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Signals from the 4th Dimension: Role of Extracellular Matrix Signaling in Synaptic Plasticity Mediating Addiction

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Signals from the 4th dimension: Role of extracellular matrix signaling in synaptic plasticity mediating addiction

By

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ALEXANDER CAMERON WANLESS SMITH. Signals from the 4th dimension: Role of extracellular matrix signaling in synaptic plasticity mediating addiction. (Under the direction of PETER W. KALIVAS).

Chronic cocaine abuse causes maladaptive neuroadaptations that underlie vulnerability to relapse, even after protracted abstinence. A great deal of work has examined mechanisms of neuroplasticity by which these occur. However, the majority of experimentation has focused on intracellular signaling cascades, while the extracellular compartment has been largely ignored. In the past decade, work has emerged in the learning and memory literature that indicates that extracellular matrix remodeling and signaling is required for adaptive forms of neuroplasticity (e.g. learning and memory), although it has not been thoroughly examined in models of maladaptive neuroplasticity. Throughout this dissertation a drug self-administration, extinction and reinstatement paradigm is used. I first examine the role of the matrix metalloproteinases (MMPs) in the nucleus accumbens core (NAcore) in both the persistent synaptic potentiation that occurs following extinction of cocaine self-administration, and in the rapid, transient potentiation that is required for cue-induced reinstatement. By measuring both the expression and activity of MMPs, this work shows that relapse to multiple classes of drugs of abuse (cocaine, nicotine, and heroin) each are accompanied by an induction of MMP activity. Furthermore, this work goes on to show that inhibiting MMP activity also reverses or blocks synaptic potentiation. A second set of experiments examines nitric oxide (NO) signaling as a mechanism of MMP activation. These experiments used biochemical examination of neuronal nitric oxide synthase (nNOS) activity following extinction and reinstatement of cocaine seeking, and a small molecule inhibitor of nNOS to determine the effects of

nNOS activity on MMP activity and relapse behavior. Finally, by using NOS1-Cre transgenic mice this work shows that selectively chemogenetically stimulating a small population of interneurons that express nNOS drives reinstatement of drug seeking. This dissertation concludes that nNOS-expressing interneurons may comprise a ‘master-switch’ by MMPs are activated, synapses are potentiated, and strongly motivated behaviors are initiated.

CHAPTER 1: INTRODUCTION

1.1 Brief Review of Glutamatergic Plasticity in Drug Addiction

Drug abuse and addiction are ancient problems, with the earliest evidence dating back to a 5000 BC Sumerian civilization that used a symbol translated as "joy" for the poppy plant which opium is derived from (Alfred R. Lindesmith, *Addiction and Opiates.* p. 207). By 2000 BC, alcohol use was widespread enough for an Egyptian priest to record the first teachings of prohibition (W.F. Crafts *et al*., *Intoxicating Drinks and Drugs*, p. 5). Now, 7000 years later, drug abuse and addiction still have large negative consequences for individuals, families, and society at large. Currently, over 22 million Americans over the age of 12 (8.5%) meet criteria for substance dependence based on the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) (1BB).

Significant effort has gone towards determining the mechanisms of synaptic plasticity that underlie the transition from recreational drug use to dependence/addiction, including substantial research on drug-induced neuroadaptations in glutamatergic inputs to the nucleus accumbens (NAc) and the dysregulation of glutamate homeostasis (Kalivas, 2009; Russo et al., 2010a; Gipson et al., 2014). Glutamatergic projections from the prefrontal cortex (PFC) to NAc are involved in determining the extent to which a behavior will be promoted or inhibited. PFC to NAc circuitry is the primary contributor to long term compulsive cocaine seeking, while the PFC to VTA projection is required for the acute reinforcing effects of cocaine (Koob and Volkow, 2010). Cocaine addiction has been described as a pathology of reward-related memory in which memories

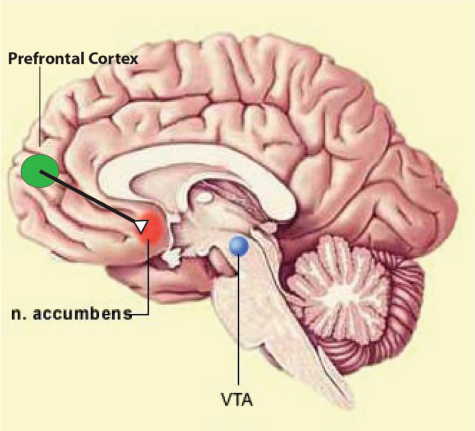


Figure 1-1. Anatomy of corticostriatal projection. Adapted from McGill university.

associated with drug use promote future drug seeking (Hyman et al., 2006). Following chronic cocaine self-administration and extinction, the balance between extrasynaptic and synaptic glutamate (glutamate homeostasis) is dysregulated in the NAc, and downregulation of extrasynaptic mGluR2/3 stimulation increases glutamate release from PFC terminals in response to action potentials (Kalivas 2009). This downregulation of mGluR2/3, in combination with downregulation of glutamate transporter 1 (GLT-1) and impaired synaptic glutamate reuptake by astrocytes, leads to hyperactivation of corticostriatal circuitry in response to cocaine-conditioned stimuli, and to glutamate overflow outside of the synaptic cleft (Kalivas, 2009). A key extrasynaptic receptor is mGluR5, which is a Gq-coupled receptor that increases intracellular free Ca^{2+} via the IP3 receptor, and activation of this receptor potentiates cue-induced reinstatement (Bespalov et al., 2005; Mitrano et al., 2008; Wang et al., 2013). Silencing the PFC via a combined infusion of the GABAb agonist baclofen and a GABAa agonist muscimol blocks cocaine-induced reinstatement and associated changes in extracellular glutamate (McFarland, 2003).

While enduring, constitutive imbalances in glutamate homeostasis are induced by all addictive drugs (Kalivas, 2009), this dysregulation is manifested in different ways following chronic exposure to different drugs. For example, in the case of cocaine and nicotine, there is prolonged potentiation of glutamatergic

synapses in the nucleus accumbens core (NAcore)(Kourrich et al., 2007; Moussawi, 2011; Gipson et al., 2013c), whereas these synapses are depotentiated following withdrawal from chronic heroin exposure (Shen et al., 2011). A major glutamatergic afferent into the accumbens arises from the medial PFC. This projection can be partially parsed into two pathways, one from the prelimbic cortex (PL) terminating in the NAcore that promotes the initiation of drug seeking for all drugs tested (McLaughlin and See, 2003; LaLumiere and Kalivas, 2008; Rocha and Kalivas, 2010; Stefanik et al., 2013a; Willcocks and McNally, 2013), and a second from the infralimbic cortex (IL) with terminals in the nucleus accumbens shell (NAshell) that promotes extinction of drug seeking for cocaine or alcohol (Peters et al., 2008; Stefanik et al., 2013b; Gass et al., 2014), but not heroin (Millan et al., 2011; Peters et al., 2013; Willcocks and McNally, 2013).

Data have recently emerged indicating that reinstatement induced by drug-associated cues to all tested classes of addictive drug, including cocaine, heroin and nicotine, is accompanied by a rapid, transient synaptic potentiation (t-SP) of glutamatergic synapses in the NAcore, characterized by enlargement of dendritic spine head diameter (d_h) and increase in the AMPA:NMDA ratio (A:N; measure of changing AMPA receptor function) (Gipson et al., 2013a; Gipson et al., 2013c; Shen et al., 2014b). Because this is a shared component of reinstatement to multiple drugs, it is viewed as a particularly promising point of pharmacotherapeutic intervention for the prevention of relapse.

1.2. The tetrapartite synapse: extracellular matrix remodeling contributes to corticoaccumbens plasticity in drug addiction

In the past decade it has been made clear that the matrix metalloproteinases (MMPs) are a family of extracellular proteases that strongly modulate synaptic plasticity (Ethell and Ethell, 2007). Hippocampal long-term potentiation is dependent on MMP activity and their cleavage of molecules that signal through integrin cell adhesion receptors (Huntley, 2012b). Physiologically, MMPs are required for a number of tasks that depend on synaptic plasticity, such as fear conditioning and spatial learning (Meighan et al., 2006; Nagy et al., 2007). Cocaine, nicotine, and heroin reinstatement are each characterized by the rapid induction of extracellular matrix-remodeling matrix-metalloproteinase (MMP) activity in the NAcore, and inhibiting MMP activity prevents reinstatement and the associated synaptic plasticity (Smith et al., 2014a). The emergence of data implicating extracellular matrix proteolysis in synaptic plasticity mediating addiction constitutes a paradigm shift away from the tripartite synapse, and towards a tetrapartite synapse, in which all four elements of synaptic architecture are considered in modeling the addicted synapse, including the presynaptic, postsynaptic, glial, and extracellular compartments (Figure 1).

1.2.1 MMP Structure and Function

MMPs are Zn^{2+}-dependent endopeptidases that degrade extracellular matrix (ECM) as well as cell-surface molecules to promote cellular reorganization. These proteins were originally discovered for their role in tumor cell invasion (Himelstein et al., 1994), angiogenesis (Yu and Stamenkovic, 2000), and wound healing (Agren et al., 1994), and more recently their contributions to

synaptic and neuronal reorganization have been elucidated (Meighan et al., 2006; Huntley, 2012b). Twenty-three distinct MMPs have been identified in the human genome, 16 of which are soluble proteins, and 7 transmembrane or GPI-anchored proteins (Huntley, 2012b), and these multidomain proteins can be further divided into subgroups based on shared domains, inserts, and substrate recognition motifs. Soluble MMPs contain an N-terminal secretory signal peptide, whereas membrane-bound MMPs do not. All MMPs contain an autoinhibitory propeptide, and all have similar catalytic regions. Most also contain a C-terminal hemopexin domain, which is attached to the catalytic domain via a flexible hinge linker. The specific structure of the hemopexin domain varies between MMPs, and confers substrate and protein binding specificity to each enzyme (Overall, 2002). Hemopexin domains often dictate subcellular localization by binding to cell surface proteins or ECM molecules, and because of the flexible nature of the hinge region, the catalytic domain can move to process substrates freely while the hemopexin domain is tethered (Collier et al., 2001). The most studied MMPs in the brain are MMPs-2, -3 and -9 (Verslegers et al., 2013). MMP-2 and MMP-9 are also referred to as Gelatinase A & B, respectively, so named for their ability to proteolytically process gelatin. Their unique ability to bind to gelatin is due to fibronectin type II (FNII) repeats within the enzymes' catalytic domain. FNII repeats recognize and bind to ECM glycoproteins that contain Arg-Gly-Asp (RGD) domains (e.g. fibronectin, laminin, thrombospondin), which are the endogenous ligands for the integrin family of cell adhesion receptors (Verslegers et al., 2013). Thus, MMPs-2/9 are able to recognize and expose integrin-signaling domains, giving them a crucial role in neuron-ECM adhesion. MMP-3 is

also called Stromelysin-1, and differs from the gelatinases in that it lacks FNII repeats, and contains a different C-terminal hemopexin domain (Sternlicht and Werb, 2001a). In addition to ECM molecules, MMPs proteolytically process a variety of growth factors, such as transforming growth factor beta (TGFß; (Yu and Stamenkovic, 2000) brain-derived neurotrophic factor (BDNF; (Mizoguchi et al., 2011b), cell surface glycoproteins (e.g. ß-dystroglycan; (Michaluk et al., 2007), cell adhesion molecules/receptors (e.g. SynCAM2; (Bajor et al., 2012), and many other proteins positioned to regulate signaling between the intra- and extra-cellular space (Huntley, 2012b).

Another major role for MMPs in the brain is in regulating the blood brain barrier (BBB), and these proteinases are sensitive to a variety of neuroinflammatory signals. Neuroinflammation describes the immune response of central nervous system tissue, normally isolated immunologically from the peripheral system by the BBB. The BBB is maintained through the collaboration of neural, glial, vascular and extracellular components and can be degraded by the aberrant action of MMPs, allowing compounds synthesized or arriving to the periphery to exert effects on CNS tissue (Kousik et al., 2012). Chronic exposure to drugs of abuse, particularly psychostimulant drugs, alters the integrity of the BBB, allowing for entry of viral and bacterial agents from the periphery that contribute to drug-induced neurotoxicity and neuroinflammation (Kousik et al., 2012; Clark et al., 2013). The neuroinflammatory action of psychostimulants can occur through a variety of mechanisms including through the disruption of tight junction channels at the BBB interface, the activation of microglia and the resulting release of pro-inflammatory cytokines, and by the aberrant activation of

enzymes that regulate the remodeling of the extracellular matrix (Kousik et al., 2012). Studies show that proinflammatory cytokines and their downstream signaling pathways potently upregulate MMP production in astrocytes and miocroglia (Gottschall and Deb, 1996; O'Shea et al., 2014). Specifically, neuron/glia co-culture studies show that pro-inflammatory cytokine release increases expression of the two main inducible MMPs, MMP-3 and MMP-9, further demonstrating the link between neuroinflammation and MMP activation (Candelario-Jalil et al., 2009).

Neuroinflammation in response to drug exposure is a growing topic of addiction research because the pharmacological reversal of drug-induced neuroinflammation in animal models of addiction can inhibit drug seeking (Scofield and Kalivas, 2014). Among the most studied examples of addictive substances that impact the integrity of the BBB and invoke a neurotoxic and neuroinflammatory responses are methamphetamine, cocaine and 3,4-methylenedioxy-N-methylamphetamine (MDMA).

1.2.3 MMP Synthesis, Release and Activation

1.2.3.1 Transcriptional regulation

For MMPs to appropriately participate in the neuronal processes including cellular reorganization and remodeling in synaptic plasticity and memory, they must be appropriately expressed, localized and temporally activated (Sternlicht and Werb, 2001b). As such, the regulation of MMP expression and activity is particularly complex and involves several well-regulated mechanisms and signaling cascades (Dzwonek et al., 2004). At the transcriptional level, MMPs and related proteins are tightly regulated with the exception of MMP-2, MMP-14

(MT1-MMP) and tissue inhibitor of metalloproteinases 2 (TIMP-2), which are less tightly restricted by transcriptional control and are co-regulated by the same transcription factors (Lohi et al., 2000). This is likely due to the fact that MMP-2 is more constitutively expressed than other MMPs. Moreover, the positive co-regulation of pro-MMP-2, MT1-MMP, and TIMP-2 also reflects the role that these three factors play in forming a complex to activate pro-MMP-2 (discussed in detail below). Apart from MMP-2, MMPs are regulated at the transcriptional level by phorbol esters, integrin derived signals, extracellular matrix proteins and stress signals (Kheradmand et al., 1998; Sternlicht and Werb, 2001b). As discussed above, MMP expression is regulated by interferons, interleukins, and growth factors, which typically induce expression of c-fos and c-jun immediate early genes whose protein products dimerize to form AP-1 (Sternlicht and Werb, 2001b) (See Figure 1). Generally AP-1 serves as a critical positive regulator of MMP expression and the promoter regions of several MMP genes contain canonical AP-1 binding sites, (Gottschall and Deb, 1996; Sternlicht and Werb, 2001b). In addition, AP-2, Sp1, Sp3 and NF-κB sites are found in several MMP promoters, speaking to the coordinated regulation of these species (Sternlicht and Werb, 2001b). As an example, MMP-9 is positively regulated by both AP-1 and NF-κB at the transcriptional level (Huntley, 2012b), and post-transcriptionally nitric oxide (NO) levels regulate the stability of MMP-9 mRNA (Dzwonek et al., 2004). Interestingly, a role for microRNAs (miRs) has been established for both regulating MMP-2/9 expression and in drug addiction. Micro-RNAs are small, non-coding RNAs that can each regulate the translation of many mRNAs. In the

dorsal striatum, miR-212 upregulates Raf1 activity and thus CREB signaling, which decreases the motivation to take cocaine (Hollander et al., 2010). While the regulation of miRs and MMPs in the brain has not been established, MMP-9 mRNA has a potential miR-212 binding site according to a TargetScan screen (targetscan.org; version 6.2), while there was no identified binding site for miR-212 on MMP-2 mRNA. MicroRNA regulation of MMP gene translation has been shown to be physiologically relevant in systems outside of the brain, most notably miR-29a regulation of MMP-2 is important in proteolysis during thoracic aortic aneurysm (Jones et al., 2011).

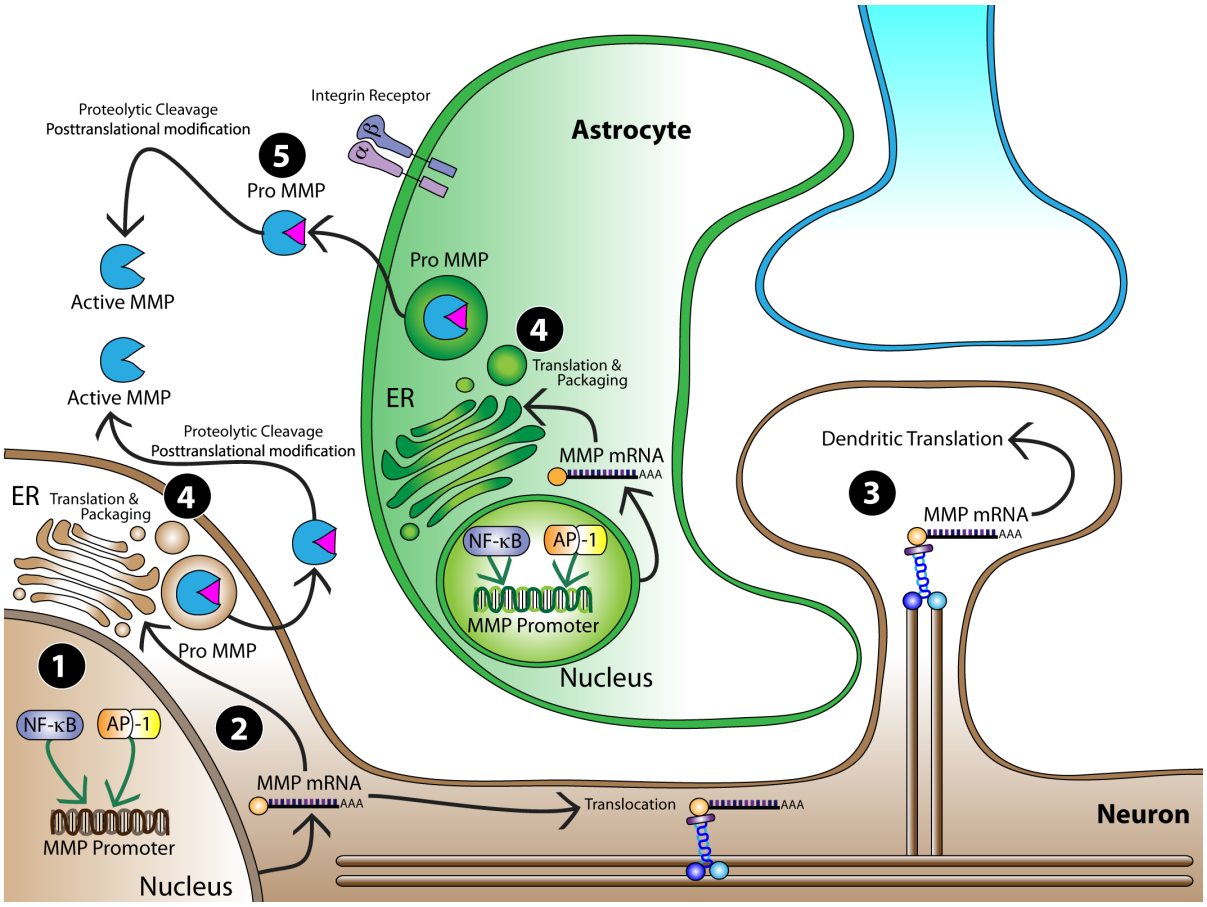


Figure 1-2. MMP expression and secretion in neuronal and non-neuronal cells. Shown here is a schematic representation of MMP expression and secretion mechanisms in neurons (brown) and astrocytes (green). **1)** In both

neurons (brown) and astroglia (green) MMP transcription is generally positively regulated by transcription factors NF-kB (blue) and AP-1 (orange/yellow). **2)** After MMP mRNA is transcribed it can be transported to the endoplasmic reticulum (ER). **3)** Alternatively, MMP mRNA can be translocated to dendritic spine heads where it is locally translated and secreted. **4)** Once translocated from the nucleus (in neurons or in astrocytes), MMP mRNA is translated at the endoplasmic reticulum and packaged and released in a pro-form. **5)** Once outside of the cell, pro-MMPs are activated in the extracellular space by proteolytic cleavage or by posttranslational modification. (Smith et al., 2015)

1.2.3.2 Release and post-transcriptional regulation

MMPs are expressed and secreted as inactive pro-enzymes, also known as zymogens. Enzymatic inactivation is maintained by the interaction of a pro-domain cysteine residue with the catalytic Zn^{2+}. When this interaction is broken, Zn^{2+} is able to fully coordinate with 3 cysteine residues in the active site, a process known as the “cysteine switch” (Loffek et al., 2011). Zymogens are processed into active MMPs through proteolytic cleavage or posttranslational modification, both of which occur through variety of mechanisms. This makes the steady state level of MMP expression at the mRNA level a relatively poor index of activity due to the large amount of regulation of pro-MMP proteins already present in the neural parenchyma (Huntley, 2012b). Beyond MMP-2, other zymogens can be cleaved by activated MMPs in the extracellular space or by serine proteases that cleave peptide bonds within the MMP pro-domain (Sternlicht and Werb, 2001b). These proteases include plasmin, tissue plasminogen activator and urokinase-type plasminogen activator, which are also important mediators of the transition from pro-MMP to an active MMP molecule (Candelario-Jalil et al., 2009).

In keeping with the activity dependent and inducible nature of MMP expression, evidence exists for the translocation of MMP-9 mRNA to the dendritic arbor with a preference for dendrites actively engaged in synaptic transmission (Dzwonek et al., 2004). Notably, MMP-9 mRNA contains specific sequence elements implicated in the translocation of other mRNAs, suggesting that this translocation plays an important role in rapidly inducible action of MMP-9 (Dzwonek et al., 2004). Furthermore, studies show that dendritic MMP-9 translation is activity dependent and contributes to the rapid increase in MMP-9 activity seen with increased excitatory neurotransmission (Dziembowska et al., 2012).

1.2.3.3 Regulation through protein-protein interactions

The activity of MMPs is regulated by a family of secreted proteins called the tissue inhibitors of metalloproteinases (TIMPs), which promote growth and act to regulate cell cycle in a variety of cell types (Mizoguchi et al., 2011a). TIMPs display substantial sequence complementarity to their MMP counterparts, and form reversible noncovalent bonds with MMPs to inactivate them (Sternlicht and Werb, 2001b). In biological systems, levels of TIMP expression are tuned to act in concert with levels of MMP expression and activation to precisely orchestrate the appropriate glycoprotein turnover rate (El Hajj et al., 2014). The TIMP family contains four members, TIMP1-4. Interestingly, members of the TIMP family differ in their ability to inhibit specific MMPs. Relatively little is known about TIMP-3 and TIMP-4. TIMP-3 is expressed at very low levels, but mRNA has been detected in cortex, cerebellum, olfactory bulb, and brain stem

(Dzwonek et al, 2004). TIMP-4 is expressed specifically by cerebellar purkinje neurons, and by neurons in specific brain stem regions (Dzwonek et al, 2004).

TIMP-1 expression is found primarily in neurons (apart from Bergman glial cells), and to date has been anatomically localized in the cortex, hippocampus, cerebellum, substantia nigra and hypothalamus (Dzwonek et al., 2004). The apparent expression pattern of TIMP1 was restricted to neuronal cell bodies in these regions with the exception of the hippocampus where dendritic localization was also observed (Rivera et al., 1997). TIMP1 expression is sensitive to several stimuli and is induced by neuronal depolarization and in certain pathological conditions including electroconvusive seizures (ECS), where the expression of TIMP1 is upregulated in both the cortex and hippocampus (Newton et al., 2003). In addition, in a cell culture model using rat cardiac fibroblasts, exposure to alcohol for 48 hours induced expression of TIMP1 (El Hajj et al., 2014). However, in the serum of human heroin addicts, levels for TIMP1 were lower than control individuals, with ratios of serum levels of MMP-2/TIMP-1, MMP-9/TIMP-1 higher in the heroin group (Kovatsi et al., 2013).

