MU-OPIOID RECEPTOR INVOLVEMENT IN COCAINE ADDICTION

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DEDICATION

I dedicate this work to my parents, MiCha and Terry Simmons whose love, support, and advice has guided me through my years and makes me who I am today, and to my sisters Mary and Terri whose love and friendship gives me strength.

“Do not go where the path may lead; go instead where there is no path and leave a trail”

Ralph Waldo Emerson

I would like to thank the members of my Graduate Committee, for their patience and insight in completing this work.

NUCLEUS ACCUMBENS MU-OPIOID RECEPTOR INVOLVEMENT IN COCAINE ADDICTION

by

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DISSERTATION

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MU-OPIOID RECEPTOR INVOLVEMENT IN COCAINE ADDICTION

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The nucleus accumbens (NAc) receives dopaminergic input from the ventral tegmental area (VTA) and is intricately involved in the reinforcing properties of cocaine. Mu-opioid receptors (MOR) are highly expressed in the NAc and act to modulate glutamatergic and dopaminergic input in response to various stimuli. Chronic cocaine self-administration may modulate MOR expression and mediate increased

craving and relapse that characterizes cocaine addiction. Chapter 3 determined MOR regulation by cocaine administration. Both contingent and non-contingent cocaine administration decrease MOR specifically in the core while delta opioid receptors (DOR) were not altered by chronic cocaine. Cocaine self-administration mediated down-regulation of MOR was β-endorphin dependent since blockade of β-endorphin prevented MOR phosphorylation, down-regulation, and endocytosis. Chapter 4 determined whether opioid receptor stimulation was sufficient to reinstate drug-seeking behaviors in extinguished animals. Both MOR and DOR specific agonists (DAMGO and DPDPE, respectively) induced cocaine seeking as did the endogenous opioids, β-endorphin and endogenously released enkephalins. Blockade of MOR decreased cocaine-primed reinstatement, indicating MOR involvement in drug-primed reinstatement. Chapter 5 identified long-term neuroadaptations possibly involved in high craving and relapse rates typically seen in humans and modeled in animals. MOR expression increased with withdrawal time indicating a potential correlate of time-dependent increases in cocaine seeking in withdrawal. To determine whether the up-regulation of MOR translated into enhanced cocaine-seeking behavior, MOR stimulated locomotor activity and cocaine-seeking/reinstatement was assessed. Locomotor

behavior in response to intra-NAc DAMGO infusions did not change after long-term withdrawal, however there were age variables that may have contributed to the negative data. When drug-seeking was assessed at 6 w withdrawal, animals had increased drug-seeking behavior compared to 1 w withdrawal animals, an effect that was potentiated by intra-NAc β-endorphin. DAMGO increased relapse to cocaine-seeking at 6 w withdrawal with no effect at 1 w withdrawal. β-endorphin primed reinstatement was similar in both groups however the effect was only significant in the 6 w withdrawal group. These findings indicate NAc MOR is regulated by cocaine self-administration and withdrawal and stimulation of MOR results in drug craving and relapse behaviors. Results further indicate a potential target in the treatment of cocaine addiction.

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LIST OF FIGURES

LIST OF DEFINITIONS

6-OHDA – 6-hydroxydopamine

α-β-end – Antibody to β-endorphin

AC – Adenylate Cyclase

ACTH – Adrenocorticotropic Hormone

AKAP84 – A-Kinase Anchoring Protein

AMPA - α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

ANOVA – Analysis of Variance

Arc – Arcuate Nucleus of the Hypothalamus

ATF2 – Active Transcription Factor

AY – Acute Yoked

BOLD fMRI – Blood Oxygenation Level Dependent Functional Magnetic Resonance Imaging

C – Celsius

cAMP – cyclic Adenosine MonoPhosphate

Coc-IgG – CSA with IgG treatment

CNQX - 6-cyano-7-nitroquinoxaline-2,3-dione

CPP – Conditioned Place Preference

CPu – Caudate Putamen

CREB – Cyclic AMP Response Element Binding Protein

CSA – Cocaine Self-Administering

cT – Cycle Threshold

CTAP – D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2

CY – Chronic Yoked

D1R – Dopamine D1 Receptor

D2R – Dopamine D2 Receptor

DALA – D-Ala2-Met5-enkephalinamide

DAMGO – D-Ala^2,N-Me-Phe^4,$glycinol^5$)-enkephalin

DAT – Dopamine Transporter

DDT – Dichloro-Diphenyl-Trichloroethane

DNA – Deoxyribonucleic Acid

DOR – Delta Opioid receptor

DPDPE – (D-Pen^2,D-Pen^5)-Enkephalin

EDTA – Ethylenediaminetetraacetic Acid

EGTA – Ethylene Glycol Tetraacetic Acid

EXT – Extinction Trained

Fischer's LSD – Fischer's Least Significant Difference

FOS B – FBJ (Finkel-Biskis-Jonkins) Osteosarcoma Oncogene B

FR1 – Fixed Ratio 1

GABA-A – Gamma Aminobutyric Acid A

GABA-B – Gamma Aminobutyric Acid B

GAPDH – Glyceraldehyde-3-Phosphate Dehydrogenase

GTPγS – guanosine 5'-O-[γ-thio]triphosphate

HA – Hemaglutanin

HC – Homecage

HEK 293 – Human Embryonic Kidney Cells 293

HPA axis – Hypothalamic Pituitary Adrenal axis

HSV – Herpes Virus Simplex

icv – Intracerebroventricular

IEG – Immediate Early Gene

ip – Intraperitoneal

kDa – kiloDalton

KOR – Kappa Opioid receptor

MAPK – Mitogen-Activated Protein Kinase

MOR – Mu Opioid Receptor

mRNA – Messenger Ribonucleic Acid

NAc – Nucleus Accumbens

NaF – Sodium Fluoride

nt – nucleotide

NDS – Normal Donkey Serum

PBS – Phosphate Buffered Saline

PC-12 – Pheochromocytoma 12 cells

PCR – Polymerase Chain Reaction

PET – Positron Emission Tomography

γPKC – Protein Kinase C

pMOR – Phosphorylated Mu Opioid Receptor

POMC – pro-opioimelanocortic

PPA – Preprotachykinin A

PPB – Preprotachykinin B

PPD – Preprodynorphin

PPE – Preproenkephalin

PVDF – Polyvinylidene Fluoride transfer membran

qRT-PCR – Quantitative Reverse Transcriptase Polymerase Chain Reaction

RNA – Ribonucleic Acid

S – Serine

SDS – Sodium Dodecyl Sulfate

SDS-PAGE – SDS-Polyacrylamide Gel Electrophoresis

SN – Substantia Nigra

SSA – Saline Self-Administering

Tukey's HSD – Tukey's Honestly Significant Difference

TTBS – Tween Tris Base Saline

VP – Ventral Pallidum

VTA – Ventral Tegmental Area

WD – Withdrawal

CHAPTER ONE

COCAINE, DOPAMINE, AND THE OPIOID SYSTEM

Introduction

Cocaine addiction is a health problem that exacts an enormous economic toll on society (Cartwright 2000; Flynn et al. 1999). Cocaine and other addictions are characterized by escalation of drug use during drug self administration and enhanced drug craving during withdrawal or abstinence, which leads to uncontrollable, compulsive use despite adverse consequences (American Psychiatric Association – *DSM-IV*, 2004). High rates of relapse to cocaine and other drugs following prolonged periods of abstinence characterize addiction to drugs (Mendelson and Mello 1996; O'Brien 1997). To effectively treat cocaine and other addictions, it is important to understand the neurobiological mechanisms behind addiction and what triggers relapse to drug use during abstinence. Studies show that cocaine and other addictions involve alterations in brain reward circuitry, especially the mesolimbic dopamine system that includes the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Dackis and O'Brien 2001; Kalivas and Volkow 2005; Koob and Le Moal 2001). These changes are thought to lead to an increased propensity for relapse during

withdrawal. However, despite numerous neurobiological changes characterized to date, few persist during abstinence and contribute to relapse.

One area of interest in cocaine addiction is the modulation of the endogenous opioid system, specifically in the NAc, a region important for relapse behavior (Leshner and Koob 1999; See 2005). Studies show that chronic cocaine modulates opioid receptor levels and activity as well as their ligands (Azaryan et al. 1996a; Hurd et al. 1992; Schroeder et al. 2003; Sivam 1989; Sweep et al. 1988; Unterwald 2001; Unterwald et al. 1992; Unterwald et al. 1994; Zubieta et al. 1996). Additionally, pharmacologic and genetic manipulation of these receptors and their ligands indicate their involvement in cocaine reinforcement and reward (Boutrel 2008; Gerrits et al. 2005; Hummel et al. 2004; Hummel et al. 2006; Schroeder et al. 2007). However, few studies have thoroughly analyzed the regulation of NAc opioid receptors in cocaine self-administration and withdrawal and their involvement in relapse behaviors. This dissertation investigates the modulation of mu opioid receptors (MOR) by cocaine self-administration, the involvement of opioid receptors in relapse behaviors, and the implication of long term MOR regulation in craving and relapse to cocaine seeking.

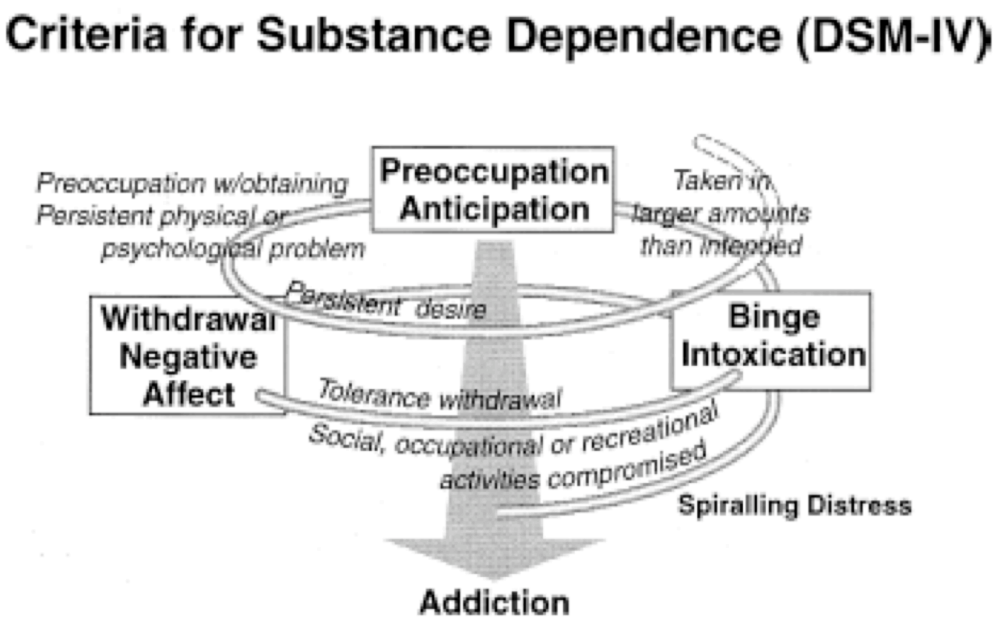


Figure 1.1 Criteria for Substance Dependence (DSM-IV). Adapted from Koob and LeMoal, 2001. Drug addiction is characterized by escalation of drug use during drug self administration and enhanced drug craving during withdrawal or abstinence, which leads to uncontrollable, compulsive use despite adverse consequences. This figure depicts the downward spiral of drug addiction where there is an increase in physical and psychological dependence, leading to compulsive use

Different brain region involvement in various aspects of cocaine addiction

As stated previously, cocaine and other drug addictions are characterized by an escalation of drug intake and craving during abstinence that ultimately leads to uncontrollable use despite adverse consequences such as los of employment, ad decrease in one's health, and loss of relationships with friends and family. Figure 1.1 depicts the downward spiral of addiction where initial drug intake results in euphoria and "high" and repeated use leads to tolerance and sensitivity paired with persistent thoughts of further drug administration (Koob and Le Moal 2001). Eventually, drug use is driven by withdrawal symptoms and increased craving during abstinence, ultimately leading to compulsive drug seeking during abstinence and uncontrollable use when taking drug. The transition from recreational drug use to addiction involves several factors including neuroadaptations brought on by chronic drug use, however the actual mechanisms behind the transition to addictive behaviors are not fully understood. To help identify changes in the brain involved in cocaine addiction, animal models of drug addiction were developed to model the different characteristics of addiction. These models provide a better

understanding of brain regions involved in various aspects of cocaine and other drug addictions. And they provide a tool to test theories on mechanisms of drug dependence and addiction.

Several brain regions are implicated in mediating the addictive properties of cocaine and in modulating craving and relapse behaviors (Figure 1.2). These regions are involved in different aspects of behavior and are connected with each other either directly or through neural circuits. Many of the connections are displayed on figure 1.2 and 1.3. The NAc, a region most implicated in modulating cocaine addiction behaviors, receives dopaminergic input from the VTA and glutamatergic input from the anterior cingulate and other prefrontal cortex regions (for review see (Koob and Le Moal 2001). The amygdala and hippocampus also innervate the NAc and prefrontal cortex regions. Various stimuli activate these brain regions leading to convergence of information onto the NAc where it exacts a motivated or adaptive response.

Cocaine inhibits monoamine transporters (serotonin, dopamine, and norepinephrine transporters), thereby increasing their presence in the synapse. Cocaine's primary salient property is through inhibition of the dopamine transporter (DAT) leading to increased dopamine release by the VTA into several brain regions, especially the NAc (Figure 1.3)

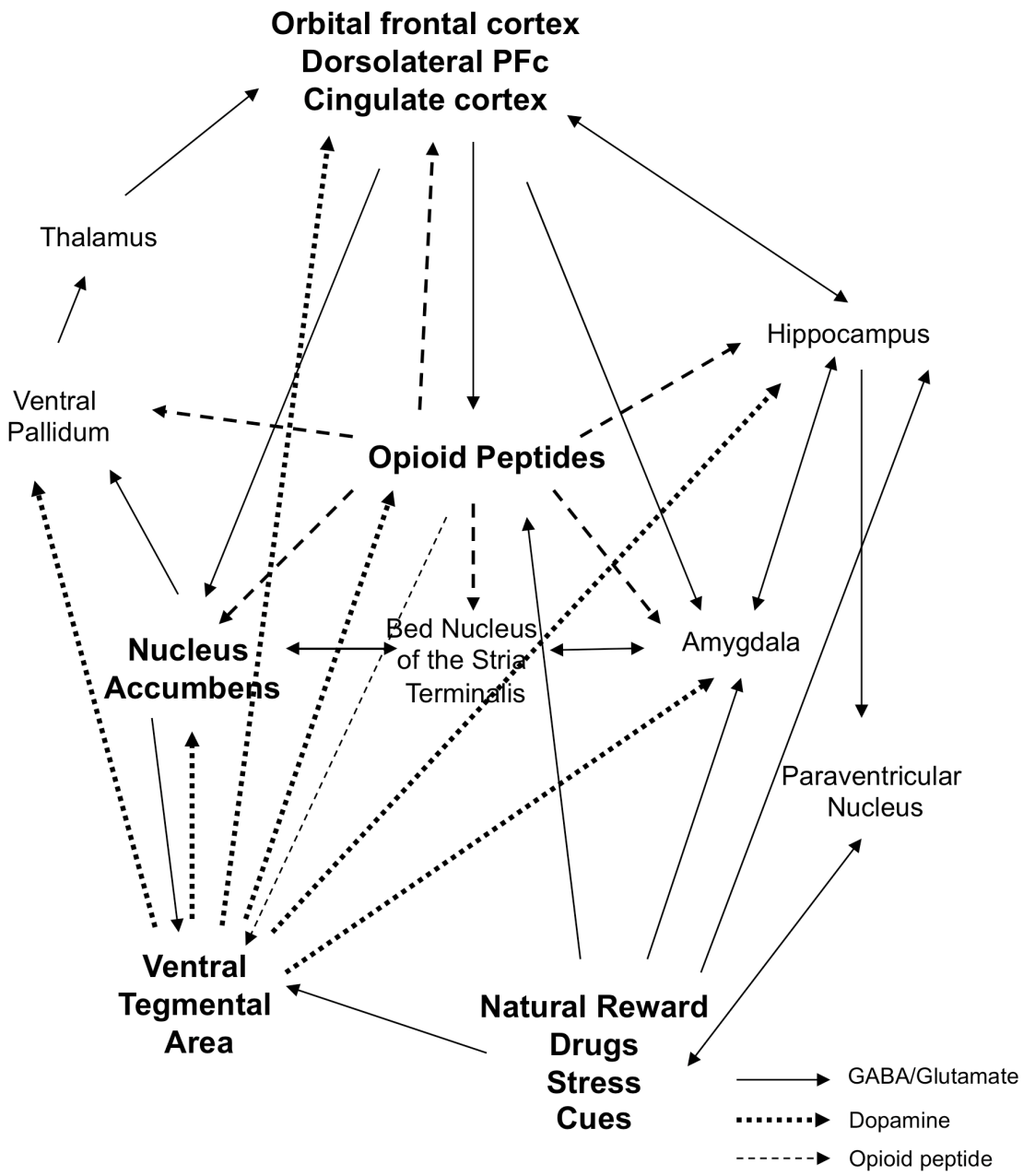


Figure 1.2 Figure depicting brain regions involved in the modulation of behavior and cocaine addiction. Natural and drug reward activate the mesolimbic dopamine system, especially the ventral tegmental area (VTA) to nucleus accumbens (NAc) pathway, thereby pairing the salience of various stimuli with the reward. Opioid peptides, especially the arcuate nucleus (Arc) release of β-endorphin, modulates the rewarding and dysphoric properties of various stimuli. Bold print type indicate nuclei most discussed by this thesis and are hypothesized to be involved in the long-term modulation of craving during drug abstinence. Adapted from Koob and LeMoal, 2001 and Kelley 2004.

(Nestler 2004; Phillips et al. 2003; Pruessner et al. 2004b; Self 1998; Spealman et al. 1999b; Stewart 2000a). Cocaine also activates opioid peptide release and expression throughout limbic nuclei involved in reward, but especially through arcuate nucleus of the hypothalamus (Arc) release of β-endorphin and through NAc and VTA release of enkephalin and dynorphin (Arroyo et al. 2000; Daunais and McGinty 1995; Hurd and Herkenham 1993; Mathieu-Kia and Besson 1998; Roth-Deri et al. 2003; Sivam 1989; Ziolkowska et al. 2006). Together, signaling from these brain regions lead to modulated processing of salient cues and adaptive motor responses with respect to cocaine administration and cues associated with cocaine.

The release of dopamine and β-endorphin into the NAc by cocaine administration increases the likelihood of further cocaine use since dopamine in the NAc is thought to increase the salience or reinforcing properties of cocaine and β-endorphin exerts/modulates the euphoric or rewarding properties of cocaine. For example, mice lacking dopamine-1 like receptors (D1R) fail to acquire cocaine self-administration and conditioned place preference (CPP) while D1R blockade decreases the reinforcing properties of cocaine (Bachtell et al. 2005; Bari and Pierce 2005; Caine et al. 2007; Cervo and Samanin 1995). Additionally, D1R and

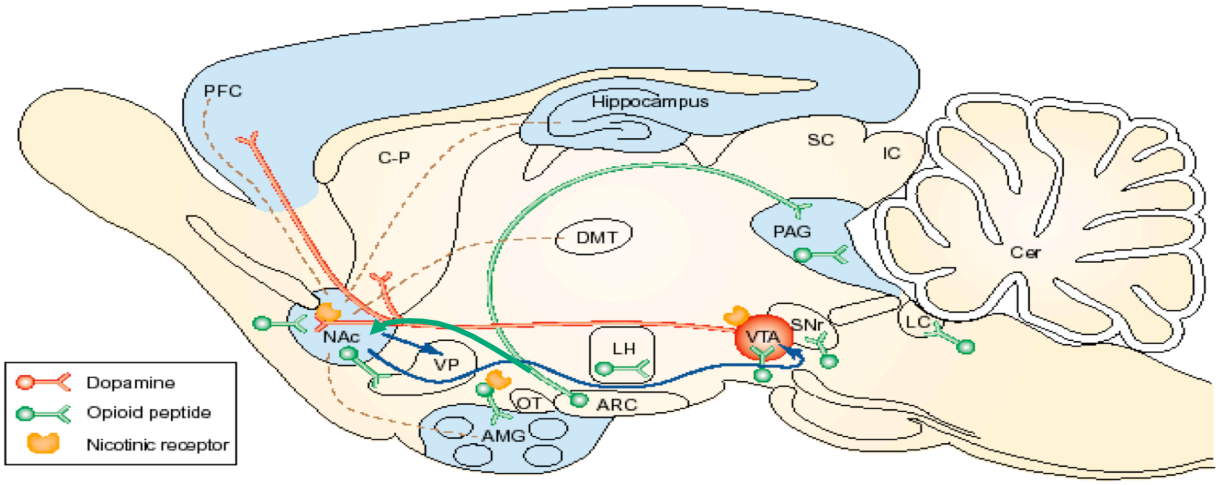


Figure 1.3 Opioid and dopamine input into the nucleus accumbens modulate behaviors involved in cocaine intake and relapse behaviors. Specifically, β-endorphin is released into the NAc from the arcuate nucleus of the hypothalamus in response to cocaine, stress, and other stimuli. Dopamine is released from the ventral tegmental area into the NAc in response to cocaine and other cocaine associated stimuli, reinforcing drug taking and drug seeking behaviors. Adapted from Nestler, 2004.

D2R (dopamine-2 like receptor) stimulation in the NAc reinstates cocaine-seeking behavior (Bachtell et al. 2005). Cocaine mediated release of β-endorphin into the NAc increases the rewarding and reinforcing properties of cocaine since blockade of β-endorphin activity in the NAc increases cocaine seeking during cocaine self-administration, decreases acquisition and expression of cocaine CPP, and attenuates acquisition of cocaine self-administration (Marquez et al. 2008; Roth-Deri et al. 2008; Roth-Deri et al. 2006; Roth-Deri et al. 2004). Together, dopamine and endogenous opioids modulate cocaine reinforcement.

Other brain regions are thought to modulate interpretation of stimuli predictive or associated with cocaine intake, thereby modulating craving and relapse behaviors in response to various stimuli. The hippocampus is involved in learning and memory hence lidocaine inhibition of the ventral hippocampus prevents the association between cocaine and contextual cues and in maintaining the discrimination between cocaine-relevant and -irrelevant contextual cues (Atkins et al. 2008). Reversible inactivation of the dorsal, but not ventral, subiculum (a substantial target of hippocampal efferents) prevents the association of contextual stimuli and cocaine since animals fail to relapse to cocaine seeking in response to cocaine-associated cues (Martin-Fardon et al. 2008). Similarly, dorsal

hippocampus inactivation interferes with acquisition and expression of cocaine CPP (Meyers et al. 2006). The amygdala is involved in the association of cues with aversive or rewarding stimuli such as pain or food or drug. In the case of cocaine, disruption of amygdala activity or blockade of D1R will block cocaine-associated cue activation of relapse behaviors (Berglind et al. 2006; Lee et al. 2005). Thus, cocaine primarily activates the VTA to NAc dopamine circuit to exert its reinforcing properties while other brain regions modulate different aspects of cocaine addiction that modulate the association of cocaine with various situations, influencing craving and relapse behaviors.

The reward pathway is important for adaptive responses to both positive and aversive stimuli since brain regions activated by various environmental cues ultimately signal through the NAc to 1) determine the salience of the stimuli and 2) determine the adaptive motor response to the stimuli. Both aversive (pain) and positive (food) stimuli are salient stimuli that animals must pay attention to in order to, for example, avoid future pain and to return to a specific location where food was previously found. Cocaine potentiates the activity of the reward pathway through its modulation of dopamine release, making cues and environmental stimuli associated with cocaine intake strongly reinforcing. Therefore

presentation of the context where an animal received cocaine or a cue paired with cocaine infusions will lead to relapse behaviors. Endogenous opioids modulate rewarding properties of cocaine and other stimuli further increasing the salience of high reward stimuli such as fatty and high salt foods and drugs of abuse.

Dichotomy of nucleus accumbens subregions

The NAc is a brain region involved in modulating behaviors related to reinforcers such as food, sex, and drugs of abuse. This region is an interface between motivation and related behavior where it integrates affective and cognitive processing from regions such as the hippocampus, prefrontal cortex and amygdala with voluntary motor behavior through the pallidum and other regions (Mogenson et al. 1980). Figure 1.3 illustrates the rat brain and brain regions that interact with the NAc (Nestler 2004). The NAc is divided into core and shell subregions based on different afferent and efferent projections (Figure 1.4a) (Heimer et al. 1991; Zahm and Brog 1992). For example, the core receives major projections from the anterior cingulate and dorsocaudal prelimbic cortices (dorsal prefrontal cortex), regions involved in mood and emotion responsivity (Britton et al. 2006; Ketter et al. 1996; Vertes 2006), while the shell receives major

afferents from the ventral prelimbic and rostral infralimbic cortices (ventral prefrontal cortex) (Brog et al. 1993; Gorelova and Yang 1997; Zahm and Brog 1992), regions involved in physiological stress responses and expression of emotion-related behaviors (Morgan and LeDoux 1999; Morgan et al. 2003; Sullivan and Gratton 2002). The subiculum receives the majority of hippocampal projections (Stewart 1997) and is thought to participate in mediating associative learning therefore modulating behavior controlled by environmental stimuli. The ventral subiculum of the hippocampus projects mainly to the shell while the dorsal subiculum projects to the core (Brog et al. 1993). Within the amygdala, a region involved in relating cues with biologically significant stimuli such as food, sex, and pain (Everitt et al. 1999), there are different populations of neurons that project preferentially to different NAc regions (Wright et al. 1996). In turn, efferents from the core preferentially project to the ventral pallidum, subthalamic nucleus, and substantia nigra while the shell preferentially project to the ventral tegmental area and ventromedial ventral pallidum (Heimer et al. 1991; Lu et al. 1998). The differential connectivity of the NAc core and shell provide different modes of information integration and signaling by the NAc core and shell.

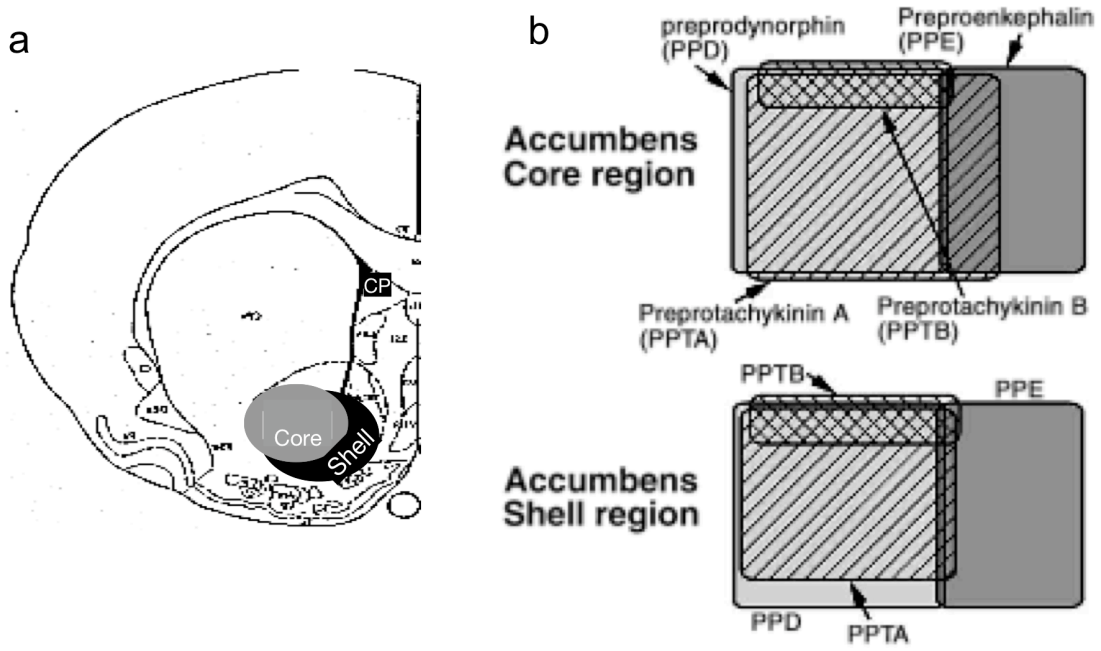


Figure 1.4 Distribution of nucleus accumbens core and shell cell types. Adapted from Furuta et al 2002. a) Diagram of a coronal section through the rat brain at 1.7 mm from bregma depicting the nucleus accumbens (NAc) core and shell subregions. The striatum contains the caudate putamen (CPu) and NAc and overall is a region involved in motor behavior and adaptive learning. b) In examining NAc projection neuronal subtypes, there is little, to no overlap of PPE (precursor for enkephalin) and PPD (precursor for dynorphin) expressing neurons of the NAc core and shell subregions. PPTA (precursor for substance P) expression is largely confined to PPD expressing neurons in the shell, with some expression in PPE neurons in the core. PPTB (tachykinin B precursor) is largely expressed in PPD neurons of both regions. Hence dynorphin, sunstance P expressing neurons are largely separate populations from enkephalin expressing neurons.

Nucleus accumbens cell distribution

The NAc consists primarily of medium spiny GABAergic neurons that have two distinct dopamine receptor expressing populations: D1-like and D2-like receptors (D1R and D2R, respectively) (Aubert et al. 2000; Lu et al. 1998; Steiner and Gerfen 1998), with some overlap (Aizman et al. 2000; Ridray et al. 1998; Schwartz et al. 1998). PPD and PPE (preprodynorphin and preproenkephalin, respectively) expressing populations are distinct and rarely overlap as shown in Figure 1.4b (Furuta et al. 2000; Lee et al. 1997). PPTA and PPTB (preprotachykinin A – precursor for substance P and preprotachykinin B – precursor for neurokinin B, respectively) along with PPD and PPE, are specific markers of projection neurons since they are not expressed in striatal interneurons and are differentially expressed, with D1R, dynorphin and substance P generally composing one projection neuron type and D2R and enkephalin positive neurons composing another. PPTB is distributed in a patch-like manner, similar to MOR expression and there is more overlap between PPTA and PPTB expressing neurons than between PPD and PPE.

As stated previously, NAc subregions differentially project to brain regions involved in processing stimuli and modulating behavior. NAc core and shell subregions project to specific regions of the VP while only the

shell projects to the VTA (Lu et al. 1998). NAc shell neurons projecting to the VTA are primarily substance P, D1R expressing while less than 3% are enkephalin, D2R expressing. In contrast, VP and substantia nigra pars compacta projecting neurons from the core and shell are D2R, PPE expressing and D1R, dynorphin expressing subtypes (Groenewegen et al. 1999; Heimer et al. 1991; Zhou et al. 2003). Further, PPE immuno-reactivity is weaker in the shell than in the core whereas PPD immuno-reactivity is highly expressed in the NAc shell and core (Furuta et al. 2002).

NAc control of motivation and associated motor behaviors

The different circuitries of the core and shell lead to a generalization of their role in behavior. The NAc core is thought to gate the transition between motivation and initiation of action to perform a conditioned behavioral response for reinforcers (Cardinal et al. 2002a; Salamone and Correa 2002; Zahm 2000). Conversely the NAc shell modulates unconditioned responses such as orientating, approach and ingestion behaviors. For example, dopamine release increases specifically in the shell, and not the core, following unpredicted consumption of a rewarding food (Bassareo and Di Chiara 1999). Alternatively, presentation of a

stimulus associated with rewarding food increases dopamine release in the core and not the shell, and further sensitizes the dopamine response in the core when the reward is consumed. Additionally, inhibition of the NAc shell with muscimol (GABA-A receptor agonist) or baclofen (GABA-B receptor agonist) and blockade of AMPA receptors with CNQX induces food intake in non-food deprived animals (Maldonado-Irizarry et al. 1995; Stratford and Kelley 1999; Stratford et al. 1998), whereas intra-NAc core infusion of AP-5, and NMDA receptor antagonist, or AMPA receptor antagonist blocks the acquisition of appetitive instrumental learning (Kelley et al. 1997; Maldonado-Irizarry and Kelley 1995a). Excitotoxic lesions of the NAc core and not the shell impair the acquisition of a conditioned Pavlovian approach response (Parkinson et al. 2000) and impair performance of conditioned response in rats lesioned after the response was trained (Cardinal et al. 2002b).

In attempting to determine core and shell differences in feeding using excitotoxic lesions, one study found that shell lesioned animals have increased body weight (Maldonado-Irizarry and Kelley 1995b), whereas core lesioned animals display significantly thinner phenotypes, however they are hyperactive in their home-cages and in a peripheral locomotor test. Interestingly, both lesion groups displayed hyperactive behaviors

compared to sham controls in the open field test. Ingestion itself was not measured.

Dopamine may be involved in the motivational activation and motor output associated with obtaining food (Aberman and Salamone 1999; Baldo et al. 2002; Salamone et al. 1994). Lesions of the dopamine system with 6-OHDA (6-hydroxydopamine) increase food intake but decreases food-reinforced instrumental behaviors (Aberman and Salamone 1999; Salamone et al. 1994). NAc shell, but not core, lesions impair VTA stimulated feeding (Trojniar et al. 2007). Both D1R and D2R antagonists suppresses locomotion when infused into the core or shell of food-deprived rats (Baldo et al. 2002). Further, D1R antagonism in the shell and D2R antagonism in the core or shell decreases the number of feeding bouts, but increases the duration of feeding. These findings support the notion that dopamine is involved in the motivation to obtain food but not the rewarding aspects of food.

NAc core and shell involvement in cocaine related behaviors

Cocaine and other drugs of abuse are thought to “hijack” the natural reward system though activation of the mesolimbic dopamine system. Activation of VTA neurons increases dopamine release into the NAc,

where they bind to dopamine receptors resulting in the reinforcing effects of cocaine (Di Chiara and Imperato 1988; Weiss et al. 1992). Exposure to cocaine and other drugs of abuse alter the processing of rewarding stimuli, resulting in a shift in reward preference (Grigson and Twining 2002).

Cocaine activates dopamine release in several forebrain structures including the NAc (Phillips et al. 2003; Pruessner et al. 2004b; Self 1998; Shalev et al. 2002b; Spealman et al. 1999b; Stewart 2000a). Studies indicate NAc regional differences in cocaine effects. Cocaine is self-administered in the NAc shell and not NAc core (Rodd-Henricks et al. 2002). Dopamine concentrations increase more in the NAc shell at lower cocaine doses with proportionately more released in the shell versus the core at higher cocaine doses (Pontieri et al. 1995). Noncontingent presentation of a cue light that was previously paired with a cocaine or morphine infusion increases dopamine in the NAc core and not shell (Bassareo et al. 2007; Ito et al. 2000). D1R and D2R dopamine receptor antagonists specifically decrease the reinforcing effects of cocaine in the NAc shell (Bachtell et al. 2005; Bari and Pierce 2005) whereas in the core, reinforcing effects of both food and cocaine are affected (Bari and Pierce 2005). An infusion of a D2R antagonist in the shell and not the core abolishes cocaine-primed reinstatement (Anderson et al. 2006). Cocaine

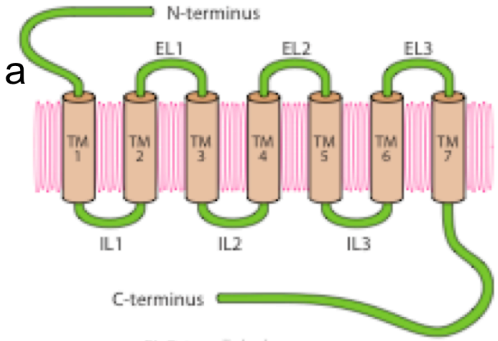
self-administration selectively abolishes long-term potentiation in the core during prolonged abstinence (Martin et al. 2006). These findings indicate different roles of NAc core and shell subregions, where the core is involved in cocaine craving and the shell is involved in cocaine reinforcement.

The Opioid System

The opioid system consists of highly homologous G-protein coupled receptors (GPCRs) belonging to the superfamily of seven transmembrane receptors (Figure 1.5a) and of peptidergic ligands that are differentially expressed and have differing affinities for the different opioid receptors (Figure 1.5b) (Davis et al. 2005; Trescot et al. 2008). Three main subtypes of receptors have been characterized: mu-, delta-, and kappa-opioid receptors (MOR, DOR and KOR, respectively). Since their discovery, intense research continues to this day in attempting to understand how opioid receptors function during acute and chronic stimulation, how endogenous peptides affect opioid receptors, and ultimately how this system modulates behavior.

Endogenous ligands to opioid receptors bind with different affinities, giving their respective “preference” for each receptor subtype.

a



b

	Mu (μ)	Delta (δ)	Kappa (κ)
	• Mu 1 – Analgesia • Mu 2 – Sedation, vomiting, respiratory depression, pruritus, euphoria, anorexia, urinary retention, physical dependence	• Analgesia, spinal analgesia	• Analgesia, sedation, dyspnea, psychomimetic effects, miosis, respiratory depression, euphoria, dysphoria, dyspnea
Endogenous Peptides			
Enkephalins	Agonist	Agonist	
β-Endorphin	Agonist	Agonist	
Dynorphin A	Agonist		Agonist
Agonists			
Morphine	Agonist		Weak agonist
Codeine	Weak agonist	Weak agonist	
Fentanyl	Agonist		
Meperidine	Agonist	Agonist	
Methadone	Agonist		
Antagonists			
Naloxone	Antagonist	Weak Antagonist	Antagonist
Naltrexone	Antagonist	Weak Antagonist	Antagonist

Figure 1.5 a) Cartoon image of the structure of opioid receptors reveal seven transmembranes with the N-terminus located extracellularly where it interacts with ligand binding. Depending on the ligand and receptor, different parts of the receptor determine binding affinity. This image was adapted from Davis et al, 2005. b) Endogenous ligands can all interact with mu opioid receptors to some extent but have higher affinity for the receptor that is indicated in red. Exogenous opioid agonists and antagonists have differing affinities for opioid receptors as well. Adapted from Trescot et al, 2008.

Enkephalins bind with 20-fold greater affinity to DOR than MOR and dynorphin is the endogenous ligand for KOR, but can interact with MOR (Chavkin et al. 1982; Simon 1991). β-endorphin has highest affinity for MOR, but can also bind with DOR (Simon et al. 1973). Since their discovery, synthetic ligands for opioid receptors were developed to more specifically target each opioid receptor for various treatments and for studies in opioid receptor function and regulation and their effects on behavior.

