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Cell-Type Specific Extracellular Matrix Signaling in the Opioid-Addicted Synapse

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Cell-Type Specific Extracellular Matrix Signaling in the Opioid-Addicted Synapse

By

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Abstract

VIVIAN CHINWENDU CHIOMA. Cell-Type Specific Extracellular Matrix Signaling in the Opioid-Addicted Synapse. (Under the direction of PETER W. KALIVAS).

To date, heroin abuse is a leading cause of drug overdose-related deaths in the United States, highlighting a need for further research elucidating effects of maladaptive neuroadaptations following prolonged heroin use. Activation of the tetrapartite synapse in nucleus accumbens core (NAcore), which comprises of pre- and post-synapse, astrocytic processes, and surrounding extracellular matrix (ECM), has been linked to increased relapse vulnerability. Specifically, degradation of ECM by activated matrix metalloproteinases (MMPs) is involved in extracellular synaptic remodeling both constitutively and transiently in extinguished and drug seeking conditions, respectively. Although increases in MMP-2,9 fluorescence can be localized to soma and dendritic processes of medium spiny neurons (MSNs) in NAcore, it is unknown which specific cell types of the tetrapartite synapse harbor changes in MMP activity under extinguished and cued reinstatement conditions (15min). I used a viral transfection strategy to label NAcore D1 and D2 MSNs and astrocytes in heroin self-administering rats, and measured localization of activated MMP-2,9 gelatinolytic puncta after FITC-gelatin microinjection under extinguished and reinstated conditions. Astrocytes exhibited reduced synaptic MMP activity co-localized with peripheral processes after extinction, but gelatinolytic activity was restored to control levels during cued heroin seeking. For D1 MSNs, I observed transient increases in MMP-9 activity localized around dendritic segments in reinstated animals compared to both saline controls and heroin-extinguished animals. D2 MSNs showed increased MMP-2 activity only in heroin-extinguished animals, but MMP-2,9 activity after 15 min reinstatement was transiently reduced to saline levels. In both cases, increased MMP activity was enhanced around dendritic spines compared to dendrite shaft. The observed changes in MMP activity

around D1 and D2 MSNs during heroin seeking are transient and reversible as gelatinolytic puncta around both cell types returned to extinguished levels after 120min of heroin seeking. I also examined effects of home-cage abstinence and heroin refraining on MMP activity selectively around D1 and D2 MSNs. Finally, I studied involvement of tissue inhibitors of metalloproteinases (TIMPs) in NAcore during reinstatement to determine if local MMP inhibition around specific cell-types is necessary for cued heroin seeking. These findings reveal how NAcore ECM signaling underlying constitutive and transient synaptic plasticity relies in part on specific cell-types.

Chapter 1: Introduction

1.1 The Opioid-Addicted Tetrapartite Synapse[1]

Opioid administration in preclinical models induces long-lasting adaptations in reward and habit circuitry (Hearing 2019, Reiner, Fredriksson et al. 2019). The latest research demonstrates that in the nucleus accumbens opioid-induced excitatory synaptic plasticity involves pre- and post-synaptic elements, as well as adjacent astroglial processes and the perisynaptic extracellular matrix. Here I outline opioid-induced modifications within each component of the tetrapartite synapse and provide a neurobiological perspective on how these adaptations converge to produce addiction-related behaviors in rodent models. By incorporating changes observed at each of the excitatory synaptic compartments into a unified framework of opioid-induced glutamate dysregulation, I highlight new avenues for restoring synaptic homeostasis that might limit opioid craving and relapse vulnerability.

1.1.1 Brief Overview of Opioid Addiction

The medicinal properties of opium, derived from the poppy plant, dates back to 3400 BC in lower Mesopotamia (Presley and Lindsley 2018). Known as the “joy plant,” opium was heavily utilized as a mystical analgesic across the globe, with opium smoking being introduced to the United States in the mid-1800s during the Gold Rush (Presley and Lindsley 2018). The current opioid epidemic began in the 1980s with prescription opioids being overly prescribed for chronic pain by misinformed physicians, and gradually expanded to recreational abuse of synthetic opioids (Salmond and Allread 2019). Today, opioids are the leading cause of drug overdose-related deaths in the United States, specifically with synthetic opioid abuse increasing most recently (i.e. fentanyl) (Brady, McCauley et al. 2016, Salmond and Allread 2019). On average, 130 Americans die every day from an opioid overdose (CDC Source), with the highest

[1]Adapted from: Kruyer A.*, Chioma V.C.*, Kalivas P.W. (2020) The Opioid Addicted Synapse. Biological Psychiatry 87(1):34-43

burden among adults aged 25-34 years (Gomes, Tadrous et al. 2018). Opioid addiction is a chronic disorder, characterized by compulsion to use drugs and a propensity to relapse, even after the acute opioid withdrawal syndrome has elapsed (Koob and Volkow 2016). Remarkably, 80% of heroin users report early use of opioid analgesics (Jones 2013), supporting the notion that opioid exposure produces long-lasting brain adaptations that can lead to compulsive drug use and seeking. Such brain adaptations manifest in clinical symptoms including impaired cognition (Wollman, Hauson et al. 2019), deficits in learning, memory, and attention (Arias, Arnsten et al. 2016), and increased impulsivity (Lee, Zhou et al. 2005, Jones, Vadhan et al. 2016). These impairments are thought to arise from pathological disruptions in prefrontal and subcortical circuits that contribute to an uncontrollable desire to use opioids (craving), and can escalate drug use and increase the likelihood of relapse (Goldstein and Volkow 2011, Schippers, Binnekade et al. 2012, Jones, Vadhan et al. 2016). An important consequence of these adaptations is that abstinent heroin users exhibit motivational bias toward heroin-related stimuli (Marissen, Franken et al. 2006, Zhao, Li et al. 2017), and elevated cue-reactivity in humans is linked to higher self-reported craving (Carter and Tiffany 1999). These cognitive changes in opioid users are long-lasting with studies pointing to drug craving and associated persistent deficits in decision-making in former opioid users after nearly two years of abstinence (Biernacki, McLennan et al. 2016). Available medications poorly address the enduring pathophysiology that causes opioid associated environmental stimuli or stress to trigger relapse in humans.

1.1.2 Preclinical Models of Opioid Addiction

Linking brain cellular pathophysiology with symptoms of human opioid use disorder (OUD) requires preclinical animal models that mimic behavioral phenotypes observed in humans with OUD. For example, rats receiving chronic non-contingent

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administration of opioids exhibit deficits in learning and memory tasks (Spain and Newsom 1991, Sala, Braida et al. 1994, Dougherty, Walsh et al. 1996, Tramullas, Martinez-Cue et al. 2007) and opioid use and abstinence in rodents involves development of social withdrawal (Lutz, Ayranci et al. 2014), anxiety- and depression-like behaviors (Haydari, Miladi-Gorji et al. 2014, Alizadeh, Zahedi-Khorasani et al. 2018), vulnerability to stressors (Bai, Belin et al. 2017), and decreased motivation for natural rewards (Zhang, Zhou et al. 2007), similar to symptoms observed in OUD (Darke, Swift et al. 1994, Scherrer, Svrakic et al. 2014, Back, Gros et al. 2015, Arias, Arnsten et al. 2016, Lubman, Garfield et al. 2018, Langdon, Dove et al. 2019, Wollman, Hauson et al. 2019). In self-administration models of OUD, rodents perform operant tasks to receive intravenous opioid infusions, often paired with a conditioning stimulus (cue). Additionally, punishment or aversive/threatening cues can be incorporated into operant training to assess an animal's loss of motivation to acquire the drug reward (Verharen, van den Heuvel et al. 2019). Animals typically undergo a withdrawal phase after self-administration with or without extinction training, before undergoing stimulus-induced opioid seeking, at which point a relapse-like state is provoked by exposure to a drug-associated context or cue, an unconditioned stressor, or a priming opioid injection. Self-administration models can also include measures of use escalation, withdrawal, extinction learning, and relapse, thus incorporating many features of OUD (Shaham, Shalev et al. 2003, Crombag, Bossert et al. 2008). In addition, rodents can be trained to self-administer natural rewards, like sucrose, and neuroadaptations occurring during self-administration, extinction or reinstated seeking of natural rewards can be compared to those observed in animals trained to self-administer opioids (Bobadilla, Garcia-Keller et al. 2017). In this dissertation introduction, I focus on the pathophysiology induced by non-contingent and self-administered opioids, and cue-induced opioid seeking, where the neurobiological adaptations are not confounded by acute drug pharmacology.

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Although dopamine strongly contributes to the reinforcing properties of all drugs of abuse including opioids (reviewed in (Solinas, Belujon et al. 2018)), a robust literature implicates dysregulated homeostasis at glutamatergic synapses in OUD-associated cognitive impairments and cue reactivity (i.e. craving) (Kalivas, Lalumiere et al. 2009, Ross and Peselow 2009). For example, glutamate levels measured by magnetic resonance spectroscopy in reward-associated brain regions in opioid-dependent users correlate positively with measures of impulsivity (Liu, Li et al. 2017), and opioid-conditioned cues evoke craving in parallel with fMRI measures of increased activity in cortico- and amygdalo-striatal glutamatergic circuits (Li, Wang et al. 2012). In the following sections, I explore the preclinical data that identify the cellular underpinnings of these OUD-induced glutamatergic adaptations found in human imaging studies.

1.1.3 The Tetrapartite Glutamatergic Synapse

Glutamatergic neurotransmission subserves cognitive functions, including learning and memory (Robbins and Murphy 2006, Platt 2007), and glutamatergic plasticity contributes to addiction- and relapse-related behaviors (Kalivas 2008). Although the contribution of glutamatergic plasticity to addiction has been more thoroughly characterized in models of psychostimulant use (reviewed in (Bobadilla, Heinsbroek et al. 2017)), important roles have been discovered for glutamatergic plasticity in many aspects of opioid addiction, including opioid-related learning (Liu, Che et al. 2017, Vatankhah, Sarihi et al. 2018), somatic withdrawal symptoms (Nakagawa and Satoh 2004, Sekiya, Nakagawa et al. 2004), and motivated drug seeking (LaLumiere and Kalivas 2008). In fact, adaptations shared between the two drug classes, particularly during cued seeking, point to the possibility of a common addiction cellular pathophysiology. However, I will also point out instances where adaptations are different between psychostimulants and opioids, and bring particular focus to opioid-

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induced excitatory synaptic adaptations in the nucleus accumbens (NA, Figure 1-1), a brain region widely identified as critical for generating motivated behaviors, including opioid use and seeking (Scofield, Heinsbroek et al. 2016). The glutamatergic cortical and subcortical NA afferents are, in part, topographically organized and make differential contributions to the shell (NAshell) and core (NAcore) subcompartments of the NA (Figure 1-1)**.** Research from different laboratories tend to focus on either the NAshell or NAcore, and I will note distinct impacts by opioids on glutamate homeostasis in the two subcompartments. Figure 1-1 illustrates the general circuitry wherein glutamatergic synapses undergo enduring and transient plasticity after opioid extinction and during provoked opioid seeking, respectively.

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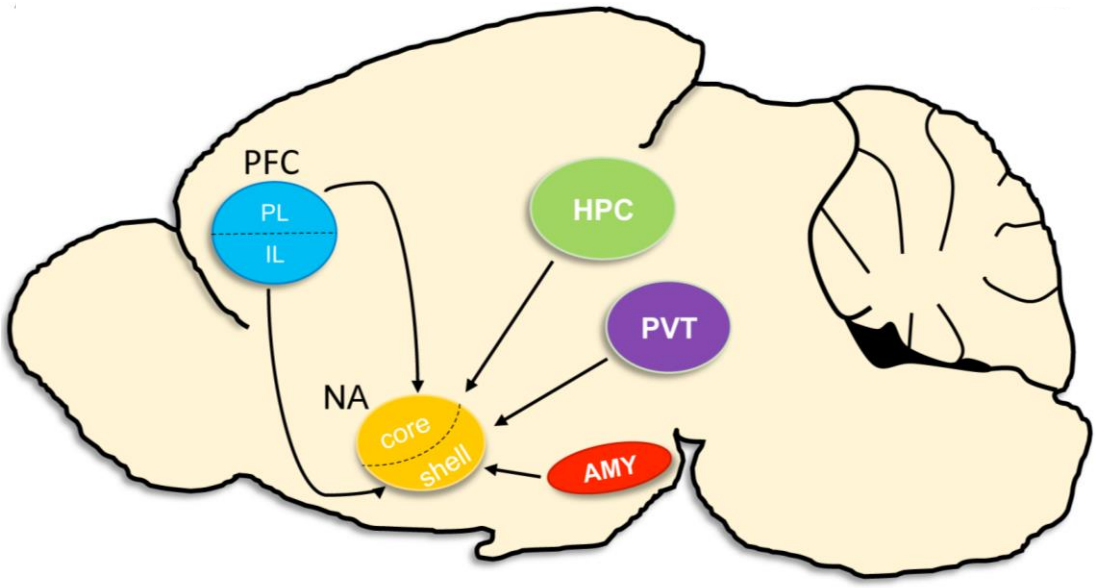

Figure 1-1. Nucleus Accumbens Glutamatergic Circuitry. Glutamatergic afferents from cortical and allocortical brain regions converge onto nucleus accumbens (NA, with core and shell subcompartments indicated. Following repeated exposure to opioids, these projections undergo maladaptive plasticity contributing to aberrant drug seeking after periods of withdrawal. Prefrontal cortex (PFC) subdivided into prelimbic (PL) and infralimbic (IL) with dashed line; hippocampus (HPC), paraventricular thalamus (PVT), and amygdala (AMY) (created by V.C. Chioma, adopted from (Kruyer, Chioma et al. 2020)).

Opioid-induced excitatory synaptic plasticity within the NA involves adaptations at multiple levels, including, but not limited to presynaptic transmitter release (LaLumiere and Kalivas 2008), the relative abundance and composition of post-synaptic receptors (Shen, Moussawi et al. 2011), and astroglial regulation of glutamate uptake and release (Shen, Scofield et al. 2014, Roberts-Wolfe and Kalivas 2015). More recently, a fourth synaptic compartment was found to be modified by opioid use, the perisynaptic extracellular matrix (ECM) (Smith, Kupchik et al. 2014). The ECM is a vast proteinaceous network that functions both as structural support and as a signaling domain to regulate post-synaptic plasticity (Huntley 2012). Considered together, these four synaptic compartments (the canonical pre- and post-synapse, astroglial perisynaptic processes and the ECM) comprise the *tetrapartite synapse* (Dityatev, Seidenbecher et

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al. 2010) (Figure 1-2) and regulate classic forms of pre- and postsynaptic plasticity, such as long-term depression and potentiation (Dityatev and Rusakov 2011). More recently, the tetrapartite synapse as an integrated unit has been shown to regulate the maladaptive plasticity produced by repeated use of many addictive drugs, including opioids (Mulholland, Chandler et al. 2016) Mulholland, Chandler et al. (2016). Below I outline the opioid-associated neuroadaptations in each of the four components, and then integrate these data back into the synaptic tetrapartite structure.

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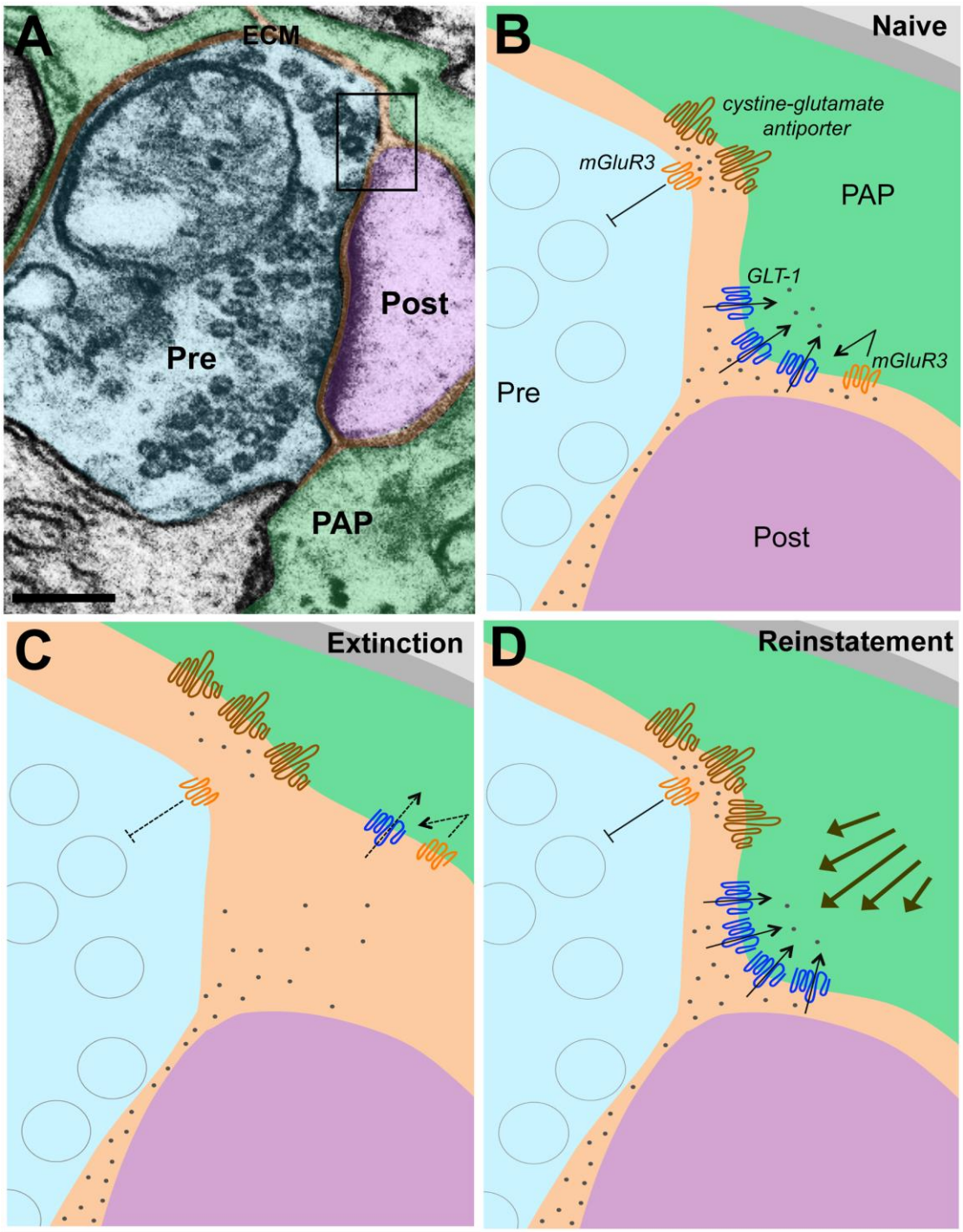

Figure 1-2. Tetrapartite Synaptic Structure and Regulation of Synaptic Glutamate Homeostasis by Astroglial Processes. (A) Tetrapartite synaptic structure demonstrated in an electron micrograph from the mouse hippocampus (scale bar = 200 nm). Presynaptic afferent (Pre) (blue) with synaptic vesicles and mitochondrion. Postsynaptic terminal (Post) (purple), with electron-dense postsynaptic density. Nearby perisynaptic astroglial processes (PAP) (green) surround the synaptic cleft. The perisynaptic extracellular matrix (orange) serves as a signaling medium between all cellular components of the synapse.

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(B) Under normal physiological conditions, glutamate transporter 1 (GLT-1) (blue) on PAPs is responsible for the majority of glutamate (small gray circles) uptake. Stimulation of astroglial metabotropic glutamate receptor 3 (mGluR3) (orange) has been linked to upregulating GLT-1 (105). Astroglial glutamate release through the cystine-glutamate antiporter (brown) can modulate release at glutamatergic terminals by stimulating presynaptic mGluR2/3 (orange) that negatively regulate presynaptic glutamate release probability. **(C)** After extinction from heroin or cocaine self-administration, NAcore astrocytes retract from the synapse, reducing their capacity for glutamate uptake through GLT-1 (blue). Downregulated expression of GLT-1 may result in part from reduced stimulation of astroglial mGluR3 (orange). Astroglial retraction may also disrupt presynaptic tone on mGluR2/3 via cystine-glutamate exchange. **(D)** During 15 minutes of cued heroin seeking, astroglial processes exhibit increased synaptic proximity and increased surface expression of GLT-1, both of which serve to attenuate seeking behavior by hindering synaptic glutamate spillover into the extracellular space. Micrograph in panel (A) was generated by AK at the Electron Microscopy Resource Center at The Rockefeller University (New York, NY) (Kruyer, Chioma et al. 2020).

1.1.3.1 Pre-synaptic Plasticity

Glutamatergic afferents to the accumbens regulate reinstated opioid seeking (Mulholland, Chandler et al. 2016, Scofield, Heinsbroek et al. 2016) and inactivation of prelimbic cortical afferents to NAcore disrupts heroin seeking, as well as the increase in extracellular glutamate produced during heroin-primed reinstatement (LaLumiere and Kalivas 2008). The increase in glutamate release evoked during heroin reinstatement can be blocked by stimulating presynaptic metabotropic glutamate receptor2/3 (mGluR2/3), which reduces presynaptic glutamate release probability (Bossert, Busch et al. 2005, Bossert, Poles et al. 2006). Moreover, deletion of mGluR2 in transgenic rats enhances heroin intake during self-administration, potentiates morphine-induced analgesia and augments naloxone-precipitated withdrawal symptoms (Gao, Jordan et al. 2018). mGluR2/3 is Gi-coupled and signaling through Gi-coupled receptors is negatively regulated by activator of G-protein signaling 3 (AGS3). Down-regulating AGS3 in NAcore, but not NAshell, potentiates mGluR2/3 signaling and decreases reinstated heroin seeking (Bowers, McFarland et al. 2004, Yao, McFarland et al. 2005). Akin to

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mGluR2/3, presynaptic cannabinoid1 (CB1) receptors regulate glutamate release probability, and intra-accumbens administration of AM251, a CB1 receptor antagonist, diminishes the duration required to extinguish morphine conditioned place preference (CPP), as well as the duration of morphine-induced reinstatement of CPP (Khaleghzadeh-Ahangar and Haghparast 2015). In addition, the CB1 receptor antagonist, AM4113, dose-dependently suppresses heroin self-administration (He, Jordan et al. 2019). Taken together, these data demonstrate that regulating glutamate presynaptic release probability in the NAcore inhibits opioid seeking.

In addition to presynaptic regulation of glutamate release, neuroadaptations in the excitability of glutamatergic neurons projecting to the accumbens can also regulate opioid use and seeking. For example, the firing frequency in glutamatergic afferents from the amygdala, but not PFC is increased after chronic morphine treatment (Yuan, Sheng et al. 2017). There are two primary subtypes of projection neurons (medium spiny neurons, MSNs) in NA that express D1- or D2-dopamine receptors (Gerfen and Surmeier 2011). In general, plasticity in excitatory signaling within the NA onto D1-MSNs is associated with enhanced opioid seeking (Lobo and Nestler 2011, Heinsbroek, Neuhofer et al. 2017), while signaling at D2-MSNs triggers extinction-related behaviors (Hearing 2019). Within the NAshell there is increased glutamate release probability at synapses on D1-MSN, while at D2-MSN synapses there is decreased release probability after repeated non-contingent morphine (Hearing, Jedynak et al. 2016). Opioid self-administration also increases glutamatergic release probability on D1-MSNs (James, Chen et al. 2013). Alternatively, morphine withdrawal increases c-Fos expression in paraventricular thalamic (PVT) projections to medial NAshell (Zhu, Wienecke et al. 2016). Specifically, PVT to NAshell D2-MSNs show increased postsynaptic currents that are normalized by depotentiating optogenetic photostimulation of PVT terminals

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synapsing onto D2 MSNs (Zhu, Wienecke et al. 2016). In a μ–opioid receptor (MOR)-null transgenic mouse, targeted rescue of MOR only in striatal D1 MSNs restores morphine-induced CPP (Cui, Ostlund et al. 2014). Similarly, remifentanil self-administration reduces sensitivity of D1-, but not-D2 MSNs, to MOR modulation in NAshell (James, Chen et al. 2013). While these studies provide insight into cell- and pathway-specific alterations associated with opioid exposure in NAshell (Figure 1-3), little is known about such adaptations in the NAcore, or how such changes in release probability are affected by extinction and reinstatement. Figure 1-3 summarizes dichotomous D1- and D2-MSN

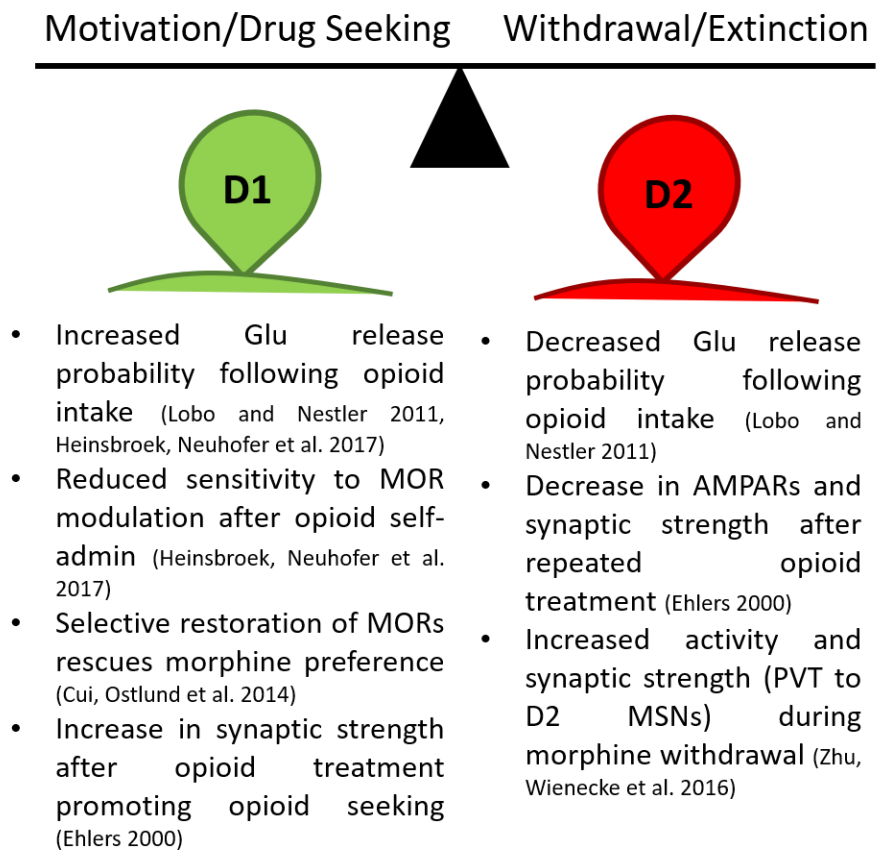

Figure 1-3. D1 vs D2 MSNs in Opioid Addiction. Emerging evidence, as discussed in this thesis, indicate cellular- and pathway-specific adaptations of these projections to NA D1 and D2 medium spiny neurons (MSNs). Schematic outlining classic D1 vs D2 dichotomous relationship with D1 MSN firing predominating during opioid seeking/motivated behavior, while D2 MSNs are activated in context of withdrawal/aversion. Bulleted are cell-type specific findings discussed throughout this dissertation (created by V.C. Chioma, adopted from (Kruyer, Chioma et al. 2020)).

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opioid-induced findings in this section as well as post-synaptic plasticity section.

1.1.3.2 Post-synaptic Plasticity

Spine morphology

On GABAergic MSNs the principal post-synaptic computational units are dendritic spines (Hausser, Spruston et al. 2000). Long-term opioid use affects postsynaptic glutamatergic plasticity and disrupting post-synaptic plasticity is sufficient to attenuate reinstated seeking induced by opioid-associated cues or opioid prime (LaLumiere and Kalivas 2008, Shen, Moussawi et al. 2011). Opioid administration in preclinical models produces actin-dependent morphological plasticity in dendritic spines (Shen, Moussawi et al. 2011), including in spine head diameter, density, and neck length, all of which indicate opioid-induced changes in spine signaling capacity (Williams and Stuart 2003, Chidambaram, Rathipriya et al. 2019). Early reports using Golgi staining found that both repeated contingent and non-contingent morphine administration produce wide-spread, enduring reductions in spine density in the NAshell (Robinson, Gorny et al. 2002). More recent 3-dimensional reconstruction of dye-filled MSNs reveals that heroin self-administration decreases spine head diameter, as well as impairs LTP and LTD in PFC projections to the NAcore (Shen, Moussawi et al. 2011, Shen and Kalivas 2013). The thinner spines observed after 14 days of extinction from heroin exhibited increased surface expression of GluN2B and reduced AMPA/NMDA ratio, measures indicating blunted capacity for synaptic plasticity (Shen, Moussawi et al. 2011). These adaptations are necessary for reinstated drug seeking initiated by heroin prime, which transiently potentiates glutamatergic inputs to MSNs, as indicated by increased spine density, spine head diameter, and field EPSCs (Shen, Moussawi et al. 2011).

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Receptor composition and density

In addition to morphological plasticity, opioid administration causes functional plasticity of excitatory synapses due to composition and abundance of post-synaptic receptor insertion. As mentioned above, morphological adaptations in NAcore spines during heroin extinction are associated with increased surface expression of post-synaptic GluN2B and reduced AMPA/NMDA receptor expression (Shen, Moussawi et al. 2011). Thus, an important mechanism for opioid-induced post-synaptic adaptations involves receptor internalization or downregulation on spines (Shen, Moussawi et al. 2011), and changes in density or composition of receptors at dendritic spines modulate synaptic output (Sjostrom, Rancz et al. 2008). For instance, ionotropic glutamate receptors on neurons in the central amygdala diffuse to dendritic compartments more proximal to the soma after non-contingent morphine administration, facilitating signal propagation (Beckerman, Ogorodnik et al. 2013). Such changes are expected to impact dendritic processing of glutamatergic and GABAergic inputs.

NMDA glutamate receptors (NMDARs) are critically involved in linking glutamate transmission with synaptic plasticity and behavior (Glass 2010) and in regulating learning and memory processes (Hansen, Yi et al. 2018). NMDA receptors are implicated in a number of opioid-mediated addictive behaviors including self-administration (Semenova, Danysz et al. 1999, Glick, Maisonneuve et al. 2001), extinction learning (Myers and Carlezon 2010), naloxone-precipitated withdrawal severity (Trujillo and Akil 1991), drug-related contextual learning (Tzschentke and Schmidt 1995), and the aversion experienced during abstinence (Myers and Carlezon 2010). Moreover, NMDARs are involved in the LTP and LTD disruption observed after heroin extinction, which is required for reinstated seeking (Shen and Kalivas 2013). Specifically, heroin self-administration produces enduring upregulation of NMDAR

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containing GluN2B subunits, and selective blockade of GluN2B in NAcore prevents reinstated heroin seeking (Shen, Moussawi et al. 2011).