TIMP-2 has been the most studied of the TIMPs. Its expression is predominantly restricted to neurons and is abundant in brain, yet is anatomically restricted to the cortex, NAc and cerebellum (Dzwonek et al., 2004). Unlike TIMP-1, TIMP-2 is not upregulated by neuronal activity or in pathological conditions (Dzwonek et al., 2004). However, methamphetamine exposure upregulates TIMP2 in the frontal cortex and nucleus accumbens (Mizoguchi et al., 2011a). Interestingly, when the upregulation of TIMP-2 is thwarted with antisense RNA inhibition, methamphetamine locomotor sensitization is increased

(Mizoguchi et al., 2011a). As discussed above for TIMP-1, heroin exposure also increases the MMP-2/TIMP-2 ratio in serum (Kovatsi et al., 2013) and in a cell culture model of human gingival fibroblasts, exposure to nicotine exposure redistributes TIMP2 to the cell surface (Zhou et al., 2007). In a series of experiments using cultured Kupffer cells (stellate macrophages isolated from the liver) from ethanol fed rats, TIMP-2 expression was elevated when compared to cultures made from control rats (Aziz-Seible et al., 2011). In a recent report we show that MMP-9 and TIMP-2 protein levels are increased in the NAcore, following cocaine-primed reinstatement of cocaine seeking (Smith et al., 2014a). However, the induction of TIMP-2 protein levels did not inhibit the increased activity of MMPs observed following cocaine-primed reinstatement (Smith et al., 2014a).

Paradoxically, apart from inhibiting the action of MMPs, TIMP proteins also form complexes with MMPs and can facilitate their activation. For example, TIMP-2 interacts with the membrane bound MT1-MMP to facilitate the formation of active MMP-2 from proMMP-2 (Shofuda et al., 1998), while MT2-MMP can activate pro-MMP-2 in a TIMP-2-independent manner (Morrison and Overall, 2006). In either a TIMP-2 dependent or independent activation, MMP-2 activated in this way activates MMP-9. In addition, TIMP-1 interacts with pro-MMP-9 and MMP-3. In this complex of proteins, residues of the C-terminal domain of pro-MMP-9 and TIMP-1 physically interact. This tertiary complex can be modified to release pro-MMP-9, leaving TIMP-1 and MMP-3 bound (Dzwonek et al., 2004).

1.2.4 MMP contributions to reward and addiction

Addiction can be described as a pathological form of learning, wherein drug associated cues and/or contexts become strong conditioned stimuli that promote a drug seeking response, even after protracted abstinence or extinction training (Hyman et al., 2006). A role for MMPs in plasticity accompanying learning and memory was posited in 2003, when Wright and colleagues found that ethanol decreased performance in a Morris water maze, and also impaired MMP-9 activity in the hippocampus (Wright et al., 2003). It has since been established that MMP-9 is activated by and required for the maintenance phase of long-term potentiation (LTP; (Nagy et al., 2006), and application of auto-active MMP-9 onto a slice is able to drive enlargement of dendritic spines and potentiate excitatory field potentials, even in the absence of LTP-inducing high frequency stimulation (Wang et al., 2008a). Given their role in LTP, it is not surprising that MMPs are involved in many forms of learning and memory, including spatial memory (Meighan et al., 2006), fear conditioning (Brown et al., 2009), avoidance learning (Nagy et al., 2007), and memory related to contextual cues (Brown et al., 2007).

Human post-mortem data indicate that MMPs may have clinical relevance in addiction. A functional polymorphism in the MMP-9 gene is associated with higher risk for alcoholism, and MMP-9 is elevated in the serum of alcohol abusers (Sillanaukee et al., 2002; Samochowiec et al., 2010), cocaine abusers had decreased MMP-9 activity in the hippocampus measured by gel zymography (Mash et al., 2007), and heroin users have significantly higher circulating MMP-2 and MMP-9 than do non-drug-using controls (Kovatsi et al., 2013). While these

clinical data are interesting, most knowledge about MMPs in reward and addiction comes from animal models.

1.2.4.1 Cocaine

Early work showed that intracerebroventricular (i.c.v.) infusion of a broad-spectrum MMP inhibitor (FN-439) blocked acquisition of cocaine conditioned place preference (CPP) when infused prior to daily conditioning sessions, and also blocked cocaine-primed reinstatement when infused immediately prior to this session (Brown et al., 2007). MMP-9, but not MMP-2 activity was increased in the medial PFC following reinstatement of CPP (Brown et al., 2008). The work described in chapters 2 and 3 below represents the most thorough examination of the role of metalloproteinases in synaptic plasticity underlying the vulnerability to relapse to cocaine seeking.

While largely untested, there are many putative mechanisms by which MMP activity may translate into to the transient synaptic potentiation associated with reinstated cocaine seeking (measured as increased AMPA:NMDA ratio and dendritic spine head diameter). The integrin family of cell adhesion receptors is particularly interesting in this regard. In a self-administration paradigm, 1 day following the last of 10 daily 2-hour sessions, the ß3-integrin subunit is downregulated, with no change in the ß1 subunit. Following 3 weeks of extinction training, the ß3 subunit is upregulated by 500%, again with no change in the ß1 subunit (Wiggins et al., 2011b). Injecting a synthetic RGD peptide daily, prior to self-admin sessions prevented the enduring ß3 subunit upregulation, and also attenuated cocaine primed reinstatement (Wiggins et al., 2011b). In addition to cleaving matrix glycoproteins to expose RGD domains that signal through a5ß3

and a5ß1 integrin receptors (Sternlicht and Werb, 2001a), MMPs are able to cause integrins to shed subunits that themselves contain RGD domains. For example MMP-2 activity may induce cell motility through proteolytic shedding of the ß1 subunit (Kryczka et al., 2012). Integrins signal primarily through integrin-linked kinase (ILK), and ILK can directly phosphorylate cofilin in order to stimulate actin polymerization and dendritic spine head growth (Kim et al., 2008). ILK also phosphorylates Ser845 of GluA1 AMPA subunits, stimulating insertion of the receptor into the synapse, which may increase AMPA:NMDA ratio (Chen et al., 2010). Behaviorally, inhibiting ILK attenuates locomotor sensitization to cocaine, as well as the increased AMPA receptor insertion that accompanies sensitization (Chen et al., 2008), but it has not yet been tested whether inhibiting

ILK during extinction training following self-administration can reduce AMPA:NMDA ratio. See figure 2 for a schematic outlining MMP signaling within the extracellular matrix.

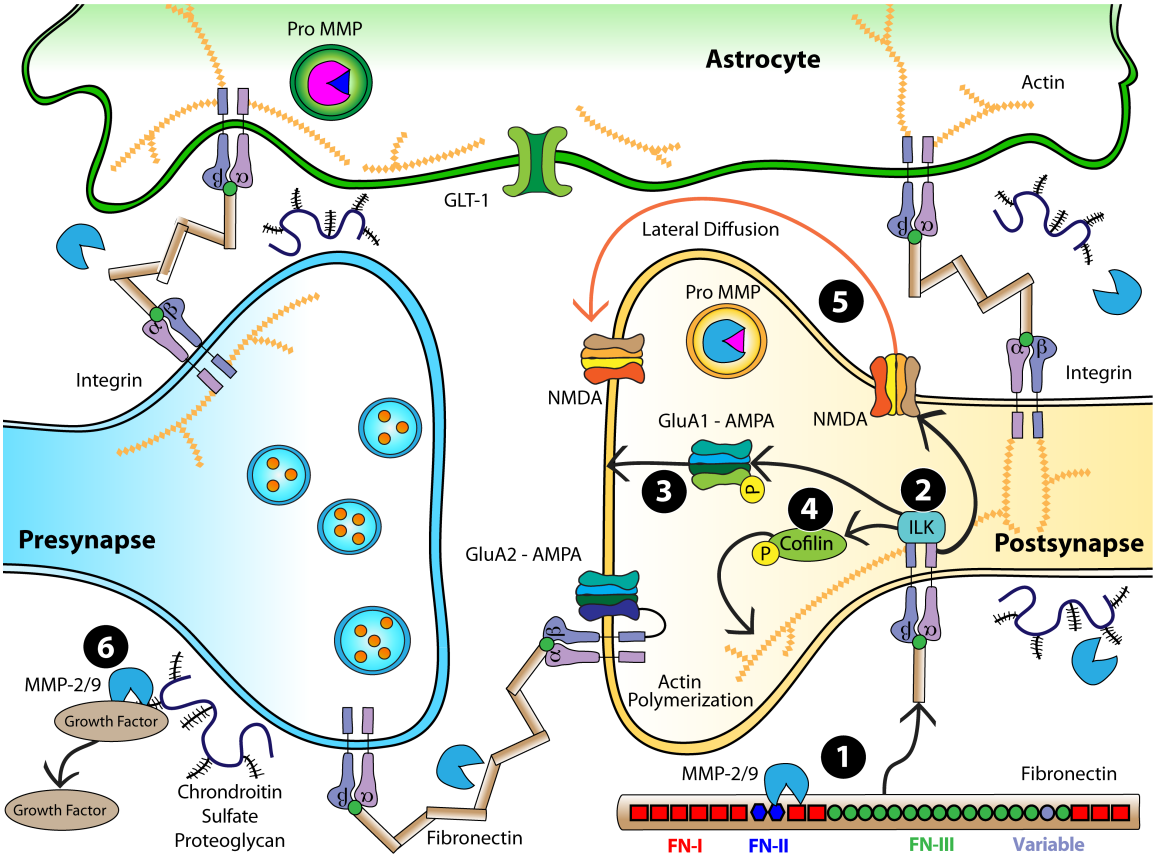


Figure 1-3: Overview of signaling in the extracellular matrix. 1) MMPs-2 and -9 signal within the ECM by proteolytically processing RGD-containing glycoproteins such as fibronectin. The brown rectangles shown here represent fibronectin, with an enlarged fibronectin molecule shown at the bottom right corner with labeled fibronectin type-1, type-2 and type-3 domains. MMPs-2 and -9 recognize FN-II repeats, allowing them to bind to and cleave fibronectin in order to expose RGD domains. **2)** Putative mechanisms by which addictive drug-induced MMP activity alters synaptic strength include signaling through integrin-linked kinase (ILK). Stimulation of ILK then promotes **3)** phosphorylation of GluA1 S845 to increase AMPA receptor insertion, **4)** phosphorylation of cofilin which stimulates actin polymerization and **5)** stimulates NMDA receptor lateral diffusion into the synapse. **6)** In addition to fibronectin, MMPs can also proteolytically process chondroitin sulfate proteoglycans, acting to liberate growth factors such

as BDNF, TGFb, NGF, and TNFa that may also act to influence synaptic plasticity. (Smith et al., 2015)

1.2.4.2 Methamphetamine

Repeated methamphetamine (METH; 2 mg/kg) exposure induces MMP-2 and MMP-9 activity in the NAcore within 2h of the last injection, and MMP-2 or MMP-9 KO mice show decreased dopamine release, and impaired behavioral sensitization in response to a METH challenge injection. Furthermore, addition of recombinant MMP-2 potentiated METH-induced dopamine release (Mizoguchi, 2007). Acutely, a large METH dose (40 mg/kg) induces MMP-9 activity within 6 hours, and this leads to proteolytic shedding of the ectodomain of intracellular adhesion molecule 5 (ICAM5), producing a soluble fragment that can signal through ß1-integrin subunits to cause cofilin phosphorylation (Conant et al., 2011). Soluble ICAM5 ectodomains have also been shown to increase frequency of mEPSCs and GluA1 Ser845 phosphorylation and surface expression, without affecting expression of GluA2 (Lonskaya et al., 2013).

Data from human imaging studies and animal models of addiction clearly demonstrate METH is potently neurotoxic (Panenka et al., 2013). Specifically, METH exposure disrupts the BBB by causing alterations in tight junction proteins (Ramirez et al., 2009) and also enhances release of pro-inflammatory cytokines including interleukin 6 and 8 (Shah et al., 2012), which may activate MMP-9 and cause aberrant degradation of the BBB (Yao et al, 2006). The importance of the neuroinflammatory response to METH exposure in the treatment of addiction has been underscored by the fact that in the laboratory setting, the systemic administration of glial modulator drugs that inhibit the release of pro-inflammatory

factors has been shown to also inhibit METH seeking in several animal models of addiction (Beardsley et al., 2010; Snider et al., 2012; Snider et al., 2013). Furthermore, METH exposure increased the expression of MMP-1 and MMP-9, which can act to degrade tight junction proteins producing structural changes to the basement BBB membrane that contribute to the neuroinflammatory response (Conant et al., 2004). In addition, studies show that METH exposure decreases expression of MMP-9 substrate laminin, indicating that MMPs may degrade the BBB by attacking the basal lamina (O'Shea et al., 2014).

1.2.4.3 Opiates

MMP-9 expression and activity are increased by acute morphine treatment, and MMP-9 is required for the development of morphine tolerance (Nakamoto et al., 2012). In the spinal cord, MMP-9 inhibition blocks morphine-induced phosphorylation of NMDA receptors, ERK1/2, and cAMP response element binding proteins, and behavioral signs of morphine withdrawal (Liu et al., 2010). Following extinction of heroin self-administration, two constituents of perineuronal nets (PNNs), tenascin R (TNR) and brevican (bcan) were downregulated in both the mPFC and accumbens, indicating increased proteolytic degradation by MMPs. Furthermore, i.c.v. infusion of a broad spectrum MMP inhibitor restored PNN composition, and attenuated cue-induced heroin reinstatement (Van Den Oever, 2010). Interestingly, we did not find an increase in MMP-2 or MMP-9 activity using *in vivo* zymography following extinction of heroin self-administration, but there was an induction of activity following 15 minutes of cue-induced reinstatement (Smith et al., 2014a). These data indicate that a third protease, possibly MMP-3, is responsible for regulating

the composition of perineuronal nets (PNNs), which are exclusively localized around GABAergic fast-spiking interneurons (FSIs). PNNs are hypothesized to be selectively localized around these interneurons because their largely anionic composition is protective against oxidative stress which results from the relatively higher metabolic requirements of fast spiking interneurons (Cabungcal et al., 2013).

1.2.4.4 Nicotine

Very little about the role of MMPs in nicotine addiction has been established. MMP activity in the accumbens core following nicotine exposure parallels cocaine: following extinction training there is a constitutive induction of gelatinolytic fluorescence, and following reinstatement there is a further induction of activity (Smith et al., 2014a). This is the only observation regarding MMPs in the nucleus accumbens following nicotine exposure; another laboratory has examined these enzymes in the hippocampus and mPFC following nicotine CPP. Natarajan and colleagues (2013) induced conditioned place preference with nicotine injections, and measured MMP-2, -3, and -9 expression following each of 5 days of acquisition of CPP, and following re-exposure to the drug-paired context after 5 days of abstinence. Inhibition of MMP activity via daily i.c.v. FN-439 infusion prior to conditioning blocked the acquisition of place preference. Following 5 days of conditioning, both MMP-2 and MMP-9 were both significantly increased in the hippocampus, but not in the mPFC, while MMP-3 remained unchanged. Following 5 days of abstinence from nicotine in the home cage, when re-exposed to the CPP apparatus there was no change in MMP-2 or MMP-9 expression, but MMP-3 expression was increased in both nicotine and saline

treated rats. This indicates a broad role for MMP-3 in reactivation of contextual memories, but does not imply a drug-specific effect (Natarajan et al., 2013).

1.2.4.5 Alcohol

Acute ethanol intoxication decreases MMP-9 activity in the hippocampus, and impairs spatial memory formation without interfering with MMP-2 activity (Wright et al., 2003). In the chronic intermittent ethanol vapor model in which rats are exposed to ethanol vapor for 14 hours per day for 4 weeks (with a target daily BAC of 200mg/dL), animals undergo repeated cycles of intoxication and withdrawal. Following 4 weeks of vapor exposure, rats allowed to self-administer ethanol during acute (6h) withdrawal display an escalation of self-administration that is indicative of a dependence-like behavioral phenotype (Walker, 2012). Chronic i.c.v. infusion of broad spectrum MMP inhibitor FN-439 during these 4 weeks blocks escalation of ethanol intake (Smith et al., 2011). Interestingly, acute FN-439 infusion only prior to post-vapor self-administration sessions also blocks this escalation, although once a rat experienced one session of post-vapor self-administration without the presence of FN-439, drinking escalated during the next session. Furthermore, in rats that received aCSF rather than FN-439 and escalated immediately following vapor exposure, acute FN-439 was not thereafter effective. This indicates that MMPs contribute to the negative reinforcement learning that occurs from ethanol consumption during acute withdrawal (Smith et al., 2011). Negative reinforcement refers to reinforcement that occurs when a response leads to the removal of a negative stimulus. This data was interpreted to enhance the hypothesis that this negative reinforcement is a learned response that requires the animal to recognize that alcohol

consumption during acute withdrawal will ameliorate withdrawal-associated negative affect, and that this learning is MMP-dependent (Smith et al., 2011; (Walker, 2012).

1.3 Contributions of neuronal nitric oxide synthase to synaptic plasticity, drug addiction and metalloproteinase activity.

The role of nitric oxide (NO) as an intercellular messenger in the brain was discovered in 1988, when NO was referred to as 'Endothelium-derived relaxing factor' (Garthwaite et al., 1988). NO was identified as a candidate retrograde neurotransmitter in 1991 (Schuman and Madison, 1991). The NO receptor, soluble guanylate cyclase (sGC), is expressed predominantly presynaptically, although it can also be detected in complex with PSD-95, suggesting a postsynaptic localization as well (Russwurm et al., 2001; Garthwaite, 2010). Soluble guanylate cyclase activity promotes cGMP formation and PKG activity. However, the reactive nitrogen species properties of NO also confer its ability to act as mediator of S-nitrosylation, an important post-translational modification for many proteins. A positive influence of NO has been shown for release of at least glutamate, GABA, dopamine, and norepinephrine (Lawrence and Jarrott, 1993; Montague et al., 1994; Li et al., 2002; West et al., 2002). In addition to the positive effects of the sGC/cGMP/PKG signaling pathway on neurotransmitter release, there are a number of targets of S-nitrosylation that also influence neurotransmitter release. For example, S-nitrosylation of syntaxin facilitates a conformational change that allows syntaxin to associate with two of its binding partners, vesicle-associated membrane protein (VAMP, a V-SNARE), and SNAP25, which allows vesicular docking at the presynaptic membrane (Palmer

et al., 2008). Furthermore, SNAP2 5 can be S-nitrosylated to increase its affinity for V-SNARE binding (Di Stasi et al., 2002).

NO is synthesized by nitric oxide synthase (NOS), There are 3 isoforms of NOS: the NOS1 gene codes neuronal NOS (nNOS), which is expressed in a subpopulation of γ-aminobutyric acid-releasing (GABAergic) interneurons known as nitrergic interneurons (Tepper et al., 2010), NOS2 is inducible NOS (iNOS), which is expressed in glia and other cell types, and is under cytokine control (Saha and Pahan, 2006), and NOS3, endothelial NOS (eNOS) is expressed by endothelial cells in blood vessels and other tissues. S-Nitrosylation has been shown to be important in regulating MMP activity (Gu et al., 2002), and specifically MMP-9 (Gu et al., 2002; Manabe et al., 2005a; Ridnour et al., 2007). Studies support a role for NO in modulating synaptic plasticity related to cocaine exposure, since NOS1 knock out mice display impaired cocaine sensitization (Itzhak et al., 1998b; Balda et al., 2009), reward (Balda et al., 2006) and cue- and context-induced CPP (Balda et al., 2006; Itzhak, 2008; Itzhak et al., 2010). Furthermore, repeated cocaine administration increases NO release in the dorsal striatum (Lee et al., 2010) and pharmacological inhibition of nNOS attenuates cocaine-mediated elevations of PFC neuronal excitability (Nasif et al., 2011).

Activity of the nNOS enzyme is negatively regulated by phosphorylation at Serine 847 (Ser847), and dephosphorylation at this residue occurs following NMDA receptor activation (Rameau et al., 2003b, 2004). Interestingly, nNOS physically interacts with the NMDA receptor subunit GluN2B through a PDZ domain interaction with postsynaptic density-95 (Cui et al., 2007), which localizes

nNOS in close proximity to elevations in internal calcium produced by activation of GluN2B-containing NMDA receptors. Once dephosphorylated at Ser847, affinity for Calmodulin (CaM) is elevated and NO production increases 50-60% (Hayashi et al., 1999) illustrating the importance of Ca^{2+}-mediated signaling in nNOS activation and production of NO. Studies show that cocaine-mediated increases in NO release in the dorsal striatum are attenuated by D1, NMDA and group I metabotropic glutamate receptor antagonists (Lee et al., 2010; Lee et al., 2011) indicating regulation of NO production by both dopaminergic and glutamatergic mechanisms (Park and West, 2009).

1.4 Statement of the problem

Cocaine addiction has long been described as the 'holy grail' of addiction research. This is because there are no current drugs approved by the FDA for the treatment of cocaine addiction. While no treatment for any addiction is 100% effective, there are at least prescribable and marginally effective treatments for alcoholism (e.g. disulfiram, topiramate), opiate addiction (e.g. buprenorphine, methadone), and nicotine addiction (e.g. varenicline), but no such treatment has been developed for psychostimulant addiction (Wallace, 1952; Kramer, 1970; Coe et al., 2005; Collins and McAllister, 2007; Kalivas and Volkow, 2011)

As previously described in the introduction, chronic cocaine exposure induces maladaptive neuroplasticity within the NAcore, and reversing these changes is seen as a favorable pharmacotherapeutic target for controlling relapse (Kalivas and Volkow, 2011). While a great deal of research has been dedicated to elucidating intracellular signaling cascades that lead to synaptic

potentiation of glutamatergic inputs onto accumbens spiny projection neurons (Kourrich et al., 2007; Conrad et al., 2008), the work described in herein is the first thorough examination of the contribution of NAcore extracellular matrix remodeling to synaptic plasticity underlying cocaine relapse. It was hypothesized that extracellular remodeling by matrix metalloproteinases contributes to both persistent and transient synaptic potentiations that underlie cue-induced reinstatement of cocaine seeking.

Furthermore, the standing model of neuroplasticity takes into account three components of each synapse: the presynaptic terminal, the postsynaptic neuron, and an astroglial cell that stabilizes the two neurons. Together, these three components form the tripartite synapse (Araque et al., 1999). Although the tripartite synapse is a beautiful representation of simple synapses, the problem is that it is just that: an oversimplification of a complex biological system. While the contribution of extracellular matrix remodeling clearly establishes a fourth component of synapses, an additional critical consideration is that intercellular non-synaptic communication that occurs via the gaseous transmitter nitric oxide is also a critical player contributing to synaptic plasticity within glutamatergic synapses. Nitric oxide is produced only by a small population of GABAergic interneurons within the accumbens, termed nitrergic interneurons. Previous work has demonstrated that cocaine affects nitric oxide production and that this drives AMPA receptors into postsynaptic densities (Lee et al., 2010; Selvakumar et al., 2014), and also that MMPs are activated by S-nitrosylation of a cysteine residue within their pro-domain (Gu et al., 2002). Thus, by integrating nitrergic signaling

into the tetrapartite synapse, I hypothesized that nNOS-expressing interneurons are critical mediators of cocaine-induced synaptic potentiation of corticostriatal circuits.

