THE ADDICTED PHENOTYPE: PROTEIN PHOSPHORYLATION STATUS AND DOPAMINE RECEPTOR RESPONSIVENESS

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DEDICATION

I devote this work to my new bride, Kimberly.
My everlasting solace.

This work is also dedicated to my parents, Ross and Sue, and brother, George.
"The best way to give advice to your children is to find out what they want
and then advise them to do it" – Harry S. Truman

Finally, the counsel and patience from my scientific mentors is greatly appreciated.
"As iron sharpens iron, so one man sharpens another" – Proverbs 27:17

THE ADDICTED PHENOTYPE: PROTEIN PHOSPHORYLATION STATUS AND DOPAMINE RECEPTOR RESPONSIVENESS

by

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PREFACE

Up-regulation of cAMP/PKA signaling by drugs of abuse may contribute to escalation and relapse, possibly by differentially altering dopamine receptor-responsiveness in the mesolimbic dopamine system. To investigate this hypothesis, our initial studies measured alterations in cAMP-dependent and –independent protein phosphorylation *in vivo* produced by chronic cocaine and heroin self-administration, changes in mesolimbic protein phosphorylation compared to individual differences in the propensity for escalating cocaine self-adminstration, and, ultimately, dopamine receptor-mediated regulation of relapse to cocaine seeking in withdrawal.

Chronic cocaine self-administration can produce either tolerance or sensitization to certain cocaine-regulated behaviors, but whether differential alterations develop in the biochemical response to cocaine is less clear. In Chapter 2, we studied cocaine-induced phosphorylation of multiple cAMP-dependent and -independent protein substrates in mesolimbic dopamine terminal regions following chronic self-administration. Changes in self-administering rats were compared to changes produced by passive yoked injection to identify regulation related to the context of behavioral reinforcement, whereas acute and chronic yoked groups were compared to identify the development tolerance or sensitization in the biochemical response to cocaine. Microwave-fixed brain tissue was collected immediately following 4 hrs of intravenous cocaine administration, and subjected to western blot analysis of phosphorylated and total protein substrates. Chronic cocaine produced region- and substrate-specific tolerance to cAMP-dependent protein phosphorylation, including phosphorylation of the AMPA $GluR_1$ receptor subunit at

serine 845 in striatal and amygdala subregions, and the NMDA NR_1 receptor subunit at serine 897 in the CA1 subregion of hippocampus. Tolerance also developed to cAMP-independent $GluR_1^{S831}$ phosphorylation in the prefrontal cortex. In contrast, sensitization to cocaine-induced phosphorylation of the pre-synaptic vesicle protein synapsin I at serine 9 developed in amygdala and hippocampal subregions, while cAMP-dependent phosphorylation of the dopamine-synthesizing enzyme tyrosine hydroxylase at serine 40 decreased in pre-synaptic striatal dopamine terminals in striatal subregions. Cocaine-induced phosphorylation of extracellular signal-regulated kinase (ERK) was dissociated from downstream phosphorylation of the transcription factor cAMP-response element binding protein (CREB) in many brain regions, and failed to develop either tolerance or sensitization with chronic administration, and failed to develop either tolerance or sensitization with chronic administration. Positive reinforcement-related correlations between cocaine intake and protein phosphorylation were found only in self-administering animals, while negative dose-related correlations were found primarily with passive yoked administration. These regional- and substrate-specific adaptations in cocaine-induced protein phosphorylation are discussed in lieu of their potential impact on the development of cocaine addiction.

In Chapter 3, we studied alterations in protein kinase A (PKA)-dependent and PKA-independent phosphorylation in multiple brain regions in rats undergoing either spontaneous or naltrexone-precipitated withdrawal (WD) from chronic intravenous heroin self-administration. Spontaneous WD from heroin self-administration produced region-specific increases in PKA-dependent $GluR_1^{S845}$ phosphorylation in the nucleus

accumbens shell, basolateral amygdala, hippocampal CA1 and CA3 regions, and premotor cortex after 24 but not 12 hrs, and there were no changes in prefrontal cortex, nucleus accumbens core or caudate-putamen. Increased $GluR_1^{S845}$ phosphorylation occurred earlier (12 hrs) in the central amygdala, ventral tegmental area, and substantia nigra. In contrast, prominent ERK phosphorylation was found in both prefrontal and premotor cortex, CA1 and CA3, caudate-putamen, and basolateral amygdala, but not in nucleus accumbens, or central amygdala in spontaneous WD. Phosphorylation of striatal CREB increased in caudate-putamen but not in nucleus accumbens, paralleling ERK rather than PKA activity in heroin WD. Naltrexone administration potentiated $GluR_1^{S845}$ and ERK phosphorylation in the central amygdala, and ERK phosphorylation in nucleus accumbens core and shell. Thus, spontaneous WD from heroin self-administration produces region- and time-dependent changes in PKA and ERK activity that could contribute to the behavioral manifestation of opiate dependence.