AMPA receptors (AMPARs) are activated at resting membrane potential and are primary mediators of postsynaptic transmission in NA (Diering and Huganir 2018). GluA1 and GluA2 AMPAR subunits undergo dynamic activity-dependent trafficking at synapses (Wang, Arora et al. 2005), and induce changes in spine morphology and synaptic strength (Russo, Dietz et al. 2010). GluA1 surface expression is increased by experience-dependent plasticity, facilitated by the fact that homomeric GluA1 AMPARs are permeable to Ca^{2+} (Clem and Barth 2006, Liu and Zukin 2007), leading to increased channel conductance, LTP, and synaptic strength (Isaac, Ashby et al. 2007, Kauer and Malenka 2007). AMPAR composition and trafficking is highly responsive to opioid administration (Glass, Lane et al. 2008, Hearing, Jedynak et al. 2016). For instance, repeated non-contingent morphine increases surface expression GluA2-lacking AMPARs in hippocampal synapses, limiting the capacity of these synapses to undergo LTD (Billa, Liu et al. 2010). Subsequent studies demonstrated that context-dependent behavioral sensitization to morphine is blocked by disrupting GluA1-phosphorylation in the hippocampus (Xia, Portugal et al. 2011), because activity-dependent phosphorylation of GluA1 is linked with its expression at the synaptic surface (Ehlers 2000). Heroin-associated cues produce similar downregulation of GluA2 in the infralimbic region of the PFC, which signals to the NAshell (Figure 1-1). However, in the absence of increased GluA1 and/or GluA3 expression, as observed in the hippocampus after non-contingent morphine (Billa, Liu et al. 2010), the overall loss of synaptic AMPARs results in synaptic depression that is necessary for cue-induced reinstatement of seeking (Van den Oever, Goriounova et al. 2008). Consistent with these findings are data showing that potentiation of AMPARs in the PFC and NAshell facilitate extinction

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learning and suppress cued reinstatement (Chen, Wang et al. 2016), while glutamate release and AMPAR stimulation mediate reinstated seeking in NAcore (LaLumiere and Kalivas 2008).

Cell subtype specificity

How the postsynaptic adaptations described above segregate according to expression of either D1 or D2 receptors is under active investigation. High levels of NMDARs in the absence of abundant AMPARs (low AMPA/NMDA) are consistent with silent synapses that cannot overcome the NMDAR Mg^{2+} block needed to generate EPSCs (Kerchner and Nicoll 2008, Dong, Taylor et al. 2017). Psychostimulants promote new synapse generation on D1-MSNs in the NAshell that are silenced when the drug is no longer available due to enhanced expression of GluN2B-containing NMDARs and reduced AMPA/NMDA (Graziane, Sun et al. 2016). During reinstatement, shuttling of Ca^{2+}-permeable AMPARs from extrasynaptic sites to synapses rapidly un-silences synapses on D1-MSNs and produces LTP (Gipson, Kupchik et al. 2013, Graziane, Sun et al. 2016). In contrast, synaptic silencing in the NAshell following repeated opioid treatment induces endocytosis of AMPARs on D2 MSNs, weakening spine structure and synaptic strength (Figure 1-3) (Shen, Moussawi et al. 2011, Graziane, Sun et al. 2016). As a consequence there is a relative increase in synaptic strength on D1- versus D2-MSNs that promotes opioid seeking (Graziane, Sun et al. 2016, Hearing, Jedynak et al. 2016). Whether a similar pattern involving post-synaptic potentiation after psychostimulants and suppressed inhibition after opioids occurs in NAcore is unknown.

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1.1.3.3 Astrocytes

Glutamate transport and release

Substantial data support a role for astrocytes in regulating synaptic structure and function (Allen 2014, Bernardinelli, Muller et al. 2014). Astrocytes express a plethora of receptors and ion channels, specifically $K_{ir}4.1$ channel, which regulates synaptic potassium buffering (Dallérac, Chever et al. 2013). Astroglial modulation of synaptic excitation occurs via glutamate uptake and release that regulates the balance between synaptic and extrasynaptic glutamate (referred to as glutamate homeostasis (Kalivas, Lalumiere et al. 2009, Coulter and Eid 2012)). Astrocytes and their perisynaptic astroglial processes (PAPs) express glutamate receptors (Aronica, Gorter et al. 2003, Skowronska, Obara-Michlewska et al. 2019), and GLT-1, the principal glutamate transporter that recovers ~90% of synaptically released glutamate (Figure 1-2) (Danbolt 2001). GLT-1, which is expressed largely on PAPs, and PAP motility regulate access of synaptically released glutamate to the extrasynaptic space and facilitate efficient excitatory synaptic transmission (Allen 2014).

Akin to other addictive drugs (Roberts-Wolfe and Kalivas 2015), contingent heroin administration reduces astroglial expression of GLT-1 in NAcore (Shen, Gipson et al. 2014, Shen, Scofield et al. 2014) (Figure 1-2C). The enduring down-regulation of GLT-1 after heroin extinction impairs clearance of synaptically released glutamate in NAcore, as shown by increased activation of extrasynaptic NMDARs (Shen, Gipson et al. 2014, Shen, Scofield et al. 2014). Down-regulated GLT-1 leads to elevated extracellular glutamate during reinstated seeking for opioids and other addictive drugs measured using *in vivo* microdialysis or glutamate biosensors (McFarland, Lapish et al. 2003, Madayag, Lobner et al. 2007, LaLumiere and Kalivas 2008). The mechanisms triggering GLT-1 downregulation are unclear, although dysregulated signaling via

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astroglial glutamate receptors may contribute since stimulation of astroglial mGluR3 increases GLT-1 protein expression (Aronica, Gorter et al. 2003) (Figure 1-2C). Thus, it seems likely that the opioid-associated reductions in activity of glutamatergic afferents to the accumbens discussed above contribute to downregulating GLT-1. It should be noted that it is not known whether GLT-1 is downregulated during the opioid self-administration or withdrawal phases of operant training.

Although cue-induced glutamate release in the NAcore is largely TTX sensitive and thus of presynaptic origin (LaLumiere and Kalivas 2008), astrocytes can also tune presynaptic transmission through astroglial glutamate release directly onto presynaptic mGluR2/3 autoreceptors (Scofield, Boger et al. 2015). Increasing astroglial glutamate release onto mGluR2/3 during cue-induced cocaine reinstatement reduces lever pressing and points to an important role for the cystine-glutamate antiporter in regulating reinstated drug seeking (Baker, Shen et al. 2002, Scofield, Boger et al. 2015) (Figure 1-2). The antiporter imports cystine in a 1:1 exchange for glutamate released extracellularly, which maintains glutamatergic tone on release-regulating presynaptic mGluR2/3 and facilitates glutathione synthesis (Forman, Zhang et al. 2009, Lewerenz, Hewett et al. 2013). Interestingly, in contrast with psychostimulant use (Knackstedt, LaRowe et al. 2009, Reissner, Gipson et al. 2015), surface expression of the catalytic subunit of the antiporter, xCT is increased in NA after heroin self-administration and extinction (Shen, Scofield et al. 2014) (Figure 1-2). Since activating the antiporter with N-acetylcysteine reduces reinstated heroin and cocaine seeking (Baker, McFarland et al. 2003, Zhou and Kalivas 2008), it is interesting to speculate that the heroin-associated increase and cocaine-associated decrease in xCT on NAcore PAPs may selectively modulate D2- or D1-MSNs (Martin, Bajo-Graneras et al. 2015), respectively, akin to the different drug-dependent effects on MSN excitability discussed above.

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In addition to cystine-glutamate exchange, other mechanisms of glutamate release by astrocytes have been reported (Woo, Han et al. 2012), but none have yet been found to contribute to glutamate plasticity observed after extinction from opioids or during reinstated seeking. For instance, while stimulating astroglial MORs increases glutamate transmission in hippocampus via the glutamate channel TREK-1 (Woo, Bae et al. 2018), this channel is also downregulated after chronic opioid use (Wang, Wang et al. 2017). However, one study has observed that activation of astrocytic MORs drives conditioned place preference and associated synaptic plasticity in hippocampus (Nam, Han et al. 2019).

Morphological plasticity

Based on the importance of glutamate uptake in cue-induced reinstatement of opioid seeking (Shen, Scofield et al. 2014) and the relatively high rates of GLT-1 surface diffusion (Murphy-Royal, Dupuis et al. 2015), our lab began investigating the synaptic proximity of astroglial processes after heroin self-administration. To do this, we determined co-registry between membrane-targeted label of astroglial cells and immunoreactivity of synaptic marker, Synapsin I. Astroglial retraction from the synapse occurs after extinction from cocaine self-administration (Scofield, Li et al. 2016, Testen, Sepulveda-Orengo et al. 2018). Similarly, after heroin extinction the synaptic proximity of the astroglial surface and immunoreactive GLT-1 is reduced (Kruyer et al. 2019) (Figure 1-2C). Thus, in addition to reduced uptake of synaptically released glutamate by down-regulated GLT-1, a diminished diffusion barrier resulting from astroglial synaptic retraction may contribute to the spillover of synaptic glutamate during reinstated heroin seeking, akin to what has been observed after extinction from cocaine self-administration (LaLumiere and Kalivas 2008, Smith, Scofield et al. 2017). Four studies support this possibility. Down-regulated GLT-1 enhances extrasynaptic stimulation of

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mGluR5 and GluN2b and promotes cocaine seeking (Gipson, Reissner et al. 2013, Smith, Scofield et al. 2017), inhibiting glutamate uptake via intracerebroventricular administration of the GLT-1 antagonist TBOA enhances acquisition of morphine place preference (Sekiya, Nakagawa et al. 2004), and activating GLT-1 using MS-153 after chronic morphine treatment attenuates the severity of naloxone-precipitated withdrawal (Nakagawa, Ozawa et al. 2001).

Recently, we found that heroin-associated cues rapidly and transiently increase the proximity of astroglial peripheral processes with the synapse ((Kruyer, Scofield et al. 2019); Figure 1-2D) and the transient re-association of the astroglial surface with NAcore synapses during heroin reinstatement appears to be compensatory. When the re-association is prevented by knockdown of the actin binding protein ezrin, which is selectively expressed in PAPs (Lavialle, Aumann et al. 2011), cue-induced heroin seeking was potentiated (Kruyer, Scofield et al. 2019). Taken together, the enduring reductions in GLT-1 expression and changes in synaptic proximity of astrocyte processes after heroin extinction, along with their morphological plasticity during cue exposure demonstrate a critical role for astrocytes in opioid seeking.

Interestingly, different striatal astrocytes respond selectively to synaptic activity on either D1- or D2-MSNs (Martín, Bajo-Grañeras et al. 2015). Thus, the broad distribution of astrocytes with varying degrees of morphological plasticity during reinstated seeking (Kruyer, Scofield et al. 2019) may reflect selective changes in synaptic proximity to one or the other MSN subtype. Selective adaptions in GLT-1 expression and astrocyte morphological plasticity in the vicinity of D1 versus D2 synapses would be expected to produce opposite outcomes on opioid seeking and is an intriguing possibility consistent with the aforementioned selective effects of chronic opioids on D1- versus D2-MSN excitability and morphology.

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While my dissertation does not investigate microglial physiology, this glial subclass also plays a key role in synaptogenesis and neuronal plasticity (Sominsky, De Luca et al. 2018, Saw, Krishna et al. 2020). Microglia undergo morphological adaptations, with constant expansion and retraction of their processes under physiological and pathological conditions (Sominsky, De Luca et al. 2018). In addition, microglial cells release a variety of factors into the central nervous system (CNS) milieu, such as glutamate, nitric oxide, cytokines, growth factors and proteases, to regulate homeostasis and respond to CNS injury (Maezawa and Jin 2010, Smith and Dragunow 2014, Miyamoto, Wake et al. 2016, Sominsky, De Luca et al. 2018). Future studies investigating the role of microglia in tetrapartite synaptic physiology in opioid-addicted behavioral states are warranted.

1.1.3.4 Extracellular Matrix

The extracellular matrix (ECM) is an extensive, proteinaceous network comprised of glycoproteins, proteoglycans, and cellular adhesion molecules (CAMs) (Bosman and Stamenkovic 2003, Wright and Harding 2009, Kaczmarek 2018). It serves as a structural scaffold that tethers neurons and glia via trans-synaptic interactions with CAMs, such as β-dystroglycan, nectin-3, and integrin receptors (Mulholland, Chandler et al. 2016, Vafadari, Salamian et al. 2016), and constitutes a signaling domain when catalytically activated (Wright and Harding 2009). Global knockdown of neuronal CAMs reduces morphine place preference, supporting ECM milieu involvement in opioid reward (Ishiguro, Liu et al. 2006). ECM degradation by matrix metalloproteinases (MMPs) permits neuronal and astroglial morphological adaptations that contribute to synaptic plasticity (Huntley 2012). MMPs are zinc-dependent endopeptidases that digest ECM proteins, such as CAMs, collagen, fibronectin, laminin as well as growth factors to facilitate synaptic remodeling, specifically morphological changes in dendritic spines and

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trafficking of NMDARs and AMPARs into the synaptic membrane (Figure 1-4) (Michaluk, Mikasova et al. 2009, Michaluk, Wawrzyniak et al. 2011, Vafadari, Salamian et al. 2016). In the following sub-sections, I will outline significant details regarding the structural and biological properties of MMP physiology and aberrant pathology.

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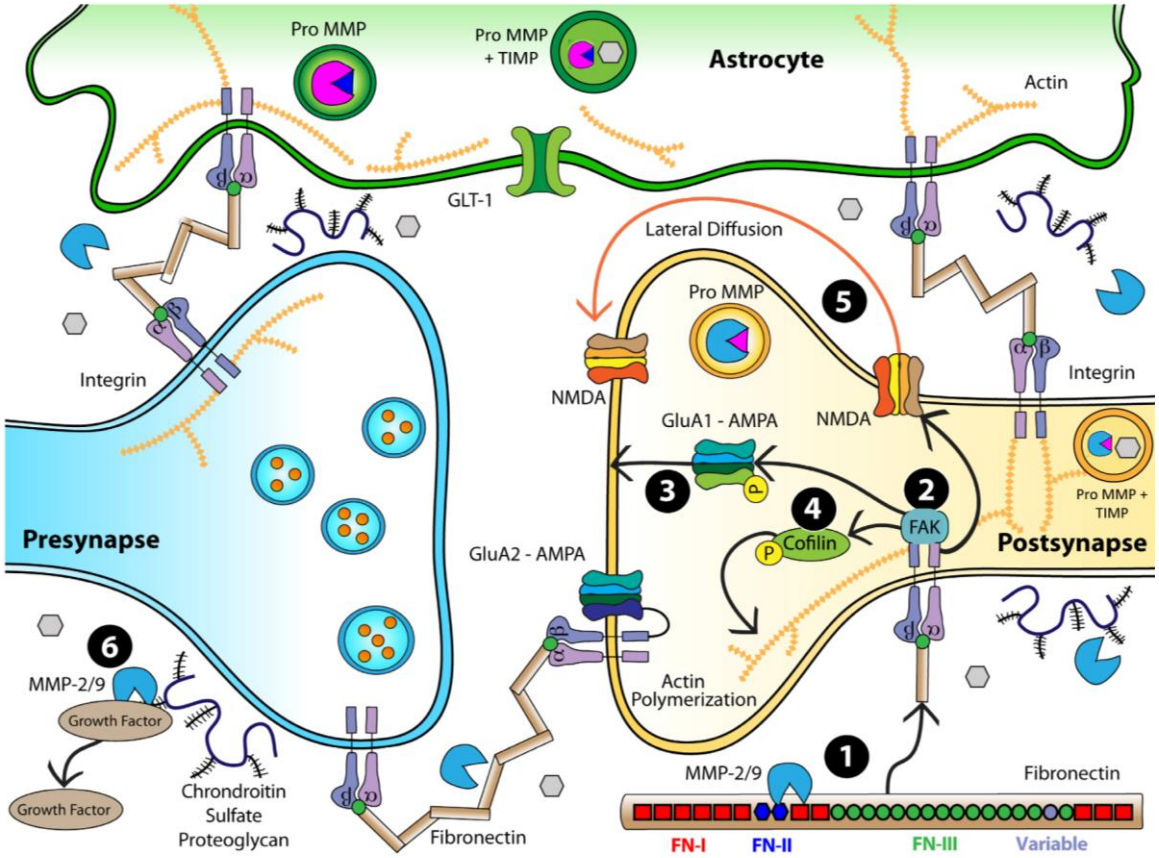

Figure 1-4. Schematic of Extracellular MMP Signaling at Tetrapartite Synapse. (1) Active MMP-2/9 proteolytically cleave ECM molecules containing FN-II repeat domains, such as fibronectin (brown rectangles). Enlarged fibronectin molecule (bottom right) contains FN-I, FN-II, and FN-III domains. Cleavage of FN-II domain reveals RGD-domain, which serves as a ligand for cellular adhesion molecules (i.e. integrins). **(2)** Increased neuronal excitability, such as from cued-drug seeking, promotes MMP activity and downstream intracellular signaling through focal adhesion kinase (FAK). Stimulation of FAK promotes many subsequent cascades including **(3)** phosphorylation of GluA1 S845 to increase AMPAR insertion at postsynaptic membrane, **(4)** phosphorylation of cofilin which regulates actin polymerization, and **(5)** translocation of NMDAR via lateral diffusion to synapse. **(6)** Active MMPs can also digest chondroitin sulfate proteoglycan to release latent growth factors involved in synaptic plasticity. Endogenous inhibitors of MMPs, tissue inhibitors of metalloproteinases (TIMPs; gray hexagons), can be co-released with MMPs in 160-200nm vesicles localized in astrocytes and somatodendritic region of neurons (modified from (Smith, Scofield et al. 2015)).

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1.2 Matrix Metalloproteinase Involvement in Synaptic Plasticity

1.2.1 Matrix Metalloproteinase: Structural and Regulatory Properties

Approximately 23 different MMPs exist in human genome, all with similar and canonical domain structures (i.e. from N-terminus to C-terminus: signal peptide, pro-peptide, catalytic domain, hinge region, and hemopexin domain), despite slightly different motifs making them distinct from each other (Beroun, Mitra et al. 2019). For instance, the gelatinase family (gelatinase A and B, MMP-2 and MMP-9, respectively) are distinct due to the type-II fibronectin domain giving them the ability to cleave fibronectin-containing proteins that contain Arg-Gly-Arp (RGD) domains (Verslegers, Lemmens et al. 2013). These RGD domains serve as endogenous ligands for integrin cell-adhesion molecules (Figure 1-4) (Verslegers, Lemmens et al. 2013, Smith, Scofield et al. 2015). The catalytic region is highly conserved across MMP species, thus why they share similar substrate profiles (Beroun, Mitra et al. 2019). The hemopexin domain is responsible for substrate and target protein interactions via connection with active site by hinge region (Huntley 2012). Majority of MMPs are secreted extracellularly (from neurons, astrocytes and microglia), except for seven that are membrane-bound by a transmembrane domain (Huntley 2012).

MMP expression is tightly regulated at the level of transcription and translation via a variety of mechanisms. AP-1 and NF-KB positively regulate MMP mRNA expression by binding at their respective promoter sites along the MMP gene (Sternlicht and Werb 2001, Dziembowska and Wlodarczyk 2012, Smith, Scofield et al. 2015). While transcriptional regulator, Yin Yang 1, can strongly repress MMP-9 transcription in rodent hippocampus (Rylski, Amborska et al. 2008). Post-transcriptionally, both nitric oxide and micro-RNA (i.e. miR-29 for MMP-2 and miR-212 for MMP-9), control stability of MMP mRNA (Löffek, Schilling et al. 2011, Smith, Scofield et al. 2015). It has also been noted

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that MMP mRNA can translocate in dendrites towards regions actively engaged in synaptic transmission, further facilitating the ability of MMPs to be released nearby sites undergoing excitatory transmission (Dzwonek, Rylski et al. 2004).

At the protein level, MMP activity is focally inhibited by reversible binding of their secreted endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs). The four isoforms of TIMPs (TIMP-1, -2, -3 and -4) bind to MMPs in 1:1 ratio at the MMP catalytic domain (Vafadari, Salamian et al. 2016). While TIMPs exhibit significant overlap in MMP inhibition, TIMP-1 has specific affinity to MMP-9 and TIMP-2 has affinity to MMP-2 (Vafadari, Salamian et al. 2016, Sánchez-Pozo, Baker-Williams et al. 2018). Like MMP-9, TIMP-1 expression is inducible upon neuronal stimulation, while TIMP-2 expression is most abundant in the adult brain and not upregulated by cellular activity (Dzwonek, Rylski et al. 2004, Smith, Scofield et al. 2015). Interestingly, either proMMP-2 or -9, is co-released with TIMP-1 in distinct 160-200 nm vesicles localized at the somatodendritic region of neurons as well as from astrocytes (Figure 1-4) (Huntley 2012, Beroun, Mitra et al. 2019). This strategic co-localization further supports the notion of focal proteolytic activity with coupled regulatory inhibition.

The balance between MMP/TIMP interaction regulates ECM proteolysis and turnover, which can contribute to physiological vs pathological MMP degradation (Arpino, Brock et al. 2015). MMP/TIMP interaction can remodel the pericellular environment by regulating the cleavage of extracellular matrix proteins, cell surface components, neurotransmitter receptors, and growth factors that mediate cell adhesion, synaptogenesis, synaptic plasticity, and long-term potentiation (Mizoguchi, Yamada et al. 2011). Independent of their ability to inhibit MMPs, TIMPs can act as growth factors and regulate cell apoptosis (Mannello and Gazzanelli 2001, Visse and Nagase 2003).

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MMPs are released as inactive enzymes (i.e. zymogens), thus embodying their digestive behavior extracellularly upon activation by disruption of cysteine-zinc interaction by either (1) cleavage of pro-domain or (2) post-translational modifications such as S-nitrosylation (Dziembowska and Wlodarczyk 2012, Smith, Scofield et al. 2017). Uniquely, MMP-2 is also activated via TIMP-2 and MMP-14 (MT1-MMP) interacting to form a tertiary complex at the cell surface membrane. Specifically, MT1-MMP dimerizes and links to TIMP-2 and pro-MMP-2, which causes cleavage of the pro-peptide region to produce mature MMP-2 (Hernandez-Barrantes, Toth et al. 2000). While MMP-9 can be activated by creating a complex with MMP-3 and tissue plasminogen activator (tPA), it can also be induced by active MMP-2 via cleavage of its pro-domain (Fridman, Toth et al. 1995, Huntley 2012). Pro-MMP-9 can also be activated by other MMPs such as 7, 13, and 26 (Toth, Chvyrkova et al. 2003).

1.2.2 Matrix Metalloproteinase: Physiology and Synaptic Plasticity

MMP-2 and MMP-9 are two of the most well-studied MMP species of the brain (Verslegers, Lemmens et al. 2013). MMP-2 is constitutively expressed, while MMP-9 is transiently inducible in an activity-dependent manner (Smith, Kupchik et al. 2014). While MMP-2 is known to be perisynaptically localized and involved in proteolytic cleavage of ECM, MMP-9 is well-characterized as an essential factor of excitatory synaptic reconfiguration (Nagy, Bozdagi et al. 2006, Gawlak, Górkiewicz et al. 2009). Most substrates of MMP-9 include CAMs such as β-dystroglycan, intercellular adhesion molecule-5 (ICAM-5), nectin-3, and neuroligin-1 (Michaluk, Kolodziej et al. 2007, Tian, Stefanidakis et al. 2007, Kaczmarek 2018). However, as indicated earlier, fibronectin-containing ECM milieu also serve as targets (Li, Liu et al. 2015). In the hippocampus, MMP-9 expression and activity are upregulated during maintenance phase of LTP at CA3-CA1 synapses (Nagy, Bozdagi et al. 2006). However, TIMP-1 abolishes this MMP-

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9-dependent LTP in the PFC (Okulski, Jay et al. 2007). As stated above, LTP is indicative of synaptic strength mediated by modification in expression and function of NMDARs (Scofield, Heinsbroek et al. 2016). Increases in MMP-9 proteolytic activity facilitates surface trafficking of NMDARs, which is mediated through integrin β1-dependent pathway (Michaluk, Mikasova et al. 2009).

In addition to changes in glutamatergic transmission and receptor expression, MMP-9 activity can facilitate dendritic spine reconstruction (Stawarski, Stefaniuk et al. 2014). Using a combinatorial 2-photon imaging and whole-cell patch clamp approach, Wang et al. established MMP-9 is both necessary and sufficient to promote spine head expansion and subsequent synaptic potentiation of field potentials in hippocampal neurons (Wang, Bozdagi et al. 2008). Szepesi et al. further endorsed this claim with data indicating MMP-9 activity-dependent formation of mature spines and insertion of postsynaptic AMPARs upon chemically induced LTP (Szepesi, Bijata et al. 2013). Interestingly, other follow-up studies appeared to observe the opposite spine effect. For instance, transgenic rats overexpressing an autoactivating mutant of MMP-9 under control of synapsin 1 promoter display elongated and thinner dendritic spines *in vivo*, specifically less mushroom spines (Michaluk, Wawrzyniak et al. 2011). This finding corroborated previous studies indicating a transformation from mushroom- to filopodia -like dendritic spines in cell culture with recombinant MMP-9 application (Bilousova, Dansie et al. 2009). In addition, live imaging of organotypic hippocampal cultures showed significant increase in spine length:width parameter (i.e. measure of spine shape) following incubation with active recombinant MMP-9 (Michaluk, Wawrzyniak et al. 2011). MMP-9 enzymatic activity causes these changes in dendritic spine morphology in dissociated cultures, but no changes in dendritic arbor (i.e. dendritic structure and number of neurites) (Michaluk, Wawrzyniak et al. 2011). As previously stated, MMP-9

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proteolytic activity influences spine morphology through involvement of β1 integrin because inhibition of this subunit completely abolishes the recombinant MMP-9-induced increase in length:width (Michaluk, Wawrzyniak et al. 2011). Pivotal work by Magnowska et al. resolved this discrepancy by nicely characterizing a key intermediary step in the MMP-9-dependent changes in dendritic spine morphology. It is transient MMP-9 proteolytic activity and subsequent TIMP-1 inhibition that causes dendritic spine maturation and maintenance of LTP (Magnowska, Gorkiewicz et al. 2016). So, while MMP-9 protease activity is needed to initiate synaptic structural and functional reconstruction (i.e. elongation), it is endogenous proteolytic inhibition that terminates the plasticity (i.e. maturation).

1.3 MMPs and Pathological Diseases

While MMPs are involved in many disease states and neuropsychiatric disorders such as schizophrenia, stress, stroke, depression, traumatic brain injury and Alzheimer's disease, below I highlight a few pathologies (i.e. cognitive abnormalities, epilepsy, and Fragile X Syndrome) given their well-studied involvement in neurogenesis and synaptic plasticity (Rivera, Khrestchatisky et al. 2010, Vafadari, Salamian et al. 2016).

1.3.1 Learning and Memory Disturbances

MMP involvement in LTP thus identifies them necessary in learning and memory formation. MMP-9 null mutant mice, but not MMP-2 exhibit impaired long-term memory (Mizoguchi, Ibi et al. 2010), and hippocampus-dependent associative learning (Nagy, Bozdagi et al. 2006). Furthermore, intracerebroventricular administration of FN-439, a broad spectrum MMP inhibitor, disrupts reconsolidation of fear memories, but not consolidation of freezing during cue presentation (Brown, Wilson et al. 2009). Many studies have also discovered MMP involvement in spatial memories. Meighan and colleagues found that MMP-9 expression is transiently increased in hippocampus during

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acquisition in Morris water maze tests, but inhibition of MMP activity with MMP-9 antisense or FN-439 alters LTP and prevents acquisition of spatial memories during water maze test (Meighan, Meighan et al. 2006). Similar results were found with direct MMP inhibition in dorsal hippocampus (Wright, Brown et al. 2007). Interestingly, TIMP-1 deficient mice fail to learn odor-related task, suggesting a necessary balance between MMPs and TIMPs is required in learning (Chaillan, Rivera et al. 2006).

Human cognitive processes also employ MMP-9. In a human model of fear conditioning, doxycycline administration (i.e. MMP-9 inhibitor) during fear acquisition reduced startle response and fear memory retention measured 1-week after testing (Bach, Tzovara et al. 2018). Furthermore, depressed individuals with impaired cognitive function exhibited decreased mRNA and protein levels of MMP-2, MMP-9, and TIMP-2 compared to healthy subjects (Bobińska, Szemraj et al. 2016). Interestingly, a positive correlation was found between mRNA and protein expression of all three genes with cognitive performance, specifically working memory, executive function, attention, verbal fluency, and auditory-verbal memory (Bobińska, Szemraj et al. 2016). A similar correlation between serum MMP-9 levels and decision-making skills are found in bulimic patients (Matsumoto, Hirano et al. 2017).

1.3.2 Epilepsy

Epilepsy is characterized by persistent bouts of seizures, which are due to genetic factors, structural/metabolic conditions in the brain, or idiopathic causes (Mizoguchi, Yamada et al. 2011, Beroun, Mitra et al. 2019). MMPs may be contributing to this condition via blood-brain barrier disruption, inflammatory processes, and/or synaptic plasticity (Beroun, Mitra et al. 2019). Studies from nearly two decades ago have indicated MMP-9 expression and activity are both upregulated in hippocampus dentate gyrus following single-dose injection of kainic acid, a preclinical chemical stimulation

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model of epilepsy (Szklarczyk, Lapinska et al. 2002). Similar results have been found 2-hr post-seizure episode induced by pentylenetetrazole, $GABA_A$ antagonist (Rylski, Amborska et al. 2009). Moreover, further studies have discovered MMP-9-deficient kainite-treated mice exhibit reduced levels of synaptic pruning compared to wildtypes. Thus, lack of MMP-9 appears protective, deeming dendritic spines more resistant to kainite-evoked pruning and decreased aberrant synaptogenesis (Wilczynski, Konopacki et al. 2008).

In humans, many studies observed significantly higher serum and cerebrospinal fluid (CSF) levels of MMP-9 in epilepsy patients compared to healthy controls, likely due to breakdown of BBB and subsequent leakage of these enzymes into the periphery (Suenaga, Ichiyama et al. 2008, Li, Wang et al. 2013, Tao Huai, Gong Yuji et al. 2020). In addition, alterations in MMP-9/TIMP-1 ratio arise in epileptic states, further contributing to increased levels of MMP-9 in serum (Suenaga, Ichiyama et al. 2008). Thus, MMP-9 as well as other inflammatory factors can be use as biomarkers of seizure disorders and potentially lead to proper diagnosis and treatment.

1.3.4 Fragile X Syndrome

Fragile X Syndrome (FXS), an extensively studied condition linked to autism, is due to full mutation of *FMR1* gene, which encodes fragile X mental retardation protein (FMRP) (Abbeduto, McDuffie et al. 2014, Vafadari, Salamian et al. 2016). Lack of FMRP produces FXS and subsequent symptoms such as intellectual disability, developmental delays and compulsive behaviors (Vafadari, Salamian et al. 2016). FMRP, a translation repressor, regulates translation of proteins involved in synaptic plasticity, hence the relationship with MMP proteolytic activity (Janusz, Milek et al. 2013). Local transport of MMP-9 mRNA to synapses is due in part to FMRP involvement. Animal models of FXS (*FMR1*-knockout) in cultured neurons and mice found increased levels of MMP-9 activity

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and mRNA at the synapse, potentially contributing to the aberrant ECM digestion and synaptic architecture (Bilousova, Dansie et al. 2009, Janusz, Milek et al. 2013). Transgenic approaches utilizing dual MMP-KO/*FMR1*-KO mice alleviated autism-like symptoms of FXS animal model (Sidhu, Dansie et al. 2014). Pharmacological approaches with minocycline (i.e. MMP inhibitor) can also reverse associated FXS behavioral symptoms as well as accompanying pathological elongated dendritic spines (Nagaoka, Takehara et al. 2016). Another pharmacological target is eukaryotic initiation factor 4E (eIF4E), which is increasingly phosphorylated in FXS patients and subsequently regulates translation of MMP-9 (Gkogkas, Khoutorsky et al. 2014). As a result, eIF4E inhibition also rescues abnormal FXS phenotype in KO mice (Gkogkas, Khoutorsky et al. 2014).

