained to receive food rewards each time a lever is pressed. Once they learn this task, food rewards are replaced by infusions of a small dose of drug through intravenous catheters. SA has higher face, construct and predictive validity compared to the CPP model, and is generally preferred in addiction studies (O'Dell and Khroyan, 2009)

**Nicotine sensitivity in adolescent females**

Adolescence is a period in which animals display high risk taking and novelty seeking behavior. During this period, the prefrontal motivational system and impulse control pathways undergo alterations to reach adult levels. The pro-motivational dopaminergic pathways are more developed and show higher activation than the inhibitory serotonergic pathways (Roesch and Olson, 2004; Volkow and Fowler, 2000; Stansfield and Kirstein, 2006; Spear 2000). This discrepancy under normal conditions aids in the adolescent trying out novel roles and responsibilities of adulthood and leaving behind playful childhood behavior. While this adaptation facilitates the development of more adult-like independent behavior, it may also be responsible for the adolescent trying the novel experience of addictive drug taking, since they experience greater reward value for the drug (Torrella et al., 2004; Leslie et al., 2004; Badanich and Kirsteina, 2004; Kelley et al., 2004).

First time use of tobacco almost always occurs in adolescents and teenagers and the addictive effects of smoking are believed to be greater during this period than during adulthood (Chambers et al., 2003; Merikangas, 2004; Adriani et al., 2002). Just a single nicotine injection produces CPP in early adolescent rats but not in adults, indicating greater sensitivity to nicotine reinforcement during this phase of life (Belluzzi et al., 2004). Some adolescents however may use smoking as a self-medication method. Adolescents with ADHD are known to smoke in order

to improve attention and cognition (Whalen et al., 2003, Odell et al., 2006). Studies have shown that adolescents who start smoking at a young age are more likely to develop long-term addiction to nicotine than those who start smoking later in life (Levin et al., 2003). Moreover, tobacco can act as a gateway drug and increase the likelihood of adolescent smokers trying illegal drugs and alcohol (Kelley and Rowan, 2004).

Apart from differences observed in response to nicotine between adults and adolescents, there are also differences in the response to nicotine between sexes. Females generally show greater sensitivity and withdrawal to nicotine than males. They also have higher motivation to acquire nicotine than males. Self-administration studies in rats have observed that female rats exhibit higher number of nicotine infusions across dose during acquisition (Collins and Izenwasser, 2004; Levin et al., 2003). These effects are independent of the stage of the estrous cycle. Females begin infusion at a lower dose, and show larger total nicotine intake than males (Chaudhri et al., 2005). These factors can account for faster addiction to nicotine in females, and the greater intensity of withdrawal symptoms when attempting smoking cessation.

**Reward circuit implicated in drug abuse**

The mesocorticolimbic dopamine pathway is believed to be responsible for generating reward and reinforcement of behavior that is required for survival in a normal animal. This circuit consists of ascending dopamine projections from the ventral tegmental area (VTA) to the nucleus accumbens (NA) and prefrontal cortex (PFC), and descending glutamatergic projections to the VTA and NA from the PFC. These dopaminergic projections are activated during the development of drug addiction, whereas the glutamatergic projections are implicated in drug craving following addiction. (Kelly and Berridge 2002; Robinson and Berridge, 2001; Robinson

and Berridge, 1993; Cousins and Salamone , 1994; Ikemoto and Panksepp, 1999; Crombag et al., 2008).

**Nucleus Accumbens (NA)**

The NA comprises of two major structures: a central core and a shell. The shell is believed to be involved in mediating incentive salience and motivation for drug obtaining behavior. The core plays a major role in expressing drug seeking behavior in response to rewarding stimulus. This structure receives dopamine input from the ventral tegmental area and glutamate inputs from the prefrontal cortex, amygdala, and hippocampus. Take together, the NA integrates inputs from limbic and cortical regions and forms a link between motivation and action (Carlezon and Thomas, 2009; Di Chiara, 2002; Di Chiara et al., 2004; Kalivas et al., 2009).

**Ventral tegmental area (VTA)**

The dopaminergic neurons of the VTA project to NA, PFC and basolateral amygdala. The release of dopamine from the VTA promotes reward-related learning (Hyman, 2005). There is an increase in dopamine release when the animal is expecting a reward and if the reward is withheld or is greater than expected there is a change in the firing patterns of the cell (Montague et al., 2004; Montague et al., 1996; Schultz et al., 1993; Hollerman and Schultz, 1998; Schultz, 1998). Dopamine release in the midbrain can thus aid in learning the relationship between a stimulus and reward and also in predicting the reward value.

**Prefrontal Cortex (PFC)**

The PFC is involved in goal selection, assigning a value to the goal and selecting an action based on the value (Miller and Cohen, 2001). The PFC receives dopaminergic inputs from

the VTA, and the phasic dopamine release acts as a gating signal that interferes with normal activity resulting in neural adaptations that lead to a pathological narrowing of goal selection and salience to those that are drug related (Volkow et al., 1993; Berke and Hyman, 2000; Redish, 2004).

**Hippocampus**

Although the hippocampus is primarily involved in learning and memory consolidation, there has been evidence that support its involvement in drug addiction. Lesions in the hippocampus impair cocaine SA and decreased response for food reward (Caine et al., 2001; Burns et al., 1993). Stimulation of the hippocampus following extinction induced reinstatement of cocaine and amphetamine SA (Vorel et al., 2001; Taepavarapruk and Phillips, 2003; Robbins et al., 2008).

**Addictive Drugs and Neural Plasticity**

Exposure to addictive drugs leads to alterations in the homeostatic parameters in order to accommodate the abnormal stimulations of the drug. Long-term exposure to nicotine leads to up-regulation of nicotinic acetylcholine receptors (nAChR) as a homeostatic response to increased desensitization of nAChRs (Buisson and Bertrand, 2001; Watkins et al., 2000). Repeated administration of cocaine, amphetamine and nicotine increased spine density and dendritic branching in the NA core and shell, and in the medial prefrontal cortex (Robinson and Kolb, 1999; Robinson and Kolb, 1997; Brown and Kolb, 2001; Gonzalez et al., 2004; Robinson and Kolb, 2004).

Addictive drugs reinforce the context and cues that are typically a part of the drug taking experience. Eventually these factors can act independently and trigger drug use. (Balfour et al.,

2000; Berke and Hyman, 2000; Di Chiara, 2000). Associative learning occurs between the drug and the environment in which it is obtained as the animal continually updates its construct of environmental saliency (Schultz et al., 1997). Thus drug seeking behavior gets woven into normal physiological adaptations and eventually begin to dominate the behavior of the animal.

**Matrix Metalloproteinases (MMPs)**

MMPs are endopeptidases belonging to the metzincin superfamily. They are typically secreted as inactive zymogens requiring zinc for their activation. They have a "pro" domain that interacts with the zinc ions in the catalytic domain, and proteolysis of the "pro" domain activates the latent MMP. MMPs are known to be involved in cell differentiation, migration and proliferation, and also the cleavage of cell surface receptors and cell-cell adhesion molecules (CAM) and ECM proteins. (Dzwonek et al. 2004, Sternlicht and Werb, 2001).

Due to their destructive nature, MMP activation is a highly controlled process that is regulated at several levels from gene transcription to enzyme activation and protein activation. Gene transcription and regulation of MMP-9 has been known to be controlled by AP-1 and NF-κB. Tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA) are enzymes that release plasmin to activate MMP-1 and 3. Some of the MMPs such as MMP-2, 3, and membrane type MMPs are known to activate other MMPs. The activity of MMPs can be inhibited by their endogenous inhibitors – Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) through the formation of tight MMP-TIMP complexes (Dzwonek et al., 2004, Wright et al., 2002; Myohanen and Vaheri, 2004; Castellino and Ploplis, 2005). Activity dependent changes in MMP levels accompany normal synaptic plasticity, and an abnormal balance between MMPs and TIMPs can result in neurological diseases such as brain tumors, multiple sclerosis,

ischemia, Alzheimer's disease, infections, and fibrosis of blood vessels (Lukes et al., 1999; Mann and Spinale, 1998).

The ECM is the major substrate for MMP activity. The ECM is a network of proteins and polysaccharides that form a scaffold around all individual cells. The ECM proteins form the underlying structural basis of tissue, and hence play a major role in influencing its morphological properties. The ECM communicates to the cells via cell adhesion molecules and cell surface receptors such as integrins, cadherins, syndecans and PSA-NCAM (Staubli et al., 1998; Kramar et al., 2001; Chan et al., 2003). Appropriate activation of these molecules can influence intracellular processes such as actin cytoskeletal remodeling, ion influx, calcium influx and gene transcription (Dityatev and Schachner, 2003; Kaczmarek et al., 2002; Yamada et al., 2001). Since MMPs are a major ECM related protein, they can regulate plasticity in neurons by mediating ECM signaling.

**Cortactin and Synaptic Plasticity**

Cortactin is an f-actin binding protein that is found highly concentrated in the postsynaptic density (PSD) of the dendritic spines. It is involved in multiple neuronal functions such as cell migration, adhesion, morphogenesis and axonal guidance (Lua and Low, 2005). It plays an important role in synaptic plasticity by stabilizing and promoting polymerization of the actin filament and thereby regulating the dendritic actin cytoskeleton. NMDA receptor activation has been shown to redistribute cortactin from the dendritic spine to the dendritic shaft (Hering and Sheng, 2003), and this allows us to use cortactin as a marker to determine the state of actin polymerization. Cortactin activity is regulated by several cell surface molecules such as N-syndecans, integrins and cadherins (Kinnunen et al., 1998; Vuori and Ruoslahti, 1995; Helwani

et al., 2004) which are in turn regulated by the ECM proteins. Thus MMPs can regulate dendritic morphology by modulating the ECM proteins.

**Matrix metalloproteinases and Synaptic Plasticity**

Although MMPs have long been implicated in neural development, it is only lately that their involvement in learning associated plasticity and cognition been appreciated. Meighan et al. have been the first to establish the critical involvement of MMPs in learning (Meighan et al., 2006). They noted that: 1. MMP-3, 9 mRNA and protein increased 4 hours following a spatial learning task, and that these increases occurred only during the active acquisition of a task. Once the task was learned, additional training did not promote MMP induction; 2. The MMP induction was NMDA receptor dependent, suggesting that MMP changes were activity dependent; 3. Inhibition of MMP interfered with the induction and stabilization of LTP, and 4. Inhibition of MMP-3, 9 by a broad spectrum MMP inhibitor FN-439 or by antisense oligo-nucleotides interfered with the acquisition of the spatial task. Wright et al. have reported increases in MMP-3 and 9 in the hippocampus and prefrontal cortex following a habituation task (Wright et al., 2004). MMP-3, 9 levels have been found to be elevated following passive avoidance conditioning, and inhibition of MMP disrupted passive avoidance memory (Olson et al., 2008; Nagy et al., 2007). MMP-2, 9 knockout mice have shown decreased CPP and sensitization following methamphetamine administration (Mizoguchi et al., 2007a, b). Together, these evidences suggest that MMPs play an important role in the acquisition and consolidation of learning.

**Consolidation and reconsolidation of memory**

Memory consolidation is thought to occur when information moves from a labile short-term state to a more permanent long-term storage. Hippocampal lesions or protein synthesis

inhibition soon after training interfered with the learning of fear conditioning and passive avoidance conditioning, but if these manipulations occurred several weeks after the initial learning of the task, no impairment was noted (Anagnostaras et al., 1999; Quevedo et al., 1999). This indicates that the long-term memory of the task is hippocampus independent. Although it is unclear where the long-term memory is stored at this time, several theories have suggested the involvement of the prefrontal cortex (Frankland and Bontempi, 2005).

The classic idea behind memory consolidation was that it was a one-time process following which the memory was stored permanently in the brain. The seminal studies conducted by Misanin and colleagues challenged this idea (Misanin et al., 1968). Misanin et al. noted that there was a renewed susceptibility of previously consolidated auditory fear memories to electroconvulsive shock following reactivation of the existing memory. This study remained relatively unnoticed until the late 1990 when the study was rediscovered and the reported phenomenon characterized as “reconsolidation” (Nader et al., 2000).

The reconsolidation hypothesis states that when a consolidated memory is recalled, it is susceptible to disruption for a few hours following reactivation. If the memory remains undisrupted, it gets reconsolidated again and can be strengthened or modified at this point. When a memory is recalled, the long-term memory trace is activated and destabilized to allow for the update of this memory trace based on new information that may be present at the time of recall.

Reconsolidation has been characterized in animals mainly using classical Pavlovian conditioning paradigms. Reconsolidation of memory has been observed following passive avoidance conditioning, radial arm maze, water maze, conditioned taste aversion, auditory fear conditioning, and contextual freezing, cocaine self-administration (Taubenfeld et al., 2001;

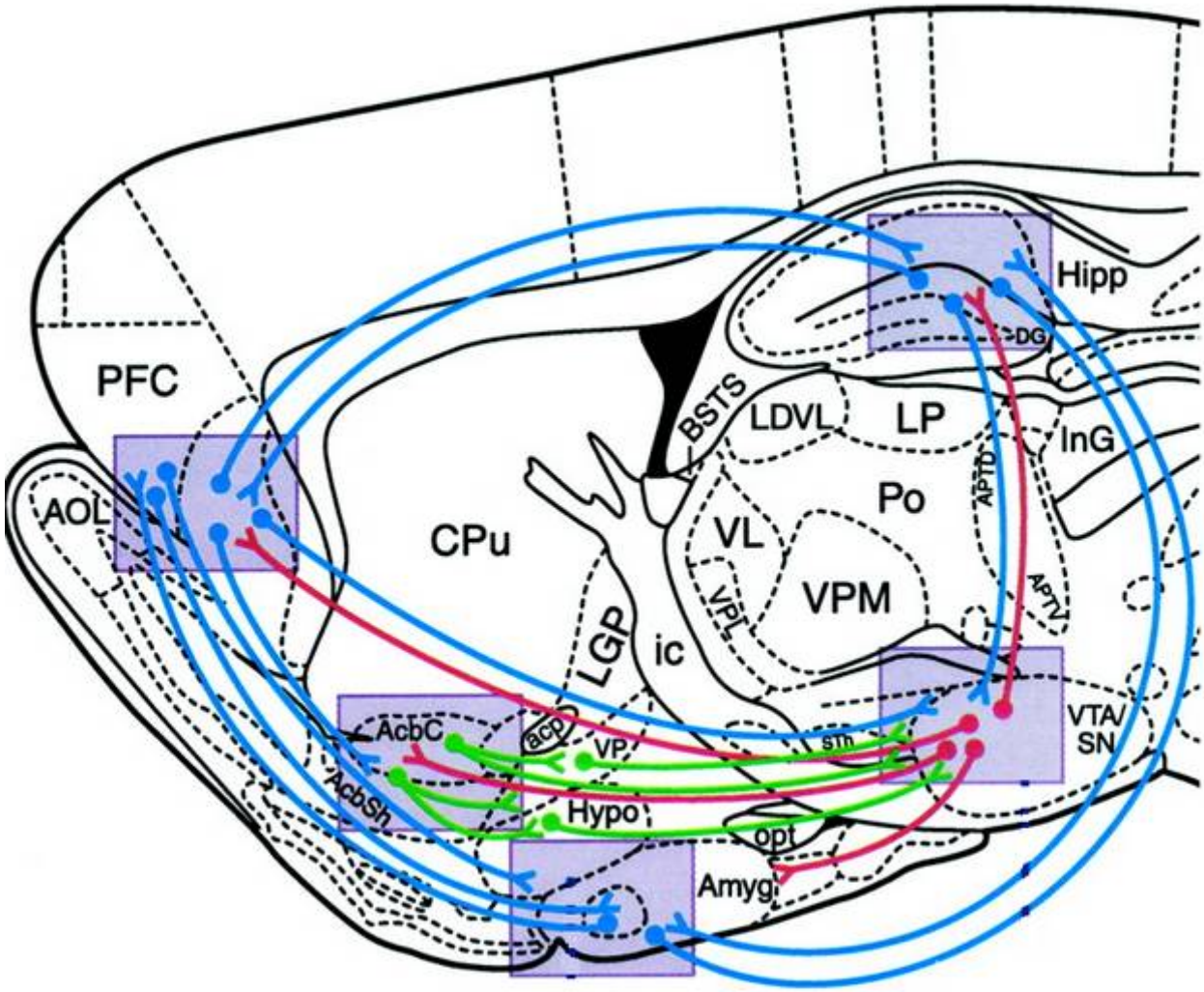
Przybyslawski and Sara, 1997; Suzuki et al., 2004; Gruest et al., 2004; Nader et al., 2000; Lee et al., 2004, 2005). These studies have shown that reconsolidation is required for the efficient learning and memory formation.