Opioid receptors are expressed both pre- and post-synaptically in various regions of the brain. Opioid receptors couple with the pertussis toxin-sensitive GTP-binding proteins Gi/Go and act to inhibit neurons through multiple downstream effectors (Figure 1.6). When presynaptic opioid receptors are stimulated, they act to inhibit neuron firing through activation of voltage gated potassium channels and inhibit neurotransmitter release through inhibition of voltage-gated calcium channels (Figure 1.6a) (Law et al. 2000). Activated Gi/Go proteins inhibit adenylate cyclase (AC) activity thereby decreasing cyclic AMP levels.

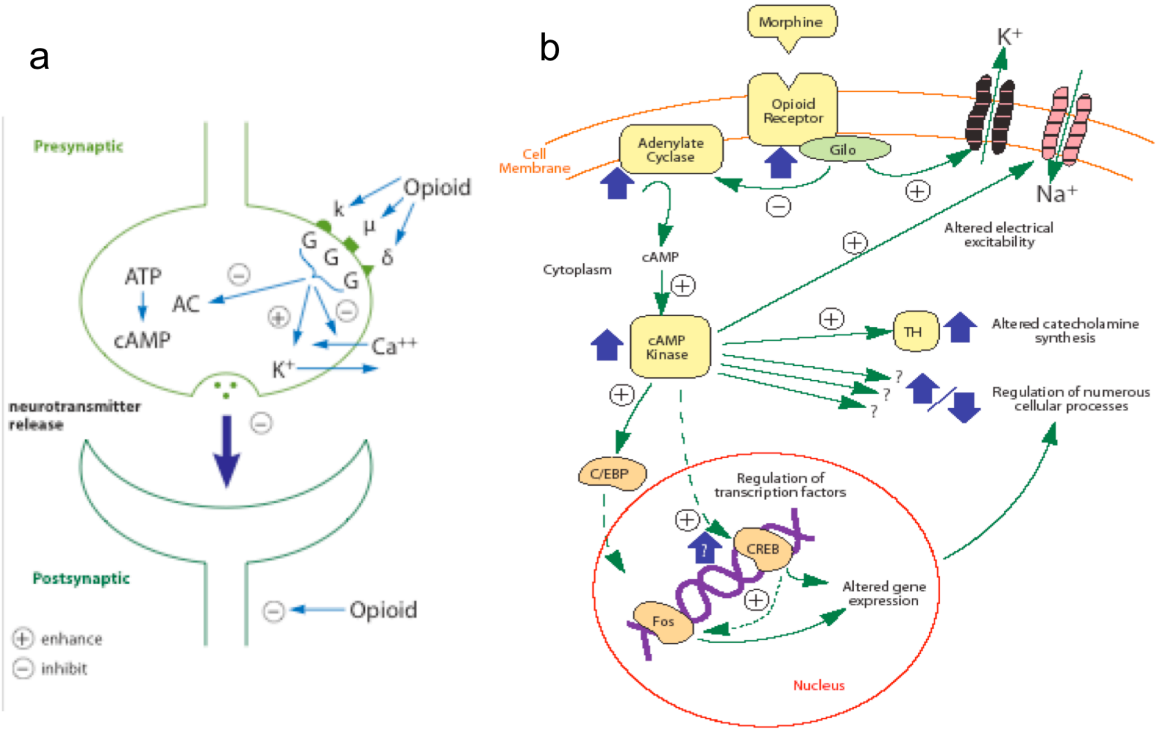


Figure 1.6 Pre- and post-synaptic signaling pathways of opioid receptors. Adapted from Trescot et al, 2008. a) Presynaptic stimulation of opioid receptors inhibits downstream signaling through G-protein mediated inhibition of adenylate cyclase activity (AC) and inhibition of calcium mediated neurotransmitter release. Opioid receptors also act to inhibit neuron activity through activation of potassium channels. b) Postsynaptic activation of opioid receptors also decreases adenylate cyclase activity thereby altering CREB and other transcription factors activity. Neuronal activity is inhibited through activation of voltage gated potassium channels and inhibition of sodium channels.

Post-synaptic opioid receptors also act to inhibit AC activity, altering downstream effector activities and gene transcription (Figure 1.6b). Opioid receptors also stimulate inwardly rectifying potassium channels, decreasing neuron firing.

Several endogenous ligands including enkephalin, dynorphin, and β-endorphin are expressed or released in the NAc (Furuta et al. 2002; Gerfen and Young 1988; Meredith et al. 1993; Svingos et al. 1998; Svingos et al. 1996; Voorn et al. 1994; Voorn et al. 1989). Dynorphin and enkephalin mRNA are found throughout MOR rich patch compartments and the matrix compartments of the striatum (Gerfen 1988). β-endorphinergic projections from the arcuate nucleus (Arc) of the hypothalamus, release beta-endorphin in the NAc (Finley et al. 1981; Watson et al. 1978). The Arc is a primary source for beta-endorphin (Bloom et al. 1978; Zakarian and Smyth 1982) where it is released into the NAc by stress or rewarding stimuli (Roth-Deri et al. 2003; Zangen and Shalev 2003). Enkephalinergic axon collaterals of GABAergic NAc neurons (Meredith et al. 1993; Svingos et al. 1998; Svingos et al. 1996) tonically release enkephalin in the NAc. Dopaminergic terminals of the ventral tegmental (VTA) co-release dynorphin (Fallon et al. 1985; Ford et

al. 2007; Van Bockstaele et al. 1994) and axon collaterals of the NAc releases dynorphin (Hara et al. 2006).

Opioid receptors are differentially expressed in the ventral striatum. NAc MOR are highly expressed in striatal patches (Mansour et al. 1995; Mansour et al. 1994; Sharif and Hughes 1989) that are enkephalinergic (Ding et al. 1996; Groenewegen et al. 1999; Guttenberg et al. 1996), but are also co-localized to a lesser degree than in the dorsal striatum with dynorphin positive neurons (Guttenberg et al. 1996). Through their preferential localization with D2R type neurons throughout the core and shell, MOR act primarily to modulate VP and not VTA (Ambrose et al. 2004; Furuta et al. 2002). On the other hand, DOR have a diffuse expression pattern throughout the brain, with higher concentrations in the striatum, NAc, and neocortex (Gouarderes et al. 1993; Sharif and Hughes 1989; Tempel and Zukin 1987). DOR are expressed on cholinergic neurons throughout the striatum (Le Moine et al. 1994) and on enkephalinergic neurons in the NAc, often apposed to terminals containing dopamine transporters (Svingos et al. 1998; 1999a). The diffuse pattern of DOR localization indicates a role in tonic modulation of NAc activity between VTA dopamine input, gluatmatergic input, and local enkephalin release. Kappa opioid receptors occur in low density in the patches of the

striatum, but are highly concentrated in the NAc (Tempel and Zukin 1987). KOR often colocalize with D1R and dynorphin containing synapses. They are also often found on terminals containing DAT, indicating their role in the modulation of DA release directly through axon collateral release of dynorphin near the synapses (Svingos et al. 2001; Svingos et al. 1999b).

The NAc Opioid System Acts Independent of Dopamine to Induce Behavior

Opioid receptors have dopamine independent actions in the NAc as shown by studies using D1R and D2R antagonists or dopamine lesions. For example, the locomotor activation effects of morphine are not antagonized by dopamine antagonists (Pert and Sivit 1977). Similarly, intra-NAc DALA (D-Ala2-Met5-enkephalinamide, enkephalin analogue) dose-dependently increases locomotor activity, and effect that is not affected by neuroleptic injection into the NAc nor by the depletion of dopaminergic input into the NAc with 6-OHDA (Kalivas et al. 1983). DALA in the NAc does not alter levels of dopamine or its metabolites (Kalivas et al. 1983). Lesioning of the dopamine system or chronic blockade of dopamine receptors potentiates locomotor activity, self-administration, and reward produced by intra-NAc DALA and systemic administration of heroin

(Johnson and Stellar 1994; Kalivas and Bronson 1985; Kalivas et al. 1983; Stinus et al. 1989; Stinus et al. 1986). Interestingly, DAMGO infusion into the NAc following 6-OHDA lesioning potentiates locomotion, however DPDPE did not and this effect was sensitive to naloxone (Churchill and Kalivas 1992), suggesting dopaminergic tone may modulate MOR activation, and not DOR. Lesioning of VTA and SN dopamine sources to the striatum increases (125I)-DPDPE binding in the NAc and striatum suggesting that enkephalins and other DOR agonists acting through DOR do not directly modulate dopaminergic afferents but regulate postsynaptic targets of the dopamine system (Dilts and Kalivas 1990). Chronic cocaine paired with neuroleptic treatment enhances NAc opioid receptor induced behavioral activity, further implicating a dopamine independent alteration of the opioid system by cocaine (Stinus et al. 1986).

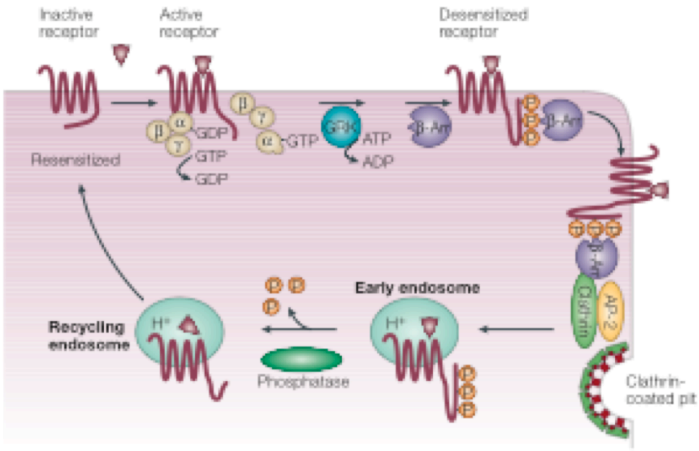
Interestingly, local opioid infusions in the NAc modulate behavior in a biphasic manner. For example, microgram doses of DAMGO or morphine infused in the NAc initially suppress locomotion but subsequently induce hyper-locomotion (Cunningham and Kelley 1992; Meyer et al. 1994). Lower doses of DAMGO decrease the latency for hyper-locomotion to occur (Meyer et al. 1994). NAc infusions of opioid agonists induce feeding behavior but also with a prolonged latency to

initiate feeding (Bakshi and Kelley 1993; Kelley et al. 2005). Similarly, intra-NAc infusions of DAMGO increase the motivation for food on a progressive ratio reinforcement schedule, and where response breakpoints are obtained after some delay (Zhang et al. 2003).

Differential regulation of MOR and DOR through ligand binding and phosphorylation

In studying the role of opioid receptors in modulating behaviors associated with cocaine use and other behaviors, it is important to understand how they are themselves regulated. G-protein coupled receptors (GPCRs) such as MOR and DOR are regulated by different mechanisms. Following ligand binding, opioid receptors are endocytosed (Figure 1.7a) (Sorkin and Von Zastrow). Typically, endocytosis of GPCRs involves phosphorylation of the receptors by G-protein regulated kinases (GRK), followed by the recruitment of β-arrestins. This recruitment leads to further recruitment of clathrins and other endocytic machinery participants that leads to endocytosis of the GPCR. Endocytosis of GPCRs by clathrin coated pits modulate receptor activity by targeting receptors to a rapid recycling pathway that resensitizes the receptor by returning the dephosphorylated receptor to the plasma membrane or by

a



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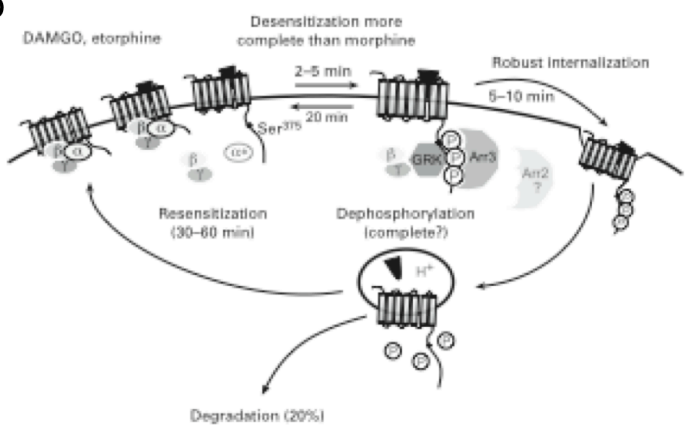

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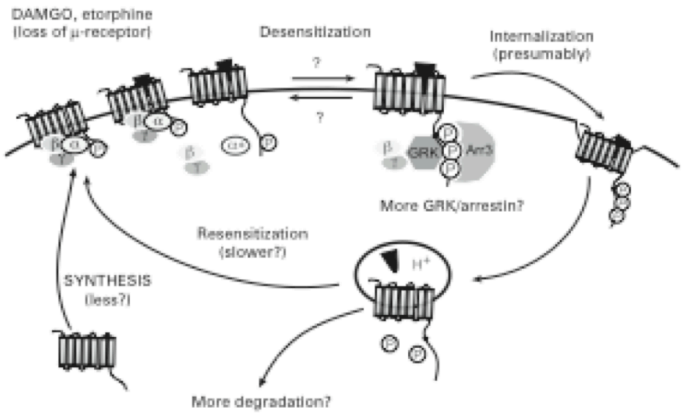


Figure 1.7 a) Recycling pathway of G-protein coupled receptors, adapted from Sorkin and von Zastrow, 2002. Following ligand stimulation, G-protein regulated kinases phosphorylate receptors leading to the recruitment of β-arrestins and other endocytic machery for clathrin coated pit mediated endocytosis. Endocytosed receptors are sent to early endosomes where either phosphatases remove phosphorylation and receptors are recycled back to the membrane or receptors are shuttled to lysosomes for degradation.
b) Typical MOR activity following acute ligand binding, adapted from Johnson et al, 2005. Typically, following acute stimulation receptors are recyled back to the membrane in a resensitized state and little MOR is shuttled for degradation.
c) It is proposed that chronic stimulation by opioids leads to a shift in the recycling pathway to one of degradation paired with potential increased synthesis to compensate for the degradation. This regulation of MOR leads to increased desensitization to compensate for chronic MOR stimulation. However, the mechanism behind the shift from MOR recycling to MOR degradation is under current investigation.

down-regulating the ligand bound receptor through proteolytic processing thus decreasing sensitivity to further stimulation (Lohse 1993; Tsao and von Zastrow 2000a).

G-protein regulated kinases (GRK) 2 and 3 phosphorylate MOR and DOR following ligand stimulation (Guo et al. 2000; Johnson et al. 2005; Lowe et al. 2002; Macey et al. 2006; Schulz et al. 2002). For DOR, phosphorylation occurs on the carboxyl tail leading to rapid internalization and subsequent desensitization through the recruitment of β-arrestins to the plasma membrane (Lowe et al. 2002; Whistler et al. 2001). Subsequently, internalized DOR are rapidly targeted to lysosomes by highly selective molecular sorting machinery for degradation (Ko et al. 1999; Tsao and von Zastrow 2000b). DOR sorting to lysosomes for degradation is ubiquitination-independent but may involve vacuolar protein-sorting proteins (Hislop et al. 2004). The preferential sorting of DOR to a degradation pathway following stimulation indicates DOR signaling is under tight regulatory control and may play a role in maintaining homeostatic states of neural circuitries, however further in vivo studies are required to delineate this supposition.

Contrary to DOR regulation, MOR are typically recycled back to the membrane following stimulation. MOR ligand binding leads to

phosphorylation of a S375 site leading to endocytosis (Figure 1.7b)(El Kouhen et al. 2001; Johnson et al. 2005). Phosphorylation of this site is ligand binding dependent and is required for endocytosis to occur. Following internalization, MOR are preferentially recycled back to the plasma membrane with restored functionality by a specific signal (MOR-derived endocytic recycling sequence) located on the carboxyl tail (Tanowitz and von Zastrow 2003). This recycling of MOR is important in the prevention of desensitization and tolerance that is often seen with drugs such as morphine (Finn and Whistler 2001; Johnson et al. 2005; Keith et al. 1998; Koch et al. 2005). With chronic stimulation of MOR, desensitization of the system through MOR down-regulation can occur (Figure 1.7c) (Johnson et al. 2005; Tao et al. 1998). Chronic stimulation of MOR decreases total MOR levels both in vitro and in vivo (Chaturvedi et al. 2001; Tao et al. 1998). In cells stably expressing MOR, pertussis toxin inactivation of Gi/Go proteins prevents MOR down-regulation whereas DOR expressing cells are unaffected (Chaturvedi et al. 2001). Studies indicate internalized MOR are sorted to early endosomes and can be degraded by lysosomes and proteasomes, however further studies are required to determine the mechanisms of MOR down-regulation (Li et al. 2001; Sternini et al. 1996).

Regulation of endogenous opioid system by cocaine

The endogenous opioid system modulates glutamatergic and dopaminergic signaling into the NAc, but more importantly endogenous opioid ligand activity at opioid receptors is able to modulate NAc output directly. Cocaine increases β-endorphin release into the NAc as do drug-related cues and stress, indicating the possibility for opioid receptor modulation of cocaine-reinforcement and relapse. The differential expression patterns of MOR and DOR lend them different mechanisms of action, with DOR more frequently modulating inhibitory and dopaminergic input to the NAc and MOR primarily modulating NAc GABAergic neurons themselves and excitatory neurotransmission (Svingos et al. 1998; 1999a; Svingos et al. 1997; Wang and Pickel 1998).

Previous reports indicate chronic cocaine administration modulates the opioid system in the NAc (for review see (Boutrel 2008; Kreek 2001; Kreek et al. 2004). Cocaine administration increases β-endorphin release into the NAc (Doron et al. 2006; Olive et al. 2001; Roth-Deri et al. 2003) an effect that is blocked by application of a D2R antagonist in the Arc of the hypothalamus (Doron et al. 2006). Whether cocaine increases extracellular enkephalin in the NAc is unknown. However, cocaine acutely increases PPE throughout the striatum (Hurd and Herkenham 1992), an

effect that is diminished with chronic cocaine (Arroyo et al. 2000; Mantsch et al. 2004; Ziolkowska et al. 2006). Dynorphin (the endogenous ligand for KOR) is not regulated by chronic binge cocaine (Daunais and McGinty 1995). β-endorphin levels are decreased immediately prior to the next scheduled cocaine self-administration session in the NAc (among other brain regions) and opioid receptor binding potential (is decreased immediately after and prior to the next scheduled cocaine self-administration session (Gerrits et al. 1999; Sweep et al. 1988; Sweep et al. 1989). These findings indicate the release of endogenous opioids during cocaine self-administration and during anticipation of cocaine self-administration since available binding sites were decreased. Alternatively, these findings indicate ligand dependent reduction in opioid receptors by cocaine self-administration.

Indeed, opioid receptors themselves are regulated by chronic cocaine administration. In rats that received chronic binge cocaine, MOR and KOR but not DOR levels were increased in the NAc (Unterwald et al. 1994). MOR stimulated GTPγS35 activity was increased as a result of chronic binge cocaine, with no change in DOR activity (Schroeder et al. 2003). However, the ability of DOR stimulation to inhibit adenylyl cyclase

(AC) activity was impaired in the NAc compared to MOR (Unterwald et al. 1993).

Withdrawal from cocaine administration also appears to modulate the endogenous opioid system. 1-day withdrawal from chronic binge cocaine decreased DOR signaling (Perrine et al. 2008) and 48-hour withdrawal altered trafficking of DOR in the NAc (Ambrose-Lanci et al. 2008). Withdrawal from cocaine self-administration did not alter enkephalin gene expression in NAc (Arroyo et al. 2000), whereas the precursor for β-endorphin, prepro-opiomelanocortin (POMC), mRNA levels are increased in animals undergoing withdrawal from cocaine self-administration (Smagula et al. 2005). Together, these findings indicate MOR is specifically modulated during cocaine self-administration and withdrawal and suggests β-endorphin activation of NAc MOR during abstinence as a promising mechanism of increased cocaine craving and subsequently relapse.

Opioid modulation of cocaine reinforcement and self-administration

Several behaviors are associated with the reinforcing effects of cocaine. As stated previously increasing locomotor behavior in response to various stimulation is a response important in adaptive behavior of an

animal as well as humans. For example, increasing foraging for food when in a state of hunger or increasing exploratory behavior to find a new food source. Conditioned place preference (CPP) is an experimental measure of an animals' ability to associate a preferred substance with environmental stimuli. This measure is thought to model an animal returning to a location where they found rewarding stimuli such as food when in a state of hunger or high fat food or sugar, highly rewarding foods. These measures involve brain regions implicated in reward processing and behavior. As such, cocaine increases locomotor behavior and produces CPP in several animal models, supporting the thought that drugs to "high-jack" the natural reward system.

Manipulation of opioid receptors in brain regions associated with the stimulating effects of cocaine modulates the rewarding and stimulatory locomotor properties of cocaine. Intracerebro-ventricle (icv), intra-NAc, or intra-VTA administration of the MOR-selective antagonist CTAP blocks cocaine CPP (Schroeder et al. 2007; Soderman and Unterwald 2008). Naltrexone, a non-specific opioid antagonist, also prevents cocaine conditioned place preference (CPP) and the locomotor stimulatory effects of cocaine (Bilsky et al. 1992; Houdi et al. 1989). Pretreatment with the kappa-opioid receptor (KOR) agonist U-50,488H (Suzuki et al. 1992) or

DOR antagonist, naltrindole, (Menkens et al. 1992; Suzuki et al. 1992) reduces cocaine CPP. Naltrindole prevents sensitization to the rewarding properties of cocaine (Shippenberg and Heidbreder 1995) and prevents the development of sensitization to the locomotor stimulating effects of cocaine (Heidbreder et al. 1996). Similarly, treatment with KOR agonist prevents sensitization to the conditioned rewarding effects of cocaine (Heidbreder et al. 1995). Mice that received icv antisense to MOR displayed reduced cocaine CPP and cocaine-mediated locomotor effects (Hummel et al. 2006). Antisense to DOR given icv also significantly attenuates cocaine-induced CPP (Suzuki et al. 1997). That opioid receptor blockade or down-regulation decreases conditioned reinforcement indicates their involvement in the rewarding effects of cocaine potentially through cocaine-induced opioid release.

Further, cocaine self-administration itself is modulated by opioid receptors. Blockade of opioid receptors with naloxone or naltrexone (non-specific opioid receptor antagonists) will decrease cocaine self-administration (Corrigall and Coen 1991). Microinjections of beta-funaltrexamine (MOR antagonist) or naltrindole-5'isothiocyanate (irreversible DOR antagonist) into the NAc attenuates responding for cocaine under a progressive ratio schedule of reinforcement in rats (Ward

et al. 2003; Ward and Roberts 2007). Chronic buprenorphine (partial MOR agonist) or methadone (MOR agonist) treatment reduces cocaine intake in humans (Vigezzi et al. 2006) and rats (Sorge et al. 2005; Sorge and Stewart 2006). Blockade of NAc β-endorphin (Roth-Deri et al. 2003) or Arc dopamine receptors (Olive et al. 2001) during cocaine self-administration results in extinction-like behaviors, indicating a decrease in the rewarding effects of cocaine. Pretreatment with a KOR agonist decreases cocaine self-administration (Mello and Negus 1998; Negus et al. 1997; Schenk et al. 1999), an effect thought to be mediated through KOR modulation of dopamine release (Heidbreder et al. 1998; Heidbreder and Shippenberg 1994; Meshul and McGinty 2000). These findings further support opioid receptor involvement in the rewarding properties of cocaine.

Circuitry involved in relapse to cocaine seeking

The three types of stimuli that will elicit relapse behaviors in human addicts and animals involve different brain pathways that ultimately converge on the NAc. The stimuli involved in relapse behaviors are: exposure to the environment where drug was previously taken or discrete cues associated with drug use (McFarland and Ettenberg 1997; Meil and

See 1997), a pharmacological agent that induces some component of the drug experience (de Wit and Stewart 1981), and environmental stressors (Ahmed and Koob 1997; Shaham et al. 2000). These distinct stimuli are processed by different brain regions and are perceived as different stimuli, however they are believed to ultimately activate a distinct circuit that culminates in drug-seeking behaviors.

Different brain regions are activated by different cues during cocaine abstinence in cocaine-addicted humans. The orbitofrontal and anterior cingulate cortices and amygdala are activated during cue-induced drug craving in cocaine addicts (Childress et al. 1999; Daglish and Nutt 2003; Kilts et al. 2001). However, the amygdala is associated with cue-induced elicitation of most motivated behaviors and is not specific to cocaine or drug craving (Everitt et al. 1999) while activation of the anterior cingulate is selective for cue-induced drug craving. Activation of the anterior cingulate tends to precede reported craving, indicating its association with mood states that precipitates craving (Volkow et al. 1999; Wexler et al. 2001). In response to a low dose cocaine challenge in human addicts, metabolic activity is increased in the anterior cingulate prior to the onset of craving, again indicating its activation is associated with mood that precipitates craving, while the ventral striatum (NAc)

showed an activation pattern associated with craving (Breiter et al. 1997). In response to stress-induced cocaine craving, abstinent cocaine-addicted patients had less activation of the anterior cingulate paired with increased activation of the striatum (Sinha et al. 2005).

Animal extinction/reinstatement testing of cocaine self-administering animals during drug abstinence allows for the modeling of drug-seeking and relapse to drug-seeking or drug use. Basically, animals are placed back in the self-administration chambers in the absence of drug and drug-paired lever pressing indicates drug seeking. Repeated testing allows the animal to dissociate drug-taking and drug-paired lever pressing (extinction training) thus the animal will stop responding on the drug-paired lever. Following extinction training, animals are tested for reinstatement of lever pressing (relapse behavior) in response to stimuli mentioned previously. This animal paradigm of extinction/reinstatement is thought to best model human craving and relapse behaviors.

Thus, in animal reinstatement models, the same limbic circuitry is activated as shown by immediate early gene (IEG) synthesis, such as c-fos and γ protein kinase C (γPKC). IEG expression increases in the basolateral amygdala, cingulate cortex, orbital cortex, and the NAc core and shell (Ciccocioppo et al. 2001; Neisewander et al. 2000) during

reinstatement testing. Fos expression increases in extinguished animals given a priming injection of cocaine (drug-induced reinstatement) in regions with dopaminergic innervation such as the VTA, dorsal striatum and central nucleus of the amygdala (Neisewander et al. 2000). Non-extinguished animals exposed to the cocaine self-administration environment (context-induced reinstatement) show increased expression in the NAc, basolateral amygdala and hippocampus. The anterior cingulate is activated by both conditions. If a cue-light is repeatedly paired with cocaine infusions during cocaine self-administration, presentation of the cue light increases γPKC in the amygdala and prelimbic cortex during reinstatement testing (Thomas and Everitt 2001). Pairing a cue light with non-contingent cocaine administration, increases γPKC in the anterior cingulate. Presentation of the cue light to animals in either condition increases expression in the NAc core.

Taken together, the prefrontal cortex, especially the anterior cingulate, is involved in the priming of reinstatement behavior in both human addicts and animal models of reinstatement ultimately through activation of the NAc core. The anterior cingulate integrates various stimuli that subsequently induces drug-seeking behavior since activation of this region precedes craving reports. As stated previously, the anterior

cingulate specifically innervates the NAc core rather than shell, thus activation of the anterior cingulate in response to various stimuli would subsequently increase glutamatergic input into the core, resulting in relapse behaviors (Cornish et al. 1999; McFarland and Kalivas 2001). Since inactivation of the core and not the shell prevents cocaine-primed reinstatement (McFarland and Kalivas 2001) and blockade of dopamine receptors in the core does not modulate cocaine-primed reinstatement (Anderson et al. 2006), NAc core appears important for craving behaviors that are independent of drug-induced dopamine release.

Stress-induced craving in cocaine addicts is a stronger stimuli in eliciting cocaine craving than low dose drug-induced craving and is associated with a shorter time to cocaine relapse (Sinha et al. 2006). Stress induced craving is associated with a decrease in activity in the anterior cingulate but increases in activity in the NAc (Sinha et al. 2005), indicating a different circuit in this activation, the hypothalamic pituitary adrenal (HPA) axis. In abstinent cocaine addicts, stress and drug-associated cues increases craving that is paired with increases in ACTH (adrenocorticotropc hormone), cortisol, prolactin and norepinephrine, indicating activation of the HPA axis (Sinha et al. 2003). The stress systems are activated by cocaine (Mello and Mendelson 1997) and drug

associated cues (Sinha et al. 2000). Stress increases β-endorphin release in the NAc (Marinelli et al. 2004) as does cocaine (Roth-Deri et al. 2003), indicating Arc neurons that release β-endorphin into the NAc during cue, drug, or stress priming represent another circuit involved in modulating relapse behaviors.

Research on opioid receptor involvement in reinstatement and relapse behaviors in rodent models and human cocaine addicts is limited. However an intact KOR system is required for stress-induced reinstatement to cocaine seeking (Redila and Chavkin 2008) and KOR antagonist blocked stress- but not cocaine-induced reinstatement of cocaine seeking (Beardsley et al. 2005). Systemic administration of the non-specific opioid antagonist, naltrexone, the partial MOR agonist, buprenorphine, or the DOR antagonist, naltrindole, reduces cocaine-primed reinstatement (Burattini et al. 2008; Comer et al. 1993; Gerrits et al. 2005). Stewart and colleagues found that opioid receptors in the VTA play a role in cocaine and heroin seeking (Stewart 1984), however this effect is thought to involve local disinhibition of dopamine neurons in the VTA leading to dopamine release in the NAc (Ford et al. 2006; Johnson and North 1992a). Similarly, through KOR modulation of dopamine release (Heidbreder and Shippenberg 1994; Maisonneuve et al. 1995;

Meshul and McGinty 2000), pretreatment with U69593, a KOR agonist, decreases cocaine-primed reinstatement to cocaine seeking (Schenk et al. 1999).

In cocaine addicts, positron emission tomography (PET) studies found increased levels of MOR binding that correlates with increased craving for cocaine during abstinence (Gorelick et al. 2005; Zubieta et al. 1996). This up-regulation persists for over 4 weeks of monitored cocaine abstinence. However, the resolution of the analysis method utilized did not allow for identification of altered NAc MOR binding. Using the Minnesota Cocaine Craving Scale, severity of craving positively correlates with MOR binding in the amygdala, anterior cingulate cortex, frontal cortex, and temporal cortex. Further, the degree of relapse severity and the time to first relapse episode positively correlates with MOR increases (Gorelick et al. 2008). Conceivably, increased MOR levels in brain regions involved in cocaine craving could mediate craving and subsequently relapse behaviors in human cocaine addicts and rodent models of addiction.

This thesis work focused on modulation of MOR by cocaine self-administration and withdrawal, and the implications for MOR modulation in the reinstatement of drug seeking behaviors. DOR was also analyzed

since DOR blockade and stimulation acts through mechanisms similar to MOR in behavior. KOR was not analyzed since these receptors primarily modulate dopamine release into the NAc through their presynaptic expression and act opposite of MOR and DOR in behavioral output. Additionally, MOR and DOR in the NAc modulates behavioral responses independent of dopamine.

CHAPTER TWO

BASIC INTRODUCTION OF METHODOLOGY AND TECHNIQUES

Behavioral Techniques

The work in this thesis utilized cocaine self-administration and locomotor assays as tools in determining the effects of various drugs on rat behavior and of cocaine on rat the brain. Cocaine self-administration allowed for analysis of changes that occurred as a result of response-contingent cocaine administration while chronic non-contingent (yoked) administration allowed for analysis of pharmacological effects of cocaine itself independent from cocaine reinforcement. Acute yoked administration determined changes that resulted from brief initial exposure to cocaine. Saline self-administration controlled for surgery, handling and procedural effects while home-cage controls served as a common baseline for all experimental groups. Locomotor studies determined the unconditioned stimulant effects of drugs as a result of cocaine use and withdrawal. Together, these tools provided a means to answer several questions as discussed in later chapters.

Cocaine self-administration paradigm

The cocaine self-administration paradigm allows one to determine behavioral and biochemical changes that result from contingent and non-contingent cocaine administration. This paradigm models human cocaine use since drug exposure is voluntary. Animals readily learn to self-administer cocaine (figure 2.1), and in some models animals will escalate their intake (Koob and Kreek 2007; Mantsch et al. 2004) and overcome adverse consequences in seeking a cocaine infusion (Pelloux et al. 2007). Further, the degree of drug-seeking behavior in animals increases with longer periods of abstinence (Grimm et al. 2002; Lu et al. 2004; Tran-Nguyen et al. 1998).

Self-administration studies discussed in this body of work involved techniques previously described (Bachtell et al. 2005; Sutton et al. 2000). In 4 h cocaine access trials, animals were trained to lever press for sucrose pellets under a food-restricted state to facilitate acquisition of lever pressing. Once animals adequately acquired lever-pressing behavior, they were fed ad libitum for at least one day and then chronic intravenous catheters were surgically implanted. Surgery for self-administration involved fitting animals with a catheter that fed 4 cm of

Sialastic tubing into the jugular vein so that the tip ended just before reaching the heart valve. The catheter line worked subcutaneously over the shoulder and exited through a back mount just below the shoulder blades so that animals could not reach the catheter and remove it.

Following recovery from surgery for 3-7 days, animals were placed in the operant chambers for self-administration. A light (house-light) illuminated the chamber when animals were first placed into the chambers, indicating drug availability. Upon depression of the drug-paired lever, the house-light turned off for a time-out period of 15 s and animals received an injection of cocaine or saline (2.5 or 5 s duration, depending on the paradigm) that was paired with illumination of a cue light above the drug-paired lever. During the time-out period, any further lever presses did not result in additional drug injections. Following the time-out period, the house light was again illuminated and the animal was free to receive another injection. Lever pressing on the inactive lever did not result in any consequences and usually acts as a measure of non-specific activity.

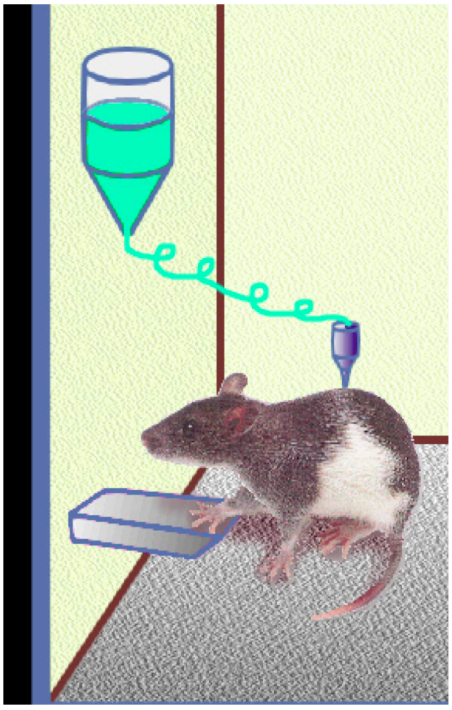


Figure 2.1 Adapted from NIDA (www.NIDA.gov). Animals are fitted with a catheter into the jugular vein allowing for intravenous cocaine self-administration. The animals are allowed to press a drug-paired lever which delivers an injection of cocaine. This paradigm allow animals to self-administer cocaine or other drugs. Drug-seeking and relapse behaviors can be measure by removing the drug so that lever pressing does not result in any consequences (extinction training). The first day of extinction training is considered drug seeking (follows variable number of days of abstinence, depending on the question being tested) since animals are attempting to receive an infusion of cocaine but are not. Once animals are extinguished, relapse (reinstatement of lever pressing) behaviors are measured by presenting animals with a drug associated cue, exposing animals to stress, or giving animals a low dose of cocaine itself or other drug.

Two basic self-administration protocols were used throughout this thesis. Animals were trained to self-administer cocaine for 4 h/d (0.5 mg/kg in 0.05 ml per 2.5 s infusion), 6 d/w for 3 w to study the effects of cocaine administration on the NAc MOR following 0 h or 24 h withdrawal. Experiments on the effects of long-term cocaine abstinence were not lever-press trained with sucrose but instead were allowed to learn to self-administer cocaine during 5 d of 10 h sessions (0.5 mg/kg in 0.025 ml per 1.25 s infusion), where stable responding was usually achieved by day 3. After a two day break in the home cage, animals were allowed to self-administer cocaine during 6 h sessions, 5 d a week, for 2 additional weeks. Long access cocaine self-administration increases the probability that animals would display addictive-like behaviors and physiology (Kippin et al. 2006; Knackstedt and Kalivas 2007; Mantsch et al. 2008).

Several other groups of animals were generated as controls for the self-administering animals. Chronic yoked animals were animals that were trained to lever press for sucrose and catheterized similar to self-administering animals. However, when placed in the operant boxes, yoked animals received cocaine non-contingently in a temporal pattern determined by a self-administering animal to determine any differences that occur as a result of the pharmacological effects of cocaine

independent of cocaine reinforcement. Acute yoked animals were trained and handled like the chronic yoked animals however they received saline passively for 3 w of testing. On the last test day, acute yoked animals received cocaine for the first time to determine any acute pharmacological effects of cocaine. For the short-term and long-term withdrawal study, control animals were home-cage and saline self-administering animals to account for handling differences.

Locomotor Behavioral Assay

The locomotor test utilized a circular chamber with 4 laser detection devices to measure horizontal locomotion. Animals were habituated to the chamber for 2 h for day one followed by 4 h test sessions consisting of 2 h habituation and 2 h test on subsequent days. Animals were treated with drugs intra-cranially with guide cannulae aimed at the NAc. Depending on the experiment, animals were either drug naïve or cocaine-trained and tested at different times of abstinence. Naïve animals were habituated to the vivarium for 5-7 d prior to surgery and allowed 7 d to recover. Cocaine trained animals were implanted with guide cannulae during catheterization and allowed 5-7 d to recover. Data were plotted in 10 m bins and 1 h bar graphs.

Biochemical Techniques

Techniques for determining gene and protein regulation included microarray analysis, qRT-PCR, and Western blot, and was used to determine changes as a result of cocaine administration. Microarray analysis was used to determine gene regulation during long-term abstinence while quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was used to determine changes in MOR mRNA that occurred immediately following cocaine self-administration. Western blot analysis was used to confirm the translation of genetic regulation at the level of protein expression. Further Western blot analysis was used to confirm specificity of an antibody to MOR following plasmid expression of a cloned rat MOR in HEK 293 cells. Immunohistochemistry was used to visualize MOR endocytosis according to published procedures (Haberstock-Debic et al. 2003).