Similar patterns of MMP-9 expression are also found in human FXS patients. For example, excessive levels of serum MMP-9 and associated abnormal dendritic spine maturation are seen in this patient population (Dziembowska, Pretto et al. 2013, He and Portera-Cailliau 2013). Many promising clinical trials hint at minocycline as a potential treatment option for FXS patients, given its ability to reduce MMP-9 expression, restore abnormal dendrite morphology and ultimately improve FXS symptoms (Paribello, Tao et al. 2010, Siller and Broadie 2012, Dziembowska, Pretto et al. 2013, Leigh, Nguyen et al. 2013).

1.4 MMPs and Opioid Addiction

Given MMP involvement in synaptic reconfiguration, their role in pathological drug addicted behaviors has been explored for over a decade. In 2003, Wright and colleagues first implicated MMP proteolytic ECM degradation in addiction, specifically alcohol-induced disruption of spatial memory due to decreased MMP-9 levels (Wright, Masino et al. 2003). Many follow-up studies after this discovery have investigated the

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role of MMPs in methamphetamines, cocaine, nicotine, and opioids (Brown, Forquer et al. 2007, Mizoguchi, Yamada et al. 2007, Brown, Forquer et al. 2008, Nakamoto, Kawasaki et al. 2012, Natarajan, Harding et al. 2013, Smith, Kupchik et al. 2014, Smith, Scofield et al. 2015) (see (Smith, Scofield et al. 2015) for review).

MMP-2 and MMP-9 of the gelatinase family, and have been investigated for their role in drug extinction and reinstatement using *in vivo* zymography, an assay in which FITC-quenched gelatin acts as a substrate for proteolytic cleavage by activated MMP-2 and 9 (see Figure 2-1 in Methods for detailed description) (Huntley 2012, Smith, Kupchik et al. 2014). Our lab showed that MMP-9 activity transiently increases during cue-induced heroin reinstatement (Smith, Kupchik et al. 2014), which is required for transient synaptic potentiation in NAcore associated with reinstated cocaine seeking (Smith, Kupchik et al. 2014). Since cued heroin seeking also potentiates NAcore MSNs, MMP-9 involvement in heroin seeking seems probable.

Chronic morphine treatment activates MMP-9 in spinal cord, which contributes to its antinociceptive effects (Nakamoto, Kawasaki et al. 2012) and attenuates symptoms of morphine withdrawal (Liu, Han et al. 2010). MMP-9 activation in both spinal cord and NA by opioids or opioid-associated cues results from increased nitric oxide synthesis, possibly secreted from microglia (Liu, Han et al. 2010). Further studies clarifying whether chronic morphine induces microglial MMP-9 and nitric oxide release in the spinal cord would contribute to our understanding of the development of morphine tolerance. Moreover, MMP-9 activation in spinal cord and NA signals into cells via β1- and β3-integrin, respectively (Liu, Han et al. 2010, Garcia-Keller, Neuhofer et al. 2019), and in NA this signaling is necessary for reinstated cocaine seeking (Garcia-Keller, Neuhofer et al. 2019). Our lab also discovered that inhibiting focal adhesion kinase (FAK) phosphorylation, a downstream β3-integrin signaling mediator, suppressed cued heroin

[1]Adapted from: Kruyer A.*, Chioma V.C.*, Kalivas P.W. (2020) The Opioid Addicted Synapse. Biological Psychiatry 87(1):34-43

seeking (Garcia-Keller, Neuhofer et al. 2019), exemplifying involvement of MMP-9-dependent intracellular cascades in synaptic plasticity. Following heroin extinction, the perineuronal net (PNN), comprised of ECM proteins such as Tenascin and Brevican, is reduced in mPFC and NA (Van den Oever, Lubbers et al. 2010). Interestingly, cued-heroin seeking restores PNN protein expression, and a broad-spectrum MMP inhibitor attenuates cued heroin seeking (Van den Oever, Lubbers et al. 2010). The apparent paradox of increasing ECM synaptic proteins simultaneous with increasing MMP-9 is possible because PNN proteins are not substrates for MMP-9 catalytic activity (Theocharis, Skandalis et al. 2016, Stamenkovic, Stamenkovic et al. 2017). Thus, this suggests the role of differential ECM milieu, in addition to MMPs, involved in synaptic plasticity facilitating heroin seeking behavior.

[1]Adapted from: Kruyer A.*, Chioma V.C.*, Kalivas P.W. (2020) The Opioid Addicted Synapse. Biological Psychiatry 87(1):34-43

Chapter 2. Statement of the Problem

Opioid addiction is a major epidemic afflicting millions of individuals in the United States with abuse ranging from prescription pain medications to illicit opioid-derived compounds (Rudd, Seth et al. 2016, Han, Compton et al. 2017, Seth, Scholl et al. 2018). Repeated opioid use dysregulates glutamate homeostasis in tetrapartite synapse of NAc, which serves as a predisposing factor to relapse behavior after periods of withdrawal from drug use (Kalivas 2009, Scofield, Heinsbroek et al. 2016). Thus far, my dissertation made clear that understanding the molecular mechanisms underpinning how the tetrapartite compartments interact to regulate one another is necessary for understanding normal synaptic plasticity, as well as the aberrant physiology induced by opioids. The preclinical data justify this research direction as a potential source of novel pharmacotherapies for OUD. However, there is need for a larger research effort into fundamental mechanisms of tetrapartite synaptic integration before we will likely be able to develop comprehensive biological rationales for reversing opioid-induced tetrapartite pathophysiology as a means to control OUD.

Given the recent discoveries on MMP involvement in addiction, there is a significant number of unknowns to be uncovered. Perhaps one approach in further understanding opioid-induced changes to extracellular matrix elements could be to elucidate cell-specific changes that arise. In NAc, 90-95% of neuronal cell populations are D1- and D2-receptor expressing GABAergic medium spiny neurons (MSNs) (Gerfen and Surmeier 2011). Classically, these two cell types regulate opposing patterns of behavior, in which D1 MSNs activate/engage behavior, while D2 inhibit behavior. In addition, astroglial cells exhibit dynamic morphological adaptations during heroin seeking (Kruyer, Scofield et al. 2019). While it is known increased MMP activity localized at the level of the soma and dendritic processes of NAcore arises during cued drug

seeking, it is unclear which accumbens cell-types are responsible for bearing this MMP activity.

The work outlined in this dissertation aims to investigate (1) which specific cell populations of the tetrapartite synapse harbor(s) MMP activity during cue-induced heroin seeking, and (2) determine the functional role of cell-specific MMP activity and its relationship to synaptic plasticity. My main hypothesis is D1-MSNs and astroglial cells exhibit increased MMP gelatinolytic activity during heroin seeking, while D2 MSNs exhibit increased MMP gelatinolytic activity following withdrawal. These data will elucidate how the microcircuit underlying t-SP relies on specific cell-types, thus pointing at potential therapeutic drug targets to treat heroin addiction.

Chapter 3. Materials and Methods

Animals and Housing Care

98 male and female Long Evans D1 and D2-Cre (+) transgenic rats (cre recombinase under control of the dopamine receptor D1 or D2 promoter; provided by National Institute of Drug Abuse) and 42 Cre (-) wildtype littermates, were individually housed using a 12:12 hr dark/light cycle with food and water made available ad libitum in a temperature and humidity-controlled environment. All experimentation occurred during the dark phase, and animals were allowed to acclimate to the vivarium environment for a week prior to surgery.

Surgery: Catherization and Virus Transfection

Rats were ~60 days old (~250 g) when they were anesthetized with isoflurane (i.e. 5% concentration during induction, 2-3% concentration maintenance during surgery) before being implanted with indwelling jugular vein catheters, and received ketorolac (2 mg/kg, ip) as a pre-operative analgesic as well as the antibiotic cefazolin (10 mg/kg, ip). Following catherization, rats were stereotaxically implanted with bilateral guide cannulae (Plastics One Inc., 18-gauge) above the NAcore (1.5mm AP, 1.8mm ML, 5.5mm DV). Dummy injectors were placed in cannulae to maintain patency for future microinjections.

Immediately following guide cannula implantation, D1 and D2 medium spiny neurons were virally transfected with AAV1-EF1a-dflox-hChR2-mCherry (Addgene; 0.75 µL/hemisphere, 0.15 µL/min, 10-minute diffusion time). Additionally, in separate wildtype animals astroglia cells were selectively labeled using a glia fibrillary acidic protein (GFAP) promoter with AAV5-GFAP-hM3dq-mCherry (University of Zurich, Zurich, Switerland; 1.0 µL/hemisphere, 0.15 µL/min, 5-minute diffusion time). For experiments in this manuscript, ChR2 and hM3dq (Gq-coupled DREADD) were solely used for

membrane-targeting of mCherry promoter and were not activated. Both viruses were injected in NAcore with 26-gauge microinjectors (Plastics One Inc.). Virus transfection occurred during surgery recovery and during the length of self-administration behavior (~4 weeks).

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Assessment and Accreditation of Laboratory Animal Care.

Drugs and reagents

Heroin HCl powder, provided by the National Institute of Drug Abuse, was dissolved in sterile normal saline. Both TIMP-1 and -2 antibodies (R&D Systems, #AF970 and #AF971, respectively) were utilized for their previously published capacities to inhibit TIMP-1 and -2 (Reikvam, Hatfield et al. 2010, Lu, Liao et al. 2019). The working dose of neutralizing antibody was a combination of equal parts of TIMP-1 (3 µg/mL) and TIMP-2 (3 µg/mL). This dose was selected based on neutralizing 50 (ND_{50}) values for both compounds (ND_{50}=1 µg/mL for TIMP-1 and ND_{50}=2.6 µg/mL for TIMP-2; obtained from product datasheet; R&D Systems). Normal Goat IgG (6 µg/mL) was used as a control reagent (R&D Systems, #AB-108-C). MMP-2i (Millipore-Sigma, #444288; 0.1 nmol; dissolved in 1% DMSO, IC_{50}=12 nM and IC_{50}=0.2 µM for MMP-9 (Rossello, Nuti et al. 2004)). MMP-9i (Millipore-Sigma, #444278; 0.1 nmol; dissolved in 1% DMSO, IC_{50}=5 nM (Levin, Chen et al. 2001)). Dye-quenched fluorescein conjugate gelatin (Thermo Fisher Scientific, #D12054; 1 mg/ml in phosphate buffered saline PBS; 1.5 µl/hemisphere) was used for *in vivo* zymography assay.

Heroin Self-administration, Withdrawal, and Cued Reinstatement

Rats underwent daily three-hour heroin self-administration sessions in two-lever operant chambers. Each active lever press delivered one 100 µg (days 1-2), 50 µg (days 3-4), or 25 µg (days 5-10) per IV infusion of heroin (FR1) paired with cues (light and tone) followed by a 20s timeout. The descending dosing protocol was used to facilitate the acquisition of heroin self-administration and to increase the number of active lever presses emitted by the end of self-administration training (Zhou and Kalivas 2008). Rats self-administered heroin for 10 days reaching criteria of ≥10 infusions/day on at least the last 3 days, followed by withdrawal (i.e. either extinction training or home-cage abstinence for 10 days). During extinction training, levers were presented and counted but did not trigger infusion of heroin or presentation of cues. Extinction proceeded for 10 training sessions or until rats reached the extinction criteria of ≤25 active lever presses for two consecutive days before a reinstatement test day. Home cage abstinence animals were handled and weighed daily and kept in behavioral room with operant chambers while their counterparts engaged in extinction training. This controlled for effects of daily handling. Tissue collection occurred either 24 hours after last day of extinction or abstinence or during a cued reinstatement test (15 or 120 mins) in which delivery of light and tone cues was restored to active lever pressing, but not heroin infusions. For one cohort of animals, after 10 days of extinction training, rats underwent "refraining" behavioral testing where rats were placed in extinguished operant chamber (15 mins) and no programmed consequences (i.e. cues or drug) were delivered with active lever pressing. Some experiments employed yoked-saline controls that were catheterized but received saline infusions in a randomized, noncontingent manner.

In vivo zymography

As demonstrated in previous work from our lab (Smith, Kupchik et al. 2014), *in vivo* zymography is utilized to assess changes in MMP function. Dye-quenched FITC-gelatin (Thermo Fisher Scientific) (reconstituted in PBS to 1 mg/mL, pH 7.2-7.4; 3.0µl of gel; 1.5µl/side) was microinjected, 2.0 mm beyond base of implanted guide cannula, with 15 mins incubation time in home cage or the operant testing environment depending on the experiment group. Subsequently, an overdose of pentobarbital (3.9 mg/mL, ip) was administered and animals were transcardially perfused with 4% paraformaldehyde (PFA). Brains were removed, placed in 4% PFA overnight for additional fixation and a vibratome was used to obtain 100 µm sections, containing the injection track and anterior commissure (AC), through the NAcore. Sections are then either mounted and coverslipped or stained via immunohistochemistry (see below) and then subsequently imaged on a Leica SP5 (Wetzlar, Germany) or Carl Zeiss LSM 880 (Oberkochen, Germany) laser scanning confocal microscope. Only slices containing both the anterior commissure (landmark for NAcore) and injection site were imaged and analyzed by ImageJ software (NIMH, Bethesda, MD) (Figure 3-1A). Fluorescence is quantified bilaterally as integrated density from four-seven sections/rat. The integrated densities were averaged for each rat and normalized to yoked-saline control values.

Immunohistochemistry

Tissue sections were obtained as described above for zymography, and cell membranes were made porous using 1x PBS with 0.5% Triton-X-100 for 10 minutes at room temperature, then blocked with 1x PBS with 2% Triton-X-100 (PBST) with 2% normal goat serum for 45 minutes. Immunohistochemistry was conducted against the mCherry reporter protein to amplify virally-labeled D1 and D2 MSNs. Primary antibodies against mCherry (Rb anti-dsRed, 1:500; Takara #632496 or Mouse anti-mCherry,

1:1000; LSBio #204825) were incubated for 48 hours at 4˚C with gentle agitation. For detection of TIMP-1 and TIMP-2 expression, sections were incubated with monoclonal primary antibodies against TIMP-1 (1:500; Abcam #18352) and TIMP-2 (1:200; Thermo Fisher Scientific #12207). Tissue virally transfected with GFAP-mCherry virus to selectively label astrocytes were incubated with synapsin I antibody (1:1000; Abcam #64581) and mCherry was visualized without antibody amplification. Following incubation with primary antibodies, slices were washed with PBST and incubated overnight at room temperature in respective species-specific fluorescent secondary antibodies (Alexa Fluor; 1:1000; Thermo Fisher Scientific). After washing in PBST, slices were mounted and coverslipped using ProLong Gold Antifade Mountant (Thermo Fisher Scientific).

Confocal Microscopy Imaging

Z-stacks were acquired using a Leica SP5 or Carl Zeiss LSM 880 laser scanning confocal microscope equipped with Argon (Ar 488 nm), Helium-Neon (He-Ne 543 nm) or Helium-Neon (He-Ne 633 nm) laser lines.

Following quantification of MMP activity at low magnification (10x), 2 or 3 sections with the highest integrated density are selected for higher magnification single cell imaging. Dendritic segments or astroglia are imaged ≤500 µm from the microinjector tip (“zymography zone”), with most images being collected between 250-500 µm frequent saturation of the fluorescent signal <250 µm from the injector tip (see Figure 3-1A). This range was selected due to majority of zymography signal being localized within this range, as well as slight differences in injector track orientation and virus expression throughout tissue section. As stated previously, cells were only imaged from brain sections containing both the microinjector track and anterior commissure in the frame to ensure that the individual cells being quantified were in NAcore subcompartment.

Dendritic spine morphology image acquisition criteria were based on parameters previously described in (Shen, Sesack et al. 2008). Only spiny dendrites located 75-200 µm from the soma and after the first branch point were imaged (see Figure 3-1B-D). Segment lengths were between 55-70 µm in length, and each dendrite segment was traced back to a different soma, thus there was one dendrite segment quantified per cell. Four to eight dendrites were imaged/animal at 63x using oil immersion objective lens at the following acquisition settings: 1024 x 256 frame size, 12-bit image resolution, 4-frame average, 0.21-µm step size, and digital zoom of 3.5x on Leica SP5 or 2.0x Carl Zeiss LSM 880 confocal microscope. Captured images were then deconvolved with Autoquant (Media Cybernetics, Rockville, MD) or processed with paired Zeiss Airyscan super-resolution deconvolution ZEN software. Following deconvolution, digital analysis of individual dendrites was processed using Imaris (version 8; Bitplane, MA) with three-dimensional (3D) space-filling Surface module to calculate % MMP gelatinolytic puncta localized within 300 nm of the dendrite surface.

Astrocytes were acquired at 63x using an oil immersion objective lens, 1024 x 1024 frame size, 12-bit image resolution, 4-frame averaging, 1-µm step size, and 1x digital zoom on Leica SP5. Three to eight astrocytes were imaged/animal. Acquired images were iteratively deconvolved 10 times (Autoquant) and digital analysis of mCherry signal intensity relative to background was used to generate a digital model of each astrocyte and corresponding extracellular MMP activity (Bitplane Imaris). All imaging and analyses were conducted by investigator blind to animal treatment groups.

The first experiments conducted are shown in figures 4-4, 4-5, 4-6 (SAL, EXT, and RST 15’ groups), and 6-2 were performed using Leica SP5 confocal. Subsequent experiments shown in figures 4-4, 4-5, 4-6 (ABS, REF 15, and RST 120’ groups), 4-9, 4-10, 5-1, 5-2, and 5-5 were imaged using Zeiss confocal.

Imaris 3D Reconstruction, Morphological Analyses and Colocalization Quantification

The following sections describe quantification methodology utilizing Imaris software analysis (see Figure 3-1E-G and 3-2 for illustration). All analyses were conducted by investigators blind to animal treatment groups.

Imaris 3D Processing: D1/D2 MSN MMP Activity Localization

Using Imaris Surface module, mCherry-labeled dendritic segments were cropped and isolated from background. The isolated dendrite was used to mask MMP-2/9 gelatinolytic puncta localized only within 300 nm from the surface of the labeled dendrite (i.e. extracellular activity). This cutoff was established due to the limit of lateral and axial resolution for confocal microscopy using Leica SP5, which is approximately 250 and 500 nm, respectively, and 140 and 400 nm, respectively, using Zeiss LSM 880 (Schermelleh, Heintzmann et al. 2010). Gelatinolytic puncta were empirically isolated by intensity-based thresholding in order to reveal active MMP-2/9 puncta located around segment of interest (≤ 300 nm) (Figure 3-1E). MMP gelatinolytic activity localized adjacent to dendrite surface was calculated by dividing volume (μm^3) of MMP puncta by volume (μm^3) of 300 nm around dendrite to obtain normalized index of MMP activity around an isolated dendrite (Figure 3-1F,G). This same approach was employed for quantification of extracellular TIMP-1/2 expression around virally labeled D1 and D2 MSNs.

In subsequent experiments described in Results section, dendritic spine head diameter was calculated for isolated mCherry-labeled dendrites previously quantified for % MMP activity adjacent to dendrite surface. The Filament module was used to semi-automatically trace dendritic shaft and identified dendritic spines as described previously (Siemsen, Giannotti et al. 2019). Spine head diameter was calculated using an automated threshold set by Imaris.

With the established filament trace, the “filament analysis” extension was used to create dendritic subcompartments to then determine MMP-2/9 puncta localized at the dendritic spine vs shaft with Imaris software. This extension separates the dendritic segment into compartments and the Surface module masks all MMP-2/9 puncta not corresponding to Imaris-defined dendritic spine heads and shaft. As indicated by (Siemsen, Giannotti et al. 2019), Imaris software utilizes the predefined boundaries of the spine heads relative to dendritic shaft and vice versa to isolate specific channels of interest in dendritic subcompartments. A 3D space-filling model of spine heads or shaft was generated and used to mask MMP-2/9 puncta not associated to either specific compartment (Figure 3-2). Using Surface module, MMP puncta localization was calculated similar to above for 1) spine head-specific and 2) shaft-specific MMP puncta localization by normalizing volume of compartment-specific MMP puncta (i.e. spine head or shaft) to volume of 300 nm around its specific compartment (Figure 3-1F,G). The spine head:shaft ratio was also determined by dividing MMP puncta localization values around each compartment.

Imaris 3D Processing: Astroglial Morphology and MMP Activity Co-localization

The digital astroglial model generated from mCherry signal was generated as described in detail by (Scofield, Li et al. 2016, Kruyer, Scofield et al. 2019) and used to mask out MMP-2/9 activity and Synapsin I signal that were not co-registered with the astroglial volume. Co-localization (astrocyte with Synapsin I, astrocyte with MMP-2/9 puncta, MMP-2/9 puncta with Synapsin I) was determined based on thresholded signal intensity in each channel. Voxels containing fluorescent signal intensity greater than noise in each channel were determined empirically within the colocalization module and were used to build a colocalization channel. The Imaris Surface Module was used to measure the colocalization channel characteristics, including net volume of colocalized

signal. Synapsin and MMP-2/9 puncta colocalization were normalized to the volume of the astrocyte from which they were generated. Surface-proximal MMP-2/9 puncta were determined by excluding colocalized signal >250nm from the astrocyte membrane and was normalized to total MMP-2/9 puncta from the same astroglial volume.

Statistical analysis:

All statistical analyses were conducted in GraphPad software (Prism ver 8). Behavioral data were analyzed by two-way ANOVA followed by Bonferroni post hoc tests for multiple comparisons. D'Agostino-Pearson normality test was used to determine if data were normally distributed. Kruskal-Wallis with Dunn's post hoc comparisons or Mann-Whitney test were used when one or more treatment groups in an experiment were found to be not normally distributed. Data found to be normally distribution were analyzed using a one- or two-way ANOVA. When two groups were compared, a two-tailed paired or unpaired Student's t-test was utilized. All D1/D2 cell-specific data was analyzed using either Nested t-test (two group comparison) or Nested ANOVA with post-hoc Bonferroni analysis when significant interaction was observed (multiple group comparison). In all cases, p values <0.05 were considered significant. All data except behavior was collected and quantified by investigators blind to animal treatment groups.

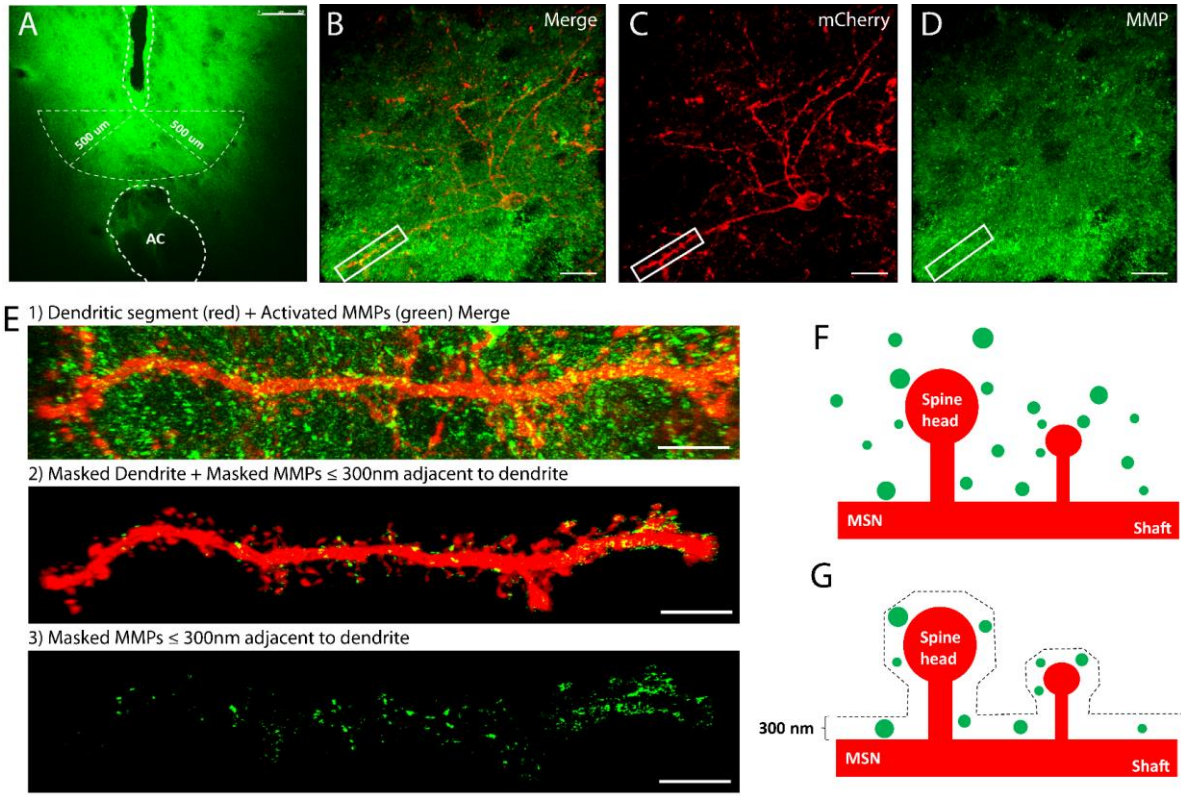

Figure 3-1. Cell-type Specific MMP-2/9 Activity Quantification Technique: Confocal Imaging and Imaris 3D Rendering. A) Representative micrograph of *in vivo* zymography assay in NAcore, 10x. White dotted lines outline the injection track and anterior commissure (AC) for ImageJ quantification. Higher magnification imaging of accumbens cell types (i.e. MSNs and astrocytes) occurs ≤500 µm from the microinjector tip ("zymography zone"), which is demarcated with white dotted semi-circle originating from microinjector tip. Scale bar=250 µm. **B)** Merge of **C)** mCherry-labeled MSN (red) surrounded by **D)** MMP activity (green) in NAcore, 63x. Dendritic spine morphology image acquisition is based on previously established criteria; white box in **B-D** indicates dendritic segment and MMP activity imaged for Imaris processing in panel E. Scale bar=20 µm. **E)** High resolution representative micrographs of Imaris 3D rendering workflow for MMP quantification around MSN, 63x. *1)* Raw confocal image of dendritic segment (red) and activated MMP gelatinolytic puncta (green). *2)* Isolated dendrite, which is used to mask out MMP puncta not within 300 nm of dendritic surface, hence within the limit of resolution (~140-250 nm) for confocal microscopy. *3)* Only masked MMPs ≤ 300 nm adjacent to dendrite shown. **F)** Model of MMP gelatinolytic puncta (green) surrounding mCherry-labeled MSN (red) with dendritic spine heads and shaft subcompartments indicated. **G)** MMP gelatinolytic puncta within 300 nm of dendrite surface were isolated and normalized to volume around dendrite in which they occupy to calculate % MMP activity around dendritic segment. Scale bar=7 µm.

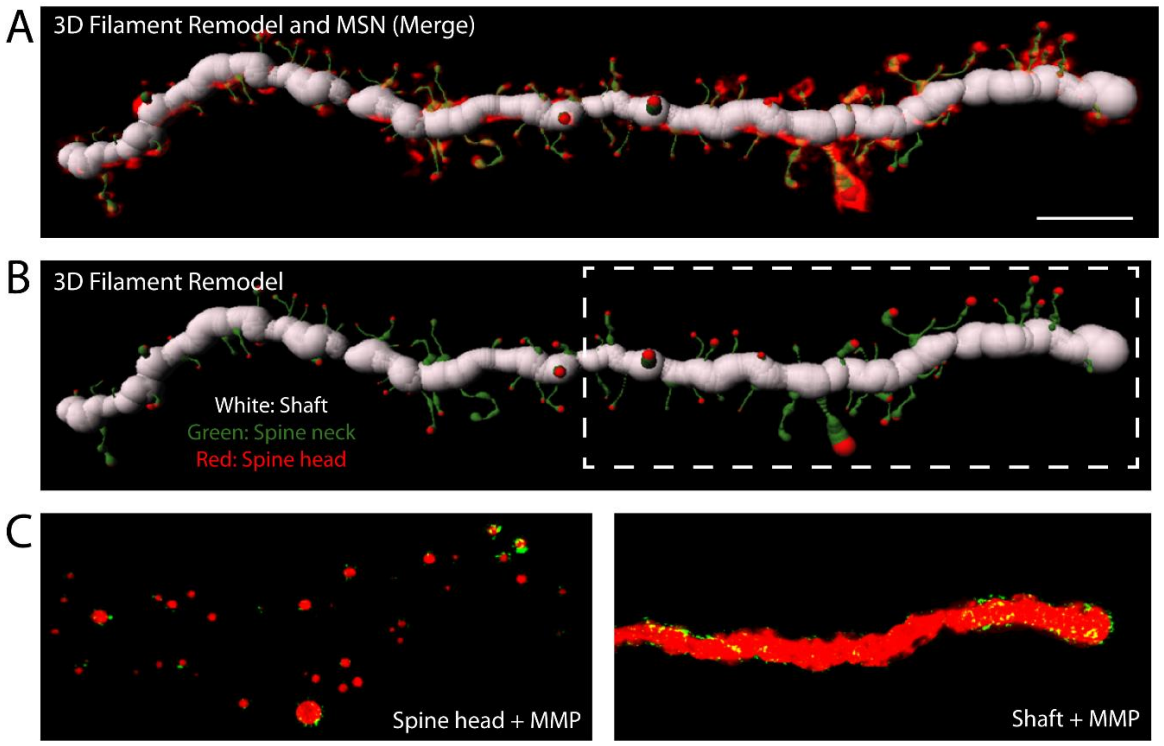

Figure 3-2. Dendritic Spine vs Shaft Subcompartment MMP-2/9 Localization Analysis. After establishing a filament trace, the "filament analysis" extension of Imaris filament module was used to isolate specific channels of interest in dendritic subcompartments (i.e. 3D filament remodel). **A)** Merge of filament remodel and isolated dendritic segment from Figure 2-1E. **B)** The 3D filament remodel recapitulates the dendritic shaft (white), spine neck (green), and spine head (red) (middle panel). White dashed box highlights region of dendrite conveyed in bottom panel. **C)** The 3D space-filling model of spine heads or shaft was used to mask MMP-2/9 puncta not associated to either the spine head (left panel) or dendrite shaft (right panel) compartments in order to calculate 1) spine head-specific and 2) shaft-specific MMP puncta localization. Scale bar=5µm.

Chapter 4. Cellular Specificity of MMP Activity around D1 and D2 MSNs in Nucleus Accumbens

Introduction

Addiction is a pervasive neuropsychiatric disease characterized by persistent use of abusive substances despite negative consequences (Koob and Volkow 2016). Opioids are the leading cause of drug overdose-related deaths with over 100,000 global deaths in 2016 (Strang, Volkow et al. 2020). Long-term use of opioids, both in clinical and pre-clinical models, dysregulates glutamatergic corticostriatal circuitry, contributing to increased relapse vulnerability and impaired ability to regulate decision-making processes (Kruyer, Chioma et al. 2020). Ventrally located in the striatum, the nucleus accumbens (NAc) serves as a critical reward processing center, mediating goal-directed and motivated behavior (Floresco 2015, Scofield, Heinsbroek et al. 2016). The NAc core subcompartment (NAcore) is comprised primarily of GABAergic medium spiny neurons (MSNs) divided into two functionally divergent classes, D1-receptor expressing and D2-receptor expressing. Classically, activating D1 MSNs promotes motivated behaviors, while D2 MSN stimulation produces the opposite (Gerfen and Surmeier 2011, Lobo and Nestler 2011, Smith, Lobo et al. 2013, Heinsbroek, Neuhofer et al. 2017).