Taken together, these results show that existing memory can be disrupted upon reactivation, and suggest that well-established drug memories may also undergo similar alterations upon reactivation, thereby presenting an unexplored therapeutic approach for the treatment of drug addiction.

**Figure 1. Mesocorticolimbic dopamine reward circuit.** Figure 1 shows the schematic representation of a rat brain sagittal section depicting pathways involved in processing of natural rewards and in neural plasticity underlying reward-related learning.

Circuitry represented in *blue* indicates long glutamatergic pathways between prefrontal cortex (*PFC*), amygdala (*Amyg*), hippocampus (*Hipp*), ventral striatum (*nucleus accumbens*), and ventral tegmental area (*VTA*). *Red circuitry* represents principal ascending mesocorticolimbic dopamine systems. *Green* descending pathways indicate primarily GABAergic descending systems. For purposes of simplicity, not all relevant circuitry is shown; Drawing of section is based on the atlas of Paxinos and Watson (1998). *AcbC*, Accumbens core; *Acb shell*, accumbens shell; *Cpu*, caudate-putamen; *VP*, ventral pallidum; *Hypo*, hypothalamus; *SN*, substantia nigra. Other abbreviations can be found in Paxinos and Watson (1998).

*Violet-shaded boxes* represent important nodes within this distributed network where NMDA/D1 receptor-mediated plasticity is proposed to be a critical substrate for behavioral adaptation and learning. Such plasticity, which may result in altered network activity, is hypothesized to mediate normal learning and memory related to natural rewards but is also a key component of addiction. (Figure and legend obtained from Kelley and Berridge, 2002).




Kelley and Berridge, 2002

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# Chapter 2

## Robust Conditioned Place Preference for Nicotine is Observed in Adolescent Female Rats

## Abstract

Conditioned place preference (CPP) is a widely used model for determining the rewarding aspects of drugs with abuse potential. Several studies have reported that nicotine was ineffective in eliciting CPP while others have observed conditioned place aversion (CPA) rather than preference for nicotine. The present study was aimed at determining the conditions that would induce CPP for nicotine in adolescent female rats. We used the biased paradigm for conditioning and our results indicate that nicotine elicits robust CPP at the dose of 0.03 mg/kg. At higher doses nicotine produced aversion and at lower doses no rewarding or aversive effects were observed. These results taken together indicate that nicotine could be a highly reinforcing substance at appropriate doses.

## 1. Introduction

Conditioned place preference (CPP) is a widely used method for determining the reward value of a substance. This procedure is especially used to determine the rewarding and reinforcing potential of addictive drugs. In this procedure the animal is trained to form an association between the drug and the context in which it is obtained. After training, the animal is given free access to both the drug paired and non-drug paired context. The context serves as a conditioned stimulus (CS) and if the drug was rewarding to the animal it spends more time in the drug paired context. Most drugs of abuse elicit robust CPP, but there have been some conflicting studies that debate the ability of nicotine to induce CPP.

Many studies report an aversive effect or no effect following nicotine CPP. Several factors such as the strain of the rat used, the dosage and route of administration of nicotine, the type of CPP paradigm, and length of training all affect the ability of nicotine to induce CPP. Studies conducted in the Hooded strain of rats show no rewarding or aversive effects to nicotine indicating that this strain might be less sensitive to the drug (Clarke and Fibiger, 1987, Shoaib et al., 1994). A wide range of nicotine doses have been show to induce both preference and aversion (Laviolette et al., 2002; Laviolette and van der Kooy, 2003a, b; Harvey et al., 2004). The type of result obtained depends on the rat strain, training paradigm and route of administration. There was no nicotine induced place preference effect at doses lower than 0.1mg/kg and doses higher than 1 mg/kg produced conditioned place aversion (CPA) across several rat strains (Fudala et al., 1985; Fudala and Iwamoto, 1986; Dewey et al., 1999; Papp et al., 2002; Horan et al., 1997; Ashby et al., 2002; Jorenby et al., 1990, Rogers et al. 2004). The biased paradigm, in which the animal receives drug injections in the non-preferred context, is thought to be more favorable for producing CPP with nicotine than the unbiased procedure

(Calcagnetti and Schechter, 1994; Acquas et al., 1989). These observations suggest that the induction of CPP by nicotine is a delicate process which depends on several parameters, but once these parameters are optimized it is possible to produce robust CPP for nicotine.

In this study we set out to determine the stimulus context and the range of nicotine doses that would elicit CPP in adolescent female rats. Our interest in this particular group stems from the fact that in humans, adolescent females show a peculiar vulnerability to nicotine (Collins and Izenwasser, 2004; Levin et al., 2003). We hoped to better understand the behavioral and molecular mechanisms that underlie this vulnerability in an animal model.

## 2. Materials and Methods

All experiments conducted adhered to the Guidelines for the Care and Use of Laboratory Animals as required by the National Institutes of Health and the protocols were subjected to the approval of Washington State University Institutional Animal Care and Use Committee.

### *2.1 Animal Housing*

Four-week-old female Sprague-Dawley rats were obtained from the vivarium. Animals were housed in groups of 4-5 per cage in a temperature/humidity controlled room, and adapted to a 12 hour light-dark cycle. They had free access to Purina laboratory rat chow and water.

### *2.2 Drugs Used*

Nicotine hydrogen tartrate was obtained from Sigma, St. Louis MO. The salt was dissolved in sterile PBS to obtain a concentration of 0.03mg/ml nicotine solution. The pH of the solution was adjusted to 7.4.

### *2.3 Conditioned Place Preference Protocol*

The CPP apparatus consisted of a wooden box (21 (W) x 21 (D) x 28 (H) cm) with two main compartments separated by a smaller compartment. One of the main compartments was painted black and the other white. The black compartment had wire mesh flooring and the white had parallel metal rods spaced 1 cm apart. The central compartment had a black wooden floor. A 15 W lamp was placed over the black compartment to compensate for high initial preference for the dark compartment. A video camera was placed directly over the apparatus to record the activity of the rats. The camera was connected to a computer where the recorded activity was

interpreted by a video tracking software that provided quantifiable information on locomotor activity, time spent on each compartment, and number of entries into a compartment.

Preconditioning - During preconditioning, the rats were placed in the central compartment and allowed free access to the entire box for 15 min. The time they spend in each compartment was noted. The animals underwent preconditioning for 2 days and the average of the two sessions was noted as the time spent in each compartment. Since we used the biased paradigm, the rats received nicotine injections in the non-preferred compartment.

Conditioning - The conditioning phase began the next day of preconditioning and at the same time. The rats received a subcutaneous nicotine injection of 1ml/kg in the non-preferred side and saline in the preferred side, and confined to the compartment for 15 min. The control group received saline injections in both the compartments. The animals were trained for 5 days and tested for conditioning on day 6.

Postconditioning - During the postconditioning phase the rat was tested for conditioning in a drug-free state. They were placed in the central compartment of the box and allowed free access to the box for 15 min and the time spent in each compartment was noted.

Relapse - To test for continued drug preference the rats were kept in their home cages for 5 days without any drug injection. On the test day, they were exposed to the CPP apparatus in a drug free state and the time spent in the nicotine-paired and saline-paired side was noted.

*2.4 Data Analysis*

The time spent on the nicotine paired compartment was be used to analyze preference. Conditioning data was expressed as mean +/- SEM of time spent on the nicotine-paired side on testing day. Two-way ANOVA and t-test have been used where appropriate.

## 3. Results

*Apparatus bias*

Since a bias to the CPP apparatus can interfere with interpreting the results of the study we first tested to see if the animals exhibited any apparatus bias in the absence of the drug (Roma and Riley 2005, Cunningham et al 2003). The experiment was initially performed in the absence of a light source over the dark compartment and animals were tested for apparatus bias by using the preconditioning procedure. The results showed a very strong bias towards the black compartment ($p<0.001$) Figure 1(A). We then introduced a 15W light source above the black compartment and this neutralized the bias Figure 1(B).

*Effective nicotine dose determination*

In order to understand the unique susceptibility of adolescent female we had to first determine the dose range of nicotine that elicits CPP in adolescent female rats. We tried several different doses as shown in Figure 2, until we observed that 0.03mg/kg of nicotine elicited robust and sustained CPP in animals. 0.1mg/kg and 0.08mg/kg of nicotine produced an aversion to the chamber where the drug was administered, and 0.01mg/kg nicotine did not elicit any behavioral changes in the animals.

*Development of CPP for nicotine*

The next aspect of CPP that we determine was the temporal characteristics of the development of preference for nicotine. We were interested in finding the minimal number of training days it took the animals to show conditioning for nicotine. Following preconditioning, the animals were conditioned for 1, 2, 3, 4 or 5 days. After each day of conditioning the animals

were tested for CPP. The results in Figure3 illustrate that the animals show significant conditioning only following 5 days of nicotine administration.

*Persistence of nicotine CPP*

To ensure that the CPP we elicited with nicotine was robust, we decided to test for CPP up to 3 weeks following conditioning. This is the only study where we used adolescent male rats since we did not want any interference from estrous-cycle related hormonal changes in the female rats to bias the outcome. The animals were trained and tested for CPP during postconditioning. The animals were once again re-exposed to the CPP chamber 2, 5, 11, and 21 days after postconditioning in the absence of any drugs. The nicotine treated animals showed a strong preference for the compartment in which the drug administered even after 21 days of abstinence from nicotine. The saline group did not show any change in preference for the compartments (Figure 4).

## 4. Discussion

The main result of this study was that nicotine elicited robust CPP in female adolescent rats. Although there have been several studies that have shown that nicotine elicits only aversion in the CPP model (Horan et al., 2007; Jorenby et al., 1990; Fudala and Iwamoto, 1986), our results indicate that nicotine can produce both aversive and rewarding effects depending on the dose administered (Goldberg et al., 1981; Laviolette and van der Kooy, 2003a, b; Harvey et al., 2004). The reason for such conflicting results for CPP with nicotine could be because at any dose nicotine has both rewarding and aversive effects. If the overall reward value is greater than the aversion at a particular dose, it translates as CPP in the contextual learning model. Even small changes in the procedure such as using a different strain or age of animals can tip the balance resulting in aversion.

The results seen in figure 2 indicated that nicotine, unlike opiates and other stimulant drugs did not produce a clear dose dependent response to conditioning (Bardo et al., 1995). We noted CPP only at 0.03mg/kg with the next higher (0.08mg/kg) and lower (0.01 mg/kg) doses being aversive or ineffective respectively. This indicates that nicotine has a step-up dose-response effect, in that, at one dose no CPP is observed but at the next dose it elicits a robust preference.

Determining the appropriate paradigm for CPP is imperative for eliciting preference with nicotine. Conflicting results can be obtained depending on whether a biased or unbiased procedure is used. Several studies have reported that CPP was elicited only when the biased procedure was used and nicotine was administered in the non-preferred compartment, and no effect was noted when paired with the preferred compartment (Carboni et al., 1989; Acquas et al.,

1989; Calcagnetti and Schechter, 1994). We used the biased paradigm in our experiments but were concerned about the effects of stress on the outcome of the results. High stress levels can lead to discrepancies in the results obtained and in general, before CPP studies are started, the animals are handled and acclimated to the experimental area for a few days to a few weeks in order to minimize their stress levels. In our experiments, we used animals that were weaned only a week prior to the start of the experiment. The stress levels in these animals could be higher than usual due to separation from the mother and due to novelty of the home cages. Our concern was that the animals were spending more time in the non-preferred chamber not because of the rewarding effects of nicotine but rather because of the anxiolytic relief provided by the drug. (Levin et al., 2007; Scheufele et al., 2000; Kassel and Unrod, 2000; Glassman, 1993)

However, as seen in figure 4, even if the animals had elevated stress levels, it did not interfere with their preference for nicotine. Since nicotine is an anxiolytic, there is a possibility that the animals preferred the anxiolytic effect of nicotine rather than the rewarding aspects. This hypothesis is unlikely because the animals continued to show a preference for the nicotine-paired compartment up to 21 days following drug administration despite probable decreases in stress and anxiety due to familiarity with the procedure.

Figure 4 is the only experiment in all of our studies that was conducted in adolescent male rats because we did not want any estrous cycle related bias of female rats to affect the outcome of the experiment. In this experiment rats were conditioned for nicotine and were tested for preference 2, 5, 11 and 21 days following testing. Between testing the animals remained in the home cage in a drug free state without undergoing extinction of the drug memory.

Our results demonstrate that nicotine induces robust CPP and could therefore be an addictive substance. These observations concur with reports from human studies implicating nicotine in the development of addiction. Although nicotine appears to have smaller rewarding effects in animals than in humans, this could be due to the absence of important contributions from environmental stimuli and other components of a cigarette in the drug-seeking and drug-taking behavior. In humans, smoking is accompanied by the formation of associations between sensory, olfactory and visual cues and these associations condition an addict to crave nicotine. The absence of the associated environmental influences could reduce the reinforcing effect of nicotine in animals. Further studies into the differences between the effects of nicotine administration and cigarette smoking in both animals and humans will be required to understand these discrepancies.