In summary, the work described herein attempts to accomplish the following goals to further our knowledge of synaptic plasticity underlying relapse to cocaine seeking:

1. **Determine whether extracellular matrix remodeling by matrix metalloproteinases contributes to cue-induced reinstatement of cocaine seeking, and the underlying neurobiological properties of this behavior.**
2. **Determine whether repeated cocaine self-administration affects nitric oxide signaling within the nucleus accumbens core.**
3. **Establish a role for nitric oxide signaling in the activation of MMPs and cue-induced reinstatement.**

James Watson wrote*: "the brain is the most complex thing we have yet discovered in our universe. It contains hundreds of billions of cells interlinked through trillions of connections. The brain boggles the mind."* But what is truly amazing about the brain is that while the same neurons you are born with are the ones you die with, your brain is not really the same brain at all. The ability of the brain to undergo constant change is the truly mind-boggling part, and it is incredibly exciting that the modern era of science has helped us to understand how these processes work and to target them for treatment when the brain's wonderful ability to change turns pathological. The work presented in this dissertation attempts to aid in this understanding by examining specific mechanisms in which maladaptive neuroplasticity occurs to create drug addiction.

CHAPTER 2: SYNAPTIC PLASTICITY MEDIATING COCAINE RELAPSE REQUIRES MATRIX METALLOPROTEINASES

Abstract

Relapse to using addictive drugs arises from pathological remodeling of excitatory synapses in the nucleus accumbens that impairs control over drug seeking behaviors. Matrix metalloproteinases (MMPs) are proteolytic enzymes responsible for degrading the extracellular matrix and thereby facilitating synaptic reorganization. Using a rat model of relapse, we found that cue-induced reinstatement of cocaine, nicotine or heroin seeking elicited a rapid, transient increase in MMP activity in the nucleus accumbens core. Also, rats withdrawn and extinguished from cocaine and nicotine, but not heroin self-administration had an enduring and stable increase in MMP activity. We discovered that MMP-2 mediated the stabilization of cocaine-induced synaptic potentiation after withdrawal, MMP-9 mediated the transient cue-induced synaptic potentiation, and the activity of both MMPs was necessary for conditioned cues to reinstate cocaine-seeking behavior. These data reveal that MMP activity is necessary for the synaptic reorganization that mediates relapse to cocaine, heroin and nicotine.

2.1 INTRODUCTION

Vulnerability to relapse is a defining characteristic of drug addiction, and controlling relapse is a primary therapeutic goal in treating addiction (Vocci and Ling, 2005). The difficulty addicts experience in controlling drug use has inspired research showing drug-induced impairments in cortical regulation of the striatal circuitry mediating motivation and habit formation (Kalivas and Volkow, 2005; Luscher and Malenka, 2011). Cocaine, nicotine or heroin abuse cause long-lasting changes at cortical glutamatergic synapses in the nucleus accumbens core (NAcore) that are necessary for relapse to occur (Robinson, 2004; Moussawi, 2011; Shen et al., 2011; Gipson et al., 2013c). Cocaine and nicotine induce enduring potentiation of glutamatergic synapses in NAcore, whereas heroin withdrawal produces enduring synaptic depression(Conrad et al., 2008; Russo et al., 2010b; Gipson et al., 2013c). While the adaptations in enduring synaptic plasticity differ between classes of addictive drug, a biomarker of relapse shared by all 3 drugs is rapid, transient potentiation of glutamatergic synapses in the NAcore that is induced in parallel with the reinstatement of drug-seeking (Anderson et al., 2008; Shen et al., 2011; Gipson et al., 2013a; Gipson et al., 2013c; Shen et al., 2014b). Although synaptic potentiation at glutamatergic synapses in NAcore is required for reinstating drug-seeking behavior, it is not understood how the long-lasting potentiation after withdrawal from cocaine or nicotine is stabilized, or how transient synaptic potentiation is initiated by cues associated with any of the 3 drugs.

Synaptic remodeling during brain development or after injury depends on the extracellular matrix (ECM), which is a proteinacious network ensheathing synapses that is degraded by a family of Zn^{2+}-dependent endopeptidases called matrix metalloproteinases (MMPs) (Huntley, 2012a). MMP-2 and -9 make up the gelatinase subfamily (Sternlicht and Werb, 2001a). Previous literature suggests that the gelatinase degradation of the ECM regulates synaptic structure and physiology by proteolytically activating growth factors (Mizoguchi et al., 2011b; Saygili et al., 2011), and processing ECM glycoproteins to expose Arginine-Glycine-Aspartic acid (RGD) domains that bind to integrins to promote AMPA glutamate receptor trafficking and actin polymerization (Cingolani et al., 2008; Huntley, 2012a). Inhibiting MMP-9 prior to high frequency electrical stimulation interferes with maintenance of hippocampal long-term potentiation, and incubating hippocampal slices in auto-active recombinant MMP-9 produces integrin-dependent long-term potentiation of evoked field potentials and enlarged spine head diameter, even in the absence of high frequency stimulation (Wang et al., 2008a). Using a relapse model of self-administration and reinstatement in rats, we hypothesized that cocaine, heroin, and nicotine alter MMP-2 and/or -9 activity, and that these enzymes are required for the transient synaptic plasticity mediating cue-induced reinstatement of drug seeking.

2.2 Methods

Animal Housing and Surgery. Male Sprague Dawley Rats (250g; Charles River) were individually housed with a 12:12 hour dark/light cycle. All experimentation occurred during the dark phase. Animals were allowed to acclimate to the

vivarium environment for 4 days prior to surgery. Rats were anesthetized with a combination of ketamine HCl and xylazine, and received ketorolac for analgesia. All rats received intrajugular catheters, and rats for microinjection experiments received intracranial catheters targeted 2mm above NAcore (+1.8 A/P, +2.1 M/L, -5.5 D/V) according coordinates from Paxinos and Watson, 2005. All rats were provided food and water *ad libitum* until 1 day prior to beginning behavioral training, at which points rats were food restricted to 25g of rat chow per day. All methods used herein comply with the NIH Guide for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

Cocaine Self-Administration and Reinstatement. After 5 days of recovery following surgery, rats underwent one overnight (12 hour) food training session. The next day, animals began daily 2-hour cocaine self-administration (SA) sessions. During SA, cocaine was delivered on an FR1 schedule with a 20s timeout following each infusion. Active lever presses that resulted in cocaine infusion simultaneously resulted in presentation of a light and tone stimulus, which serve as cocaine-conditioned cues. An inactive lever was also provided to control for non-motivated responding. Following 10 SA sessions (≥ 10 infusions/day), rats began extinction training, during which all programmed consequences were removed from lever pressing. Extinction training lasted at least 10 days, or until two consecutive days ≤ 25 active lever presses. Reinstatement was induced by presentation of light/tone cues following an active lever press. Microinjections of an MMP inhibitor or vehicle were given 15 minutes

prior to beginning reinstatement in most cases, or 15 minutes prior to gel infusion for zymography experiments. For behavioral experiments (Fig.1), a within-subjects crossover design was used; In this paradigm, each rat received each condition (MMP-2i, MMP-9i, or Vehicle) according to a latin square design. Rats were required to meet extinction criteria prior to each reinstatement test. These sessions lasted 120 minutes. For zymography, spine morphology, and AMPA:NMDA ratio (A:N) experiments reinstatement sessions were 15 minutes long, at which point rats were sacrificed for further measurements.

In Vivo Zymography. Because MMPs are secreted in inactive pro-forms and catalytically activated within the ECM, activity assays are preferable to immunoblotting for protein content. We used an *in vivo* zymography assay to directly measure MMP activity. Dye-quenched gelatin is an MMP-2/9 substrate which has been intramolecularly quenched with FITC fluorophores such that it cannot fluoresce until proteolytically processed by MMP-2 or MMP-9 (Bozdagi et al., 2007). The amount of fluorescence produced forms a linear relationship with incubation time and MMP activity. Dye-quenched FITC-Gelatin (Molecular Probes, Eugene, OR) was reconstituted in PBS at 1 mg/ml pH 7.2-7.4. 3.0µl of gel (1.5µl/side) was infused and allowed to incubate for 15 minutes prior to perfusion. Rats were perfused with 4% paraformaldehyde (PFA) and brains were placed in 4% PFA for 90 minutes for additional fixation. Brains were mounted on a vibratome and sliced into 50µm sections through the NAc. Sections were mounted and coverslipped. Imaging took place on a Leica LSM510 confocal microscope. Fluorescence was excited with a 488nm Argon laser, and emissions

were filtered to 515-535nm. Images were taken through a 10x objective with a 0.3 numerical aperture. Only slices in which the injection site and anterior commissure could be visualized in the same frame were imaged. ImageJ (NIH) was used to quantify images. The injection tract was masked out to control for wound-healing related induction of MMP activity, and an integrated density of fluorescence was measured.

Western Blotting. Rats were rapidly decapitated at t=0 or t=15. The NAc was dissected and homogenized in RIPA lysis buffer containing 1.0% SDS and protease/phosphatase inhibitors. Homogenate was centrifuged at 4°C for 5 minutes at 10,000 x g. Supernatant was collected and protein concentration was determined via a biconchinic acid assay (Thermo Scientific). 30µg protein was added to each lane of 10% Bis-Tris gels (Bio-Rad), and transferred to nitrocellulose membranes via the Invitrogen iBlot transfer system. Primary antibodies were used for MMP-2 (1:1500, Abcam), MMP-9 (1:500, Millipore), and TIMP2 (1:1000, Abcam) and HRP-conjugated goat anti-rabbit secondary was used at 1:10,000. GAPDH was used as a loading control for MMPs-2 and -9, and Calnexin was used for TIMP-2. A Kodak Image Station was used to visualize and quantify protein expression.

Quantification of dendritic spine head morphology. Spine morphology was performed as described in detail elsewhere (Shen et al., 2008). Briefly, brains were sliced into 200µm sections through the NAc, and a Helios gene gun (Bio-Rad) was used to label sections with DiI-coated tungsten particles. Imaging

occurred on a Zeiss LSM510 confocal microscope. DiI was excited using a Helium/Neon 543nm laser. Dendrites chosen for imaging were between 75-200 µm from the soma and after the first branch point. Images of dendrites were taken through a 63x oil immersion objective (Plan-Apochromat, Zeiss; NA = 1.4, WD = 90 µm) at 0.1µm intervals along the z-axis. Images were deconvoluted via Autoquant prior to analysis (Media Cybernetics, Bethesda, MD), and then a 3-D perspective was rendered by the Surpass module of Imaris software package (Bitplane; Saint Paul, MN). The smallest quantifiable diameter spine head was 0.143µm.

Slice preparation for electrophysiology. Rats were anesthetized with ketamine HCl (1 mg/kg Ketaset, Fort Dodge Animal Health, Iowa) and decapitated. The brain was removed from the skull and 220µm thick coronal NAc sections were obtained using a vibratome (VT1200S Leica vibratome; Leica Microsystems, Wetzlar, Germany). Slices were immediately placed into a vial containing artificial cerebrospinal fluid (aCSF) and a mixture of 5 mM kynurenic acid and 50 µM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5). Slices were incubated at 32°C for 30-40 minutes and then stored at room temperature.

In vitro whole cell recording. All recordings were collected at 32˚C (controlled by TC-344B, Warner Instrument Corporation, Hamden, Connecticut) in the dorsomedial NAcore. Inhibitory synaptic transmission was blocked with picrotoxin (50 µM). Multiclamp 700B (Axon Instruments, Union City, CA) was used to record excitatory postsynaptic currents (EPSCs) in whole cell patch-clamp configuration.

Glass microelectrodes (1-2 MΩ) were filled with cesium-based internal solution (in mM: 124 cesium methanesulfonate, 10 HEPES potassium, 1 EGTA, 1 MgCl2, 10 NaCl, 2.0 MgATP, and 0.3 NaGTP, 1 QX-314, pH 7.2-7.3, 275 mOsm). Data were acquired at 10 kHz, and filtered at 2 kHz using AxoGraph X software (AxoGraph Scientific, Sydney, Australia). To evoke EPSCs a bipolar stimulating electrode (FHC, Bowdoin, Maine) was placed ~300 μm dorsomedial to the recorded cell to maximize chances of stimulating prelimbic afferents. The stimulation intensity chosen evoked ~50% of maximal EPSC. Recordings were collected every 20 sec. Series resistance (Rs) measured with a 2 mV depolarizing step (10 ms) given with each stimulus and holding current were always monitored online. Recordings with unstable Rs, or when Rs exceeded 10 MΩ were aborted.

Measuring the AMPA/NMDA ratio. Recordings started no earlier than 10 min after the cell membrane was ruptured, to allow diffusion of the internal solution into the cell. AMPA currents were first measured at -80 mV to ensure stability of response. The membrane potential was then gradually increased to +40 mV. Recording of currents resumed 5 min after reaching +40 mV to allow stabilization of cell parameters. Currents composed of both AMPA and NMDA components were then obtained. Then D-AP5 was bath-applied (50 μM) to block NMDA currents and recording of AMPA currents at +40 mV was started after 2 min. NMDA currents were obtained by subtracting the AMPA currents from the total current at +40 mV.

Statistical Analysis All statistics were done using GraphPad Prism Version 6. 120 minute reinstatement sessions for behavior analysis were analyzed using One-Way ANOVAs with Bonferroni post-hoc tests. Zymography measurements were analyzed using either paired or unpaired t-tests, depending on which was appropriate for the experiment. A:N data were analyzed using t-tests with Bonferroni's correction for planned comparisons. D_h measurements were analyzed using a Two-Way ANOVA with time point and inhibitor dose as factors.

2.3 RESULTS

Cocaine, heroin, and nicotine reinstatement induced MMP activity within the NAcore. In order to quantify MMP-2 and MMP-9 proteolytic activity within the NAcore, we microinjected a FITC-quenched gelatin peptide that fluoresces following cleavage by MMP-2 or MMP-9 (Bozdagi et al., 2007) in a linear manner over 60 min after administration (Figure 2-7). Rats were trained to self-administer cocaine, heroin, or nicotine for 10 days during which drug infusion was paired with a light/tone compound conditioning stimulus. Lever pressing was then extinguished over the next 10 days during drug withdrawal (Figure 1a). This self-administration protocol resulted in rats achieving stable levels of active lever pressing for all three drugs that was significantly greater than inactive lever pressing, and the extinction protocol reduced active lever pressing to a criterion of <25% of the active lever pressing during self-administration (Figure 2-8). FITC-gelatin was bilaterally microinjected into NAcore, and 15 minutes later animals were sacrificed just prior to initiating cue-induced reinstatement of cocaine-, heroin- or nicotine-seeking, or after 15, 45, or 120 min of reinstatement (Figure

1b). MMP-2/9 activity was also quantified following reinstatement initiated by a noncontingent cocaine injection (10mg/kg, ip). Gelatinase activity was increased in NAcore ~2.5-fold in cocaine-extinguished compared with yoked-saline rats, and 15 min of cue-induced reinstatement of cocaine seeking caused a further ~2-fold increase (Figure 1b-e). The cue-induced increase in MMP-2/9 activity was transient and returned to pre-reinstatement levels by 120 min after initiating the reinstatement session (Figure 1b). Nicotine affected MMP-2/9 activity similarly to cocaine, but with a smaller magnitude increase. Following extinction of nicotine self-administration, MMP-2/9 activity was increased by ~60% compared with yoked saline animals, and after 15 minutes of cue-induced reinstatement, activity was increased an additional ~50%. In contrast with cocaine or nicotine, heroin-trained subjects did not show increased gelatinolytic fluorescence following extinction, but akin to the other two addictive drugs, cue-induced reinstatement of heroin seeking caused a ~2.6-fold induction of MMP-2/9 activity. Reinstatement induced by a noncontingent cocaine injection also increased MMP2/9 activity. However, the increase was delayed and appeared at 45 minutes after administering a cocaine priming (Figure 1b). In fact, at 15 min after administering noncontingent cocaine to initiate a reinstatement session MMP2/9 activity was actually reduced compared to levels measures in cocaine-extinguished animals. This delayed increase in MMP-2/9 activity during cocaine-induced reinstatement parallels the pattern of reinstated active lever pressing that is minimal during the first 15 minutes after cocaine-primed reinstatement, and is significantly increased by 45 minutes after cocaine administration (Shen et al., 2014a). Similarly, the rise in MMP2/9 activity after 15 min of cued reinstatement for all three drugs

parallels a rapid increase in active lever pressing (Gipson et al., 2013a; Gipson et al., 2013c).

Gelatinolytic fluorescence in NAcore was organized around cell soma, and could often be seen outlining primary dendrites (Figure 1i,g). Moreover, somatic fluorescence always co-labeled with the neuronal marker NeuN (Figure 1h). To evaluate whether other regions of the striatal complex showed similar increases in MMP-2/9 activity, we microinjected FITC-gelatin into the dorsal striatum and the nucleus accumbens shell (NAshell) of yoked saline controls or rats extinguished from cocaine self-administration that were reinstated for 15 min by conditioned cues. Increases in fluorescence were not measured in either region following 15 min of cue-induced reinstatement of cocaine seeking compared with yoked saline rats (Figure 2-9).

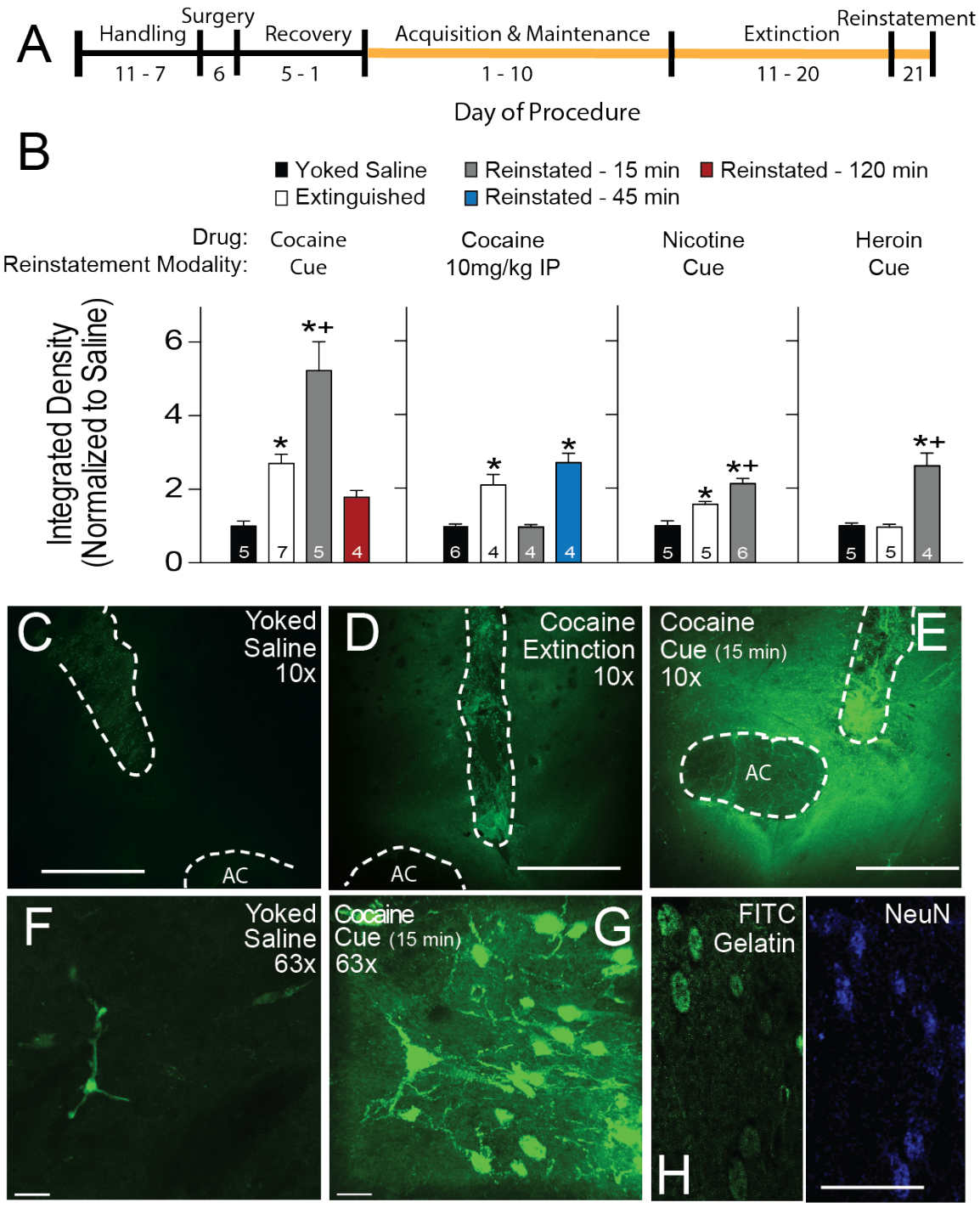


Figure 2-1: Cocaine, nicotine, and heroin reinstatement induced MMP activity in the NAcore. **a)** *Outline of the rat self-administration-extinction-reinstatement protocol.* ***b)*** *Gelatinase activity was increased following extinction from cocaine self-administration, and further increased 15 minutes following cue-induced reinstatement, and returned to baseline by 120 min; one-way ANOVA* $F_{(3,17)} = 17.80$, $p < 0.0001$. *Nicotine self-administration and extinction resulted in a similar pattern of*

*changes as cocaine, albeit with smaller magnitude, one-way ANOVA $F_{(2,13)} = 19.70$, $p = 0.0001$. Heroin did not induce MMP activity following extinction, but cue-induced reinstatement produced a 2.6 fold induction in MMP activity, one-way ANOVA $F_{(2,11)} = 25.19$, $p < 0.0001$. Reinstatement induced by a 10mg/kg cocaine priming injection showed a rapid decrease in MMP activity, followed by a delayed increase following 45 minutes of reinstatement, one-way ANOVA $F_{(3,14)} = 23.42$, $p < 0.0001$. N in bars indicates number of animals, and each animal constitutes the average of 4 slices in which the injection site could be clearly imaged in the same frame as the anterior commissure (AC). * $p < 0.05$ compared to yoked-saline using a Bonferonni test for multiple comparisons; + $p < 0.05$ compared to extinguished.* ***c,d,e)*** *Representative 10x magnification micrographs of FITC-gelatin fluorescence in yoked-saline, cocaine extinguished and cue-reinstated NAcore. Dashed lines show areas masked-out from quantification and included nonspecific fluorescence associated with damage at the injection site and the AC. Scale bar= 500 µm.* ***f,g)*** *Example 63x magnification micrograph in yoke-saline and cue-reinstated cocaine animals showing some fluorescence around primary dendrites. Scale bar = 20 µm. Shows double labeling of fluorescent puncta with the neuronal marker NeuN. Scale bar= 100 µm. N inside bar is number of animals, 5 slices were averaged per animal, and data was normalized to yoked-saline mean. All experiments were conducted by myself, except for the nicotine experiment, in which I did the surgeries, Cassie Gipson ran the animals, and I did the microinjections, perfusions, imaging, and analysis.*

MMP-2 activity was constitutively up-regulated following extinction, and MMP-9 activity was transiently induced by reinstating cocaine seeking.

Since the reinstatement of cocaine seeking produced the largest induction of MMP activity, we used cocaine-trained rats to investigate the possibility that MMP-2 and MMP-9 may have selective effects on drug-induced synaptic plasticity. Since FITC-quenching is relieved by proteolytic activity of either MMP-2 or MMP-9, we used selective pharmacological inhibitors of MMP-2 (1.0 nmol/side) and MMP-9 (0.1 nmol/side) to determine which MMP was mediating the increased fluorescence following cocaine extinction and reinstatement (Levin, 2001). The enduring basal increase in fluorescence in cocaine extinguished compared to yoked-saline subjects was abolished by intra-NAcore microinjection

of MMP-2, but not MMP-9 inhibitor (Figure 2a). Conversely, the increase in fluorescence induced by 15 min of cued reinstatement was reduced by the MMP-9, but not MMP-2 inhibitor (Figure 2b). The increase in fluorescence at 45 min following reinstatement elicited by a cocaine priming injection was also reduced by an MMP-9 inhibitor (Figure 2c). This pattern of gelatinase expression is consistent with previous work showing that the brain constitutively expresses MMP-2 activity, while MMP-9 is transiently induced by external stimuli (Verslegers et al., 2013). Also, the differential effect of MMP-2 and MMP-9 blockade on extinguished versus reinstated MMP activity supports the selectivity of the individual inhibitors (Levin, 2001).