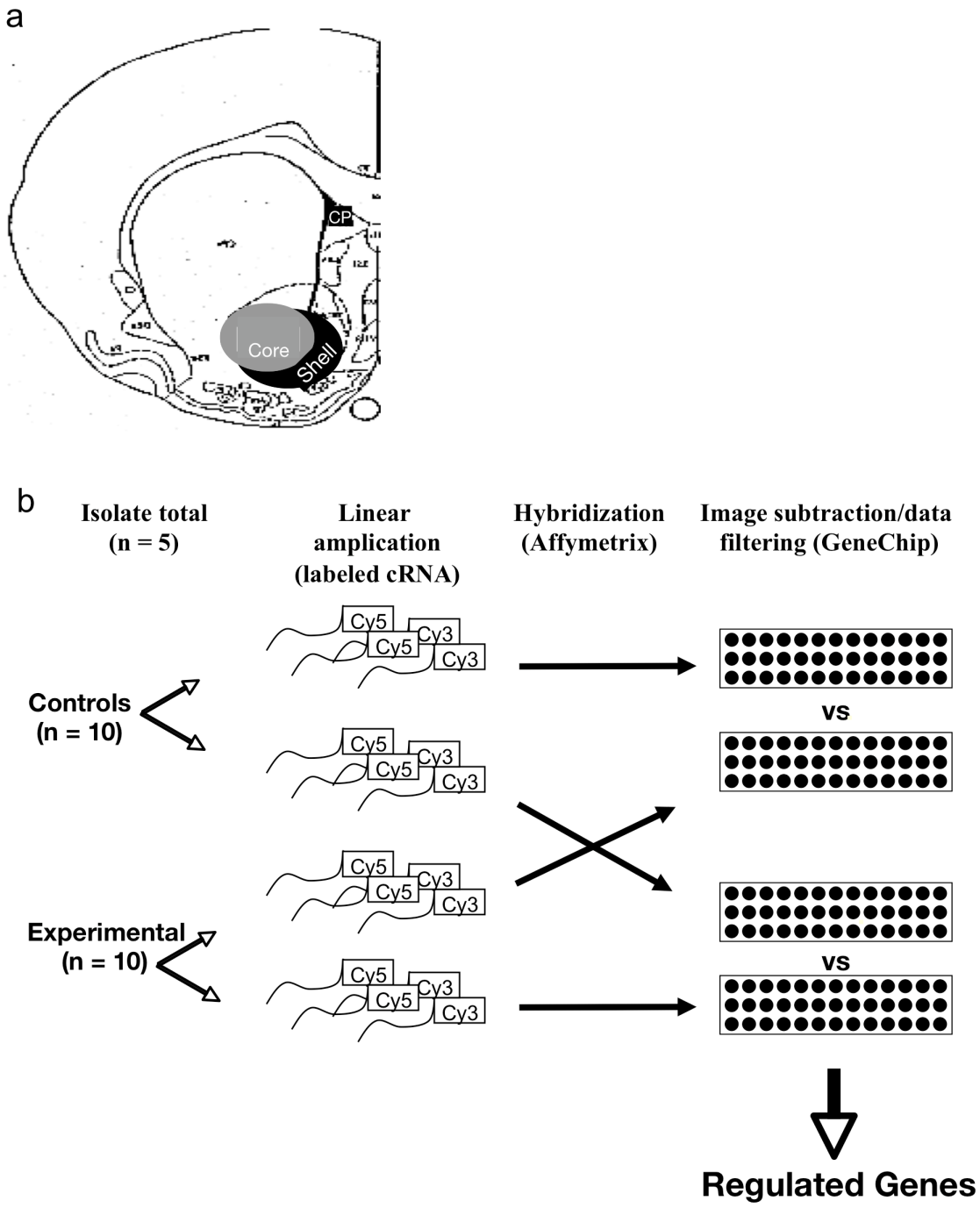


Figure 2.2 (a) Diagram of NAc regions dissected for microarray, quantitative RT-PCR, and Western blot analysis. (b) Cartoon diagram of microarray tissue samples and hybridization.

Microarray Analysis

This part of the study was initiated prior to the start of this thesis project as a means of generating leads for further study. It was through the microarray analysis that MOR was identified as a gene of interest regulation at the protein level. Microarray analysis allowed for identification of genes that were altered compared to controls as a result of withdrawal from cocaine self-administration. This technique involved allowing animals to self-administer cocaine for 3 w (10 h/d for 1 w, 6 h/d for 2 w). Following self-administration, animals were counter-balanced for drug intake and divided into 1w and 6w withdrawal and compared with age-matched homecage controls. The behavioral and Western blot data included animals that were generated by others in the laboratory at Yale University and animals generated by this thesis work at UT Southwestern Medical Center. All animals used for microarray analysis were generated at Yale University. Following the respective withdrawal or test periods, animals were euthanized and several brain regions were dissected with regions from 5 animals pooled for each microarray chip, for a total of 2 replicates (Figure 2.2). Microarray analysis was conducted by the Genomics Institute of the Novartis Research Foundation (La Jolla, CA). Briefly, RNA is isolated from the samples are amplified once using RT-

PCR to create labeled cRNA. The labeled samples are then hybridized to microarray gene chips containing several combinations of small parts of known and unknown genes of the entire rat genome. Levels of fluorescence are compared between groups. NAc core and shell were the focus of this study. Changes identified at 6 weeks withdrawal were used to screen for persistent changes in gene expression with a 1.5-fold change in expression as criteria.

Western blot analysis

Animals used for Western blot analysis were euthanized by either microwave fixation or rapid decapitation. Microwave fixation was used in the short-term withdrawal study since these animals were generated as a lab collaboration for a tissue bank that allowed for the analysis of several proteins of interest, both phosphorylated and non-phosphorylated forms. This technique fixes the state of proteins at the moment of exposure and decreases noise generated in phosphorylated proteins as a consequence of handling and degradation during dissection. Rapid decapitation was utilized when animals were cannulated or microwave fixation was unavailable (short-term withdrawal and long-term abstinence studies, respectively). Following euthanasia, brains were removed and dissected

in ice-cold PBS. NAc core and shell were placed in individual, labeled tubes and immediately homogenized in homogenization buffer: 320mM sucrose, 5nM Hepes buffer (pH to 7.4), 50mM NaF, 1% SDS, 1:100 dilution each of phosphatase inhibitor cocktail I and II and Protease inhibitor cocktail (Sigma, St Louis, MO) or frozen on dry ice and stored at -80C until homogenization was done. The Lowry protein assay (BioRad) was used to determine protein concentrations and 20 ug samples were aliquoted and stored at -80 degrees C until Western blotting. Western blotting allows for separation of proteins on acrylamide gels, based on their molecular weights (Figure 2.3). For proteins analyzed by these studies, 10% acrylamide gels were used. 10X Tris-Glycine-SDS buffer (BioRad) was diluted to 1X using sterile water (2 L final volume) and poured into the upper and lower dams. Samples were loaded into individual wells after they were flushed with buffer. Gels were ran at 60V overnight at room temperature (approximately 20 hours). Transfer buffer was prepared fresh the following morning before completion of the gel run (to allow buffer to cool to 4C prior to use): 400ml 10X Tris-Glycine (BioRad), 800ml methanol, 2800ml sterile water. Polyvinylidene fluoride (PVDF) membranes were prepared in 100% methanol (soak for 3 minutes) and followed by incubation in transfer buffer (approximately 1 hour).

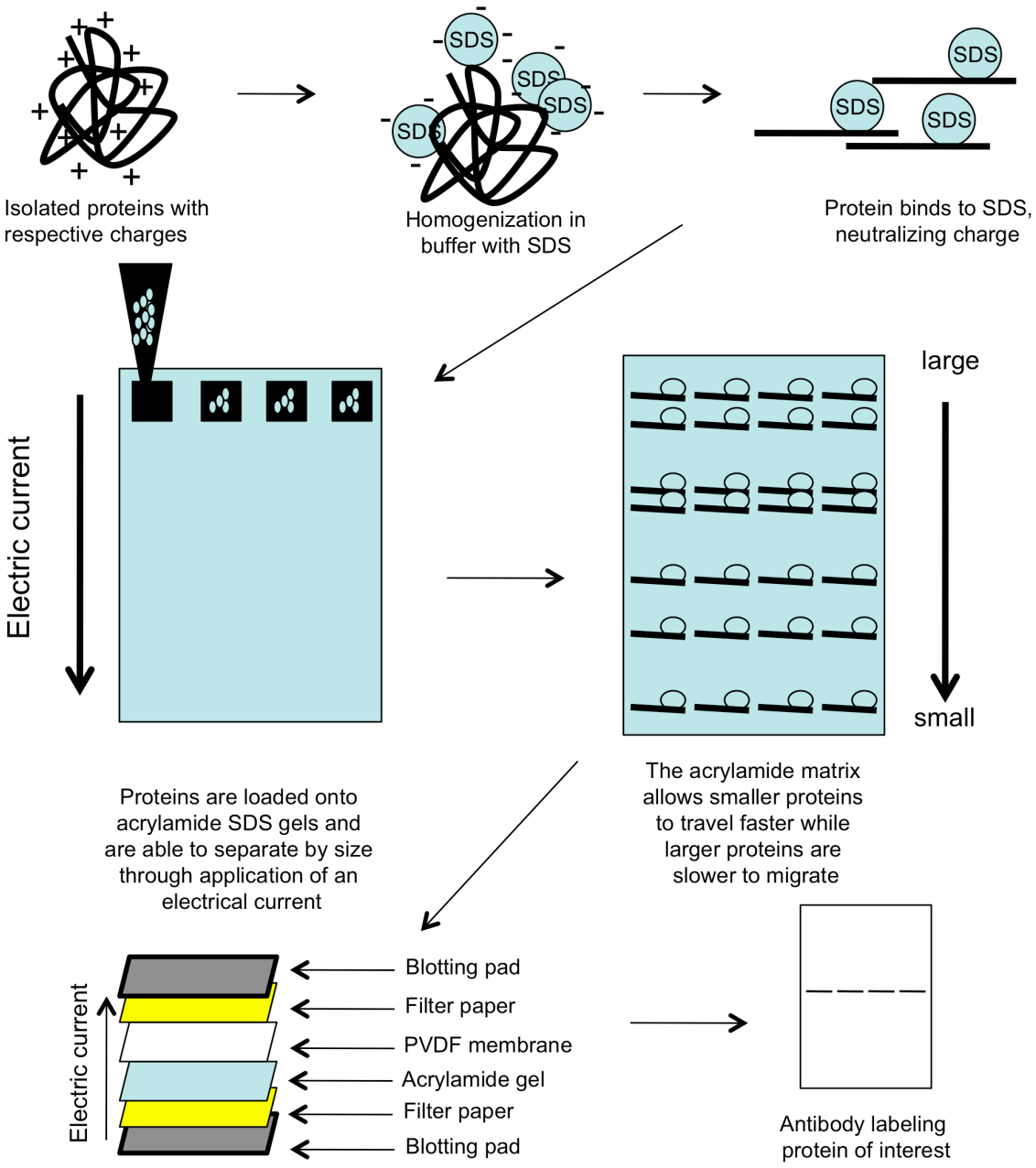


Figure 2.3 Simplified schematic of Western blotting of proteins using SDS PAGE. Proteins are homogenized in buffer containing SDS which binds to the positively charged proteins, neutralizing their charge. This interaction allows proteins to be separated by size alone by loading samples onto a polyacrylamide gel and applying an electrical current that causes the protein to migrate down the gel. Once proteins reaches desired separation (as visualized with a molecular weight ladder that is run along with the samples, proteins are transferred to a PVDF membrane using an electrical current. The membrane is then washed and labeled with an antibody against the desired protein and visualized using chemiluminesence.

Blotting pads and filter paper were also soaked in transfer buffer prior to transfer. Acrylamide gels were then transferred to PVDF membranes overnight at 4C at 45V (~30mA) for approximately 20 hours. Membranes were blocked with 5% milk in TTBS 4C overnight, followed by primary antibody overnight at 4C (MOR, DOR, pMOR, AKAP84, Kv4.2, or β-tubulin), and then respective secondary antibody after washes at room temperature (1hr). Chemiluminesence was used to visualize bands on hyperfilm (Amersham). X-ray film of protein blots were densitized and experimental samples were expressed as a percent change from averaged control values.

Quantitative RT-PCR Analysis

qRT-PCR was used to determine changes of MOR mRNA immediately following cocaine administration in an attempt to determine a mechanism behind MOR regulation by cocaine. Animals were euthanized by rapid decapitation and brains were dissected for several brain regions. Individual samples were immediately homogenized in RNA-STAT-60 (600 ul) and frozen on dry ice and stored at -80C until mRNA was extracted. Chloroform (160 ul) was added to each sample, vortexed, and allowed to separate for 2-3minutes at room temperature, followed by 4 C

centrifugation for 15 minutes at 14,000 RPM. The aqueous layer was isolated and 4 ul linear acrylamide was added to help precipitate the nucleic acids. Isopropanol (400 ul) was added, vortexed, and allowed to precipitate at -20C for 30 minutes or overnight. The samples were centrifuged for 10 minutes at 4 C at 14,000 RPM and the supernatant removed. Pellets (mRNA) were washed by adding 500 ul of 75% ethanol, vortexing briefly, and centrifuging the samples at 4C for 5 minutes at 14,000 RPM. Ethanol was carefully removed and the pellet allowed to dry. The pellet was then resuspended in 20ul depC H2O and heated for 8-10 minutes at 50 C with vortexing every 2 minutes. Concentration was checked using a nano-drop (Thermo Fischer Scientific). Samples utilized for analysis were those with wavelength ratios of 260/280 more than 1.65.

RNA was treated according to instructions in Invitrogen's DNase treatment kit. 17ul of the least concentrated sample was used as the normalizing determinant. RNA concentration of all samples were normalized to the least concentrated sample. 2 ul 10X Turbo DNase Buffer (Invitrogen) was added to each tube, followed by 1 ul of TURBO DNase enzyme (Invitrogen). Samples were incubated at 37 degrees C for 30 minutes. 2 ul DNase inactivating reagent was added and the samples

centrifuged at 10,000 x g for 1.5 minutes at 4 degrees C. The supernatant was isolated and stored in a fresh tube.

cDNA was prepared using Invitrogen's Superscript III kit. 8 ul of RNA was added to a PCR tube. 1 ul of hexamers and 1 ul of dNTP was added per sample and heated to 65 degrees C for 5 minutes. Samples were cooled on ice and Superscript III was added as 1 ul of each of the following: 10X Superscript III buffer, MgCl, DTT, RNase out, and Superscript III. Samples were diluted to 200 ul total and stored at -80 degrees C until qRT-PCR was done.

Primers were generated to range from 100-150nt in length and to have a GC content of 40-70% with a melting temp of near 60 degrees C. The primers used for MOR were 5'-CAACTTGTCCCACGTTGATG-3' and 3'-AAGGTACCAGTGTCGGTAAT-5', so that the end product mapped to the N-terminal transmembrane 1 spanning region of MOR and yielded a product that was 119 nt in length. The primers used for the internal standard, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), were 5'-AACGACCCCTTCATTGAC-3' and 3'-CCACGACTCATACAGCACCT-5' and yielded a product that was 136 nt in length. 5ul of cDNA was used in triplicate for each gene analyzed. Dissociation curves were generated to

ensure production of a single product and the products were run on an 0.8% agarose gel as a secondary confirmation.

Immunohistochemistry

This procedure was used to visualize hemaglutanin (HA)-tagged MOR in the NAc following various treatments. Viral mediated expression of HA-tagged MOR was required since MOR labeling in the NAc is primarily dendritic and diffuse, making it difficult to quantitate any changes that might occur as a result of cocaine self-administration or other manipulation. In the fentanyl study, animals were treated with fentanyl or saline subcutaneously (sc) 3 d post HSV-MOR infusion and were euthanized 20 minutes later. Saline and cocaine self-administering animals received intra-NAc infusions of HSV-MOR prior to their last self-administration session. Animals were given 2 d off to allow viral expression to reach peak levels. Immediately prior to the last self-administration session, animals were treated with an antibody to β-endorphin in one NAc core hemisphere and contralateral treatment with IgG. Animals then self-administered saline or cocaine and were euthanized immediately after this session. All animals were euthanized by with chloral hydrate and transcardially perfused with ice-cold 1X PBS

(BioRad) for 5 m followed by 4% paraformaldehyde for 15 m. Brains were removed and placed in 4% paraformaldehyde overnight at 4 degrees C. Brains were then cryoprotected by equilibration in 20% sucrose in PBS at 4 degrees C followed by 30% sucrose in PBS at 4 degrees C.

Brains were sectioned and detected as described previously (Haberstock-Debic et al. 2003). Briefly, 45um thick slices were cut using a cryostat and washed in PBS. Slices were treated with 1% peroxidase/ PBS for 30 m and blocked with 3% Normal Donkey Serum (NDS; Jackson Labs, Bar Harbor, Maine) in PBS containing 0.3% Triton X-100 (Sigma, St. Louis, MO) for 1 h at 25 degrees C. Free-floating sections were incubated for 36 h with rabbit primary anti-HA.11 (1:250; Covance, Princeton, NJ) at 4 degrees C. Sections were washed and labeled with Cy3-Donkey-anti-rabbit IgG (1:200; Jackson Labs). Sections were mounted on slides after washing with PBS and fixed with Dent's fixative and coverslipped.

HA-tagged MOR labeled cell bodies and processes were counted across 10-16 sections, counting 15-30 cell bodies and 25-60 cell processes, keeping left and right hemispheric NAc separate since each animal received IgG or anti-β-endo in contralateral NAc core. In fentanyl and saline sc treated animals, left and right cell counts were pooled and only 8-10 sections were counted. Highly punctate expression of HA-

tagged MOR was considered endocytosed and counts were expressed as percent endocytosed (punctate) compared to total counts for cell bodies and processes.

CHAPTER THREE:

MODULATION OF MU OPIOID RECEPTORS IN NUCLEUS ACCUMBENS BY BETA ENDORPHIN RELEASE DURING COCAINE SELF-ADMINISTRATION

(in preparation)

Simmons, D; Buzin, N; Larson, E; Graham, DL; Edwards, S; Bachtell, RK; Neve, R; von Zastrow, M; Self, D

Introduction

Drug addiction is characterized by compulsive drug use despite adverse consequences and is thought to involve the dysregulation of brain reward circuitries (Dackis and O'Brien, 2001; Kalivas and Volkow, 2005; Koob and Le Moal, 2001). It is believed that addiction related behaviors reflect a shift in the hedonic set-point that regulates normal homeostatic behaviors (Koob and Le Moal). The opioid system is intricately involved in the modulation of appetitive behavior (Cunningham et al. 1997) as well as cocaine-related behaviors (Bakshi and Kelley 1993), indicating a potential role for the endogenous opioid system in cocaine addiction. Cocaine produces numerous neurobiological changes in several brain regions that

are implicated in cocaine addictive behaviors, however the functional consequences of many changes remain to be fully understood.

Cocaine increases dopamine levels in the nucleus accumbens (NAc) through activation of the ventral tegmental area (VTA), and elicitation of cocaine seeking behaviors are seen through activation of this circuit (Phillips *et al*, 2003; Pruessner *et al*, 2004; Self and Nestler, 1998; Shalev *et al*, 2002; Spealman *et al*, 1999; Stewart, 2000). Chronic cocaine modulates neuronal activity and several proteins involved in dopamine signaling (Anderson and Pierce 2005; Ben-Shahar et al. 2007; Thomas et al. 2001). Cocaine also modulates NAc opioid receptor expression via dopamine receptor activity (e.g., Unterwald et al., 1992), however the mechanism for this regulation is unknown.

Chronic cocaine administration modulates the opioid system in the NAc (for review see (Boutrel 2008; Kreek 2001)). β-endorphin is released during cocaine self-administration and non-contingent cocaine (Roth-Deri et al. 2003). β-endorphin levels are decreased in the NAc (among other brain regions) after cocaine self-administration (Sweep et al. 1989) and opioid receptor binding potential is decreased immediately after and prior to the next scheduled cocaine self-administration session (Gerrits et al. 1999). These findings indicate that β-endorphin release during cocaine

self-administration drives a ligand-dependent reduction in opioid receptors. However, rats that received chronic binge cocaine from experimenter-delivered injections show increased mu-opioid receptor (MOR) but not delta-opioid receptor (DOR) levels in the NAc (Unterwald et al. 1994). MOR stimulated GTPγS35 activity also increases as a result of chronic binge cocaine, with no change in DOR activity (Schroeder et al. 2003), however, the ability of DOR, and not MOR, stimulation to inhibit adenylyl cyclase activity is impaired in the NAc (Unterwald et al. 1993). 1-day withdrawal from chronic binge cocaine decreases DOR signaling (Perrine et al. 2008) and 48-hour withdrawal alters trafficking of DOR in the NAc (Ambrose-Lanci et al. 2008).

Whether cocaine increases extracellular enkephalins in the NAc is unknown, but cocaine acutely increases preproenkephalin expression throughout the striatum (Hurd and Herkenham 1992), although this acute effect is diminished with chronic cocaine administration (Arroyo et al. 2000; Mantsch et al. 2004). Conversely, the precursor for β-endorphin, pro-opiomelanocortin (POMC), mRNA levels are increased in animals undergoing long-term withdrawal from cocaine self-administration (Smagula et al. 2005). Together, these findings indicate MOR may be

differentially modulated during cocaine self-administration and non-contingent cocaine administration.

The mechanisms of opioid receptor regulation is currently under intense investigation. Studies indicate MOR are typically recycled back to the membrane following stimulation and endocytosis, whereas DOR are preferentially shuttled to lysosomes for degradation (Ko et al. 1999; Lohse 1993; Tanowitz and von Zastrow 2003; Tsao and von Zastrow 2000b; Whistler et al. 2001). Several studies indicate MOR phosphorylation at the serine 375 site occurs in response to ligand stimulation and that this phosphorylation increases ligand dependent endocytosis (El Kouhen et al. 2001; Schulz et al. 2004). Morphine and fentanyl increases phosphorylation at S375 site of MOR (Petraschka et al. 2007) and mutation of this site decreases endocytosis by DAMGO stimulation. Mice lacking β-endorphin fail to show increased pMOR in response to partial sciatic nerve ligation compared to wild-type littermates (Petraschka et al. 2007) suggesting that β-endorphin preferentially phosphorylates this site. However, MOR phosphorylation, endocytosis, and degradation in response to cocaine has not been conducted.

This project investigated whether cocaine self-administration modulates the expression of MOR and DOR in the NAc. MOR was

decreased by chronic cocaine administration in a β-endorphin dependent manner, since blockade of β-endorphin activity at MOR prevented MOR phosphorylation, endocytosis, and down-regulation. DOR was not regulated by chronic cocaine administration, however it was acutely decreased following acute cocaine administration, supporting previous findings that DOR stimulation preferentially shuttles DOR for degradation rather that recycling back to the membrane. MOR was not affected acutely, further giving credence to the hypothesis that chronic stimulation of MOR shifts the normal recycling pattern following MOR stimulation to one of degradation.

Materials and methods

Animals and surgery

Male Sprague-Dawley rats (weighing 250-300) were ordered from Charles-River Kingston, RI, USA and housed individually in wire cages in a climate-controlled environment (21 degrees C). Animals were under a 12:12h light:dark cycle (lights on at 7:00 AM) and fed ad libitum until testing began. Animals were cared for in accordance of the National

Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals* and IAACUC.

After 1 week of habituation to the vivarium, animals were maintained on a restricted diet at 85% original body weight and trained to lever press for 45 mg sucrose pellets in operant chambers (Med-Associates, Georgia, VT). Animals were trained on a fixed-ratio 1 (FR1) reinforcement schedule where each lever press resulted in dispensing of a sucrose pellet until acquisition criteria was achieved (3 consecutive d of 100 pellets consumed). This procedure was used to facilitate subsequent acquisition of cocaine self-administration. Upon reaching criterion, animals were fed ad libitum for at least 24 h prior to surgery implantation of a chronically indwelling catheter in the jugular vein as described previously (Edwards et al, 2006). Catheters were flushed daily with 0.2 ml of heparinized (20IU/ml) bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml) to prevent infection and maintain patency. Antibiotic ointment was applied daily to the catheter exit wound to prevent infection.

For NAc core injections, an intra-cranial, 26-guage bilateral guide cannula was aimed at the NAc (±1.5 mm lateral; 1.7mm anterior to bregma; -3.7 ventral to dura with the level skull; (Paxinos and Watson 1998), and held in place with 4 jewelers screws and dental cement.

Dummy cannulae were cut flush with the tip of the guide cannulae while infusion cannulae (33 gauge) were cut to extend 3 mm beyond the guide cannulae tip, and dummy cannulae remained in place until the day of intracranial virus or antibody infusion. Animals were allowed 5-7 d to recover prior to start of experiment.

Cocaine self-administration

Animals were trained on an FR1 schedule as previously described (Edwards et al. 2007). Briefly, the house light was on when animals were placed in the operant chamber (indicating drug-availability) and each lever press on the drug-paired lever resulted in an intravenous (iv) drug injection of cocaine (0.5mg/kg/ 0.05ml) or saline (0.09% saline in 0.05ml) delivered over 2.5 s in daily 4 h sessions for 3 w (6 d/w) (CSA and SSA, respectively). Depression of the drug-paired lever resulted in a drug injection paired with illumination of a cue-light above the lever and extinguishing of the house-light for a time-out period of 15 s. Any drug-paired lever pressing during the time-out period did not result in further infusions, but were counted. A second lever (inactive lever) did not result in any consequences, but was recorded. Chronic yoked (CY) cocaine

animals received passive non-contingent cocaine injections in a temporal pattern determined by their self-administering paired animal. Acute yoked (AY) animals received saline injections passively in a temporal pattern determined by their yoked self-administering partner on all but the last session when they received cocaine injections for the first time. Age- and group-matched untreated controls remained in their home cages and were handled daily (HC).

Measurement of protein in nucleus accumbens tissue homogenates

Immediately after or 24 h after the last test session, animals for protein analysis were euthanized by microwave fixation (5 kW, 1.5s, Murimachi Kikai Co., LTD. Tokyo, Japan). Brains were rapidly dissected and tissue samples were obtained for NAc core and shell and CPu using 12 and 14 gauge punches from chilled coronal brain slices (1.5mm thick). Samples were immediately homogenized by sonication in lysis buffer (320mM sucrose, 5nM Hepes, 50mM NaF, 1 mM EGTA, 1mM EDTA, 1% SDS, with protease inhibitor cocktail and phosphatase inhibitor cocktails I and II diluted 1:100; Sigma, St. Louis, MO), boiled for 5 m, and stored at -

80 degrees C until protein concentration was determined by the Lowry method.

20 ug of each sample was subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide in Tris/Glycine/SDS buffer (BioRad, Hercules, CA), followed by transfer to polyvinylidene membranes (PVDF; Amersham, Piscataway, NJ) by electrophoretic transfer at 4 degrees C. Membranes were blocked overnight in 5% nonfat milk in Tris-buffered saline in 1% Tween (TTBS) at 4 degrees C and incubated in primary antibody for MOR and DOR (1:500 and 1:2000; Chemicon, Temecula, CA) and pMOR (1:2000; Cell Signaling, Danvers, MA) for 24 h at 4 degrees C. Membranes were washed with TTBS 3 times for 15 m each and labeled with species-specific peroxidase-conjugated secondary (BioRad) for 1 h at 25 degrees C. Membranes were washed 3 times for 15 m each with TTBS and detected by chemiluminesence detection (SuperSignal West Dura; Pierce, Rockford, Il). Following detection, membranes were stripped and reprobed for β-tubulin (1:25,000; Upstate, Charlottesville, VA) for an internal standard of protein loading and membrane transfer. Immunoreactivity was quantified by densitometry (Scion Image, NIH, Bethesda, MD) under conditions linear over at least a 3-fold concentration.

Anti-β-endorphin/ IgG treatment and measurement of protein in nucleus accumbens homogenates

Animals were trained and implanted with a guide cannula directed above the NAc core as described above and a catheter for self-administration as described above (CSA and SSA groups only). Prior to the last self-administration session, animals were treated with an antibody to β-endorphin (α-β-end) in NAc core of one hemisphere and contralateral NAc core IgG. Animals were allowed to self-administer cocaine or saline and euthanized by rapid decapitation immediately after the self-administration session. Brains were rapidly dissected and tissue was collected as described above. 20 ug samples were subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide and electrophoretically transferred. Detection of MOR (1:2000; ABCAM, Cambridge, MA) and pMOR (1:2000; Cell Signaling) was done for 24h at 4 degrees C. Membranes were washed and labeled with species-specific peroxidase-conjugated secondary (BioRad). Following chemiluminesence detection (ECL Plus; Amersham), membranes were stripped and reprobed for β-tubulin as an internal standard of protein changes.

Quantitative RT-PCR for MOR

Animals for mRNA analysis were euthanized by rapid decapitation, brains removed and tissue treated as described previously (Graham et al. 2007). Briefly, tissue samples were subjected to homogenization in RNA-STAT60 (IsoTex Diagnostics; Friendswood, Texas) and RNA was extracted according to the manufacturer's instructions. DNAase-treated (Ambion; Austin, TX) RNA samples were converted to cDNA using Superscript III (Invitrogen; Carlsbad, CA). Cycle thresholds (cT) were calculated from triplicate reactions using the second derivative of the reaction. MOR cT values were normalized by subtracting GAPDH cT values, which showed no regulation by cocaine. Fold changes were calculated by subtracting the mean normalized control cT values from cT values for individual samples. Primer sequences for MOR and GAPDH were 5'-CAACTTGTCCCACGTTGATG-3' and 3'-AAGGTACCAGTGTCGGTAAT-5', so that the end product mapped to the N-terminal transmembrane 1 spanning region of MOR and yielded a product that was 119 nt in length. The primers used for the internal standard, GAPDH (glyceraldehydes-3-phosphate dehydrogenase), were 5'-AACGACCCCTTCATTGAC-3' and 3'-CCACGACTCATACAGCACCT-5'

and yielded a product that was 136 nt in length. 5ul of cDNA was used in triplicate for each gene analyzed.

Immunohistochemistry

Animals were trained and implanted with a guide cannula aimed above NAc core site for virus and antibody injection and catheter for self-administration as described above. Prior to the last self-administration session, animals were injected with an HSV vector for expression of HA-tagged MOR and placed back in their homecages for two days to allow viral expression to reach peak levels. 3 d after viral injection (last day of self-administration), animals were pretreated with α-β-end and IgG in contralateral NAc core infusions and allowed to self-administer cocaine or saline. Immediately after the session, animals were anesthetized with chloral hydrate and transcardially perfused with ice-cold 1X PBS (BioRad) for 5 m followed by 4% paraformaldehyde for 15 m. Brains were removed and placed in 4% paraformaldehyde overnight at 4 degrees C. Brains were then cryoprotected by equilibration in 20% sucrose in PBS at 4 degrees C followed by 30% sucrose in PBS at 4 degrees C.

For verification of sensitivity to MOR-mediated receptor internalization, another set of animals were implanted with cannulae aimed at the NAc core and allowed 7 d to recover. Following recovery, animals were bilaterally injected with HSV expressing HA-tagged MOR and allowed for viral expression to reach peak levels by d 3. Animals were given subcutaneous vehicle or 0.05 mg/kg fentanyl (potent MOR agonist) (Zhang et al. 2000). Animals were anesthetized with an overdose of chloral hydrate 20 minutes later and transcardially perfused as described above.

Brains were sectioned and detected as described previously (Haberstock-Debic et al. 2003). Briefly, 45um thick slices were cut using a cryostat and washed in PBS. Slices were treated with 1% peroxidase/ PBS for 30 m and blocked with 3% Normal Donkey Serum (NDS; Jackson Labs, Bar Harbor, Maine) in PBS containing 0.3% Triton X-100 (Sigma, St. Louis, MO) for 1 h at 25 degrees C. Free-floating sections were incubated for 36 h with rabbit primary anti-HA.11 (1:250; Covance, Princeton, NJ) at 4 degrees C. Sections were washed and labeled with Cy3-Donkey-anti-rabbit IgG (1:200; Jackson Labs). Sections were mounted on slides after washing with PBS and fixed with Dent's fixative and coverslipped.

HA-tagged MOR labeled cell bodies and processes of self-administering animals were counted across 10-16 sections, counting 15-30 cell bodies and 25-60 cell processes, keeping left and right hemispheric NAc separate since each animal received IgG or anti-β-endo in contralateral NAc core. For fentanyl/saline treated animals, 8-10 sections were analyzed and left and right hemisphere counts were pooled. Highly punctate expression of HA-tagged MOR was considered endocytosed and counts were expressed as percent endocytosed (punctate) compared to total counts for cell bodies and processes. Experimenter was blind to group assignment and treatment.

Confirmation of MOR antibody specificity

HEK 293 cells were grown to 75% confluence and lipofectamine 2000 (Invitrogen) was used to express a plasmid containing MOR clone (generous gift from Lisa Monteggia, PhD; UT Southwestern Medical Center, Dallas, Texas). Control was lipofectamine treatment alone and no treatment. 24 h after transfection, cells were collected and subjected to SDS-PAGE on 10% polyacrylamide gels as described above. PVDF

membranes were labeled for MOR (1:500; Chemicon) and detected using chemiluminesence (SuperWest Dura; Pierce).

Data Analysis

Densitized immunoblots were expressed as a percentage of the mean for untreated control values to normalize data across blots. Protein levels were corrected for loading and transfer differences (β-tubulin) and the ratio of pMOR/ MOR were done. Data were analyzed by simple main effects ANOVA on protein. Post-Hoc tests utilized Fischer's least significant differences (LSD) for pair-wise comparison. Student's t-test comparisons were used between controls to determine whether or not to pool control groups. Immunohistochemistry data were analyzed by 2-factor ANOVA of treatment X cell region and significant interactions were followed up using simple main effects ANOVA on cell region. Post-Hoc tests utilized Fischer's LSD for pair-wise comparison and student's t-test comparisons were used between controls.

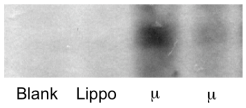


Figure 3.1 Expression of the rat clone of MOR in HEK 293 cells shows specific labeling of MOR using Western blot analysis.

Results

Confirmation of MOR Western blot

Figure 3.1 shows Western blot results for HEK 293 cells expressing rat MOR clone. MOR was at apparent molecular weight 65-80 kDa, as previously described (Christoffers et al. 2003; Garzon et al. 1995).

Regulation of MOR but not DOR by cocaine self-administration

Figure 3.2a shows the behavioral and euthanasia schedule for groups generated in this part of the experiment. Figure 3.2b shows the self-administration pattern for cocaine (CSA) and saline (SSA) self-administering animals. There was no difference between SSA and HC MOR levels, therefore these data were pooled into a common control for comparison to experimental groups. Immediately following the last session, MOR levels were significantly regulated (Figure 3.2c; $F_{3,76} = 4.41$, $p = 0.007$). NAc core MOR levels were significantly down-regulated in both CSA and CY (chronic yoke) animals (Fischer's LSD: $p = 0.004$ and $p = 0.006$, respectively), but not in AY (acute yoke) compared to controls. NAc core MOR levels were normalized 24 h later (Figure 3.2d; $F_{2,46} = 0.11$, p = NS). DOR was only acutely down-regulated in NAc core (Figure 3.2e; $F_{3,64} = 3.156$, $p = 0.031$; Fischer's LSD: $p = 0.003$) and not regulated 24 h

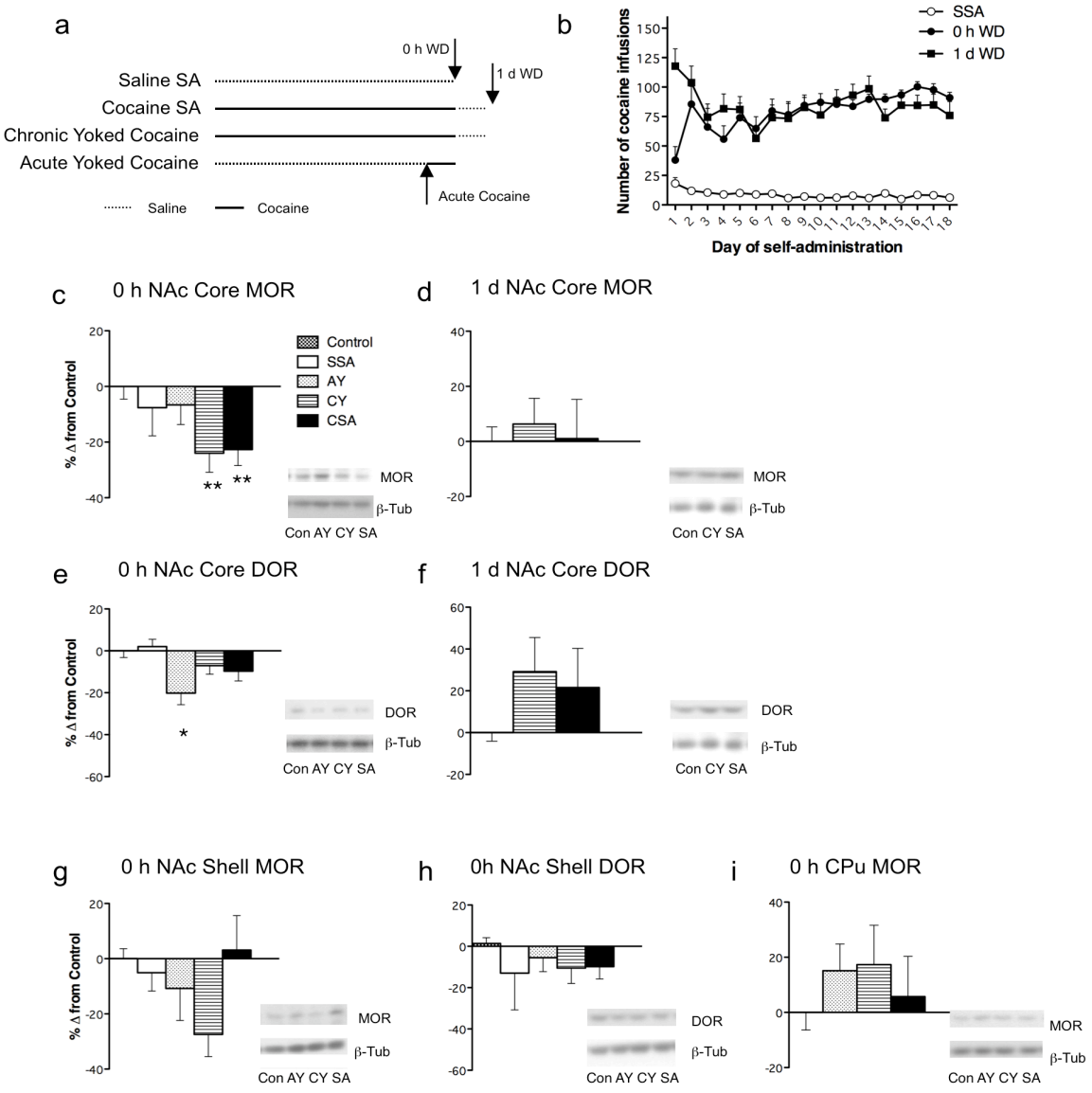


Figure 3.2 (a) Self-administration schedule for 0 h and 1 d withdrawal groups. (b) Number of cocaine or saline infusions animals self-administered during the 3 week trial. (c) Animals that self-administered cocaine and their chronic yoked partners had significantly less MOR in the Nac core compared to controls. (d) Levels of MOR were not different between groups by 1 d withdrawal. (e) Acute exposure to cocaine decreased DOR levels in the core with no regulation at 1 d withdrawal (f). (g-i) MOR levels were not regulated in the shell or CPu and DOR was not regulated in the shell at 0 h withdrawal. Symbols indicate differs compared to control (* $p < 0.05$, ** $p < 0.01$) by Fischer's LSD *post hoc* tests.

later (Figure 3.2f; $F_{2,41} = 1.45$, p = NS). MOR and DOR were not regulated in NAc shell (Figure 3.2g; $F_{3,67} = 2.175$, p = NS, Figure 3.2h; $F_{3,71} = 0.924$, p = NS) and MOR was not regulated in the CPu (Figure 3.2i; $F_{3,39} = 0.565$, p = NS) at 0 h withdrawal. These findings indicate MOR protein levels are decreased by both contingent and non-contingent chronic cocaine specifically in NAc core. The acute DOR effect agrees with previous literature that shows stimulation of DOR results in preferential sorting to lysosomes for degradation (Turchan et al. 1999; Whistler et al. 2002). Additionally, [(3)]- H-Tyr-Tic psi [CH2-NH]Phe-Phe-OH (highly selective DOR antagonist) binding of DOR decreases in response to acute, and not chronic binge, cocaine treatment (Turchan et al. 1999).