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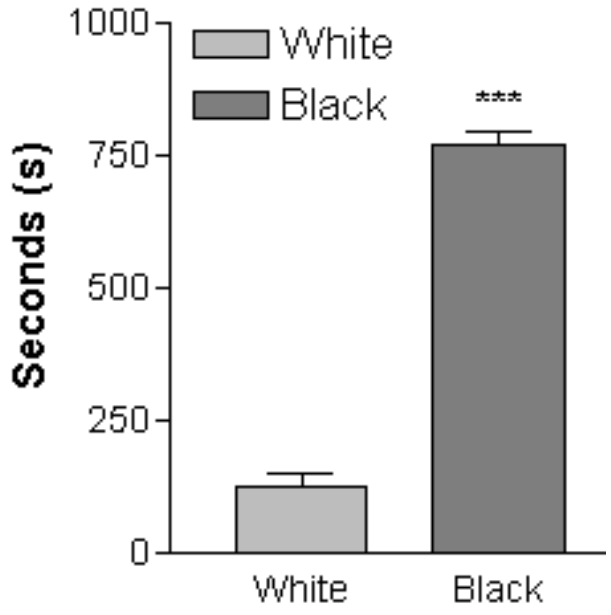
**Figure 1. Initial preference in the CPP chamber.** Graph represents the time spent by animals in the white and black compartment of the CPP chamber. The data is an average of 2 trials recorded for 15 mins; N=6. (A) Unpaired Two tailed t-test analysis shows a significant preference for the black compartment (***$p<0.0001$). (B) No significant preference was noted between black and white chambers following the introduction of a light source above the black compartment.

Figure 1

(A)


1000
750
500
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Seconds (s)
White
Black
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White
Black

(B)

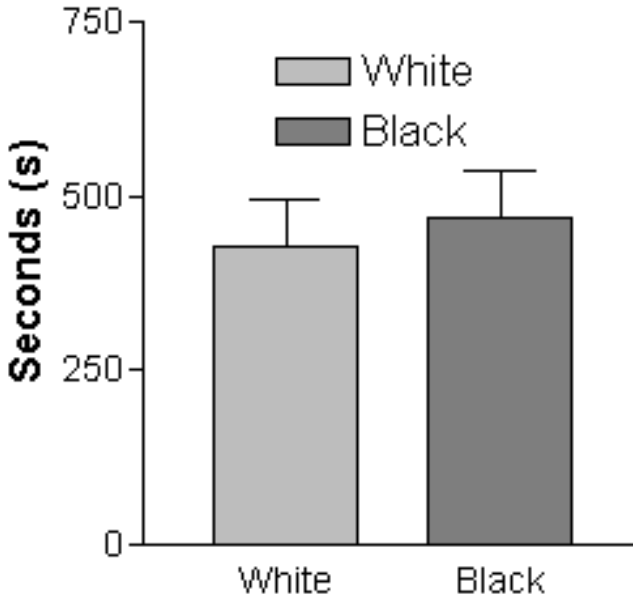

750
500
250
0
Seconds (s)
White
Black
White
Black

**Figure 2. CPP following different doses of nicotine.** Graph represents the preference shown for the nicotine paired compartment following different dose of nicotine administration. Line represents the initial preference for a compartment in the absence of nicotine. N=4 for each group. Two tailed t-test analysis indicated a significant decrease from initial preference following conditioning with 0.1mg/kg nicotine (*$p<0.05$). A significant increase from initial preference was observed following conditioning with 0.03mg/kg nicotine (**$p<0.003$).

Figure 2

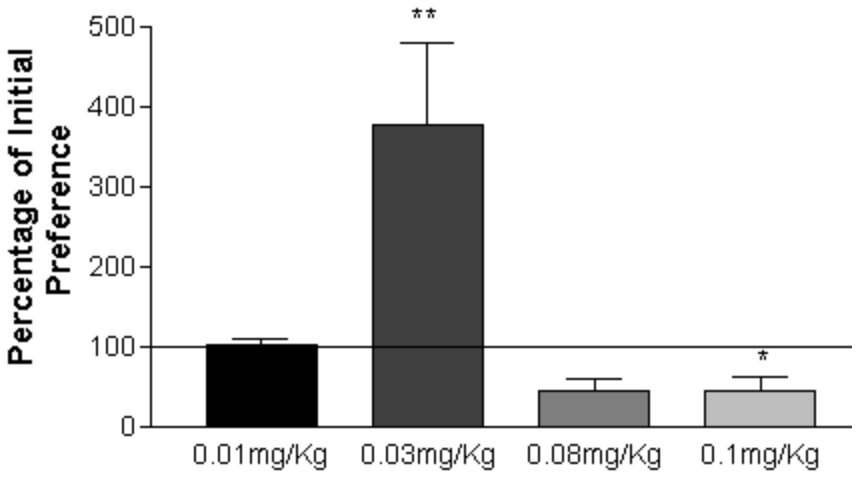

Percentage of Initial Preference
500
400
300
200
100
0
**
*
0.01mg/Kg
0.03mg/Kg
0.08mg/Kg
0.1mg/Kg

**Figure 3. Development of CPP over 5 days of nicotine injection.** The graph shows the preference of the nicotine and saline groups to the drug paired compartment after each day of CPP training. 2-way ANOVA indicated a significant interaction between (treatment x day) ($p<0.04$). Bonferroni post-test showed a significant increase in preference for nicotine paired chamber ($*p<0.05$) after 5 days of CPP.

Figure 3

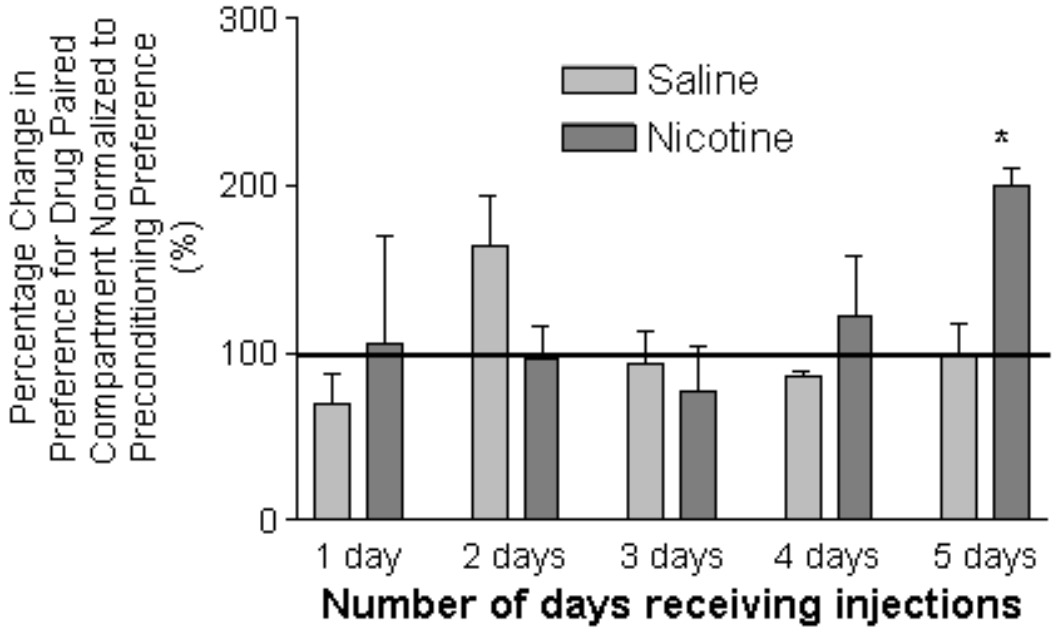
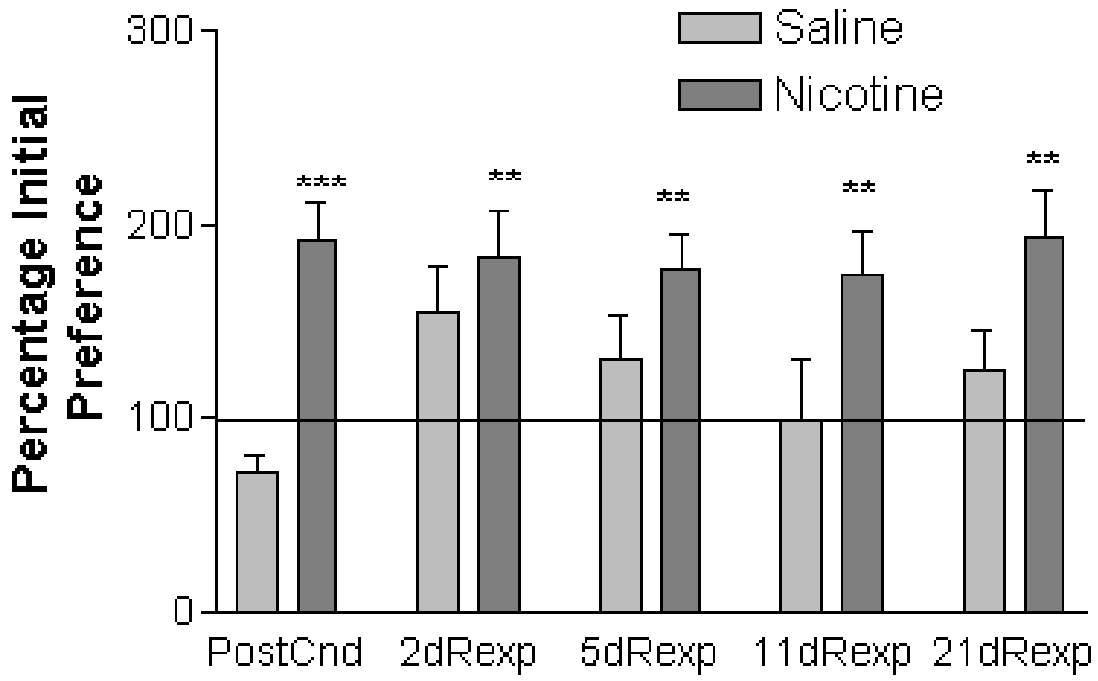

Percentage Change in Preference for Drug Paired Compartment Normalized to Preconditioning Preference (%)
300
200
100
0
Saline
Nicotine
*
1 day
2 days
3 days
4 days
5 days
Number of days receiving injections

**Figure 4. Persistance of Nicotine CPP.** This graph shows the preference for the nicotine paired compartment following conditioning. PostCnd = postconditioning preference for the nicotine paired compartment. 2dRexp, 5dRexp, 11dRexp, 21dRexp = re-exposure to the CPP chamber in the absence of drugs 2, 5, 11, and 21 days following post conditioning. N=8 for nicotine group; N=5 for saline group. Paired t-test shows a significant increase in preference for the nicotine paired compartment at postconditioning (***p=0.0005), 2 days following postconditioning (**p=0.0045), 5 days after postconditioning (**p=0.0016), 11 days after postconditioning (**p=0.0049) and 21 days after postconditioning (**p=0.0029).

Figure 4


Saline
Nicotine
Percentage Initial Preference
300
200
100
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**
**
PostCnd
2dRexp
5dRexp
11dRexp
21dRexp

# Chapter 3

**Matrix Metalloproteinase Activity is Required for Nicotine Conditioned Place Preference Acquisition and Context Dependent Relapse in Adolescent Female Rats.**

## Abstract

Synapses undergo activity-dependent plasticity during the acquisition of a task, which is believed to be the cellular mechanism underlying learning. Reconfiguration of the ECM is required for synaptic plasticity, and the primary candidate involved in controlling this process is a family of endopeptidases called matrix metalloproteinases (MMPs). Our hypothesis is that MMP mediated synaptic plasticity occurs during the acquisition of drug related learning and during re-exposure to the drug related context. Results show that transient changes in MMPs -2, 3, and 9 occur in the hippocampus and prefrontal cortex following context dependent learning of nicotine conditioned place preference (CPP). Inhibition of MMPs during nicotine conditioning interfered with the development of CPP. Elevation in MMP-3, but not MMP-2 and MMP-9 expression, accompanied re-activation of previously learnt drug related memory. Changes in the activity of cortactin, an actin regulatory cytoskeletal protein, were also observed during the acquisition of CPP in the prefrontal cortex and hippocampus, but not following re-exposure to the drug context. These results suggest that MMPs – 2, 3, and 9 are involved in facilitating intracellular and extracellular events required for synaptic plasticity and memory consolidation.

## 1. Introduction

Addiction is characterized by persistent behavioral, psychological and physiological changes that manifest as dependence, tolerance, withdrawal and relapse in the addict. The addictive process, like all learning-dependent events, is reliant on changes in synaptic plasticity and underlying alterations in the structural remodeling of synaptic connections (Mansvelder and McGehee, 2000; Robinson and Kolb, 2004). Drugs of abuse, which intercede to modify these learning-dependent changes in synaptic structure and function, consolidate an association between the drug and its related context, such that even after prolonged abstinence, drug related cues are sufficient in triggering drug seeking behavior (Thomas and Malenka, 2003; Jones and Bonci, 2005; Kauer and Malenka, 2007).

Consolidated memory is subject to reconsolidation following activation, and is labile at this time (Debiec et al., 2002; Milekic and Alberini, 2002; Artinian et al., 2007). Studies have shown that disruption of critical biochemical processes that are requisite for the reconsolidation process leads to an attenuation of drug seeking behavior (Lee et al., 2005; Miller and Marshall, 2005; Lee et al., 2006). Thus, targeting molecules involved in consolidation and reconsolidation might represent a useful strategy for blunting persistent drug dependence.

Matrix metalloproteinases (MMPs) make up a family of over 25 distinct, zinc dependent, endopeptidases that can degrade various molecules, including extracellular matrix (ECM) proteins, cell adhesion molecules, and various growth factors and thereby affect signaling between the ECM proteins and nearby cells. These interactions are typically involved in regulating cytoskeletal reorganization and transcriptional activity. The activity of MMPs is a highly controlled process, modulated by Tissue Inhibitors of MMPs (TIMPs) (Sternlicht and

Werb, 2001; Clark et al., 2007) and activating proteases like plasmin. Inhibition of MMPs during spatial learning has been shown to elevate levels of hippocampal cortactin, a cytoskeletal protein involved in actin polymerization (Meighan et al., 2006) and dendritic spine remodeling. MMP-3 levels were raised during passive avoidance conditioning and blockade of MMPs resulted in learning impairment of the task (Olson et al., 2008). MMP-3 and MMP-9 are involved in spatial learning in the Morris water maze (Wright et al., 2007), and also in the stabilization of LTP during hippocampal plasticity (Nagy et al., 2006; Meighan et al., 2007). Inhibition of MMPs disrupted consolidation and reconsolidation of cocaine induced conditioned place preference (CPP) (Brown et al., 2007). These data demonstrate that MMPs play a significant role in cognitive function. Since drug associated learning employs at least some of the same mechanisms as normal learning, inhibition of synaptic restructuring by blocking MMP activity would be expected to interfere with consolidation and reconsolidation of the drug-context association, resulting in the decay of the drug memory.

Although it has been shown that MMPs are involved in the formation and maintenance of drug associated memory, the type of MMP involved and their pattern of activation is unknown. Moreover, studies have yet to look at MMP changes during drug acquisition and relapse in the hippocampus and prefrontal cortex (PFC) – two regions of the brain primarily involved in encoding and consolidating information from environmental stimuli, and in long term memory storage and reward value assignment to an experience (Laroche et al., 2000; Miller and Cohen, 2001; Kringelbach, 2005).