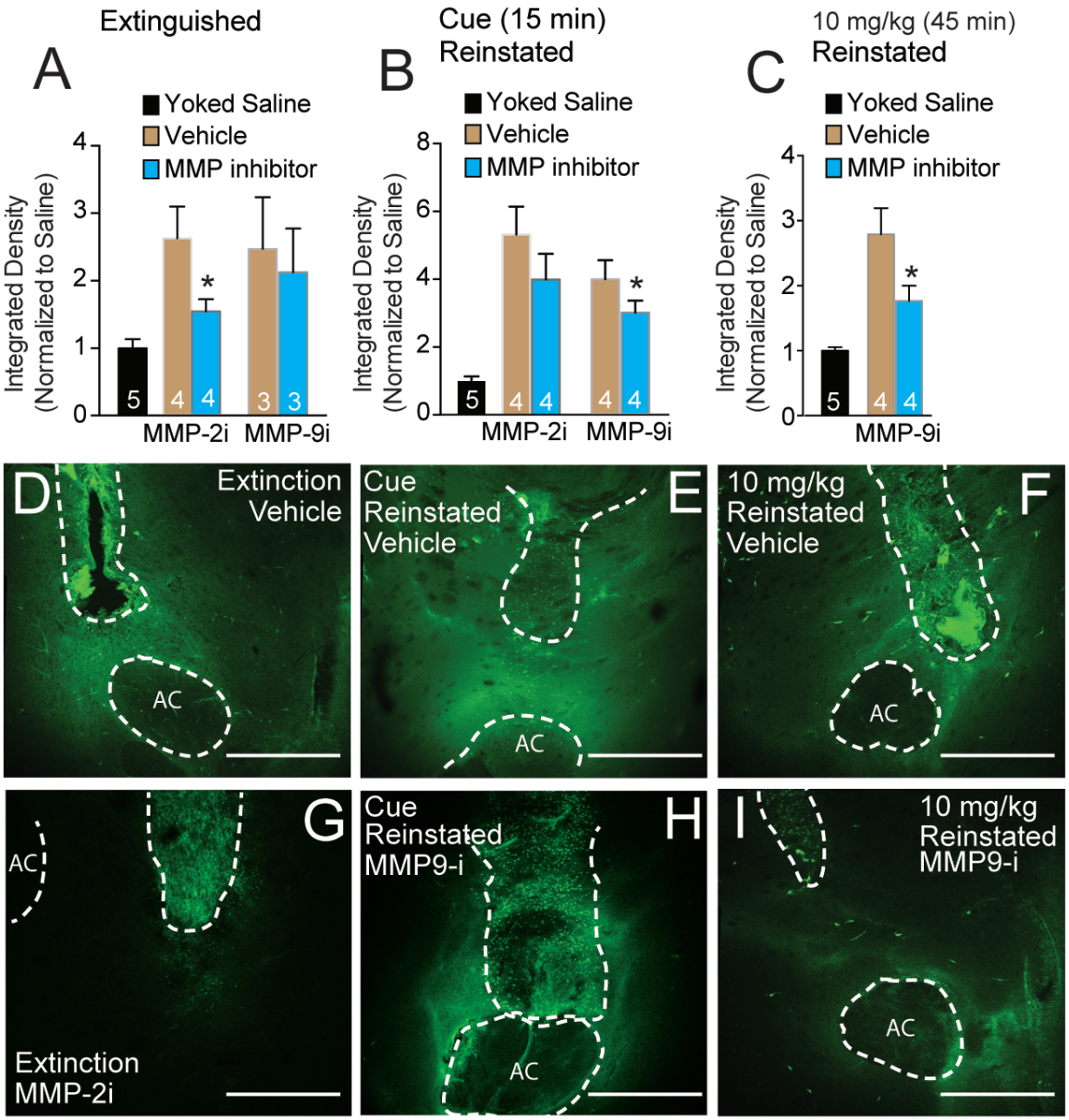


Figure 2-2. MMP-2 was constitutively induced following extinction of cocaine self-administration, and MMP-9 was transiently induced by cues associated with cocaine. *Animals received unilateral vehicle injection and contralateral injection of either MMP-2 or MMP-9 inhibitor. Opposite hemispheres were paired and data were analyzed via paired Student's t-test.* ***a)*** *MMP-2 inhibitor (MMP-2i; 1 nmol) decreased gelatinase activity following extinction compared to vehicle injection into the contralateral NAcore,* $t_{(3)} = 3.72$, $p = 0.034$, *while MMP-9i (0.1 nmol) was without effect. Yoked saline data shown for comparison are from panel 1c.* ***b)*** *MMP-9i, but not MMP-2i, reduced gelatinase activity 15 minutes following cue-induced reinstatement.* $t_{(3)} = 3.47$, $p = 0.040$. ***c)*** *MMP-9 inhibition reduced fluorescence induced 45 minutes after a cocaine-priming injection* $t_{(3)} = 3.77$, $p = 0.037$. *For all representative fluorescent micrographs injection damage (encompassed by dashed line) and AC were masked out for quantifying fluorescence. Scale bar= 500µm. * $p < 0.05$, comparing*

vehicle with inhibitor used a paired Student's t-test. N inside bar is number of animals, 5 slices were averaged per animal, and data was normalized to the yoked-saline mean. All experiments were conducted myself.

Role of increased MMP-2 or MMP-9 synthesis in mediating increased proteolytic activity. We next determined if the increases in MMP-2 or MMP-9 proteolytic activity in NAcore were associated with changes in protein content. Neither the enduring increase in MMP-2, nor the transient increase in MMP-9 activity after 15 min of cued reinstatement of cocaine seeking was accompanied by a change in whole cell MMP-2 or MMP-9 protein (Figure 3a) or mRNA (Figure 2-10) content in the NAcore. Similarly there was no change in the TIMP-2 content, an endogenous protein that binds to and negatively regulates MMP-2 activity (Brew et al., 2000). Since the MMP-2 and MMP-9 antibodies recognize both the active and inactive proMMP-2/9 proteins, the increase in MMP-2 and MMP-9 activity shown in figure 1 likely results from protein activation rather than increased protein synthesis (Huntley, 2012a).

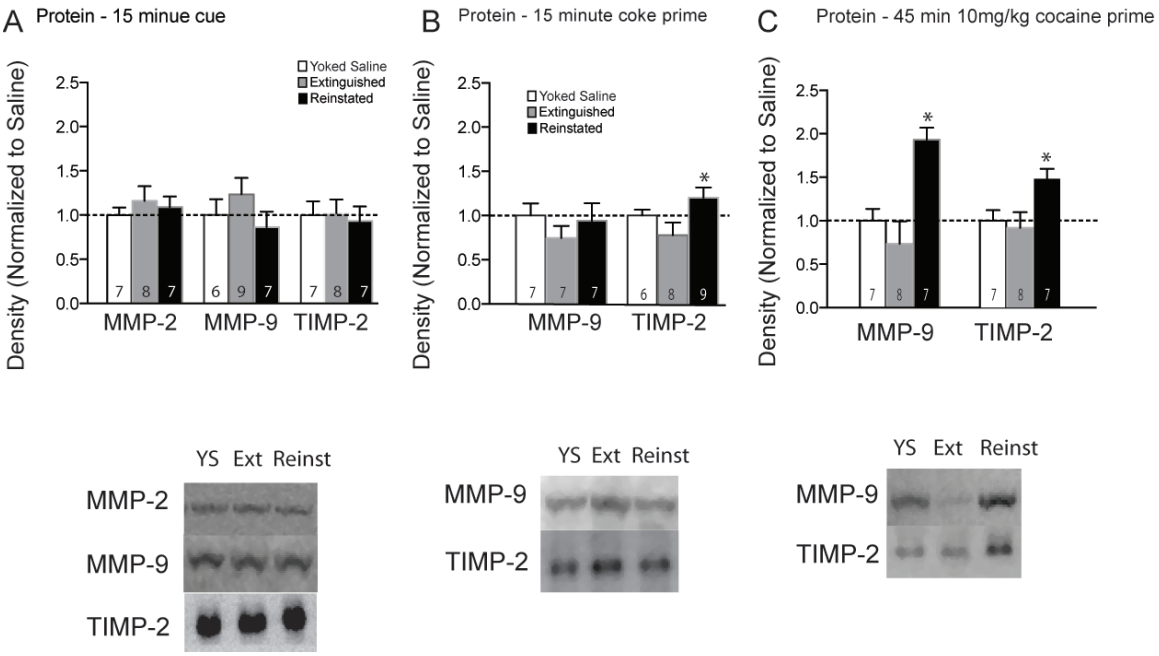


Figure 2-3: Cue-induced activation of MMP-2/9 does not involve changes in protein expression, but cocaine-induced activation does. *NAcore was harvested from yoked saline controls (YS), rats extinguished from cocaine (Ext), or rats reinstated for 15 min by conditioned cues or for 15 min and 45 min by a noncontingent cocaine injection (Rein; 10 mg/kg, ip).* ***a)*** *Cue-induced reinstatement did not alter NAcore protein levels of MMP-2, MMP-9 or TIMP-2.* ***b)*** *Fifteen min after cocaine-induced reinstatement the level of MMP-2 and MMP-9 were not altered, but the level of TIMP-2 was elevated in cocaine-reinstated animals compared to cocaine extinguished subjects; one-way ANOVA* $F_{(2,20)} = 3.756$, $p = 0.0412$. ***c)*** *After 45 min of cocaine-induced reinstatement, the levels of MMP-9 and TIMP-2 were elevated; MMP-9 one-way ANOVA* $F_{(2,19)} = 10.35$, $p = 0.0009$; *TIMP-2 one-way ANOVA* $F_{(2,19)} = 4.306$, $p = .0287$. * $p < 0.05$, *comparing yoked saline with reinstated protein levels using a Newman-Kuels post hoc.* + $p < 0.05$, *comparing extinguished with reinstated protein levels. All experiments were conducted myself.*

Although cue-induced increases in MMP activity were not associated with changes in protein expression, MMP-9 levels in NAcore were increased in parallel with elevated MMP-9 activity at 45 min after a cocaine priming injection (Figure 3c). While MMP-9 content was altered at 15 min after a cocaine injection, the level of TIMP-2 was increased compared with cocaine-extinguished rats at 15 min (Figure 3b), and elevated compared with both extinguished and yoked saline animals at 45 min after a cocaine priming injection (Figure 3c).

These protein data are consistent with the reduced MMP-2 activity found at 15 min following noncontingent cocaine priming injection in figure 1b resulting in part from elevated TIMP-2, and the increase in MMP-9 activity at 45 min after cocaine in figure 2c arising in part from increased MMP-9 expression. Moreover, the increase in MMP-9 protein at 45 min after a noncontingent cocaine injection is consistent with a previous study showing elevated MMP-9 in the prefrontal cortex one hour after reinstating conditioned place preference with an acute cocaine injection(Brown et al., 2008).

Gelatinase inhibition decreased reinstatement-associated transient synaptic potentiation in the NAcore. Withdrawal from cocaine self-administration is associated with long-term synaptic potentiation in NAcore excitatory synapses (Robinson and Kolb, 2004; Moussawi et al., 2009; Wolf and Ferrario, 2011), and after 15 min of cue-induced reinstatement, NAcore synapses undergo further, transient potentiation (Anderson et al., 2008; Gipson et al., 2013a). Studies examining the effect of cocaine use on synaptic plasticity have employed morphological measurements of spine density and head diameter (d_h), and electrophysiological measures of synaptic strength (assessed as the ratio of AMPA to NMDA currents; A/N). We used whole-cell patch clamp of medium spiny neurons (MSN) in the NAcore to determine if the enduring and reinstatement-induced increases in A/N depended on MMP-2 or MMP-9 activity (Figure 2-4a). Following vehicle microinjection into the NAcore, A/N in cocaine-extinguished animals was elevated compared to yoked-saline rats, and was further elevated 15 min after initiating cue-induced reinstatement (Figure 2-4b).

The enduring increase in A/N in cocaine-extinguished rats was restored to yoked-saline levels by the MMP-2, but not MMP-9 inhibitor. Either the MMP-2 or MMP-9 inhibitor reduced the elevated A/N initiated by 15 min of cue-induced reinstatement to levels equivalent to those measured after cocaine extinction. Neither inhibitor significantly affected A/N in yoked-saline subjects (Figure 2-11a). There was also no affect of the MMP inhibitors on spontaneous excitatory postsynaptic current (sEPSC) frequency or amplitude in any of the three treatment groups (Figure 2-11d,e). These data demonstrate that the enduring increase in A/N measured in cocaine extinguished animals required MMP-2 activity, while the transient elevation in A/N produced during cued reinstatement necessitated both MMP-2 and -9 activity.

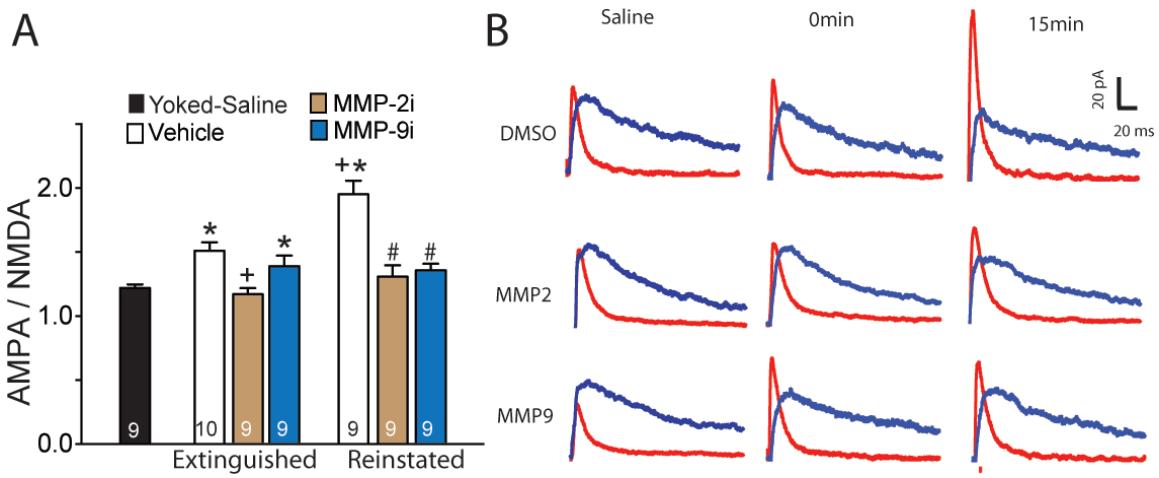


Figure 2-4. Gelatinase inhibition reduced the AMPA:NMDA ratio (A/N) following extinction from cocaine self-administration and reinstatement. a) *A/N that was elevated by cocaine extinction was reduced by MMP-2i, while the transient increase in A/N during reinstatement was reduced by either MMP-2i or MMP-9i, one-way ANOVA* $F_{(6,57)} = 13.08$, $p < 0.0001$. * $p < 0.05$, *compared to yoked-saline vehicle, using a post-hoc Newman-Keuls test.* + $p < 0.05$ *compared to extinguished vehicle.* # $p < 0.05$, *compared to reinstated vehicle. N inside bar is number of cells recorded.* ***b)*** *Representative EPSCs of patched medium spiny neurons (MSNs) in the NAcore show elevated AMPA currents following extinction and reinstatement, and normalization by MMP-2 and/or MMP-9 inhibition. Red line indicates AMPA current,*

blue line indicates NMDA current. Non-truncated NMDA currents are shown in figure 2-11. All electrophysiology was conducted by Yonatan Kupchik.

Next, we diolistically labeled MSNs with lipophilic DiI followed by 3-dimensional reconstruction of high-density confocal images to determine if the changes in spine morphology produced after extinction from cocaine self-administration or after cue-induced reinstatement depended on MMP-2 and MMP-9 activity (see Figure 2-12 for representative DiI filled neurons). Following vehicle injection into the NAcore, there was an increase in d_h after extinction from cocaine self-administration compared to yoked-saline, and a further increase 15 min after cue-induced reinstatement of cocaine seeking (Figure 2-5a). Akin to effects on elevated A/N in figure 2-4a, the long-lasting increase on d_h in extinguished animals depended on MMP-2 activity, and the increase produced by 15 minutes of cued reinstatement depended on both MMP-2 and MMP-9 activity (Figure 2-5a). However, in contrast with the measurement of A/N in extinguished subjects, the MMP-2 inhibitor reduced d_h below levels in yoked-saline animals (Figure 2-5a). The changes in mean d_h were also reflected in cumulative frequency plots where a leftward shift relative to yoked saline is produced by smaller d_h, and a shift to the right by larger d_h (Figure 2-5e,f). Thus, in extinguished rats the vehicle treated subjects frequency curve was shifted to the right relative to yoked saline. MMP-9 inhibition also shifted the d_h frequency curve to the right, while MMP-2 inhibition shifted the d_h to the left of yoked-saline animals (Figure 2-5e,f). Also consistent with the analysis of mean d_h in figure 5a, inhibiting either MMP-2 or MMP-9 in reinstated rats shifted the frequency plot to the left compared to the vehicle group and were equivalent to yoked-saline rats (Figure 2-5e,f).

Spine density did not fully parallel changes in d_h. Spine density was elevated in cocaine-extinguished compared to yoked-saline rats, but no further elevation was produced by cued reinstatement (Figure 2-5b). While MMP-2 inhibition reduced both the extinguished and reinstated increase in spine density to control levels, MMP-9 inhibition was without effect under either condition. The lack of effect by inhibiting MMP-9 and the reduced density after MMP-2 inhibition is also reflected in spine density cumulative frequency plots (Figure 2-12c). Combined with the measure of d_h, these data indicate that reinstatement is associated with transiently increasing the size (d_h) of existing spines, not creating new spines, and that MMP-2 activity supports the enduring increase in the number of spines in extinguished animals that are enlarged by MMP-9 activity during cued reinstatement. Neither MMP inhibitor affected d_h or spine density in yoked-saline animals (Figure 2-13b,c).

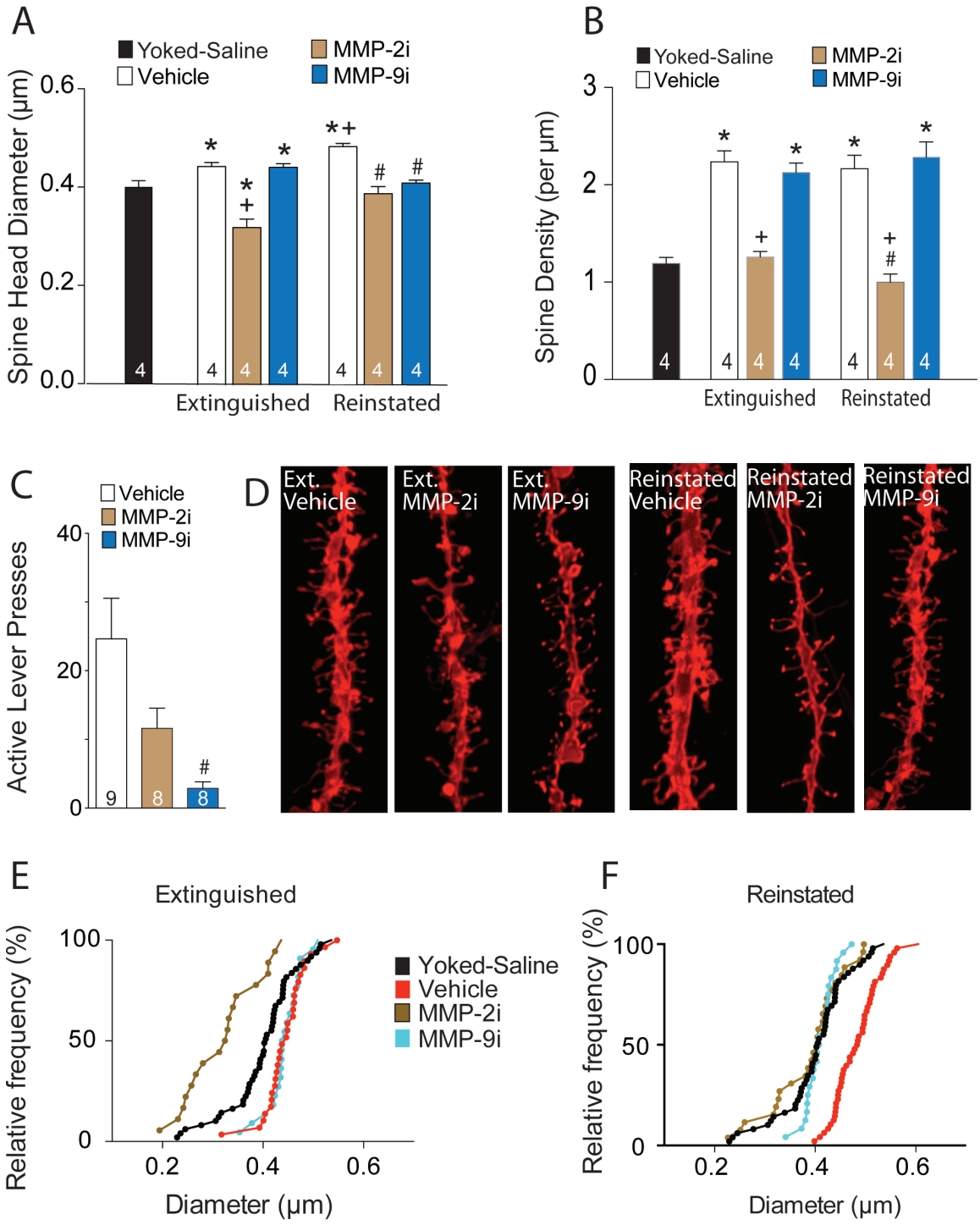


Figure 2-5. Gelatinase inhibition affects dendritic spine head diameter (d_h) similarly to A/N. a) *The enduring elevation of d_h by cocaine extinction was reduced by intra-NAcore microinjection of an MMP-2i, while the transient increase in d_h during reinstatement was reduced by microinjection of either an MMP-2i or -9i, one-way ANOVA $F_{(8, 27)} = 11.68$, $p < 0.0001$.* ***b)*** *The increase in spine density produced in*

extinguished animals was blocked by MMP-2i, not MMP-9i, one-way ANOVA $F_{(8, 27)} = 13.47$, $p < 0.0001$. ***c)*** *MMP-9i, but not MMP-2i blocked behavioral manifestation of reinstatement over 15 minutes* ***d)*** *Representative micrographs of quantified dendritic segments in each group.* ***e,f)*** *Frequency plots of dh revealed the same pattern of change by MMP-2 and MMP-9 inhibition as the mean analysis.* $p < 0.05$, compared to yoked saline vehicle, using Bonferonni planned comparison post-hoc test (panels A and B). + $p < 0.05$ compared to extinguished vehicle, # $p < 0.05$, compared to reinstated vehicle. N inside bar is number of animals. These experiments were performed in collaboration with Cassie Gipson and Michael Scofield. I personally ran all animals and performed the initial diolistic labeling. Drs Gipson and Scofield helped with the imaging and analysis.*

MMP-2 and -9 activity were both required for cue-induced reinstatement behavior. The data in figures 1 and 2 show that extinction from cocaine self-administration elevated MMP-2 activity, and that cue-induced reinstatement produced a further transient increase in MMP-9 activity. Figures 3 and 4 show that acute inhibition of MMP-2 reversed persistent synaptic potentiation produced by cocaine self-administration, while inhibition of either MMP-2 or MMP-9 blocked the transient potentiation initiated by cocaine-associated cues. Given the strong association previously reported between reinstated behavior and synaptic potentiation in accumbens MSNs (Gipson et al., 2013a), and the dependence of synaptic potentiation on MMP-2 and MMP-9 activity in figures 3 and 4, we hypothesized that inhibitors of MMP-2 and MMP-9 would attenuate cue-induced reinstatement. Indeed, for the A/N and spine morphology measurements in figures 3 and 4 where reinstatement was only quantified for 15 min before the animals were sacrificed, both MMP-2 and MMP-9 inhibitors partially or completely reduced reinstated cocaine seeking compared with vehicle mincroinjection into the NAcore. To fully evaluate the involvement of MMP-2 and MMP-9 in cue-induced reinstatement, we used a counterbalanced within-subjects

crossover design, and infused 2.5% DMSO vehicle, MMP-2 inhibitor, or MMP-9 inhibitor into the NAcore 15 minutes before initiating a 120 min reinstatement session. Rats microinjected with vehicle showed stable reinstated active lever presses over the three trials (Figure 2-16). Inhibition of either MMP-2 or MMP-9 caused a dose-dependent reduction in cue-induced reinstatement compared to vehicle control injections (Figure 6b,c). MMP-9 inhibition also dose-dependently reduced cocaine-primed reinstatement (Figure 6d). In contrast, neither drug significantly reduced the reinstatement of lever pressing for sucrose pellets (Figure 6f). A lack of effect by MMP inhibition on reinstated sucrose seeking is consistent with the lack of synaptic potentiation in the NAcore during cue-induced sucrose reinstatement (Gipson et al., 2013a), and supports the specificity of MMP inhibition for drug-associated synaptic adaptations and reinstated drug seeking.