Regulation of MOR mRNA by cocaine administration

Given that only MOR was regulated by chronic cocaine administration, quantitative RT-PCR was done for MOR mRNA in the NAc to determine a regulatory mechanism behind this regulation. Figure 3.3a shows the self-administration schedule for animals used in this experiment. Cocaine intake for the last week was not different from animals in Figure 3.2. An 0.8% agarose gel was run of RT-PCR products for MOR and GAPDH (Figure 3.3b). Single products were seen and no

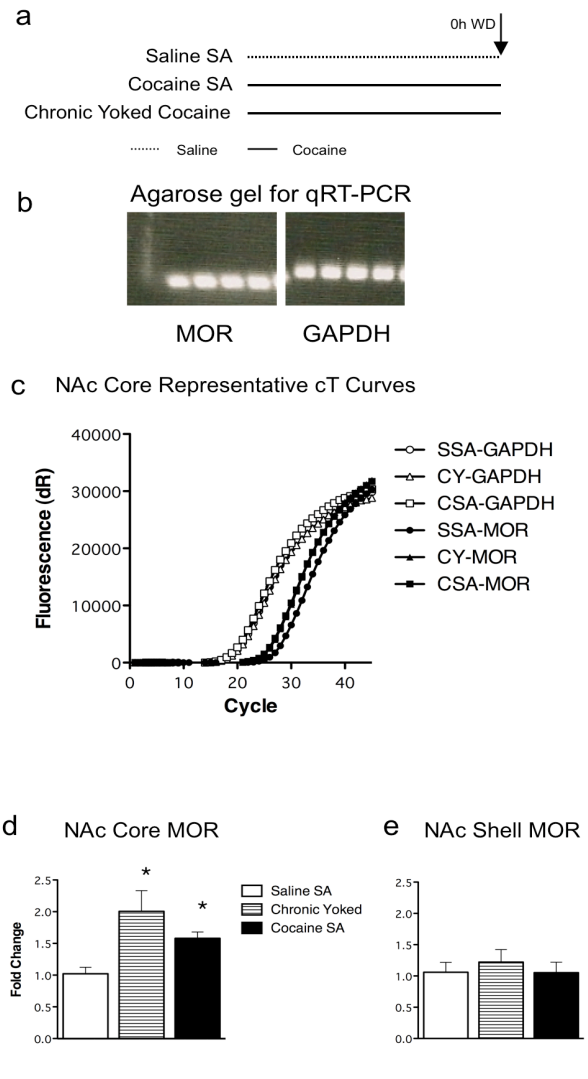


Figure 3.3 (a) Self-administration schedule for cDNA bank animals. (b) Representative gel of MOR and GAPDH PCR products to check for primer dimers and single product production. (c) Representative cT curves of GAPDH and MOR for SSA, CY, and CSA groups. (d) Levels of MOR were increased in CY and CSA animals compared to control in the NAc core. (e) MOR mRNA was not regulated in the NAc shell. Symbols indicate (* $p < 0.05$) differs from control by Fischer's LSD *post hoc* tests.

primer dimers were present. Representative cT and dissociation curves are shown in Figure 3.3c for SSA, CY, and CSA animals. In contrast to protein levels, Figure 3.3d shows that MOR mRNA was significantly increased in CSA and CY animals compared to controls ($F_{2,11} = 7.606$, $p = 0.008$). There was no regulation of MOR mRNA in the shell (Figure 3.3e; $F_{2,11} = 3.253$, p = NS).

Mechanism behind MOR down-regulation during cocaine administration

Previous studies in vitro show that chronic stimulation of MOR by specific agonists leads to a GRK-dependent phosphorylation and results in a shift in its recycling pathway to a degradation pathway, resulting in an overall decrease in MOR levels (Chaturvedi et al. 2001; Macey et al. 2006). This experiment determined whether MOR was being phosphorylated during cocaine administration by quantifying pMOR levels relative to total MOR, and if blockade of endogenous β-endorphin activation of MOR would prevent cocaine induced down-regulation of MOR. Figure 3.4a shows pMOR levels immediately after the last self-administration session normalized to untreated controls. Total pMOR levels were not significantly regulated with chronic cocaine administration ($F_{3,62} = 2.051$, p = NS); however, given the down-regulation of total MOR

protein by chronic cocaine self-administration, the ratio of pMOR/MOR was analyzed (Figure 3.4b). Relative pMOR levels were significantly increased by cocaine ($F_{3,56} = 3.024$, $p = 0.04$). As predicted, relative pMOR levels were increased in AY animals (Fischer's LSD *post hoc* test; $p = 0.05$), suggesting that endogenous opioids are released by cocaine administration. Accordingly, relative pMOR levels were significantly increased in CSA animals (Fischer's LSD *post hoc* test; $p = 0.01$). Interestingly, CY animals did not have a significant increase in relative pMOR levels, indicating a different mechanism of MOR regulation by non-contingent cocaine.

To further determine β-endorphin as the ligand chronically stimulating MOR, resulting in its down-regulation, another set of animals were given NAc pretreatments with an antibody to β-endorphin prior to the last self-administration session to determine if β-endorphin is involved in MOR binding and subsequent down-regulation. Figure 3.4c shows the self-administration schedule and treatment. Figure 3.4d shows the self-administration data for this group of animals. This experiment replicated above findings that chronic cocaine down-regulated NAc core MOR (Figure 3.4e; Coc-IgG) and that pretreatment with an antibody to β-endorphin prevented MOR down-regulation ($F_{3,48} = 2.868$, $p = 0.046$). In

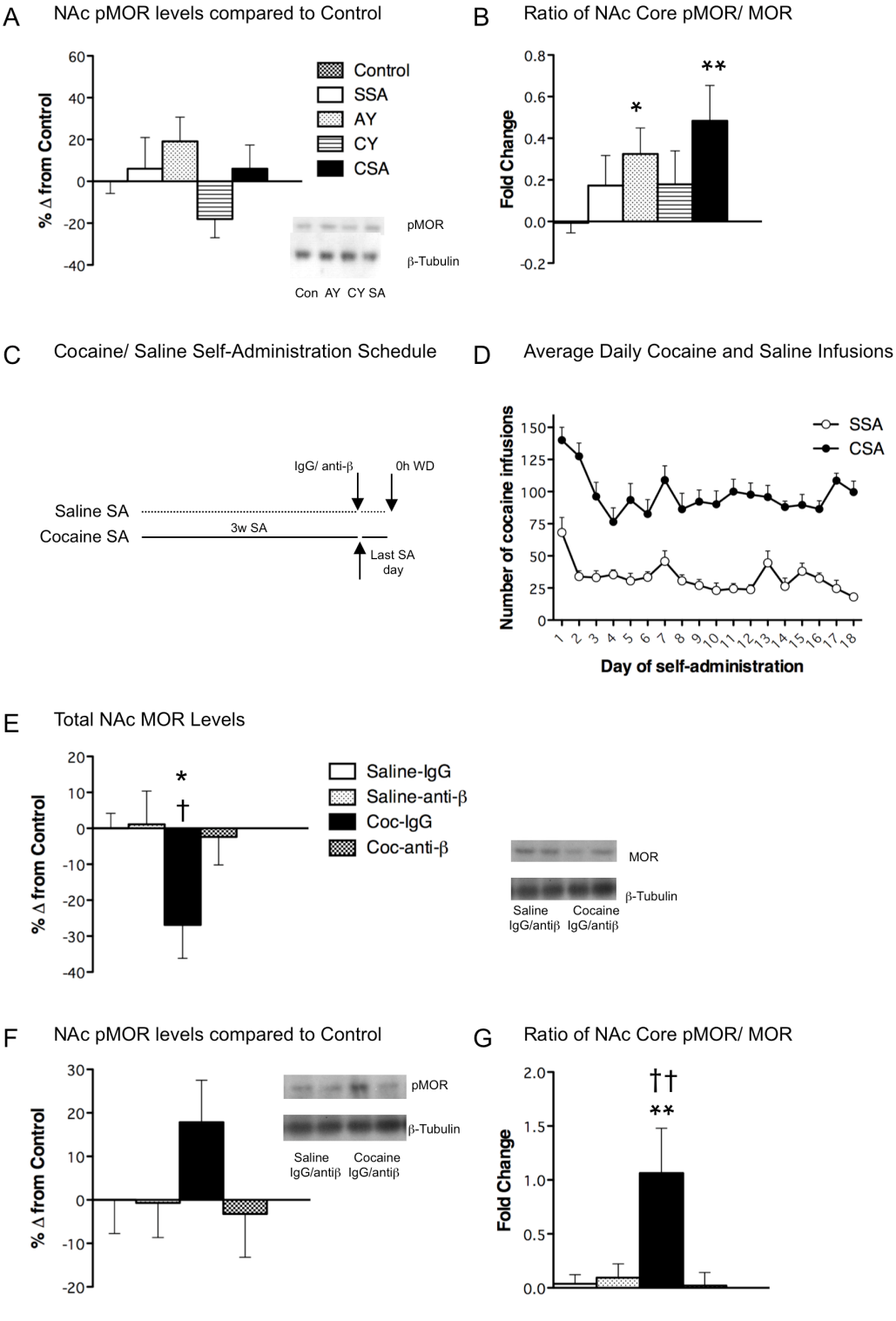

A
NAc pMOR levels compared to Control
% Δ from Control
Control
SSA
AY
CY
CSA
pMOR
β-Tubulin
Con AY CY SA
B
Ratio of NAc Core pMOR/ MOR
Fold Change
C
Cocaine/ Saline Self-Administration Schedule
IgG/ anti-β
0h WD
Saline SA
Cocaine SA
3w SA
Last SA day
D
Average Daily Cocaine and Saline Infusions
Number of cocaine infusions
SSA
CSA
Day of self-administration
E
Total NAc MOR Levels
% Δ from Control
Saline-IgG
Saline-anti-β
Coc-IgG
Coc-anti-β
MOR
β-Tubulin
Saline IgG/antiβ
Cocaine IgG/antiβ
F
NAc pMOR levels compared to Control
% Δ from Control
pMOR
β-Tubulin
Saline IgG/antiβ
Cocaine IgG/antiβ
G
Ratio of NAc Core pMOR/ MOR
Fold Change

Figure 3.4 (a) Acute or chronic cocaine did not increase pMOR when normalized to β-tubulin alone. However, considering the total decrease in MOR by chronic cocaine, the ratio of pMOR to MOR was taken (b) revealing that relative pMOR was increased by acute cocaine and cocaine self-administration, but not chronic yoked cocaine (c) Animals were trained to self administer cocaine or saline for 3 w. (d) Self-administration of cocaine was not different from previous animals, although saline self-administration activity was slightly higher than previous animals. (e) Following antibody treatment, there was not effect of treatment on MOR in SSA animals. IgG control treated CSA animals had significantly less MOR than control animals. Treatment with an antibody against β-endorphin abolished cocaine induced down-regulation of MOR. (f) pMOR was slightly increased in IgG CSA animals however anti-β-endorphin treatment blocked any phosphorylation. (g) When relative pMOR levels were considered, there was a significant increase in pMOR in IgG CSA animals and a blockade of this increase by anti-β-endorphin treatment. Symbols indicate differs from control (* $p < 0.05$, ** $p < 0.01$) and († $p < 0.05$, †† $p < 0.01$) differs from other groups by Fischer's LSD *post hoc* tests.

saline SA animals, there was no effect of α-β-end or IgG treatment on MOR levels. In NAc core of Coc-IgG animals, MOR was significantly different from all treatments (Fischer's LSD *post hoc* test; Sal-IgG: p = 0.022, Sal-α-β-end: p = 0.014, Coc-α-β-end: p = 0.033). pMOR was not altered in saline SA animals with either treatment and a trend for an increase in pMOR was seen in Coc-IgG relative to pooled saline treatments (Figure 3.4f; $F_{2,62}$ = 2.528, p = 0.088). The increased relative pMOR seen in the previous experiment (Figure 3.4b) was replicated as seen Figure 3.4g ($F_{3,46}$ = 4.75, p = 0.006). Coc-IgG pMOR was significantly increased compared to Sal-IgG and to Sal-α-β-end (Fischer's LSD *post hoc* test; p = 0.003 and p = 0.007, respectively). The increase in relative pMOR was a blocked by α-β-end pretreatment (Fischer's LSD *post hoc* test; p = 0.003).

Visualization of MOR endocytosis by fentanyl

MOR phosphorylation can lead to receptor endocytosis, and experiments were designed to test this hypothesis using HA-tagged MOR over-expression and methods validated previously (Haberstock-Debic et al. 2003). As a proof of principal, cannulated naïve animals given HSV-HA-MOR infusions were challenged with a 0.05 mg/kg sub-cutaneous (sc)

injection of the highly potent MOR agonist fentanyl. Following quantification of cell bodies and dendritic processes of saline and fentanyl treated animals (Figure 3.5a and 3.5b), there was no main effect of treatment ($F_{1,7} = 2.034$, $p = 0.197$) but there was a trend for a main effect of counts in cell bodies and dendrites ($F_{1,1} = 4.895$, $p = 0.063$) and an interaction between treatment and counts in cell bodies and dendrites ($F_{1,7} = 10.58$, $p = 0.014$). There was a trend for an increase in fentanyl induced endocytosis of HA-tagged MOR in cell bodies, however this was not significant ($F_{1,7} = 4.979$, $p = 0.061$) and there was no effect in dendritic processes ($F_{1,7} = 0.436$, $p = 0.53$). Detection of internalized MOR by fentanyl may have required a longer incubation time or a higher dose of fentanyl. Since the overall goal of determining the facility of detecting endocytosed MOR was accomplished (Figure 3.5a), the subsequent experiment was carried out.

Visualization of β-endorphin-dependent cocaine-induced MOR endocytosis

Previous studies showed that β-endorphin is released during cocaine administration (Roth-Deri et al. 2004) and that blockade of β-endorphin activity decreased the reinforcing properties of cocaine (Roth-

Deri et al. 2003). It was hypothesized that chronic stimulation of MOR by β-endorphin during cocaine self-administration results in endocytosis of MOR and alternative sorting of MOR for degradation during cocaine self-administration. Figure 3.5c shows the HSV-amplicon vector used to express HA-tagged MOR while Figure 3.5d shows the behavioral and treatment schedule for animals in this experiment. Figure 3.5e shows the self-administration data for saline and cocaine trained animals. Figure 3.5f shows representative confocal images of cell bodies cocaine self-administering animals that were treated with IgG in one hemisphere NAc and α-β-endorphin in the contralateral NAc core. There was a highly significant main effect of treatment (Figure 3.5g; $F_{3,16} = 7.139$, $p = 0.003$). There was a significant effect of treatment in endocytosed cell bodies ($F_{3,16} = 6.896$, $p = 0.003$). A significant increase in endocytosed HA-tagged MOR was observed in cell bodies of cocaine self-administering animals compared to saline/IgG controls (Fischer's LSD *post hoc* test; $p = 0.015$) and saline/α-β-end (Fischer's LSD *post hoc* test; $p = 0.001$), an effect that was abolished by α-β-end treatment (Fischer's LSD *post hoc* test; $p = 0.005$). There was also a trend for α-β-end treatment to decrease basal endocytosis of MOR in saline animals (Fischer's LSD *post hoc* test; $p =$ NS), suggesting basal β-endorphin tone that promotes internalization.

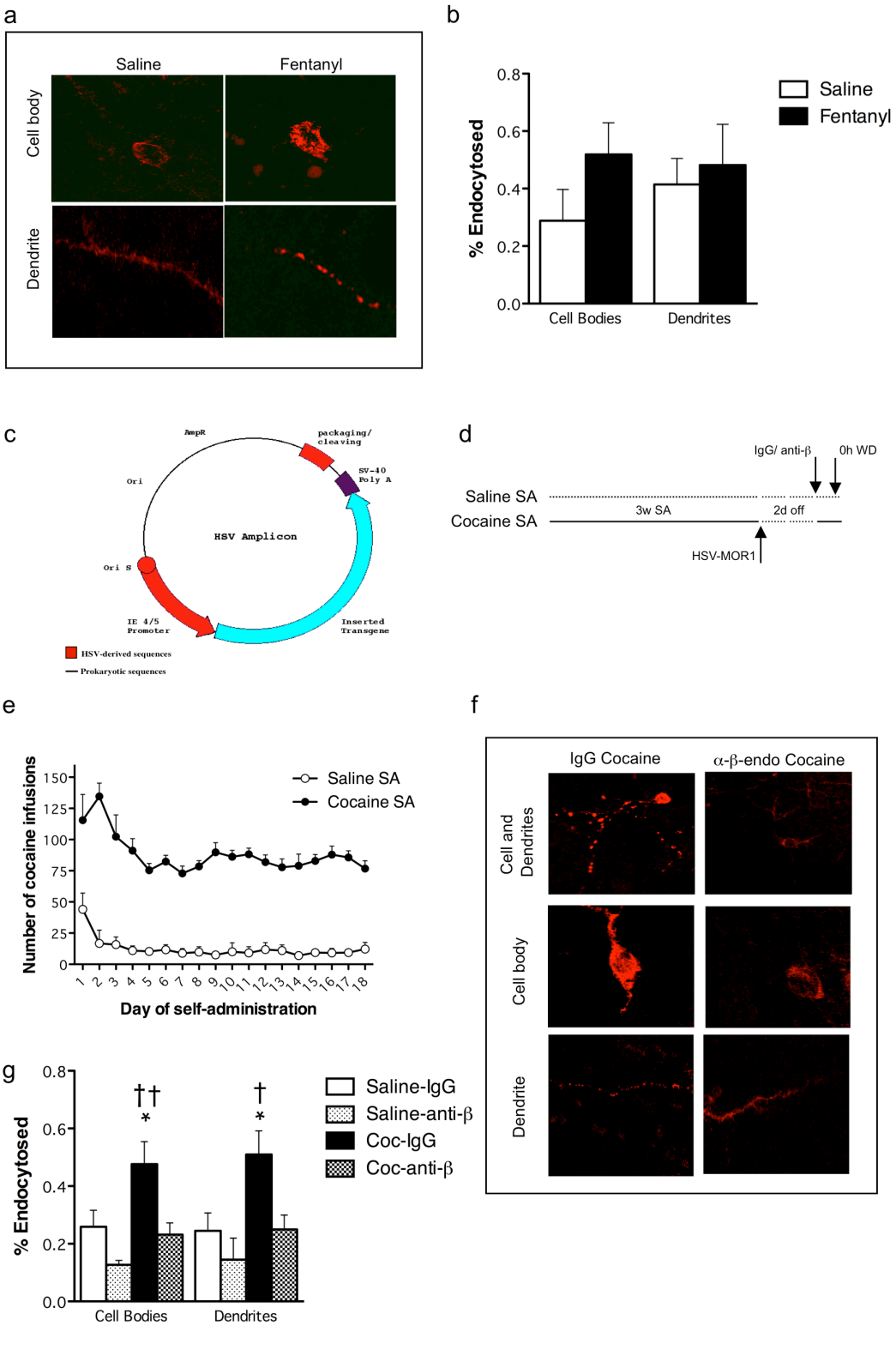

a
Saline
Fentanyl
Cell body
Dendrite
b
% Endocytosed
0.8
0.6
0.4
0.2
0.0
Saline
Fentanyl
Cell Bodies
Dendrites
c
AmpR
packaging/
cleaving
Ori
SV-40
Poly A
HSV Amplicon
Ori S
IE 4/5
Promoter
Inserted
Transgene
HSV-derived sequences
Prokaryotic sequences
d
IgG/ anti-β
0h WD
Saline SA
Cocaine SA
3w SA
2d off
HSV-MOR1
e
Number of cocaine infusions
150
125
100
75
50
25
0
Saline SA
Cocaine SA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Day of self-administration
f
IgG Cocaine
α-β-endo Cocaine
Cell and
Dendrites
Cell body
Dendrite
g
% Endocytosed
0.8
0.6
0.4
0.2
0.0
††
†
*
*
Saline-IgG
Saline-anti-β
Coc-IgG
Coc-anti-β
Cell Bodies
Dendrites

Figure 3.5 (a) Representative confocal images of cell bodies and dendrites of animals treated with vehicle or fentanyl and stained for HA-tagged MOR show punctate expression as a result of fentanyl treatment, indicating endocytosis. (b) Quantification of percent of cell bodies and dendrites exhibiting endocytosed MOR were not significantly different between groups. However, the number of animals per group were low and this study demonstrated the technique was useful in the detection of endocytosed HA-tagged MOR. (c) Cartoon of virus vector used for the expression of HA-tagged MOR that was validated previously. (d) The self-administration schedule for SSA and CSA was the same as that for previous animals with the exception of a "break" for the infusion of virus and time given for expression to reach peak levels prior to the last self-administration day. (e) Levels of cocaine and saline self-administration was not different from previous animals. (f) Representative confocal images reveal punctate expression in the cell body of CSA animals pretreated with IgG and more diffuse expression of HA-tagged MOR when pretreated with an anti-body to β-endorphin. (g) Quantification of percent of cell bodies and dendrites endocytosed following each treatment reveal that cocaine causes increased endocytosis of HA-tagged MOR that is prevented by blocking β-endorphin activity in the NAc core. Symbols indicate differs from control (* $p < 0.05$) and († $p < 0.05$, †† $p < 0.01$) differs from other groups by Fischer's LSD *post hoc* tests.

There was also a significant effect of treatment on endocytosed MOR dendritic processes ($F_{3,17} = 5.236$, $p = 0.01$). Again, there was no effect of α-β-end treatment on saline animals, while cocaine self-administration increased HA-tagged MOR endocytosis compared to saline/IgG (Fischer's LSD *post hoc* test; $p = 0.013$) and saline/α-β-end (Fischer's LSD *post hoc* test; $p = 0.002$). This effect was reversed by pretreatment with α-β-end, thus blocking β-endorphin stimulation of MOR endocytosis (Fischer's LSD *post hoc* test; $p = 0.011$).

Discussion

This study found that chronic cocaine administration, both contingent (CSA) and non-contingent (CY), decreased MOR levels specifically in NAc core. Relative phosphorylation of MOR was increased in acute yoked (AY) and cocaine self-administering (CSA) animals but not chronic yoked (CY) animals, indicating activation of MOR by acute cocaine exposure and by cocaine self-administration, but that during chronic involuntary cocaine exposure a different mechanism alters MOR. The down-regulation of MOR was β-endorphin dependent since blockade of β-endorphin activity in the NAc core prevented MOR phosphorylation and down-regulation by cocaine self-administration. Further, blockade of β-

endorphin activity prevented increased MOR endocytosis during cocaine self-administration. Acute exposure to cocaine also decreased DOR expression in NAc core, but this effect was not seen following chronic cocaine administration. This finding is not surprising since DOR stimulation results in its endocytosis and preferential sorting to the lysosome for degradation.

Opioid receptors and their ligands are centrally located to modulate cocaine's effects in the NAc and potentially elicit the reinforcing effects of cocaine itself. Cocaine's inhibition of monoamine transporters, especially the dopamine transporter (DAT), results in an increase in dopamine concentrations at the synapse where dopamine can interact with D1-like and D2-like receptors. NAc MOR are coexpressed with D2-R where they can synergize with D2R activity. Interestingly, chronic cocaine administration potentiates D2R stimulated cocaine seeking following extinction of drug-paired lever pressing (Edwards et al. 2007), a time when β-endorphin release was shown to also occur (Roth-Deri et al. 2003), indicating possible cross-talk between MOR and D2R. DOR, on the other hand, are expressed largely on axon terminals of the NAc, either in colocalization with DAT or on terminals apposed to DAT containing terminals, thereby allowing direct modulation of dopamine release and

control presynaptic secretion of other neurotransmitters whose release may influence or be influenced by extracellular dopamine, as well as on spines of GABAergic projection neurons where they can modulate NAc-mediated activity (Svingos et al. 1999a). However, DOR was not regulated past acute cocaine exposure indicating less of a role in increasing relapse behaviors following chronic cocaine.

Previous studies found variable results on MOR regulation by cocaine. Acute binge cocaine up-regulates MOR mRNA (Yuferov et al. 1999), while continuous osmotic minipump delivery of cocaine transiently increases NAc MOR mRNA starting at day 2, peaking at day 3, and normalized by day 4 (Azaryan et al. 1996a; Azaryan et al. 1998). However, chronic binge cocaine for two days did not change MOR mRNA in another study (Rosin et al. 2000). [3H]DAMGO binding to MOR in NAc is increased on day 4 but not changed at 7 or 14 days of continuous cocaine. Similarly, another study found no change in NAc MOR following 14 days of an escalating binge cocaine paradigm (Bailey et al. 2005a), despite earlier studies that found chronic binge cocaine increased [3H]DAMGO binding (Unterwald et al. 1992; Unterwald et al. 1994), indicating differential regulation depending on the paradigm used. Conversely, human cocaine abusers who died with cocaine or its

metabolites present at death had decreased MOR binding in the striatum (Hurd and Herkenham 1993). Similarly this project found MOR levels decreased immediately following both cocaine self-administration and yoked cocaine administration, a regulation that possibly is mediated through different mechanisms. Overall, the findings of this study using cocaine self-administration better follows the human report where addicts that had cocaine present at death had decreased MOR binding in the striatum.

That the down-regulation of MOR that was not paired with increased relative pMOR levels in CY animals could reflect the paradigm of cocaine delivery. Cocaine self-administration models voluntary cocaine use as seen in humans while chronic yoked administration determines pharmacological effects produced by drug administration itself. MOR down-regulation occurs in both CSA and CY groups that would indicate this effect is pharmacological. However, when relative pMOR levels were analyzed, phosphorylated MOR was increased in only self-administering and acute yoked animals and not chronic yoked animals. The difference in pMOR activation may reflect different mechanisms of MOR modulation with involuntary cocaine administration that is considered stressful. Chronic stress alters the hypothalamic-pituitary-adrenal axis (HPA) [for

review see (Koob and Kreek 2007)). In humans, psychological trauma decreases MOR binding in the NAc (Liberzon et al. 2007) and in rodents, limbic (olfactory tubercle, NAc, and septum) MOR is down-regulated by the highly stressful platform method of sleep-deprivation (Fadda et al. 1991). Similarly, MOR binding is decreased in NAc and striatum by prenatal stress (Insel et al. 1990; Sanchez et al. 1996), while MOR mRNA is up-regulated by stress induced by morphine withdrawal (Zhou et al. 2006). Further studies are required to determine the mechanism behind this differential regulation of MOR by chronic yoked cocaine administration.

Cocaine administration increases β-endorphin release in NAc, thus increasing relative pMOR in acute yoked and cocaine self-administering animals, an effect that is potentially lost in CY animals through an unknown compensatory mechanism. Studies indicate chronic stimulation of MOR by agonists results in desensitization and in some cases a shift in the regulatory pathway, from recycling to degradation (Celver et al. 2004; Chaturvedi et al. 2001). The S375 site of MOR is ligand binding dependent and is involved in MOR endocytosis. Truncation or mutation of this site prevents MOR endocytosis (El Kouhen et al. 2001). This project showed that phosphorylation of MOR by cocaine self-administration is

through β-endorphin since antibody blockade of β-endorphin prevents MOR phosphorylation. Accordingly, MOR down-regulation was prevented by pretreatment with the antibody, further indicating chronic stimulation of MOR by β-endorphin during cocaine self-administration results in a shift in the normal recycling pattern of MOR, leading to desensitization and down-regulation. This is the first study that showed chronic cocaine increases MOR endocytosis in vivo and the role β-endorphin plays in MOR phosphorylation, endocytosis, and degradation during cocaine self-administration.

Previously it was found that [3H]DAMGO binding increased with chronic binge cocaine in the NAc (Unterwald et al. 1992) and that DAMGO stimulated [35S]GTPγS binding increased with chronic binge cocaine (Schroeder et al. 2003), indicating increased MOR levels in the NAc. The difference between data presented here and the previous studies may be explained by altered affinities of MOR for its specific ligand or differential coupling of MOR to its downstream signaling cascade. One study in support of this hypothesis analyzed monkey brain [3H]DAMGO binding and [35S]GTPγS activity. This study found lower [3H]DAMGO binding sites in the central nucleus of the amygdala that was associated with higher DAMGO stimulated [35S]GTPγS binding relative to other amygdala

nuclei. Whereas in the medial nucleus of the amygdala, the reverse was true (Daunais et al. 2001), indicating differential coupling to an effector system that is not dependent on MOR expression levels.

Another difference between our findings and previous reports is in the use of different techniques to measure MOR levels. Western blot analysis does not differentiate membrane expressed MOR versus internal stores. A shift in the recycling pattern of MOR to a degradation pathway during chronic stimulation would lead to an overall decrease in MOR levels despite increases in MOR mRNA. Potentially, the regulation of MOR may be streamlined so that higher levels are expressed on the membrane whereas internal stores are decreased. This is supported by the increased mRNA expression, possibly indicating that as MOR are being degraded, synthesis of MOR is increased with a rapid shuttling of the receptors to the membrane, which leads to a decrease in internal MOR stores.

In conclusion, this study showed that NAc MOR are differentially regulated by cocaine, depending on contingency. Cocaine self-administration decreased MOR levels in the NAc core through β-endorphin activity at MOR since antibody pretreatment against β-endorphin prevented phosphorylation of MOR and normalized MOR concentrations

to control levels. Cocaine self-administration increased MOR endocytosis through β-endorphin since antibody blockade of β-endorphin prevented MOR endocytosis. Chronic yoked cocaine administration decreased MOR levels but not through β-endorphin mediated phosphorylation, since relative pMOR levels were not increased following cocaine administration. The down-regulation of MOR during chronic yoked cocaine without increased relative pMOR levels indicates an alternative adaptation that may be associated with stress that is β-endorphin independent. Both cocaine paradigms revealed normalized MOR by 24 h after the last cocaine administration session, potentially through up-regulation of mRNA driven MOR expression. Acute cocaine exposure results in MOR phosphorylation but not regulation of total MOR. The decreased MOR by chronic cocaine self-administration may participate in dysphoria that is associated with cocaine cessation and indicate a neuroadaptation that contributes to cocaine addiction.

CHAPTER 4:

MOR AND DOR MODULATION OF COCAINE-SEEKING BEHAVIOR

Simmons, D; Self, DW
(accepted in Neuropsychopharmacology, 2009)

Introduction

Drug addiction involves dysregulation in brain reward circuitry leading to compulsive drug use (Dackis and O'Brien 2001; Kalivas and Volkow 2005; Koob and Le Moal 2001). In addition to drug reward, the mesolimbic dopamine system plays an integral role in relapse to drug-seeking behavior, as stimuli that elicit drug seeking also activate dopamine neurons in the ventral tegmental area (VTA) leading to dopamine release in forebrain regions, such as the nucleus accumbens (NAc) (Phillips et al. 2003; Pruessner et al. 2004a; Self and Nestler 1998a; Shalev et al. 2002a; Spealman et al. 1999a; Stewart 2000b). Opioid receptors also play a role in relapse to cocaine seeking in animal models, as systemic treatment with naltrexone inhibits cocaine seeking elicited by exposure to cocaine-associated cues (Burattini et al. 2008). Similarly, cocaine-primed cocaine seeking is blocked by systemic administration of the partial mu-opioid receptor (MOR) agonist buprenorphine, the delta-opioid receptor (DOR)

antagonist naltrindole, or the nonspecific opioid antagonist naltrexone (Comer et al. 1993; Gerrits et al. 2005).

Stewart (Stewart 1984) and colleagues found that opioid receptors in the VTA play a role in reinstatement of cocaine and heroin seeking, as intra-VTA morphine treatments trigger drug seeking in an extinction/ reinstatement paradigm, an animal model of relapse. This effect is thought to involve local disinhibition of dopamine neurons in the VTA, leading to dopamine release in the NAc (Ford et al. 2006; Johnson and North 1992b; Leone et al. 1991). In contrast, earlier studies suggest that opioid receptors localized in the NAc do not play a role in drug seeking, as intra-NAc morphine treatments fail to reinstate cocaine or heroin seeking (Stewart and Vezina 1988; Tang et al. 2005), and blockade of NAc MOR with the selective MOR antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-THr-NH_2) failed to significantly alter cocaine-primed reinstatement of cocaine seeking (Tang et al. 2005). However, endogenous opioid peptides, such as β-endorphin, are released in the NAc by cocaine, and stressful situations (Roth-Deri et al. 2004; Roth-Deri et al. 2003; Zangen and Shalev 2003), events that trigger reinstatement of cocaine seeking, and intra-NAc morphine infusions induce a conditioned place preference {van der Kooy, 1982).

Opioid receptors are highly expressed by NAc neurons (Mansour et al. 1995; Mansour et al. 1987), and local opioid infusions in the NAc modulate behavior in a biphasic manner. Thus, microgram doses of (D-Ala^2,N-Me-Phe^4,glycinol^5)-enkephalin (DAMGO) (MOR agonist) or morphine infused in the NAc initially suppress locomotion but subsequently induce hyper locomotion (Cunningham and Kelley 1992; Meyer et al. 1994). Lower doses of DAMGO decrease the latency for hyper-locomotion to occur (Meyer et al. 1994). As doses of agonists used in these locomotor studies are similar to those used in prior reinstatement studies, it is possible that the behavioral suppressive effects masked the potential of NAc opioid receptor stimulation to trigger reinstatement of drug-seeking behavior. Moreover, NAc infusions of opioid agonists induce feeding behavior but also with a prolonged latency to initiate feeding (Bakshi and Kelley 1993; Kelley et al. 2005). Similarly, intra-NAc infusions of DAMGO increase the motivation for food on a progressive ratio reinforcement schedule, and where response breakpoints are obtained after some delay (Zhang et al. 2003). Therefore, it is possible that these delayed motivational effects reflect the initial suppressive effects of high-dose MOR agonist infusions.

In this study, the role of NAc opioid receptors in reinstatement of cocaine-seeking behavior using MOR- and DOR-selective ligands and endogenous opioid peptides was investigated. We found that NAc infusions of MOR and DOR agonists effectively reinstate cocaine seeking through selective actions at their respective receptors. Stimulation of NAc opioid receptors by the endogenous peptides, β-endorphin and enkephalins, also induced cocaine-seeking behavior. The results clearly establish that either MOR or DOR stimulation in the NAc is sufficient to elicit cocaine seeking behavior, and that MOR receptors play an important role in cocaine-primed relapse. These findings also suggest that persistent neuroadaptations in NAc opioid receptors following chronic cocaine use could contribute to drug seeking behavior in prolonged abstinence.

Materials and Methods

Animals and housing conditions

Male Sprague-Dawley rats weighing 225-275g (Charles River Laboratories, Kingston, NY) were individually housed in wire cages with food and water available *ad libitum*, except during lever press training. Experiments were conducted during the light cycle of a 12:12-h light/dark

cycle (lights on at 0700 hours) in accordance with guidelines established by the National institute of Health and the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

Sucrose Lever Press Training and Surgery

Lever press training, self-administration, and reinstatement testing were performed in operant test chambers (Med-Associates, East Fairfield, VT). Chambers were equipped with two response levers and an infusion pump as described earlier (Edwards et al. 2007). Animals were food-restricted to prevent weight gain and trained to lever-press for sucrose pellets on a fixed ratio 1 (FR1) reinforcement schedule until an acquisition criteria of 100 sucrose pellets consumed for 3 consecutive tests was reached. Following lever-press training, animals were fed *ad libitum* for at least 1 day before surgery. Animals were anesthetized and implanted with a chronic indwelling catheter into the jugular vein that exited subcutaneously on the back. An intracranial, 26-guage bilateral guide cannula was aimed at the NAc (±1.5 mm lateral; 1.7mm anterior to bregma; -5.7 ventral to dura with the level skull) or caudate putamen (CPu) (±1.5 mm lateral; 1.7 mm anterior to bregma; -3.2 mm ventral to dura) (Paxinos and Watson 1998). Dummy and infusion cannulae (33

gauge) were cut to extend 1 mm beyond the guide cannulae tip, and dummy cannulae remained in place until the day of intracranial drug infusion. Animals were allowed 5-7 days to recover before starting the experiment.