The guiding hypothesis for these studies is that transient alterations in MMP levels are required for the consolidation and reconsolidation of drug memories and that inhibition of MMP activity will disrupt the acquisition and context dependent relapse of CPP to nicotine in

adolescent female rats. To address this, we examined the levels of MMP molecules implicated in cognition - MMPs 2, 3, 9, along with the intracellular cytoskeletal protein cortactin, in the hippocampus and PFC during acquisition and context dependent relapse of nicotine CPP. Cortactin is an f-actin binding protein that is involved in the stabilization and polymerization of the dendritic cytoskeleton in an activity dependent manner. Cortactin levels correspond to the state of actin polymerization and this makes it an ideal marker of dendritic plasticity (Hering and Sheng, 2003). Cortactin function is dependent on ECM signaling which is in turn affected by MMPs (Vuori and Ruoslahti, 1995; Helwani et al., 2004). Hence we hypothesized that cortactin levels would be dependent on MMP activity in the ECM and were therefore interested in monitoring its activity following nicotine CPP. Next, we determined whether inhibition of MMP activity with a broad spectrum MMP inhibitor, FN-439, could disrupt acquisition of drug-associated learning. Our specific interest in nicotine as the addictive molecule stems from its position as the leading cause of preventable mortalities in the world. Since adolescent females exhibit greater vulnerability to the development of dependence to nicotine (Perkins et al., 1999; Trauth et al., 2000; Levin et al., 2003; Lynch, 2006), all our experiments were performed on female adolescent rats.

## 2. Materials and Methods

All experiments conducted adhered to the Guidelines for the Care and Use of Laboratory Animals as required by the National Institutes of Health and the protocols were subjected to the approval of Washington State University Institutional Animal Care and Use Committee.

### *2.1 Animal Housing*

Four-week-old female Sprague-Dawley rats were obtained from the vivarium. Animals were housed in groups of 4-5 per cage in a temperature/humidity controlled room, and adapted to a 12 hour light-dark cycle. They had free access to Purina laboratory rat chow and water.

### *2.2 Drugs Used*

Nicotine Hydrogen Tartrate was obtained from Sigma, St. Louis MO. The salt was dissolved in sterile PBS to obtain a concentration of 0.03mg/ml nicotine solution. The pH of the solution was adjusted to 7.4.

### *2.3 Conditioned Place Preference Protocol*

The CPP apparatus consisted of a wooden box (21 (W) x 21 (D) x 28 (H) cm) with two main compartments separated by a smaller compartment. One of the main compartments was painted black and the other white. The black compartment had wire mesh flooring and the white had parallel metal rods spaced 1 cm apart. The central compartment had a black wooden floor. A 15 W lamp was placed over the black compartment to compensate for high initial preference for the dark compartment. A video camera was placed directly over the apparatus to record the activity of the rats. The camera was connected to a computer where the recorded activity was

interpreted by a video tracking software that provided quantifiable information on locomotor activity, time spent on each compartment, and number of entries into a compartment.

*Preconditioning* - During preconditioning, the rats were placed in the central compartment and allowed free access to the entire box for 15 min. The time they spend in each compartment was noted. The animals underwent preconditioning for 2 days and the average of the two sessions was noted as the time spent in each compartment. Since we used the biased paradigm, the rats received nicotine injections in the non-preferred compartment.

*Conditioning* - The conditioning phase began the day after preconditioning and at the same time of day. The rats received a subcutaneous nicotine injection of 1ml/Kg in the non-preferred side and saline in the preferred side, and confined to the appropriate compartment for 15 min. The control group received saline injections in both the compartments. The animals were trained for 5 days and tested for conditioning on day 6.

*Postconditioning* - During the postconditioning phase the rat was tested for conditioning in a drug-free state. They were placed in the central compartment of the box and allowed free access to the box for 15 min and the time spent in each compartment was noted.

*Relapse* - To test for relapse the rats were kept in their home cages for 5 days without any drug injection. On the test day, they were exposed to the CPP apparatus in a drug free state and the time spent in the nicotine-paired and saline-paired side was noted.

*2.4 Tissue preparation*

Three hours following postconditioning rats were guillotined and the hippocampi from each hemisphere were extracted along with the prefrontal cortex. The tissue was flash frozen in liquid

nitrogen and stored in a -80°C freezer until all samples were collected. Tissues were then weighed and homogenized on ice in homogenization buffer. (50mM Tris HCl pH 7.6, 150 mM NaCl, 5mM CaCl2, 0.05% Brij 35, 0.02% $NaN_3$). Homogenates were centrifuged at 12000RCF for 10 minutes at 4°C and the supernatant was retained for immunoblotting.

*2.5 Western Immunoblotting*

20µl of the supernatant were mixed 1:1 with 2 × Laemlli sample buffer plus ß-mercaptoethanol. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently transferred on to a nitrocellulose membrane. Following transfer, membranes were preblocked in 5% milk/Tris-buffered saline before addition of primary antibody. Membranes were incubated in primary antibody overnight at 4°C [1:2000, MMP-9 (Abcam, Cambridge, MA, USA); 1:2000, MMP-3 (RDI, Flanders, NJ, USA); 1:1000, MMP-2 (Chemicon, Temecula, CA, USA); 1:2000, cortactin (Upstate, Charlottesville, VA, USA)]. After rinsing in alternating washes of Tris-buffered saline and TTBS (0.1% Tween 20 in Tris-buffered saline), blots were incubated for 2 h with a 1:10 000 dilution of horseradish peroxidase-conjugated secondary antibody and rinsed again in Tris-buffered saline/TTBS. The membrane was incubated for 2 minutes in SuperSignal (Pierce, Rockford, IL, USA) subsequently exposed to the phosphoroimager for visualization. Signal intensity per volume was quantitated using TotalLab Image Analysis software (ADInstruments Inc., Colorado Springs, CO, USA).

*2.6 Intracerebroventricular (i.c.v) cannula placement*

The rats were anesthetized using ketamine hydrochloride (100mg/kg im.) and a unilateral intracerebroventricular (i.c.v.) guide cannula was inserted as described by Pederson et al., 1998. After retraction of the scalp, a hole was drilled through the skull 1 mm posterior to Bregma and

1.5 mm lateral to the midline, and a PE-60 guide cannula with a 2 mm beveled tip was inserted and held in place using holding screws and dental cement. The scalp was sutured and animals were allowed to recover for 5 days. Before the beginning of the CPP experiment, the cannula placement was confirmed by a drinking response elicited by icv. administration of angiotensin II (Sigma Chemical, St.Louis, MO. 100pmol in 2µl of sterile artificial cerebrospinal fluid vehicle. All infusions of the inhibitor and angiotensin II were made over the duration of 20s.

*2.7 MMP inhibitor administration in vivo*

Fifteen minutes before behavioral testing rats received i.c.v. infusions of MMP inhibitor FN-439 (Sigma, St Louis, MO, USA) dissolved at 7µg in 1µl artificial CSF (aCSF; 124 mm NaCl, 3 mm KCl, 1.24 mm $Na_2PO_4$, 1.3 mm $MgSO_4$, 2.0 mm $CaCl_2$, 26 mm $NaHCO_3$, 10 mm d-glucose) and infused at a rate of 2 µL over 20s followed by a 30s rest period.

*2.8 Data Analysis*

The time spent on the nicotine paired compartment was be used to analyze preference. Data was analyzed using a two-way ANOVA followed with a Bonferroni post hoc test. The MMP inhibitor data was analyzed using a t-test. All analysis were performed using the GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA)

## 3. Results

*MMPs in the hippocampus following each day of CPP training:*

In order to evaluate the MMP changes that accompany nicotine CPP, hippocampal homogenates were assayed for the levels of MMPs 2, 3 and 9 by western blotting. Changes in the pro forms of MMP-2 (72 kDa), MMP- 3 (57/59 kDa doublet) and MMP-9 (92/89 kDa doublet) were detected.

Figure 1A shows a significant increase in pro-MMP-2 in both saline and nicotine treated groups on days 1 and 4 of CPP training compared to home cage controls. These groups showed significantly different levels of MMP-2 from one another on days 2 and 5. On day 2 the pro-MMP-2 levels of nicotine treated rats were lower than the saline treated rats but were significantly higher than the saline group on day 5. The middle panel shows that during the initial acquisition of the conditioning task there was a significant decrease in pro-MMP-3. However, by the end of 5 days of conditioning both nicotine and saline treated animals exhibited a similar surge in pro-MMP-3 protein. The right panel shows that the levels of pro-MMP-9 in the nicotine and saline groups start well below the naïve group but gradually increase during the later days of conditioning. On days 4 and 5 the pro-MMP-9 levels of the nicotine group dramatically increased over both saline and naïve groups. Representative bands for each pro-MMP are shown in Figure 1B.

*MMPs in the prefrontal cortex following each day of CPP training:*

The saline and nicotine treated groups exhibited (Figure 2A) a significant increase in pro-MMP-2 levels in the prefrontal cortex on day 4. Similar to the hippocampus, pro-MMP-3 levels significantly decreased on conditioning days 3 and 4. However, unlike the hippocampus no late surge in pro-MMP levels was observed on day 5. The levels of pro-MMP-9 in the prefrontal cortex exhibited little change during conditioning. The only significant change was a modest decrease in the nicotine group on day 2. Representative bands for each pro-MMP are shown in Figure 2B.

*Cortactin in the hippocampus following CPP training:*

Cortactin levels decreased significantly for both the saline and nicotine groups during the first two days of conditioning and then returned to control levels on days 3 and 4 (Figure 3A). A late decrease in cortactin levels on day 5 was selectively observed for the nicotine treated animals suggesting that at this time point, the nicotine groups are undergoing synaptic plasticity events. Representative bands for cortactin are shown in Figure 3B.

*Cortactin in the prefrontal cortex following CPP training:*

The pattern of cortactin changes in the PFC were similar to that found in the hippocampus (Figure 4A). The levels in the prefrontal cortex fell dramatically below baseline on days 1 and 2 in both nicotine and saline groups and climbed back up to baseline by day 5. Unlike the hippocampus no late drop in the nicotine group was observed on day 5. Representative bands for cortactin are shown in Figure 4B.

*Inhibition of MMPs during acquisition of CPP:*

Intracerebroventricular infusions of the broad spectrum inhibitor FN-439 or aCSF was administered 15 minutes prior to nicotine conditioning on each of the five days of conditioning. The results show that the aCSF treated group developed normal CPP while the inhibitor treated group did not show a significant difference from preconditioning values and thus developed no nicotine CPP (Figure 5).

*Conditioned place preference for nicotine following re-exposure to the CPP chamber:*

There was a significant increase in preference for the nicotine paired compartment following 5 days of nicotine conditioning (170.1% ± 11.12). The preference for the nicotine paired compartment became even more robust when the animal was kept away from the drug context for 5 days and then re-exposed to the CPP chamber (273.9% ± 14.24) (Figure 6).

*MMPs in the hippocampus following re-exposure to the CPP chamber:*

Five days after the post conditioning test neither pro-MMPs-2 nor -9 exhibited any changes from baseline in both re-exposed and not re-exposed groups suggesting that these proteins are not involved in reconsolidation of an activated memory (Figure 7A). However, rats that were re-exposed to the CPP chamber showed a selective and marked increase in pro-MMP-3 levels in both saline and nicotine groups indicating an involvement of MMP-3 in synaptic plasticity. Representative bands for pro-MMP-3 are shown in Figure 7B.

*MMPs in the prefrontal cortex following re-exposure to the CPP chamber:*

In the prefrontal cortex, no significant changes in pro-MMPs were noted between saline and nicotine treatment groups, or between groups that were re-exposed to the CPP chamber and those that were not (Figure 8A). However, we were able to detect dramatic changes in the 45kDa active form of MMP-3 (Figure 8B). Representative bands for active MMP-3 are shown in Figure 8C. Both nicotine and saline groups showed a large increase in active MMP-3 levels following re-exposure to the CPP chamber while no such changes were evident in the non-re-exposed groups. The surge in the active MMP-3 in the prefrontal cortex following re-exposure suggests an involvement of MMP-3 in the reconsolidation process.

*Cortactin levels in the hippocampus following re-exposure:*

No differences were noted in the levels of cortactin in both the re-exposed and not re-exposed groups or in saline and nicotine groups (Figure 9).

*Cortactin levels in the prefrontal cortex following re-exposure:*

As with the hippocampus there were no changes in cortactin levels in the prefrontal cortex among the various groups (Figure 10).

## 4. Discussion

There are numerous reports supporting the involvement of MMPs in synaptic plasticity and memory consolidation and reconsolidation (Meighan et al., 2006; Nagy et al., 2006; Brown et al., 2008). However the temporal pattern of expression of MMPs following learning is not clearly understood. We have shown that transient changes in MMP- 2, 3, and 9 occur during the active learning and consolidation of a contextual dependent task in the hippocampus and prefrontal cortex. Inhibition of MMPs by the broad spectrum MMP inhibitor FN-439 interfered with the development of nicotine CPP. Furthermore, our results show that MMP-3, but not MMP-2 or MMP-9, is involved in synaptic plasticity following re-activation of consolidated memory. Finally, cortactin, a marker for the state of the dendritic cytoskeleton, shows significant decreases during early stages of acquisition of the CPP task and a drug dependent change on day 5, following the acquisition of the CPP task.

The pattern of MMP expression in the hippocampus following each day of conditioning suggests that two different types of learning are ongoing. One is context depended learning and the other is drug dependent learning. The nicotine and saline treated rats show significantly different expression of MMP-2 on days 2 and 5 and MMP-9 on days 4 and 5. This change in the expression pattern of MMP-2 and MMP-9 due to nicotine suggests that these proteins are involved in accommodating drug related learning. No such differential protein expression pattern between nicotine and saline groups was observed in MMP-3 levels. On days 3-5 when the animals was actively learning and consolidating the contextual task, the proMMP-3 (57-59kDa) levels in both the nicotine and saline groups increased significantly. When the animals were re-exposed to the drug associated context following a 5 day withdrawal in the home-cage, MMP-3 levels increased dramatically in the hippocampus and prefrontal cortex indicating that MMP-3

was involved in synaptic reconsolidation or retrieval of previously acquired memory. Brown et at., have reported increases in MMP-9 levels in the PFC following cocaine primed reinstatement of CPP (Brown et al. 2008). In our re-exposure experiments, the animals did not receive a drug priming injection and this could explain the absence of MMP-9 changes in the PFC. These results suggest an involvement of MMP-3 in general learning that occurs due to handling and novelty resulting from exposure to the CPP apparatus, while MMP-9 changes appear more specific to drug related learning. Inhibition of MMP activity with a broad spectrum MMP inhibitor resulted in the disruption of CPP thus verifying our contention that MMP activation is requisite for drug-related learning. This further suggests that MMPs are involved in the development of CPP for nicotine.