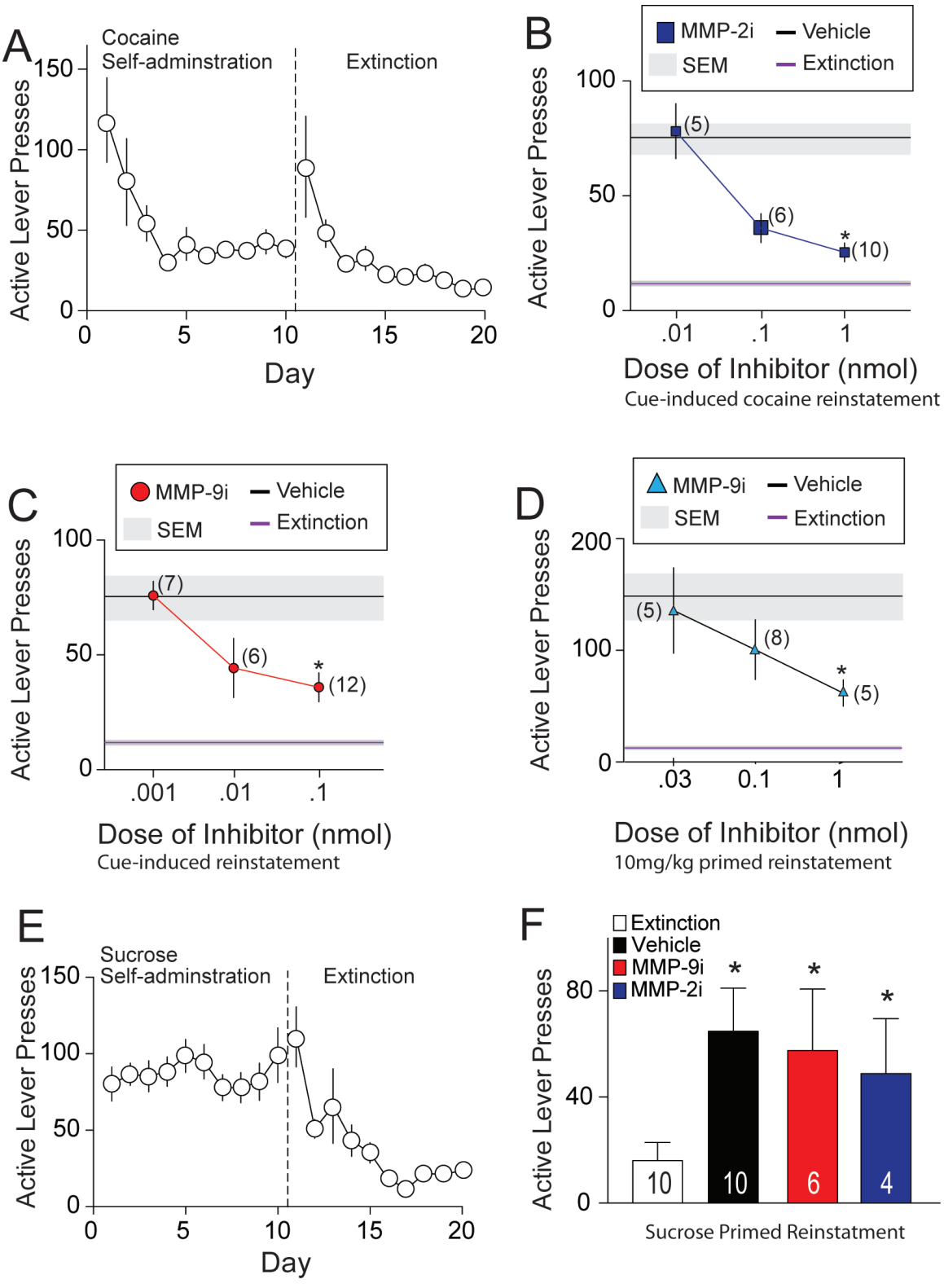


Figure 2-6. Administration of either MMP-2i or MMP-9i into the NAcore produced a dose-dependent decrease cue-induced reinstatement. ***a)*** *Active lever pressing during daily cocaine self-administration and extinction sessions.* ***b,c)***

Bilateral microinjection of MMP-2i or MMP-9i into the NAcore dose-dependently decreased active lever pressing in response to cocaine-conditioned cues over a two-hour reinstatement session. Two-Way ANOVA revealed a main effect for dose of inhibitor selectively on active lever pressing, treatment dose $F_{(7,172)} = 11.61$, $p < .0001$, active vs inactive lever $F_{(1, 172)} = 140.0$, $p < .0001$, interaction $F_{(7, 172)} = 8.023$, $p < .0001$. Bonferonni's post-hoc test revealed significant attenuation of active lever pressing following .1 nmol of MMP-9 inhibitor and following 1 nmol MMP-2 inhibitor. No dose of either inhibitor had any effect on active lever pressing. ***d)*** *Bilateral microinjection of MMP-9i dose-dependently decreased active lever pressing in response to a cocaine priming injection over a two-hour reinstatement session. Two-Way ANOVA dose $F_{(4, 97)} = 11.28$, $p < .0001$, lever $F_{(1, 97)} = 71.14$, $p < .0001$, interaction $F_{(4, 97)} = 11.28$, $p < .0001$.* ***e)*** *Daily active lever pressing during sucrose self-administration and extinction training.* ***f)*** *Intra-NAcore microinjection of either MMP-2i (1 nmol/side) or MMP-9i (0.1 nmol/side) failed to reduce cue-induced reinstatement of sucrose seeking. Kruskal-Wallis$_{(4,30)} = 10.61$, $p = 0.014$. * $p< 0.05$ compared to extinction, + $p < .05$ compared to vehicle, # $p < .05$ compared to paired inactive responding, using Bonferonni's post hoc test. N inside bar is number of animals. All experiments were conducted myself.*

2.4 DISCUSSION

Our data show that the rapid, transient induction of gelatinase activity in NAcore is a biomarker for cue-induced reinstatement that is shared between three distinct chemical classes of addictive drug, including cocaine, heroin, and nicotine. We also found that reinstatement induced by a noncontingent cocaine injection resulted in a similar, albeit delayed gelatinase activation. Furthermore, the increase in gelatinase activity by either cue or acute cocaine depended on activating MMP-9. In contrast with transiently increased gelatinase activity being a consistent biomarker for reinstated drug seeking, animals extinguished from cocaine and nicotine self-administration show increased gelatinase activity in NAcore, but there was no activation in heroin-extinguished subjects. This distinction between heroin, cocaine and nicotine is paralleled by other measures of synaptic plasticity at excitatory synapses in NAcore where cocaine and

nicotine extinguished rats show increases in both AMPA/NMDA and d_h or spine density (Gipson et al., 2013a; Gipson et al., 2013b), and heroin extinguished rats show either no change or reductions (Shen et al., 2011). When a neurobiological trait is shared between different classes of addictive drug it would seem to increase the likelihood that this trait contributes to one or more of the shared behaviors used to identify addiction, such as the vulnerability to relapse. Thus, the fact that the transient synaptic potentiation and gelatinase activation produced during a reinstated behavior that is designed to model relapse was shared between cocaine, heroin and nicotine supports a role for this type of transient synaptic plasticity in relapse. Conversely, the lack of consistency between addictive drugs at inducing enduring synaptic potentiation and gelatinase activity argues that these adaptations are drug specific and therefore less likely to contribute to the behaviors characterizing addiction that are shared between addictive drugs.

While the transient increase in MMP activity induced by cocaine associated cues was prevented by microinjecting a MMP-9 inhibitor into the NAcore, the enduring synaptic potentiation in NAcore following extinction from cocaine self-administration was reversed by intra-NAcore microinjection of an MMP-2 inhibitor. A necessary role for MMP-9 activity in synaptic plasticity is consistent with studies showing that both electrically and chemically induced synaptic potentiation in the hippocampus requires MMP-9 activity (Wang et al., 2008b; Szepesi et al., 2013). In contrast, to our knowledge this is the first report of

MMP-2 involvement in synaptic potentiation. Although the transient, reinstatement-associated increase in MMP activity was MMP-9 dependent, we found the enduring rise in MMP-2 activity was also necessary for cue-induced behavioral reinstatement and the associated increases in A/N and d_h. Interestingly, while transient MMP-9 dependent synaptic potentiation involved increases in A/N and d_h, there was not a parallel increase in spine density. However, the enduring increase in spine density in extinguished rats was MMP-2 dependent. One interpretation of this dissociation between d_h and spine density is that the enduring increased density of spines can be readily and transiently potentiated during reinstatement by increased MMP-9 activity. Thus, changes in spine density reflect a more permanent alteration in steady state synaptic connectivity.

Consistent with our data showing involvement of MMPs in addiction, clinical studies reveal that MMP-9 gene expression is altered in the brain of cocaine addicts (Mash et al., 2007), the serum of heroin addicts (Kovatsi et al., 2013), and that an MMP-9 gene polymorphism is associated with alcohol dependence (Samochowiec et al., 2010). In animal models, intra-ventricular injection of a nonselective MMP inhibitor reduces reinstated cocaine conditioned place preference, heroin seeking, and ethanol consumption by dependent rats (Brown et al., 2007; Van Den Oever, 2010; Smith et al., 2011). Supporting a general involvement of MMPs in addiction pathology, we showed that nicotine and heroin reinstatement was also associated with increased MMP activity in NAcore.

We did not investigate the specific signaling mechanism(s) whereby constitutively increasing MMP-2, or the transient cue-induced elevation of MMP-9 mediates either synaptic potentiation or reinstated cocaine seeking. There exist a number of known MMP-2 and MMP-9 protein substrates in the ECM that have been shown to play a role in animal models of addiction, and are possible candidate MMP signaling substrates. For example, MMP-2 and/or MMP-9 catabolically activate secreted brain-derived growth hormone (BDNF)(Mizoguchi et al., 2011b), and BDNF in the nucleus accumbens is both increased following cocaine self-administration and promotes reinstated cocaine seeking (Graham et al., 2007). Moreover BDNF is well characterized to induce synaptic potentiation in many brain regions (Bramham, 2008). Another example is the activation of thrombospondin by MMP-2 and -9. The thrombospondin receptor, the $\alpha 2\delta 1$ auxiliary subunit of voltage-gated calcium channels (Bauer et al., 2010), is upregulated in the nucleus accumbens following withdrawal from self-administered cocaine (Reissner et al., 2011), and thrombospondin binding to $\alpha 2\delta 1$ induces synaptogenesis (Eroglu et al., 2009). Finally, MMP-2 and -9 processing of ECM proteins such as laminin, fibronectin or thrombospondin reveals an RGD motif that binds to integrins (Ethell and Ethell, 2007). Binding of integrins promotes the activity of NMDA receptors, insertion of AMPA receptors into the synapse, and actin elongation, a necessary step in spine extrusion and expansion (Cingolani et al., 2008; Michaluk, 2009; Chen et al., 2010). Moreover, the beta-3 subunit of integrin is up-regulated in the accumbens following extinction from cocaine self-administration, and providing exogenous RGD to

prevent ECM proteins from binding integrins inhibits cocaine-induced reinstatement (Wiggins et al., 2011a). Clearly, there are a number of viable avenues to explore in future studies to understand how the constitutive and cue-induced increases in MMP activity mediate enduring and transient LTP in MSNs that underlies reinstated cocaine seeking.

While our data strongly implicate MMP-2, and especially MMP-9 in both behavioral reinstatement and reinstatement-associated synaptic plasticity in the NAcore, there were interesting deviations from literature-based expectations. For example, TIMP-2 was increased at both 15 and 45 min noncontigent injection of cocaine (Smith et al., 2014b). The elevation of TIMP-2 at 15 min is consistent with the reduction in MMP activity we observed after 15 min of cocaine-induced reinstatement and the established role of TIMP-2 to inhibit MMP-2 activity(Brew et al., 2000). However, the increase at 45 min of cocaine-induced reinstatement was not expected since MMP-9 activity was increased and TIMP-2 also inhibits MMP-9 in some studies; although it forms a preferential complex with MMP-2 (Lambert et al., 2004). Also, we did not identify the mechanism by which MMP-2 and MMP-9 are constitutively and transiently elevated, respectively. A number of possibilities can be pursued in future studies. For example, nitrosylation of either protein is known to promote the active over inactive conformation of the protein(Manabe et al., 2005b), and nitric oxide is elevated after withdrawal from chronic cocaine and methamphetamine administration (Lee et al., 2010). Also, previous studies have strongly implicated spillover of synaptic glutamate in the NAcore as a necessary mediator of reinstated cocaine seeking(Kalivas, 2009),

and increasing intracellular calcium levels by stimulating either NMDA receptors or mGluR5 would be expected to promote MMP-2/9 (Lee et al., 2011).

In summary, we have identified a specific and necessary role for MMP-2 and MMP-9 activity in the enduring vulnerability to relapse that depends on MMP induction of both constitutive and transient excitatory synaptic potentiation in the NAcore. Moreover, the transient increase in MMP-9 was observed across multiple classes of addictive drug, including cocaine, heroin and nicotine. Taken together, these data open study of the extracellular matrix signaling domain as a potential research theme for understanding and treating substance abuse disorders.

Supplemental figures for Chapter 2: Synaptic plasticity mediating cocaine relapse requires matrix metalloproteinases:

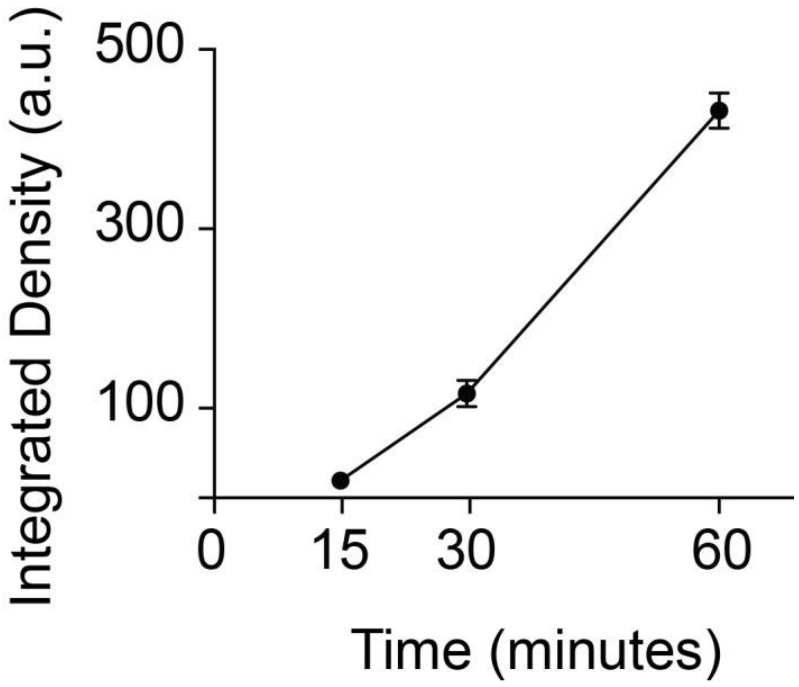


Figure 2-7. Control FITC-gelatin substrate experiments show linearity of fluorescence over time after injection. The catabolism of the FITC-gelatin substrate shows linear increases in gelatinolytic fluorescence over 15 - 60 minutes incubation *in vivo*. Injections were made into the dorsal hippocampus due to relatively higher constitutive MMP activity compared with the NAcore or striatum. N= 3 at each time point. I conducted all aspects of this experiment.

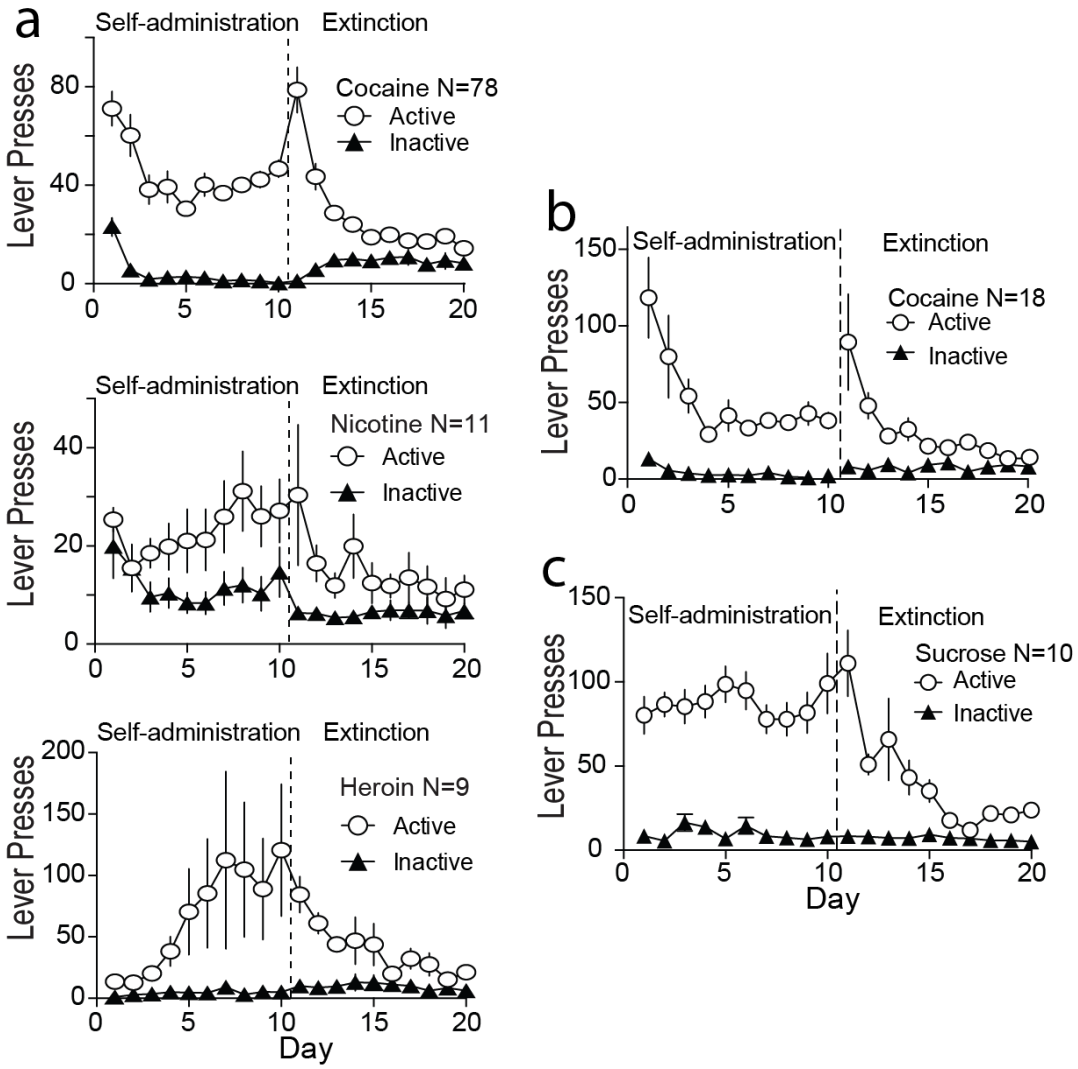


Figure 2-8. Lever pressing during self-administration and extinction of cocaine, nicotine and heroin. a) Active and inactive lever pressing data for animals that were used for in vitro measurements of zymography, Western blotting, electrophysiology or dendrite morphology in figures 1 and 2. b) Training data for rats used in cocaine studies in figure 2e,f. c) Training data for rats used in sucrose studies in figure 2g. I conducted all aspects of this experiment, except for nicotine self-administration and extinction, which were performed by Dr. Cassandra Gipson.

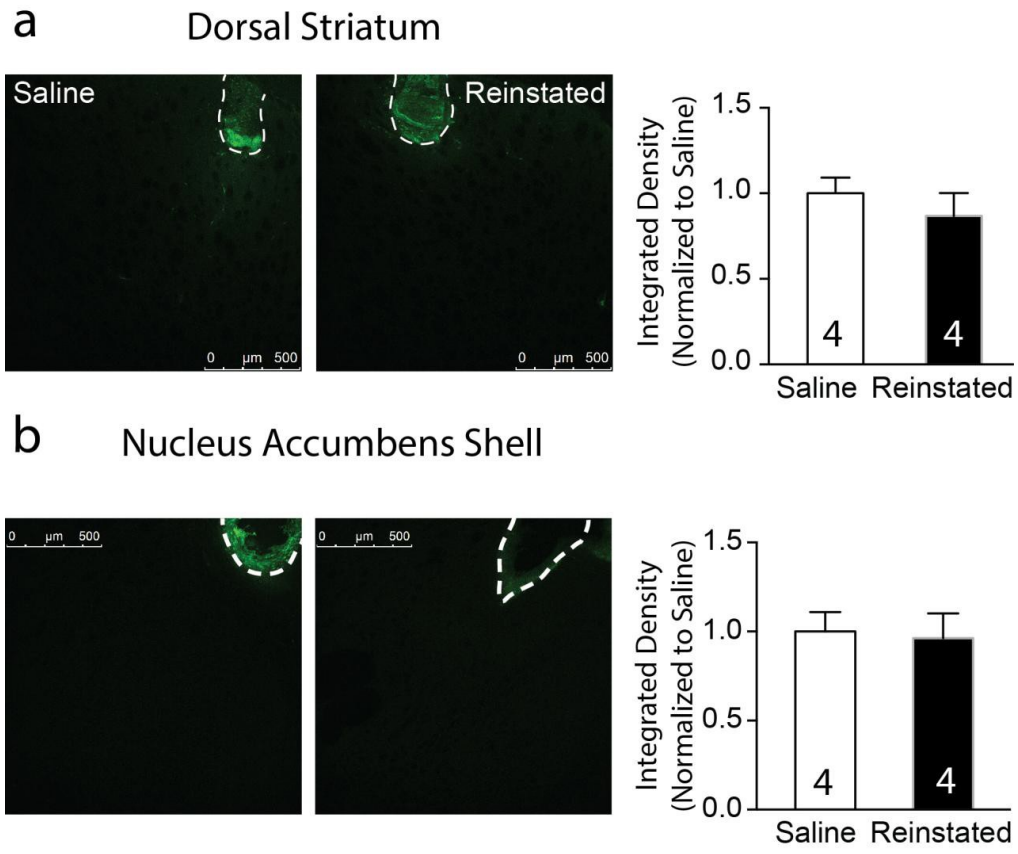


Figure 2-9. Lack of increased MMP-2 and MMP-9 activity in the dorsal striatum or accumbens shell following cue-induced reinstatement of cocaine seeking. There was no change in gelatinolytic fluorescence in either the dorsal striatum or the nucleus accumbens shell between yoked-saline controls and animals that underwent 15 minutes of cue-induced reinstatement. This indicates anatomical specificity for MMP-dependent plasticity within within the striatum is largely confined to the NAcore. Dashed lines on the micrographs encompass the injection site that was masked-out for quantification. Unpaired Student's t-test revealed no significant effect of reinstatement on fluorescence, dorsal striatum $t(4) = 1.411$, $p > 0.05$, NAshell $t(4) = 0.2112$, $p > 0.05$. I conducted all aspects of this experiment.