Cocaine self-administration and within session-reinstatement testing

Animals were tested in a within-session extinction/reinstatement paradigm as described previously (Bachtell et al. 2005). Briefly, animals self-administered cocaine (0.5 mg/kg in 0.1 ml over 5 s, time-out period 15 s) in daily 4 h sessions for 5-6 days/week until a criteria of 3 consecutive days of <10% variance in mean cocaine intake was reached (~3 weeks). During the 5 s injection, a cue light above the lever was illuminated, whereas the house light was turned off for the entire injection and time-out period. Subsequently, animals were trained in the within-session extinction paradigm that consisted of 1 h cocaine availability followed by 3 h extinction conditions in which only response-contingent injection cues were available. Animals extinguished responding to criteria of ≤ 5 responses at either the drug-paired or inactive lever for the final hour of the session for at least three consecutive sessions, while maintaining a minimum of 15 self-administered cocaine injections with ± 10% variability

in the first hour of the session. Mean cocaine self-administration on the test day was 25 ± 0.74 (NAc) and 26 ± 0.95 (CPu) injections/h. Test days were conducted with an intra-NAc infusion of MOR and DOR agonists alone (0.5 μl/side over 2 min) or in combination as sequential antagonist/agonist infusions (1.0 μl/side total volume) immediately prior to the final hour of the test session. For cocaine priming experiments, animals received NAc or CPu antagonist infusions followed immediately by iv priming with saline (0.4 ml) or cocaine (2.0 mg/kg in 0.4 ml). Following each test day, animals returned to within-session extinction training until stable self-administration and extinction criteria were reached for at least two consecutive sessions before the next test. Animals received a maximum of eight intracranial test infusions. See Figure 4.1 for overview.

Locomotor testing

Some animals trained in the within-session reinstatement paradigm were given 1 week off from cocaine self-administration and tested for locomotor responses to agonist infusions in the NAc or CPu using peak doses for reinstatement that were selective for the drug-paired lever. The locomotor testing apparatus consisted of a circular-shaped plexiglass

arena with 12 cm wide metal floors (Med-Associates) with four pairs of photocells located at 90° intervals around the 1.95 m perimeter to record locomotor activity. Animals were habituated for 2 h in the dark followed by an intra-NAc or intra-CPu drug infusion and returned to the chambers for 2 h of subsequent testing. Testing of each drug was randomized and performed on consecutive days. Animals received five injections in locomotor tests.

Histological Confirmation of injection sites

Animals were anesthetized with chloral hydrate, and cresyl violet (0.3 μl) was infused into the NAc or CPu through the guide cannula. Animals were immediately decapitated and brains removed. Slices (0.8 mm thick) were collected throughout the forebrain and analyzed under a dissecting microscope for the location of the infusion sites according to the coordinates of Paxinos and Watson (Paxinos and Watson 1998).

Drugs

Drugs used were DAMGO, DPDPE ((D-Pen^2,D-Pen^5)-Enkephalin), CTAP, β-endorphin, met-enkephalin, and thiorphan (Bachem Bioscience Inc., King of Prussia, PA), and naloxone and naltrindole (Sigma-Aldrich,

Atlanta, GA). Ligands were dissolved in 0.9% sterile saline except thiorphan, which was dissolved in 1:4 DMSO/saline. Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Research Triangle Park) and dissolved in 0.9% sterile saline.

Statistical Analysis

As not all animals completed an experiment, data were analyzed using a two-factor mixed regression analysis (SAS 9.1.3) of treatment X lever, followed by main effects analysis of each lever separately. *Post-Hoc* tests utilized one- or two-tailed Dunnett's tests where appropriate for comparison with controls and Tukey's honestly significant difference test for pair-wise comparison where appropriate. Locomotor data were analyzed by one-way repeated measures ANOVA on treatment for each hour tested. *Post hoc* tests utilized Dunnett's one-tailed test for comparison with controls.

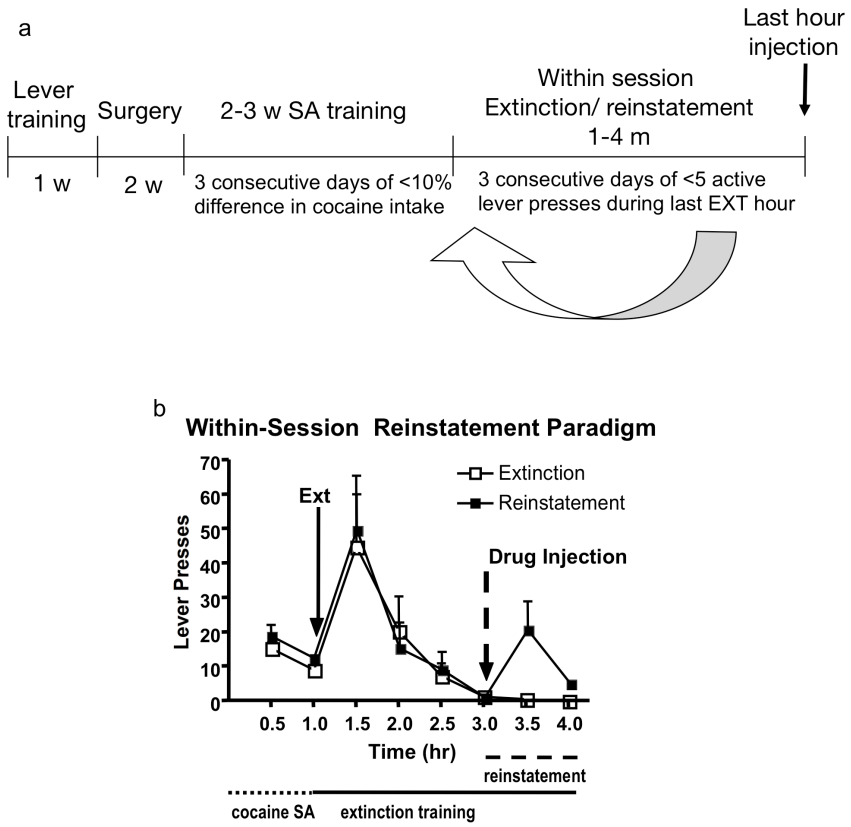


Figure 4.1 (a) Self-administration and extinction/reinstatement schedule for the within session reinstatement paradigm. Animals were lever press trained for sucrose pellets then surgerized to implant a catheter for self-administration and a guide cannula for NAc core infusions. Following recovery, animals trained to self-administer cocaine until stable and then entered the within-session extinction phase of the paradigm. (b) An example of within session extinction shows that animals self-administered cocaine for 1 hour followed by 3 hours of extinction. When criteria was reached, test day (reinstatement) consisted of 1 hour of cocaine self-administration, 2 hours of extinction, an intra-NAc core infusion of drug and 1 hour testing of the effect of drug on reinstatement of responding.

Results

MOR and DOR involvement in reinstatement

We first determined whether NAc infusions of the MOR-selective agonist, DAMGO, and the DOR-selective agonist, DPDPE, could reinstate non-reinforced drug-paired lever responding following extinction of cocaine seeking. Intra-NAc infusions of DAMGO produced an inverted-U shaped dose-response curve (Figure 4.2a) for non-reinforced responding on the drug-paired but not inactive lever (dose x lever: $F_{6,161} = 2.37$, $p = 0.032$), with a main effect of both dose ($F_{6,161} = 3.52$, $p = 0.003$) and lever ($F_{1,161} = 46.01$, $p < 0.001$). DAMGO induced moderate peak rates of responding at very low doses (1-3 ng/side) when compared with vehicle infusions without increasing inactive lever responding, whereas higher doses (10 ng/side) led to reduced responding (drug-paired lever: $F_{6,66} = 3.33$, $p = 0.006$; inactive lever: $F_{6,66} = 1.13$, $p =$ NS). Similarly, intra-NAc infusions of DPDPE produced an inverted U-shaped dose-response curve (Figure 4.2b), but induced greater responding and at higher doses of 300-3000 ng/side (dose: $F_{6,113} = 12.09$, $p < 0.001$; lever: $F_{6,113} = 28.16$, $p < 0.001$). Unlike DAMGO, DPDPE induced substantial and significant lever pressing of both drug-paired and inactive levers compared with vehicle (drug-paired lever: $F_{6,40} = 11.37$, $p < 0.001$; inactive lever: $F_{6,40} = 3.34$, $p = 0.009$).

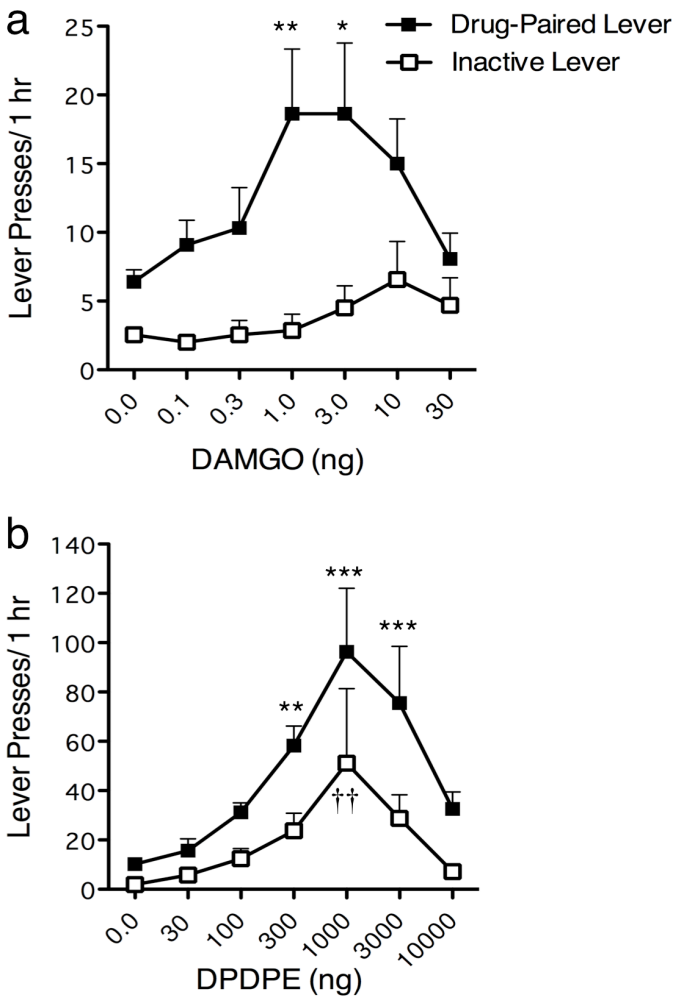


Figure 4.2 Intra-NAc infusions of (a) the mu-opioid receptor selective agonist DAMGO or (b) the delta-opioid receptor selective agonist DPDPE increase non-reinforced drug-paired lever responding in a within-session reinstatement procedure. Data represent the mean ± SEM for doses of DAMGO (n = 9-27) animals/treatment) and DPDPE (n = 5-22 animals/treatment). Symbols indicate drug-paired lever (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) or inactive lever (†† $p < 0.01$) differs from vehicle-infused controls by Dunnett's *post hoc* tests.

Inactive lever responding significantly increased only at the peak dose for drug-paired lever responding (1000 ng/side).

Antagonist inhibition of agonist mediated reinstatement

To determine whether DAMGO-stimulated reinstatement of cocaine seeking was mediated by MOR stimulation in the NAc, we tested the ability of the MOR-selective antagonist, CTAP, to block DAMGO-primed reinstatement using the lowest effective dose from the earlier experiment (1 ng/side). Intra-NAc pretreatment of CTAP dose-dependently blocked DAMGO-primed reinstatement (Figure 4.3a; dose x lever: $F_{6,172} = 7.82$, $p < 0.001$), with a main effect of dose ($F_{6,172} = 7.78$, $p < 0.001$) and lever ($F_{1,172} = 123.36$, $p < 0.001$). Non-reinforced responding at the drug-paired lever was blocked with maximally effective doses as low as 0.1 ng/side of CTAP (drug-paired lever: $F_{6,67} = 8.59$, $p < 0.001$; inactive lever: $F_{6,67} = 1.19$, $p =$ NS). Similarly, we tested the DOR-selective antagonist, naltrindole, against the lowest effective dose for DPDPE-induced reinstatement that did not increase inactive lever responding (300 ng). Figure 4.3b shows that intra-NAc treatment of naltrindole reduced DPDPE-primed reinstatement in a dose-dependent manner, achieving control levels at

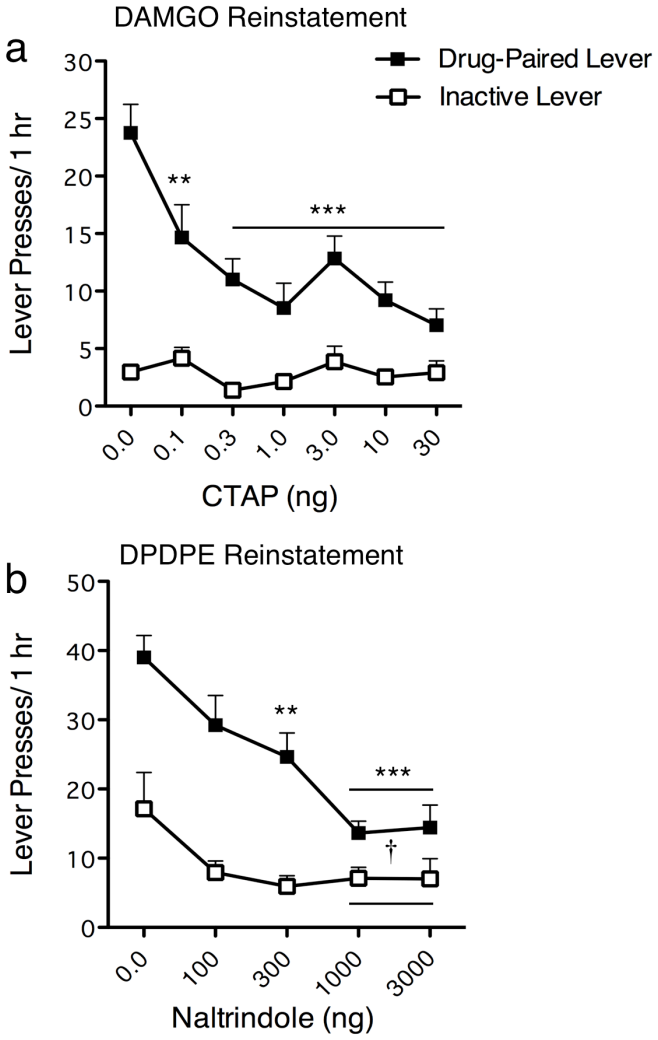


Figure 4.3 Intra-NAc pretreatment with (a) the mu-opioid receptor selective agonist antagonist CTAP followed by 1ng DAMGO and (b) the delta-opioid receptor selective antagonist naltrindol followed by 300 ng DPDPE dose-dependently attenuates reinstatement of cocaine seeking. Data represent the mean ± SEM for doses of DAMGO/CTAP (n = 13-22 animals/treatment) and DPDPE/naltrindol (n = 18-20 animals/treatment) combinations. Symbols indicate drug-paired lever (** $p < 0.01$, *** $p < 0.001$) or inactive lever († $p < 0.05$) differs from agonist/vehicle-infused controls by Dunnett's *post hoc* tests.

1000 ng/side (dose x lever: $F_{4,139} = 2.85$, $p = 0.026$; dose: $F_{4,139} = 11.35$, $p < 0.001$; lever: $F_{1,139} = 55.54$, $p < 0.001$). Drug-paired lever responding was significantly attenuated starting at 300 ng/side, with the maximal suppression at 100 ng/side ($F_{4,58} = 11.63$, $p < 0.001$). Naltrindole produced some mild suppression of responding on the inactive lever (inactive lever: $F_{4,58} = 2.48$, $p = 0.05$).

Drug specificity and antagonist inhibition of cocaine mediated reinstatement

To determine whether DAMGO- and DPDPE-induced cocaine seeking was specific to MOR or DOR blockade, MOR and DOR agonists and antagonists were tested in a cross-blockade experimental design. Animals were given intra-NAc infusions of DAMGO (1 ng/side) or DPDPE (300 ng/side) following pretreatment with maximally effective doses of CTAP (30 ng/side), naltrindole (1000 ng/side), or vehicle. Figure 4.4a shows that DAMGO-induced reinstatement of drug-paired lever responding was selectively blocked by CTAP, but not naltrindole, when compared with vehicle (treatment x lever: $F_{2,82} = 5.09$, $p = 0.008$; treatment: $F_{2,82} = 4.84$, $p = 0.01$; lever: $F_{1,82} = 68.65$, $p < 0.001$). CTAP significantly attenuated drug-paired lever responding ($F_{2,29} = 5.61$, $p =$

0.009), with no effect on inactive lever responding ($F_{2,29} = 0.40$, p = NS). Conversely, Figure 4.4b shows that DPDPE-induced reinstatement was selectively blocked by naltrindole but not CTAP (treatment: $F_{2,40} = 8.83$, $p < 0.001$; lever: $F_{1,40} = 39.01$, $p < 0.001$), with attenuation on the drug-paired lever ($F_{2,13} = 5.65$, $p = 0.017$) and a trend for reduction in lower responding on the inactive lever ($F_{2,13} = 2.87$, $p = 0.092$). Together, these results indicate that selective stimulation of either MOR or DOR in the NAc is sufficient to independently trigger cocaine-seeking behavior.

Given that cocaine injections are known to increase endogenous opioid release in the NAc, we tested whether MOR or DOR in the NAc plays a role in cocaine-primed reinstatement of cocaine-seeking behavior. Animals were given NAc pretreatments with vehicle, CTAP, or naltrindole immediately prior to an iv cocaine injection (2 mg/kg) in the reinstatement paradigm. As the peak dose of CTAP (30 ng against 1 ng DAMGO) had no effect on cocaine-primed reinstatement (data not shown), we tested a higher dose of CTAP (3 μg/side) more commonly used in intracranial studies (Soderman and Unterwald 2008; Tang et al. 2005), along with the 1 μg/side dose of naltrindole. The affinity of CTAP for MOR (2.36 ± 0.46 nM) is 15.7 times lower than that of naltrindole for DOR (0.15 ± 0.01 nM) (Bonner et al. 2000; Clayson et al. 2001; Pelton et al. 1986; Portoghese et

al. 1988), indicating that relatively higher amounts of CTAP than naltrindole may be required to inhibit endogenous opioid activity at MOR than DOR. Furthermore, the doses of CTAP and naltrindole used were roughly molar equivalents (5.4 uM and 4.8 uM, respectively). Intra-NAc pretreatment with CTAP significantly reduced cocaine-primed reinstatement compared with vehicle pretreatment (Figure 4.4c), whereas pretreatment with naltrindole did not (treatment x lever: $F_{2,82} = 4.17$, $p = 0.019$; treatment: $F_{2,82} = 5.12$, $p = 0.008$; lever: $F_{1,82} = 62.51$, $p = 0.001$). CTAP significantly attenuated drug-paired lever responding in response to an iv cocaine prime without affecting inactive lever responding (drug-paired lever: $F_{2,17} = 7.08$, $p = 0.006$; inactive lever: $F_{2,17} = 0.38$, $p =$ NS). These findings indicate that endogenous opioid release in the NAc contributes to cocaine-primed reinstatement of cocaine seeking through activation of MOR but not DOR.

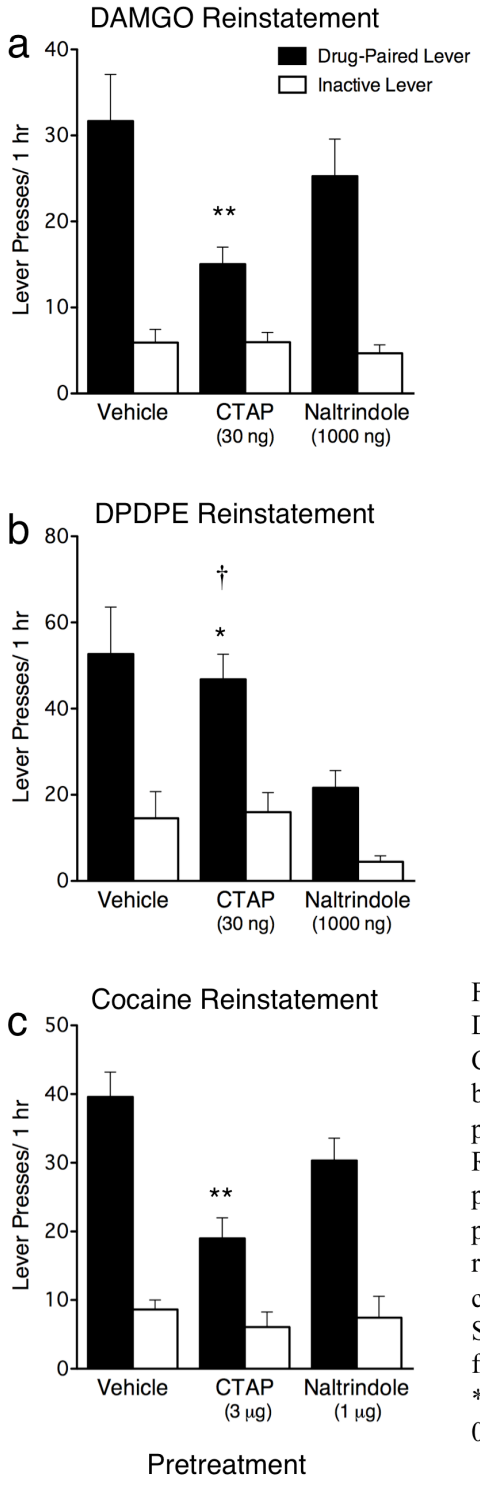


Figure 4.4 (a) Reinstatement induced by 1 ng DAMGO is blocked by intra-NAc pretreatment with CTAP and not naltrindol. (b) Reinstatement induced by 300 ng DPDPE is blocked by intra-NAc pretreatment with naltrindol and not CTAP. (c) Reinstatement induced by intravenous cocaine priming (2mg/kg) is blocked by intra NAc-pretreatment with CTAP and not naltrindol. Data represent the mean ± SEM agonist/antagonist combinations (n = 12-40 animals/treatment). Symbols indicate drug-paired lever responses differ from agonist/vehicle infused controls (* $p < 0.05$, ** $p < 0.01$) or CTAP differs from naltrindol († $p < 0.05$) by Tukey's HSD *post hoc* tests.

Endogenous opioid peptides reinstate cocaine seeking in the NAc

The next set of experiments determined the ability of endogenous opioids to reinstate cocaine seeking using intra-NAc infusions β-endorphin and met-enkephalin. Intra-NAc infusions of β-endorphin dose-dependently reinstated responding at the cocaine-paired lever, with effective doses ranging from 100 to 1000 ng/side (Figure 4.5a; dose x lever: $F_{4,103} = 4.07$, $p = 0.004$; dose: $F_{4,103} = 12.11$, $p < 0.001$; lever: $F_{1,103} = 51.27$, $p < 0.001$). β-endorphin infusions significantly increased drug-paired lever responding ($F_{4,42} = 8.82$, $p < 0.001$), with minor increase in inactive lever responding at the highest dose ($F_{4,42} = 7.32$, $p < 0.001$). In contrast, intra-NAc infusions of met-enkephalin failed to reinstate cocaine seeking up to doses as high as 10 μg/side (Figure 4.5b; $F_{5,102} = 0.43$, p = NS). As earlier studies used enkephalin derivatives, suggesting that enkephalins are degraded too rapidly to produce effects in behavioral tests {Kalivas and Bronson, 1985; Phillips et al, 1983}, we used the enkephalinase inhibitor, thiorphan, to determine if the accumulation of endogenously released enkephalins would reinstate cocaine seeking. Intra-NAc thiorphan infusions effectively reinstated responding to levels similar to β-endorphin (Figure 4.5c; dose: $F_{4,92} = 6.77$, $p < 0.001$; lever: $F_{1,92} = 63.27$, $p < 0.001$). Thiorphan induced prominent responding on the drug-paired lever ($F_{4,36} = 4.55$, $p = 0.004$),

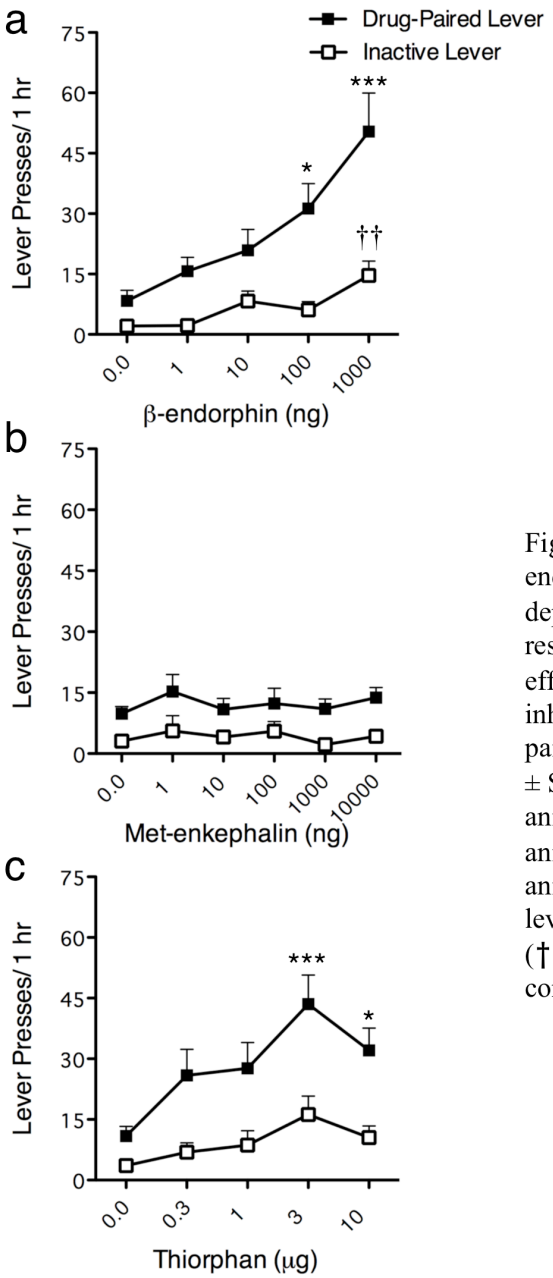


Figure 4.5 Intra-NAc infusions of (a) the endogenous opioid peptide β-endorphin dose-dependently reinstates drug-paired lever responding, (b) whereas met-enkephalin has no effect. (c) Intra-NAc infusion of the enkephalinase inhibitor thiorphan significantly increases drug-paired lever responding. Data represent the mean ± SEM for doses of β-endorphin (n = 10-15 animals/treatment), met-enkephalin (n = 7-11 animals/treatment), and thiorphan (n = 10-15 animals/treatment). Symbols indicate drug-paired lever (* $p < 0.05$, *** $p < 0.001$) or inactive lever (†† $p < 0.01$) differs from vehicle-infused controls by Dunnett's *post hoc* tests.

with minor increases in responding on the inactive lever at the peak dose of 3.0 ug that approached significance ($F_{4,36} = 2.48$, $p = 0.061$). These findings indicate that either MOR-preferring (β-endorphin) or DOR-preferring (enkephalins) endogenous opioid peptides in the NAc are capable of eliciting cocaine-seeking behavior.

Receptor specificity of endogenous opioid-induced reinstatement of cocaine seeking

Although β-endorphin and enkephalins preferentially interact with MOR and DOR respectively, they also interact with other opioid receptors. We tested the ability of 3 ug CTAP, 1ug naltrindole, and the less specific opioid antagonist naloxone to block β-endorphin- and thiorphan-induced reinstatement. Animals were given NAc infusions of maximally effective doses of β-endorphin (1 μg/side) or thiorphan (3 μg/side) immediately following vehicle, CTAP (3 μg/side), naltrindole (1 μg/side), or naloxone (10 μg/side) pretreatments. Figure 4.6a shows that β-endorphin-induced reinstatement of cocaine seeking was selectively attenuated by CTAP or naloxone, but not naltrindole (treatment x lever: $F_{3,74} = 4.83$, $p = 0.004$; treatment: $F_{3,74} = 10.45$, $p < 0.001$; lever: $F_{1,74} = 66.94$, $p = 0.001$), specifically reducing responding on the drug-paired lever ($F_{3,27} = 12.63$, p

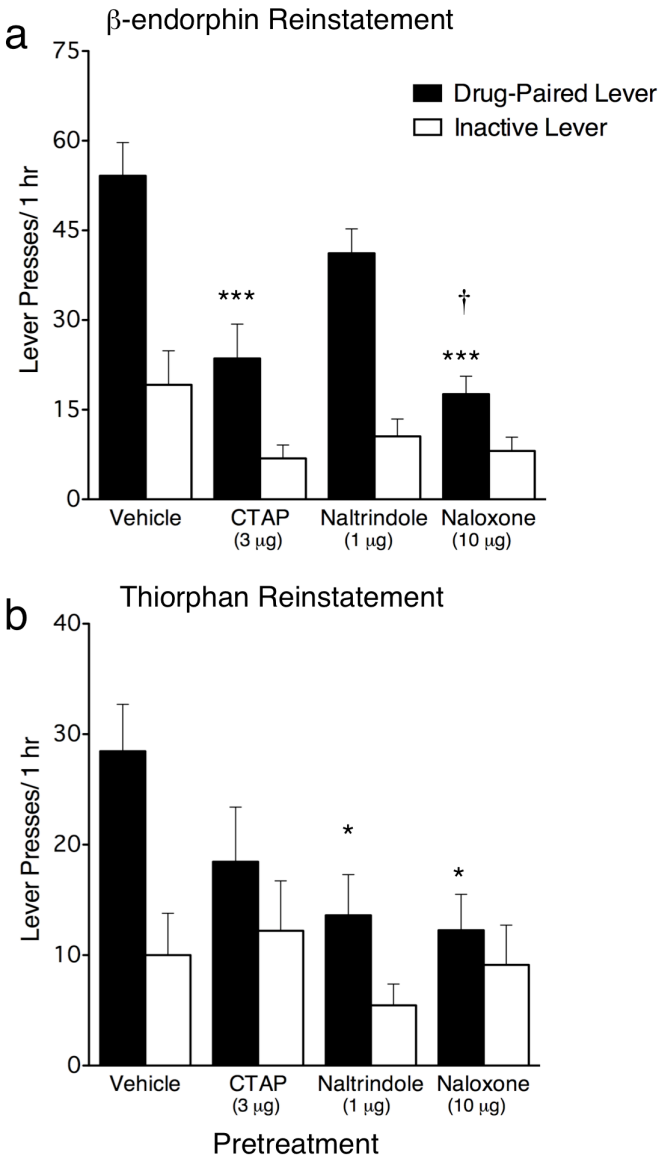


Figure 4.6 (a) Reinstatement induced by 1 ug β-endorphin is blocked by intra-NAc pretreatment with CTAP or naloxone, but not naltrindol. (b) Reinstatement induced by 3 ug thiorphan is blocked by intra-NAc naltrindol or naloxone, but not CTAP. Data represent the mean ± SEM for β-endorphin/antagonist (n = 9-17) animals/treatment) and thiorphan/ antagonist (n = 8-14 animals/treatment) combinations. Symbols indicate drug-paired lever responses differ from agonist/vehicle-infused controls (* $p < 0.05$, *** $p < 0.001$) or differs from naltrindol († $p < 0.05$) by Tukey's HSD *post hoc* tests.

< 0.001) and not the inactive lever ($F_{3,27} = 0.94$, p = NS). Conversely, Figure 4.6b shows that reinstatement elicited by the enkephalinase inhibitor, thiorphan, was blocked selectively by naltrindole or naloxone, but not significantly by CTAP (treatment: $F_{3,69} = 5.55$, $p = 0.002$; lever: $F_{3,74} = 4.83$, $p = 0.004$). Naltrindole and naloxone reduced thiorphan-induced responding on the drug-paired lever and not inactive lever (drug-paired lever; $F_{3,26} = 4.45$, $p = 0.012$; inactive lever: $F_{3,26} = 2.23$, p = NS). Thus, the endogenous opioid peptide, β-endorphin, reinstates cocaine seeking through the selective activation of NAc MOR, whereas elevations in endogenous enkephalin levels trigger cocaine seeking primarily through DOR activation, consistent with their preference for these receptors.

Regional specificity for MOR- but not DOR-induced reinstatement of cocaine-seeking behavior

To determine whether MOR and DOR stimulation of cocaine seeking was specific to the NAc, or due to potential spread up the cannulae shaft, we infused effective doses of all agonists 2.5 mm dorsal to the NAc site in the CPu, a region shown to have similar expression patterns of opioid receptors as the NAc. Although none of the MOR-acting agonists or the enkephalinase inhibitor induced reinstatement in the

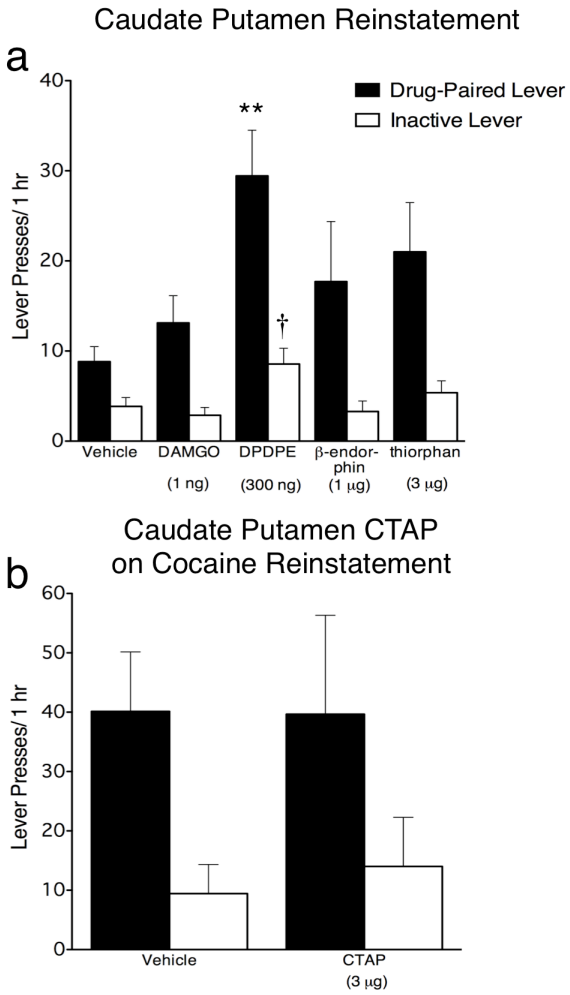


Figure 4.7 (a) Effective NAc doses of DAMGO, β-endorphin, and thiorphan are ineffective at reinstatement when infused in the CPu, while intra-Cpu DPDPE induces significant drug-paired and inactive lever responding. (b) Pretreatement with intra-CPu infusions of CTAP has no effect on reinstatement induced by intravenous cocaine priming (2mg/kg). Data represent the mean ± SEM for doses of agonists (n = 7-18 animals/treatment) and cocaine/antagonist (n = 6-8 animals/treatment) combinations. Symbols indicate drug-paired lever responses (** $p < 0.01$) or inactive lever responses († $p < 0.05$) differ from vehicle -infused controls by Dunnett's *post hoc* tests

CPu (Figure 4.7a), CPu infusions of DPDPE were sufficient to stimulate responding (treatment x lever: $F_{4,97} = 2.41$, $p = 0.05$; treatment: $F_{4,97} = 6.51$, $p < 0.001$; lever: $F_{1,97} = 40.91$, $p < 0.001$), with increases in responding on both the drug-paired lever ($F_{4,38} = 4.61$, $p = 0.004$) and inactive lever ($F_{4,38} = 3.43$, $p = 0.017$). It should be noted, however, that the 300 ng/side dose of DPDPE elicited twice as much responding in the NAc than in the CPu. In addition, intra-CPu pretreatment with CTAP at a dose that blocked cocaine-primed reinstatement in the NAc failed to alter cocaine seeking when infused in the CPu (Figure 4.7b) compared with vehicle-pretreated animals (treatment x lever: $F_{1,14} = 0.08$, $p = NS$; treatment: $F_{1,14} = 0.09$, $p = NS$; lever: $F_{1,14} = 10.21$, $p = 0.01$). Together, these data indicate that MOR involvement in reinstatement of cocaine seeking is specific to the NAc, whereas DOR in both sites are capable of triggering this behavior.