In Chapter 4 we studied PKA-dependent $GluR_1^{S845}$ phosphorylation and ERK phosphorylation mediated by ERK kinase in striatal subregions in an animal model of cocaine craving. Here, animals with chronic cocaine self-administration experience were re-exposed to the self-administration test chambers for 1 hr in the absence of cocaine to measure phosphorylation induced by the environmental context paired with cocaine reinforcement. After 1 day WD, $GluR_1^{S845}$ levels were elevated in both self-administering and yoked groups in the nucleus accumbens shell, but this effect persisted only in self-administering animals after 3 weeks WD. In the nucleus accumbens core, context-induced phosphorylation of both $GluR_1^{S845}$ and ERK increased from early to late WD

from chronic cocaine self-administration, implicating this region in mediating the intensification of cocaine craving with longer periods of abstinence. These differential region- and substrate-specific adaptations to withdrawal- and context-induced protein phosphorylation could underlie the maintenance of cocaine addiction by exacerbating the potential for drug relapse in withdrawal.

Finally, as a behavioral correlate, studies in Chapter 5 sought to compare these changes in protein phosphorylation status with alterations in dopamine-receptor mediated regulation of relapse to cocaine seeking in withdrawal. Here, the cocaine-addicted phenotype was modeled in rats based on individual differences in preferred levels of cocaine intake and a propensity for relapse in withdrawal. Since these cocaine-taking and -seeking behaviors are strongly but differentially regulated by postsynaptic dopamine D_1 and D_2 receptors in the mesolimbic system, we determined whether the development of cocaine addiction would be related to differential sensitivity in functional D_1 and D_2 receptor responses. Using a population of 40 outbred Sprague-Dawley rats trained to self-administer cocaine for 3 weeks, we found that animals with higher preferred levels of cocaine intake exhibited a vertical and rightward shift in the self-administration dose-response function, and were more resistant to extinction from cocaine self-administration, similar to phenotypic changes reported in other models of cocaine addiction. After 3 weeks of withdrawal from cocaine self-administration, high intake rats were subsensitive to the ability of the D_1 agonist SKF 81297 to inhibit cocaine-seeking behavior, but supersensitive to cocaine seeking triggered by the D_2 agonist quinpirole, when compared to low intake rats. Additionally, high intake rats developed profound increases in

locomotor responses to D_2 receptor challenge from early to late withdrawal times, whereas low intake rats developed increased responsiveness to D_1 receptor challenge. In a second experiment, responses to the mixed D_1/D_2 agonist apomorphine and the NMDA glutamate receptor antagonist MK-801 failed to differ between low and high intake rats. These findings suggest that cocaine addiction is specifically related to differential alterations in functional D_1 and D_2 receptors that mediate opposing influences on cocaine-seeking behavior. Alterations in dopamine receptor signaling may issue from an enduring up-regulation of cAMP/PKA signaling in drug withdrawal.

The potential for reduced D_1 receptor (G_s/G_{olf}-coupled) sensitivity to account for tolerance to cocaine-regulated PKA-dependent protein phosphorylation during self-administration (Chapter 2) is discussed in Chapter 6. Conversely, it is possible that PKA up-regulation in cocaine WD (Chapter 4) could account for the paradoxical sensitization of subsequent G_i/G_o-coupled D2 receptor responses. Thus, addiction-related alterations in D_1 and D_2 dopamine receptor responses may ultimately involve complex reciprocal interactions between adaptations in PKA signaling pathways that differentially influence D_1 and D_2 receptor signaling.

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LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

Δ	Delta
BLA	Basolateral Amygdala
CA1	Cornu Ammonis Hippocampal Field 1
CA3	Cornu Ammonis Hippocampal Field 3
CaMK	Calcium-Calmodulin-Dependent Protein Kinase
cAMP	Cyclic Adenosine Monophosphate
CEN	Central Amygdala
CPU	Caudate Putamen
CREB	Cyclic AMP Response Element Binding Protein
CSA	Cocaine Self-administration
D_1	Dopamine D_1 Receptor
D_2	Dopamine D2 Receptor
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular Signal-Regulated Kinase
Fisher's LSD	Fisher's Least Significant Difference
FosB	FBJ (Finkel-Biskis-Jonkins) Osteosarcoma Oncogene B
GluR1	AMPA Glutamate Receptor Subunit 1
HSA	Heroin Self-administration
IP	Intra-peritoneal
MEK	ERK kinase