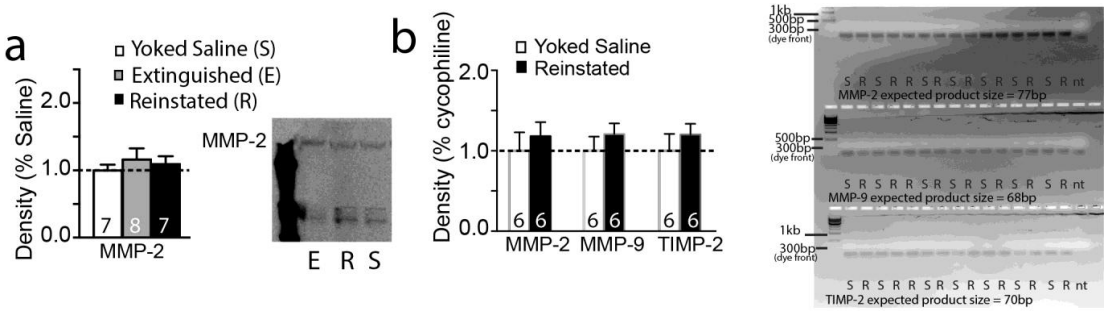


Figure 2-10. There were no changes in protein concentration of MMP-2 or mRNA of each MMP-2, MMP-9, or TIMP-2 a) MMP-2 protein was quantified in NAcore tissue obtained from yoked saline, cocaine extinguished and after 15 min of cued reinstatement in cocaine-trained rats. There was no difference between treatment groups using a one-way ANOVA. b) mRNA content quantified by PCR. Paired Student's t-tests did not reveal any significant differences between groups. MMP-2 $t_{(10)} = 0.6321$, $p > .05$, MMP-9 $t_{(10)} = 0.934$, $p > .05$, TIMP-2 $t_{(10)} = 0.814$m $p > .05$. S = Yoked Saline, R = Reinstated. I conducted all aspects of this experiment.

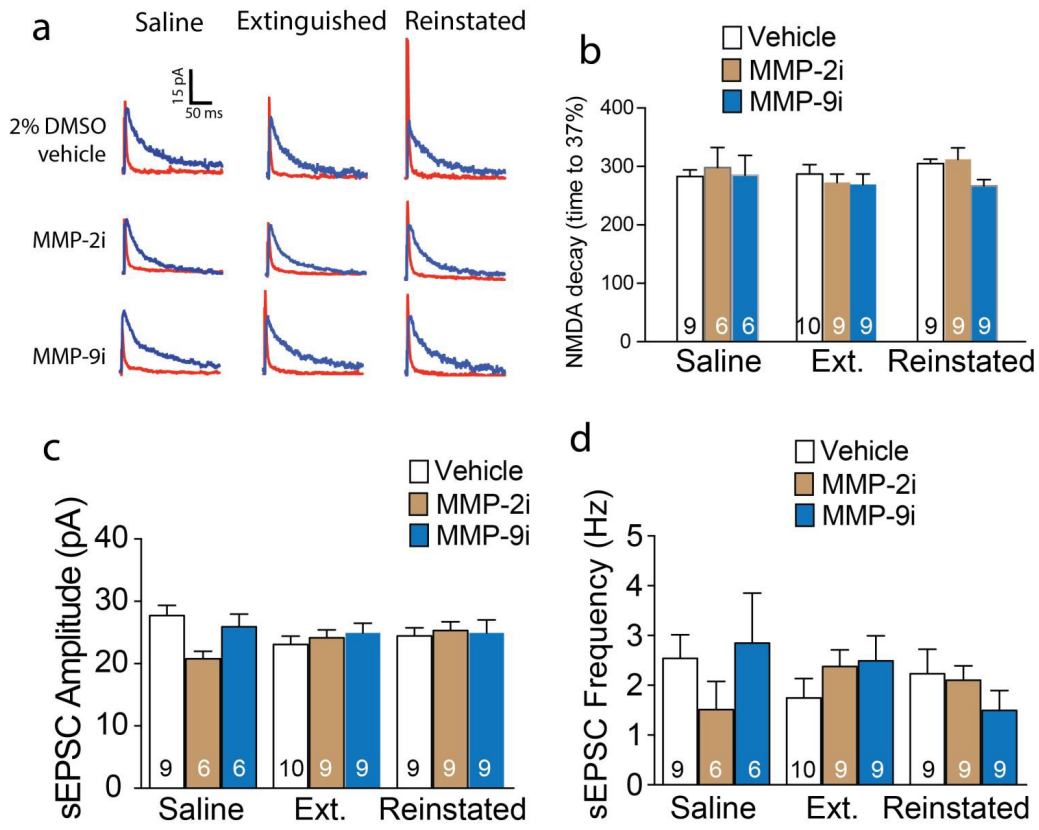


Figure 2-11. MMP inhibition did not have an effect on NMDA decay time or sEPSCs. a) Representative traces showing full length AMPA and NMDA current recordings. b) No effect of MMP inhibition on NMDA decay time. One-way ANOVA $F_{(8,67)} = 0.83$, $p > .05$. c) MMP inhibition did not alter sEPSCs amplitude in any condition. One-way ANOVA $F_{(8,67)} = 1.34$, $p > .05$. d) MMP inhibition did not alter sEPSCs frequency in any condition. One-way ANOVA $F_{(8,67)} = 0.88$, $p > .05$. N is shown in bars as number of neurons recorded (panels a, d, e) or animals quantified (panels b, c) with each animal being the average of 6-12 neurons. I trained the animals on self-administration, extinction, and reinstatement, and Dr. Yonatan Kupchik performed the electrophysiological recordings.

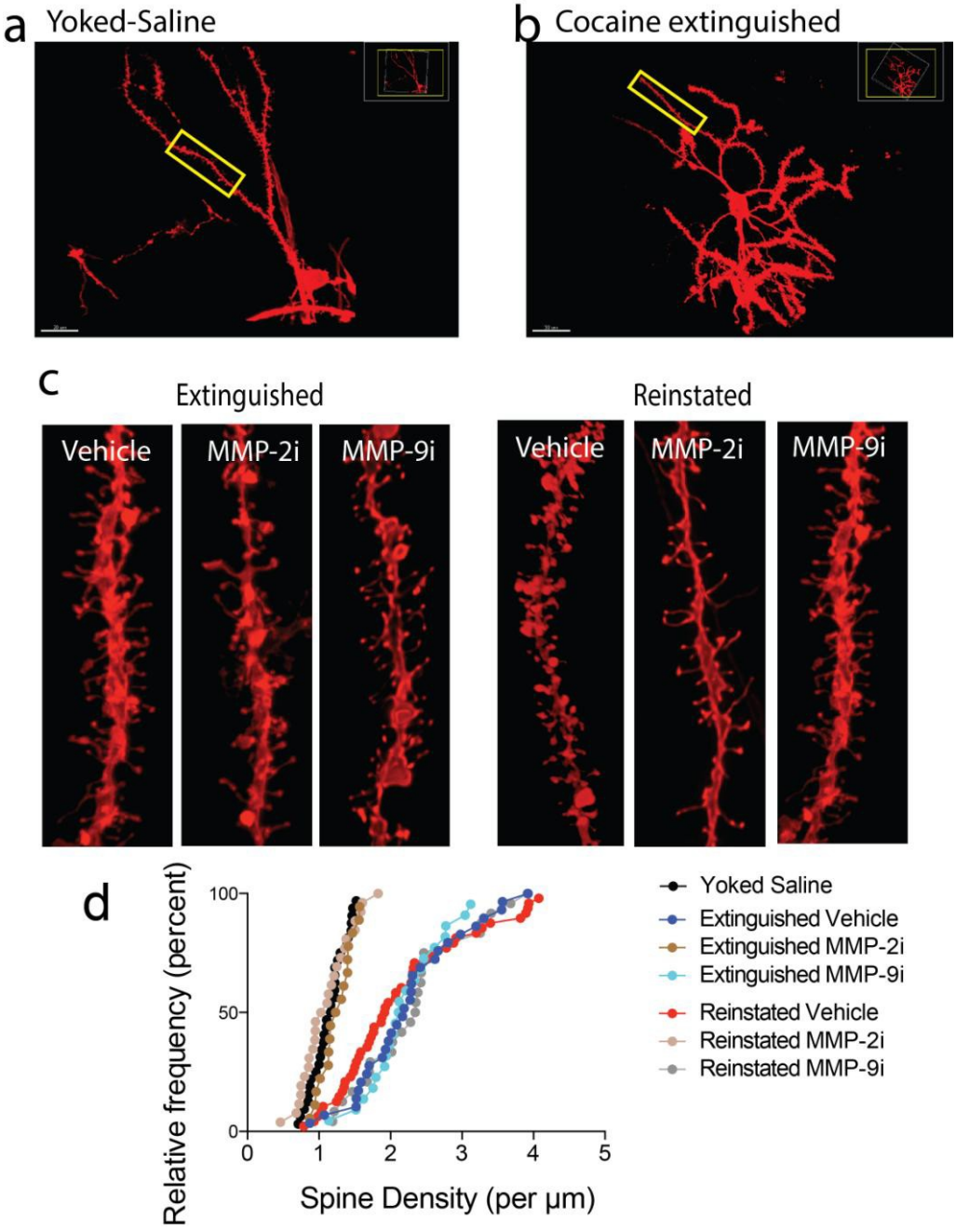


Figure 2-12 Representative images and dendritic spine density frequency plot a,b) For dendritic spine analysis, images of entire neurons were taken at 1 µm resolution, and then 45-55 µm segments located between 75-200 µm from the soma and after the first branch point were imaged at 0.1 µm resolution for 3-dimensional reconstruction and morphological analysis. The yellow rectangle indicates the location of the dendritic segment that was imaged from these neurons. c) Representative micrographs of quantified dendritic segments from each group. d) Shows cumulative frequency distribution for dendritic spine density. There was a noticeable shift to the right in extinguished cocaine treated

animals relative to yoked saline indicating greater spine density in cocaine-extinguished rats (see statistical analysis of mean values in figure 5). While MMP-2 inhibition reversed this effect in cocaine-extinguished subjects, MMP-9 inhibition was ineffective. Reinstatement did not alter the distribution relative to extinguished vehicle treatment, and MMP-2, but not MMP-9 inhibition returned the reinstated cocaine distribution to yoked saline distribution. Drs. Cassandra Gipson and Michael Scofield assisted in the imaging and analysis of these data.

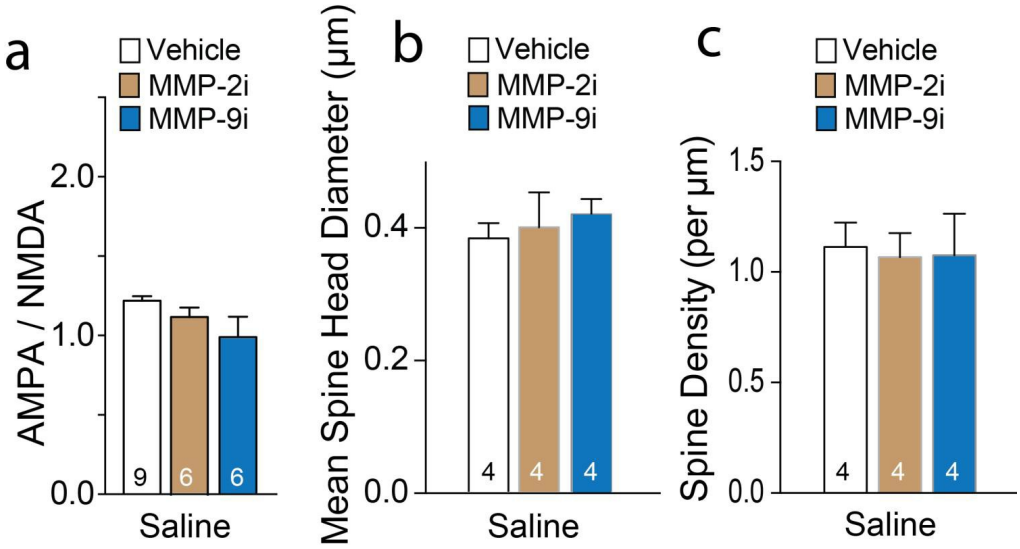
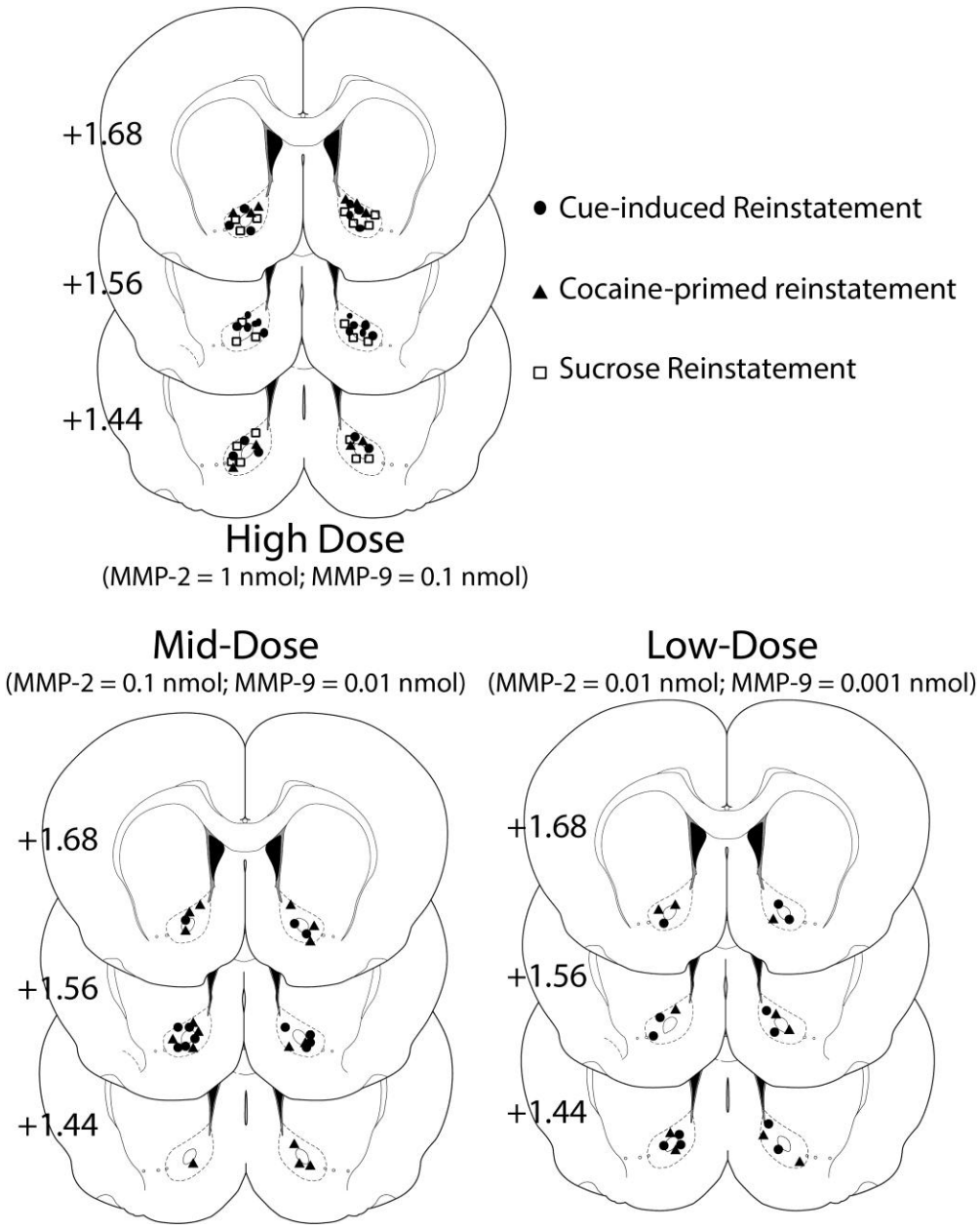


Figure 2-13. MMP inhibition did not affect synaptic strength in yoked saline animals. a) Shows A/N following vehicle, MMP-2 or MMP-9 inhibition in yoked-saline controls. One-way ANOVA revealed $F(2,19)= 3.41$, $p > .05$. b) Neither MMP-2 nor MMP-9 inhibition affected spine head diameter in yoked-saline controls. One-way ANOVA revealed $F(2,9) = 1.01$, $p > .05$. c) Neither MMP-2 nor MMP-9 inhibition affected spine density in yoked-saline controls. One-way ANOVA $F(2,9) = 0.12$, $p < .05$. Dr. Yonatan kupchik assisted with electrophysiological recordings, and Drs. Cassandra Gipson and Michael Scofield assisted with spine analysis.



Appendix 2-14. Histological verification of microinjection sites for animals microinjected with vehicle or MMP inhibitors prior to reinstatement. Rats with injection cannula outside of the NAcore were excluded from behavioral analysis. I performed all aspects of this experiment.

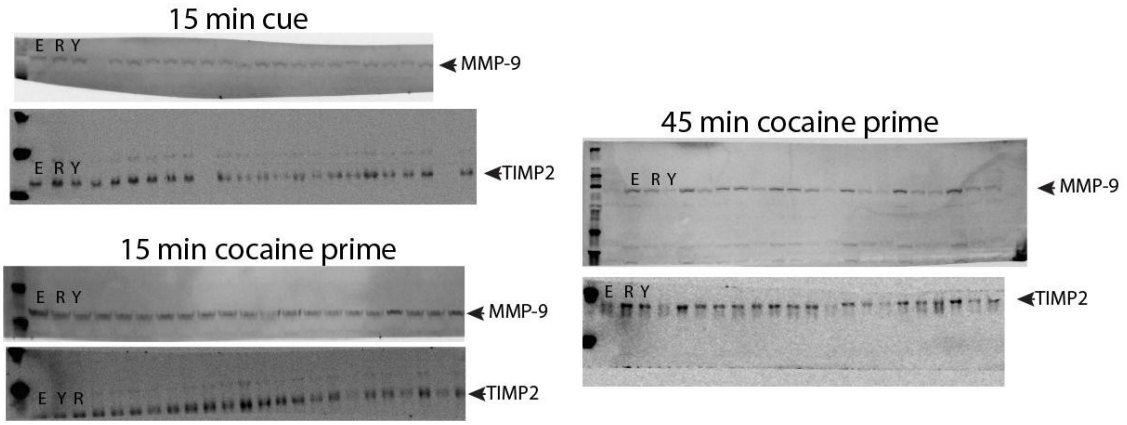
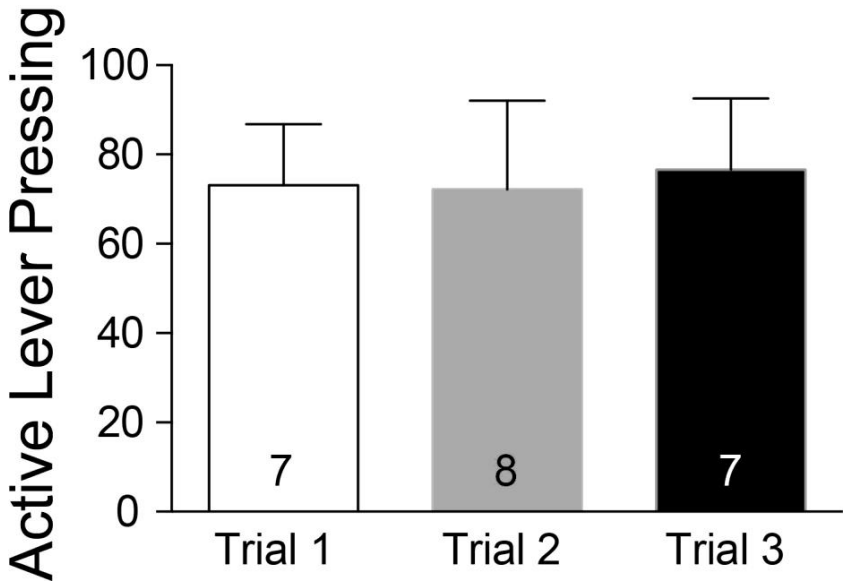


Figure 2-15. Full-length western blots corresponding to truncated blots shown in Figure 1h. ERY pattern repeated across the gel.
E- extinguished, R- reinstated, Y- yoked saline. I performed all aspects of this experiment.



Appendix 2-16. Lever pressing following vehicle microinjection in dose-response analysis in Figure 2 was stable across three reinstatement trials. The experiments in figures 3-2e-f were conducted as a within-subject crossover design consisting of 3 reinstatement trials per animal (unless a microinjection cannula became clogged, in which case only 2 trials were conducted. These data show the response to vehicle when it was randomly given in the first, second or third trial, and that there is no difference in vehicle reinstatement across 3 trials. The data argue against the possibility that the data if figure 6 were influenced by the order of drug injection across trials. One- Way ANOVA revealed $F(2, 22) = 0.02$, $p > .05$. I performed all aspects of this experiment.

Chapter 3: nNOS-expressing interneurons in the nucleus accumbens core drive reinstatement of cocaine seeking

Abstract

Chronic cocaine use results in a synaptic potentiation of glutamatergic synapses in the nucleus accumbens core (NAcore) that underlies vulnerability to relapse following extinction. Matrix metalloproteinases (MMPs) are extracellular matrix remodeling enzymes that promote synaptic plasticity, and inhibition of MMPs results in reversal of cocaine-induced potentiation of synapses in the NAcore. One mechanism by which MMPs are activated is through S-Nitrosylation via nitric oxide (NO), which is produced by a small population of GABAergic interneurons in the NAcore. We utilized a cocaine self-administration reinstatement paradigm, and evaluated the role of nitrergic signaling in relapse-associated MMP activity, synaptic potentiation, and behavior. Inhibition of neuronal nitric oxide synthase (nNOS) blocked reinstatement behavior and the associated increase in MMP activity. Finally, NOS1-Cre transgenic mice were used to chemogenetically target a Gq-DREADD to nNOS-expressing interneurons, and we show that selectively activating these receptors is able to drive both MMP activity an reinstatement behavior. This indicates that this small (~1%) population of interneurons within the NAcore may be a master switch by which synaptic potentiation and relapse behavior are initiated.

3.1 Introduction

Drug addiction is a chronic relapsing disorder characterized by a loss of control over intake, even after protracted abstinence (Kalivas, 2005). The nucleus accumbens core (NAcore) serves as a gateway between limbic and motor information, and its afferents from the prefrontal cortex are involved in initiating goal directed behaviors (Kalivas, 2009). Glutamatergic plasticity within the corticostriatal projection originating in the prelimbic cortex and terminating in the NAcore underlies the vulnerability to reinstatement. Decreased basal extrasynaptic glutamate provides less tone on mGluR2/3 receptors on presynaptic terminals, and primes this system for an activity-dependent spillover of synaptic glutamate in response to cocaine-conditioned cues (Kalivas 2009). This large presynaptic release of glutamate is accompanied by decreased function of glial glutamate reuptake through GLT-1, and leads to overflow of glutamate outside of the synapse (Reissner et al., 2015). There are numerous potential extrasynaptic targets for glutamate, and of these both mGluR5 and GluN2B receptors are particularly attractive candidates in controlling vulnerability to relapse (Bespalov et al., 2005; Mitrano et al., 2008; Shen et al., 2011; Wang et al., 2013) Postsynaptic neurons in the NAcore exist in a persistently potentiated state, and undergo a further rapid, transient synaptic potentiation following cue-induced reinstatement (Gipson et al., 2013; Smith et al., 2014).