Opioid agonist induction of locomotor behavior in cocaine-trained animals

Following 1-week withdrawal from cocaine self-administration and reinstatement testing, the locomotor response to intracranial infusions of DAMGO, DPDPE, β-endorphin, and thiorphan was tested using doses that produced peak and primarily drug-paired lever responding when infused in

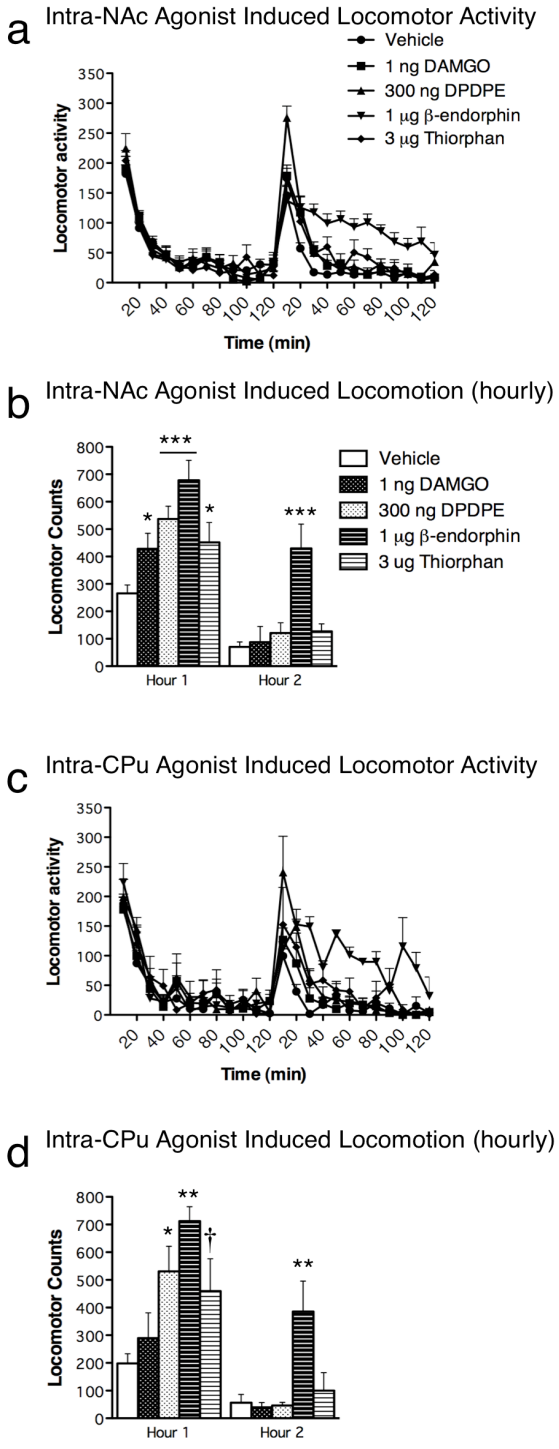


Figure 4.8 The Effects of reinstating doses of agonist treatments on horizontal locomotion in cocaine trained animals. (a) Timeline of locomotor behavior during habituation for 2 h and following intra-NAc infusion of opioid agonist. (b) All agonists increase locomotor behavior for 1 h when infused in the NAc while β-endorphin activity remains elevated during the second h of testing. (c) Timeline of locomotor behavior in response to intra-CPu infusions of opioid agonists. (d) Only DPDPE and β-endorphin increased locomotor responding in the CPu with a trend for thiorphan to increase locomotion. Data represent the mean ± SEM for NAc (n = 7-13 animals/treatment) and CPu (n = 4-5 animals/ treatment). Symbols indicate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) differs from vehicle-infused controls by Dunnett's *post hoc* tests.

the NAc. Figure 4.8a and 4.8b shows that all treatments increased locomotion for 1 h after infusion into the NAc when compared with vehicle infusions ($F_{4,46} = 8.429$, $p < 0.001$), whereas only β-endorphin increased locomotion for at least 2 h after infusion ($F_{4,41} = 8.258$, $p < 0.001$). Thus, the lower doses of DAMGO and DPDPE that triggered cocaine seeking produced psychomotor effects without the delay typically observed with higher doses in earlier studies. Figure 4.8c and 4.8d shows that very similar locomotor responses were produced by infusions of DPDPE and β-endorphin in the CPu ($F_{4,16} = 5.427$, $p = 0.006$), with a trend for thiorphan to increase locomotor activity during the first hour ($p = 0.059$). In contrast, intra-CPu infusions of DAMGO failed to significantly increase locomotion. Together, these findings suggest that although psychomotor activation may accompany reinstatement of cocaine seeking with NAc infusions, similar locomotor responses with CPu infusions are dissociated from cocaine seeking in many cases.

Injection Sites

Figure 4.9 illustrates the localization of all infusion sites in the NAc and CPu used in this study. Fourteen animals were eliminated from NAc

studies, and three animals were eliminated from CPu studies, due to misplacement of one or both cannulae.

Discussion

This study found that the selective stimulation of either MOR or DOR in the NAc is sufficient to reinstate cocaine-seeking behavior in rats following extinction of cocaine self-administration. Thus, NAc infusions of either the MOR-selective agonist, DAMGO, or the DOR-selective agonist. DPDPE, effectively elicited cocaine-seeking responses on the drug-paired lever that delivered cocaine injections during prior self-administration. The threshold dose for eliciting cocaine seeking was 300 times lower with DAMGO (1 ng/side) than with DPDPE (300 ng/side), whereas DPDPE induced greater peak rates of responding and was associated with generalized but lower rates of responding on the inactive lever. This later effect with DPDPE could be related to psychomotor activation rather than motivation for cocaine, or an inability to appropriately discriminate the drug-paired from inactive levers with increased DOR stimulation, although a lower dose of DPDPE selectively induced responding at the drug-paired lever. Both of these metabolically stable opioid peptide agonists produced an inverted U-shaped dose-response curve indicating that higher doses

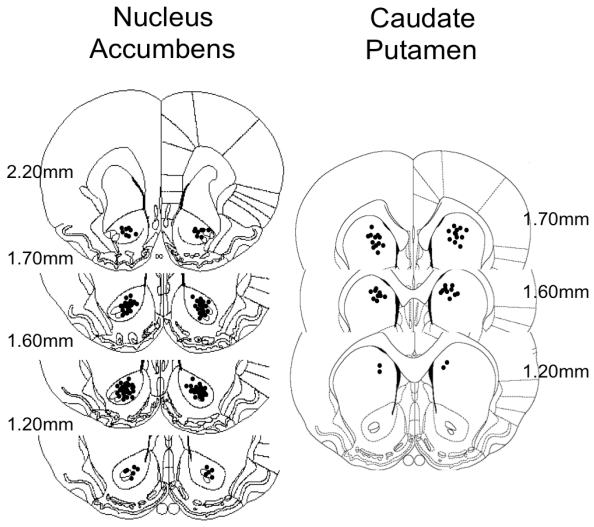


Figure 4.9 Localization of infusion sites in the medial NAc and CPu (+ 1..2 through +2.2 mm from Bregma, Paxinos and Wason, 1998).

were ineffective, and potentially explaining the failure to detect morphine-induced reinstatement of drug seeking at microgram doses used in earlier studies (Stewart and Vezina 1988; Tang et al. 2005).

In contrast, intra-NAc infusions of the endogenous opioid peptide β-endorphin induced cocaine seeking with a monophasic dose-response curve up to 1 μg/side, possibly reflecting the sensitivity of this peptide to metabolic degradation. Importantly, the reinstating effects of both DAMGO and β-endorphin were blocked by the MOR-selective antagonist CTAP, and not by the DOR-selective antagonist naltrindole. The ability of DAMGO and β-endorphin to reinstate cocaine seeking was localized to the NAc, as the failure of more dorsal CPu infusions to reinstate responding negates the possibility of diffusion along the cannulae shaft or into the cerebral ventricles. These data firmly establish that MOR in the NAc mediate relapse to cocaine-seeking behavior. Although the stimulation of MOR in dorsomedial CPu is ineffective, dorsolateral CPu sites could be involved in cocaine seeking, given that the inactivation of this site reduces cocaine-seeking behavior (See et al. 2007).

In addition, infusions of CTAP into the NAc, but not the CPu, attenuated cocaine-primed reinstatement, possibly relating to the ability of cocaine to increases endogenous β-endorphin release in the NAc (Olive et

al. 2001; Roth-Deri et al. 2003). Higher doses of CTP (3μg) were required to attenuate cocaine-primed reinstatement than DAMGO-primed reinstatement (30ng), possibly reflecting higher concentrations of cocaine-induced β-endorphin release relative to the very low doses of DAMGO that were effective (1-3 ng/side). The high dose of CTAP that attenuated cocaine-primed reinstatement also blocked β-endorphin-primed reinstatement that required a higher dose range (0.1-1 μg/side) than found with DAMGO.

Cocaine-induced β-endorphin release in the NAc is blocked by dopamine receptor antagonist infusions in the arcuate nucleus of the hypothalamus (Doron et al. 2006), the primary source for β-endorphin innervation of the NAc. β-Endorphin release in the NAc also is induced by exposure to footshock stress, or the unmet expectation of cocaine reward under extinction conditions (Roth-Deri et al. 2003; Zangen and Shalev 2003), situations that elicit cocaine-seeking behavior. Thus, taken together with our findings, β-endorphin stimulation of MOR in the NAc could contribute to cocaine seeking elicited by cocaine priming, exposure to cocaine-associated environments, and stressful events. In contrast, an earlier study found that NAc pretreatment with CTAP does not block cocaine-primed reinstatement using longer acting intraperitoneal cocaine

priming-injections (Tang et al. 2005), whereas effective blockade was found using shorter acting intravenous cocaine priming in our study. Another difference could involve the use of within- *vs* between-session extinction/reinstatement paradigms.

In contrast to DAMGO and β-endorphin, cocaine seeking induced by DPDPE was blocked by pretreatment with the DOR- but not the MOR-selective antagonist in the NAc. NAc infusions of the enkephalinase inhibitor thiorphan (to elevate endogenous enkephalins) also reinstated cocaine seeking, and the effect was blocked by DOR antagonist pretreatment, although marginal (non significant) attenuation also was found with the MOR antagonist potentially relating to enkephalin activity at MOR. DOR stimulation in more dorsal CPu sites with DPDPE also induced a moderate degree of cocaine seeking, but with greater efficacy in the NAc. Moreover, the reinstating effect of DPDPE in the CPu was accompanied by a significant inactive lever responding, an effect not found with this DPDPE dose in the NAc, and potentially relating to psychomotor activation, as discussed above. In this regard, infusions of the enkephalinase inhibitor thiorphan in the CPu failed to reinstate cocaine seeking, and had no effect on inactive lever responding in either striatal site. Together, the double dissociation with MOR- and DOR-selective

ligands clearly indicates that MOR and DOR in the NAc mediate cocaine seeking through distinct and independent mechanisms.

Interestingly, blockade of DOR in the NAc failed to attenuate cocaine-primed reinstatement of cocaine seeking. Whether cocaine increases extracellular enkephalins in the NAc is unknown, but cocaine acutely increases preproenkephalin expression throughout the striatum (Hurd and Herkenham 1992), although this acute effect is diminished with chronic cocaine administration (Arroyo et al. 2000; Mantsch et al. 2004). One study found that systemic administration of naltrindole decreases cocaine self-administration but only at doses that also suppressed locomotor behavior (de Vries et al. 1995). Another study showed reduced lever pressing for cocaine irrespective of reinforcement schedule (Reid et al. 1995), and intra-NAc infusions of an irreversible DOR-alkylating analog of naltrindole (Portoghese et al. 1990) decreased responding for cocaine on a more demanding progressive ratio schedule of reinforcement (Ward and Roberts 2007), suggesting generalized effects on motor performance. In contrast, icv administration of the naltrindole analog strongly reduced heroin administration while only modestly decreasing cocaine self-administration on a less-demanding fixed-ratio reinforcement schedule (Martin et al. 2000), suggesting that endogenous DOR activity plays little

role in the effects of cocaine. Similarly, our results are consistent with the notion that endogenous release of enkephalins in the NAc does not contribute to cocaine-primed reinstatement of cocaine seeking, but further tests are needed to determine whether cocaine seeking induced by stress or cocaine-associated cues involves endogenous enkephalinergic activity at NAc DOR.

Intra-NAc infusions of MOR and DOR agonists at doses that effectively reinstated cocaine seeking also increased horizontal locomotion, with β-endorphin infusions producing prolonged effects over 2 hour of testing. Infusions of all treatments into the dorsomedial CPu also increased locomotion to similar levels, with the exception of the MOR agonist DAMGO, whereas only the DOR agonist DPDPE triggered cocaine seeking in this region. Although these data support the notion that DPDPE-induced reinstatement may be related to psychomotor activation, the dissociation of locomotor activity and cocaine seeking with infusions of β-endorphin and thiorphan in the CPu suggests that the reinstating effects of these treatments in the NAc are not related to generalized psychomotor activation. Moreover, although it could be argued that DAMGO-induced reinstatement is related to psychomotor activation, the lack of increases in inactive lever responding with DAMGO infusions suggests that

reinstatement reflects motivational rather than motor effects. In contrast to reinstatement of cocaine seeking, NAc infusions of higher doses of DAMGO (0.25-2.5 μg) induce a delayed increase in locomotion and preference for sucrose and high-fat foods often after a period of behavioral suppression (Cunningham and Kelley 1992; Meyer et al. 1994; Zhang et al. 2003; Zhang and Kelley 1997), whereas we found that very low doses induce locomotion and cocaine seeking without delay. These findings suggest that lower doses of DAMGO could be employed to elicit appetitive behavior without delay in future studies.

Although MOR and DOR are coupled to similar intracellular signaling pathways, their distinct involvement in modulating drug-seeking behavior can be attributed to differences in their subanatomical distribution. MOR are largely expressed extrasynaptically on dendrites and dendritic shafts of GABAergic and cholinergic cells within striatal patches (Svingos et al. 1997; Wang and Pickel 1998) in which they modulate excitatory and GABAergic input to NAc neurons (Gracy et al. 1997). Presynaptic MOR can also modulate the release of GABA onto NAc neurons (Svingos et al. 1997). DOR can either directly or indirectly modulate dopamine release through expression on dopamine terminals or on GABAergic terminals apposed to dopamine terminals. DOR can also

modulate postsynaptic responses in spiny neurons that receive dopamine input (Svingos et al. 1999a). MOR co-localizes predominantly with preprotachykinin positive neurons in patch compartments that constitute the direct striatal output, and more rarely with preproenkephalin positive neurons of striatal matrix that constitute the indirect output (Furuta et al. 2002). The differential expression patterns of MOR and DOR lend them different mechanisms of action, with DOR more frequently modulating inhibitory and dopaminergic input to the NAc and MOR primarily modulating NAc GABAergic neurons themselves (Svingos et al. 1999a; Svingos et al. 1997; Wang and Pickel 1998).

Chronic-primed reinstatement of cocaine seeking requires glutamatergic neurotransmission in the NAc core (Cornish and Kalivas 2000; McFarland et al. 2003) and dopaminergic neurotransmission in the NAc shell (Anderson et al. 2003), although direct dopamine receptor stimulation in the medial NAc core elicits greater cocaine seeking than that in the shell or lateral core region (Bachtell et al. 2005; Schmidt et al. 2006). Although we did not compare core with shell subregions in this study, the ability of the MOR antagonist CTAP to block cocaine-primed cocaine seeking suggests that β-endorphin is released in the vicinity of the medial NAc. Given that the locomotor activating effects of intra-NAc

MOR- and DOR-selective agonists are not attenuated by dopamine depletion or chronic dopamine receptor blockade (Churchill and Kalivas 1992; Stinus et al. 1986), it is likely that cocaine seeking elicited by MOR and DOR stimulation is mediated independent of dopamine release in the NAc. Furthermore, dopamine depletion leads to supersensitivity to MOR but not DOR agonist infusions in locomotor tests (Churchill and Kalivas).

Chronic cocaine administration modulates opioid receptor expression in the NAc (for review see (Boutrel 2008; Kreek 2001)), suggesting that changes in these receptors could modulate the propensity for relapse during cocaine withdrawal. Free β-endorphin levels are decreased in the NAc and other brain regions within 1 day withdrawal from cocaine self-administration, potentially reflecting the depletion of endogenous stores (Sweep et al. 1989). Similarly, opioid receptor binding decreases immediately after and prior to the next scheduled cocaine self-administration session (Gerrits et al. 1999), possibly reflecting the release of endogenous opioids during cocaine self-administration or a compensatory response of opioid receptors to release of endogenous opioids during cocaine self-administration. Chronic cocaine administered in a daily binge pattern transiently increases MOR but not DOR density and MOR-stimulated [35S]GTPγS binding in the NAc (Schroeder et al.

2003; Unterwald et al. 1992). However, the ability of DOR, but not MOR, stimulation to inhibit adenylyl cyclase activity is impaired in the NAc following chronic cocaine (Unterwald et al. 1993), and this impairment persists for at least 1 day of cocaine withdrawal (Perrine et al. 2008), coinciding with increased internalization of DOR in NAc neurons (Ambrose-Lanci et al. 2008). Although these changes could modify the ability of MOR and DOR to trigger cocaine relapse in early cocaine withdrawal, we reported that MOR, and not DOR, levels in the NAc core progressively increase from 1 to 6 weeks of withdrawal from chronic cocaine self-administration (Self et al. 2004), and the effect is accompanied by increases in the precursor for β-endorphin, pro-opiomelanocortin, in the arcuate nucleus of the hypothalamus (Smagula et al. 2005). These findings suggest that progressive increases in MOR signaling in the NAc contribute to time-dependent increases in cocaine seeking behavior in cocaine withdrawal when animals are exposed to cocaine-paired environments or stressful conditions (Grimm et al. 2001; Sorge and Stewart 2005; Tran-Nguyen et al. 1998).

Human studies also support a relationship between increased MOR and cocaine craving in abstinence. Thus, MOR binding measured by positron emission tomography is increased in striatal and cortical regions

in abstinent cocaine addicts and positively correlates with measures of cocaine craving (Gorelick et al. 2005; Zubieta et al. 1996). In subsequent studies, the up-regulation in MOR binding was found to persist for up to 12 weeks of abstinence and positively correlate with the amount of prior cocaine use (Gorelick et al. 2005). Moreover, the up-regulation of MOR in abstinence served as an independent predictor of time to relapse in cocaine addicts, and positively correlated with amount of cocaine use during the first month of relapse (Gorelick et al. 2008). While limitations in detection precluded examination of MOR exclusively in the NAc, our animal data suggest that such long-lasting increases in MOR could functionally increase the propensity for cocaine relapse (Self et al. 2004).

In this regard, treatment with the opioid receptor antagonist naltrexone in combination with behavioral therapy decreased cocaine use over time (Schmitz et al. 2001). When threefold higher doses of naltrexone were utilized in combination with psychosocial treatment, the severity of cocaine use decreased (Pettinati et al. 2008). In response to an acute cocaine dose, addicts reported decreased “good effects” and “crash” when treated with naltrexone (Kosten et al. 1992; Sofuoglu et al. 2003), although naltrexone reportedly does not decrease subjective reports of craving elicited by cocaine-associated cues (Modesto-Lowe et

al. 1997). Our findings suggest that blockade of MOR and DOR in the NAc contributes to the therapeutic potential of naltrexone in the treatment of cocaine addiction.

CHAPTER FIVE:

EFFECTS OF LONG-TERM MODULATION OF MU OPIOID RECEPTORS DURING ABSTINENCE FROM COCAINE SELF-ADMINISTRATION

Simmons DL, Sutton MA, Hoppenot R, Walker JR, Self DW

Introduction

Drugs of abuse produce numerous neuroadaptations (Bailey et al. 2005b; Baker et al. 2003; Mantsch et al. 2004; Spangler et al. 1996; Unterwald 2001) however few persist during abstinence where the propensity for relapse is high (Grimm et al. 2003; Grimm et al. 2002; Lu et al. 2004; Tran-Nguyen et al. 1998). Several studies have discovered neurobiological changes associated with chronic drug use. Most of these studies focus on pharmacological neuroadaptations directly produced by repeated drug exposure, leading to the phenomena of tolerance and sensitization (Koob and Le Moal 2001; Nestler and Aghajanian 1997). However, few investigations have been able to uncover the mechanisms of relapse behavior with increasing periods of abstinence when most neurobiological changes return to normal. Given that drug-seeking and drug craving persist (or increase) despite long periods of abstinence,

many current theories suggest that relatively long-term neuroadaptations in limbic brain regions underlie the propensity for relapse in addicted individuals (Grimm et al. 2003; Grimm et al. 2002; King et al. 1999; Neisewander et al. 1994; Tran-Nguyen et al. 1998; White and Kalivas 1998). Only a few studies have addressed neuroadaptations during longer periods of abstinence, and thus, further research is necessary for understanding neuroadaptations underlying persistent drug craving that increase an individual's propensity for relapse.

Animal models of drug craving, such as the reinstatement paradigm, have been developed to understand the mechanisms of relapse to drug-seeking behavior. This model provides an objective measure of operant events associated with drug self-administration, and has face validity because similar stimuli trigger drug craving in humans and drug seeking in animals. Animals can be tested during prolonged periods of forced abstinence in this paradigm, allowing for increased insight into the neuroadaptations that may contribute to increased propensities for relapse. The level of drug-seeking behavior is indicated by the amount of effort (lever pressing) exerted by animals to self-administer the drug when reinforcement is withheld. This behavior measures both the magnitude and persistence of drug-paired lever responding during extinction testing

in the absence of drug, and by reinstatement of this responding following extinction, through presentation of various stimuli. Three stimuli commonly used to reinstate drug-seeking behavior in animals following extinction of drug self-administration are drug-associated cues, stress, and low priming doses of the self-administered drug. These stimuli are thought to induce relapse, at least in part, by their ability to elevate dopamine levels in the NAc, which has been well-characterized in drug reward paradigms and represents an important player in the neural circuit involved in relapse to drug seeking (Self and Nestler 1998b; Shalev et al. 2002b; Spealman et al. 1999b; Stewart 2000a).

Using the reinstatement paradigm, investigators have shown that the level of cocaine-seeking behavior progressively increases with longer periods of forced abstinence (Grimm et al. 2001; Tran-Nguyen et al. 1998). This model, referred to here as the “Cocaine Abstinence Effect”, is thought to reflect time-dependent increases in cocaine craving that lead to increased relapse rates during prolonged abstinence. The model also represents the phenomenon of incentive sensitization, whereby drug-associated stimuli (environmental context, conditional cues) show enhanced ability to stimulate craving as abstinence proceeds. Using a rat model where cocaine-seeking behavior progressively increases with

longer withdrawal times, this study was initialized by utilizing microarray profiling to identify changes in gene expression that coincide with this behavioral phenomenon. Among other changes, this study found that levels of mu-opioid receptor (MOR) in the rat NAc core progressively increase over several weeks of cocaine withdrawal. The purpose of this study was to further characterize changes in MOR in the NAc during cocaine withdrawal, and test the functional role of MOR in regulating addictive behavior.

Methods

Animals and Surgery

Animals were ordered from Charles-River Kingston and housed in the animal research facility in accordance with NIH standards and IACUC at UT Southwestern Medical Center. Following 5-7 days of habituation, animals were implanted with a catheter into the jugular vein for cocaine self-administration. Animals were allowed to recover 3-5 d prior to start of experiment. For NAc core infusions, animals were implanted with a guide cannula aimed at the core (±1.5 mm lateral; 1.7mm anterior to bregma; -

5.7 ventral to dura with the level skull) (Paxinos and Watson 1998) and allowed 5-7 d to recover prior to starting the experiment.

Self-administration

Animal catheters were attached to a drug line that fed out to a syringe pump so that depression of the drug-paired lever resulted in an injection of 0.5mg/kg cocaine in 0.1 ml over 2.5 s. Injections were paired with a 2.5 s illumination of a cue light above the drug-paired lever and extinguishing of the house-light for 15 s (time-out) during which no further injections were allowed. Following the time-out period, the house light was again illuminated indicating drug availability. Animals were allowed to self-administer cocaine for 10 h a day (5 d) during their dark cycle for 1 week, followed by 6 h a day access for 2 weeks (5 d/w). This paradigm allowed animals to learn to self-administer cocaine without sucrose lever press training and resulted in stable responding by the end of the first week.

Following cocaine self-administration, animals remained in their home-cages but were handled regularly. Animals were counter-balanced for drug-intake and assigned to 1 w or 6 w withdrawal groups (1wWD or 6wWD, respectively) or 1 w or 6 w extinction groups (1wExt or 6wExt).

Extinction animals were placed back in the operant boxes with no drug-available following 3 or 38 d forced abstinence in their home cage (1wExt or 6wExt, respectively). Lever pressing was recorded for 5 d of 6 h sessions, but no drug was delivered. The first day of extinction training measured drug-seeking behavior induced by drug associated context. Following several days of extinction training, animals eventually extinguished responding on the drug-paired lever allowing for reinstatement testing. On test day (7 d or 42 d) animals extinguished for 5 h followed by presentation of the cue light at regular 5 m intervals of 2.5 s each. Responding was recorded during this last hour of the test session as a measure of cue-induced reinstatement of drug seeking. Age and group matched control animals remained in their home-cage for the duration of the experiment, but were handled daily (homecage control, HC). Animals were all euthanized on day 10 or 45 for 1w (1wWD or 1wExt) or 6 w (6wWD or 6wExt) groups, respectively.

Tissue collection

Animals were euthanized by rapid decapitation and brains removed. Brains were dissected using a brain slicer (Braintree) and tissue punches of NAc core and shell were taken using 12- and 14- gauge syringe

needles that were filed flush and edges sharpened. Unilateral samples were placed in separate labeled tubes for Western blot analysis or pooled (n = 6 each) for microarray analysis (Novartis) and immediately frozen on dried ice. Tissue was stored at -80° C until analysis could be done.

Microarray Analysis

Pooled samples were sent to Novartis for preparation and analysis. Briefly, samples were pooled, homogenized, and mRNA isolated. RNA was amplified once to create Cy3 and Cy5 labeled cRNA and hybridized onto Affymetrix chips. 1.5 fold changes relative to home-cage controls were detected.

Western blot Analysis

Tissue punches were homogenized by sonication in lysis buffer (320mM sucrose, 5nM HEPES, 50mM NaF, 1 mM EGTA, 1mM EDTA, 1% SDS, with protease inhibitor cocktail and phosphatase inhibitor cocktails I and II diluted 1:100; Sigma, St. Louis, MO), boiled for 5 min at 90°C, and stored at -80 degrees C until protein concentration was determined by the Lowry method.

20 ug of each sample was subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide in Tris/Glycine/SDS buffer (BioRad), followed by transfer to polyvinylidene membranes (PVDF; BioRad) by electrophoretic transfer at 4 degrees C. Membranes were blocked ovenight in 5% nonfat milk in Tris-buffered saline in 1% Tween (TTBS) at 4 degrees C and incubated in primary antibody for MOR and DOR (1:500 and 1:2000 respectively; Chemicon) for 24h at 4° C. Membranes were washed with TTBS 3 times for 15 m each and labeled with species-specific peroxidase-conjugated secondary (BioRad) for 1 h at 25º C. Membranes were washed 3 times for 15 m each with TTBS and detected by chemiluminesence detection (SuperDura-West: Pierce). Following detection, membranes were stripped and reprobed for β-tubulin (1:25,000; Upstate) for an internal standard of protein changes. Immunoreactivity was quantified by densitometry (Scion Image, NIH) under conditions linear over at least a 3-fold concentration.

Locomotor testing

The locomotor testing apparatus consisted of a circular-shaped plexiglass arena with 12 cm wide metal floors (Med-Associates) with four pairs of photocells located at 90-degree intervals around the 1.95 m perimeter to

record locomotor activity. Animals were habituated for 2-h in the dark followed by an intra-NAc drug infusion and returned to the locomotor chambers for 2-h of subsequent testing. Testing of each drug was randomized and done on consecutive days. Animals received no more than 4 injections each.

Data Analysis

Data were analyzed by simple main effects analysis of variance (ANOVA). Multiple comparisons tests used Fischer's *post hoc* least-significant-difference (LSD) test when appropriate.

Results

Self-administration, Extinction, and Reinstatement Behavior

Figure 5.1a shows the self-administration schedule for animals tested for effects of long-term abstinence (6 w Ext) on behavior compared to short-term withdrawal (1 w Ext). Animals in the long-term withdrawal group (6 w Ext) exhibited significantly higher drug-seeking behavior during compared to short-term withdrawal animals (1w Ext) as seen in Figure 5.1b. This data replicated previous reports where longer periods of abstinence

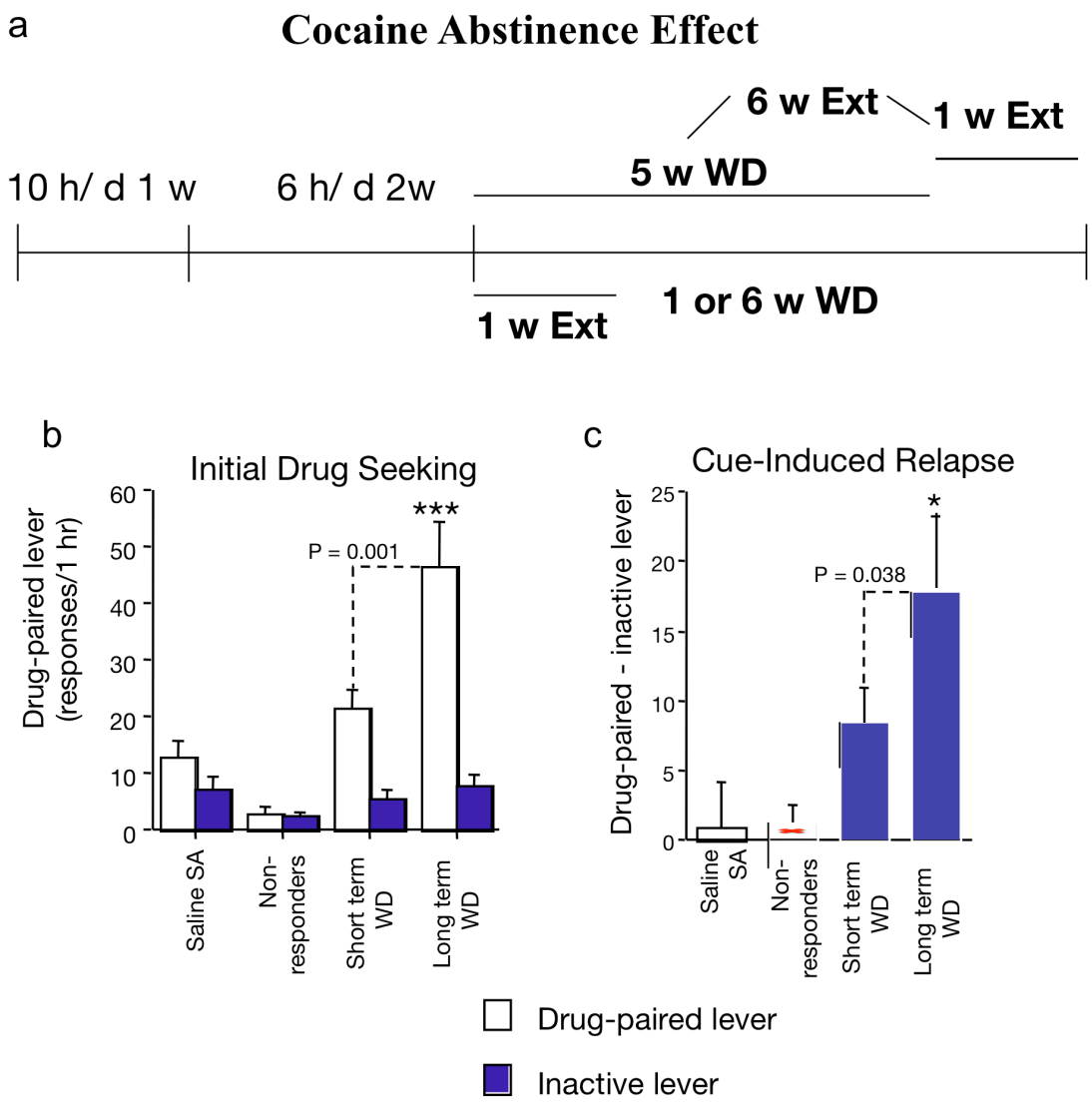


Figure 5.1 Diagram of cocaine self-administration, withdrawal, and extinction for experiments used in this chapter. Animals were allowed to self-administer cocaine for 3 weeks. Animals were counter-balanced for drug intake and divided into 4 groups: 1 w WD, 1 w Ext, 6 w WD or 5 w WD + 1 w Ext (6 w Ext). The extinction groups allowed for the behavioral test of drug seeking and reinstatement to drug-seeking. The withdrawal groups were analyzed for gene and protein changes. (b) 1 w Ext animals started testing 3 days after the last self-administration session and 6 w Ext animals were tested 38 days after their last session so that by the end of testing, they had the same amount of total withdrawal as did the 1 and 6 w WD groups. (b) During the first hour of the first test session, animals with long-term withdrawal sought cocaine significantly more than did short term withdrawal, as indicated by the number of drug-paired lever responses exerted. (c) After 1 w of extinction training, animals in long-term withdrawal reinstated significantly to lever pressing in response to non-contingent presentation of the cue-light previously paired with cocaine infusions compared to short term withdrawal animals.

resulted in increased drug-seeking behavior (Grimm et al. 2001; Grimm et al. 2002). Following 5 days of extinction training, animals in both groups extinguished drug-paired lever pressing to baseline. Cue-induced reinstatement of drug-paired lever pressing was examined by non-contingent presentation of the cue light during the last hour of the last extinction session. Long-term withdrawal animals (6 w Ext) reinstated to the cue-light significantly more than short-term withdrawal animals (1 w Ext) as seen in Figure 5.1c. Now that the behavioral phenotype was induced, physiological correlates were determined.

Genes Altered by Long-Term Withdrawal and Confirmation at the Protein Level

Several genes were regulated however this project's focus was on long-term changes seen at 6wWD. Genes shown in figure 5.2a show gene changes that had antibodies available commercially and that also changed at the protein level. In the NAc shell, the voltage-gated potassium channel of isoform 4.3 (Kv4.3) had increased mRNA but decreased protein at long-term withdrawal. In the NAc core, A-kinase anchoring protein 84 (AKAP-84), a splice variant of AKAP121 showed

a

Regulated genes	mRNA	Protein
NAc Shell		
Kv 4.3	↑	↓
NAc Core		
AKAP 84	↓	↑
MOR	↑	↑

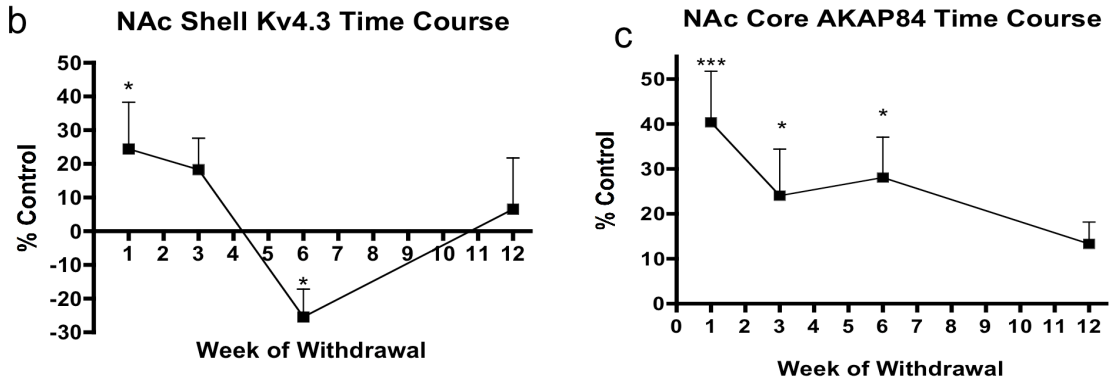


Figure 5.2 (a) Microarray analysis identified several genes regulated by 6 w WD, however, of those that could be measured by Western blot, only the voltage-gated potassium channel Kv4.3 was confirmed at the protein level in NAc shell, A-kinase anchoring protein AKAP84 and mu-opioid receptor MOR in NAc core. (b) Timecourse of Kv4.3 regulation does not parallel with the cocaine abstinence effect. (c) AKAP84 regulation steadily decreases with withdrawal in a way that does not parallel with increased craving during prolonged abstinence.

decreased mRNA, but increased protein levels. MOR was increased in the NAc core at both mRNA and protein levels.

Temporal regulation of identified proteins

To determine the temporal regulation of these confirmed protein changes, Western analysis was conducted on animals that underwent 1, 3, 6, and 12 weeks of withdrawal following the same self-administration schedule as shown in Figure 5.1a. As seen in Figure 5.2b and Figure 5.2c, AKAP-84 and Kv4.3 had temporal regulation patterns that did not correlate with the behavioral phenotype seen shown in Figure 5.1b and Figure 5.1c, that is increased drug-seeking behavior with longer abstinence periods. However, MOR protein levels started to increase at 3wWD and persisted out to 12wWD (Figure 5.3a), paralleling the incubation of craving responses in the Cocaine Abstinence Effect seen by other groups. Figure 5.3b shows representative blots for MOR and Figure 5.3c and 5.3d shows that DOR was not regulated at any time-point. This up-regulation of MOR coupled with work shown in previous chapters support MOR as a potential mediator of increased craving and subsequent relapse seen in rodents and potentially in humans. Therefore subsequent

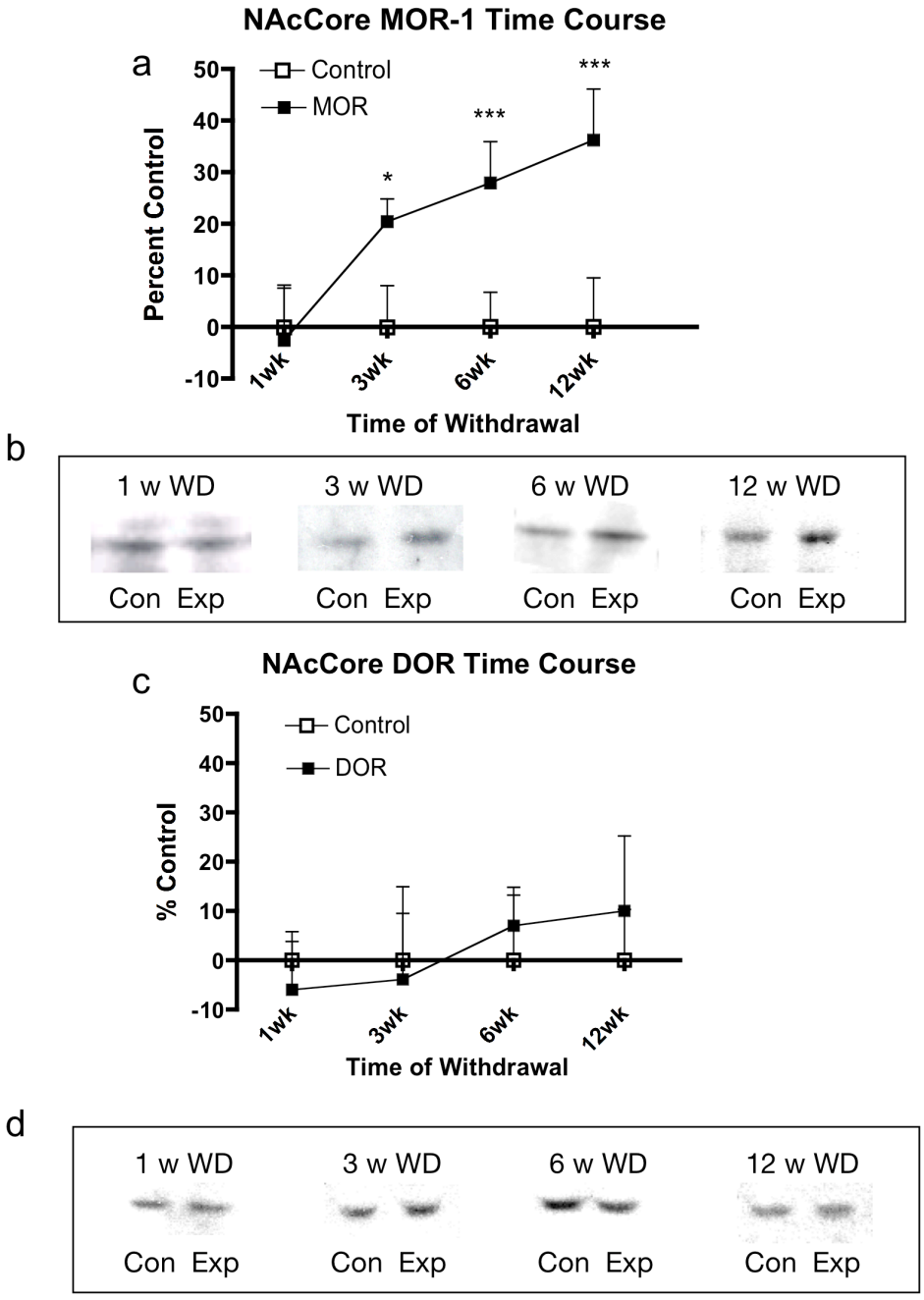


Figure 5.3 (a) Timecourse of MOR regulation indicate MOR starts to increase at 3 w WD and persists out to 12 w WD. (b) Representative blots of control and WD animals. (c) DOR was not regulated at any time point. (d) Representative blots for DOR in control and WD groups.

studies set out to determine whether the up-regulated MOR were functional and translated into a behavioral phenotype.