Following chronic heroin administration, underlying synaptic plasticity mechanisms produce constitutive changes at the tetrapartite synapse (pre- and post-synapse, astroglial processes, and extracellular matrix (ECM)) contributing to impending aberrant drug seeking behavior (Kruyer, Chioma et al. 2020). It has been previously shown that persistent heroin use causes impaired long-term potentiation (LTP) and depression (LTD) (Shen and Kalivas 2013), reduced glutamate clearance (Shen, Scofield et al. 2014), changes in presynaptic strength (Hearing, Graziane et al. 2018), and alterations in postsynaptic receptor composition (Shen, Moussawi et al. 2011,

Graziane, Sun et al. 2016). These enduring neuroadaptations facilitate glutamate spillover in NAcore during cue-induced reinstatement of drug seeking behavior, and activate downstream processes contributing to transient synaptic potentiation (t-SP) on medium spiny neurons (MSNs) (Gipson, Kupchik et al. 2013, Mulholland, Chandler et al. 2016). t-SP can be quantified as increases in dendritic spine head diameter (d_h) and AMPA receptor currents of MSNs in response to drug-associated cues. Specifically, D1 MSNs exhibit potentiated dendritic spine d_h and AMPA:NMDA ratios in cocaine cue reinstating animals, while D2 MSNs exhibit elevated spine d_h and AMPA:NMDA ratios related to extinction training during cocaine-withdrawal (Bobadilla, Heinsbroek et al. 2017, Roberts-Wolfe, Bobadilla et al. 2018).

MSN potentiation is meditated by parallel transient activation of extracellular matrix metalloproteinases (MMPs), which are endopeptidases that cleave ECM milieu, a proteinaceous signaling network ensheathing the synapse (Wang, Bozdagi et al. 2008). MMP-2 and -9 belong to the gelatinase family and their activity is necessary for many events associated with synaptic plasticity, such as changes in dendritic spine morphology, NMDA receptor lateral translocation and AMPA receptor insertion into postsynaptic membranes (Michaluk, Mikasova et al. 2009, Stawarski, Stefaniuk et al. 2014, Szepesi, Hosy et al. 2014). As it pertains to addiction, transient increases in catabolic MMP-9 activity in NAcore is both necessary and sufficient to reinstate drug seeking to cocaine-conditioned cues and initiate the associated potentiation in markers of t-SP i.e. AMPA/NMDA ratio and dendritic spine diameter (Smith, Kupchik et al. 2014, Garcia-Keller, Neuhofer et al. 2019). The observed increase in MMP-9 activity can be localized at the neuronal level, both surrounding the soma and extending out along dendritic processes (Smith, Kupchik et al. 2014). While MMPs are induced around single

cells during transient drug seeking, it is unknown which cell-types of NAcore harbor this cue-induced MMP activity and whether this serves any functional significance.

To determine this, my project utilized a heroin self-administration, extinction and reinstatement (RST) model, where an animal learned to engage an active lever to receive an intravenous infusion of heroin paired with light + tone cues. Conditioned cues were later restored in the absence of heroin after multiple days of withdrawal. D1 and D2 transgenic rats expressing Cre recombinase were virally transfected with Cre-dependent mCherry virus to quantify MMP activity specifically around mCherry-labeled D1 and D2 MSNs in NAcore. I hypothesize that D1-MSNs exhibit increased MMP localization during heroin seeking, while D2 MSNs exhibit increased MMP localization following extinction.

Results

Heroin Self-Administration, Withdrawal, and Cue-Induced Seeking

Animals were trained to self-administer saline or heroin for 10 days, subsequently withdrawn for another 10 days (extinguished or home-cage abstinence), and then presented with heroin-associated cues for reinstatement testing (Figure 4-1A). There were no differences in heroin self-administration behavior (i.e. active lever presses or infusions) between D1/D2-cre (+) rats (Figure 4-1B,C). In the behavioral tests described, all lever responding was recorded for 15 mins, except for RST 120 min test. Both rat lines exhibited similar and increased cued heroin seeking behavior (i.e. active lever presses) compared to their extinction baseline and refraining (re-exposure to extinguished environment) (Figure 4-1D). Both rat genotypes also reinstated similarly during 120 min cued reinstatement (Figure 4-1E). No difference observed in inactive lever pressing during self-administration or across behavioral tests between genotypes (Figure 4-2).

In addition, no sex differences were observed in heroin self-administration behavior and infusions (Figure 4-3A,B). Compared to their extinction baseline, refraining, and 120 min reinstatement behaviors, both sexes exhibited similar and statistically significant heroin seeking behavior (i.e. active lever presses) (Figure 4-3C,E). No difference observed in inactive lever pressing during self-administration or across behavioral tests between sexes (Figure 4-3D,F).

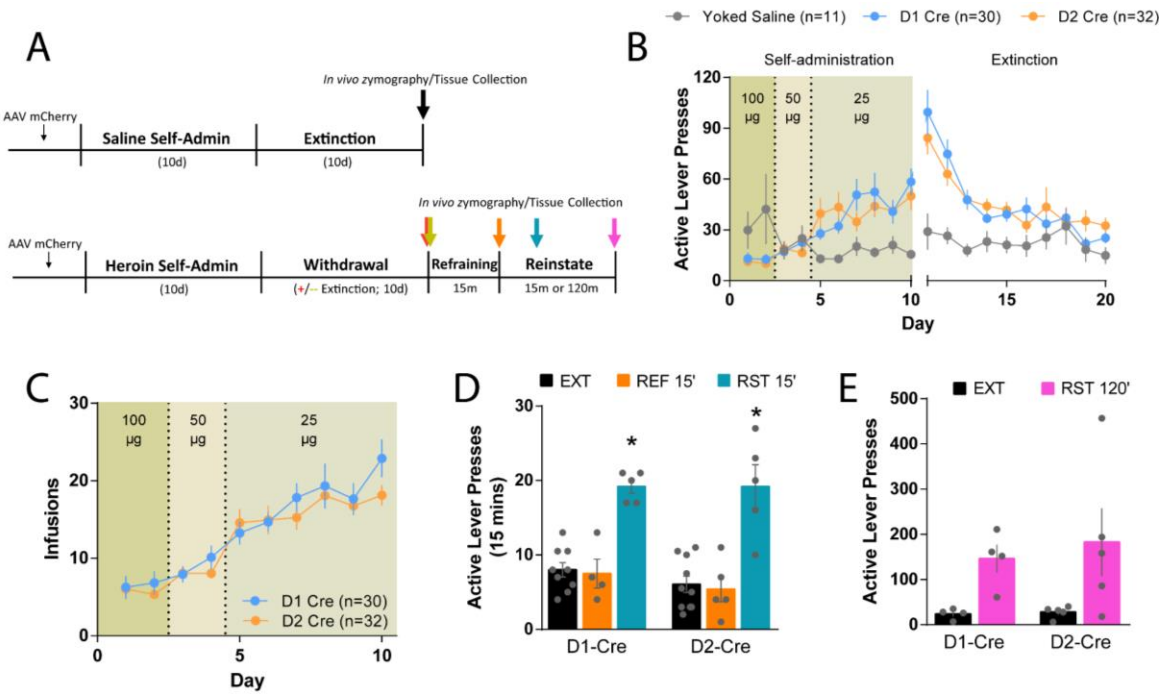

Figure 4-1. Heroin Self Administration, Withdrawal, and Behavioral Responding in D1- and D2- Cre (+) Animals. A) Experimental timeline outlining heroin self-administration, withdrawal, and reinstatement. **B)** Time course of active lever pressing in D1- and D2-cre animals during heroin self-administration and extinction. Significant difference found in active lever presses between yoked saline and both D1- and D2-cre rat genotypes during self-administration and extinction (2-way ANOVA; effect of group treatment $F_{(2,60)}=3.935$, $p=0.0248$; effect of time $F_{(19,1140)}=7.487$, $p<0.0001$; interaction $F_{(38,1140)}=2.617$, $p<0.0001$). No difference in active lever presses between D1-cre and D2-cre rats trained to self-administer heroin (2-way ANOVA; effect of genotype $F_{(1,52)}=0.0405$; $p=0.8413$; effect of time $F_{(19,988)}=18.94$; $p<0.0001$; interaction $F_{(19,988)}=1.026$; $p=0.4275$). **C)** No differences in escalation of heroin infusions during self-administration between D1 and D2-cre rats (2-way ANOVA; effect of genotype $F_{(1,52)}=1.592$; $p=0.2127$; effect of time $F_{(9,468)}=29.70$; $p<0.0001$; interaction $F_{(9,468)}=0.8974$; $p=0.5274$). **D)** Cue-induced reinstatement of heroin-seeking increased active lever presses over 15 mins similarly in D1- and D2-cre rats (2-way ANOVA; interaction $F_{(3,15)}=15.92$; $p<0.0001$). **E)** Cue-induced reinstatement of heroin-seeking increased active lever presses over 120 mins similarly in D1- and D2-cre rats (2-way ANOVA; effect of genotype $F_{(1,7)}=0.1772$; $p=0.6864$; EXT/RST $F_{(1,7)}=11.07$; $p=0.0126$; interaction $F_{(1,7)}=0.1512$; $p=0.7089$). Each dot in bar represents an animal. Data are shown as mean ± SEM. *$p<0.05$ comparing EXT and REF to RST 15' or RST 120' using Bonferroni post hoc test.

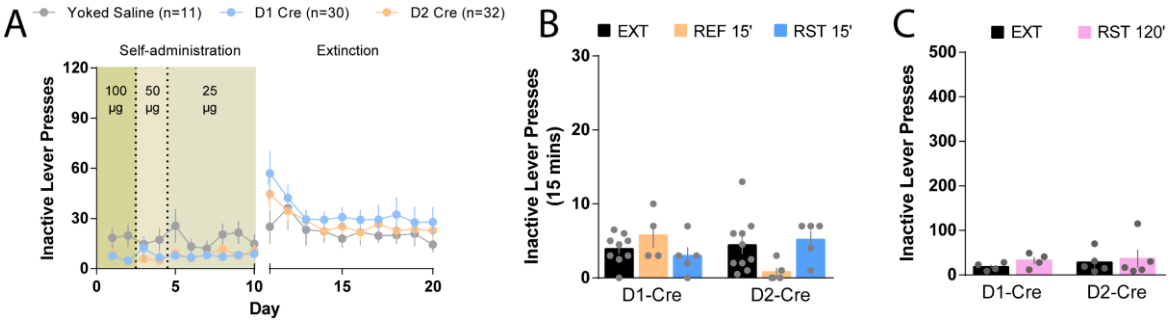

Figure 4-2. Inactive lever pressing for Heroin Self-Administration and Behavioral Tests in D1- and D2- Cre (+) Animals. A) Time course of inactive lever pressing in D1- and D2-cre animals during heroin self-administration and extinction. No significant differences were found between yoked saline and both D1- and D2-cre rat genotypes during self-administration and extinction (2-way ANOVA; effect of group treatment $F_{(2,60)}$=0.2355, p=0.7909; effect of time $F_{(19,1140)}$=8.612, p<0.0001; interaction $F_{(38,1140)}$=1.268, p=0.1297). No difference in inactive lever presses between D1-cre and D2-cre rats trained to self-administer heroin (2-way ANOVA; effect of genotype $F_{(1,52)}$=0.4209; p=0.5194; effect of time $F_{(19,\ 988)}$=16.02; p<0.0001; interaction $F_{(19,988)}$=0.4989; p=0.9639). **B)** No difference in inactive lever presses during behavioral tests (2-way ANOVA; effect of genotype $F_{(5,22)}$=2.159; p=0.0959; effect of test $F_{(1,22)}$=0.2245; p=0.6403; interaction $F_{(5,22)}$=1.372; p=0.2729). **C)** No difference in inactive lever presses during 120 min RST (2-way ANOVA; effect of genotype $F_{(1,7)}$=0.1626, p=0.6988, EXT/RST $F_{(1,7)}$=2.184, p=0.1830, interaction $F_{(1,7)}$=0.1624, p=0.6990). Each dot in represents an animal. Data are shown as mean ± SEM.

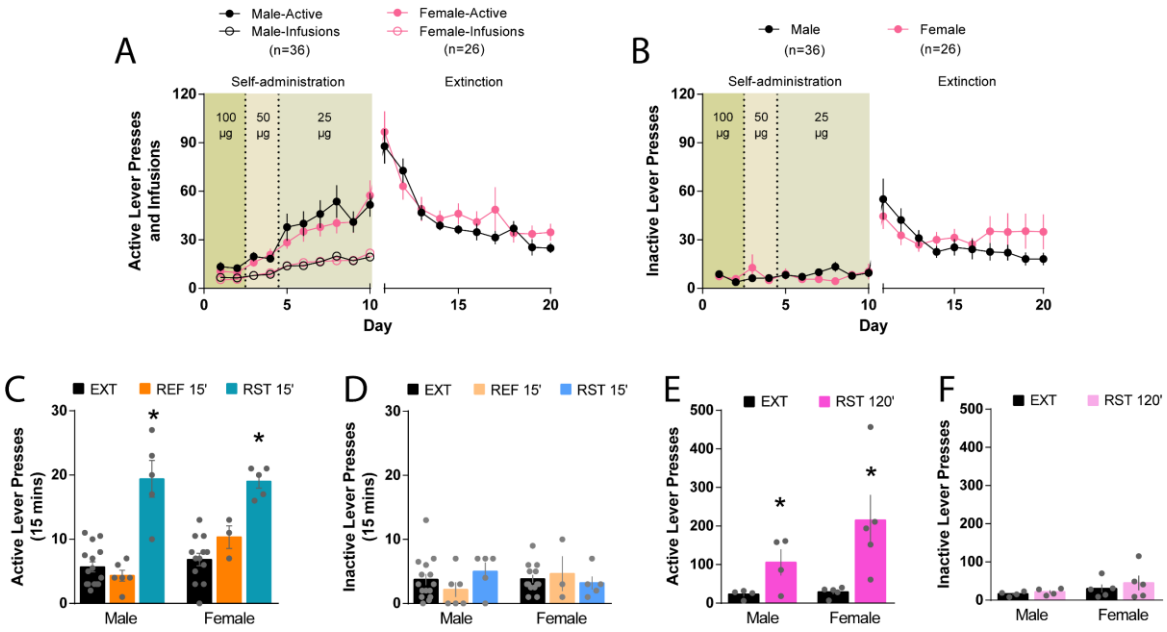

Figure 4-3. Male and Female Heroin Self-Administration and Behavioral Responding. A) Time course of active lever pressing for male and female animals during heroin self-administration and extinction. No differences were found in active lever pressing (2-way ANOVA; effect of sex $F_{(1,52)}$=0.0258; p=0.8730; effect of time $F_{(19,988)}$=18.43; p<0.0001; interaction $F_{(19,988)}$=0.8989; p=0.5846) or infusions (2-way ANOVA; effect of sex $F_{(1,\ 52)}$=0.0306; p=0.8619; effect of time $F_{(9,468)}$=28.81; p<0.0001; interaction $F_{(9,468)}$=0.5055; p=0.8708). **B)** No difference in inactive lever presses between males and females during self-administration and extinction (2-way ANOVA; effect of sex $F_{(1,52)}$=0.1460, p=.7040), but an effect of time $F_{(19,988)}$=16.07; p<0.0001), interaction $F_{(19,988)}$=1.699; p=0.0309). **C)** Cue-induced reinstatement of heroin-seeking increased active lever presses over 15 mins similarly in male and female rats (2-way ANOVA; interaction $F_{(3,15)}$=16.48; p<0.0001). **D)** No difference in inactive lever presses during behavioral tests (2-way ANOVA; effect of sex $F_{(3,15)}$=0.9348; p=0.4482; effect of test $F_{(1,15)}$=0.5007; p=0.4900; interaction $F_{(3,15)}$=0.09737; p=0.9603). **E)** No difference in active lever pressing between sexes during 120 min RST (2-way ANOVA; effect of sex $F_{(1,7)}$=1.758; p=0.2265, EXT/RST $F_{(1,7)}$=13.05; p=0.0086, interaction $F_{(1,7)}$=1.931; p=0.2072). **F)** No difference in inactive lever pressing between sexes during 120 min RST (2-way ANOVA; effect of sex $F_{(1,7)}$=1.325; p=0.2875, EXT/RST $F_{(1,7)}$=1.922; p=0.2082, interaction $F_{(1,7)}$=0.4033; p=0.5456) Each dot in represents an animal. Data are shown as mean ± SEM. *p<0.05 comparing EXT and REF to RST 15' or RST 120' using Bonferroni post hoc test.

D1 and D2 MSN-specific MMP Localization in NAcore

Following chronic heroin self-administration and extinction training, transient increases in MMP-9 activity in NAcore are elicited after 15 mins of cued heroin seeking compared to heroin-extinguished and saline control rats (Smith, Kupchik et al. 2014). Although increases in MMP-2,9 fluorescence can be observed along medium spiny neurons (MSNs) at the soma and dendritic processes in accumbens, it is unknown which specific cell types are responsible for this MMP proteolytic activity under heroin-extinguished and cued reinstatement conditions. I hypothesized that D1-receptor expressing MSNs exhibit increased pericellular localization of MMP gelatinolysis during transient cued heroin seeking, while D2-receptor expressing MSNs exhibit increased localization following extinction training. To evaluate cell-type specific MMP gelatinolytic puncta localization around MSNs following withdrawal (with or without extinction training) or cue-induced heroin seeking, male and female D1 and D2 cre (+) rats were virally transfected with cre-dependent mCherry vector and underwent *in vivo* zymography to assess MMP proteolytic activity under various behavioral tests and withdrawn conditions. Animals were trained to self-administer yoked saline or heroin for 10 days during which infusion of heroin was paired with a light/tone compound conditioning cue. Subsequently, animals underwent extinction training or home-cage abstinence for 10 days. Finally, after extinction some rats were returned to operant environment for one of the following behavioral tests: 1) refraining (REF; 15 min), 2) transient cued seeking (RST; 15 min), and 3) prolonged cued seeking (RST; 120 min). FITC-quenched gelatin was bilaterally microinjected into NAcore 24 hours after the last day of withdrawal (extinguished; EXT or abstinent; ABS) or immediately prior to behavioral testing. Yoked saline animals were sacrificed 24 hours after last withdrawal day. MMP-2/9 gelatinase activity was significantly increased in NAcore only in transient (15 min) heroin seeking

rats compared to all other groups (Figure 4-4A). The same trend is seen when groups are separated by D1- or D2-cre genotypes (Figure 4-4B,C). Interestingly, no difference in MMP-2/9 activity was seen between extinguished, abstinent or refraining groups.

To assess cell-type specific extracellular MMP-2/9 proteolytic activity, mCherry-labeled MSNs were imaged with confocal microscopy and digitally reconstructed with Imaris software analysis to measure punctate gelatinolytic MMP activity around D1 and D2 MSNs. For D1 MSNs, I observed increased MMP-2,9 gelatinolytic puncta localized with dendritic surfaces in transient cue-reinstated animals (15 min) compared to yoked saline controls (Figure 4-5). These data further support the well-studied perspective that D1 MSNs promote drug seeking behavior. There was no difference in MMP-2/9 activity around D1 MSNs associated with withdrawal, with or without extinction training. For D2 MSNs, I observed increased MMP-2,9 gelatinolytic punctate signal in heroin-extinguished animals, but MMP-2,9 activity after 15 min reinstatement was reduced to saline control levels (Figure 4-6). I subsequently tested whether prior extinction training was mediating localization of D2 MSN MMP activity by placing rats in home cage abstinence (10 days) after self-administration. I found that constitutive MMP activity around D2 MSNs was dependent on previous extinction training as there was a reduction in MMP localization after 10 days of home cage abstinence where animals were handled daily but not returned to the operant chamber for extinction training (Figure 4-6). Interestingly, after two hours of cued heroin seeking, MMP gelatinolytic activity was restored to extinguished levels. In addition, there is no difference between MMP activity localized around D2 MSNs in heroin extinguished and heroin-refraining animals (Figure 4-6). These findings provide further evidence of D2 MSNs involvement in promoting extinguished responding, while suppressing reward seeking. Importantly, there was no

difference in total MMP activity per z-stack across EXT, REF 15', RST 15' and RST 120' groups between D1 and D2 MSN cell-types (Figure 4-7).

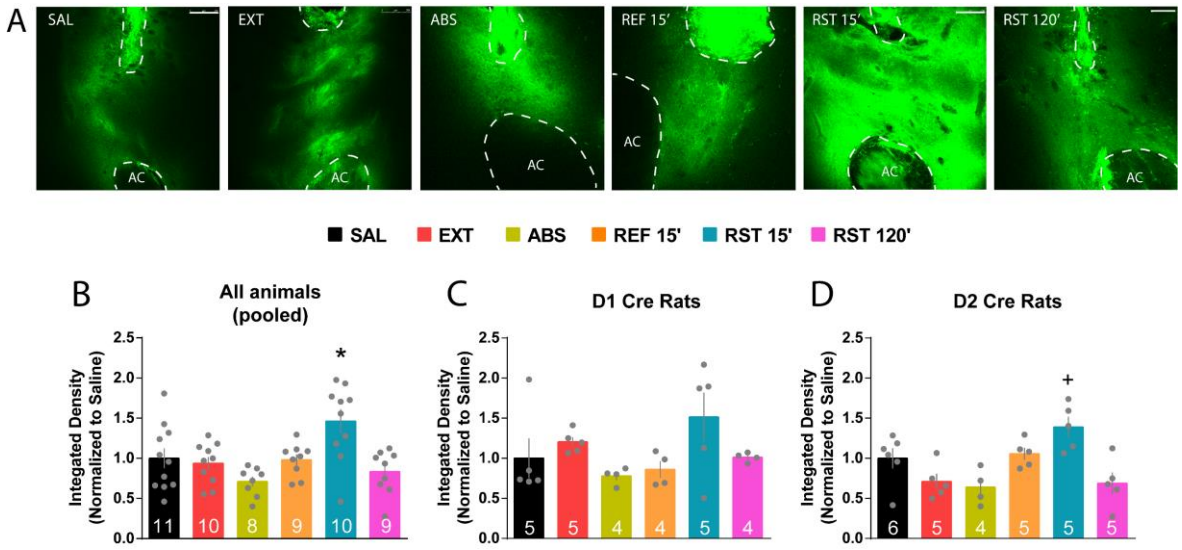

Figure 4-4. MMP-2/9 Activity in NAcore During Withdrawn and Heroin Seeking Behaviors. A) Representative micrographs of MMP-2/9 activity in NAcore following withdrawal (with or without extinction) and during behavioral seeking tests. **B)** Increased MMP-2/9 proteolytic activity in NAcore during transient heroin seeking compared to other behavioral groups in pooled analysis of both D1 and D2-cre rats (1-way ANOVA; $F_{(5,51)}$=5.802; p=0.0003). **C)** Similar trend is seen in D1 Cre rats (1-way ANOVA; $F_{(5,21)}$=2.043; p=0.1138) and **D)** D2 Cre rats (1-way ANOVA; $F_{(5,24)}$=6.015; p=0.0010). N in bars indicates number of animals with an average of four-seven NAcore slices/rat. Data was normalized to its respective yoked saline mean. Data are shown as mean ± SEM. *p<0.05 comparing EXT, ABS, REF 15', RST 120' to RST 15' using Tukey post hoc test in pooled data. $^{+}$p<0.05 comparing EXT, ABS, RST 120' to RST 15' using Tukey post hoc test in D2 cre rat analysis. Scale bar=250 µm.

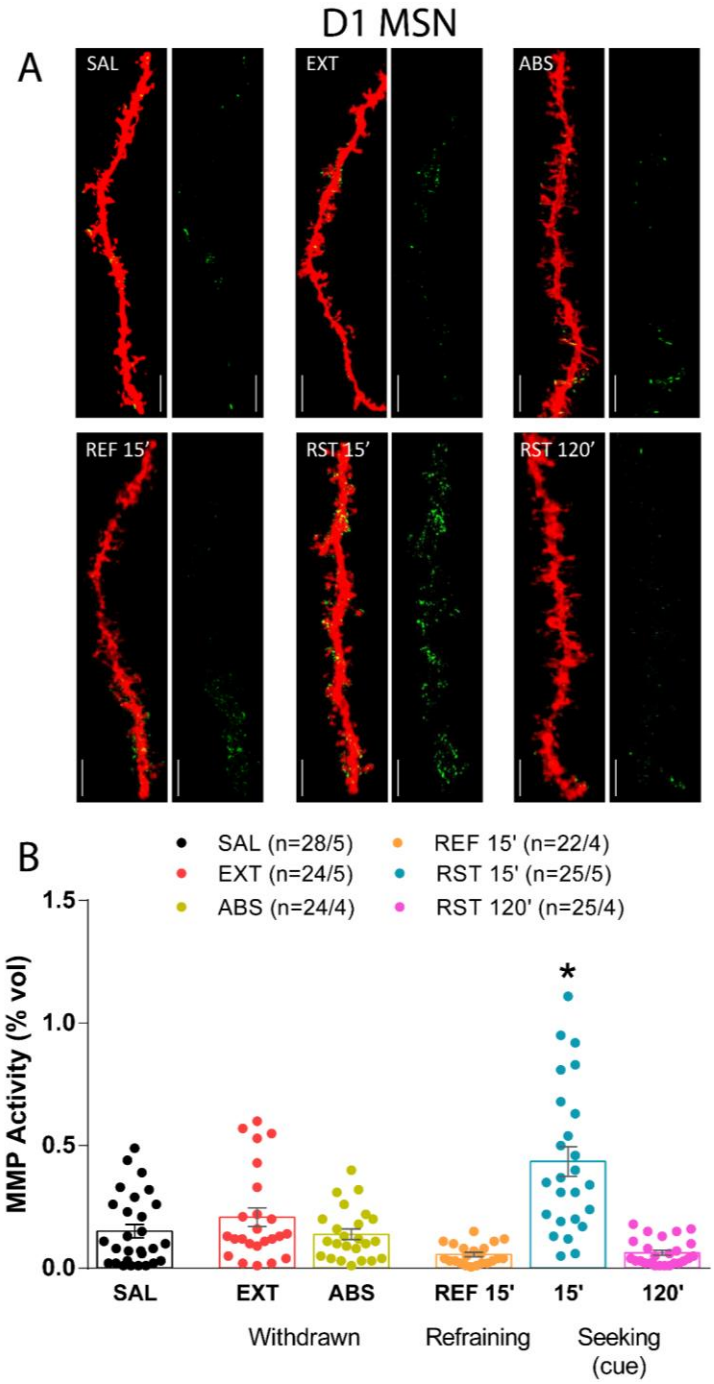

Figure 4-5. Transient and Reversible MMP-2/9 Gelatinolytic Puncta Around D1 MSNs During Cued Heroin Seeking. A) Representative images of 3D reconstruction of mCherry-labeled dendritic segments (red) and MMP gelatinolytic puncta (green) localized within 300nm of dendrite surface for each group SAL, EXT, ABS, REF 15', RST 15' and RST 120'. **B)** Transient heroin seeking induces significant increase in active MMP puncta localization around D1 MSNs (Nested ANOVA; $F_{(4,17)}$=6.410; p=0.0025). No differences observed between extinction- and abstinent-associated withdrawal (Nested ANOVA; $F_{(2,11)}$=0.6229; p=0.5543). Data are shown as mean ± SEM. N represents number of neurons quantified over number of animals in each condition. *p<0.05 compared RST 15' to SAL; using Bonferroni post hoc test. Scale bar=7 µm.

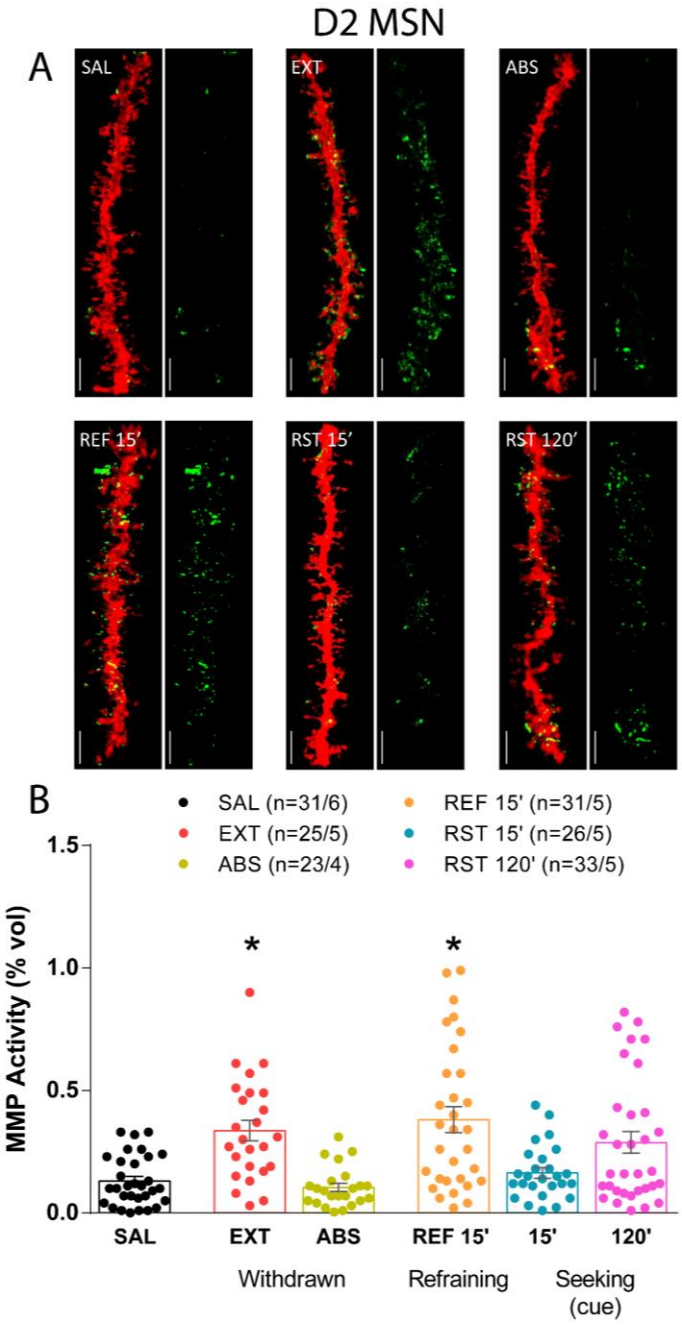

Figure 4-6. Constitutive MMP-2/9 Gelatinolytic Puncta around D2 MSN Following Heroin Extinction. A) Representative images of 3D reconstruction of mCherry-labeled dendritic segments (red) and MMP gelatinolytic puncta (green) localized within 300nm of dendrite surface for each group SAL, EXT, ABS, REF 15', RST 15' and RST 120'. **B)** Constitutive effects of extinction training induced increase in active MMP puncta localization around D2 MSNs (Nested ANOVA; $F_{(5,\ 24)}$=6.496; p=0.0006). A trend towards an increase in MMP activity was observed after two-hour RST compared to saline control (Nested ANOVA; p=0.0763). No difference between heroin extinguished and heroin-refraining animals ($t_{(24)}$=0.6915; p<0.9999). Data are shown as mean ± SEM. N represents number of neurons quantified over number of animals in each condition. *p<0.05 comparing EXT and REF 15' to SAL; using Bonferroni post hoc test. Scale bar= 5 µm.