Recent findings from our lab have established a role for matrix metalloproteinases (MMPs)-2 and -9 in mediating both these persistent and transient synaptic potentiations (Figures 2-4, 2-5; Smith et al., 2014). Specifically,

constitutively upregulated MMP-2 activity is responsible for driving persistent synaptic potentiation following extinction, and cue-induced reinstatement produces a transient induction of MMP-9 activity that drives transient synaptic potentiation (Figures 2-1, 2-2). MMPs-2 and -9 can each be activated through S-nitrosylation of a cysteine residue within their pro-domain via nitric oxide. Furthermore, we have recently shown that the selective mGluR5 agonist (RS)-2-Chloro-5-hydroxyphenylglycine (CHPG) is capable of robustly initiating reinstatement of cocaine seeking and transient synaptic potentiation even in the absence of cues (Cassandra Gipson & Peter Kalivas, April 15, 2015). CHPG stimulates mGluR5 receptors that in turn cause the release of Ca^{2+} from internal stores, however, the mechanism by which CHPG elicits reinstatement or affects cells has not been studied.

The primary neural components of the NAcore are medium spiny neurons (MSNs), which are projection neurons that make up approximately 95% of the neurons in the NAcore (Tepper et al., 2010). The remaining 5% of neurons are interneurons, of which there are 3 main types: 1) cholinergic interneurons that are also called tonically active interneurons or giant interneurons, 2) fast-spiking GABAergic low-threshold spike interneurons that are also characterized by their high expression of Parvalbumin, and 3) GABAergic interneurons that co-express neuronal nitric oxide synthase (nNOS), somatostatin, and neuropeptide Y (NPY) (Tepper et al., 2010). nNOS is an enzyme that synthesized nitric oxide (NO) from the substrate L-arginine, and is physically coupled to NMDA receptors via a PDZ interaction with PSD95 (Cui et al., 2007). nNOS is Ca^{2+}-sensitive, due to its

dependence on Calmodulin (CaM) binding for activity to occur (Rameau et al., 2003a). NO is a freely diffusible gaseous neurotransmitter, that canonically signals through the sGC/cGMP/PKG pathway (Haley et al., 1992; Gabach et al., 2013), but can also react with thiol groups of cysteine or methionine residues to S-Nitrosylate a large number of proteins, such as MMPs. Very little is known about the effects of cocaine on NO signaling. In the dorsal striatum, NO efflux is increased following 7 days of experimenter-administered cocaine and 7 days of withdrawal (Lee et al., 2010). In the nucleus accumbens shell, cocaine sensitization requires the S-nitrosylation of Stargazin, and AMPA trafficking protein that regulates surface expression of GluA1 AMPA subunits (Selvakumar et al., 2014). In the current experiments, we test the hypothesis that Ca^{2+} signaling within nNOS-expressing interneurons induces NO efflux, S-nitrosylation of MMPs, synaptic potentiation, and reinstatement of cocaine seeking. Our results show that inhibition of nNOS is capable of attenuating both cue- and CHPG-induced reinstatement in rats. By utilizing NOS1-Cre transgenic mice, we selectively chemogenetically target nNOS-expressing interneurons, and show that stimulation of this cell population is able to drive not only MMP activity and transient synaptic potentiation, but can cause reinstatement of cocaine seeking even in the absence of cocaine-conditioned cues.

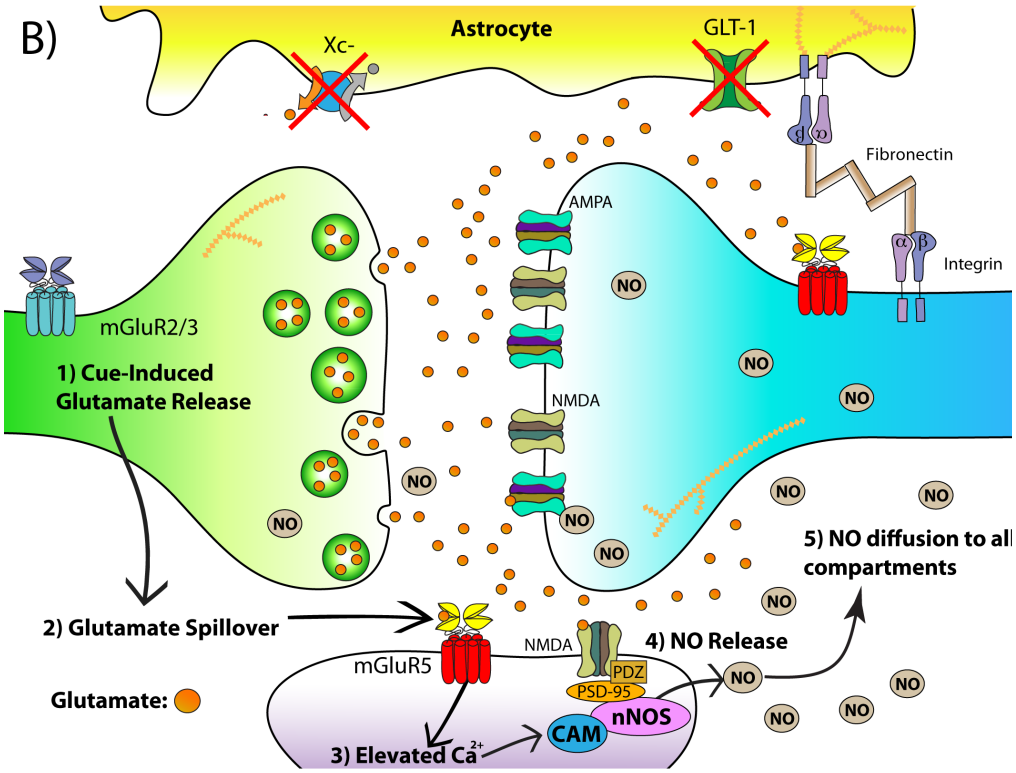


Figure 3-1. Proposed model of glutamatergic spillover leading to nitric oxide production. Cocaine conditioned cues stimulate a large glutamate from cortical afferents in the NAcore. While this glutamate is targeted for a dendritic spine on a medium spiny neuron, dysregulation of glutamate homeostasis allows glutamate to spill out of the synapse and affect nearby nNOS-expressing interneurons. Once nNOS is created, it is able to freely diffuse and affect physiology in each the presynaptic terminal, the medium spiny neuron, and in the extracellular space.

3.2 Methods

Animal Housing and Surgery. Both rats and mice were used. Male Sprague-Dawley Rats (250g; Charles River) and NOS1-Cre transgenic mice (Jackson Labs #017526 B6.129-$^{Nos1tm1(cre\_Mgmj}$/J) were individually housed with a 12:12 hour dark/light cycle. All experiments occurred during the dark phase, and animals were allowed to acclimate to the vivarium environment for 4 days prior to surgery. Rats were anesthetized with a combination of ketamine HCl and xylazine, and received ketorolac for analgesia. All rats received intrajugular

catheters, and rats for microinjection experiments received intracranial cannula targeted 2 mm above the NAcore for rats, 1 mm above the NAcore for mice (rats +1.8 A/P, +2.1 M/L, -5.5 D/V; mice +1.5 A/P, +1.3 M/L, -3.6 D/V) (Paxinos and Watson, 2007). Animals were food restricted to 25g of rat chow per day. All methods used comply with the NIH Guide for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

Drugs Used. Drugs used include N-Propyl L-Arginine hydrochloride (NPLA; Tocris; nNOS K_i = 57 nM. 3158-fold selectivity over iNOS, 149-fold specificity over eNOS). (*RS*)-2-Chloro-5-hydroxyphenylglycine (CHPG; Tocris). Clozapine N-Oxide (CNO), the synthetic ligand for DREADD receptors.

Drug Self-Administration and Reinstatement. After 5 days of recovery from surgery, rats underwent one overnight (12 hour) food training session. The next day, animals began daily 2-hour self-administration (SA) sessions. During SA, cocaine was delivered using an FR1 schedule with a 20 s timeout following each infusion. Active lever presses that resulted in cocaine infusion simultaneously resulted in presentation of a compound light (above the active lever) and tone (2900 Hz) conditioning stimulus. An inactive lever was also provided to control for non-motivated responding. Following 10 SA sessions at ≥10 infusions/day, rats began extinction training, during which all programmed consequences were removed from lever pressing. Extinction training lasted at least 10 days, or until

two consecutive days ≤ 25 active lever presses. Reinstatement was induced by presentation of light/tone cues following an active lever press. Microinjections of NPLA, CHPG (10μg), CNO (1μg), or vehicle, or systemic injections of CNO (3mg/kg) or vehicle were given 15 minutes prior to beginning reinstatement in most cases, or 15 minutes prior to gel infusion for zymography experiments. For behavioral experiments, a within-subjects crossover design was used. In this paradigm, each rat received each condition according to a Latin square design. Rats were required to meet extinction criteria prior to each reinstatement test. Reinstatement sessions lasted 120 minutes in the behavioral experiment, and for zymography experiments reinstatement sessions were 15 minutes long, at which point rats were anesthetized with 100mg pentobarbital, then transcardially perfused with 4% paraformaldehyde for further measurements. For western blotting experiments, rats underwent 15 minute reinstatement sessions and then were sacrificed via rapid decapitation.

In Vivo Zymography. Because MMPs are secreted in inactive pro-forms and catalytically activated within the ECM, activity assays are preferable to immunoblotting for protein content for assessing changes in MMP function (Kupai et al., 2010). We used an *in vivo* zymography assay to directly measure MMP activity. Dye-quenched gelatin is an MMP-2/9 substrate containing intra-molecularly quenched FITC fluorophores that cannot fluoresce until proteolytically processed by MMP-2 or MMP-9 (Bozdagi et al., 2007). The amount of fluorescence produced forms a linear relationship with incubation time and MMP activity Figure 2-7). Dye-quenched FITC-Gelatin (Molecular Probes,

Eugene, OR) was reconstituted in PBS at 1 mg/ml pH 7.2-7.4. 3.0µl of gel (1.5µl/side) was microinjected 15 minutes prior administering an overdose of pentobarbital (100 mg/kg, ip) and beginning transcardial perfusion of 4% paraformaldehyde (PFA). Brains were removed, placed in 4% PFA for 90 minutes for additional fixation, a vibratome was used to obtain 50 µm sections through the NAc. Sections were mounted and coverslipped. Fluorescence was excited with a 488nm Argon laser, emissions filtered to 515-535nm, and images obtained through a 10x objective with a 0.3 numerical aperture (Leica confocal microscope). Only slices in which the injection site and anterior commissure could be visualized in the same frame were imaged. ImageJ (NIH) was used to quantify images. All quantified images contained the anterior commissure, which was masked to prevent being quantified, but provided a landmark for the NAcore. MMP activity is induced as part of the acute inflammatory response to tissue damage from the microinjector, and thus the microinjector tract was readily visible in all quantified sections due to equivalent high fluorescence in all treatment groups (Figure 1). This tract was also masked to eliminate quantifying any MMP activity caused by microinjection-induced acute damage. Fluorescence was quantified bilaterally as integrated density from four sections per rat, and the integrated densities were averaged within each rat and normalized to yoked-saline control values.

Western Blotting. Rats were rapidly decapitated after extinction of cocaine self-administration or yoked-saline, or following 15 or 45 minutes following cued or

cocaine-primed reinstatement. The NAcore was dissected and homogenized in RIPA lysis buffer containing 1.0% SDS and protease/phosphatase inhibitors. Homogenate was centrifuged at 4°C for 5 minutes at 10,000 x g. Supernatant was collected and protein concentration was determined via a biconchinic acid assay (Thermo Scientific). 30µg protein was added to each lane of 10% Bis-Tris gels (Bio-Rad), and transferred to nitrocellulose membranes via the Invitrogen iBlot transfer system. Primary antibodies were used for nNOS (1:1000, Millipore #AB5380), phospho-Ser847 nNOS (1:1000, Abcam #16650) and HRP-conjugated Goat anti-Rabbit secondary antibody was used at 1:10,000. GAPDH was used as a loading control. A Kodak Image Station was used to visualize and quantify protein expression.

Immunoprecipitation. Protein-A dynabeads were washed in cold PBS, and MMP-2 (Millipore #19015) or MMP-9 antibody (Millipore #19016) were added at 1:1000 dilution and allowed to incubate for 1h at RT. Beads were washed 3 times for 5 minutes each in cold PBS containing 0.2% Triton-X100 (PBST). Protein A was crosslinked to antibodies with 5mM bis(sulfosuccinimidyl)suberate (BS3) for 30 minutes, and the crosslinking reaction was quenched via 1M Tris-HCl for 15 minutes. NAcore samples were prepared as whole-cell lysates in RIPA buffer with protease and phosphatase inhibitors. 45µg of protein was added for detection of MMP-2, or 100µg of protein for MMP-9 in 200µl RIPA buffer containing protease and phosphatase inhibitors. Beads were washed 3 minutes for 5 minutes each in cold PBST. Elution occurred via 50mM Glycine at pH 2.8,

and samples were heated to 50^{o}C for 10 minutes. Western blotting was performed above, and membranes were probed with an antibody against S-nitrosocysteine (Sigma Aldrich #N5411). Membranes were then stripped via RestorePLUS western stripping buffer (Life Technologies #46428) for 10 minutes at 37^{o}C, and re-probed using the same antibody that was used for the immunprecipitation. SNO-cysteine content was normalized to total protein for quantification.

Statistics: All statistics were performed using GraphPad Prism version 7. Two-hour reinstatement sessions were analyzed using a repeated measures One-Way ANOVA. Zymography data were analyzed using paired t-tests, with opposite hemispheres forming pairs.

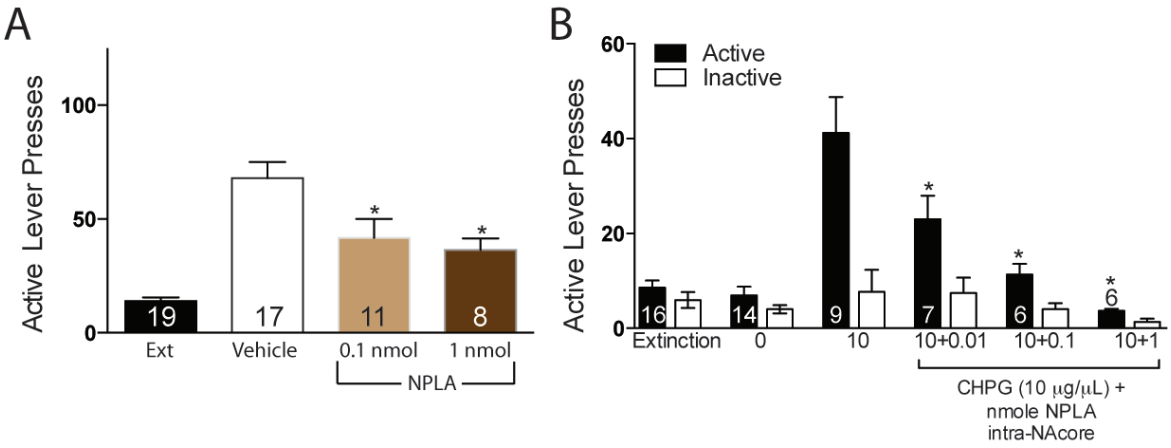
3.3 RESULTS

nNOS inhibition attenuates both cue-induced and CHPG-induced reinstatement

The role of nNOS and nitrergic signaling in hippocampal LTP was established over two decades ago (Haley et al., 1992), however, its role in synaptic plasticity contributing to cocaine reinstatement has never been thoroughly evaluated. We hypothesized that nNOS production of NO and nitrergic signaling are important for reinstatement of cocaine seeking and the associated induction of MMP activity. To test this hypothesis, we utilized the pharmacological nNOS inhibitor N-propyl-L-arginine (NPLA) to measure the effects of nNOS inhibition on cue-

induced reinstatement and the associated induction of MMP activity in rats. We used a counterbalanced within-subjects crossover design and infused 1nmol NPLA or aCSF vehicle 15 minutes prior to a reinstatement session. We found that NPLA significantly attenuated cue-induced reinstatement (Figure 3-2a). We then sought to examine whether CHPG may be acting through an nNOS-dependent mechanism to elicit reinstatement. Again using a counterbalanced within-subjects design, we infused 10µg CHPG in a cocktail with 0.001, 0.1, or 1.0 nmol NPLA, and found that nNOS inhibition is capable of blocking CHPG-induced reinstatement in a dose-dependent manner (Figure 3-2B).

Figure 3-2. nNOS inhibition via NPLA inhibits both cue-induced and CHPG-induced reinstatement behavior. *A) Intra-NAcore microinjection of 1 nmol NPLA significantly reduced reinstatement compared to vehicle. One-Way*



ANOVA revealed a significant difference between groups ($F(3, 50) = 19.83$, $p < .0001$). Bonferonni's post-hoc test revealed a significant difference between extinction and vehicle ($p < .0001$), and between vehicle and 0.1 nmol NPLA ($p = .0338$), and between vehicle and 1 nmol NPLA ($p = .0018$) but not between extinction and NPLA. B) NPLA reduced CHPG-induced reinstatement in a dose-dependent manner. One-Way ANOVA revealed $F(5, 53) = 14.67$, $p < .0001$. Bonferonni's post-host test revealed that all three doses of NPLA significantly reduced active lever pressing, and only the smallest (.01) dose significantly reinstated compared to extinction. The cue-induced reinstatement was conducted by myself. Contributions to the CHPG experiment are as follows: I did

the surgeries with help from Michael Scofield, the NARC ran the animals, Cassie Gipson did the microinjections prior to reinstatement, and I analyzed the data.

nNOS inhibition also attenuates both persistent and transient inductions of MMP activity

We previously reported that MMP-2 is constitutively upregulated following extinction, and MMP-9 is transiently induced following cue-induced reinstatement, but had not explored how these inductions occur. A between-subject design was used to perform *in vivo* zymography to measure MMP activity 24 hours following extinction or 15 minutes after initiation of cue-induced reinstatement. NPLA was able to reduce MMP activity following extinction and both forms of reinstatement (Figure 3-3). This indicates for the first time that nNOS activity following cocaine self-administration contributes to MMP-mediated synaptic plasticity driving reinstatement.

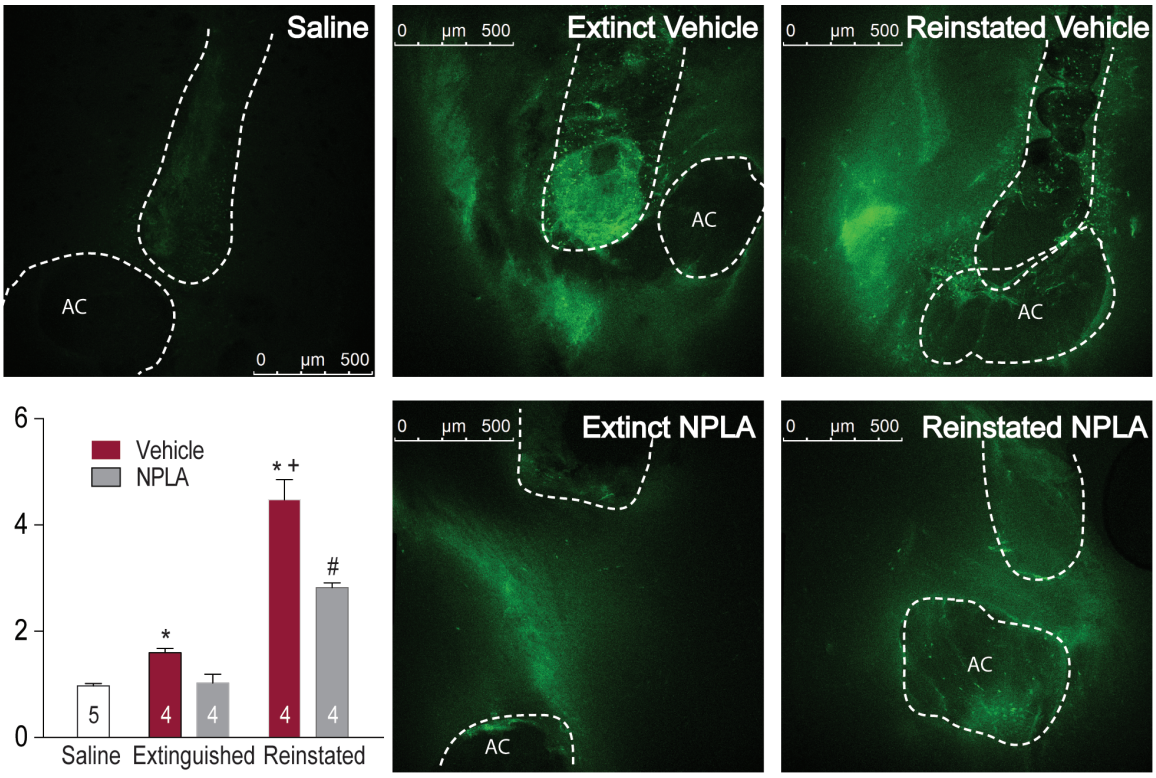


Figure 3-3. nNOS inhibition attenuates MMP activity both following extinction and reinstatement. *Each animal received unilateral vehicle and contralateral NPLA infusion intra-NAcore, and 100µm sections were analyzed for gelatinolytic fluorescence. Opposite hemispheres formed pairs. Extinction paired t-test t(3) = 3.616, p = .0363. Reinstated paired t-test t(3) = 5.085, p = .0147. All experiments were conducted myself.*

nNOS activity and MMP nitrosylation are increased following cocaine exposure

nNOS is partially regulated by phosphorylation of serine 847, which decreases its affinity for CaM and activity. We found that following both extinction and cue-induced reinstatement, Ser847 is significantly dephosphorylated, indicating increased enzymatic activity and NO production (Figure 3-4A) (Rameau 2007. Antibodies specific for S-nitrosylated forms of MMPs are not

commercially available. Thus, in order to determine whether this increase in NO efflux was directly consequential for S-nitrosylation of MMPs, we immunoprecipitated either MMP-2 or MMP-9, and probed for S-nitroso-cysteine. We found that MMP-2 S-nitrosylation is significantly increased following reinstatement, with a trend (p = .07) towards an increase following extinction (Figure 3-4B). No change was detected in S-nitrosylation of MMP-9. Limitations of this strategy include the inability to distinguish critical nitrosylated cysteines from irrelevant ones.