MMPs are known to alter intracellular signaling to affect cytoskeletal restructuring of the neuron (Michaluk et al., 2009; Nagy et al., 2006; Hering and Sheng, 2003). Cortactin, an actin regulatory protein is concentrated in dendritic spines of hippocampal neurons and is involved in regulating activity dependent changes in dendritic morphology. There is a functional relation between MMP activity and cortactin expression. Increases in MMP activity has been shown to decrease cortactin expression (Meighan et al., 2006). Our results show changes in cortactin expression in the hippocampus and prefrontal cortex on the first 2 days of conditioning and thereby indicate that the dendritic cytoskeleton is actively undergoing restructuring on these days irrespective of drug treatment. Learning aspects independent of the drug treatment seem to be triggering cortactin function at this time point. Cortactin showed no changes from baseline during the later days of conditioning when the animal completed learning the task, or following re-exposure to the drug context. However a significant difference was noted between the nicotine and saline groups on day 5 of acquisition suggesting a drug dependent effect. These results imply

that by the end of 5 days of conditioning, synaptic changes have been completed and that nicotine interferes with any elevations of cortactin that occur during post-conditioning. There is also the possibility that cytoskeletal modification at the later stages of learning could be occurring at a different time point than those used for tissue collection in this study or cortactin changes may be highly localized in the hippocampus or prefrontal cortex making it difficult to detect in gross dissections of these sections.

Our observations are consistent with the current theories of consolidation and reconsolidation of memory. According to this theory, recently acquired memory is thought to be consolidated and stored in the hippocampus. As the memory mature, it becomes less dependent on the hippocampus and the memory trace gets moved to other areas of the brain such the prefrontal cortex (Frankland and Bontempi, 2005). The results that we noted during nicotine conditioned place preference learning shows that MMPs in the prefrontal cortex do not fluctuate as much as the MMPs in the hippocampus during the acquisition of the task. However, when the animals were re-exposed to the CPP chamber, MMP-3 levels in the hippocampus and prefrontal cortex showed a large increase. This suggests that during the consolidation of CPP the memory trace is actively being formed in the hippocampus and it has not yet been transferred to the prefrontal cortex. The high levels of MMP-3 in the both the hippocampus and prefrontal cortex during re-exposure suggests that the memory trace is being moved to the prefrontal cortex, and at the time point we harvested the brain the memory is residing in both the locations. It is possible that if the memory was reactivated at a much later time point it would have become independent of the hippocampus altogether.

Although in this study we were only interested in the involvement of MMPs -2, 3 and 9 in a contextual learning paradigm, there is no reason to believe that other MMPs in the brain are

not involved in this process as well. A particularly appealing candidate is MMP-7. MMP-7 has been known to be involved in regulating dendritic spine structure in the hippocampus (Bilousova et al., 2006). It is also implicated in affecting the structure and function of neurons by modulating synaptic proteins at the active zone, which are involved in synaptic vesicle recycling (Szklarczyk et al., 2007).

In our MMP inhibition studies, we purposely employed FN-439, a broad spectrum MMP inhibitor, because we did not know which MMPs besides MMPs 2, 3, and 9 might be involved in the remodeling process. Since it is likely that limited number of the brain MMPs are involved in CPP learning, a more selective inhibition strategy may be effective at blunting CCP. Given the widespread role of MMPs in multiple physiological processes such an approach wound seem necessary if MMP inhibition can ever be considered seriously as a therapeutic option for the treatment of addictive behavior. The use of virally delivered siRNAs that inhibit specific MMP gene transcripts might represent a more focused treatment approach. The results presented here support our hypothesis that MMPs are involved in the learning of a nicotine-associated contextual memory. MMP activity is crucial for cytoskeletal reorganization and consolidation of new memories. Any irregularities in MMP activity leads to deficits in learning and memory. Further investigation of up-stream and down-stream regulatory molecules involved in MMP expression and activity will provide a better understanding of the contributions of these proteinases to synaptic plasticity and learning and memory formation.

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**Figure 1. MMPs in the hippocampus following each day of CPP training**. (A) MMP proteins were assayed in the rat hippocampus by Western blotting. Pro-MMP levels in the hippocampus 3 hours following CPP testing is shown in the figure. Data has been normalized to naïve protein levels and are represented as mean ± SEM of percentage MMP protein levels in naïve rats following each day of CPP training. Data was analyzed using 2 way ANOVA followed by Bonferroni post-test. Line represents MMP levels in naïve animals on corresponding groups. * represents comparisons between nicotine and saline groups vs. naïve animals; + represents comparisons between nicotine and saline groups on the same day. 3 comparisons were made: 1. Comparisons between saline and naïve animals over the 5 days; 2. Comparisons between nicotine and naïve animals over the 5 days and; 3. Comparisons between nicotine and saline groups over the 5 days for each of the MMP proteins. N= 4-8 for saline; n = 5-8 for nicotine and N = 4-5 for naive group.

Pro-MMP-2: There was a significant interaction between (saline/naïve treatment group x day, $p=0.0004$) and independent saline/naïve treatment ($p<0.0001$) and day effect ($p<0.0001$). There was a significant interaction between (nicotine/naïve treatment group x day, $p<0.0001$) and independent nicotine/naïve treatment ($p<0.0001$) and day effect ($p<0.0001$) in the MMP-2 protein. There was a significant interaction between (saline/nicotine x day, $p<0.0001$) and an independent effect of training day ($p<0.0001$).

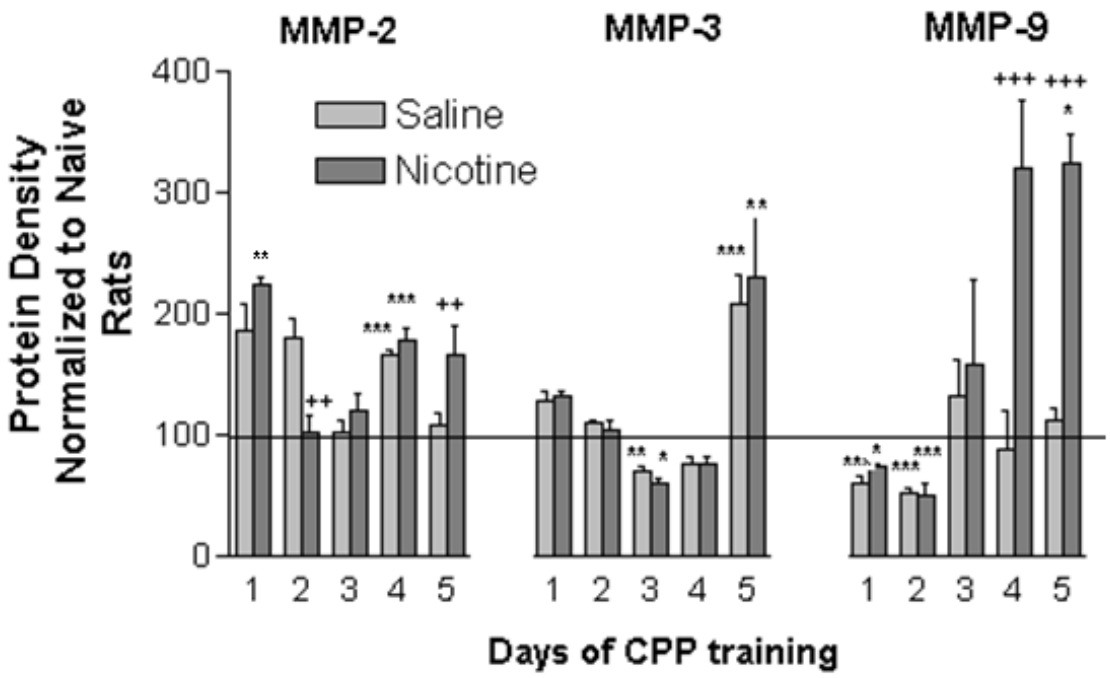
Pro-MMP-3: There was a significant interaction between (nicotine/naïve treatment group x day, $p<0.0001$) and independent day effect ($p<0.0001$). There was a significant interaction between (saline/naïve treatment group x day, $p<0.0001$) and independent day effect ($p<0.0001$). There was a significant independent day effect ($p<0.0001$) in the (saline/nicotine x day) but no significant difference between nicotine and saline groups on each day of training.

Pro-MMP-9: There was a significant interaction between (nicotine/naïve treatment group x day, $p<0.0001$) and independent day effect ($p<0.0001$). There was a significant interaction between (saline/naïve treatment group x day, $p<0.0001$) and independent day and treatment effect ($p<0.0001$). There was a significant interaction between (saline/nicotine x day, $p=0.0002$) and an independent effect of training day ($p<0.0001$) and treatment ($p<0.0001$).

(B) Shows the protein density of representative bands from western blots in the hippocampus.

Figure 1

(A)




(B)

| MMP-2 | Day1 | Day2 | Day4 |
|---|---|---|---|
| Naïve | | | |
| Saline | | | |
| Nicotine | | | |

| MMP-3 | Day3 | Day5 |
|---|---|---|
| Naïve | | |
| Saline | | |
| Nicotine | | |

| MMP-9 | Day1 | Day2 | Day4 | Day5 |
|---|---|---|---|---|
| Naïve |  |  |  |  |
| Saline |  |  |  |  |
| Nicotine |  |  |  |  |

**Figure 2. MMPs in the prefrontal cortex following each day of CPP training**. See figure 1 for full explanation of terminology. N= 4-8 for saline; n = 5-8 for nicotine and N = 4-5 for naive group.

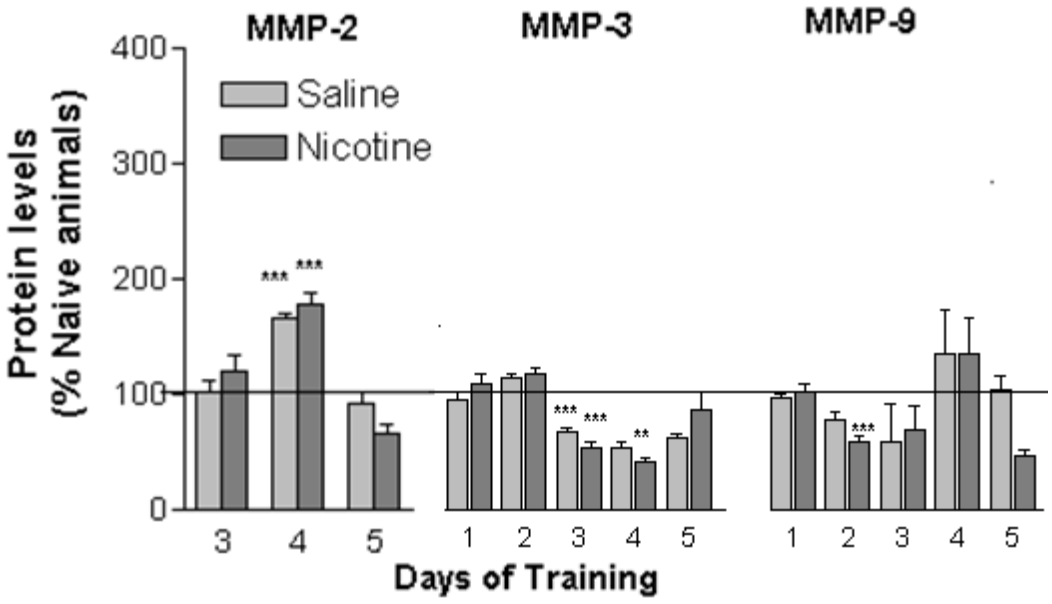
(A) Pro-MMP-2: There was a significant interaction between (saline/naïve treatment group x day, $p=0.0002$) and independent saline/naïve treatment ($p=0.0008$) and day effect ($p<0.0001$) in the MMP-2 protein. There was a significant interaction between (nicotine/naïve treatment group x day, $p=0.0008$) and independent nicotine/naïve treatment ($p=0.0002$) and day effect ($p<0.0001$).

Pro-MMP-3: There was a significant interaction between (saline/naïve treatment group x day, $p=0.0023$) and independent day effect ($p<0.0001$) and saline/naïve treatment effect ($p=0.0015$) in MMP-3 protein. There was a significant interaction between (nicotine/naïve treatment group x day, $P<0.001$) and independent day and treatment effect ($p<0.0001$).

Pro-MMP-9: 2-way ANOVA (saline/naïve treatment group x day) showed an independent day effect ($p<0.0001$) in MMP-9. There was a significant interaction between (nicotine/naïve treatment group x day, $P<0.001$) and independent day effect ($p<0.001$).

(B) Shows the protein density of representative bands from western blots in the PFC.

Figure 2 (A)




(B)

| MMP-2 | Day 3 | Day 4 |
|---|---|---|
| Naive | | |
| Saline | | |
| Nicotine | | |

| MMP-3 | Day 3 | Day 4 |
|---|---|---|
| Naive | | |
| Saline | | |
| Nicotine | | |

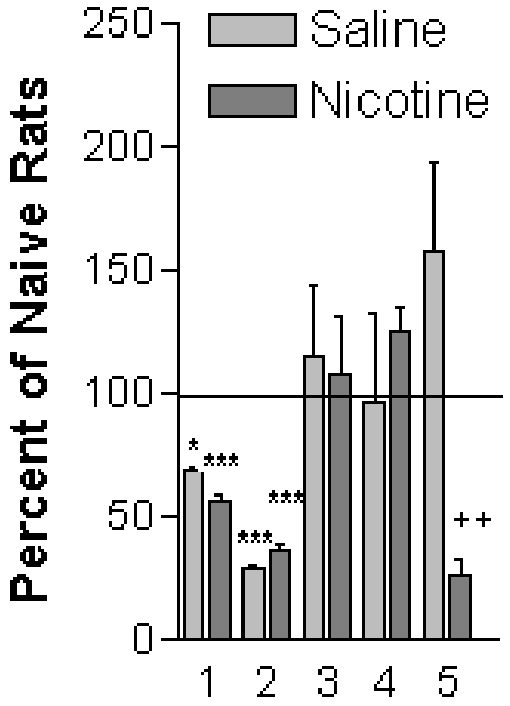
| MMP-9 | Day 2 |
|---|---|
| Naive | |
| Saline | |
| Nicotine | |

Figure 3. Cortactin in the Hippocampus following CPP training. (A) Data has been normalized to naïve protein levels and are represented as mean ± SEM of percentage MMP protein levels in naïve rats following each day of CPP training. n = 4-8 saline group; n = 5-8 nicotine group; n = 4 naïve group Cortactin levels in the hippocampus 3 hours following CPP testing. Line represents cortactin levels in naïve animals on each day. (A) Statistical analysis by 2 factor ANOVA indicate an interaction between treatment group and day. The nicotine and saline groups showed a significant decrease compared to naïve group on days 1 and 2 (nicotine/naïve treatment x day, ***$p<0.001$ on days 1 and 2) (saline/naïve treatment x day, *$p<0.05$ on day 1 and ***$p<0.001$ on day 2). There was a significant interaction between (nicotine/saline treatment group x day). Bonferroni post test showed a significant difference between nicotine and saline groups on day 5 (++$p<0.001$).

(B) Shows the protein density of representative bands from western blots in the hippocampus.