Functional regulation of MOR during withdrawal

The next experiment was designed to determine whether increased MOR levels translated into up-regulation of MOR function. Figure 5.4 shows the strategy for answering this question. Animals were allowed to self-administer cocaine as previously described, counter-balanced for drug intake, and divided into 1w or 6w WD groups. Following withdrawal, animals were tested for functional regulation of MOR using the MOR specific agonist, DAMGO (1ng), to stimulate MOR induced locomotion. The DOR-specific agonist, DPDPE (300ng), was used as a positive control of opioid receptor stimulation induced locomotion that is not under the control of cocaine withdrawal effects. DOR was not regulated with cocaine withdrawal therefore DPDPE-induced locomotion should not be different between withdrawal groups. Following locomotor testing, animals were tested for MOR-stimulated drug seeking and reinstatement to drug-seeking behavior using 1 ng DAMGO or 1 ug β-endorphin (endogenous MOR ligand). Figure 5.4b shows the timeline for the first test day where initial drug seeking in extinction and reinstatement could be measured and

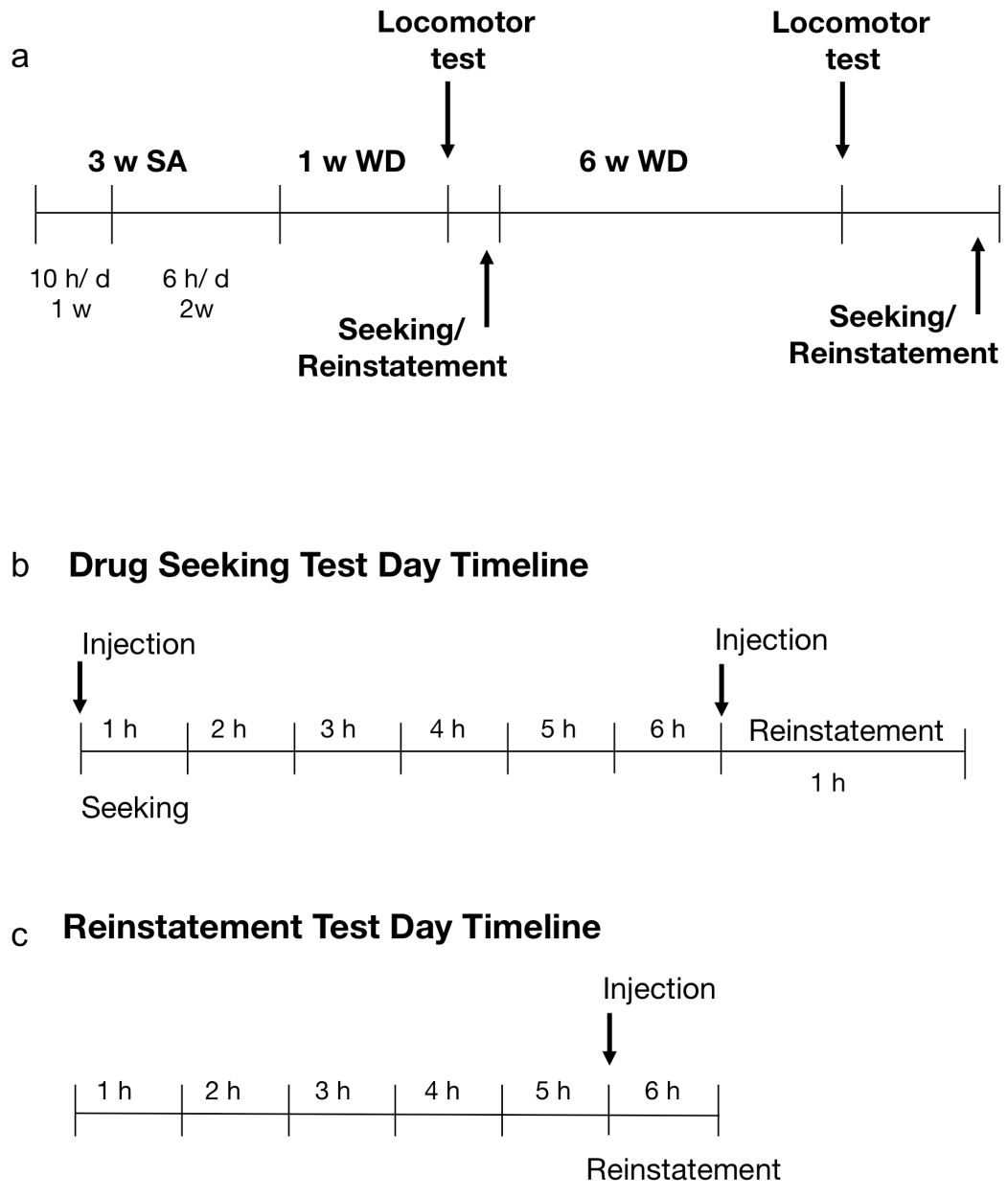


Figure 5.4 (a) Diagram of cocaine self-administration and subsequent testing following 1 w and 6 w WD. (b) Diagram of test day to measure drug seeking and subsequently reinstatement. (c) Reinstatement of drug seeking on a subsequent test day.

Figure 5.4c show the subsequent test day where only reinstatement was tested.

Prior to carrying out this experiment, drug naïve animals were cannulated for drug injections into the NAc core and tested for locomotor behavior. One, 3, and 10 ng doses of DAMGO were tested along with 1 μg β-endorphin, 3 μg met-enkephalin, 300 ng DPDPE, and 3 μg thiorphan. Figure 5.5 shows that all drugs tested increase locomotor behavior in drug naïve animals with the exception of met-enkephalin. Figure 5.5a shows that DAMGO dose dependently increased locomotor behavior. DPDPE also increased locomotor behavior (Figure 5.5b) as did β-endorphin (Figure 5.5c). Figure 5.5d shows that thiorphan but not met-enkephalin increased locomotor behavior in a similar pattern as β-endorphin. These findings were expected since locomotor behavior was increased in cocaine-trained animals (Chapter 4, Figure 4.8), but were tested in drug naïve animals to make sure that locomotor behavior was not different across paradigms and treatments. Figure 5.5e shows behavior for all drugs over the first hour of testing. This project is a work in progress, therefore for the purposes of this thesis, only DAMGO and DPDPE were tested in locomotor behavior during withdrawal since these are potent and

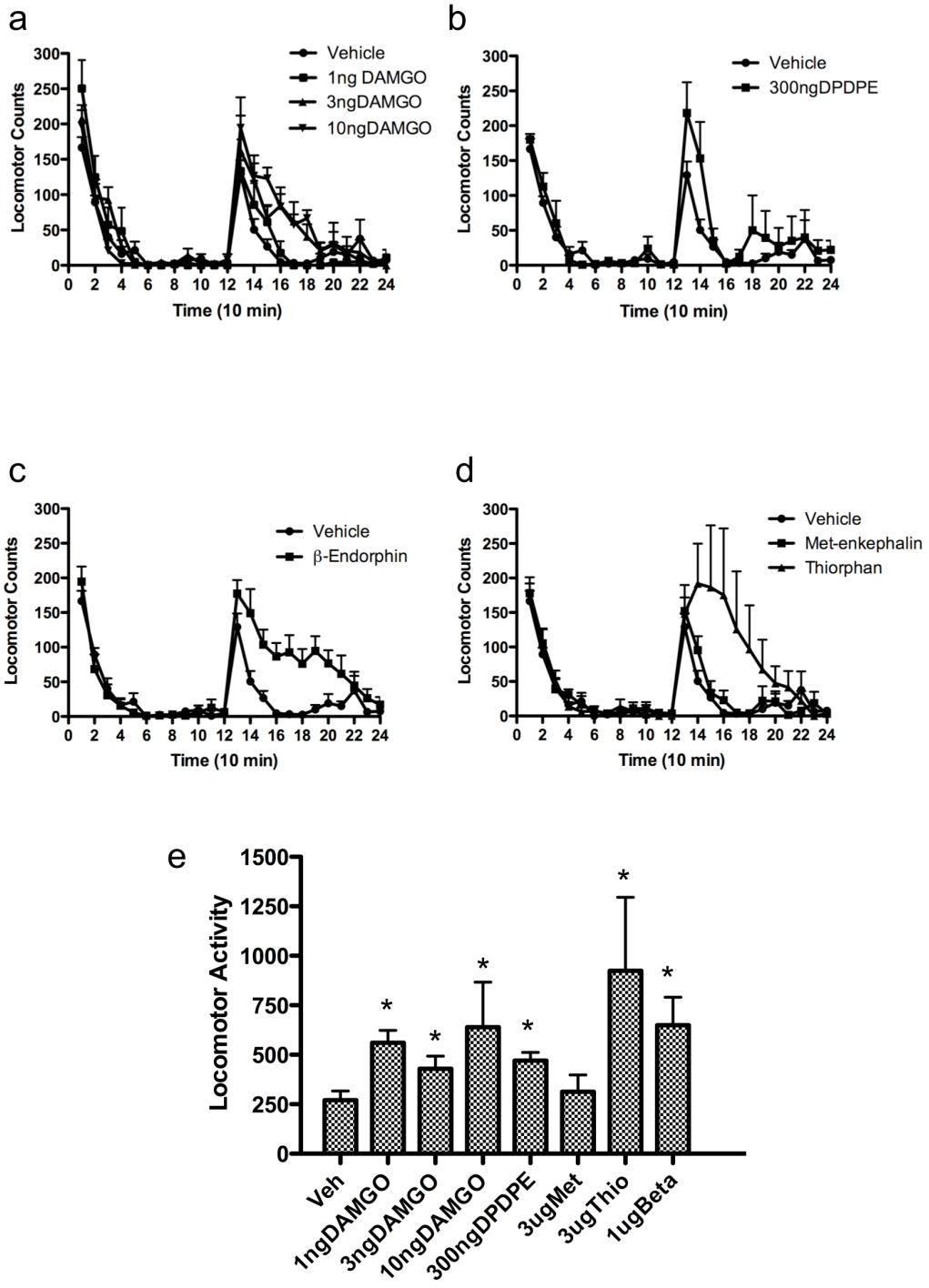


Figure 5.5 Timecourse of locomotor counts for (a) DAMGO, (b) DPDPE, (c) β-endorphin, and (d) thiorphin and met-enkephalin. (e) Locomotor activity for first hour of testing shows all drugs increased behavioral activity compared to vehicle and met-enekephalin.

specific agonists for MOR and DOR, respectively. Future studies would test endogenous ligands.

Figure 5.6a shows locomotor behavior during the first test hour for all animals tested following short- or long-term withdrawal (1 w WD and 6 w WD, respectively). Figures 5.6b-d show locomotor patterns for all groups tested with different DAMGO doses while Figure 5.6e shows patterns for DPDPE. These data were further divided for better visualization in Figure 5.7. Figure 5.7a shows locomotor data for 1 ng DAMGO in 1 w and 6 w WD CSA animals. There was no difference between groups despite the increased MOR expression seen at 6 w WD. However there appeared to be different basal locomotor levels between groups as seen in the habituation phase (first 2 h) of the test. Figure 5.7b compares behavior in 6 w WD CSA animals in response to 1 ng DAMGO infusion and vehicle. There was no increase in locomotor responses to intra-NAc DAMGO infusions. Figure 5.7c compares 1 and 6 w WD CSA animals following 3 ng DAMGO infusions. There was no difference between groups and again, 3 ng DAMGO did not increase locomotor behavior compared to vehicle in 6 w WD CSA animals (Figure 5.7d). Figure 5.7e shows no difference in 1 and 6 w WD CSA animal behavior in

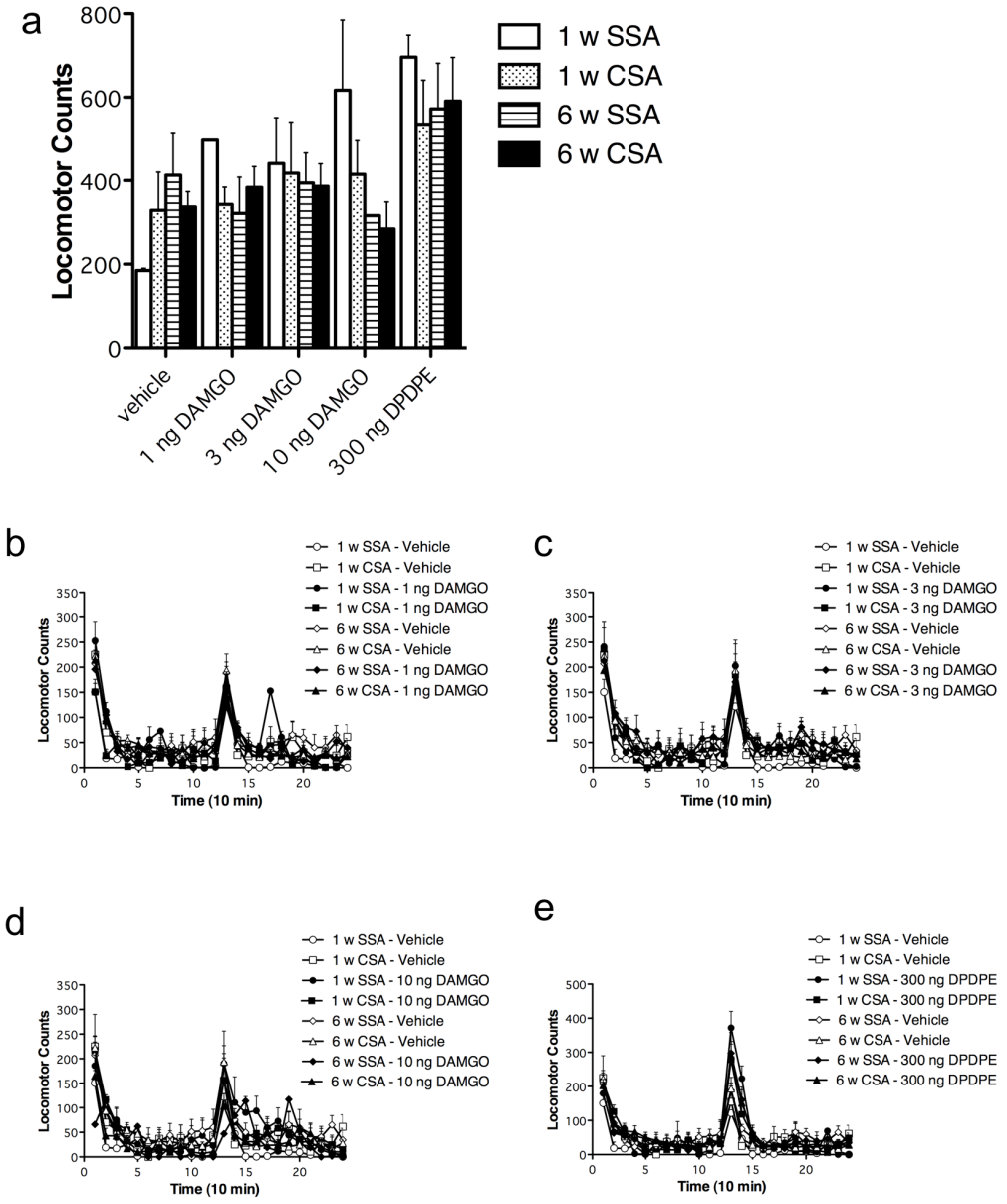
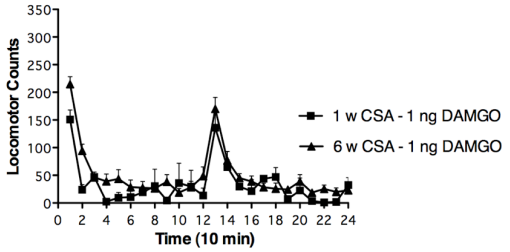


Figure 5.6 (a) Timecourse of locomotor behavior for 1 hour following drug injection in all groups tested reveal no effect of treatment or condition. (b) Timecourse of locomotor habituation (2 h) and test (2 h) after 1 ng DAMGO. (c) Timecourse of locomotor habituation and test for 3 ng DAMGO, (d) 10 ng DAMGO, and (e) 300 ng DPDPE.

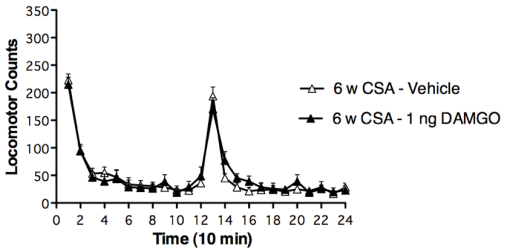
response to intra-NAc core 10 ng DAMGO infusions, with an apparent basal locomotor difference between groups seen during habituation. Figure 5.7f reveals a suppression by 10 ng DAMGO on behavior in 6 w WD CSA animals compared to vehicle infusions. As expected, 300 ng DPDPE infusions increased locomotor behavior similarly between 1 w and 6 w WD animals (Figure 5.7g) and increased behavior in 6 w WD animals compared to vehicle infusions (Figure 5.7h).

The negative data in response to DAMGO infusions may indicate age and cocaine mediated differences between groups. Indeed, the locomotor response to DAMGO seen in naïve animals was lost in these CSA animals. In addition, there were some apparent basal locomotor differences between 1 w and 6 w WD CSA animals at 1 and 10 ng DAMGO (Figure 5.7a and 5.7e). In analyzing the data for all these groups, it was determined that in many cases, locomotor baselines (vehicle infusion) were different in the groups since 6 w WD animals were much older than 1 w WD animals and also there appeared to be locomotor baseline differences between saline and cocaine self-administering animals (Figure 5.8a). When average locomotor behavior was normalized to vehicle injections in SSA animals at both withdrawal time points, there was an effect of group ($F_{1,4} = 20.360$, $p < 0.001$) and a trend for an effect

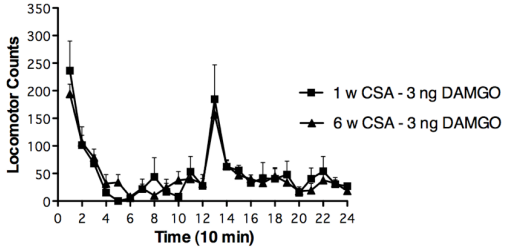
a 1 ng DAMGO in 1w and 6w WD CSA Animals



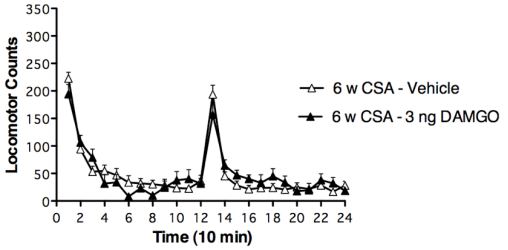
b 1 ng DAMGO in 6w WD CSA animals



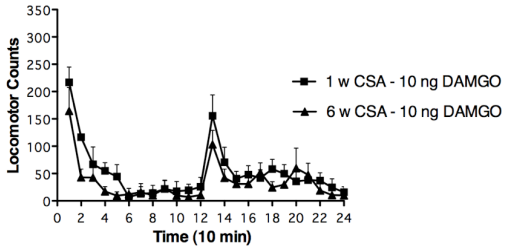
c 3 ng DAMGO in 1w and 6w WD CSA Animals



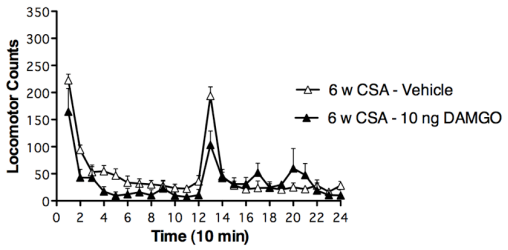
d 3 ng DAMGO in 6w WD CSA animals



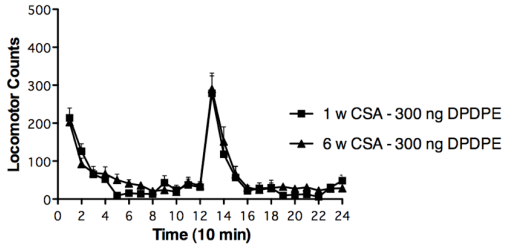
e 10 ng DAMGO in 1w and 6w WD CSA Animals



f 10 ng DAMGO in 6w WD CSA animals



g 300 ng DPDPE in 1w and 6w WD CSA Animals



h 300 ng DPDPE in 6w WD CSA animals

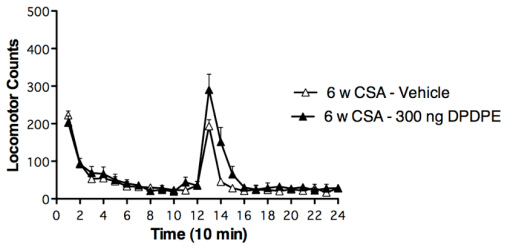


Figure 5.7 Timeline of locomotor data for 1 w and 6 w WD CSA animals in response to drug treatment. (a) Locomotor behavior in response to 1 ng DAMGO infusion into the NAc for 1 w and 6 w WD CSA animals reveal basal loocomotor differences between groups. (b) 6w WD CSA animals did not increase locomotor activity in response to 1 ng DAMGO, compared to behicle treatment. (c) Locomotor data for 3 ng DAMGO in 1 w and 6 w WD CSA animals showed no difference between groups and (d) 3 ng DAMGO did not increase behavior compared to vehicle infusions at 6 w WD. (e) 10 ng DAMGO treated animals showed differences in basal locomotor data between 1 w and 6 w WD CSA animals (f) with suppression of locomotor behavior at 6 w WD compared to vehicle. (g) 300ng DPDPE stimulated locomotor behavior were not different between 1 and 6 w WD CSA animals and (h) 300 ng DPDPE increased locomotor behavior compared to vehicle in 6 w WD CSA animals.

of dose ($F_{4,24}$ = 2.438, p = 0.075, NS). These findings justify normalizing the locomotor data to vehicle infusions since SSA, as well as CSA, groups appear to have baseline differences possibly due to age. Therefore, Figures 5.8b and 5.8c account for baseline differences between groups by normalizing the behavioral data to vehicle injections in each group. In 1 w WD animals that self-administered cocaine, responses to DAMGO and DPDPE infusions were blunted as seen in Figure 5.8b (group: $F_{1,4}$ = 13.352, p = 0.001, drug: $F_{4,22}$ = 2.251, p = 0.07, drug x group: $F_{4,22}$ = 1.241, p = NS). There was a significant difference in only in saline self-administering animals in response to 10 ng DAMGO (Fischer's LSD *post hoc* test: $p < 0.05$) or 300 ng DPDPE (Fischer's LSD *post hoc* test: $p <$ 0.05) infusion into the NAc core compared to vehicle. The other doses were also increased, however the number of animals in each group were low and more animals are required for further analysis.

Overall, in animals that self-administered cocaine, it appeared that they were less sensitive to MOR and DOR infusions since their behavior was significantly less compared to saline self-administering animals at 1w WD. In 6w WD animals, there was an effect of drug (Figure 6.9c; $F_{4,59}$ = 2.525, p = 0.05) but not group ($F_{1,4}$ = 1.011, p = NS), with only 300ng DPDPE increasing locomotor behavior. There was an overall lack of

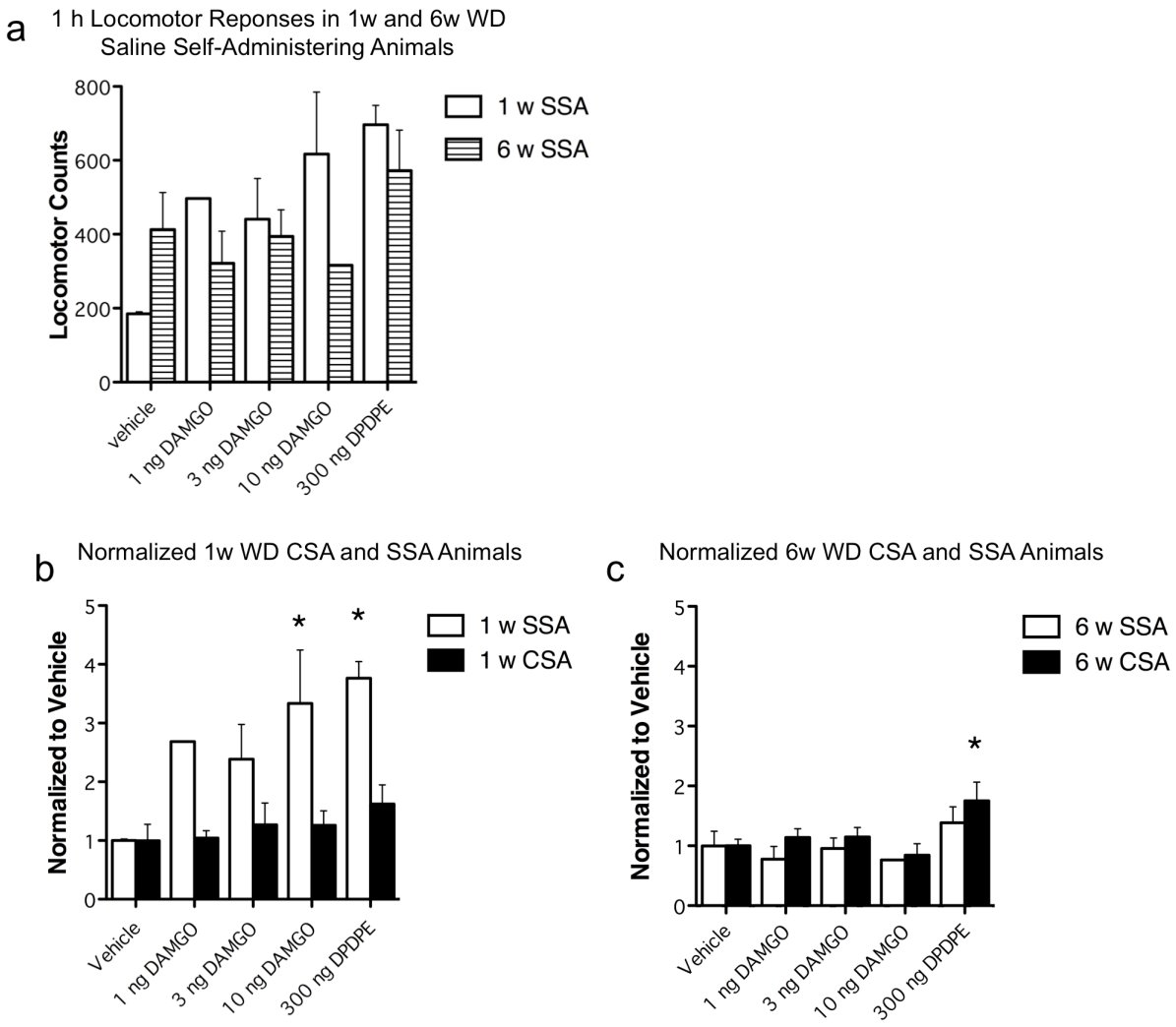


Figure 5.8 (a) 1 h locomotor counts for 1 w and 6 w SSA groups show baseline differences between animals. (b) and (c) data are normalized to averaged vehicle responses 1 and 6 w WD animals, respectively. (b) DAMGO and DPDPE increased locomotor behavior in SSA 1w WD animals but this effect was blunted in 1 w CSA animals. (c)Animals in 6 w WD had an overall lack of behavioral response to agonist infusion indicating a possible age effect on locomotor behavior.

effect of drug in both groups on locomotor behavior that may be attributed to age in the animals. However, these data are preliminary and the numbers per group are low therefore further experiments are required before any conclusion can be reached. In the future, animals should be started in the self-administration paradigm at an earlier age and possibly using 3w WD (where MOR first starts to increase) compared to 3d WD since these are time-points that other studies have utilized and may alleviate the flattening of locomotor behavior that was seen in these studies at 6 w WD.

Drug-Seeking Behavior and Reinstatement Induced by MOR agonists

Following locomotor testing, cocaine trained withdrawal animals were placed back in the self-administration chambers following an intra-NAc core infusion of 1ng DAMGO, 1ug β-endorphin, or vehicle (see Figure 5.9a for timeline example). Responding on the drug-paired and inactive levers was recorded in the absence of cocaine, indicating drug-seeking behavior. Animals were allowed to extinguish responding for an additional 5 hours and then tested for reinstatement of drug-seeking behavior with an injection of 1 ng DAMGO, 1 ug β-endorphin, or vehicle. Animals were tested on a subsequent day for reinstatement by another dose of drug

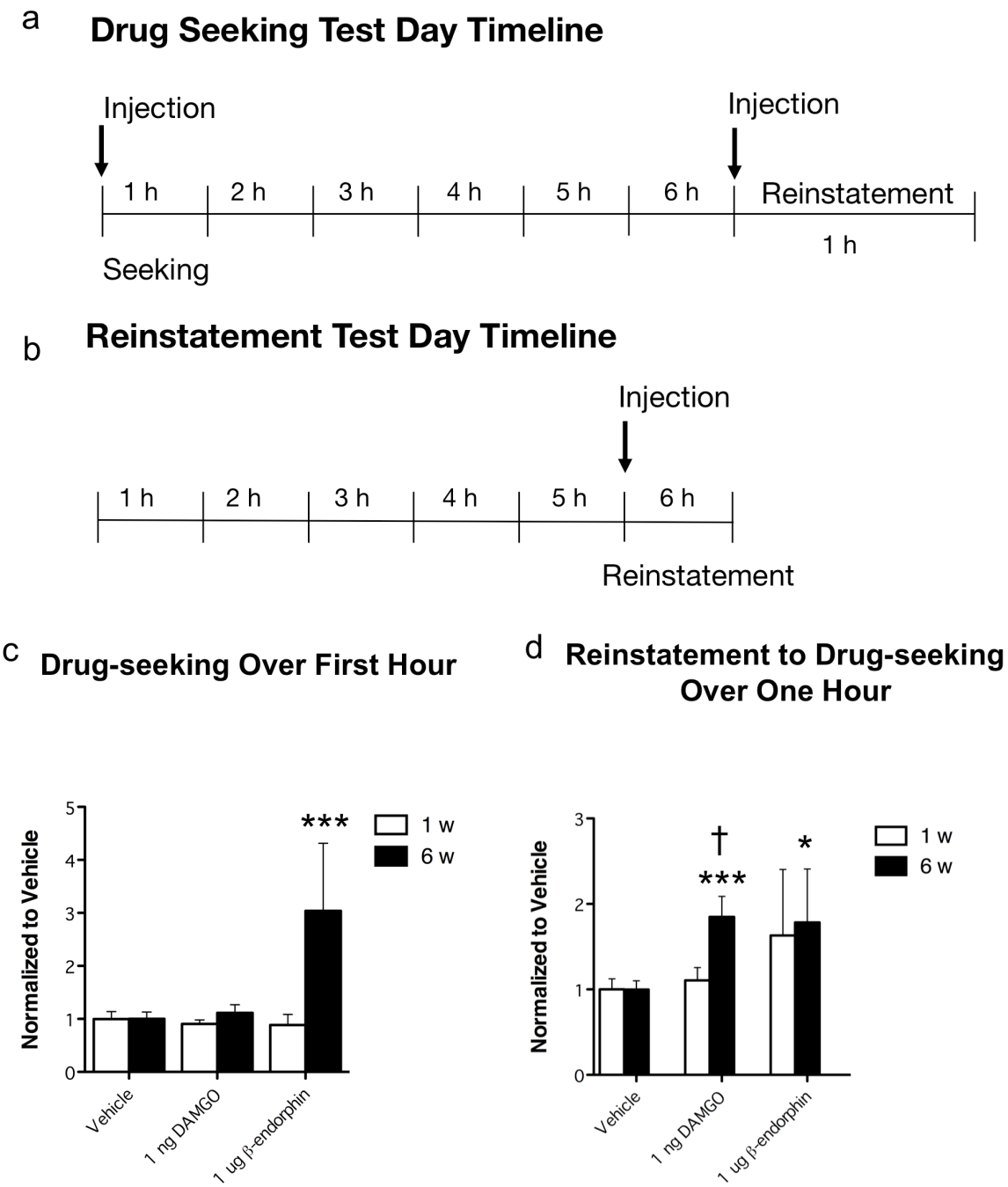


Figure 5.9 (a) Diagram of drug seeking test day with reinstatement after 5 h extinction and (b) diagrams an additional day of reinstatement following 5 h extinction. (c) Drug seeking measured over the first hour revealed β-endorphin increased drug-seeking at 6 w WD with no effect at 1 w WD. DAMGO had no effect in either group. (d) DAMGO and β-endorphin reinstated drug-seeking in 6w WD animals only indicating potential desensitization of MOR at 1 w WD. Symbols indicate differs from vehicle (* $p < 0.05$, *** $p < 0.001$) and 6w WD differs from 1 w WD DAMGO and vehicle infusions († $p < 0.05$).

(see Figure 5.9b for example). For reasons described in the previous paragraph, responding was normalized to vehicle behavioral measures.

When animals were tested for cocaine seeking behavior immediately following their respective withdrawal time periods, there was a main effect of withdrawal time (Figure 5.9c; $F_{1,2} = 15.541$, $p < 0.001$) and treatment ($F_{2,48} = 7.133$, $p = 0.002$) and an interaction of withdrawal time and treatment ($F_{2,48} = 8.234$, $p = 0.001$). DAMGO infusions (1 ng) did not potentiate drug-seeking compared to vehicle treated animals in either 1 w or 6 w WD groups. However, β-endorphin (1 μg) potentiated drug seeking in 6 w withdrawal animals compared to 1 w WD animals and compared to vehicle treatment (Fischer's LSD *post hoc* test: $p = 0.002$).

Following extinction training, animals reinstated to cocaine-seeking behavior when treated with DAMGO or β-endorphin (Figure 5.9d; treatment: $F_{2,66} = 5.371$, $p = 0.007$, treatment x withdrawal time: $F_{2,66} = 2.379$, $p = 0.01$), however there was no effect of withdrawal time on reinstatement of drug-seeking behavior ($F_{1,2} = 2.02$, $p =$ NS). DAMGO stimulation at 6 w WD significantly increased reinstatement to cocaine seeking (Fischer's LSD *post hoc* test: $p = 0.001$), as did β-endorphin (Fischer's LSD *post hoc* test: $p = 0.043$) when compared to vehicle treated controls. Reinstatement of cocaine seeking in 6 wWD animals by DAMGO

was different from all other treatments except β-endorphin (Fischer's LSD *post hoc* test: $p < 0.01$). There was no significant behavioral result at the 1 w WD time-point for reinstatement of cocaine seeking, however there was a trend for an increase by β-endorphin treatment. More animals are required to determine if this is a real effect. These findings indicate that β-endorphin stimulation of MOR induces greater cocaine seeking after 6 w WD compared to 1 w WD, and that either DAMGO or β-endorphin stimulation of MOR effectively reinstates cocaine seeking at 6 but not 1 w WD. While a decrease in NAc MOR levels was not found after 1 w WD, the lack of effect of MOR agonists at this time suggests that MOR may still be in a desensitized state.

Discussion

The initial goal of this chapter was to identify a change in the NAc, a region important for cocaine self-administration and relapse behaviors, that paralleled the behavioral phenotype of increased craving during cocaine abstinence, the so called "Cocaine Abstinence Effect". This study showed that with long-term withdrawal (6 weeks), initial drug seeking following withdrawal and relapse to drug seeking following extinction training increases compared to short term withdrawal (1 week). This

finding was first reported by other groups and is a reliable model of human drug addict craving and relapse behaviors (Grimm et al. 2001; Tran-Nguyen et al. 1998).

Following validation of the behavioral paradigm, genetic changes were identified in NAc core and shell subregions using microarray analysis (in collaboration with Novartis) followed by confirmation of gene changes at the level of protein expression using Western blot. Microarray profiling identified several changes in gene expression, many of which were not changes at the level of protein expression. Protein confirmation studies were limited by antibody availability for Western blot tests. With time, other protein targets may present themselves when antibodies are commercially available. For those genes that were altered at the protein level, it was interesting to discover that changes in gene expression did not always parallel changes in protein expression. In the NAc shell, Kv4.3 (voltage-gated A-type potassium channel) was increased at the mRNA level, but decreased at the protein level at six weeks of withdrawal. Conversely, NAc core AKAP-84 (A-kinase anchoring protein 84, a splice isoform of AKAP121 that binds to the regulatory subunits of protein kinase A for mitochondria), was decreased at the mRNA level and increased at the protein level at six weeks of withdrawal. When the temporal regulation

of these protein changes were analyzed, Kv4.3 was increased 1 week after cocaine self-administration cessation, decreased at 6 weeks of withdrawal, and then normalized by 12 weeks of withdrawal. AKAP84 was high at 1 week of withdrawal, but steadily normalized by 12 weeks of withdrawal. Because these changes did not positively correlate with the Cocaine Abstinence Effect, they were not considered as targets for further analysis as modulators of cocaine addictive behaviors.