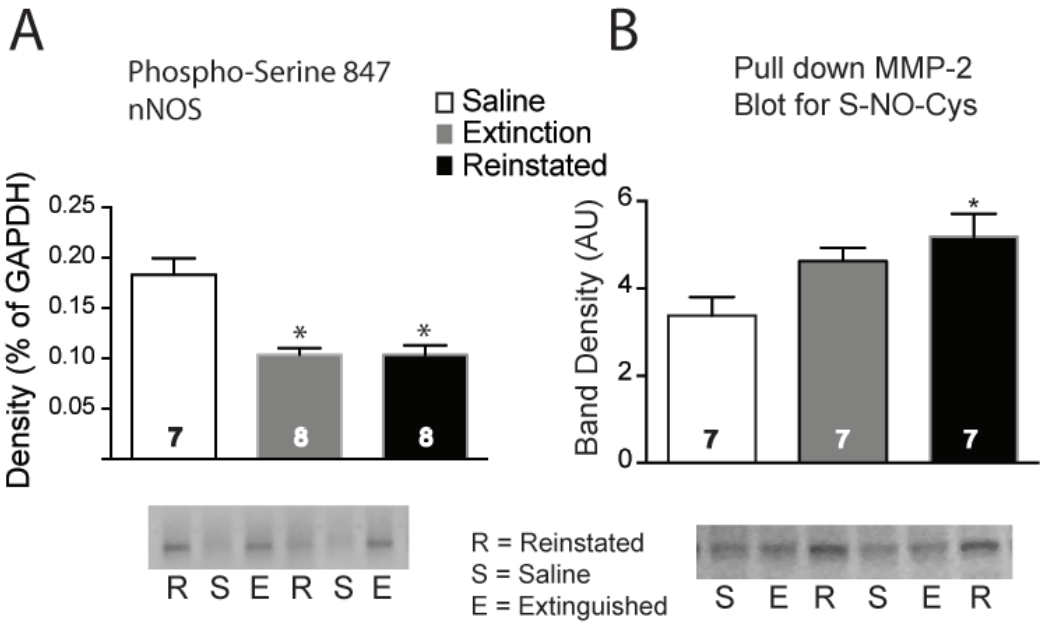


Figure 3-4. Biochemical data indicates increased NO production and increased MMP-2 S-nitrosylation. ***A)*** *One-Way ANOVA shows that nNOS serine 847 is significantly dephosphorylated following extinction and reinstatement of cocaine seeking (F(2, 20) = 16.50, p < .0001). Bonferroni's post-hoc confirmed that both of these are significantly decreased compared to saline, with no difference between extinction and reinstatement.* ***B)*** *One-Way ANOVA shows an increase in MMP-2 S-nitrosylation following reinstatement. (F(2, 18) = 4.694, p = .0229). Bonferroni's post-hoc confirmed that reinstatement is significantly increased compared to yoked-saline controls (p = .0229), while extinction exhibited a trend that did not reach significance. Western blot experiments were conducted myself, immunoprecipitation was done with the aid of Michael Scofield.*

NOS1-Cre transgenic mice allow selective chemogenetic targeting of nNOS+ interneurons

Discovering a necessary role for nNOS in cue- and CHPG induced reinstatement then led us to question whether nNOS activity is sufficient to drive MMP activity and reinstatement behavior. In order to target these cells specifically, we used NOS1-Cre transgenic mice (Jackson Labs), and Cre-dependent AAV2-hSyn-DIO-hM3D(Gq), a Gq-coupled DREADD virus to selectively express these designer receptors in nNOS-expressing interneurons. We first used immunohistochemistry to validate that the mCherry tag on the receptor specifically colocalized with immunolabeled nNOS interneurons (Figure 3-5).

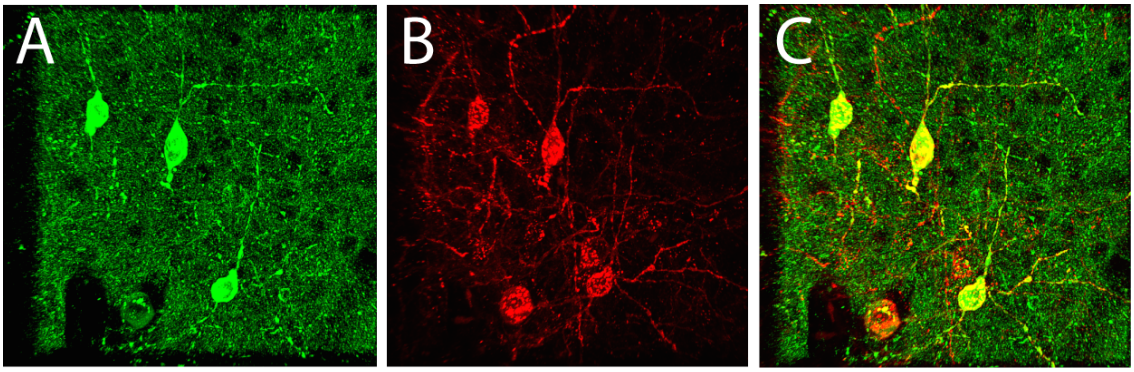


Figure 3-5. Gq-DREADD Virus is selectively transduced into nNOS-expressing interneurons in NOS1-Cre mice. A) Immunohistochemistry for nNOS. **B)** mCherry tag expressed by AAV2-hSyn-DIO-HM3Dq-mCherry. **C)** Merge of the two channels showing colocalization and selective expression.

Gq-DREADD stimulation of nNOS expressing interneurons induces MMP activity throughout the accumbens

We used our *in vivo* microelectrode biosensor to confirm that stimulation of Gq-DREADD with clozapine N-oxide (CNO) increases efflux of NO (Figure 3-6). We hypothesized that increased NO efflux would lead to increased MMP activity, and performed *in vivo* zymography experiments in which one hemisphere of drug-naïve mice received CNO treatment, the contralateral hemisphere received vehicle treatment in a counterbalanced manner, and opposite hemispheres were compared using a paired t-test. Results showed that stimulating Gq-coupled receptors selectively in nNOS-containing neurons induced MMP activity throughout the NAcore. This indicated that nNOS-expressing interneurons (approximately 1% of neurons in the accumbens) are capable of globally modifying synaptic strength and plasticity throughout the accumbens. Importantly, it was possible that stimulating this cell population induces MMP activity in a manner independent of their nitrergic properties, and so we then infused either a cocktail of CNO and NPLA, or a cocktail of CNO and vehicle prior to performing zymography, and determined that this effect was entirely nNOS-dependent (Figure 3-6).

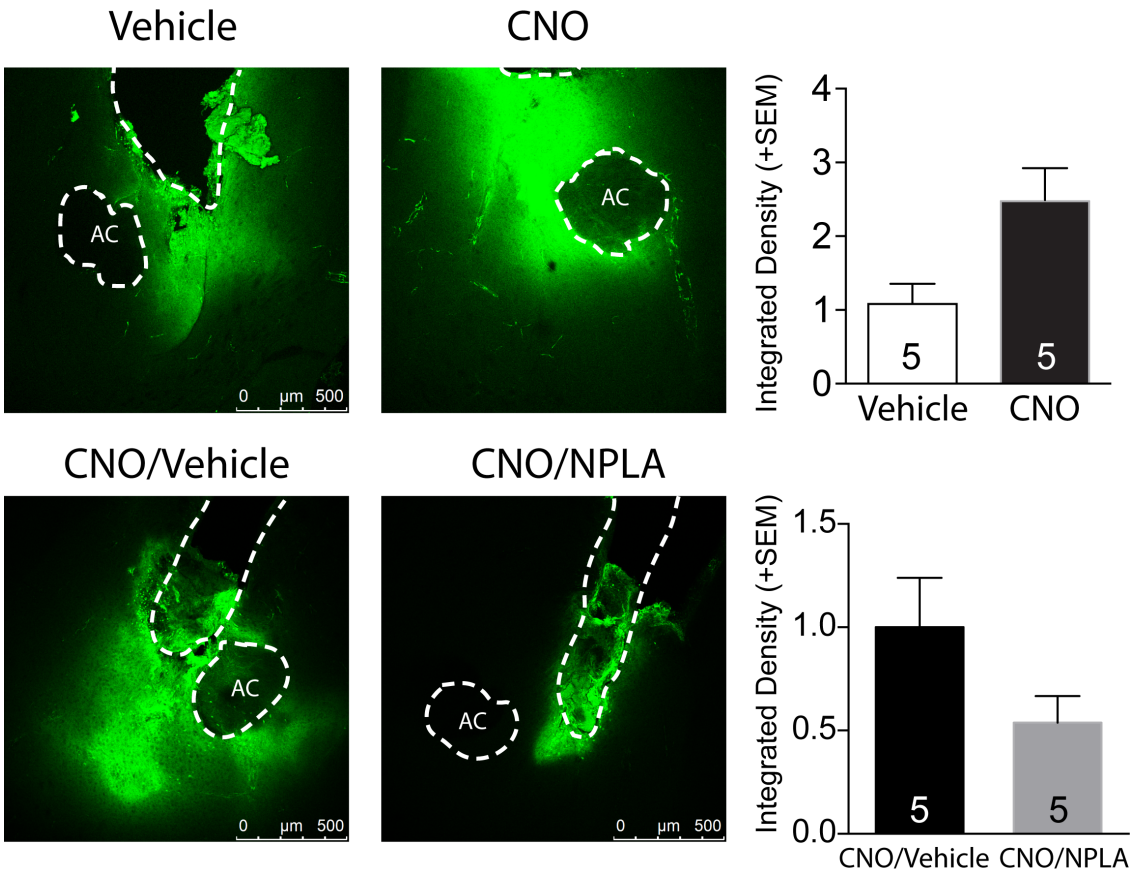


Figure 3-6. Gq stimulation of MMP activity. *CNO significantly elevated gelatinolytic fluorescence compared to vehicle treated hemispheres in naïve NOS1-Cre mice. Paired t-test t(4) = 3.019, p = .0392. This effect was nNOS-dependent, since NPLA significantly reduced CNO-induced fluorescence, paired t-test t(4) = 2.968, p = .0412. Michael Scofield helped with surgeries, I performed injections and completed the zymography protocol.*

nNOS interneurons drive reinstatement of cocaine seeking in the absence of cues

Finally, we hypothesized that Gq-stimulation of nNOS-expressing interneurons would potentiate cue-induced reinstatement. Using a within-subjects counterbalanced crossover design, we injected either 3 mg/kg CNO (i.p.) or 0.5% DMSO vehicle into mice that have undergone cocaine self-administration and

extinction to criteria. Gq-stimulation not only potentiated cue-induced reinstatement 3-fold (Figure 3-7A), but was also capable of inducing reinstatement on its own in the absence of cues, in a similar manner to CHPG (Figure 3-7B).

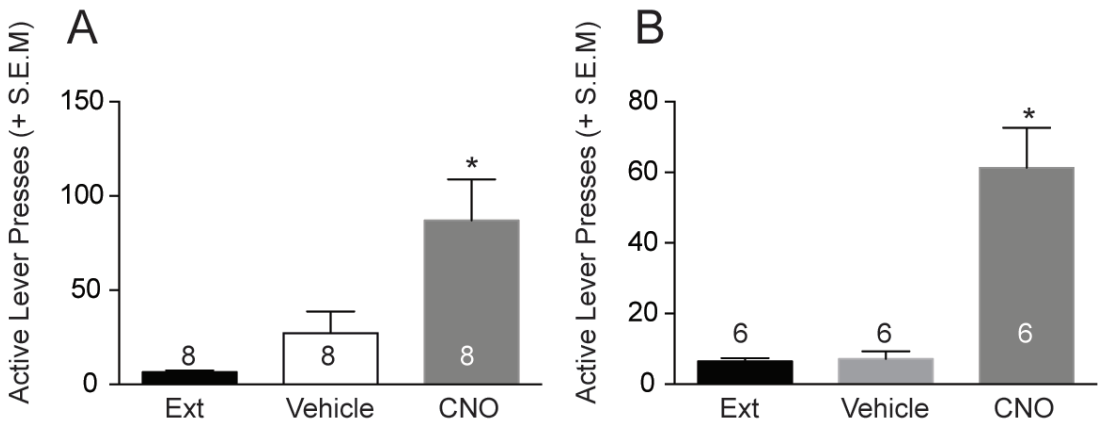


Figure 3-7. Gq-DREADD stimulation drives reinstatement. *A) Shows cue-induced reinstatement data, One-Way ANOVA revealed a significant difference between groups (F(3,6) = 17.51), p = .0104. Bonferonni's post-hoc test revealed a significant difference between vehicle and CNO p = .0392. B) Shows extinction data, One-Way ANOVA revealed that CNO treatment significantly drives reinstatement, F(3, 6) = 23.40, p = .0105. Contributions for these experiments are as follows: I conducted the surgeries, Jasper Heinsbroek ran the self-administration and reinstatement, and I analyzed the data.*

3.4 DISCUSSION

In the present study we examine the hypothesis that nitrergic signaling contributes to reinstatement of cocaine seeking via activation of MMPs. We showed previously that glutamatergic synapses in the NAcore are persistently potentiated following extinction of cocaine self-administration, and that this was reversed by inhibition of MMP-2 (Figures 2-4, 2-5). Furthermore, cue-induced reinstatement is associated with a rapid, transient potentiation of these synapses, measured by both dendritic spine head diameter and AMPA/NMDA ratio, and

that this potentiation is dependent on MMP-9 activity (Figures 2-4, 2-5). We hypothesized that nitrergic signaling is critical for activating MMPs and driving synaptic potentiation that led to reinstatement. Our data show that inhibition of nitric oxide production via nNOS attenuates cue-induced reinstatement, and also attenuates the induction of both MMP-2 and MMP-9 that persists following extinction and coincide transiently with reinstatement, respectively. Additionally, data included here show that intra-NAcore infusion of the mGluR5 agonist CHPG robustly induces reinstatement (Figure 3-2), and that this effect is completely dependent on nNOS activity. Biochemical data indicated a persistent stimulatory dephosphorylation of nNOS Ser847 following extinction of cocaine self-administration (Figure 3-4), indicating a persistent increase in NO release. This signal was also detected as an increase in S-Nitrosylation of MMP-2 (Figure 3-4), but could not be detected on MMP-9.

Because nNOS is expressed only in a small population of GABAergic interneurons that comprise approximately 1% of striatal neurons, we then examined the potential for these cells to modulate reinstatement themselves. Previously, most work in the NAcore has examined medium spiny neurons, the major projection neurons of the striatum, and their afferent and efferent connections. By using NOS1-Cre transgenic mice, we were able to selectively target Gq-coupled DREADD receptors to this small cell population. By inducing Ca2+ release from internal stores with the DREADD agonist CNO, we were able to induce MMP activity throughout the NAcore (Figure 3-6), and drive reinstatement behavior even in the absence of conditioned cues (Figure 3-7).

This indicates that this small population of nitrergic neurons may comprise a 'master-switch' for triggering the synaptic potentiation onto MSNs that underlies the vulnerability to reinstatement.

These findings are consistent with previous reports of NO-dependent plasticity contributing to drug addiction. In a conditioned place preference paradigm, systemic treatment with an nNOS inhibitor prior to daily conditioning sessions abolished acquisition of CPP (Itzhak et al., 1998a), and nNOS inhibition immediately following a reinstatement session impaired the subsequent reinstatement session, indicating that NO is important for memory reconsolidation (Itzhak, 2008). In the dorsal striatum, NO efflux is persistently increased following 7 days of systemic cocaine injection and 7 days of withdrawal (Lee et al., 2010), a finding that parallels what our biochemical data indicate occurs in the accumbens core. Most recently, Selvakumar et al (2014) showed that S-nitrosylation of the AMPA trafficking protein Stargazin in the accumbens shell was required for cocaine sensitization and insertion of GluA1 AMPA receptors. In addition to regulating AMPA trafficking, NO also S-nitrosylates AMPA receptors directly at cysteine 875, which in turn stimulates phosphorylation of serine 831 to increase single-channel conductance and endocytosis (Selvakumar et al., 2013).

Nitrergic signaling is particularly well positioned to modulate synaptic plasticity on large networks of cells because of its ability to participate in volume transmission, and because of the reactive nitrogen species properties of NO. Volume transmission describes transmission that occurs outside of the synaptic

cleft, so called because the molecule diffuses through the volume of extracellular space (Sykova, 2004). NO is a gaseous molecule that, while it does not fit many formal definitions for neurotransmitters, was originally characterized as a retrograde neurotransmitter, but is now simply recognized as an intercellular transmitter (Schuman and Madison, 1991). Once synthesized by nNOS, NO freely diffuses away from its point of origin, and is unhindered by lipid bilayer cell membranes. Diffusion will continue until NO reacts with an encountered molecule via S-nitrosylation or another nitrosative/oxidative mechanism, until it becomes oxidized into the free radical peroxynitrite, or until it reacts with other NO molecules to reduce to a much more stable nitrate/nitrite species. Volume transmission is dependent on the amount of tortuosity, or hindrance to diffusion, of a compartment such as the extracellular space. Within the extracellular space, the major contributors to tortuosity are glycoproteins such as fibronectin, laminin, and collagen (Sykova, 2004). These glycoproteins are the primary component of the extracellular matrix, and have long been recognized as important substrates for MMPs. Thus, increased glycoprotein degradation following S-nitrosylation of MMPs by NO may represent a feed-forward mechanism by which these interneurons reduce the tortuosity of the brain region, in turn promoting S-nitrosylation of further synaptic contents such as Stargazin or AMPA receptors.

Dysregulation of glutamate homeostasis following chronic cocaine experience causes a massive release of glutamate in response to cocaine-conditioned cues, and combined with decreased glial glutamate reuptake, this release is hypothesized to induce glutamate spillover out of the synaptic cleft

(Kalivas, 2009; Reissner et al., 2015). Once outside of the synaptic cleft, glutamate is able to act on extrasynaptic receptors such as mGluR5, which stimulates synaptic potentiation, and in turn, reinstatement (Wang et al., 2013). While most reports posit that glutamate spillover exerts an effect through stimulation of mGluR5 directly on MSNs, the current data indicate that glutamate spillover onto nearby nNOS-expressing interneurons may be the most important player. Glutamate spillover is also confined by the properties of tortuosity and anisotropy in a similar manner as NO (Sykova, 2004). Thus, constitutively increased NO/MMP activity may facilitate glutamate spillover by degrading the extracellular matrix and thus decreasing the tortuosity of the extracellular space. While CHPG and Gq-DREADD stimulation of nNOS were both able to induce MMP activity, the current studies did not address whether afferent glutamatergic projections were synapsing directly onto nNOS-expressing interneurons, or whether glutamate spillover from synapses onto MSNs was stimulating these cells. Experiments to directly test these two hypotheses are important future directions. Furthermore, an important future direction will be to examine the mechanism by which MMP-9 is activated, because data here indicate that it is not through S-nitrosylation. MMP-9 can be activated by multiple proteases, the most studied of which is tissue plasminogen activator (tPA), as well as MMP-2.

In conclusion, activity of nNOS-expressing interneurons is both necessary and sufficient for reinstatement of cocaine seeking. Furthermore, manipulating nNOS activity bidirectionally is also able to modulate MMP activity that has previously been shown to be required for drug-induced synaptic plasticity. These

data indicate that approximately 1% of neurons in the accumbens core may constitute a master-switch for region-wide synaptic potentiation which underlies the vulnerability to relapse.

Chapter 4: Summary, conclusions, and future directions

The study of synaptic architecture has been a popular focus in neuroscience for decades. The most accepted current model is the tripartite synapse, in which a presynaptic neuron synapses onto a postsynaptic neuron, and this connection is stabilized by astrocytic endfeet. This dissertation offers data suggesting a fourth component of the synapse: the extracellular matrix. Historically, even the existence of the extracellular matrix (ECM) within the brain was once a controversial topic. Here, I show that matrix metalloproteinases (MMPs), the primary regulators of the ECM, are important modulators of synaptic plasticity contributing to drug addiction. Specifically, upregulated MMP-2 activity following extinction of cocaine self-administration drives a persistent synaptic potentiation compared to yoked-saline controls, and MMP-9 drives a rapid, transient synaptic potentiation upon the initiation of cue-induced reinstatement. Furthermore, there is evidence of volume transmission that is not synaptic, but rather intercellular transmission between neighboring cells, even of different neuronal types. I highlight two forms of volume transmission: 1) nitric oxide (NO) signaling, which is based on free diffusion of the gaseous molecule through the cell membranes and the extracellular space, and 2) glutamate spillover, which is dependent on the quantity of glutamate exocytosis, and the ability of glutamate to diffuse through the extrasynaptic space without reuptake. Both of these, and all other types of volume transmission are dependent on tortuous barriers to

diffusion. The term ‘volume transmission’ describes any type of transmission that does not rely on synaptic transmission, but relies on diffusion through the volume of the extrasynaptic space. Tortuosity is defined as “hindrance to diffusion”, and describes how easily a molecule can diffuse through the extracellular space (ECS). Two primary contributors to tortuosity in the ECS are the polyelectrolyte ECM molecules such as fibronectin that are degraded by MMPs, and the ensheathment of synapses by glial endfeet. Thus, increased MMP activity and decreased tortuosity allows more permissive diffusion parameters. My data show that mGluR5 stimulation of nNOS-expressing interneurons results in increased NO production and MMP activity. I propose a feed-forward system in which glutamate spillover increases NO production, leading to an increase in MMP activity, which in turn decreases ECS tortuosity via cleavage of ECM molecules, which allows for more glutamate overflow. There are a number of future experiments that could further elucidate glutamate/NO interactions.

4.1 Does glutamate spillover contribute to nitric oxide production?

In order to test whether corticostriatal glutamate overflow contributes to increased NO production, I would use an electrochemical biosensor method similar to that described in chapter 3, but with the addition of a stimulating electrode placed in the prelimbic cortex. I hypothesize that stimulating corticostriatal efferents in a drug-naïve animal will evoke NO release, and that this will be potentiated by TBOA. Furthermore, this glutamate-evoked NO release will be potentiated in animals with a history of cocaine self-administration and

extinction, and this effect would be rescued by a chronic ceftriaxone treatment that has been shown to normalize GLT-1 expression and glutamate reuptake (Knackstedt et al., 2010).

4.2 Is nitric oxide production and MMP activity permissive for glutamate spillover?

If MMP activity does indeed decrease tortuosity of the ECS in response to drug-induced nitric oxide, MMP inhibitors should decrease the ability of glutamate to spill out of the synapse. A method for measuring glutamate spillover has been established in our lab (Shen et al., 2014b). Thus, I propose to use inhibitors against MMP-9 and nNOS prior to measuring spillover. I hypothesize that MMP-9 inhibition will have a greater and more rapid effect than nNOS inhibition, due to the direct effect of MMP-9 activity on ECM composition and tortuosity. I further hypothesize that injection of Chondroitinase-ABC, a commercially available enzyme that destroys chondroitin sulfate proteoglycans, would increase synaptic glutamate spillover.

Interestingly, I would hypothesize that inhibition of glutamate spillover would facilitate increased synaptic signaling, which would contradict previous data showing a decrease of AMPA:NMDA ratio following MMP inhibition. An alternative explanation for this is that a decrease in extrasynaptic NMDA receptor stimulation following MMP inhibition results in an increased AMPA:NMDA ratio. It is also possible that increased glutamate spillover also results in stimulation of

extrasynaptic AMPA receptors that augments AMPA current moreso than NMDA current, leading to an increased ratio.

4.3 Do MMPs contribute to the retraction of astrocytic end feet following cocaine self-administration and extinction?

The contacts between astrocytic endfeet and synapses or neurons are very dynamic. In some normal physiological processes, like sleep, the interstial space swells by as much as 60%, indicating a retraction of astrocytic processes (Xie et al., 2013). Unpublished preliminary data from our lab shows a decrease in colocalization between astrocytic membranes and synapsin-expressing presynaptic terminals, indicating decreased contact of synapses by astrocytes and thus decreased tortuosity. I hypothesize that MMP cleavage of neuron-glia adhesion molecules is required for this retraction, and that acute inhibition of MMP-2 or MMP-9 would restore synaptic contacts between these cells. Furthermore, I hypothesize that prolonged inhibition of MMP-2/9 would result in a restoration of GLT-1 expression following chronic cocaine.

4.4 Is MMP activity permissive for synaptic penetration by NO and S-nitrosylation of synaptic proteins?

If MMP activity and subsequent degradation of ECM surrounding corticostriatal synapses increases diffusibility of NO through the ECS, I hypothesize that inhibiting constitutively increased MMP-2 activity following

extinction will decrease S-nitrosylation of syntaxin-1 presynaptically, and Stargazin postsynaptically.

In conclusion, the work presented here contributes novel information to a rapidly advancing field by examining the extracellular matrix in the nucleus accumbens core as an important signaling domain in synaptic plasticity underlying drug addiction. Furthermore, I go on to identify a cell population which previously has not been studied in this brain area, and discover that this small cell population is able to bidirectionally modulate reinstatement behavior, indicating that it may constitute a ‘master-switch’ to control addictive behaviors.

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