NAc	Nucleus Accumbens
NAC	Nucleus Accumbens Core
NAS	Nucleus Accumbens Shell
NR1	NMDA Receptor Subunit 1
P	Phosphorylated
PAGE	Polyacrylamide Gel Electrophoresis
PFC	Prefrontal Cortex
PKA	Protein Kinase A/cAMP-Dependent Protein Kinase
PKC	Protein Kinase C
PMC	Premotor Cortex
PVDF	Polyvinylidene fluoride
S, SER	Serine
SA	Self-administering
SDS	Sodium Dodecyl Sulfate
SN	Substantia Nigra
T, THR	Threonine
TDMAC	Tridodecylmethyl Ammonium Chloride
TH	Tyrosine Hydroxylase
TYR	Tyrosine
VTA	Ventral Tegmental Area
WD	Withdrawal
YK	Yoked

CHAPTER 1

General Introduction

The mesolimbic dopamine system

The mesolimbic dopamine system consists of dopaminergic neurons in the ventral tegmental area (VTA) and their target neurons in forebrain regions including the nucleus accumbens, prefrontal cortex, amygdala, and hippocampus (Swanson 1982) (Koob and Swerdlow 1988). This system is the major neural substrate for the reinforcing effects of virtually all drugs of abuse, including opiates and psychostimulants (Berridge and Robinson 1998; Spanagel and Weiss 1999; Pierce and Kumaresan 2006). In addition, this system is activated by subsequent exposure to drug-related environmental cues, stress, and other pharmacological stimuli that trigger relapse to drug seeking during withdrawal (Self and Nestler 1998); (Shalev et al. 2002). Release of dopamine from mesolimbic terminals acts on two major classes of post-synaptic receptors that are distinguishable by their opposite modulation of intracellular cyclic AMP (cAMP) formation (Lachowicz and Sibley 1997). D_1-like receptors (D_1 and D_5) are positively coupled to adenylyl cyclase and stimulate cAMP formation, whereas D_2-like receptors (D_2, D_3, and D_4) are negatively coupled to adenylyl cyclase to reduce cAMP levels. In the nucleus accumbens, D_1 and D_2 receptor expression largely segregates into distinct neuronal populations that project to different brain regions (Lu et al. 1998) (Steiner and Gerfen 1998).

Protein phosphorylation in the mesolimbic dopamine system

D_1 and D_2 dopamine receptor signaling involves several membrane-delineated and second messenger pathways, but their regulation of cAMP activity ultimately influences phosphorylation of specific cAMP-regulated phosphoproteins by protein kinase A (PKA). PKA phosphorylates a wide variety of protein substrates, including the AMPA receptor subunit $GluR_1$ at Ser 845. In turn, PKA-mediated $GluR_1^{S845}$ phosphorylation potentiates excitatory currents mediated by AMPA receptors (Roche et al. 1996; Banke et al. 2000) and promotes AMPA receptor insertion in synaptic membranes, ultimately leading to an enhancement of synaptic plasticity (Esteban et al. 2003; Mangiavacchi and Wolf 2004). NMDA glutamate receptor activity is similarly enhanced by PKA-mediated phosphorylation of NR1 subunits at serine 897 (Tingley et al. 1997; Westphal et al. 1999). Importantly, substantial crosstalk exists between the cAMP/PKA and Ras/Raf/MEK/ERK pathways (for review, see Sweatt 2001). In this pathway, PKA can either activate MEK through Rap1 and B-Raf (Vossler et al. 1997), or inhibit MEK signaling by preventing MEK's activation by Raf-1 (C-Raf) (Sevetson et al. 1993; Dumaz and Marais 2005). As a consequence, the ERK signaling cascade is activated in response to dopamine receptor activation (Yan et al. 1999) and plays a synergistic role in PKA-mediated CREB activation (Grewal et al. 2000; Zanassi et al. 2001). The acute biochemical response to cocaine involves increases in both cAMP-dependent and cAMP-independent protein phosphorylation via convergent dopamine D_1 and NMDA glutamate receptor signaling in striatal neurons (Nishi et al. 2000) (Snyder et al. 2000; Valjent et al.

2004) Mattson et al. 2005; (Mattson et al. 2005; Valjent et al. 2005). Together, these and other protein phosphorylation events represent post-translational protein modifications that could allow for both the induction and long-term expression of neuroadaptations necessary for the proposed molecular "switch" from intitial drug abuse to the pathological state of drug addiction.