Figure 3

(A)




(B)

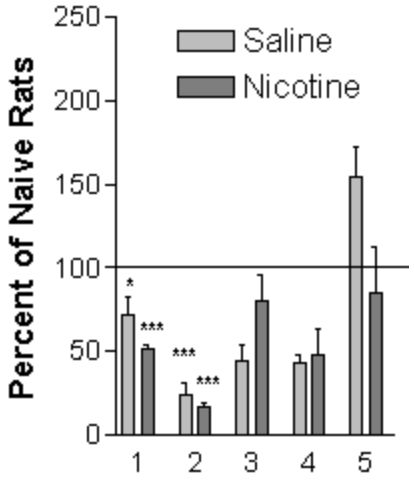
| Cortactin | Day1 | Day2 | Day5 |
|---|---|---|---|
| Naive | | | |
| Saline | | | |
| Nicotine | | | |

**Figure 4. Cortactin in the prefrontal cortex following CPP training.** See figure 1 for full explanation of terminology. (A) n = 4-8 saline group; n = 5-7 nicotine group; n = 4 -7 naïve group. Cortactin levels in the hippocampus 3 hours following CPP testing. Line represents cortactin levels in naïve animals on each day. Statistical analysis by 2 factor ANOVA indicate an interaction between treatment group and day. The nicotine and saline groups showed a significant decrease compared to naïve group on days 1 and 2 (nicotine/naïve treatment x day, ***$p<0.001$ on days 1 and 2) (saline/naïve treatment x day, *$p<0.05$ on day 1 and ***$p<0.001$ on day 2).

(B) Shows the protein density of representative bands from western blots in the PFC.

Figure 4

(A)




(B)

| Cortactin | Day1 | Day2 |
|---|---|---|
| Naïve |  |  |
| Saline |  |  |
| Nicotine |  |  |

**Figure 5. MMP inhibitor interferes with the development of CPP.** The graph shows the preference for the nicotine paired compartment after 5 days of conditioning. Line represents initial preference for the compartment in the absence of the drug. N=5 in each group. Unpaired t-test show a significant increase in preference for the nicotine paired compartment in the aCSF treated group (*$p<0.02$) but not in FN-439 treated groups.

Figure 5

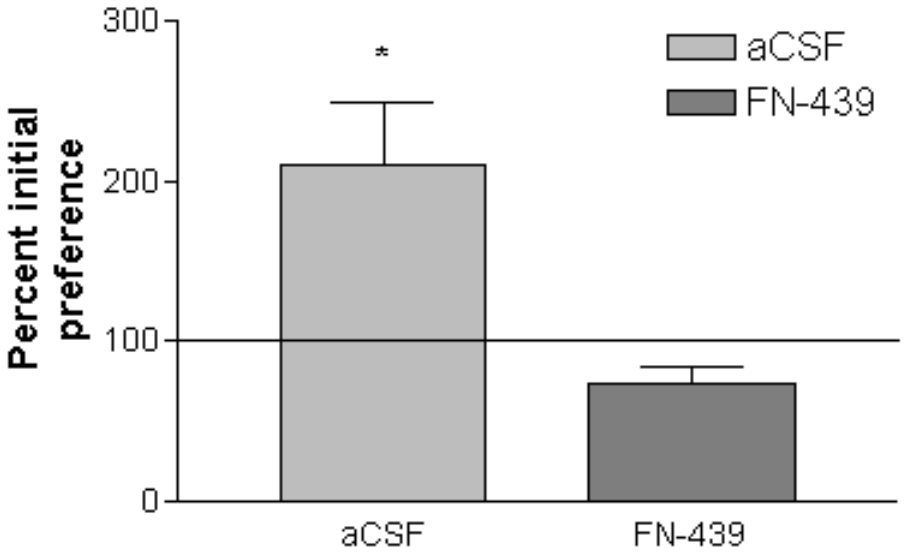

300
200
100
0
Percent initial preference
*
aCSF
FN-439
aCSF
FN-439

**Figure 6. Conditioned place preference for nicotine following re-exposure to CPP chamber**. Data has been normalized to preconditioning preference and is represented as mean ± SEM of percentage change is preference for drug paired side. Line represents initial preference for the drug paired compartment. PostCnd = preference for drug paired compartment following 5 days of nicotine conditioning. ReExp = preference for drug paired compartment 5 days after postconditioning. There is a significant increase in preference following re-exposure compared to postCnd (***P=0.0004; unpaired two tailed t-test).

Figure 6

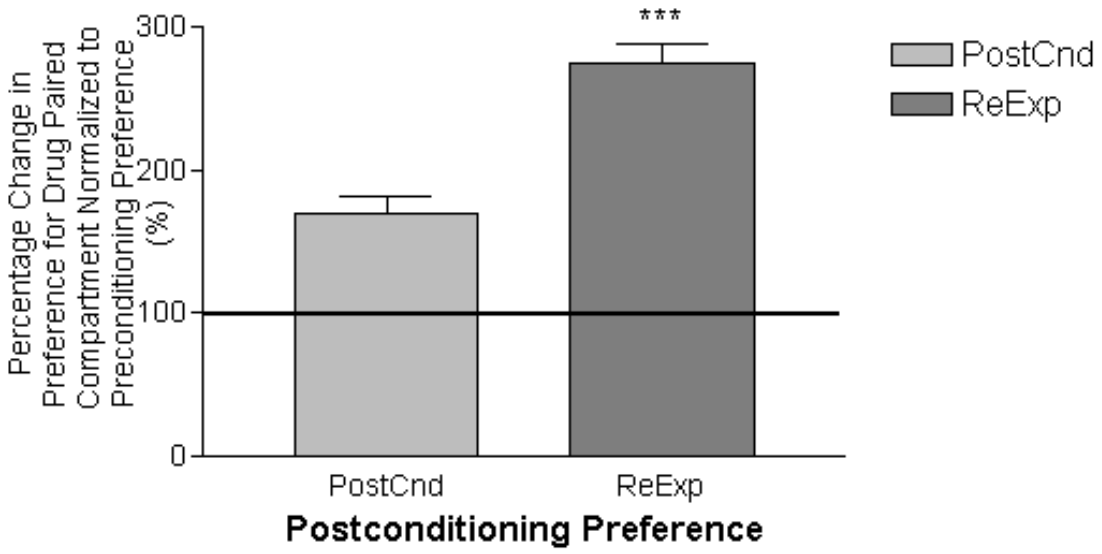
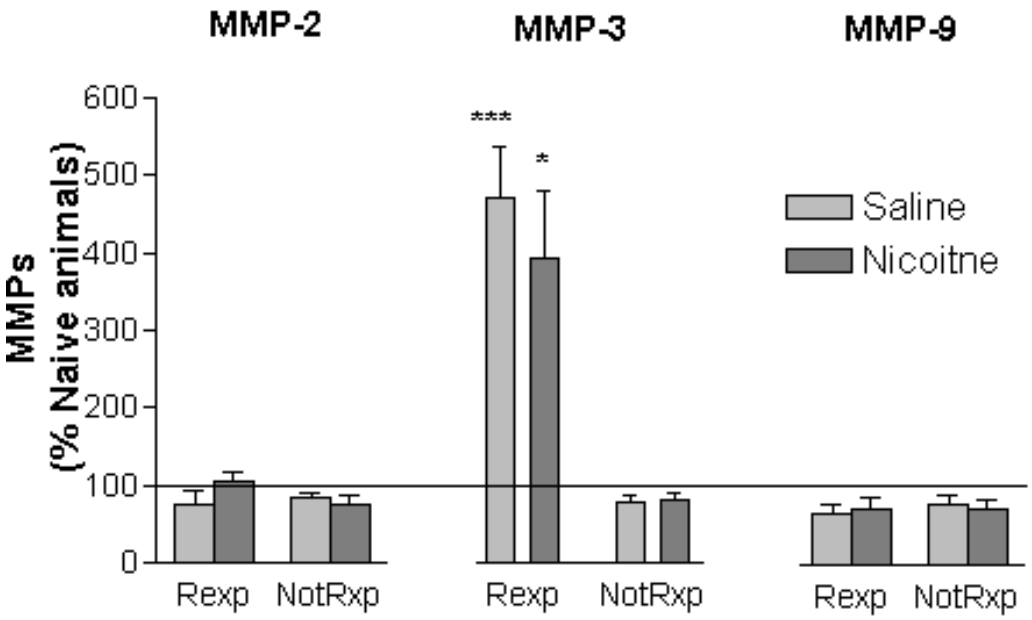

***
300
200
100
0
Percentage Change in Preference for Drug Paired Compartment Normalized to Preconditioning Preference (%)
PostCnd
ReExp
Postconditioning Preference
PostCnd
ReExp

**Figure 7. MMPs in the hippocampus following re-exposure.** (A) Data has been normalized to naïve protein levels and are represented as mean ± SEM of percentage MMP protein levels in naïve rats. N= 4 in naïve group; N=7 nicotine and saline groups. Rexp = group that was re-exposed to the CPP chamber 5 days following postconditioning. NotRxp = group that was not re-exposed to the CPP chamber 5 days following postconditioning. Line represents MMP levels in naïve animals. Statistical analysis by 2 factor ANOVA (saline/nicotine treatment x chamber exposure) indicated an independent chamber exposure effect ($p<0.0001$). A significant interaction was noted in the (saline/naïve treatment vs. exposure, $p<0.0009$) along with an independent treatment effect ($P=0.0065$).

(B) Shows the protein density of representative bands from western blots in the hippocampus.

Figure 7

(A)



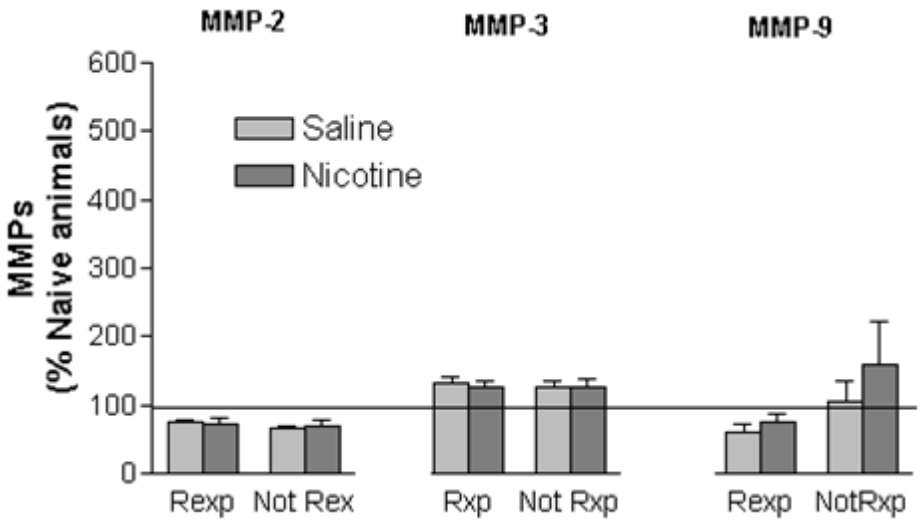

(B)

| MMP-3 | Re-exposed | Not re-exposed |
|---|---|---|
| Naive | | |
| Saline | | |
| Nicotine | | |

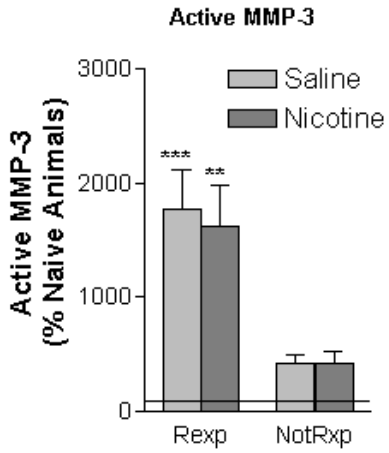
**Figure 8. MMPs in the prefrontal cortex following re-exposure.** See Figure 7 for full explanation of terminology. (A) 2-way ANOVA showed no significant differences among the levels of pro-MMPs among groups. (B) A 45KDa active MMP-3 form was apparent along with the proMMP-3 form in the prefrontal cortex. Statistical analysis by 2 factor ANOVA (saline/nicotine treatment x chamber exposure) indicated an independent chamber exposure effect ($p<0.0001$). (C) Shows the representative protein bands from western blots of 45kDa active MMP-3 of in the PFC.

Figure 8

(A)




(B)




(C) 45KDa active MMP-3

| MMP-3 | Re-exposed |
|---|---|
| Naïve | |
| Saline | |
| Nicotine | |

**Figure 9. Cortactin levels in the hippocampus following re-exposure.** Data has been normalized to naïve protein levels and are represented as mean ± SEM of percentage cortactin protein levels in naïve rats. N= 4 in naïve group; N=7 nicotine and saline groups. No significant changes were noted between or within groups.

Figure 9

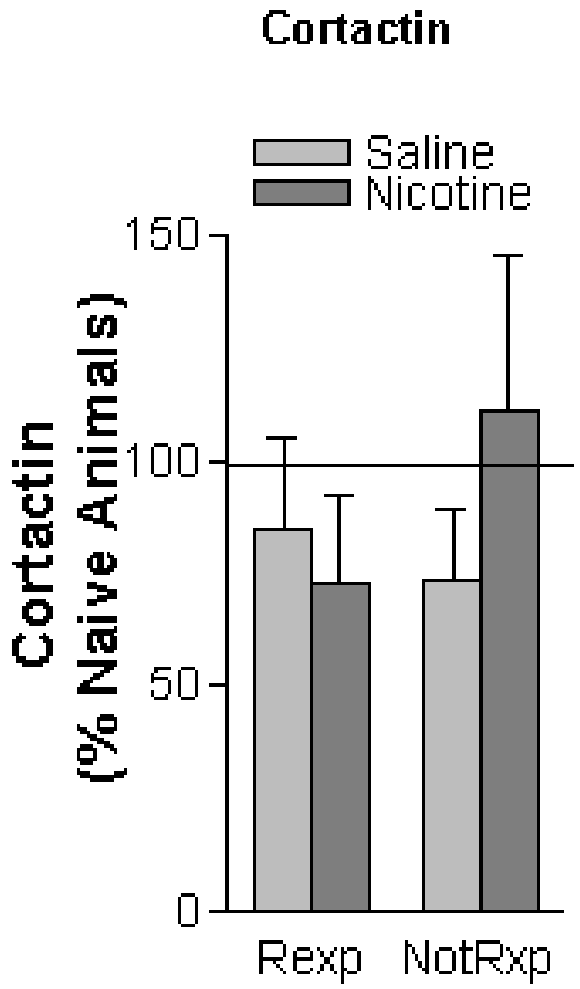
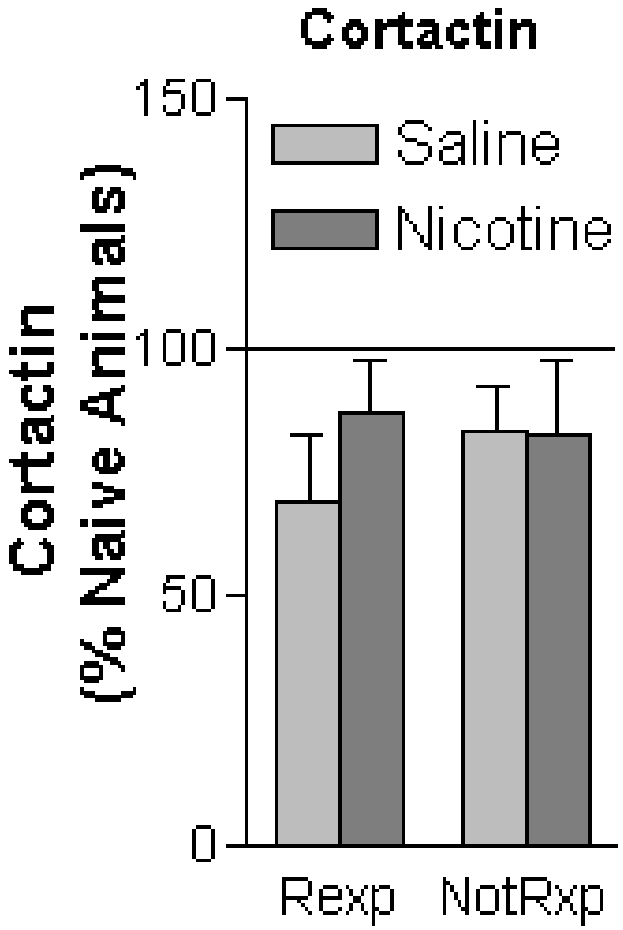

Cortactin
Saline
Nicotine
150
100
50
0
Cortactin
(% Naive Animals)
Rexp
NotRxp

**Figure 10. Cortactin levels in the prefrontal cortex following re-exposure.** Data has been normalized to naïve protein levels and are represented as mean ± SEM of percentage cortactin protein levels in naïve rats. N= 4 in naïve group; N=7 nicotine and saline groups. No significant changes were noted between or within groups.