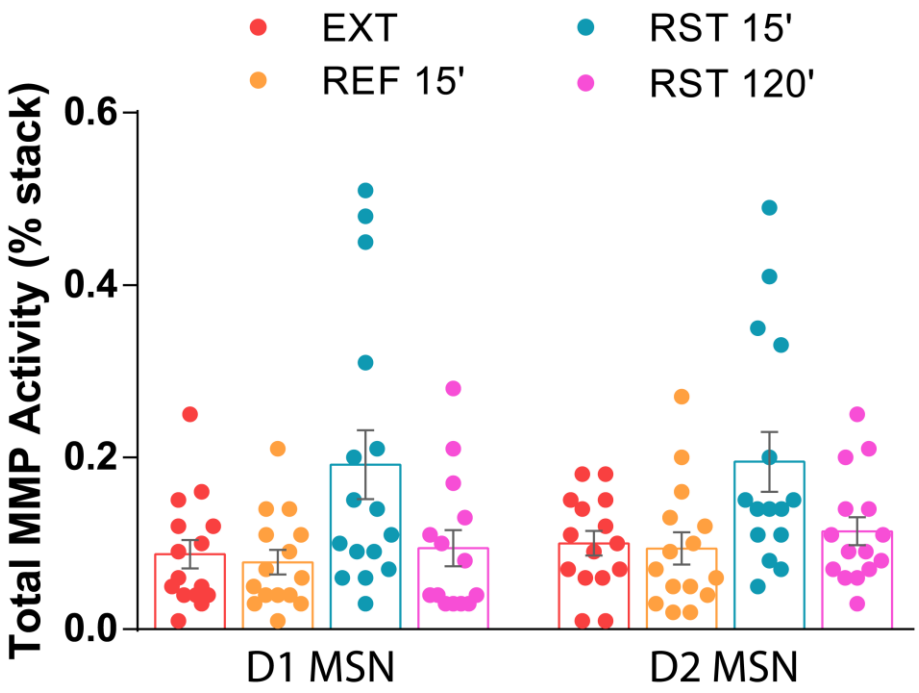

Figure 4-7. No Difference in Total MMP Activity Across Groups Between D1 and D2 MSNs. Total MMP activity quantified in z-stack of EXT, REF 15', RST 15' and RST 120' groups did not differ between D1 and D2 mCherry-labeled MSNs (2-way ANOVA; effect of cell type, $F_{(1, 112)}$=0.577; p=0.4510; effect of group, $F_{(3, 112)}$=8.639; p=0.4510; interaction, $F_{(3, 112)}$=0.04193; p=0.9885. Three to four stacks were quantified per animal. Data are shown as mean ± SEM.

Dendritic Subcompartment-Specific MMP Gelatinolytic Activity Around D1 and D2 MSNs

To determine whether MMP-2/9 gelatinolytic activity localized around dendritic segments is specific to dendritic spine heads or shaft, previously processed D1 and D2 MSNs from saline, extinguished, and cued heroin seeking rats (15 min) were further analyzed for dendritic spine morphology and subcompartment-specific MMP-2/9 localization. Previous studies have evaluated heroin-induced changes in synaptic strength by measuring dendritic spine head diameter (d_h) of medium spiny neurons, irrespective of cell-type (Shen, Moussawi et al. 2011). Given the cell-type specific nature of my project, I adapted this standard morphological analysis to determine heroin-related single cell adaptations to MMP localization. For D1 MSNs, there was a potentiation in d_h in transient heroin seeking animals compared to yoked saline and heroin-extinguished animals (Figure 4-8A). As an extension of these analyses, I quantified fluorescent MMP-2/9 gelatinolytic activity specifically at dendritic spine head and shaft subcompartments. I discovered an overall increase in MMP activity around both dendritic spine head and shaft subcompartments of D1 MSNs in heroin seeking rats compared to saline controls and heroin-extinguished rats (Figure 4-8B). Interestingly, increases in MMP proteolytic activity were enriched at dendritic spine heads in reinstating animals, which is indicated by an elevated ratio of spine head:shaft MMP puncta localization (Figure 4-8C). However, there was no correlation between % spine head MMP localization and the corresponding spine head diameter (Figure 4-9). For D2 MSNs, there was no statistical difference in d_h across groups, however, there was a trend towards depotentiation in d_h in heroin seeking animals compared to saline and heroin-extinguished animals (Figure 4-8D). I discovered an overall increase in MMP activity around both dendritic spine heads and shaft of D2 MSNs in heroin-extinguished rats compared to yoked saline and

heroin seeking rats (Figure 4-8B). Interestingly, there was also enriched MMP proteolytic activity at dendritic spine heads in heroin-extinguished animals (Figure 4-8C), supporting the notion of MMP involvement in synaptic reconfiguration.

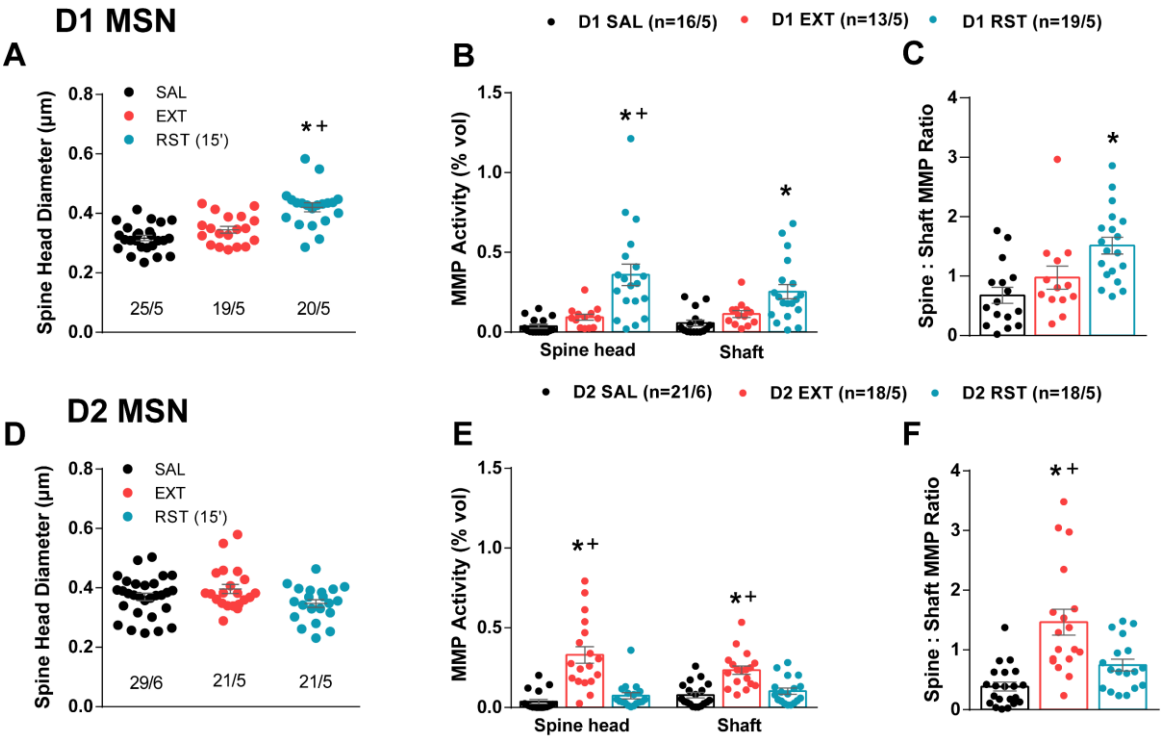

Figure 4-8. MMP Activity around D1 and D2 MSNs is Spine Head-Enriched in Heroin Seeking and Withdrawn Conditions, Respectively. A) D1 MSNs exhibit potentiated spine head diameter during transient heroin seeking (Nested 1-way ANOVA; $F_{(2,\ 12)}$=8.280; p=0.0055). **B)** MMP gelatinolytic puncta is increased in both spine head and shaft subcompartments of D1 dendritic segments during transient heroin seeking (Nested 1-way ANOVA; spine head $F_{(2,\ 12)}$=11.80; p=0.0015; shaft $F_{(2,\ 12)}$=7.766; p=0.0069). **C)** Spine:Shaft MMP ratio revealed enhanced spine head-specific MMP gelatinolytic puncta around D1 MSNs during cue-induced RST (Nested 1-way ANOVA, $F_{(2,\ 12)}$=7.491; p=0.0077). **D)** No difference D2 MSN spine head diameter across groups (Nested ANOVA; $F_{(2,\ 13)}$=2.455; p=0.1246). **E)** MMP gelatinolytic puncta is increased in both spine head and shaft subcompartments of D2 dendritic segments under extinguished conditions (Nested 1-way ANOVA; spine head $F_{(2,\ 53)}$=25.08; p<0.0001; shaft $F_{(2,\ 13)}$=11.69; p=0.0012). **F)** Spine:Shaft MMP ratio revealed enhanced spine head-specific MMP gelatinolytic puncta around D2 MSNs following extinction (Nested 1-way ANOVA; $F_{(2,\ 54)}$=15.66; p<0.0001). Data are shown as mean ± SEM. N represents number of neurons quantified over number of animals in each condition. *p<0.05 comparing RST to SAL, $^{+}$p<0.05 comparing RST to EXT, *p<0.05 comparing EXT to SAL for D2 MSNs using Bonferroni post hoc test.

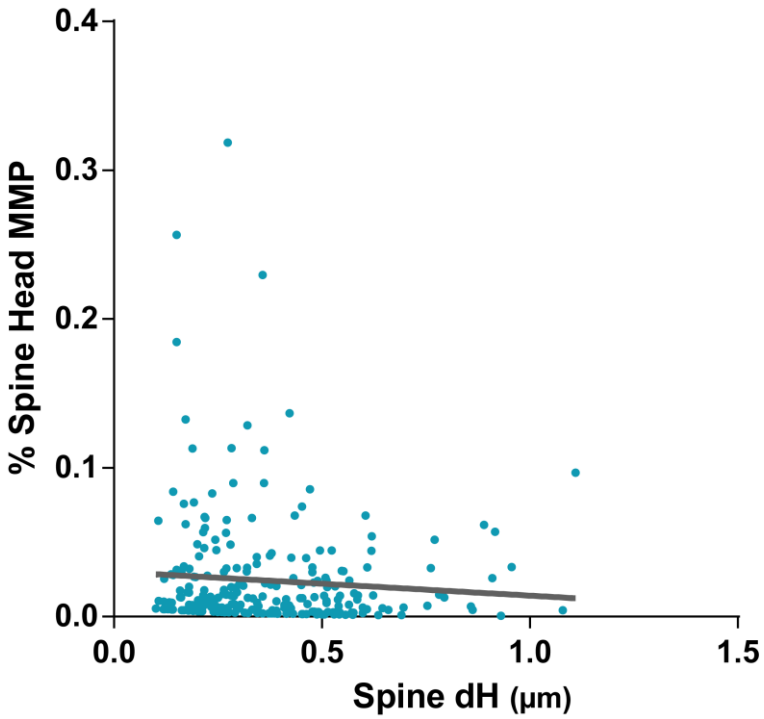

Figure 4-9. No Relationship Between % Spine head-specific MMP Localization and Spine Head Diameter of D1 Reinstated Cells. Potentiated dendritic spine head diameters of D1 MSNs during reinstatement does not correlate with % spine head MMP gelatinolytic puncta (Linear regression; r^2=0.006518; p=0.1936)

MMP-2/9 Specificity Around D1 and D2 MSNs in Seeking and Extinguished Animals

To determine if transient and enduring increases in MMP activity around D1 and D2 MSNs, respectively, are MMP-2- or MMP-9-dependent, I utilized specific pharmacological inhibitors of each respective gelatinase under extinguished and cued heroin seeking conditions in D1 and D2 cre rats (Figure 4-10A). The placement of all microinjection cannula tips used for all brain sections imaged for MSNs can be found in Figure 4-10B. D1 or D2 cre rats received intra-NAcore pre-treatment of vehicle (Veh, 1% DMSO) in one hemisphere and MMP-2i or -9i (0.1 nmol for either inhibitor) in the contralateral hemisphere 15 mins prior to cue-induced reinstatement session (15 min) for D1 cre rats or 30 min prior to sacrifice 24 hrs after the last extinction session in D2 cre rats. I first examined tissue at low magnification, and compared to Veh treatment, both MMP-2i and MMP-9i produced a trend towards decreased overall MMP activity in NAcore of D1 cre rats (Figure 4-10C). Compared to Veh treatment, only MMP-2i reduced overall MMP activity in NAcore of D2 cre extinguished rats. There is no difference in extinction-associated accumbens MMP activity with MMP-9i (Figure 4-10D). Single cell analysis for cell-type specific effects revealed that for D1 MSNs, MMP-9i, but not MMP-2i, reduced gelatinolytic activity compared to vehicle during cued heroin seeking (Figure 4-11A), consistent with previous reports of involvement of MMP-9 activity in transient synaptic plasticity (Michaluk, Kolodziej et al. 2007). For D2 MSNs, MMP-2i, but not MMP-9i, significantly reduced gelatinolytic activity compared to vehicle following extinction training (Figure 4-11B), consistent with the reported constitutive nature of MMP-2 activity at excitatory synapses (Verslegers, Lemmens et al. 2013).

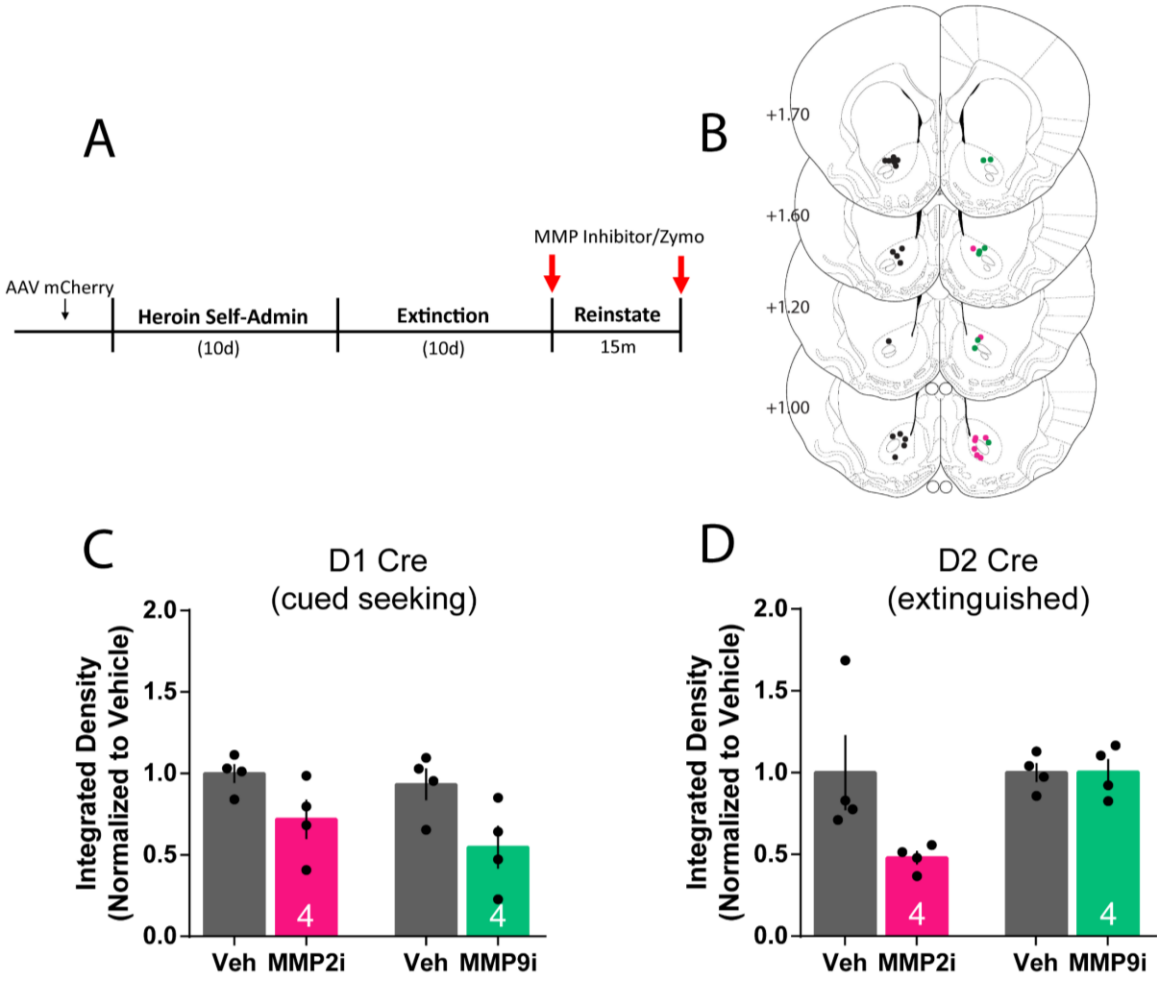

Figure 4-10. MMP Activity in NAcore is Affected by MMP-2 and -9 Inhibition Following Withdrawal and Transient Heroin Seeking. A) Experimental timeline outlining heroin self-administration, extinction, and reinstatement. **B)** Histological locations of microinjector tip for animals injected with vehicle (black) or MMP-2i (pink) or -9i (green). Animals received unilateral vehicle injection and contralateral injection of either MMP-2 or MMP-9 inhibitor. **C)** Both MMP-2i and -9i injection (0.1 nmol each) produced a trend towards a decrease in NAcore MMP activity during cued heroin seeking in D1 Cre rats (Paired t-test; MMP-2i: $t_{(3)}$=2.999, p=0.0577; MMP-9i: $t_{(3)}$=2.418, p=0.0944). **D)** MMP-2i injection (0.1 nmol each) produced a trend towards a decrease in MMP activity following extinction in D2 Cre rats. No difference was observed with MMP-9i (Paired t-test; MMP-2i: $t_{(3)}$=2.323, p=0.1028; MMP-9i: $t_{(3)}$=0.0403, p=0.9704). N in bars indicates number of animals with an average of four-seven NAcore slices/rat. Data was normalized to its respective yoked saline mean. Data are shown as mean ± SEM.

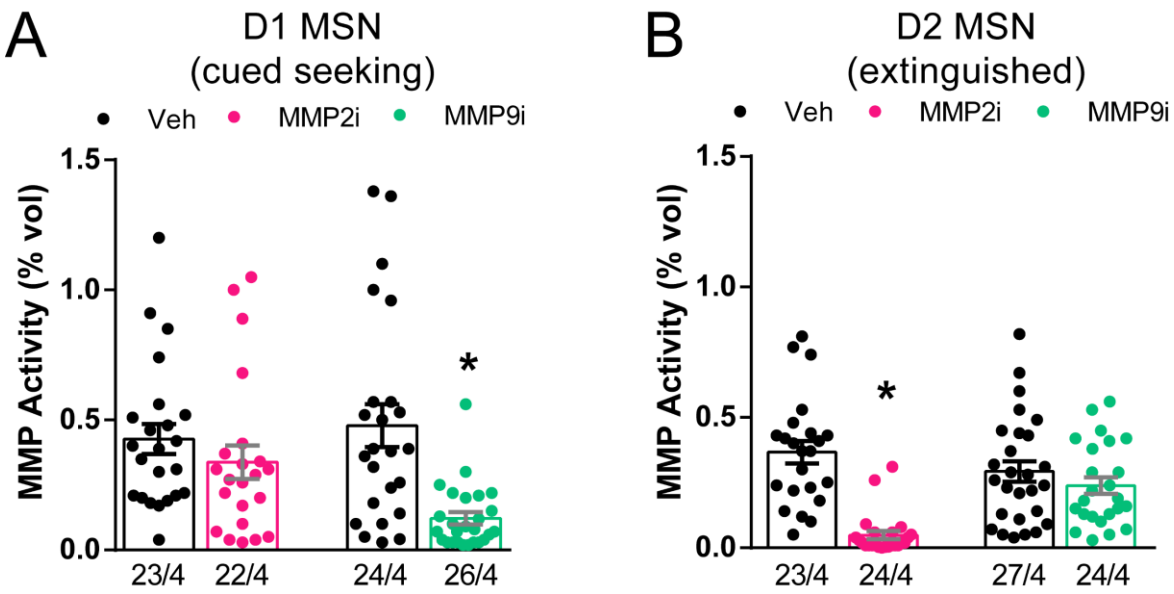

Figure 4-11. MMP Activity Around D1 MSNs During Cued Heroin Seeking is MMP-9 dependent, while MMP Activity Around D2 MSNs After Withdrawal is MMP-2 dependent. A) Transient MMP gelatinolytic activity around D1 MSNs during cued-heroin seeking is MMP-9 dependent (Nested t-test; Veh/MMP9i, $F_{(1,48)}=18.52$, $p<0.0001$; Veh/MMP2i, $F_{(1,6)}=0.68$, $p=0.45$). **B)** Constitutive MMP gelatinolytic activity around D2 MSNs following extinction is MMP-2 dependent (Nested t-test; Veh/MMP2i, $F_{(1,45)}=49.17$, $p<0.0001$; Veh/MMP9i; $F_{(1,6)}=0.5387$, $p=0.4907$). Data are shown as mean ± SEM. N represents number of neurons quantified over number of animals in each condition.

Table 1-1. Summary I: D1 and D2 MSN-specific MMP Gelatinolytic Activity

Cell Type	Extinguished	Abstinent	Refraining 15′	Reinstated 15′	Reinstated 120′	MMP-2 Inhibition	MMP-9 Inhibition
D1 MSN	No change (compared to SAL)	No change	No change	Increased	No change	No change	Decreased (RST 15′)
D2 MSN	Increased (compared to SAL)	No change	Increased	No change	Increased	Decreased (EXT)	No change

Discussion

In these experiments, I endeavored to determine the cellular specificity of MMP gelatinolytic activity around cell types of the NAcore. I discovered enhanced proteolytic puncta, corresponding to MMP-9 activity, around D1 MSNs during transient cued heroin seeking, while MMP-2 gelatinolytic puncta surrounded D2 MSNs following constitutive extinction-related withdrawn conditions. Importantly, MMP gelatinolytic puncta were enriched at dendritic spine head compartments in both D1 and D2 MSNs under heroin-reinstated and -extinguished conditions, respectively. These data support a growing body of literature describing MMP enzymatic involvement in synaptic reconstruction in animal models of pathological learning.

For D1 MSNs, I observed transient and reversible MMP-9 gelatinolytic activity that was accompanied by overall spine head diameter potentiation. While these measurements did not correlate (Figure 4-9), it nonetheless supports the notion of D1 MSN activation driving motivated and goal-directed behaviors. Previous studies have characterized D1 MSN involvement in locomotor sensitivity, sucrose consumption, and use of addictive drugs (Lobo, Covington et al. 2010, Lobo and Nestler 2011, Calipari, Bagot et al. 2016). Prior consumption of cocaine selectively strengthens excitatory synaptic inputs onto D1 MSNs in a manner coinciding with locomotor sensitization (Pascoli, Turiault et al. 2012). While it is unknown whether similar synaptic adaptations arise in D1 MSNs after opioid exposure, my data reveal MMP-9-dependent plasticity in

the extracellular environment of this cell subpopulation of NAcore in response to presenting cues paired with heroin delivery that lead to motivated seeking.

For D2 MSNs, I observed constitutive MMP-2 activity under extinguished conditions (i.e. 24 hours after last extinction session) that was not accompanied by overall spine head diameter morphological changes. These data support previous findings of NAc D2 MSNs involvement in withdrawal and behavioral inhibition of drug seeking (Bock, Shin et al. 2013, Calipari, Bagot et al. 2016, Heinsbroek, Neuhofer et al. 2017, Roberts-Wolfe, Bobadilla et al. 2018). Lack of D2 MSN activation enhanced motivation to seek cocaine, while direct stimulation suppresses cocaine self-administration (Bock, Shin et al. 2013). *In vivo* imaging of D2 MSNs revealed quiescent firing during expression of cocaine place preference, while active cocaine refraining behavior produced potentiated AMPA:NMDA current ratios (Calipari, Bagot et al. 2016, Roberts-Wolfe, Bobadilla et al. 2018). Following repeated opioid exposure, NAshell D2 MSNs produce silence synapses via internalization of AMPA receptors, resulting in immature dendritic spine architecture and poor synaptic efficiency (Graziane, Sun et al. 2016). D2 MSNs also encode prior unfavorable outcomes which is consistent with our finding that MMP-2 activity is selectively elevated around D2-MSNs after extinction training and during refraining (Zalocusky, Ramakrishnan et al. 2016).

What do these findings mean? A number of speculations can be proposed, mostly revolving around the central concept that MMPs serve as essential components of the extracellular matrix to regulate synaptic adaptations. One hypothesis is MMP activity may be a biomarker of active and dynamic synaptic reconfiguration. Specifically, insertion or translocation of glutamate receptors to the post-synaptic membrane, dendritic spine head potentiation, or instructive signaling to terminate ongoing cascades (Huntley 2012). Similar to c-fos, MMP induction indicates increased excitatory

neurotransmission, and a high resolution *in vivo* zymography assay adapted to capture synaptic activity at dendritic spine heads could reveal active extracellular signaling required to sustain or initiate cellular reconstruction.

Another postulation is MMP cleavage may be creating an ECM microenvironment that promotes refraining and seeking around D2 MSNs and D1 MSNs, respectively. Following heroin withdrawal, ECM constituents, Tenascin R and Brevican, are downregulated in NAcore, but restored when heroin-associated cues are presented (Van den Oever, Lubbers et al. 2010). While overall MMP gelatinase activity in NAcore is unchanged after heroin extinction, the downregulation of these proteins could be facilitating extinction in a cell-specific fashion. It is known that integrins, cell-adhesion molecules (CAMs), tightly interact with the ECM to modify ECM composition and morphology (Park and Goda 2016). MMP catalytic products, RGD peptides, bind to integrin molecules to initiate intracellular signaling cascades that correspond to changes in synaptic plasticity. Specifically, β1 and β3 integrin, the most abundant in the brain, exhibit fluctuations in NAcore expression with cocaine-administration (Wiggins, Pacchioni et al. 2009, Wiggins, Smith et al. 2011). D1- and D2 MSN-specific regulation of drug addicted behavior likely involve integrins, as β1 is considered to maintain enduring processes, while β3 is involved in transient synaptic plasticity (Garcia-Keller, Neuhofer et al. 2019). Further investigation of specific ECM proteins, such as CAMs and fibronectin, and their regulation of drug-associated behaviors is warranted.

Finally, MMP distribution at dendritic spine heads vs shaft may be attributed to differential glutamatergic inputs from prelimbic or infralimbic cortex during seeking and withdrawal. During cued drug seeking, robust excitatory drive from prelimbic cortex causes increased glutamate release at striatal synapses and subsequently induces nitric oxide production, which then nitrosylates inactive MMP enzymes and promotes drug

seeking (Smith, Scofield et al. 2017). In addition, glutamatergic inputs from infralimbic cortex are necessary to inhibit drug seeking in extinguished rats (Peters, LaLumiere et al. 2008). However, further experimentation is required to determine the direct relationship between excitatory drive and onset of MMP activity, and if this is exclusive to corticostriatal projections mediating heroin seeking.

A remaining open question is whether these cell-specific findings apply to natural rewards, such as sucrose. While these studies utilized saline as controls, sucrose-based studies could indicate whether cell-type specific MMP localization is unique to pathological rewards. It is unknown whether overall gelatinase activity is increased during sucrose reinstatement, however, it is unlikely because while MMP-2 and -9 pharmacological inhibition abolishes cocaine seeking, it does not affect cue-induced sucrose seeking (Smith, Kupchik et al. 2014). Nonetheless, future studies may reveal underlying mechanisms of these differential processes, and aspects of how physiological MMP activity becomes pathological.

In summary, these studies I discovered MMP species-specific and cell-type specific adaptations that contribute to constitutive and transient forms of plasticity. Taken together, my findings provide a unique perspective of single cell MMP-2/9 proteolytic localization and how this contributes to synaptic reconfiguration. These discoveries could potentially serve as a step towards individualized treatment options that focus on restoration of specific cellular aberrations in individuals suffering from addiction.

Chapter 5: TIMP Regulation of MMP Activity During Heroin Seeking

Introduction

Tissue inhibitors of metalloproteinases (TIMPs) are the known endogenous inhibitors of MMPs. In order to counteract MMP activity and maintain ECM integrity, TIMPs bind reversibly in a 1:1 ratio to the catalytic site of enzymatically active or the pro-form of MMPs (Dzwonek, Rylski et al. 2004). Independent of their inhibitory properties, TIMPs have the ability to act as growth factors and regulate cell apoptosis (Mannello and Gazzanelli 2001, Visse and Nagase 2003). The four homologous isoforms of TIMPs, 1-4, exhibit some overlap in MMP target species, but efficacy of MMP inhibition varies across TIMPs (Arpino, Brock et al. 2015). The most well-studied of this family are TIMP-1 and TIMP-2. While either TIMP can inhibit the gelatinases (MMP-2 and -9), TIMP-1 has greater affinity to MMP-9, while TIMP-2 has stronger preference for MMP-2 (Vafadari, Salamian et al. 2016, Sánchez-Pozo, Baker-Williams et al. 2018). Paradoxically, TIMP-1 and TIMP-2 can also form complexes with proMMP-9 and proMMP-2, respectively, to facilitate their activation (Shofuda, Moriyama et al. 1998, Dzwonek, Rylski et al. 2004). Like MMP-9, TIMP-1 expression is inducible upon neuronal stimulation, while TIMP-2 expression is most abundant in the adult brain and not upregulated by cellular activity (Dzwonek, Rylski et al. 2004, Smith, Scofield et al. 2015). Interestingly, either proMMP-2 or -9, can be co-released with TIMP-1 in distinct vesicles localized at the somatodendritic region of neurons as well as from astrocytes (Huntley 2012, Beroun, Mitra et al. 2019), thus further supporting the physiological balance of focal proteolytic activity with coupled regulatory inhibition.

TIMPs are also involved in adaptations to dendritic morphology. Seemingly conflicting studies report direct application of autoactive MMP-9 can expand spine head

diameters, while other studies indicate MMP-9 incubation produces thin, filopodia-like spines (Wang, Bozdagi et al. 2008, Michaluk, Wawrzyniak et al. 2011, Szepesi, Bijata et al. 2013). A seminal discovery in this field found that while MMP-9 initiates synaptic plasticity to produce thin spines, it is subsequent TIMP-1 inhibition that terminates the plasticity to produce mature, mushroom spines (Magnowska, Gorkiewicz et al. 2016). Hence, both proteins are necessary for synaptic reconstruction and maintaining a productive balance in ECM degradation.

In addition to their role in synaptic physiology, TIMP expression is altered in pathological addiction behaviors. Reduced TIMP-1 levels are observed in the serum of human heroin addicts compared to healthy individuals, with serum level ratios of MMP-2/TIMP-1, MMP-9/TIMP-1, and MMP-2/TIMP-2 higher in heroin-exposed individuals, suggesting excessive MMP activity in this population (Kovatsi, Batzios et al. 2013). While TIMP-2 protein expression is increased in NAcore with cocaine-primed reinstatement, it is unchanged following cocaine withdrawal and cue-induced cocaine reinstatement, despite increased MMP-2/9 activity (Smith, Kupchik et al. 2014). Although TIMP expression is unaltered in NAcore with this paradigm, it is unknown whether underlying cell-type specific differences in expression exist or if TIMP function is affected during cued heroin seeking. In the previous chapter, I discovered cell-specific MMP-2/9 gelatinolytic activity around D1 and D2 MSNs, which may be due to specific TIMP expression. Thus, I hypothesize TIMP-1 and -2 expression will be increased around D1 MSNs following extinction, while increased around D2 MSNs during transient heroin seeking.