NAc core MOR, on the other hand, was increased both at the mRNA and protein level. This finding, paired with the timeline for increased MOR expression, where increases started at 3 weeks of withdrawal and persisted out to 12 weeks of withdrawal, indicated an up-regulation of overall MOR synthesis through transcriptional up-regulation and subsequent translation of MOR. Further, the steady increase in MOR expression that paralleled the addicted behavioral phenotype indicated this receptor as a promising target for further studies as a modulator of addictive behaviors. It is predicted that as MOR increases, so does craving and the propensity for relapse. Changes in MOR protein expression can be transcriptionally driven, the translational machinery could be primed for compensatory protein expression, or trafficking could be altered for increased expression. Since the findings in this study were

during long periods of time, the increased mRNA and protein regulation would indicate altered protein expression through translational changes in addition to gene driven expression. However, further studies are required to answer this question.

To determine if up-regulated MOR protein was functionally expressed, studies were carried out to differentiate a behavioral phenotype of higher MOR expression at long-term withdrawal from non-regulated MOR at short-term withdrawal. MOR agonist infusions into the NAc core were utilized to stimulate functional MOR at both time periods. Specifically, DAMGO, the MOR specific agonist, and β-endorphin, the endogenous peptide for MOR were used to determine their effects on MOR-stimulated locomotor behavior, cocaine-seeking behavior, and reinstatement of cocaine seeking. Results were preliminary, however they indicate a strong possibility for MOR stimulation increasing drug-seeking and relapse behaviors during long-term withdrawal. Stimulation of MOR by β-endorphin, but not DAMGO, potentiated initial cocaine seeking at 6 w withdrawal and not at one week of withdrawal indicating a time-dependent alteration in MOR function following cocaine self-administration and withdrawal. Both DAMGO and β-endorphin reinstated drug-seeking behavior in six-week withdrawal animals compared to vehicle infusions.

There was a trend for β-endorphin increasing cocaine seeking during reinstatement tests in one-week withdrawal animals, although this was not significant. Interestingly, DAMGO did not reinstate cocaine-seeking behavior at one of week withdrawal. Although preliminary, this finding paired with the non-significant β-endorphin finding indicate MOR at this time point may be in a desensitized state. Further research is required to answer this possibility.

This study found that long-term up-regulation of MOR protein in NAc core rises slowly from early to late withdrawal and is accompanied by increases in mRNA levels. Interestingly, earlier reports of changes in MOR protein levels in rodents were not paralleled by changes in MOR mRNA levels with chronic cocaine (Azaryan et al. 1998; Azaryan et al. 1996b). Instead, MOR mRNA was found to increase transiently upon experimenter-administered cocaine, peaking at day 3, and returning to baseline by day 4, whereas [3H]DAMGO binding to MOR in NAc was increased on day 4 but not changed at days 7 or 14 of continuous cocaine delivered by osmotic minipump (Azaryan et al. 1998). Another study found no change in NAc MOR protein following 14 days of an escalating binge cocaine paradigm (Bailey et al. 2005a), despite earlier studies that found chronic binge cocaine increased [3H]DAMGO binding (Unterwald et

al. 1992; Unterwald et al. 1994). Withdrawal from escalating binge cocaine increases [(3)H] Tyr-D-Ala-Gly-MePhe-Gly-ol ([(3)]DAMGO) binding to MOR in limbic brain regions, including the anterior cingulate and CPu (Bailey et al. 2005a). Alternatively, another group found that MOR are down-regulated following withdrawal from chronic intermittent cocaine in the NAc core and limbic cortical layer as shown by [(125)I]DAMGO binding (Sharpe et al. 2000). These studies indicate MOR regulation differs across studies and paradigms during cocaine administration and withdrawal. However, these studies utilized the more stressful experimenter delivered cocaine whereas this thesis project utilized cocaine self-administration, a model of human cocaine consumption.

The significance of MOR up-regulation during cocaine withdrawal is revealed by the human literature. Human cocaine addict studies find a correlation with MOR regulation in key brain regions and craving as well as with time to first relapse and severity of relapse. A positron emission tomography (PET) study found that male cocaine addicts had increased levels of MOR binding that positively correlated with their self-reported increased craving for cocaine during abstinence (Gorelick et al. 2005; Zubieta et al. 1996). This up-regulation persisted for over 4 weeks of monitored cocaine abstinence. Using the Minnesota Cocaine Craving

Scale, severity of craving positively correlated with MOR binding in the amygdala, anterior cingulate cortex, frontal cortex, and temporal cortex. Further, the degree of relapse severity and the time to first relapse episode positively correlated with MOR increases (Gorelick et al. 2008). Human studies report findings that parallel data reported in this thesis. In human cocaine abusers who died with cocaine or its metabolites present at death had decreased MOR binding in the striatum (Hurd and Herkenham 1993). Similarly, this thesis work found decreased MOR immediately following cocaine administration (Chapter 3), an effect that normalized by 24 hour after session, and was accompanied by increased MOR mRNA. Together with data shown in this chapter, where MOR protein levels gradually increase during increased periods of abstinence, indicate an up-regulated expression system for MOR in the NAc core that is driven by mRNA. However, further studies are required to determine the mechanism behind increased MOR expression during withdrawal. These findings further implicate MOR as a potential modulator of cocaine craving during abstinence.

Several studies in rodents indicate MOR as an integral part of modulating cocaine reward and addiction. When MOR are down regulated using antisense oligodeoxynucleotides to MOR (AS ODN),

conditioned place preference (CPP) and cocaine sensitization are decreased (Hummel et al. 2006). Similarly, blockade of MOR with the MOR specific antagonist, CTAP (D-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH2) given icv decreases CPP, locomotor sensitization, cocaine induced hyperactivity (Schroeder et al. 2007). Further, pretreatment with CTAP in the NAc core during conditioning with cocaine prevents CPP (Soderman and Unterwald 2008). The locomotor activating effects of cocaine are reduced in MOR KO animals (Chefer et al. 2004; Yoo et al. 2003), however these effects can differ depending on mouse strain (Hummel et al. 2004). Cocaine self-administration is decreased in MOR knock-out mice (Mathon et al. 2005 2005). Reinstatement of cocaine seeking by cocaine priming progressively attenuates with repeated pretreatment with the opioid antagonist, naltrexone (Gerrits et al. 2005) and cue-induced reinstatement is blocked by naltrexone (Burattini et al. 2008). Further, pretreatment with buprenorphine dose-dependently suppresses cocaine-primed reinstatement (Comer et al. 1993). These findings paired with our finding of increased MOR during withdrawal indicate the involvement of MOR in mediated craving and relapse seen in humans and rodents.

An understanding of the corticolimbic circuit involvement in cocaine addiction is important in delineating NAc core involvement in relapse

behaviors. In human cocaine addicts taking cocaine, BOLD fMRI imaging revealed a negative correlation in anterior cingulate and NAc activity but increased activity when in a state of craving (Risinger et al. 2005). Cue induced cocaine craving also increases activity in the anterior cingulate (Childress et al. 1999; Garavan et al. 2000; Wexler et al. 2001) and NAc (Breiter et al. 1997) of cocaine addicts. The NAc core receives major projections from the anterior cingulate and dorsocaudal prelimbic cortices (dorsal prefrontal cortex) (Gorelova and Yang 1997; Zahm and Brog 1992), whereas the shell receives input from the ventral prelimbic and rostral infralimbic cortices (ventral prefrontal cortex) (Gorelova and Yang 1997; Zahm and Brog 1992). Microdialysis in rats self-administering cocaine show increased dopamine release in the NAc shell compared to baseline whereas in the core this effect was less substantial (Hedou et al. 1999). Cocaine administration significantly decreases dorsal and ventral prefrontal cortical activity yielding a significant negative relationship between dopamine levels in the ventral and dorsal medial prefrontal cortices and dopamine concentrations in the shell and core of the NAc (Hedou et al. 1999). Hence, dorsolateral glutamate release into the core is thought to be more specific in the expression of cocaine and other

psychostimulant related behaviors while dopamine is more involved in the shell (Hedou et al. 1999; Pierce and Kalivas 1997; Pierce et al. 1998).

Further, the chronic up-regulation of NAc MOR and increased drug seeking and reinstatement of drug seeking demonstrated in this project correlates with many findings found in human studies. Cocaine abusers that are abstinent from cocaine use have increased MOR binding in several brain regions that positively correlate with self-report cocaine craving (Zubieta et al. 1996). MOR binding is increased in the anterior cingulate and striatum 1-4 d after the last cocaine use and positively correlated with severity of cocaine craving. The percent of days spent taking cocaine prior to admittance to the trial was correlated with higher MOR levels in the anterior cingulate, and anterior cingulate MOR levels remained elevated out to 12 weeks (Gorelick et al. 2005). All three modes of reinstatement: stress, cue, and drug, are thought to converge on the anterior cingulate that then signals through the NAc core (Kalivas and McFarland 2003). MOR alterations in the core would modulate responsiveness through β-endorphin signaling. Increased glutamatergic signaling into the NAc from the anterior cingulate during cocaine craving paired with MOR activation in the NAc would increase the likelihood for relapse to occur.

Increased MOR levels can mediate increased craving induced by stress, drug-associated cues, or small doses of cocaine since all of these stimuli increase β-endorphin release in the NAc. Any stimulation by beta-endorphin would potentiate craving since higher concentrations of available MOR binding sites are present during withdrawal, thus heightening the craving state and increasing the likelihood of relapse. In fact, increased MOR levels served as an independent predictor of time to relapse in cocaine addicts (Gorelick et al. 2008). As such, high dose buprenorphine, a partial MOR agonist, significantly reduces cocaine and opiate use in outpatients dependent on cocaine and opiates (Montoya et al. 2004). The high dose of buprenorphine would occupy much of the available MOR binding sites (Zubieta et al. 2000), therefore decreasing the euphoric properties attributed to cocaine by β-endorphin release in the NAc (Roth-Deri et al. 2008). Additionally, occupation of MOR binding sites would modulate anterior cingulate glutamatergic signaling that would otherwise further increase drug craving, since the anterior cingulate of cocaine addicts exhibits increased activity that is associated with cocaine craving (Daglish and Nutt 2003; Goldstein and Volkow 2002).

Cocaine addiction treatments based on targeting opioid receptors are under current investigation. Naltrexone, an opioid receptor antagonist,

in conjunction with cognitive behavioral therapy decreases craving in human cocaine addicts (Grassi et al. 2007) and decrease cocaine use (Schmitz et al. 2001). In response to an acute cocaine dose, addicts reported decreased "good effects" when treated with naltrexone (Sofuoglu et al. 2003). However, naltrexone did not decrease subjective reports of craving when shown cues associated with cocaine use (Modesto-Lowe et al. 1997). When 3-fold higher doses of naltrexone were utilized in combination with psychosocial treatment, severity of cocaine use decreased (Pettinati et al. 2008). Chronic buprenorphine or methadone (MOR agonist) treatment reduced relapse to cocaine use in humans (Peles et al. 2006; Vigezzi et al. 2006) and intake in rats (Leri et al. 2004; Sorge et al. 2005). Further, in heroin addicts with ongoing cocaine addiction, there is an "endorphin deficiency" (Schluger et al. 2001), pointing to a mechanism for buprenorphine and methadone mediated decrease in drug use. By maintaining agonist binding to MOR during drug abstinence, craving may be decreased or alleviated. MOR activity in response to stress-induced or drug-induced β-endorphin release may be blocked through a mechanism of opioid tolerance and cross-tolerance (Dole et al. 1966; Kreek 2000).

Methadone causes rapid receptor internalization (Keith et al. 1998) and causes greater agonist-induced MOR desensitization (Yu et al. 1997), which may account for decreased euphoria experienced with cocaine use. Buprenorphine has a slow onset and long duration, which may further alleviate craving during cocaine abstinence. Buprenorphine was shown to reduce heroin and cocaine self-administration in addicted men and also decreased the severity of the addiction (Gastfriend 1993; Montoya et al. 2004). Chronic buprenorphine treatment decreases MOR levels in several limbic brain regions (Belcheva et al. 1993) while naltrexone increases MOR expression (Lesscher et al. 2003). Hence it is hypothesized that chronic buprenorphine treatment paired cognitive behavioral therapy in cocaine addicted individuals would lead to normalization of the opioid system allowing for progressively less occurrences of craving and relapse and finally cessation of cocaine use. In summary, these studies support our findings that MOR is involved in relapse to cocaine seeking and that modulation of this receptor during prolonged abstinence may increase the propensity for relapse indicating MOR as a viable target in the treatment of cocaine addiction.

CHAPTER SIX:
CONCLUSIONS AND DISCUSSION

This thesis work studied the regulation of MOR by cocaine self-administration and withdrawal, and the involvement of opioid receptors in relapse to cocaine seeking. Immediately following chronic voluntary or involuntary cocaine administration, MOR protein was decreased whereas DOR was not. Regulation of MOR in cocaine self-administering animals was mediated through chronic β-endorphin stimulation of MOR, since relative pMOR levels were increased. Further support of this finding is that blockade of β-endorphin interaction with MOR through use of an antibody against β-endorphin prevented MOR phosphorylation, endocytosis and down-regulation. Regulation of MOR during non-contingent cocaine administration appears to be through a different mechanism since relative pMOR was not increased following cocaine exposure. Evidence suggests stress may decrease MOR expression through a pathway not involving β-endorphin itself. In contrast, both contingent and non-contingent cocaine administration were associated with increased mRNA for MOR indicating compensatory up-regulation of MOR transcription during cocaine exposure allowing for nomalization of

MOR levels by one day and one week of withdrawal. Additionally, this thesis showed that NAc MOR and DOR stimulation reinstates cocaine-seeking behavior further implicating opioid involvement in cocaine addiction. Withdrawal from cocaine self-administration increased MOR mRNA and protein in the NAc and stimulation of these receptors potentiated cocaine seeking and relapse to cocaine seeking.

In general it is postulated that long-lasting neuroadaptations occur in the mesolimbic dopaminergic reward circuit during drug use that mediates addictive behaviors and subsequently craving and relapse behaviors during abstinence. Ways in which repeated drug use can cause neuroadaptations include altered transcription of genes, altered processing of RNA, altered translation of mRNA into proteins, altered processing of proteins, and changes in protein trafficking, to name a few. This project found a neuroadaptation (MOR regulation) in the NAc that occurs during cocaine self-administration and ultimately modulates craving and relapse behaviors. Figure 6.1 is a hypothetical model of what occurs during cocaine self-administration and withdrawal.

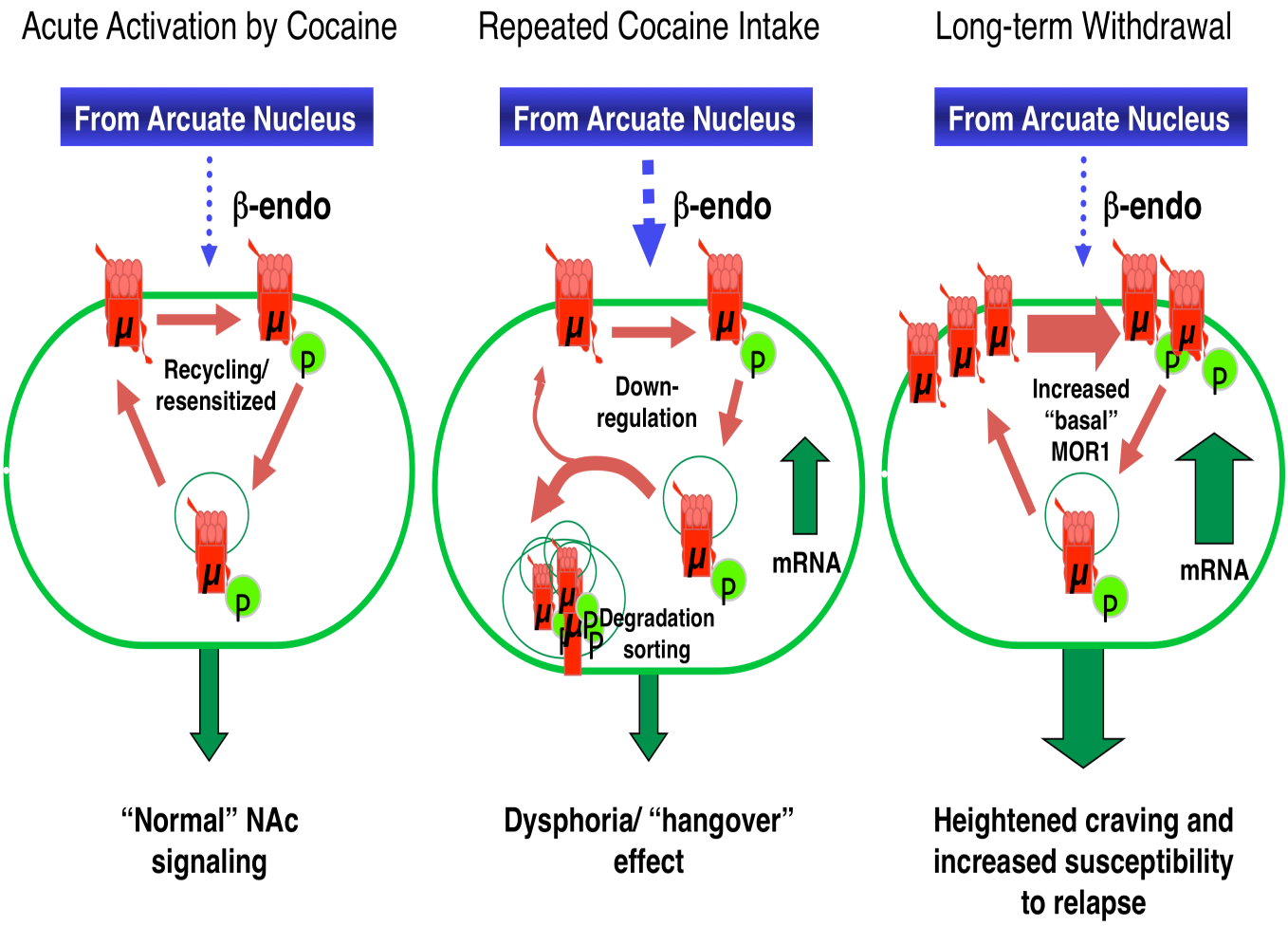


Figure 6.1 Hypothetical model of NAc MOR regulation during cocaine self-administration and withdrawal. Acute cocaine administration releases beta-endorphin into the NAc where it stimulates MOR. This acute stimulation of MOR results in normal signaling, phosphorylation, endocytosis, and recycling back to the membrane in a resensitized state. However, with chronic cocaine administration, beta-endorphin in chronically released into the NAc where MOR recycling shifts to one of degradation. This increase in MOR degradation is accompanied by increased MOR mRNA synthesis. Cessation of cocaine at this time contributes to dysphoria and a "hang-over" effect. With long periods of abstinence, the MOR transcription and synthesizing machinery remains up-regulated indicating a new set-point for MOR synthesis. Hence MOR levels increase and this increase is associated with heightened craving during abstinence in response to any beta-enorphin stimulation by stress or rewarding stimuli.

With acute cocaine administration, MOR are stimulated by β-endorphin release from the arcuate nucleus of the hypothalamus (Arc). This stimulation results in ligand-binding dependent phosphorylation of the serine 375 site of MOR resulting in the recruitment of β-arrestins and supporting endocytic machinery to internalize the stimulated receptors. Following endocytosis, the receptors are dephosphorylated and preferentially sorted back to the membrane for subsequent stimulation. With chronic cocaine administration, β-endorphin is chronically being released into the NAc, thus chronically stimulating MOR, resulting in a shift in the recycling pattern of MOR. This shift results in greater degradation of MOR rather than recycling back to the membrane. This shift is paired with increase MOR mRNA synthesis to compensate for this change in MOR regulation. Upon cessation of cocaine, an individual is in a state of dysphoria and with longer abstinence individuals often find themselves in a state of depression. However, MOR expression becomes normalized by 1 day and 1 week of withdrawal since the synthesis of MOR was upregulated during chronic cocaine exposure. This represents a new "set-point" for an individual: an overall up-regulation of the MOR expression system. This regulation accounts for the steady increase in MOR levels in the NAc with longer periods of abstinence. Hence, during long-term

withdrawal, there is an increase of MOR so that any β-endorphin release in response to stress, cues, or other stimuli would potentiate MOR signaling, thereby increasing craving and the potential for relapse.

In the search for molecular mechanisms of cocaine and other drug addictions, the cAMP (cyclic Adenosine-Mono-Phosphate) pathway received much attention and may tie in to MOR regulation. In the NAc, repeated drug administration increases enzyme activity levels of adenylate cyclase (AC) and cAMP-dependent kinase (PKA) while decreasing protein levels of the inhibitory G-protein (Nestler et al. 1990; Terwilliger et al. 1991), leading to an overall increase in cAMP-dependent signal transduction, an event that is most obvious when drug is removed. However, despite similar changes in this system across several drugs of abuse and other neurological disorders, the mechanisms behind this phenomenon remains to be fully understood.

Drug stimulation of inhibitory G-protein coupled receptors such and MOR activates $G_{i/o}$ GTP-binding proteins, leading to their dissociation from MOR into $G_{i/o}\alpha$ and $G\beta\gamma$ dimers (Birnbaumer et al. 1990; Childers et al. 1992). $G\alpha$ acts to inhibit AC, resulting in decreased cAMP levels (Birnbaumer et al. 1990). However, with chronic drug administration cAMP levels accumulate (Ammer and Schulz 1993; Avidor-Reiss et al.

1995), $G_{i/o}$ proteins become down-regulated (Nestler et al. 1990), and AC and PKA activity increases to compensate for chronic drug exposure. This accumulation is through pertussis toxin sensitive $G_{i/o}$ protein activation since cAMP accumulation in response to chronic stimulation is prevented by pertussis toxin treatment (Avidor-Reiss et al. 1996). This phenomenon is called AC superactivation where the removal of inhibitory G-protein receptor stimulation results in hyperactivity of the AC system or AC "overshoot" as a result of increased AC enzyme activity, cAMP accumulation, and accompanying alterations (Childers 1991; Nestler 1992). The mechanism behind this phenomenon is not fully understood, but is thought to involve alterations in gene transcription and subsequent protein expression.

Hence, subsequent studies show the activation of CREB (cAMP Response Element Binding Protein), a transcription factor activated by PKA, increases in response to chronic cocaine and other drugs of abuse (Carlezon et al. 1998; Nestler 1992; 2001; Walters et al. 2003). Repeated cocaine or morphine treatment cause several cAMP-related neuroadaptations through altered transcription of genes by CREB (Nestler 2001). Generally, it is thought that up regulation of the cAMP pathway and CREB in the NAc mediates tolerance and dependence to drugs of abuse

while deltaFosB mediates increased sensitization to drugs (Nestler 2004). CREB and related proteins mediate the effects of cAMP second messenger pathways on gene expression (Mayr and Montminy). CREB is activated through PKA phosphorylation of CREB (pCREB), allowing it to interact with cAMP Response Element (CRE) sites on genes to regulate their transcription (with the help of several other regulatory and binding proteins). The importance of this common mechanism across several drug addictions is demonstrated by findings where manipulation of CREB activity modulates drug reward and dependence. For example, CREB knock-out mice show attenuated opiate physical dependence and withdrawal (Maldonado et al. 1996). Viral-mediated over-expression of CREB in the NAc decreases the rewarding properties of morphine and cocaine whereas blocking CREB activity increases them (Barrot et al. 2002; Carlezon et al. 1998). Further, CREB over-expression decreases reinforcing properties of natural rewards such as sucrose, modeling behavior seen in human drug addicts. Similarly, mice over-expressing CREB show reduced sensitivity to drugs while those expressing mCREB show the opposite (McClung and Nestler 2003; Newton et al. 2002; Sakai and Kidokoro 2002). Thus, AC superactivation by chronic drug exposure would increase activation of CREB, changing regulation of gene targets

and subsequent neural signaling and processing. Overall, this would lead to changes in behavioral responses to drugs of abuse.

Several genes are shown to have CRE-sites and may be regulated by CREB, however in the NAc, these genes are not fully identified (Mayr and Montminy 2001). One gene best characterized in the NAc is dynorphin, where chronic cocaine and other drugs induces dynorphin expression in the NAc and is CREB dependent (Carlezon et al. 1998). In relation to MOR, there is a putative CRE-binding site (Lee and Lee 2003). PC-12 cells (pheochromocytoma cells) endogenously express MOR and the CRE-site was identified in the promotor region of MOR. To determine how MOR mRNA are increased by chronic stimulation, researchers stimulated AC activity and examined CRE-site binding by CREB and CREB-binding protein (CBP). Forskolin induction of AC and subsequent activation of CREB led to increased CREB and CBP binding to the endogenous MOR promotor, as shown by chromatin immunoprecipitation. When a dominant-negative CREB (CREB that can not be activated through phosphorylation) was introduced, forskolin-mediated MOR gene induction was blocked. Whether this occurs in vivo remains to be demonstrated, however this finding indicates a potential mechanism for

MOR regulation during chronic cocaine administration and subsequently in withdrawal.

Few studies have determined the effect of cAMP pathway changes on cocaine self-administration and relapse behaviors. Using intra-NAc infusions of cAMP analogs that activate (S_p-cAMPS) or inhibit (R_p-cAMPS) PKA, our group demonstrated that inhibition of PKA activity reduced cocaine self-administration and reinstated cocaine seeking behavior following extinction training (Self et al. 1998). Further, PKA inhibition shifted the dose response curve for cocaine to the left. These findings are consistent with enhancement of cocaine's effects by prevention of cAMP pathway activation. Using PKA activator infusions, cocaine self-administration base-lines increased and the dose response curve for cocaine shifted to the right, indicating tolerance and decreased sensitivity to cocaine. Hence, increased cAMP pathway activity and subsequent CREB activation causes tolerance and reduced sensitivity to drugs.

Another group used PKA activators (S_p-cAMPS) and inhibitors (R_p-cAMPS) in the NAc to see if this alters the motivation to self-administer cocaine (Lynch and Taylor 2005). Using a progressive ratio of cocaine reinforcement, where animals must work harder for each subsequent cocaine injection, PKA activation increased the motivation to obtain

cocaine whereas PKA inhibition decreased it. These behaviors persisted for several days after the NAc drug infusions, further implicating cAMP system in modulating addictive behaviors.

However, AC superactivation does not persist long enough to account for the addicted phenotype where the propensity for relapse increases with longer periods of abstinence (The Cocaine Abstinence Effect). In cells, AC superactivation is eventually lost following removal of drug (Avidor-Reiss et al. 1996). In vivo, increased PKA activity in the NAc was observed following 7 days cocaine treatment (Hope et al. 2005). This increase persisted for 7 days post-cocaine treatment, but was not apparent at 21 days when cocaine sensitization was still present. In animals self-administering cocaine, PKA activity was also increased in the NAc 1d and 30 d after cocaine abstinence, but not at 90 days, whereas AC activity was not changed (Lu et al. 2003). This would indicate downstream modifications by PKA activation of CREB leads to the addicted phenotype, potential modifications such as MOR up-regulation.

Neural implications of modulated MOR expression during chronic cocaine and long-term withdrawal can be extrapolated from several studies. Chronic stimulation of MOR by opioids decreases opioid receptor binding sites (Noble et al. 2000) and is accompanied by increased MOR

mRNA synthesis (Lee and Lee 2003; Yoshikawa et al. 2000). Endocytosis is important in the prevention of tolerance and dependence (Finn and Whistler 2001), hence superactivation of AC is not seen in cocaine withdrawal, despite the implications that chronic MOR stimulation during cocaine administration would intuitively cause this. Typically, phosphorylation and endocytosis prevents the occurrence of tolerance that is seen with chronic morphine treatment (Whistler et al. 1999). However, with compensatory up-regulation of the MOR expression system, the decrease in MOR seen immediately following cocaine self-administration becomes normalized by 1 day and 1 week of withdrawal and continues to increase during withdrawal. This increase in MOR expression, preliminarily shown to be functionally increased (Chapter 5), indicates a persistent up-regulation in the NAc. Thus stimulation of MOR during long periods of cocaine abstinence would result in reinstatement of drug seeking as seen in animals that were treated with inhibitors of PKA (Self et al. 1998), where animals reinstated to cocaine seeking after treatment with R_p-cAMPS.

Interestingly, MOR are regulated in humans addicted to various drugs of abuse, indicating the same limbic circuitry is involved in rewarding and addictive processes and that there may be a similar pharmacological

target between groups in the modulation of craving and relapse. Similar to the cAMP pathway that is increased in opiate, psychostimulant, and other drug administration in animal models of drug addiction, MOR is regulated by several salient stimuli: Food, stress, sucrose, ethanol, heroin, etc. in humans. For example, naltrexone administered during alcohol intoxication decreases the positive reinforcing stimulant effects and increases the sedative effects of alcohol (McCaul et al. 2000; Swift et al. 1994) and alcoholics consume less alcohol during naltrexone treatment (Drobes et al. 2003; O'Malley et al. 2002). Naltrexone reduces alcohol drinking, craving and relapse rates in alcoholics (O'Brien et al. 1996; O'Malley 1996). Similar to cocaine administration, alcohol administration increases beta-endorphin levels in several brain regions (Gianoulakis et al. 1983; Schulz et al. 1980). Further, in alcoholics abstaining from alcohol intake, there is a positive correlation with MOR binding potential (BP) in the anterior cingulate and the Beck Depression Inventory (BDI), a measure of mood (Bencherif et al. 2004). Another study found significant up-regulation of MOR in the ventral striatum (including the NAc) by PET imaging after 1 – 3 weeks of abstinence in alcoholic patients (Heinz et al. 2005). This increase remained elevated out to 5 weeks of abstinence and MOR levels positively correlated with the intensity of alcohol craving.

Heroin addicts have increase MOR BP in the anterior cingulate compared to controls, that is reduced when treated with buprenorphine (Zubieta et al. 2000). In healthy male smokers who abstain from smoking for one night, smoking denicotinized cigarettes increases activation of MOR neurotransmission in the anterior cingulate compared to nonsmoking controls (Scott et al. 2007). Further, MOR binding potential decreases when smoking a regular cigarette indicating modulation of opioid function by nicotine and cigarette smoking. In animal models, nicotine induces alterations in opioid peptide protein and mRNA in several brain regions (Houdi et al. 1998). Naloxone treatment precipitates a withdrawal syndrome in rats chronically treated with nicotine (Carboni et al. 2000), indicating chronic modulation of opioid receptors and/or AC superactivation. These and several other studies implicate MOR in the rewarding properties of drugs and in the modulation of craving and relapse behaviors.

Cocaine inhibits monoamine transporters (serotonin, dopamine, and norepinephrine transporters), thereby increasing their presence in the synapse. Cocaine's primary salient property is through inhibition of the dopamine transporter (DAT) leading to increased dopamine release by the VTA into several brain regions, especially the NAc (Nestler 2004; Phillips

et al. 2003; Pruessner et al. 2004b; Self 1998; Spealman et al. 1999b; Stewart 2000a). Drugs of abuse all increase dopamine release into the NAc through various pathways, ie morphine disinhibits VTA dopamine release into the NAc whereas cocaine increases dopamine presence in the synapse once released. The increased presence of dopamine in the NAc indicates salient cues to an animal or person to pay attention to specific stimuli or rewards to either avoid or repeat a behavior. Dopamine is also important for goal directed behaviors, instrumental responding for reinforcement, and feeding (Hnasko et al. 2006; Robinson et al. 2007; Robinson et al. 2006). However, when dopamine production is blocked in mice, they still display behaviors associated with positive reinforcement of cocaine. For example, dopamine deficient mice display CPP for cocaine (Hnasko et al. 2007). This effect is not blocked by D1R antagonist, however, fluoxetine, a serotonin transporter inhibitor, produced CPP in dopamine deficient mice and not wild-type controls, indicating cocaine induced CPP in dopamine deficient mice is through serotonin. This is supported by the finding that preventing dopamine neuron firing with pretreatment with the D2R antagonist, quinpirole, prevents both cocaine and fluoxetine CPP in these mice. Hence other systems are capable of compensating for dopamine in mediating rewarding properties of various

stimuli such as drugs of abuse, however dopamine in the dorsal striatum is important in normal behavior as shown by rescue studies (Palmiter 2008).

The NAc core is thought to gate the transition between motivation and initiation of action to perform a conditioned behavioral response for reinforcers (Cardinal et al. 2002a; Salamone and Correa 2002; Zahm 2000). Conversely the NAc shell modulates unconditioned responses such as orientating, approach and ingestion behaviors. For example, dopamine release is increased specifically in the shell, and not the core, following unpredicted consumption of a rewarding food (Bassareo and Di Chiara 1999). Alternatively, presentation of a stimulus associated with the rewarding food increased dopamine release in the core and not the shell, and further sensitized the dopamine response in the core when the reward was consumed. For cocaine, it is self-administered in the NAc shell and not NAc core (Rodd-Henricks et al. 2002), indicating an alternative mechanism for cocaine reinforcement through the NAc core. Dopamine concentrations increase more in the NAc shell at lower cocaine doses with proportionately more released in the shell versus the core at higher cocaine doses (Pontieri et al. 1995). Noncontingent presentation of a cue light that was previously paired with a cocaine or morphine infusion increased DA in the NAc core and not shell (Bassareo et al. 2007; Ito et al.

2000), indicating more of a role in cocaine craving through the NAc core. D2R antagonist in the shell and not the core abolishes cocaine-primed reinstatement (Anderson et al. 2006) whereas MOR antagonism blocked cocaine primed reinstatement (Chapter 4). Cocaine self-administration selectively abolishes long-term potentiation in the core during prolonged abstinence indicating an increased "depressed" state of the NAc core potentially through MOR up-regulation (Martin et al. 2006). These findings support different roles of NAc core and shell subregions, where the core is involved in cocaine craving and the shell is involved in cocaine reinforcement.

Cocaine also activates opioid peptide release and expression throughout limbic nuclei involved in reward, but especially through arcuate nucleus of the hypothalamus (Arc) release of β-endorphin and through NAc and VTA release of enkephalin and dynorphin (Arroyo et al. 2000; Daunais and McGinty 1995; Hurd and Herkenham 1993; Mathieu-Kia and Besson 1998; Roth-Deri et al. 2003; Sivam 1989; Ziolkowska et al. 2006). Together, signaling from these brain regions lead to modulated processing of salient cues and adaptive motor responses with respect to cocaine administration. There is a balance between dopamine and opioid signaling in the NAc in response to drugs and environmental stimuli.

Dopamine in the NAc is thought to increase the salience or reinforcing properties of cocaine and β-endorphin exerts/modulates the euphoric or rewarding properties of cocaine. For example, mice lacking dopamine-1 like receptors (D1R) fail to acquire cocaine self-administration and conditioned place preference (CPP) while D1R blockade decreases the reinforcing properties of cocaine (Bachtell et al. 2005; Bari and Pierce 2005; Caine et al. 2007; Cervo and Samanin 1995). Cocaine mediated release of β-endorphin into the NAc increases the rewarding and reinforcing properties of cocaine since blockade of β-endorphin activity in the NAc increases cocaine seeking during cocaine self-administration, decreases acquisition and expression of cocaine CPP, and attenuates acquisition of cocaine self-administration (Marquez et al. 2008; Roth-Deri et al. 2008; Roth-Deri et al. 2006; Roth-Deri et al. 2004). Together, dopamine and endogenous opioids modulate cocaine reinforcement. However, during abstinence, MOR appear to modulate drug craving.

That MOR stimulation would cause relapse behaviors was demonstrated in chapter 4. DAMGO and β-endorphin stimulation in NAc reinstates cocaine-seeking behavior in animals that have extinguished responding for cocaine. The specificity of these ligands for MOR clearly establish that the NAc is an important target in the modulation of cocaine

seeking behaviors by endogenous opioid activity. Thus, MOR up-regulation in long-term withdrawal could underlie increasing craving and the propensity for relapse. Previous studies report β-endorphin activity is decreased in cocaine addicts, which could reflect depletion of endogenous stores during cocaine use, and would indicate less stimulation of MOR during early abstinence periods. After longer withdrawal, MOR up-regulation could exacerbate the craving response to stress, drug associated cues, and drugs that increase β-endorphin release, and subsequently the likelihood for relapse.

These findings indicate targeting MOR during cocaine abstinence may increase the success rate of treating cocaine addiction. By binding to MOR with agents that have slower kinetics than β-endorphin, available binding sites for β-endorphin binding as a result of stress or drug-associated cues would decrease the likelihood of relapse. Methadone increases MOR endocytosis, further decreasing available MOR binding sites. Combinatorial treatment of different MOR ligands may be beneficial in the treatment of cocaine addiction. It is hypothesized that after chronic MOR ligand treatment, the up-regulation of the MOR expression system and activation may become normalized, however further studies are required to answer this question.

Future studies in analyzing the mechanisms of MOR modulation of craving and relapse would lead to better treatment strategies for cocaine and potentially other addictions. In the rat model of self-administration, it would be important to determine if anterior cingulate MOR expression also changes by cocaine self-administration and withdrawal, and whether β-endorphin infusions into the anterior cingulate modulate seeking behaviors through altering neurotransmitter release in the NAc core. To further determine the mechanisms behind cocaine self-administration mediated regulation of MOR, it would be interesting to use chromatin immunoprecipitation to determine any alterations in MOR gene regulation. Isolating synaptoneurosomes from 1 versus 6 w WD animals and measuring MOR expression levels would indicate differences in synaptic MOR expression during withdrawal. Alternatively, using biotinylation to label surface MOR in brain slices from cocaine self-administering animals at various withdrawal time points would allow specific detection of MOR that is expressed on the membrane versus internal stores. Electrophysiological measurements of NAc activity in response to DAMGO, β-endorphin, DPDPE stimulation and other treatments during long-term withdrawal would determine NAc activity differences that are

independent of complicated, and potentially confounding, behavioral measures.

VITAE

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