Figure 10


Cortactin
Cortactin (% Naive Animals)
150
100
50
0
Saline
Nicotine
Rexp
NotRxp

# Chapter 4

## General Discussion

## General Discussion

Recently, the Harding and Wright lab discovered that changes in the expression of a family of extracellular matrix (ECM) degradative enzymes called the matrix metalloproteinases are required for several types of learning such as spatial learning, passive avoidance conditioning, habituation and novel object exploration (Meighan et al., 2006; Olson et al., 2008; Wright et al., 2004,2006). Other studies have since reported results supporting the involvement of MMPs in LTP and synaptic plasticity (Meighan et al., 2007; Nagy et al., 2006; Mizoguchi et al., 2007a, b; Wang et al. 2008). These results suggest that MMP activity may be a general and required event for any activity involving synaptic remodeling. Since the development of drug dependence requires synaptic plasticity, we hypothesized that MMPs are involved in this process (Robinson and Kolb, 1997; Robinson and Kolb, 1999; Brown and Kolb, 2001; Gonzalez et al., 2004; Robinson and Kolb, 2004).

During the development of drug related learning the neural connections in the brain undergo remodeling in order to consolidate the drug memory. When existing drug memory is activated, it initiates reconsolidation in which the original memory trace is temporarily plastic thus allowing for the trace to be strengthened or modified. Both consolidation and reconsolidation phases require synaptic restructuring and we believe that MMPs are involved in this synaptic restructuring process (Przybyslawski and Sara, 1997; Suzuki et al., 2004; Gruest et al., 2004; Nader et al., 2000; Lee et al., 2004, 2005). Inhibition of MMPs during consolidation and reconsolidation in various learning paradigms resulted in the disruption of active learning and previously learnt memory (Meighan et al., 2006, 2007; Olson et al., 2008; Wright et al., 2007). The ability to disrupt the reconsolidation process thus presents an opportunity to interfere

with synaptic plasticity and could provide new therapeutic options in the treatment of learned pathologies like drug addiction, phobias, or stress disorders.

### *Nicotine and Conditioned Place Preference*

Our goal was to understand the mechanisms that underlie the neuroplasticity that occurs in adolescent female smokers and to use the conditioned place preference model to study it. The first step was to develop a procedure that produced CPP for nicotine. There were many studies reporting that it was not possible to produce CPP with nicotine but our results showed otherwise (Jorenby et al., 1990; Fudala and Iwamoto, 1986; Laviolette and van der Kooy, 2003a, b). The data presented in chapter 2 demonstrates that nicotine produces robust and persistent CPP in adolescent female rats. The animals required 5 consecutive days of nicotine administration to develop CPP. Once conditioning was established the preference for nicotine persisted for at least 21 days following conditioning. In agreement with our findings other studies have shown that the nicotine preference lasted for over 40 days after initial nicotine conditioning (Liu et al., 2008; Cohen et al., 2005).

### *MMPs and Plasticity*

The results reported in chapter 3 show that transient changes in MMPs -2, 3, and 9 occur in the hippocampus and prefrontal cortex during the acquisition of the place preference task. The pattern of expression of the MMPs varies with each day of conditioning and depends on the type of drug treatment the animal has received. The relationship between changes in MMPs and

physical remodeling of dendritic spines can be inferred from our observations since the levels of cortactin, a dendritic cytoskeletal plasticity marker, were altered concomitantly. Cortactin showed a decrease in levels in the hippocampus and prefrontal cortex during initial learning of the conditioning task. Two factors appear to be essential for drug related learning to occur: 1. There is a change from baseline in the pattern of expression of MMPs and 2. The MMP changes are transient. Supporting this assertion are observations that MMPs are involved in various types of learning and that inhibition of MMPs prevented the learning of environmentally relevant information (Meighan et al., 2006; Mizoguchi et al., 2007a; Nagy et al., 2007; Olson et al., 2008; Wright et al., 2004). Furthermore, sustained increase in MMP has been implicated in neuropathology. Elevation in MMP levels have been observed following neuroinflammation and cerebrovascular diseases such as brain injury and hemorrhage, cerebral ischemia, and in Alzheimer's disease and multiple sclerosis (Rosenberg, 1995; Anthony et al., 1997; Deb and Gottschall, 1996; Yang et al. 2007). Following brain injury MMPs are activated as part of the cellular repair process by inflammatory factors and immune cells to facilitate their migration across the blood-brain barrier (Armao et al., 1997; Leppert et al., 1995). Initial elevations in MMPs that occur soon after injury are believed to be involved in disassembling the ECM and initiating apoptosis of damaged neurons (Heo et al. 1999; Gu et al.,2002), whereas MMPs elevations that occur at later time points are thought to be involved in angiogenesis and neurogenesis (Lee et al. 2006; Wang et al. 2006). However, sustained elevations in MMPs leads to continual break down the ECM and basal lamina of capillaries, and interferes with the repair process by increasing the permeability of the blood-brain barrier, and permitting neurotoxic substances to enter the brain (Belayev et al., 1996; Rosenberg et al., 1990, 1998; Rosenberg and Navratil, 1997; Yang and Betz, 1994). These observations suggest that transient, moderate MMP

activation with appropriate temporal distribution is required for normal physiological functioning of the brain. The results that we have reported show that the pattern of expression of each MMP varies with each of the days of conditioning, further reflecting the careful regulation of MMPs that is required for encoding information. Together these results strongly suggest that changes in the availability of MMPs are required for drug related learning and plasticity. Although our studies have focused on MMP 2, 3 and 9, the MMP family has over 25 different proteinases, and the involvement of other secreted or membrane-bound MMPs in drug related learning should not be discounted.

### *Hypothetical Model of MMP Involvement in Learning Associated Synaptic Plasticity*

Our lab has developed a model to understand the role of MMPs at the synaptic level during the acquisition of a learning paradigm. LTP is the cellular counterpart of learning and during the development of LTP synapses undergo morphological alteration to improve the efficiency of communication between neurons such that there is enhanced neurotransmitter release in the presynaptic terminal and greater responsiveness to the released neurotransmitters at the postsynaptic dendrite.

During LTP in the hippocampus, there is an increased release of glutamate neurotransmitter resulting in the activation of postsynaptic AMPA and NMDA receptors.Activation of these receptors results in calcium entry into the cell and the calcium can trigger MMP secretion by promoting NF-κB activity (Dzwonek et al. 2004; Sternlicht and Werb, 2001). Postsynaptic increases in intracellular calcium can also increase gene transcription of MMPs, and activate signaling molecules for cytoskeletal reorganization and receptor localization.

Increased levels of calcium could activate calpain, a calcium dependent protease that is implicated in destabilizing the cytoskeleton through cortactin degradation (Huang et al. 1997). MMPs can also degrade cell adhesion molecules such as integrins, cadherins and NCAMs that are involved in the formation and maintenance of LTP (Sternlicht and Werb, 2001; Ronn et al., 1995; Bahr 2000). Degradation of the CAMs can result in cytoskeletal destabilization and thereby spine retraction (Figure 1). Morphological alterations of dendritic spines and increases in the number of small spines have been observed during the induction of LTP. (Marrone and Petit, 2002; Carlisle and Kennedy, 2005; Sala 2002; Wang et al., 2008) Furthermore, MMPs can activate growth factors such as TNF-α and BDNF and these factors are known to play an important role in synaptic plasticity (Bramham and Messaoudi, 2005; Pang and Lu, 2004; Binder and Scharfman, 2004; Marini et al., 2004). TNF-α can activate NF-κB, creating a positive feedback loop resulting in a sustained increase in MMP levels. Thus MMPs could be vitally involved in facilitating synaptic plasticity events.

***MMPs and Drug Associated Learning***

Drug addiction is increasingly considered to be an aberrant form of learning and memory formation (Hyman, 2005). While normal learnt memory can be lost or forgotten over time due to injury or deficits in brain functioning, drug related learning has the opposite problem – the drug associations are so firmly ensconced in the brain that they continue to affect behavior even after prolonged periods of drug abstinence. The persistence of drug memory makes it extremely difficult for an addict to refrain from drug use even after the withdrawal symptoms have abated as exposure to drug related cues trigger intense craving for the drug. There are several

therapeutic drugs available to aid in nicotine abstinence but the success rates of these products have been abysmal. A more reliable and effective means of treatment is required for the prevention and cure of nicotine addiction.

Plasticity at the synaptic level (e.g. dendritic spine morphology) appears to be essential for drug associated learning to occur. Our results show that MMPs 2 and 9 are required for the acquisition of drug dependent learning whereas MMP-3 is involved in encoding learning in general. We hypothesize that elevations in MMP disrupt ECM-cell adhesion contacts resulting in altered signaling and destabilization of the actin cytoskeleton of the dendrite spine. Inhibition of all MMP activity with a broad spectrum MMP inhibitor prevented acquisition of relevant drug cues by interfering with MMP dependent plasticity events required for learning. These observations support our central hypothesis that MMP activity is required for drug associated learning and memory formation.

***Implication of the major findings***

Our results taken alongside other observations suggest that MMPs play an important role in memory formation and modification. Due to the labile nature of even well established long-term memory, we now have a new strategy for addressing maladaptive learnt associations (Centonze et al., 2005). The targeting of MMPs for therapeutic intervention is particularly attractive because numerous MMP inhibitors, some of which are blood-brain barrier permeate, have been developed and have passed FDA toxicological evaluations. Pharmacological manipulation using MMP inhibitors can affect a wide variety of physiological and pathological events that require synaptic plasticity. MMP inhibitors may have utility in the treatment of several

neurodegenerative diseases such as Alzheimer's disease, Parkinson's, and multiple sclerosis where abnormal synaptic activity has been observed (Nalivaeva et al., 2008; Fong et al., 2008; Choi et al., 2008). Thus therapeutic MMP modulation may provide a novel treatment option for a wide range of neurological diseases.

***Future Direction***

We have only just begun to understand the involvement of MMPs in memory consolidation and reconsolidation and there are a great many questions to answer. Little is known about the temporal and spatial characteristics of MMP activation or the type of MMP that a particular behavioral task may activate. Most of the research involving MMPs in the brain has focused only on MMPs 2, 3 and 9 but addressing the activity of other MMPs in the brain, their interactions with each other, along with their upstream and down-stream targets needs to be characterized in order to better understand and exploit the system for therapeutic purposes.

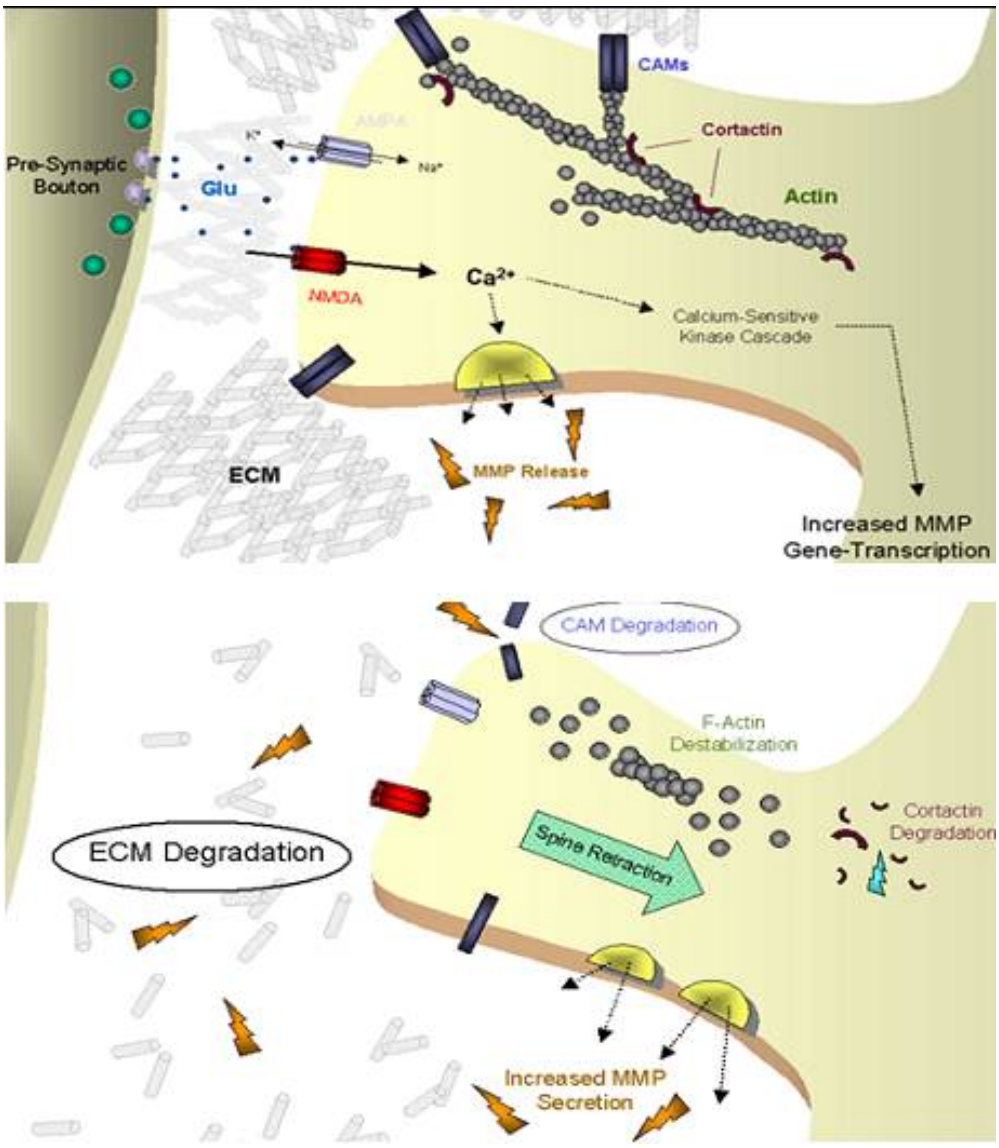



Figure 1. Model of MMP involvement in synaptic plasticity. (Top) LTP increases $Ca^{2+}$ flow into the cell leading to MMP secretion into the ECM. (Bottom) MMPs interfere with ECM-cell signaling resulting in cytoskeletal destabilization.