Results

TIMP-1/2 Inhibition Does Not Affect Extinguished Heroin Seeking or Associated MMP Gelatinolytic Activity

TIMPs are known endogenous inhibitors of MMPs, and while previous studies observed no change in expression of TIMP-2 during transient cocaine seeking (Smith, Kupchik et al. 2014), this does not address possible changes in TIMP inhibitory function under these conditions. To elucidate the role of TIMP involvement in refraining-associated MMP activity, D1- and D2-cre (-) Long Evans wildtype rats were trained on heroin self-administration, extinguished and pre-treated concurrently with neutralizing TIMP-1 and -2 antibodies (6 µg/mL) in NAcore 15 min prior to refraining test (30 min) and *in vivo* zymography assay (Figure 5-1A,B). Animals did not exhibit any difference in active lever presses during refraining with TIMP-1/2 antibody treatment compared to their extinction baseline (Figure 5-1C). In addition, TIMP-1 and -2 inhibition did not affect MMP-2/9 activity in refraining animals compared to untreated control rats (Figure 5-1D,E). Importantly, dual TIMP-1 and -2 treatment does not affect locomotor activity (Figure 5-F).

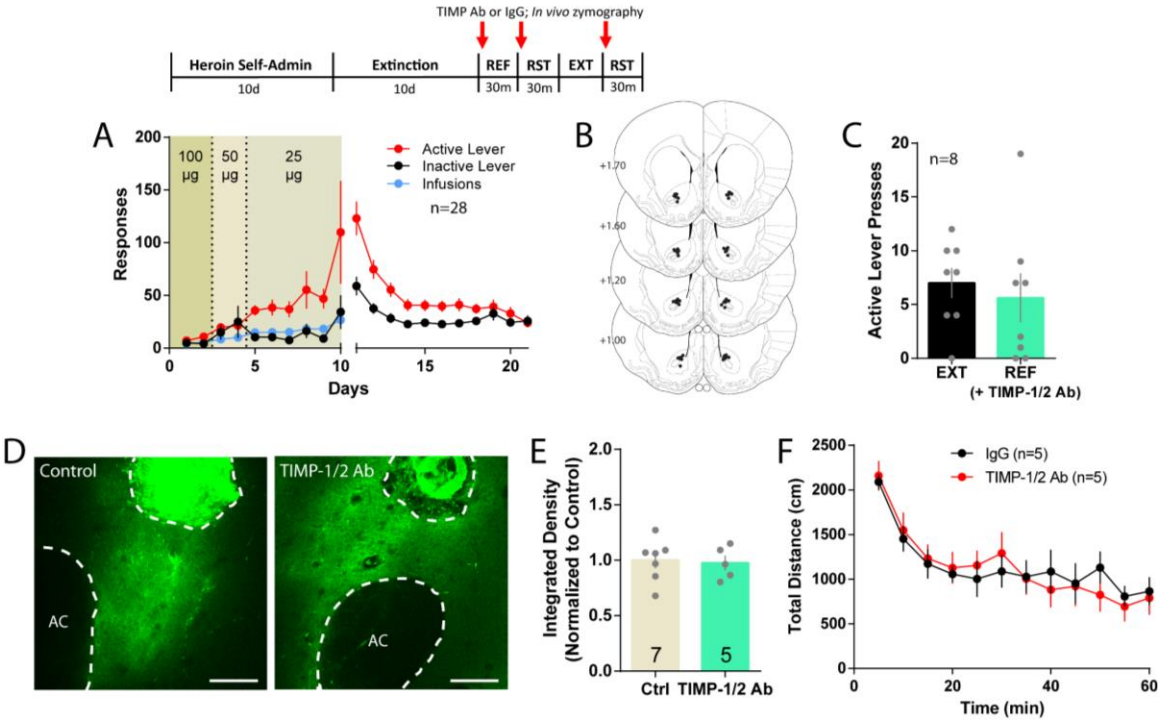

Figure 5-1. TIMP-1/2 Inhibition Does Not Affect Refraining Behavior or Associated MMP Activity. A) Experimental timeline outlining heroin self-administration, extinction, and reinstatement. Rats were trained to self-administer heroin and responding to the heroin-paired cues was subsequently extinguished. **B)** Histological locations of microinjector tip for animals injected with IgG (6 µg/mL) or combined TIMP-1 and -2 antibody (6 µg/mL) (black) in NAcore. **C)** No difference in refraining behavior with TIMP-1/2 antibody pre-treatment in NAcore compared to extinction (Paired t-test; $t_{(7)}$=0.8295; p=0.4342). **D)** Representative fluorescent micrographs of NAcore *in vivo* MMP activity in refraining controls and TIMP-1/2 antibody-treated animals. Injection track and anterior commissure masked out with dashed line. **E)** No difference in MMP activity, quantified as integrated density, in refraining controls compared to TIMP-1/2 antibody-treated animals (Unpaired t-test; $t_{(10)}$=0.2400; p=0.8152). **F)** Open field locomotor activity showed no difference in total distance (cm) traveled between IgG and TIMP-1/2 antibody-treated animals (2-way ANOVA; $F_{(1,4)}$=24.09; p=0.4329). Scale bar=250 µm. N in bars indicates number of animals with an average of four-seven NAcore slices/rat. Data was normalized to control (i.e. untreated refraining animals) mean. Data are shown as mean ± SEM.

TIMP-1/2 Inhibition Potentiates Cue-induced Heroin Seeking and Associated MMP Gelatinolytic Activity

To determine the role of TIMP involvement during cue-induced heroin seeking, cre(-) Long Evans wildtype animals were pre-treated with either IgG control or combined

TIMP-1 and -2 neutralizing antibodies in NAcore (6 µg/mL), using within-subject crossover design, 15 min prior to cued heroin reinstatement (30 min) and *in vivo* zymography assay (Figure 5-1A). Compared to IgG controls, TIMP-1/2 inhibition enhanced cued heroin seeking (Figure 5-2A). In addition, active lever pressing during the first 15 min of the reinstatement session was increased in TIMP-1/2 group compared to IgG controls (Figure 5-2B). Enhanced heroin seeking behavior corresponded with potentiated MMP activity in TIMP-1/2 antibody-treated animals compared to IgG controls (Figure 5-2C,D). There was no difference in cued heroin seeking behavior when either neutralizing TIMP antibody was given on its own compared to IgG (Figure 5-3). These data suggest possible adaptations in TIMP-1 and -2 inhibitory properties that may be promoting heroin seeking behavior.

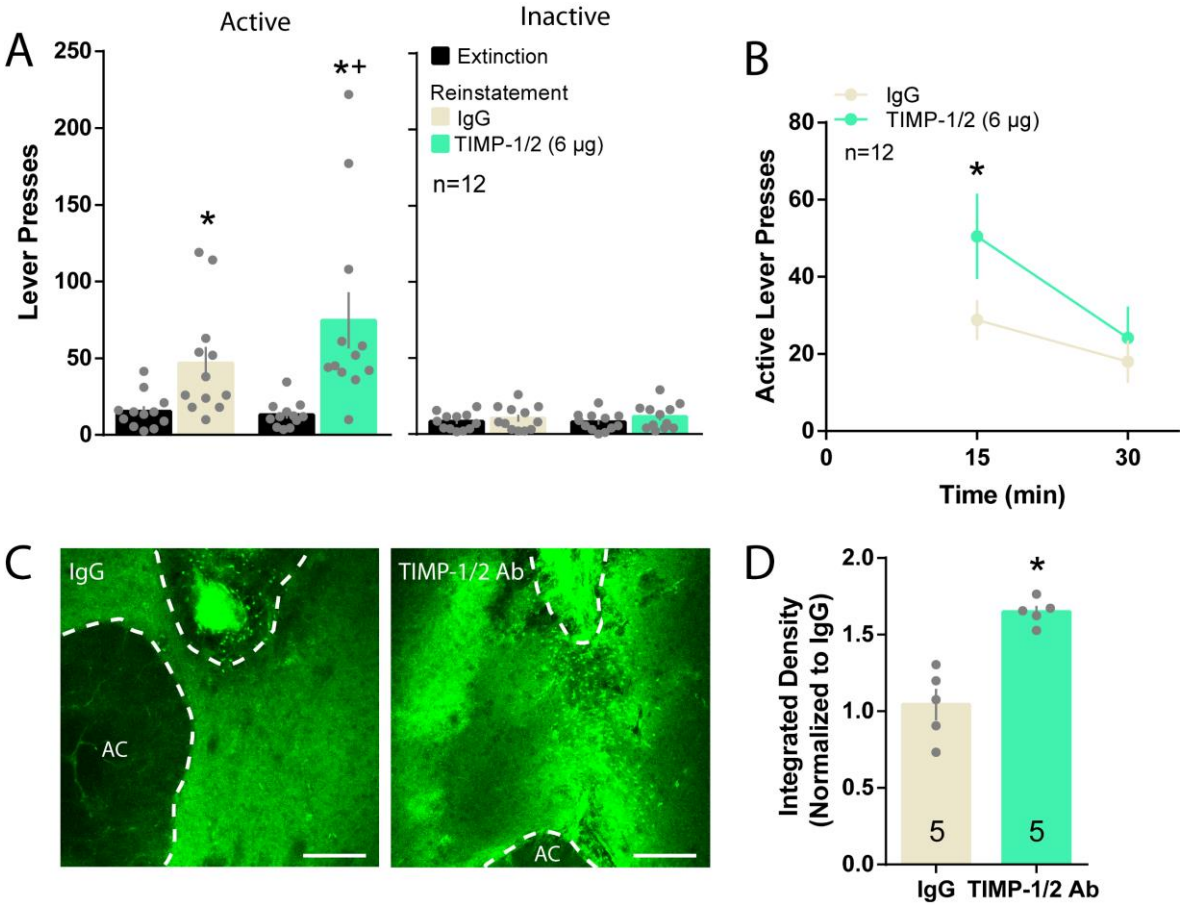

Figure 5-2. Dual TIMP-1/2 Inhibition Potentiates Cue-Induced Heroin Seeking and MMP Proteolytic Activity. A) Reinstated animals (t=30 min) received IgG or TIMP-1/2 antibodies microinjected in NAcore in randomized crossover design. Combined TIMP-1/2 inhibition significantly potentiates cued heroin seeking compared to IgG (2-way ANOVA; effect of treatment (IgG/TIMP) $F_{(1,11)}$=6.700; p=0.0252; EXT/RST $F_{(1,11)}$=11.56; p=0.0059; interaction $F_{(1,11)}$=6.650; p=0.0256). No difference in inactive lever presses during cue-induced heroin seeking after IgG or TIMP-1/2 antibody microinjection in NAcore (2-way ANOVA; $F_{(1,11)}$=0.3372; p=0.5731). **B)** Time course of reinstatement session revealed enhanced heroin seeking in first 15 min compared to IgG controls (2-way ANOVA; effect of treatment $F_{(1,22)}$=1.748; p=0.1998; effect of time $F_{(1,22)}$=28.40; p<0.0001; interaction $F_{(1,22)}$=4.939; p=0.0368). **C)** Representative fluorescent micrographs of NAcore *in vivo* MMP activity in reinstating IgG and TIMP-1/2 antibody-treated animals. Injection track and anterior commissure masked out with dashed line. **D)** Dual TIMP-1/2 inhibition significantly potentiates MMP-2/9 enzymatic activity in NAcore during cue-induced heroin reinstatement (Unpaired t-test; $F_{(4,4)}$=7.944; p=0.0005). Scale bar=250 µm. N in bars indicates number of animals with an average of four-seven NAcore slices/rat. Data was normalized to IgG control mean. Data are shown as mean ± SEM. *p<0.05 compared to extinction, $^{+}$p<0.05 compared to IgG using Bonferroni post hoc test.

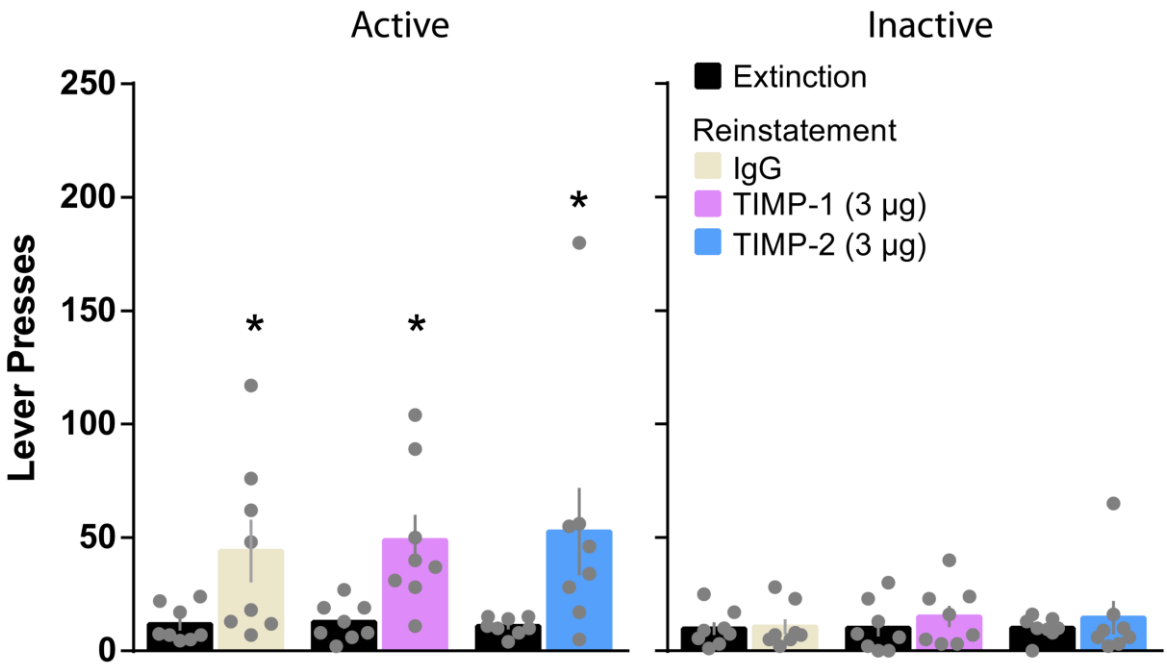

Figure 5-3. Single dose administration of TIMP-1 or -2 Ab does not significantly affect heroin reinstatement compared to IgG control. A) No difference in cued heroin seeking between IgG or TIMP-1 or TIMP-2 antibody administration in NAcore (2-way ANOVA; effect of treatment $F_{(2, 14)}$=0.1720; p=0.8437; EXT/RST $F_{(1, 7)}$=9.607, p=0.0173; interaction $F_{(2,14)}$=0.2145; p=0.8096). No difference in inactive lever presses during cue-induced heroin seeking after IgG or TIMP-1 or -2 antibody microinjection (2-way ANOVA; effect of treatment $F_{(2,14)}$=0.1827; p=0.8350; EXT/RST $F_{(1,7)}$=1.818; p=0.2196; interaction $F_{(2,14)}$=0.1743; p=0.8419). Data are shown as mean ± SEM. *p<0.05 compared to extinction.

Cell-type Specific TIMP-1/2 Expression around D1 and D2 MSNs

In the previous chapter, I described cell-type specific MMP gelatinolytic activity around D1 and D2 MSNs following withdrawal and cued heroin seeking conditions. Based on the role TIMPs play in regulating MMP activity and drug seeking, I hypothesized that TIMPs may also display cell-specific expression around D1 and D2 MSNs, possibly contributing to the specific gelatinolytic activity observed around these dichotomous cell types in NAcore. To determine if TIMPs also exhibit cell-type specific

expression, D1- and D2-cre (+) rats were virally transfected with cre-dependent mCherry construct and subsequently underwent heroin self-administration and extinction, followed by cue-induced reinstatement for 15 min (Figure 5-4). Yoked saline animals were used as a control and sacrificed 24 hours after last extinction session. Brain sections from NAcore of yoked saline, extinguished and heroin seeking D1 and D2 rats were collected and stained for TIMP-1 and -2 immunoreactivity (Figure 5-5 for reps). For D1 MSNs, TIMP-2 immunoreactive puncta were elevated around D1 MSNs in extinguished rats compared to yoked saline and reinstated groups (Figure 5-5C). A similar trend was found for TIMP-1 immunoreactivity around D1 MSNs (Figure 5-5A). For D2 MSNs, increased TIMP-1 and TIMP-2 immunoreactive puncta were observed around D2 MSNs during transient heroin seeking compared to yoked saline and extinguished rats (Figure 5-5B,D). In addition, there was a trend towards higher TIMP-2 expression around D1 MSNs compared to D2 MSNs after extinction training. Interestingly, TIMP-1 and -2 expression around D2 MSNs during heroin seeking was higher than expression around D1 MSNs, suggesting selective TIMP mediated reductions in MMP activity around D2 MSNs; thereby facilitating heroin seeking behavior (Figure 5-5). Importantly, there was no difference in total TIMP-1 and -2 expression per z-stack across SAL, EXT, and RST 15’ groups between D1 and D2 MSN cell-types (Figure 5-6).

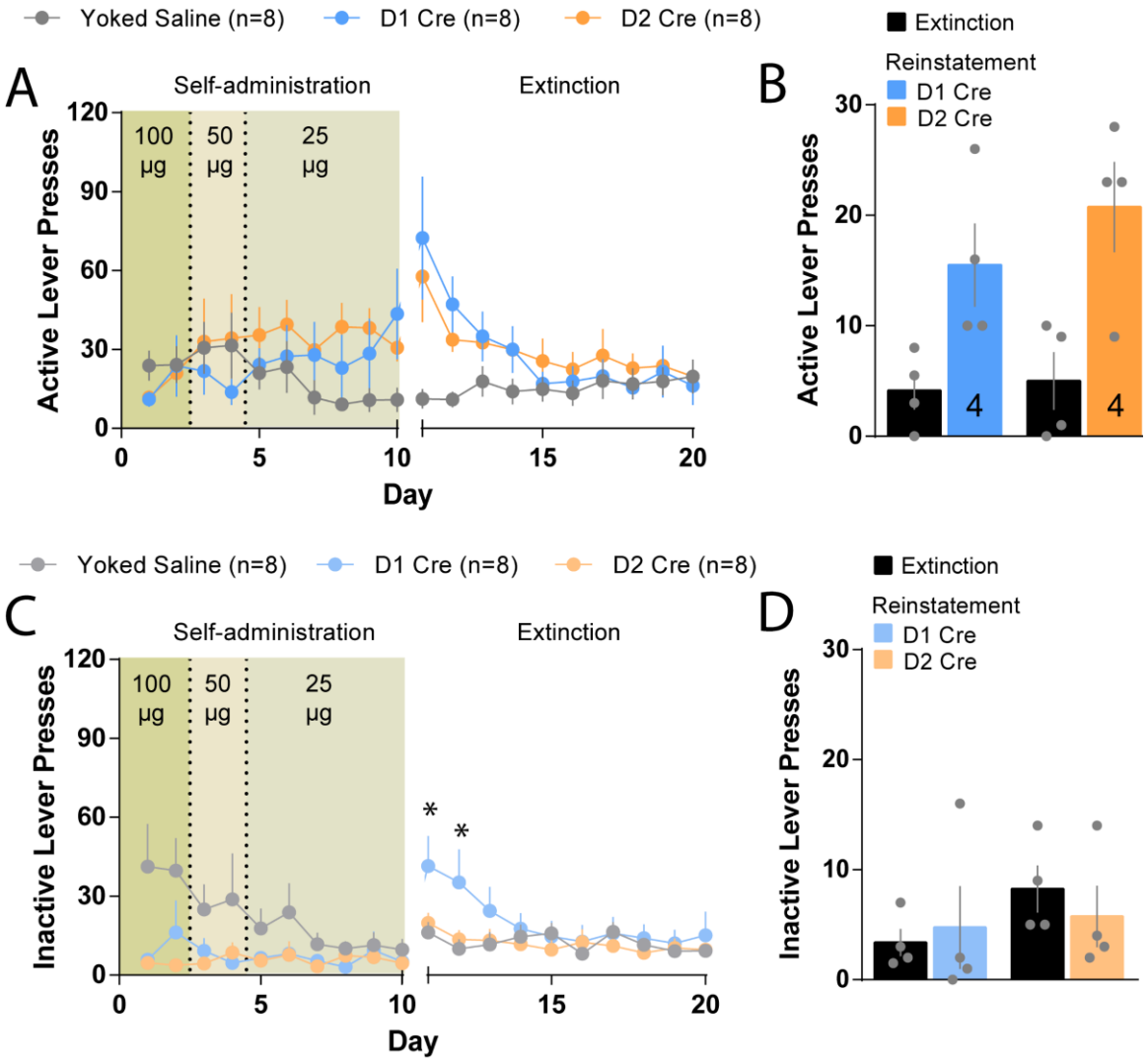

Figure 5-4. D1- and D2-Cre Heroin Self-Administration and Reinstatement Behavior **A)** Time course of active lever pressing in yoked saline and D1- and D2-cre animals during heroin self-administration and extinction. No difference in active lever presses between D1-cre and D2-cre rats trained to self-administer heroin (2-way ANOVA; effect of genotype $F_{(1,14)}$=0.3738; p=0.6820; effect of time $F_{(19,266)}$=3.704; p<0.0001; interaction $F_{(19,266)}$=0.6698; p=0.8474). **B)** Cue-induced reinstatement of heroin-seeking increased active lever presses over 15 mins similarly in D1- and D2-cre rats (2-way ANOVA; effect of genotype $F_{(1,6)}$=0.5776; p=0.4761; EXT/RST $F_{(1,6)}$=44.49; p=0.0005; interaction $F_{(1,6)}$=1.157; p=0.3233). **C)** Time course of inactive lever pressing in D1- and D2-cre animals during heroin self-administration and extinction. No difference in inactive lever presses between rat genotype (effect of genotype $F_{(1,13)}$=1.071; p=0.3196), but significant effect of time ($F_{(19, 247)}$=5.673; p<0.0001); interaction ($F_{(1, 5)}$=1.842; p=0.0192). **D)** No difference in inactive lever presses during behavioral tests (2-way ANOVA; effect of genotype $F_{(1,6)}$=0.7328; p=0.4248; EXT/RST $F_{(1,6)}$=0.1397; p=0.7214; interaction $F_{(1,6)}$=0.1.658; p=0.2453). Each dot in represents an animal. Data are shown as mean ±

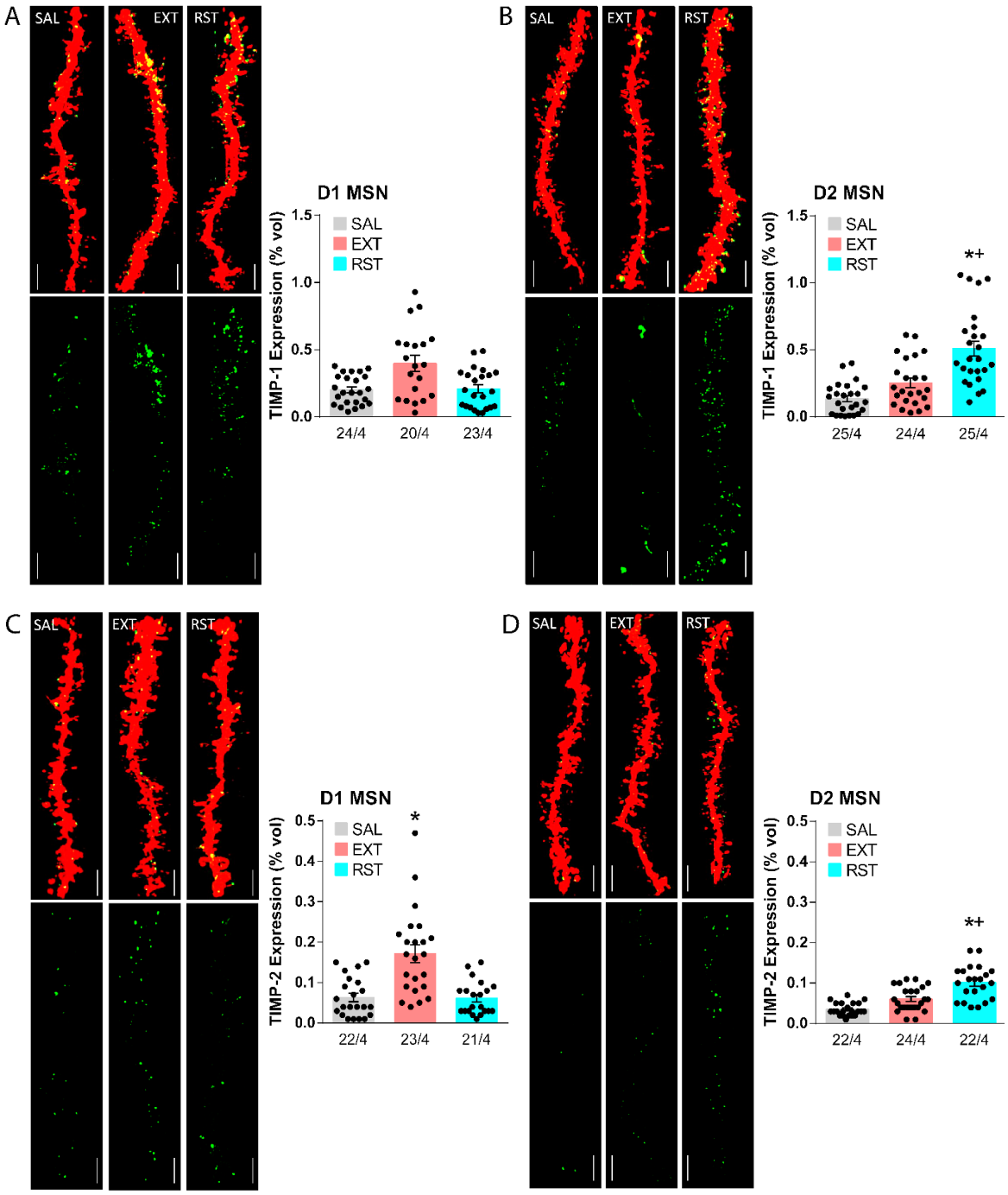

Figure 5-5. D1 and D2 MSN-specific localization of TIMP-1 and -2 immunoreactive puncta following extinction and transient heroin seeking. Representative images of 3D reconstruction of mCherry-labeled dendritic segments (red) and TIMP-1 or -2 puncta (green) localized within 300nm of dendrite surface for each group SAL, EXT, and RST 15' are shown beside each corresponding graph. **D1 MSNs: A)** No difference in TIMP-1 expression around D1 MSNs across groups (Nested 1-way ANOVA; $F_{(2,9)}$=1.318; p=0.3147). **C)** Increased TIMP-2 immunoreactivity around D1 dendritic segments under extinguished conditions compared to saline and RST (Nested 1-way ANOVA; $F_{(2,9)}$=7.202; p=0.0136).

D2 MSNs: B) Increased TIMP-1 and **D)** TIMP-2 immunoreactivity around D2 dendritic segments during cued heroin seeking (Nested 1-ANOVA; TIMP-1, $F_{(2,9)}$=19.08; p=0.0006; TIMP-2, $F_{(2,71)}$=17.63; p<0.0001). TIMP-1 and 2 expression is elevated around D2 MSNs during heroin seeking compared to D1 MSNs (TIMP-1, Nested t-test; $F_{(1,6)}$=9.034; p=0.0238; TIMP-2, Nested t-test; $F_{(1,47)}$=9.911; p=0.0029). Trend towards increased TIMP-2 expression around D1 MSNs under extinguished conditions compared to D2 MSNs (Nested t-test; $F_{(1,6)}$=4.729; p=0.0726). Data are shown as mean ± SEM. N represents number of neurons quantified over number of animals in each condition. *p<0.05 compared to SAL, $^{+}$p<0.05 compared to EXT using Bonferroni post hoc test. Scale bar= 5 µm.

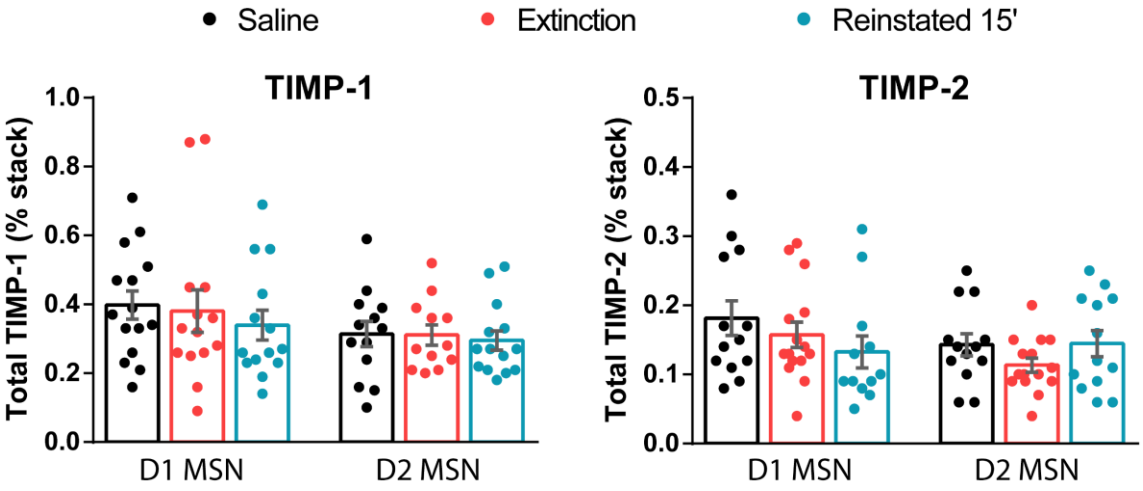

Figure 5-6. No Difference in Total TIMP Expression Across Groups Between D1 and D2 MSNs. Total MMP activity quantified in z-stack of SAL, EXT, and RST 15' groups did not differ between D1 and D2 mCherry-labeled MSNs (2-way ANOVA; effect of cell type, $F_{(1, 75)}$=2.319; p=0.1320; effect of group, $F_{(2, 75)}$=1.219; p=0.3012; interaction, $F_{(2, 75)}$=1.039; p=0.2762. Three to four stacks were quantified per animal. Data are shown as mean ± SEM.

Table 1-2. Summary II: D1 and D2 MSN-specific MMP Actvity vs TIMP Expression

	MMP Activity		TIMP-1/2 Expression	
Cell Type	**Extinguished**	**Reinstated 15'**	**Extinguished**	**Reinstated 15'**
D1 MSN	No change (compared to SAL)	Increased	Increased (compared to SAL)	No change
D2 MSN	Increased (compared to SAL)	No change	No change (compared to SAL)	Increased

Discussion

In the present study, TIMP-1/2 functionality was investigated based on the hypothesis that local MMP inhibition around specific cell-types is involved in cued heroin seeking. I discovered TIMP-1/2 inhibition in NAcore only significantly impacts behavioral responding during transient cued heroin seeking, not heroin refraining. This could be due to basal level MMP-2/9 activity seen in heroin-withdrawn conditions compared to heroin reinstated rats, thus TIMPs may not necessarily be inhibiting excessive MMP activity in this steady state (Smith, Kupchik et al. 2014). These findings fit nicely with observations that indicate TIMP-1 inhibition terminates synaptic plasticity producing mature spines (Magnowska, Gorkiewicz et al. 2016), hence if TIMPs are inhibited then MMP-2/9 gelatinase activity is unregulated and cued heroin seeking is exacerbated. It is conceivable that chronic treatment of TIMP-1/2 from extinction day 1 could produce protracted lever pressing in withdrawal due to excessive MMP activity and unregulated synaptic reconstruction, whereas at the end of extinction when synaptic plasticity may already be established. Another interesting aspect of this experiment is that combined administration of TIMP-1 and -2 antibody potentiates cued responding as opposed to single administration of either antibody alone. This could be due to the promiscuous nature of TIMPs and overlap in target substrates (Dzwonek, Rylski et al. 2004). While TIMP-1 has preference for MMP-9, and TIMP-2 has greater affinity for MMP-2, there is some overlap with other MMP species (Vafadari, Salamian et al. 2016). It is possible both MMP-2 and MMP-9 need to targeted simultaneously given their role in mediating cued reinstatement (Smith, Kupchik et al. 2014).

I also discovered cell-specific expression of immunoreactive TIMP-1 and -2 puncta around D1 and D2 MSNs in NAcore following extinction and transient cued heroin seeking. The rationale behind this experiment was to determine if local inhibition

of MMP activity around these cell-types mediated heroin seeking. In the previous chapter, D2 MSNs exhibited increased MMP-2 activity only in heroin-extinguished animals, but MMP-2,9 activity after 15 min reinstatement was reduced to saline control levels. This was an interesting observation considering overall MMP activity in NAcore is increased during transient cued reinstatement, suggesting focal regulation of MMPs at the cellular level. For D1 MSNs, I observed increased TIMP-2 immunoreactivity around D1 dendritic segments under extinguished conditions compared to saline controls and reinstated groups. For D2 MSNs, I observed increased TIMP-1 and TIMP-2 immunoreactivity around D2 dendritic segments during transient heroin reinstatement compared to saline controls and heroin-extinguished rats. Importantly, D2 MSNs exhibited greater TIMP-2 localization compared to D1 MSNs during cued heroin seeking, suggesting selective regulation around D2 MSNs to promote drug seeking mediated by D1 MSN activation. These data oppose the cell-type specific MMP localization data in Chapter 4, conveying the dichotomous relationship between MMPs and their endogenous inhibitors. Selective expression of either TIMP-1 or -2 protein around D1 and D2 MSNs may be indicative of MMP-2 or-9-specific regulation in further contributing to expression of motivated seeking. Despite data suggesting no change in TIMP-1 and -2 protein expression in cued reinstatement compared to saline and extinguished groups (Figure 5-6) (Smith, Kupchik et al. 2014), these cell-specific data suggest TIMPs play an active role in drug seeking behavior.

The data described above rely on immunohistological quantification of immunoreactive TIMP puncta, and not necessarily interaction between with MMP-2 and -9. The ideal experiment would be to measure colocalization of MMP gelatinolytic puncta and TIMP protein via fluorescence resonance energy transfer (FRET), however, these data may be confounded by the fact that TIMP-1 and TIMP-2 can form complexes with

proMMP-9 and proMMP-2, respectively, for activation (Shofuda, Moriyama et al. 1998, Toth, Chvyrkova et al. 2003, Dzwonek, Rylski et al. 2004, Sánchez-Pozo, Baker-Williams et al. 2018).

Given the role of TIMP-1 in spine maturation, it is of interest to determine if dendritic spine heads exhibit increased localization with TIMP-1 or TIMP-2 during cued heroin seeking, and if this corresponded to cell-type specific morphology (Chapter 4). Many studies indicate the importance of TIMP-1 regulation of MMP activity and termination of synaptic plasticity (Okulski, Jay et al. 2007). At the molecular level, TIMP1 mRNA is rapidly upregulated following LTP induction and onset of seizure activity, ensuring neuroprotection from excessive proteolytic damage (Rivera, Tremblay et al. 1997). Behaviorally, aberrations in TIMP function or expression affect fear-potentiated startle, spatial memory, and other forms of cognitive function (Jaworski, Boone et al. 2005, Jourquin, Tremblay et al. 2005, Chaillan, Rivera et al. 2006, Baba, Yasuda et al. 2009). ECM integrity depends on balance between MMP degradation and TIMP inhibition, thus TIMP function is a critical component of synaptic plasticity. Therapies targeting this system may be beneficial in restoring physiological processes that mediate adaptive behaviors.

Chapter 6: Astroglial Morphological Plasticity and MMP Activity in Nucleus Accumbens

Introduction

Tetrapartite synaptic physiology involves ongoing interaction between pre and post-synapse, astroglial processes, and the ECM at excitatory synapses (Dityatev, Seidenbecher et al. 2010). Our understanding of synaptic plasticity arose from many studies that have focused on ECM signaling around dendritic spines (Michaluk, Wawrzyniak et al. 2011, Stawarski, Stefaniuk et al. 2014, Szepesi, Hosy et al. 2014). Given the plastic nature of this synaptic element, it is known that MMP-9 activity induces morphological adaptations in response to neuronal activity (Vafadari, Salamian et al. 2016). Recent discoveries also characterize astrocytes as dynamic synaptic components with transiently evoked morphological changes (Perez-Alvarez, Navarrete et al. 2014, Kruyer, Scofield et al. 2019). Astroglial cells were classically known as support cells due to their ability to maintain extracellular homeostasis in their microenvironment (Deitmer 2004, Scofield and Kalivas 2014, De Pittà, Brunel et al. 2016). Peripheral astroglial processes make contact with thousands of synapses, maintaining integrity with neurons to facilitate inter-communication and neuronal metabolism (Bushong, Martone et al. 2002, Parpura and Verkhratsky 2012). In addition, astrocytes play a role in synaptic formation and transmission (Eroglu and Barres 2010). In the nucleus accumbens, astroglia regulate excitatory synaptic plasticity through release and uptake of extrasynaptic glutamate via the cysteine-glutamate exchanger and glutamate transporter (GLT-1), respectively, strategically located at perisynaptic peripheral processes (Anderson and Swanson 2000, Kalivas 2009, Takahashi, Foster et al. 2015). Chronic use of drugs of abuse, such as cocaine, heroin and nicotine, reduce function and expression of both the exchanger (not seen with heroin) and GLT-1, contributing to

glutamate spillover and increased relapse propensity (Knackstedt, LaRowe et al. 2009, Knackstedt, Melendez et al. 2010, Gipson, Reissner et al. 2013, Shen and Kalivas 2013, Reissner, Gipson et al. 2015). Downregulation of the exchanger causes low basal glutamate levels (unknown for heroin) and increased presynaptic glutamate release probability, due to lack of tonic stimulation of presynaptic mGluR2/3 autoreceptors (Moussawi, Pacchioni et al. 2009). Moreover, impaired GLT-1 function results in poor glutamate clearance and aberrant glutamate homeostasis, which is further exacerbated with excessive glutamate release during cue-induced reinstatement (McFarland, Lapish et al. 2003, LaLumiere and Kalivas 2008, Gipson, Reissner et al. 2013). However, functional restoration of these two glutamate transporters with compounds such as, N-acetylcysteine or ceftriaxone, suppresses cued drug seeking (Zhou and Kalivas 2008, Knackstedt, Melendez et al. 2010, Reichel, Moussawi et al. 2011, Shen, Scofield et al. 2014). Interestingly, subsequent studies found that activation of Gq-coupled signaling cascades in NAcore astrocytes using DREADD receptors decreases both ethanol and cocaine seeking (Bull, Freitas et al. 2014, Scofield, Boger et al. 2015). These data further support the role of gliotransmission in inhibiting aberrant motivation to seek abusive substances.

Emerging evidence indicate astroglial morphological plasticity also mediates drug seeking behavior. Following both cocaine and heroin self-administration and extinction, synaptic proximity of astroglial processes is reduced, indicated by decreased co-registry of the astrocyte membrane and presynaptic marker Synapsin I (Scofield, Li et al. 2016, Kruyer, Scofield et al. 2019). However, synaptic contact is rapidly restored during transient cued heroin seeking. This motility is mediated by astroglial actin-binding protein, Ezrin. Thus ezrin knock down reduces astroglia-synapsin colocalization, and consequently exacerbates cued heroin seeking (Kruyer, Scofield et al. 2019). These

findings indicate compensatory involvement of astrocytes in mediating motivational responding.

Matrix metalloproteinases (MMP) have been well-studied for their role in synaptic plasticity and reconstruction by digesting the extracellular matrix to permit morphological adaptations (Huntley 2012). MMP-2 and -9 (gelatinases) are released from astrocytes, microglia and neurons, and the most well-studied in the addiction field (Könnecke and Bechmann 2013, Smith, Scofield et al. 2015), and it is plausible they may play a role in dynamic astrocyte morphological plasticity observed during transient cued heroin reinstatement. Previous *in vitro* studies discovered MMP-2, not MMP-9, involvement in astrocyte migration (Ogier, Bernard et al. 2006); however it remains unknown how *in vivo* MMP gelatinase activity plays a role in active astroglial plasticity during transient behaviors.

To determine if the morphological plasticity associated with heroin extinction and cued reinstatement involves MMP activation, I utilized the previously described heroin self-administration, withdrawal and reinstatement model, and virally transfected rats with AAV-GFAP-mCherry vector to quantify MMP-2/9 activity specifically around mCherry-labeled astroglial cells in NAcore. I hypothesize that astrocytes exhibit increased MMP gelatinolytic activity during heroin seeking, potentially contributing to NAcore synaptic astroglial plasticity. Together, these data may reveal an underlying MMP-mediated pathway that contributes to compensatory astroglial regulation of cue-induced heroin reinstatement.

Results

Astroglial Plasticity and MMP-2/9 Localization During Heroin Seeking and Withdrawal

Previous work from our lab has found that synaptic retraction of NAcore astroglia after heroin self-administration and extinction is partially reversed during transient cued heroin seeking, restoring synaptic proximity of peripheral processes (Kruyer, Scofield et al. 2019). Given the dynamic nature of astrocyte motility and cued-induced plasticity, it is plausible MMPs contribute to migration of astrocyte processes via regulation of actin cytoskeleton (Ogier, Bernard et al. 2006). While MMP-2 gelatinolysis has been implicated in *in vitro* astrocyte migration, it is unknown whether *in vivo* MMP-2/9 activity is involved using in heroin self-administration and cued reinstatement. To determine if MMP gelatinolytic activity around astrocytic processes is involved in astrocyte synaptic morphology, male and female cre (-) Long Evans wildtype rats were labeled with a membrane-bound fluorescent reporter for subsequent immunohistochemical analysis (Figure 6-2). Rats were trained on heroin self-administration or yoked saline (10 days), extinguished, and some were reinstated to cues previously associated with heroin infusions (15 min) with concurrent FITC gelatin injection for *in vivo* zymography assay (Figure 6-1). Yoked saline animals were used as a control and sacrificed 24 hours after last extinction session. As expected, animals re-exposed to cues during reinstatement significantly pressed the active lever compared to inactive lever and their extinction baseline (Figure 6-1). Brain sections from NAcore of yoked saline, heroin-extinguished and heroin seeking rats were collected and stained for presynaptic marker Synapsin I and imaged using confocal microscopy. Following astrocyte image acquisition, Imaris 3D processing was utilized to quantify total MMP gelatinolytic puncta co-registry with astrocyte membrane as well as co-registration of MMP-2/9 puncta and Synapsin I

(Figure 6-2). These measurements were normalized to the volume of each respective mCherry-labeled astrocyte. As previously shown (Kruyer, Scofield et al. 2019), co-registration of the astroglial membrane with Synapsin I was reduced after heroin extinction (Figure 6-3), indicating low synaptic contact after withdrawal. Importantly, synaptic contact is restored during transient cue-induced heroin seeking, as shown in (Kruyer, Scofield et al. 2019) (Figure 6-3A). As previously reported, there was no difference in total synapsin I across groups, supporting changes in astrocyte proximity and not number of synaptic contacts (Figure 6-3C) (Kruyer, Scofield et al. 2019). Interestingly, co-registration of MMP-2/9 puncta and Synapsin I was reduced under extinguished conditions, however, cue-induced heroin seeking increased co-registration of MMP puncta with Synapsin I (Figure 6-3B). Quantification of total overall MMP-2/9 fluorescence showed a trend towards an increase in the reinstated group, consistent with previous findings of elevated MMP activity during drug seeking (Figure 6-3D) (see chapter 4 and (Smith, Kupchik et al. 2014). These data suggest possible MMP-2/9 involvement at synapses re-associating with dynamic astroglial processes during heroin seeking.

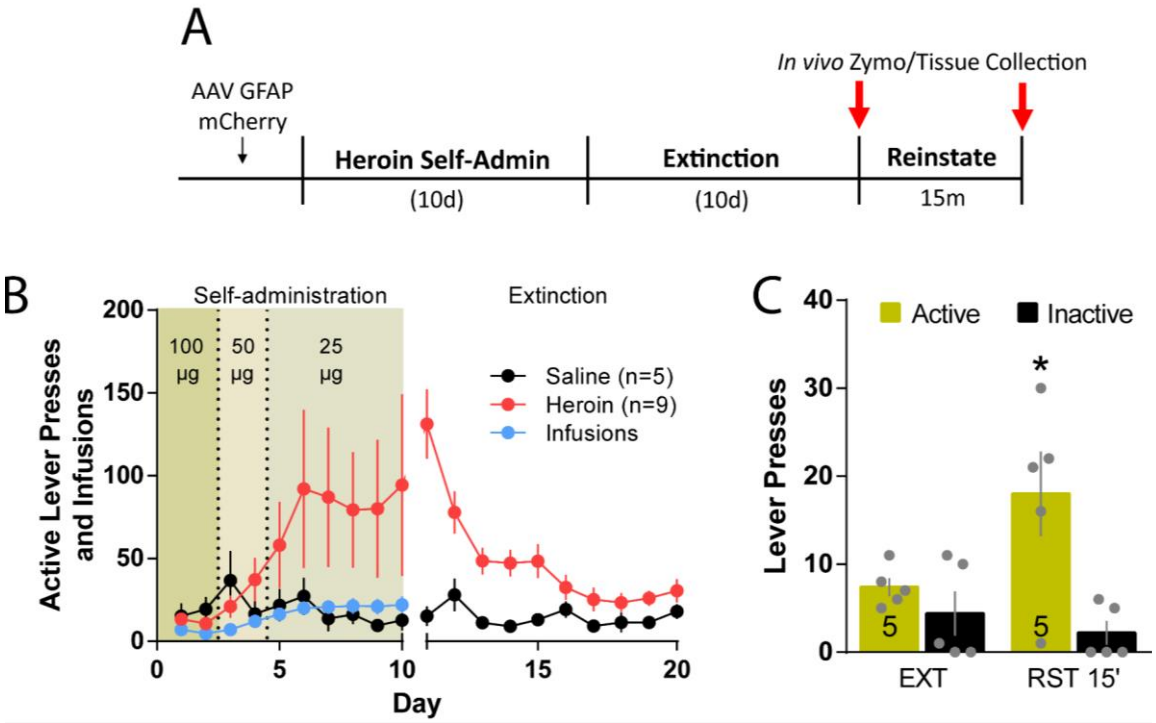

Figure 6-1. Heroin Self-Administration, Extinction, and Reinstatement Behavior. A) Experimental timeline outlining heroin self-administration, extinction, and reinstatement. Rats were trained to self-administer heroin and were subsequently extinguished to the cues associated with heroin infusions. **B)** Time course of active lever pressing in wildtype animals during heroin self-administration and extinction. **C)** Cued heroin seeking induced significant active lever presses in rats (2-way ANOVA; Inactive/Active $F_{(1,4)}$=31.94; p=0.0048; EXT/RST $F_{(1,4)}$=5.805; p=0.0736; interaction $F_{(1,4)}$=5.184; p=0.0851). N in bars indicates number of animals. Data are shown as mean ± SEM. *p<0.05 compared to inactive using Bonferroni post hoc test.

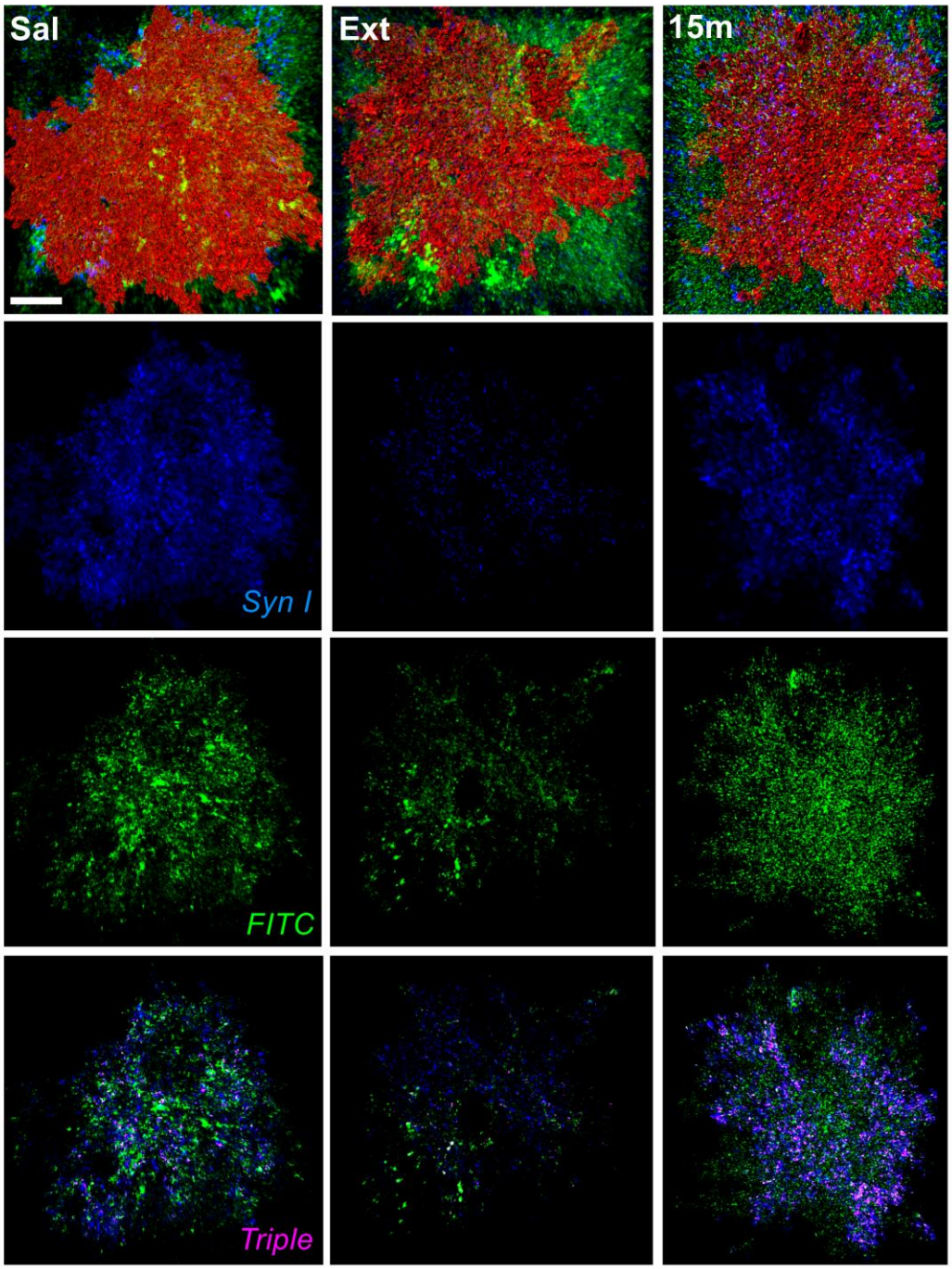

Figure 6-2. Imaris Quantification Workflow for MMP-Synapsin I colocalization. Representative micrographs of digitized 3D model of mCherry-labeled astrocyte (red) for each treatment group (Sal, Ext, and Rst 15 m). Synaptic proximity is measured by co-registry of astroglial membrane and synapsin I immunoreactivity (blue). Following heroin extinction, synaptic contact is reduced, but restored to control levels during transient heroin seeking. Green fluorescence indicating MMP activity (i.e FITC) within 250 nm of virally-labeled astrocyte membrane is masked to quantify co-localization of MMP activity (green) with Synapsin I (blue) from the region occupied by the astrocyte in the top panel. This triple co-localization is shown in pink, indicating increased MMP activity around synapses re-associated with astrocyte processes during heroin reinstatement.

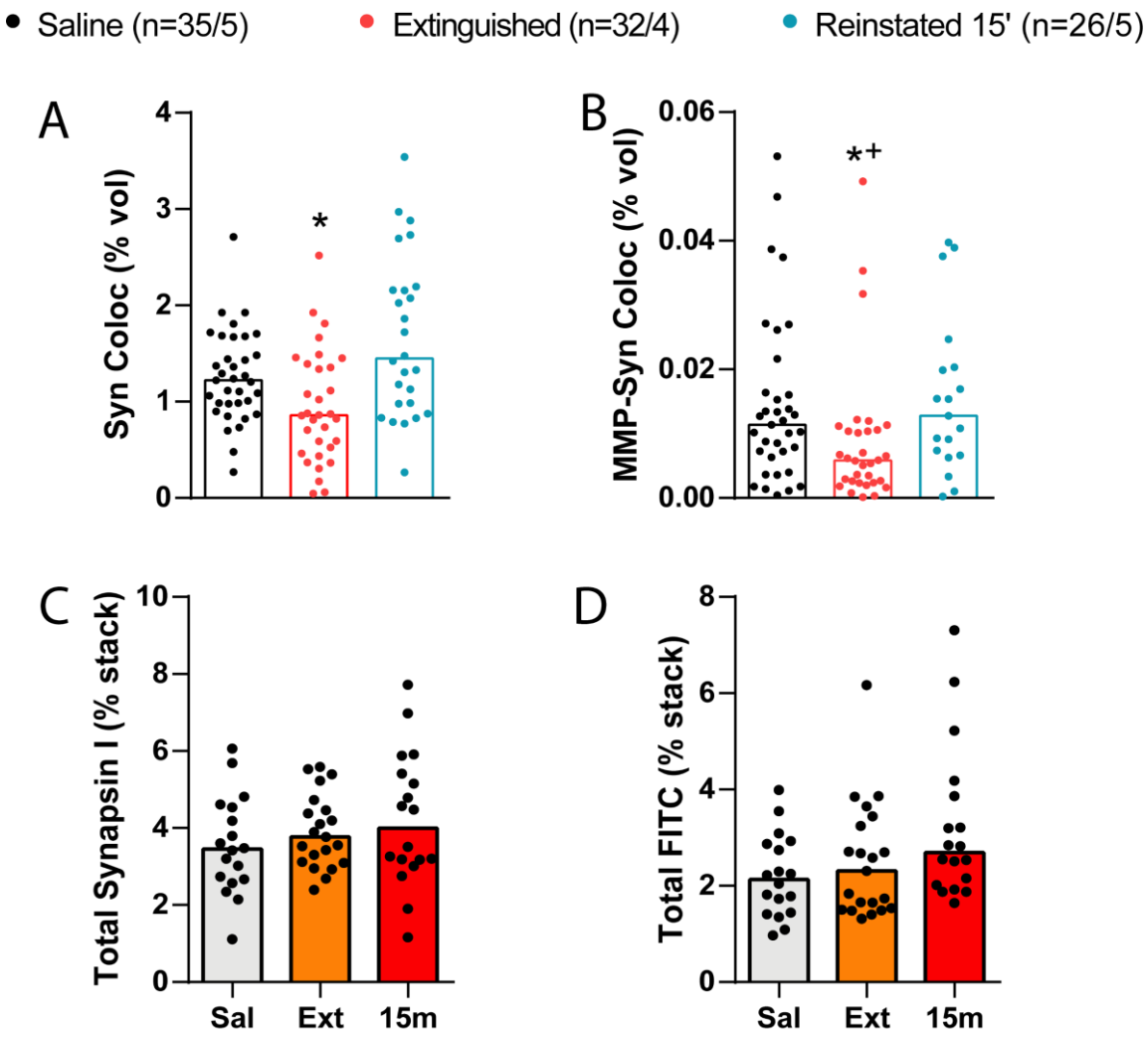

Figure 6-3. MMP Gelatinolytic Puncta Colocalize with Astroglial Peripheral Processes in NAcore During Transient Heroin Seeking. A) Synaptic contact between astrocyte membrane and presynaptic marker synapsin I was reduced after heroin extinction, while cued heroin seeking transiently restores peripheral astroglial processes to synapse (Kruskal-Wallis=13.32; p=0.0013). **B)** MMP gelatinolytic puncta and synapsin co-registry was reduced under extinguished conditions, while transiently increased during cue-induced heroin seeking (Kruskal-Wallis=12.80; p=0.0017). **C)** No difference in total Synapsin I across groups; normalized to z-stack volume (Kruskal-Wallis=1.605; p=0.4483). **D)** Trend towards increase in total MMP activity in cued heroin seeking group; normalized to z-stack volume (Kruskal-Wallis=5.208; p=0.0740). N represents number of cells quantified over number of animals in each condition. Data are shown as median. *p<0.05 compared to SAL, $^{+}$p<0.05 compared to RST using Dunn post hoc test. Data were processed and quantified in collaboration with Anna Kruyer, PhD.

Discussion

In the experiments described above, I endeavored to determine whether gelatinolytic activity of MMP-2/9 was involved in transient astrocytic plasticity during cued heroin seeking. First, by using confocal microscopy and digital rendering of NAcore astrocytes, I recapitulated previous findings that astroglial synaptic contact is reduced after heroin extinction, and transiently restored during cue-induced heroin reinstatement to saline control levels (Kruyer, Scofield et al. 2019). MMP-2/9 proteolytic activity around the astrocyte membrane was significantly reduced in heroin-extinguished rats compared to saline controls. Remarkably, co-registry of MMP gelatinolytic puncta and Synapsin I was decreased under extinguished conditions, while cue-induced heroin seeking produced an increase in synapsin-MMP co-registration. These findings suggest MMP-2/9 involvement at synapses transiently re-associated with astrocyte processes during heroin seeking using a rodent model of heroin self-administration. Dynamic astroglial motility during active heroin seeking is compensatory and negatively regulates drug seeking behavior (Kruyer, Scofield et al. 2019). It is possible MMP-2/9 activity facilitates this compensatory mechanism to allow for adaptations in astroglial morphology during transient seeking. This postulation raises the question: Is MMP-2/9 activity necessary for astrocytic plasticity? Previous data suggest MMP-2/9 activity is necessary for drug seeking behavior and potentiation of synaptic strength (i.e. AMPA:NMDA current ratios and dendritic spine head morphology) (Smith, Kupchik et al. 2014). However, it is unknown whether transient astroglial motility during heroin seeking is contingent upon MMP-2/9 gelatinolytic activity. Thus, further studies need to determine the necessity of MMP-2/9 co-registry with peripheral processes and if synaptic proximity is still restored when MMP-2/9 activity is inhibited.

To that end, MMP-2/9-mediated ECM digestion may initiate intracellular signaling to Ezrin, an astrocyte-specific actin-binding protein, which when phosphorylated enables membrane protrusions and motility (Kawaguchi, Yoshida et al. 2017). Previous studies have found constitutive pericellular MMP-2 proteolytic activity associated with actin-rich filopodia and lamellipodia of migrating astrocytes, indicating a link between gelatinolytic degradation and actin reorganization (Ogier, Bernard et al. 2006). Further evaluation of whether ezrin phosphorylation and subsequent astrocyte migration is MMP-2/9-dependent would elucidate outside-in signaling that contributes to drug seeking behavior (Luo, Carman et al. 2007) (Garcia-Keller et al. 2020, submitted).

The relationship between compensatory astrocyte motility and transient synaptic potentiation in NAcore medium spiny neurons during heroin seeking still remains unclear. Interestingly, specific striatal astrocytes selectively respond to activity of either D1 or D2 MSNs, suggesting pre-defined neuron-to-astrocyte synaptic communication (Martín, Bajo-Grañeras et al. 2015). In Chapter 4, I discovered D1 MSNs exhibit increased extracellular MMP gelatinolytic activity during transient cued heroin seeking, similar to perisynaptic astrocyte processes. Thus, it is plausible MMPs may be mediating synaptic proximity between D1 and D2 MSNs and astrocytes after cued seeking and heroin withdrawal (see proposed model in Chapter 7). As findings continue to emerge, a greater understanding of tetrapartite synapse plasticity could reveal therapeutic targets to treat symptoms of addiction.

Chapter 7: Conclusions and Future Directions

The neuroscience community has made great advances in our understanding of synaptic transmission. Previously known as the tripartite synapse, comprised of pre- and post-synapse and perisynaptic astroglial processes, a fourth component, the extracellular matrix, has been incorporated to reflect adaptations in this vast proteinaceous structural scaffold and signaling domain (Dityatev, Seidenbecher et al. 2010). Recent acceptance of the tetrapartite synapse has ignited numerous studies exploring the dynamics of this system in the role of synaptic plasticity, specifically as it pertains to learning and memory (Meighan, Meighan et al. 2006, Nagy, Bozdagi et al. 2006, Wright, Brown et al. 2007, Huntley 2012). To expound upon these studies, my dissertation utilizes a cell-specific approach to understand dynamics of extracellular matrix signaling as they pertain to opioid addiction. Here, I show that matrix metalloproteinases (MMPs) exhibit cell-type specific activity around components of the tetrapartite synapse, specifically D1- and D2-receptor expressing medium spiny neurons (MSNs) and astrocytes in nucleus accumbens core (NAcore). I discovered enduring MMP-2 proteolytic activity around D2 MSNs following extinction training, while MMP-9 proteolytic activity localized around D1 MSNs was transiently present during cue-induced heroin seeking. For astroglia cells, I observed reduced MMP gelatinolytic activity around synapses exhibiting decreased contact with peripheral processes under extinguished conditions, however, synaptic MMP-2/9 enzymatic activity was restored as astroglial processes returned to synapse during cued heroin seeking. Furthermore, I determined how tissue inhibitors of metalloproteinases (TIMPs) functionally regulate heroin seeking behavior with pharmacological manipulation and single cell immunoreactivity quantification. In these studies, dual inhibition of TIMP-1/2 enhances cued heroin seeking and associated MMP activity. I found that TIMP-1 protein preferentially localizes around D1 MSNs following heroin-extinction, while TIMP-1/2

immunoreactivity harbors around D2 MSNs during transient cue-induced heroin seeking. These findings oppose the aforementioned cell-type specific MMP gelatinolytic activity localized around D1 and D2 MSNs, providing mechanistic support for how these cell types promote drug seeking and withdrawal/behavioral inhibition, respectively.

Constitutive Cell-type Specific Tetrapartite Plasticity

Following chronic use of opioids, maladaptive morphological and functional changes arise at glutamatergic synapses in NAcore, and these adaptations occur uniquely in each component of the tetrapartite synapse (Hearing 2019, Kruyer, Chioma et al. 2020). Prolonged opioid exposure impairs NAcore glutamate homeostasis due to downregulation of astroglial GLT-1 and disrupted tone onto mGluR2/3 autoreceptors, which negatively regulate synaptic glutamate release (Robbe, Bockaert et al. 2002, Shen and Kalivas 2013). Opioid extinction training possibly reduces presynaptic excitation of NAcore D1 MSNs, while increasing stimulation of D2 MSNs to facilitate inhibitory drug seeking behavior, i.e. refraining (Figure 7-1B). This hypothesis is based on previous findings utilizing a cocaine-refraining paradigm (Roberts-Wolfe, Bobadilla et al. 2018). Other studies employing non-contingent morphine administration observed morphine-induced weakening of D2 MSN synaptic inputs from infralimbic cortex to nucleus accumbens shell (NAshell) circuitry (Graziane, Sun et al. 2016, Hearing, Jedynak et al. 2016). Although, infralimbic-NAshell projections are more involved in regulating extinguished responding and behavioral inhibition than NAcore, it is also possible that some projections from the prelimbic cortex may innervate D2 MSNs to mediate this behavior (Vertes 2004, Peters, LaLumiere et al. 2008). The proposed increased glutamate release onto D2 MSNs activates mGluR5 receptors on local neuronal nitric oxide synthase (nNOS) interneurons, which then release NO and activate MMP-2 via S-nitrosylation (Dziembowska and Wlodarczyk 2012, Smith, Scofield et al.