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# Appendix A

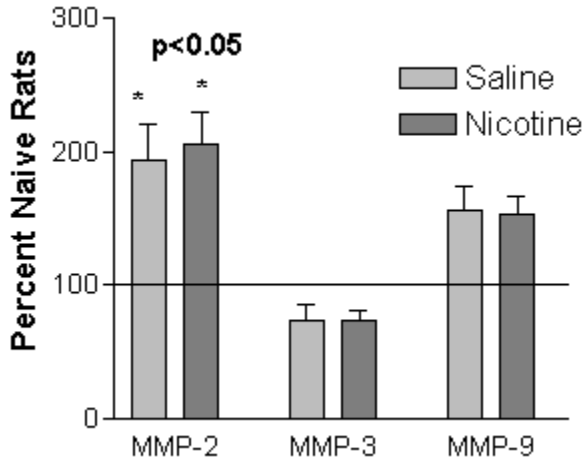
## Home Cage Nicotine Injection Induces Synaptic Plasticity

**Appendix 1. Home cage nicotine injection induces synaptic plasticity**

In this experiment, adolescent female rats were given subcutaneous (s.c.) nicotine injections in the home cage for 5 consecutive days while the control group received saline injections at the same time each day. The next day, the animals were sacrificed and the hippocampus and prefrontal cortex were collected. Western blotting was done to quantify the levels of MMPs – 2, 3, 9 and cortactin in these brain regions.

**Figure 1. MMPs in the hippocampus following 5 days of home cage drug administration.** Levels of MMP-2, 3, 9 were assayed using the Western blotting. N = 6 in each group. Line represents MMP levels in naïve animals. One way ANOVA analysis indicates a significant increase in pro-MMP-2 levels (*p<0.05) in both nicotine and saline treated animals. (B) Shows representative pro-MMP-2 protein bands.

(A)

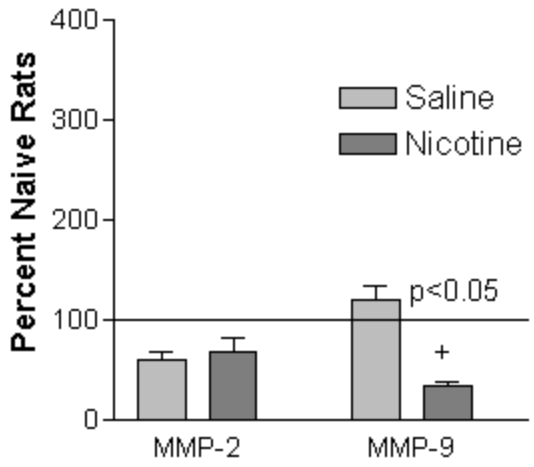



(B)

| MMP-2 | 5 day Home cage |
|---|---|
| Naïve |  |
| Saline |  |
| Nicotine |  |

**Figure 2. MMPs in the prefrontal cortex following 5 days of home cage drug administration.** Levels of MMP-2, and 9 were assayed using the Western blotting. N = 6 in each group. Line represents MMP levels in naïve animals. One way ANOVA analysis indicates a significant decrease in pro-MMP-9 in nicotine treated group compared to saline (+$p<0.05$). (B) Shows representative pro-MMP-9 protein bands.

(A)




(B) MMP-9 Home Cage

| MMP-9 | 5 day home cage |
|---|---|
| Naïve | |
| Saline | |
| Nicotine | |

**Figure 3. Cortactin in the hippocampus following 5 days of home cage drug administration.** Levels of cortactin were measured by Western blotting. N = 6 in each group. Line represents cortactin levels in naïve animals. One way ANOVA analysis indicates no significant change in cortactin in the hippocampus.

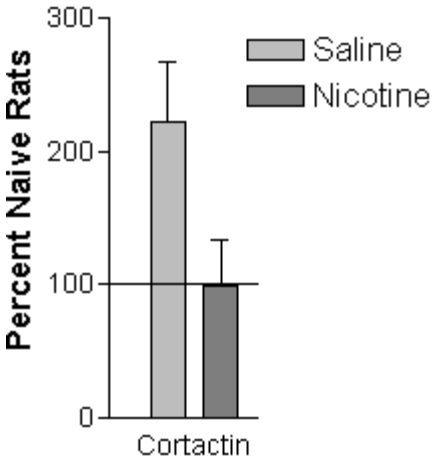
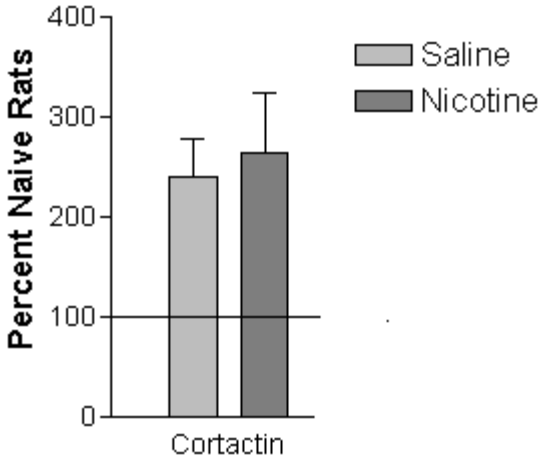



**Figure 4. Cortactin in the prefrontal cortex following 5 days of home cage drug administration.** Cortactin levels were measured using the Western blotting. N = 6 in each group. Line represents cortactin levels in naïve animals. One way ANOVA analysis indicates no significant changes in cortactin.



## Results

The results obtained were: 1.pro- MMP-2 levels in the hippocampus increased following 5 consecutive days of nicotine and saline administration; 2. pro-MMP-9 levels in the nicotine treated rats showed a decrease compared to saline and naïve animals in the prefrontal cortex; 3. there was no change in cortactin levels in both the hippocampus and the PFC.

## Discussion

Pro-MMP-2 levels increase in the hippocampus irrespective of the drug treatment that the animal underwent suggesting that MMP-2 changes are due to the novelty of the drug administration procedure itself rather than for the drug. Nicotine and saline treated groups show differential activation of pro-MMP-9 in the prefrontal cortex. Pro- MMP-9 levels in the nicotine group were significantly lower than the saline group indicating the involvement of MMP-9 in drug related learning. These results compared with the changes that occur following CPP show differences in the temporal and spatial characteristics of activation of MMPs. This is consistent with the idea that different MMPs are employed at various time-points for acquiring diverse information and associations. However, much more in-depth research of MMP activity is required to fully understand the involvement of MMPs in synaptic plasticity.

# Appendix B

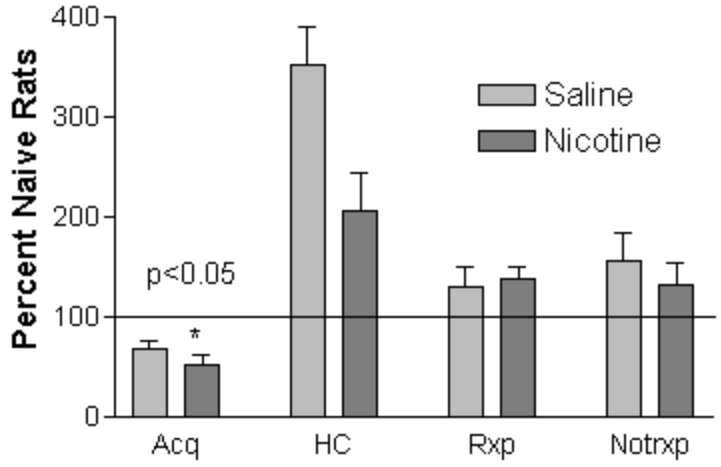
TIMP-1 and TIMP-2 Changes Following Nicotine Treatment

**Appendix 2. TIMP-1 and TIMP-2 changes following nicotine treatment.**

Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) are a family of four secreted protein that reversibly bind MMPs in a 1:1 fashion and inhibit the degradative activity of MMPs. Individual TIMPs vary in their ability to bind various MMPs. TIMPs, especially TIMP-1 and TIMP-2 have been implicated in cell grown, proliferation, migration, apoptosis and plasticity related to learning (Jourquin et al. 2005, Rivera et al. 1999, 2002) Therefore we were interested in examining TIMP activity during drug associated learning.

**Figure 1. TIMP-1 in the hippocampus.** Graph represents TIMP-1 in the hippocampus. N = 6-8. Acq = TIMP levels at postconditioning. HC = TIMP after 5 days of home cage drug injection. Rxp = TIMP levels during re-exposure to the CPP chamber 5 days after postconditioning. Notrxp = TIMP levels in animals that were not re-exposed to the CPP chamber 5 days after postconditioning. Line = TIMP levels of naïve animals. One way ANOVA analysis indicated a significant decrease in TIMP-1 levels of the nicotine treated group following CPP training (*$p<0.05$). (B) Shows representative TIMP-1 29kDa bands.

(A)

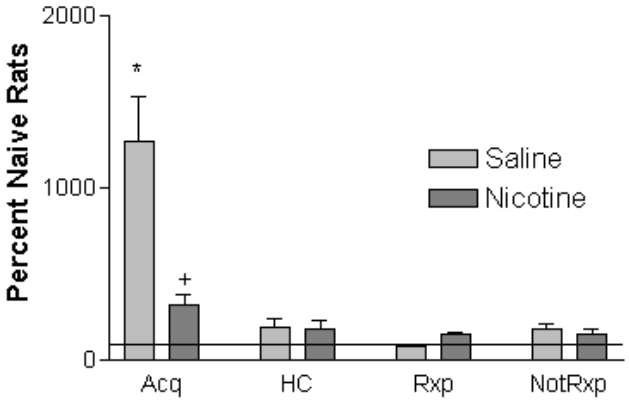


(B)

| TIMP-1 | Acq |
|---|---|
| Naïve |  |
| Saline |  |
| Nicotine |  |

**Figure 2. TIMP-1 in the prefrontal cortex.** Graph represents TIMP-1 in the PFC. N = 6-8. Acq = TIMP levels at postconditioning. HC = TIMP after 5 days of home cage drug injection. Rxp = TIMP levels during re-exposure to the CPP chamber 5 days after postconditioning. Notrxp = TIMP levels in animals that were not re-exposed to the CPP chamber 5 days after postconditioning. Line = TIMP levels of naïve animals. One way ANOVA analysis indicated a significant decrease in TIMP-1 levels of the nicotine treated group following CPP training (*p<0.05). One way ANOVA analysis indicated a significant decrease in TIMP-1 levels of the nicotine treated group following CPP training (*p<0.05). (B) Shows representative TIMP-1 29kDa bands.

(A)

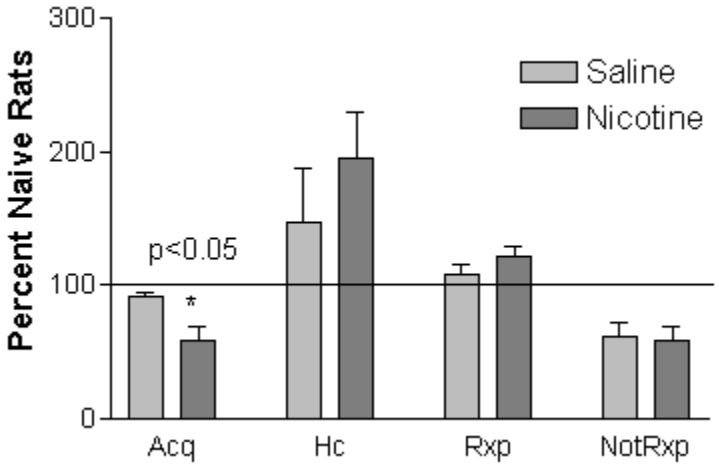



(B)

| TIMP-1 PFC | Acq |
|---|---|
| Naïve |  |
| Saline |  |
| Nicotine |  |

**Figure 3. TIMP-2 in the hippocampus.** Graph represents TIMP-2 in the hippocampus. N = 6-8. Acq = TIMP levels at postconditioning. HC = TIMP after 5 days of home cage drug injection. Rxp = TIMP levels during re-exposure to the CPP chamber 5 days after postconditioning. Notrxp = TIMP levels in animals that were not re-exposed to the CPP chamber 5 days after postconditioning. Line = TIMP levels of naïve animals. (B) shows representative 21 kDa TIMP-2 band after CPP training.

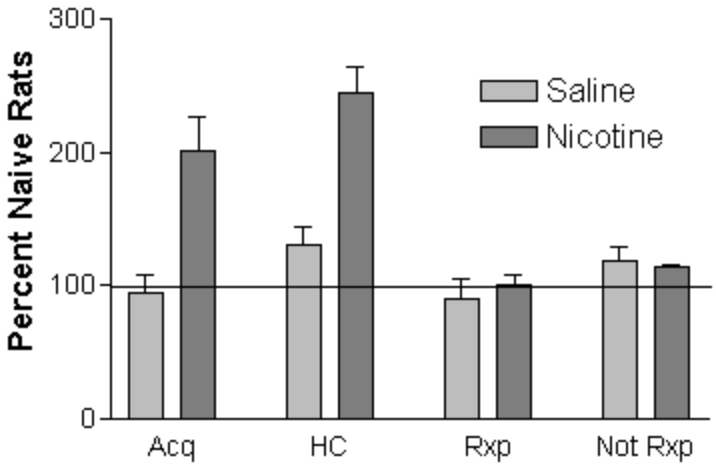
(A)




(B)

| TIMP-2 | Acq |
|---|---|
| Naïve | |
| Saline | |
| Nicotine | |

**Figure 4. TIMP-2 in the prefrontal cortex.** Graph represents TIMP-2 in the PFC. N = 6-8. Acq = TIMP levels at postconditioning. HC = TIMP after 5 days of home cage drug injection. Rxp = TIMP levels during re-exposure to the CPP chamber 5 days after postconditioning. Notrxp = TIMP levels in animals that were not re-exposed to the CPP chamber 5 days after postconditioning. Line = TIMP levels of naïve animals. No significant changes in TIMP-2 were noted in the prefrontal cortex.




**Results and discussion**

Our results show that TIMP-1 changes in both the hippocampus and prefrontal cortex following acquisition. The nicotine treated group showed significantly decreased TIMP-1 and TIMP-2 levels in the hippocampus following drug acquisition. In the prefrontal cortex nicotine and saline groups showed significant differences in their expression pattern. These results suggest that TIMP-1 changes in the hippocampus are specific to drug related learning. The increase in TIMP-1 in the saline but not nicotine group could mean that the nicotine injections are interfering with learning and therefore TIMP related plasticity in the PFC.

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