2017). This produces the enduring MMP-2 activity observed around D2 MSNs under extinguished conditions (i.e. 24 hours after last extinction session), likely contributing to potentiated D2 MSN synaptic strength following extinction training (Roberts-Wolfe, Bobadilla et al. 2018). However, it is unclear how enduring this MMP-2 gelatinolysis is and whether it can be manipulated to affect extinction behavior. MMP-2 proteolytic activity subsequently stimulates integrin-dependent intracellular cascades to phosphorylate focal adhesion kinase (p-FAK), a signaling molecule that mediates expression of postsynaptic glutamate receptors (Park and Goda 2016, Garcia-Keller, Neuhofer et al. 2019). It is possible these activated D2 neurons innervate ventral pallidal GABAergic (VP_{GABA}) to promote heroin refraining (Heinsbroek, Bobadilla et al. 2020).

In addition to reduced expression of glutamate transporters, astrocytes exhibit reduced synaptic contact, further contributing to dysregulated glutamate balance following heroin exposure (Kruyer, Scofield et al. 2019). The timeline of when retraction has fully occurred is unclear, but it is plausible that earlier on in extinction training MMP-2/9 activity may be detectable at synapses in close proximity to astroglial processes. It is also unclear how astroglial retraction directly relates to MMP localization around D1 and D2 MSNs, however, it is possible these are two independent processes working in concert to facilitate enduring processes during heroin withdrawal (Figure 7-1B). Astrocyte retraction contributes to aberrations in glutamate homeostasis, which primes the tetrapartite synapse for potential relapse (Scofield, Li et al. 2016, Kruyer, Scofield et al. 2019). Whereas D1 and D2 MSN-specific MMP-2/9 localization and subsequent alterations in spine head morphology mediate active behavioral inhibition after withdrawal. Thus, similar to how different chemical classes of abused drugs induce distinct enduring synaptic modifications, components of the tetrapartite synapse can also

exhibit dissociable functions in maintaining inhibited drug seeking in the absence of conditioned cues capable of inducing drug seeking.

Transient Cell-type Specific Tetrapartite Plasticity

During transient drug seeking, glutamate spillover is increased causing subsequent activation of MMP-2 and -9, which degrades the extracellular matrix and permits transient plasticity at tetrapartite synapse (Smith, Scofield et al. 2017). I discovered increased gelatinase activity at synapses actively re-associating with perisynaptic astroglial processes (PAPs) as well as at dendritic spine heads of D1 MSNs during cue-induced heroin seeking. It is plausible that astroglial plasticity occurs in a cell-type specific manner to mediate heroin seeking behavior (Martín, Bajo-Grañeras et al. 2015). The observed MMP-2/9 activity around astroglial processes may be a consequence of astrocytes being within synaptic proximity to D1 MSNs. Astrocytes harboring cue-induced changes in MMP activity may make preferential contact with D1 MSNs during transient heroin seeking to act as insulation against synaptically released glutamate, and thus prevent spillover into extrasynaptic space (Figure 7-1C) (Rusakov 2001, Parpura and Zorec 2010, Zorec, Araque et al. 2012). Thus, retraction from D1-MSNs permits spillover and stimulation of transient synaptic potentiation (t-SP), while the restoration of synaptic proximity is compensatory. Alternatively, astroglial peripheral processes may return to the synapse to release TIMP-1 or -2 to terminate D1 MSN-mediated t-SP and promote dendritic spine maturation (Rivera, Tremblay et al. 1997, Magnowska, Gorkiewicz et al. 2016). Future studies are warranted to disentangle these possible cell-specific mechanisms. Furthermore, astrocytes are also able to communicate with D2 MSNs (Martín, Bajo-Grañeras et al. 2015). During cued seeking, a complimentary or alternate hypothesis is that astrocytes may restore proximity selectively to presynaptic terminals of D2 synapses. Gliotransmission onto mGluR2/3

autoreceptors decreases release probability, and thus lack of glutamatergic stimulation of D2 MSNs promotes transient drug seeking (Heinsbroek, Neuhofer et al. 2017). This lack of neurotransmission also contributes to reduced MMP-2/9 activity and increased TIMP expression around D2 MSNs. This proposed microcircuit describes cell-type specific neuronal-astrocyte interaction within the dynamic tetrapartite synapse contributing to expression of active drug seeking behavior. Further investigation is warranted to determine if astrocytes exhibit a predilection for one synapse or the other following withdrawal or relapse. These perturbations may serve as therapeutic targets to precisely ameliorate underlying pathological aberrations in synaptic physiology in the addicted brain.

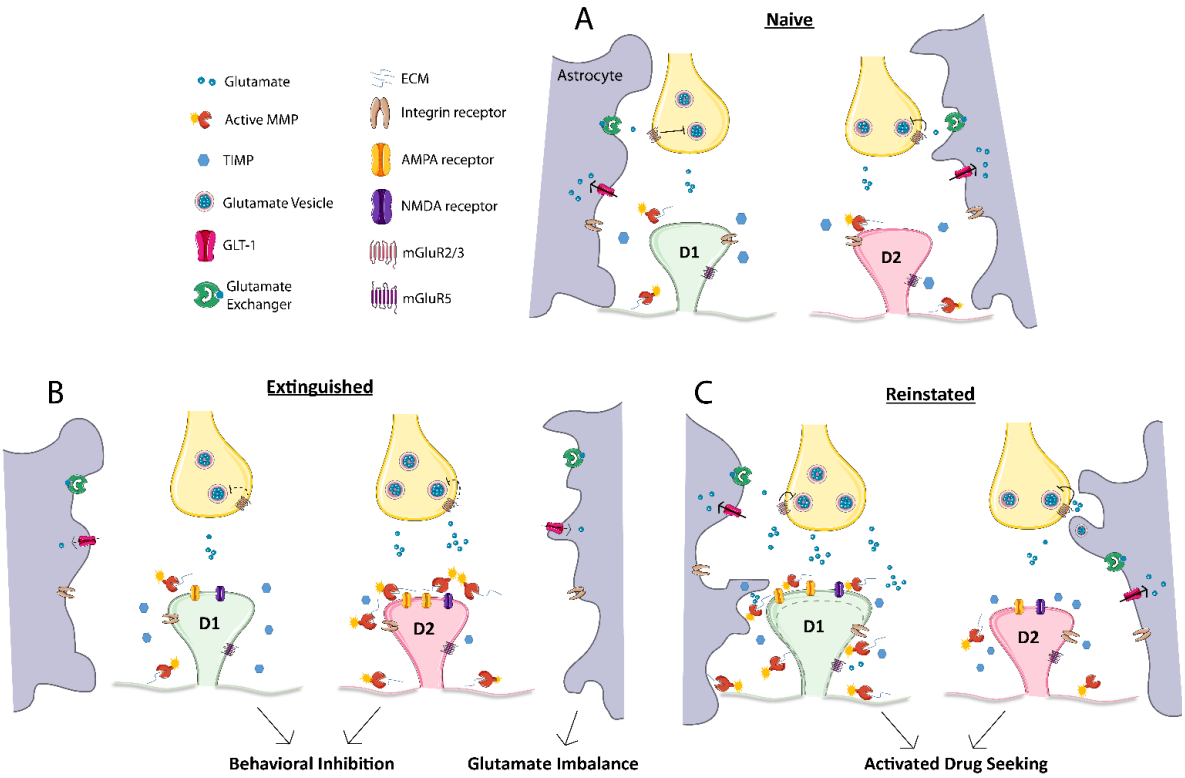

Figure 7.1 Proposed Cell-type Specific Tetrapartite Synapse Plasticity in Nucleus Accumbens. A) The tetrapartite synaptic structure comprises of pre- and postsynapse, perisynaptic astroglial processes, and surrounding extracellular matrix. Under normal, drug naïve conditions, glutamate homeostasis is maintained by constant uptake and release via function of astroglial GLT-1 and cysteine-glutamate exchanger, respectively. Glutamate release onto mGluR2/3 negatively regulates presynaptic glutamate release probability. Basal levels of MMP-2/9 activity surround postsynaptic D1 and D2-receptor expressing medium spiny neurons (MSNs). **B)** After extinction from heroin self administration, astroglial processes retract from the synapse, impairing GLT-1 glutamate clearance and presynaptic tone onto mGluR2/3. To facilitate inhibitory drug seeking behavior, presynaptic glutamate release onto D2 MSNs is increased. Selective D2 MSN stimulation induces constitutive MMP-2 activation, digestion of ECM proteins that bind to integrin molecules and activate insertion of AMPA receptors through intracellular signaling cascades. The direct link between astrocyte retraction and D1 and D2 MSN plasticity is unclear, however, it is possible these are distinct mechanisms mediating extinction-induced plasticity. **C)** During transient cued heroin seeking, increased glutamate release indirectly stimulates inactive MMP-9 around D1 MSNs. Transient MMP-9 activity around D1 MSNs results in dendritic spine head expansion and AMPA receptor insertion via integrin-mediated signaling. Additionally, astroglial processes rapidly restore synaptic contact differentially at D1 and D2 synapses. D1 MSN-communicating astrocyte processes exhibit increased MMP gelatinolytic activity, and possibly function to reduce glutamate spillover into extrasynaptic space or release TIMPs to terminate synaptic plasticity. D2 MSN-communicating astrocyte processes may preferentially associate with pre-synaptic terminals to release glutamate onto mGluR2/3 to consequently promote drug seeking behavior.

Is Extracellular Matrix Degradation Permissive, Instructive, or Restrictive of t-SP: chicken or the egg?

It is possible that it is all three. The vast nature of ECM milieu serves not only a structural scaffolding unit, but it is also a dynamic structure that undergoes constant remodeling to facilitate cell growth, migration, and homeostatic conditions (Theocharis, Skandalis et al. 2016). The ECM microenvironment exhibits complex and diverse expression around neurons, consequently this may affect how individual dendritic spines respond to extracellular signals such as from MMP catalytic degradation (Bonnans, Chou et al. 2014). In the case of t-SP, numerous studies have reported transient increases in synaptic strength via measurements of glutamate receptor transmission and dendritic spine head and astroglial morphology (Gipson, Reissner et al. 2013, Kruyer, Scofield et al. 2019), with additional studies indicating that MMPs are important modulators of this synaptic plasticity (Smith, Kupchik et al. 2014). Given the nature of those experiments as well as my own, it is difficult to determine when exactly these adaptations occur in relation to onset of MMP-2/9 activity. This proteolytic activity exhibits permissive properties in that disruption of structural barriers (i.e. cell-adhesion molecules) permits spine head growth, insertion of glutamate receptors to the postysynaptic membrane, and astrocyte motility (Ogier, Bernard et al. 2006, Michaluk, Kolodziej et al. 2007, Wang, Bozdagi et al. 2008, Michaluk, Mikasova et al. 2009, Szepesi, Hosy et al. 2014). Thus, it is plausible that MMP gelatinase activity degrades ECM molecules hindering reconstruction of D1 MSNs and peripheral astroglial processes during a drug seeking event. Identification of specific ECM milieu and MMP-9 substrates that function to hinder mobility would be an interesting observation supporting this phenomenon. In addition, pharmacological inhibition of the gelatinases would provide insight to MMP-dependent morphological adaptations endorsing cued-heroin seeking.

The instructive nature of ECM signaling is quite interesting in that it relies in part on the byproducts of proteolytic digestion, which themselves contain biologic activity. For instance, MMP-2/9 degradation of fibronectin molecules reveals usually cryptic RGD domains, which serve as a ligand for binding to αVβ3 integrin receptors (Ruoslahti 1996, Verslegers, Lemmens et al. 2013). Integrin activation then induces a host of downstream cascades involving focal adhesion kinase (FAK) and cofilin, which promote actin polymerization and changes in AMPA receptor composition of postsynaptic membrane surface, and transient drug seeking behavior (Park and Goda 2016, Garcia-Keller, Neuhofer et al. 2019). Unpublished data from the lab characterized cell-type specific intracellular expression of phosphorylated FAK and cofilin, and these findings corroborate my observations indicating t-SP only in D1 MSNs during reinstated drug seeking (Garcia-Keller et al. 2020, unpublished observations). Further evaluation of whether MMP-2/9-dependent β3 integrin signaling similarly regulates actin dynamics and subsequent astrocyte migration to select synapses would broaden our understanding of outside-in extracellular signaling and how it contributes to cue-induced drug seeking behavior (Luo, Carman et al. 2007).

Finally, depending on the nature of instructive signaling employed, extracellular matrix regulation can also be deemed as restrictive to t-SP, particularly through the function of tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 can be co-released with MMP-2/9 in small vesicles localized in the somatodendritic compartment and dendritic spines, suggesting tightly coupled regulation of their trafficking in neurons (Sbai, Ferhat et al. 2008). TIMPs terminate excessive MMP enzymatic activity to maintain ECM integrity, hence why these molecules play an equally important role as their counterparts in synaptic plasticity (Okulski, Jay et al. 2007, Arpino, Brock et al. 2015). As discovered in (Magnowska, Gorkiewicz et al. 2016), TIMP inhibition is

necessary to promote spine head functional and morphological maturation, unsilencing of excitatory synapses, and maintenance of long-term potentiation. This group found that excessive, uncontrolled MMP activity produces elongated, immature spines (Magnowska, Gorkiewicz et al. 2016). Thus, restriction of aberrant MMP activity by terminating their function and induced plasticity benefits synaptic homeostasis and stability. This is consistent with my findings where TIMP-1/2 inhibition in NAcore exacerbates heroin seeking (Chapter 5). While dendritic morphology was not assessed, it is likely these cells exhibit a greater proportion of thin spines than mushroom-like due to excessive gelatinolysis. Interestingly, previous studies from our lab show that MMP-9 inhibition prevents spine head enlargement and suppresses drug seeking behavior. This may seem contradictory to the previous point, but preventing MMP activation before the onset of cue-induced neuronal activity would prevent the initiation of plasticity altogether, hence there would be no subsequent need for TIMP inhibition. While not robust, I observe measurable TIMP expression around a proportion of D1 MSNs during transient heroin seeking. This mechanism is likely terminating the transient synaptic plasticity in this cell population (i.e. producing potentiated spine heads) and preventing the pathological behavior from persisting and promoting within trial extinction of lever pressing. A similar restrictive mechanism employs the collagen-associated proteoglycan decorin, which acts a reservoir for sequestered transforming growth factor-beta (TGF-β) (Imai, Hiramatsu et al. 1997). Once ECM milieu is digested by MMPs, TGF-β is released and inhibits MMP gene expression. This negative feedback mechanism limits MMP expression and thus restricts the extent of synaptic plasticity (Imai, Hiramatsu et al. 1997, Sternlicht and Werb 2001). In a way, these restrictive mechanisms could be deemed as therapeutic. While such mechanisms may not prevent the initiation of drug relapse by cues, they could be useful as relapse-terminating agents.

In summary, ECM signaling is quite dynamic and may employ permissive, instructive, and restrictive properties to modify synaptic efficiency. All three mechanisms work in concert to produce informative updates to ongoing adaptations in the ECM microenvironment. Unless high-resolution, real-time imaging of this dynamic microenvironment can be conducted, we are left to postulate the temporal sequence of events during transient synaptic potentiation and other forms of plasticity.

Normalizing Aberrant Tetrapartite Synaptic Homeostasis in Opioid Addiction

The studies described in previous chapters convey enduring adaptations in each of the four synaptic compartments in NAc following repeated non-contingent or self-administered opioids, and opioid seeking is associated with rapid and transient plasticity within each compartment. In some instances, the adaptations in one compartment have been experimentally linked to adaptations in other compartments (Figure 1-2). This is most clear with astroglial retraction and downregulated GLT-1, which mediates presynaptic glutamate spillover, induction of MMP activity, and morphological and physiological postsynaptic potentiation. Although much work remains to fully characterize how the tetrapartite synaptic compartments interact to regulate plasticity, it is clear that each of the four compartments are altered by opioid use and that these alterations regulate the initiation and intensity of opioid-seeking in rodent models of OUD.

Our developing understanding of tetrapartite synaptic physiology and pathophysiology has led to novel drug development strategies targeted to adaptations in each tetrapartite compartment. In the presynaptic compartment, the most tractable possibility is using mGluR2/3 agonists to reduce opioid seeking, although concerns have been raised due to parallel decreases in seeking of natural rewards (Bossert, Busch et al. 2005, Bossert, Poles et al. 2006). Promising postsynaptic receptor pharmacological

manipulations include blocking mGluR5, which prevents conditioned morphine reward (Popik and Wróbel 2002). Additionally, intracerebroventricular NMDAR and AMPAR antagonism during context extinction impairs morphine-induced reinstatement of conditioned place preference (CPP) and increases NAc c-fos expression (Siahposht-Khachaki, Fatahi et al. 2016, Siahposht-Khachaki, Fatahi et al. 2017, Siahposht-Khachaki, Ezzatpanah et al. 2018). Also, heroin cue-induced and primed reinstatement and accompanying increases in spine density in NAcore are impaired by ifenprodil, a GluN2B antagonist (Shen, Moussawi et al. 2011).

Several studies focus on reversing glutamate spillover following chronic opioid use by restoring function and/or expression of GLT-1 with N-acetylcysteine (NAC) or ceftriaxone, and thereby reducing heroin cue and prime reinstatement (Zhou and Kalivas 2008, Shen, Gipson et al. 2014, Hodebourg, Murray et al. 2018). Other compounds such as propentofylline (methylxanthine adenosine uptake inhibitor) and clavulanic acid (β-lactam antibiotic) enhance expression of GLT-1 and inhibit morphine place preference (Sweitzer, Schubert et al. 2001, Schroeder, Tolman et al. 2014). It remains unknown whether these astroglial-targeting restorative agents are effective at preventing changes in astroglial synaptic proximity that contribute to cued opioid seeking. Interestingly, preliminary studies show that chronic NAC treatment (5 consecutive days, 100 mg/kg, ip) does not reduce cued seeking-induced MMP-2/9 activity in NAcore of reinstating animals, despite suppressed lever pressing (Figure 7-2). Instead, chronic NAC treatment potentiates gelatinase activity in heroin-naïve animals, which peaks at 7 days after treatment, but persists for up to 10 days after treatment. These effects are not apparent after acute treatment of NAC (Figure 7-2). Interestingly, this persistent, but reversible increase is MMP-2-mediated, consistent with the constitutive functionality of MMP-2 activity (Figure 7-2). This increase is not mediated via a nitric oxide-dependent pathway,

suggesting an alternative, indirect pathway activating MMP-2 activity (Figure 7-2). Further investigation is warranted to determine whether these changes are D1 or D2 MSN-specific in naïve animals and if heroin exposure disrupts the homeostatic balance between these cell-types.

Finally, therapeutics based on opioid-induced adaptations in the ECM seem furthest from development given the difficulties encountered in clinical trials using MMP-9 inhibitors in treating neuroinflammation and cancer progression (Vafadari, Salamian et al. 2016, Winer, Adams et al. 2018). However, promising results from my dissertation indicate impetus to approach potential therapies in a cell-type specific manner. Similar methods are employed in single cell assessment of neuronal activity in subthalamic nucleus of levodopa-induced dyskinetic Parkinson's disease (PD) patients (Ozturk, Kaku et al. 2020). These patients exhibit unique electrophysiological firing patterns compared to unmedicated PD patients, thus providing insight into a pathological neural signature that can hopefully be therapeutically restored (Ozturk, Kaku et al. 2020). While cell-type specific transcriptional changes evoked by chronic drug use have been identified (Avey, Sankararaman et al. 2018, Bhattacherjee, Djekidel et al. 2019, Brenner, Tiwari et al. 2020), to date, human studies evidencing abnormal single cell dynamics in brain regions associated with addiction have not been conducted. Resolution of such aberrant neural firing patterns would provide precise insight to targetable "problem cells/networks" that require selective therapeutic intervention.

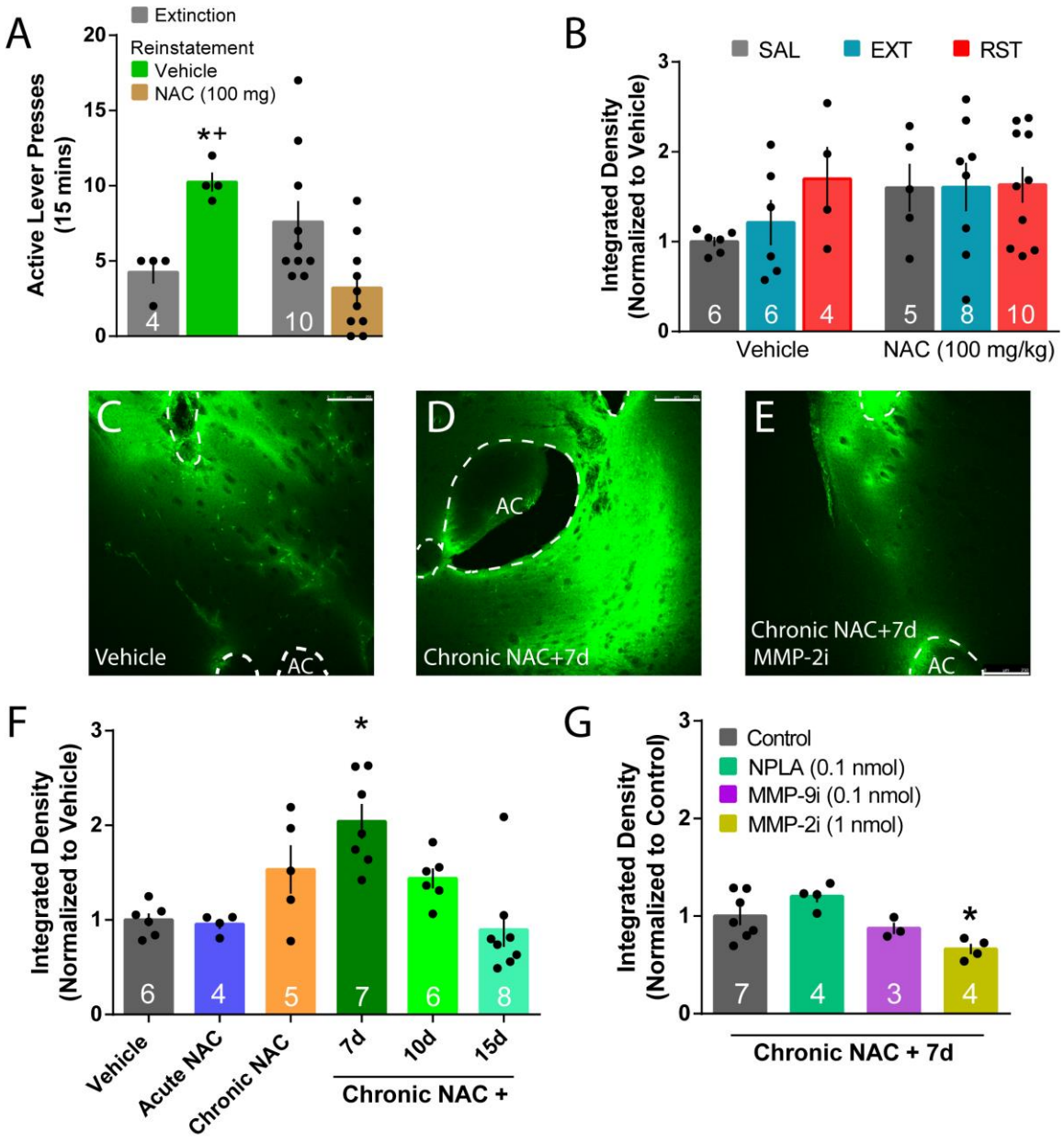

Figure 7-2. Chronic Systemic NAC Administration Induces Constitutive NAcore MMP-2 Activity in Heroin Naïve Animals. **A.** Chronic NAC (100 mg/kg, 5 days) administration suppressed transient heroin seeking behavior (2-way ANOVA; effect of treatment $F_{(1,12)}$=1.208, p=0.2933; EXT/RST $F_{(1,12)}$=0.5995, p=0.4537; interaction $F_{(1,12)}$=25.33, p=0.0003). **B.** No difference in MMP activity across SAL, EXT, or RST groups treated with vehicle or NAC (1- way ANOVA; $F_{(5,33)}$=1.286, p=0.2936). **C-E.** Representative micrographs of MMP gelatinolytic activity in NAcore in vehicle, chronic NAC+7d, and chronic NAC+7d with MMP-2i heroin-naïve animals. **F.** Constitutively increased NAcore MMP activity 7 days after chronic NAC treatment (1-way ANOVA; $F_{(5,30)}$=7.761, p<0.0001). **G.** Constitive gelatinolysis 7 das after chronic NAC treatment is MMP-2-dependent (1-way ANOVA; $F_{(3,14)}$=6.531, p=0.0055). *p<0.05 compared to extinction, $^{+}$p<0.05 compared to NAC using Bonferroni post hoc test; *p<0.05 compared to vehicle, acute NAC, and 15d using Bonferroni post hoc test. Scale bar=250 µm.

Future Directions

A new focus that has emerged from my studies is possible cell-specific neuron-astrocyte interactions driving synaptic plasticity during cued heroin seeking. It is known that striatal astrocytes selectively respond to activity of either D1 or D2 MSNs, suggesting pre-defined neuron-to-astrocyte synaptic communication (Martín, Bajo-Grañeras et al. 2015). Because I observed increased synapsin colocalization at synapses harboring MMP-2/9 activity during cue-induced heroin reinstatement, one would speculate these synapses are D1 MSNs based on my observations from Chapter 4, but this is yet to be determined. Unpublished data from our lab suggests GLT-1 is not restored to astrocyte processes regaining close proximity to the synapse, suggesting this mechanism may not be directly involved in the drug seeking process (Kruyer et al. 2020, unpublished observations). This finding supports the notion of discrete and dissociable astroglial mechanisms that can be employed during transient heroin seeking. It would be interesting to determine if GLT-1 restoration was synapse-specific and involved focal MMP activity in this microenvironment. Novel bioengineering approaches to elucidate communicating neuro-glial ensembles after withdrawal and transient heroin seeking would be an major advancement in our understanding of synaptic plasticity and the tetrapartite synapse being a part of a larger connectome (Ganguly and Trigun 2020).

A key limitation of my work is the inability to monitor these dynamic changes in real-time. Future studies could resolve this with high resolution *in vivo* live cell imaging with modalities such as two-photon microscopy. This approach permits visualization of synaptic adaptations, such as dendritic spine head expansion and changes in spine density within minutes of the inducing stimulus (Sigler and Murphy 2010). With this set-up, direct application of FITC gelatin can be utilized to monitor real-time dynamics between MMP activity and specific cell types mediating synaptic plasticity during

transient drug seeking (Underly, Levy et al. 2017). These findings would provide spatiotemporal precision to the permissive and instructive properties of MMP activity in contributing to synaptic reconstruction. Another innovative approach could utilize genetically modified dendrites containing membrane tagged MMP-2/9 vesicles that when released and activated within synaptic proximity emit fluorescence. The most exciting aspect of recent advancements in imaging techniques is the ability to evaluate individual synapses, and thus make direct implications on the relationship between presynaptic activity and postsynaptic function (Sanderson, Georgiou et al. 2020). Combined use of modified pre- and postsynaptic membrane tags grants the ability to investigate the relationship between glutamate receptor trafficking and probability of presynaptic neurotransmitter release at individual synapses (Sanderson, Bradley et al. 2018). Future studies utilizing this methodology may investigate the strength of specific glutamatergic inputs onto D1 MSNs and how activity-dependent synaptic plasticity involves MMP induction. Obtaining a better understanding of the dynamic nature of the tetrapartite synapse would better equip us with knowledge of synaptic physiology and how it is affected by drugs of abuse.

Ultimately, these preclinical findings would serve as a great steppingstone to pursue translational research regarding MMP activity in opioid-addicted human subjects. Previous studies observed increased serum ratios of MMP-2/TIMP-1, MMP-2/TIMP-2 and MMP-9/TIMP-1 levels in heroin users compared to healthy controls, and this aberration was not restored within 21 days of abstinence (Kovatsi, Batzios et al. 2013). Armed with this knowledge, future translational studies can be conducted to elucidate neurobiology targets that may be causing persistent MMP-2/9 expression and how this is affecting functionality in various brain regions. MMP-2/9 expression and activity may serve as a clinical biomarker indicating acute or chronic neuropathology mediating opioid

addiction. The goal of this work would be to develop safe, clinically efficacious MMP inhibitors to counteract opioid-induced aberrations in ECM integrity in addicted individuals.

Some of the results presented in my dissertation may pose as confounding given the nature of the *in vivo* zymography assay, specifically how MMP fluorescence increases linearly over time (Smith, Kupchik et al. 2014). While I am capturing a "snapshot" of active synaptic processes, I believe my findings are valid for the following reasons. In addition to remaining blind to treatment groups during experimentation and data collection, the cell-specific observations I have made follow a unique pattern that does not seem solely due to random chance. 1) Overall MMP activity in NAcore increases during reinstatement irrespective of rat genotype, but this increase is not reflected around D2 MSNs. 2) Overall MMP activity in NAcore does not change after extinction compared to saline controls, but I observe increased MMP activity around D2 MSNs. 3) Total MMP activity normalized to z-stack does not change across groups between D1 and D2 MSN micrographs (Figure 4-7). 4) These D2 MSN-MMP activity adaptations oppose the observed cell-specific TIMP-1 and -2 immunoreactivity following extinction and during cued heroin seeking, suggesting focal inhibition around D2 MSNs to mediate drug seeking. 5) Total TIMP immunoreactivity normalized to z-stack does not change across groups between D1 and D2 MSN micrographs (Figure 5-6). 6) The increased MMP gelatinolytic puncta localization is spine head-enriched in both D1 and D2 MSNs which is consistent with previous studies (Szklarczyk, Lapinska et al. 2002, Wang, Bozdagi et al. 2008) . If this observation was due to chance, then it would be expected that the dendrite shaft would harbor the bulk of MMP fluorescent activity given the larger surface area compared to dendritic spine heads. In addition, MMP localization around individual spine heads, irrespective of size, also does not necessarily follow this

theory, as there is no correlation between spine head diameter and MMP gelatinolytic localization with my methodology (see Figure 3-2C and Figure 4-9). 7) Lastly, for astroglial cells, saline and reinstated groups exhibit similar MMP-synapsin I colocalization, despite higher overall total MMP fluorescence in the reinstatement group. This suggests astroglial proximity dynamics involving ECM signaling that is not due to increased MMP gelatinolytic signal in the NAcore.

Conclusions

In conclusion, the research presented here outlines exciting discoveries examining how cell-type specific extracellular matrix signaling in nucleus accumbens core underlies pathological synaptic plasticity in opioid addiction. While gelatinase activity around certain cell types may seem like a small drop in the big pond that is addiction, my dissertation provides novel insight towards the inner workings of tetrapartite synapse physiology during withdrawal and relapse. Importantly, it conveys the necessity of investigating aberrations in specific cell types, thus it is imperative to expound upon these findings to reveal tractable therapeutic targets to alleviate symptoms of drug addiction.

Chapter 8: References

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