Neuroadaptations in dopamine receptor-regulated cAMP signaling cascades within the mesolimbic dopamine system may be involved in escalating drug intake

Chronic exposure to drugs of abuse, including cocaine and heroin, produce strikingly similar alterations in dopamine receptor signaling pathways in the nucleus accumbens. These changes are characterized by decreases in inhibitory G proteins and by increases in adenylyl cyclase and cAMP-dependent protein kinase (PKA) (Terwilliger et al. 1991) (Self et al. 1995). Together, all of these changes contribute to a generalized up-regulation of accumbens PKA activity. These neuroadaptations may contribute to escalating drug self-administration related to an addicted phenotype, because experimental activation of PKA in the accumbens, or inactivation of inhibitory G proteins, both lead to similar increases in drug self-administration (Self et al. 1994) (Self et al. 1998). Importantly, these tolerance-like effects were mediated through sustained, but not acute, PKA activation, suggesting compensatory adaptations to a chronic elevation in PKA activity.

The addicted phenotype

The transition from initial drug use to an addicted state is characterized by an escalation in the amount of drug consumed during self-administration, and enhanced drug craving in drug withdrawal leading to compulsive drug-seeking behavior (American Psychiatric Association – *DSM-IV*, 2004). These complex behavioral changes have been modeled in variations of the rodent self-administration paradigm in attempts to classify "addicted" from "non-addicted" animals (Ahmed and Koob 1998a; Ahmed et al. 2000; Piazza et al. 2000; Sutton et al. 2000). The addicted phenotype can be selected from outbred populations based on individual differences in preferred levels of drug intake (Piazza et al. 2000). Most importantly, escalating drug intake has been specifically related to increased drug seeking during withdrawal (Sutton et al. 2000); therefore, both addictive traits are encompassed in a single subpopulation of outbred rats. These behavioral differences suggest that differences also exist in the neuronal substrates that regulate these behaviors. The elucidation of neurobiological differences underlying the transition to escalating drug intake and increased drug-seeking, whether induced by chronic drug use, or due to an inherent predisposition, is of vital importance to our ultimate ability to understand drug addiction and develop effective treatments.

Regulation of cocaine intake and cocaine seeking by D_1 *and* D_2 *dopamine receptors*

Cocaine intake is highly regulated by both D_1 and D_2 classes of dopamine receptors. Pretreatment with either D_1 or D_2 agonists reduces cocaine self-administration, but differentially alters self-administration patterns (Self et al. 1996; Caine et al. 1999). Conversely, pretreatment with D_1 and D_2 antagonists increase cocaine intake under

unrestricted access conditions (Corrigall and Coen 1991). These studies suggest that both D_1 and D_2 receptors contribute to inhibitory regulation of cocaine intake during self-administration. In contrast, D_1 and D_2 receptors mediate opposite effects on cocaine seeking during withdrawal, as measured by the extinction/reinstatement model of drug relapse in rats (Self et al. 1996; De Vries et al. 1999). Selective stimulation of D_2 receptors strongly induces cocaine seeking (renewal of responding on a lever previously associated with cocaine self-administration, but without cocaine delivery), whereas selective D_1 receptor stimulation is without effect, even at doses that potentiate locomotor activity. Moreover, stimulation of D_1 receptors attenuates cocaine's ability to reinstate further cocaine seeking (cocaine priming), whereas stimulation of D_2 receptors facilitates cocaine-induced reinstatement (Self et al. 1996). A similar D_1/ D_2 dichotomy may also regulate craving in humans (Haney et al. 1998; Haney et al. 1999). Together, these studies suggest that D_2 receptors contribute to craving elicited by low doses of drugs, drug-related cues, stress, and other stimuli that activate the mesolimbic dopamine system (Self 1998). In contrast, high D_1 receptor tone may diminish craving.

Hypothesis

Our hypothesis suggests that as animals become addicted, they also become less sensitive to D_1 receptor-mediated reward, and escalate their drug intake to compensate for the reduced D_1 tone. In contrast, they may be more sensitive to D_2 receptor-induced drug seeking, resulting in a propensity for craving and relapse. This hypothesis is based on the

dichotomous role of D_1 and D_2 receptors in cocaine seeking, as well as hypothetical alterations in D_1 and D_2 receptor responses as a consequence of drug-induced neuroadaptations in D_1 and D_2 signaling pathways (up-regulation of the cAMP/PKA pathway in addiction may preclude D_1 and sensitize D_2 responses via reciprocal negative and positive feedback, respectively). Thus, we initially characterized neuroadaptations in protein phosphorylation status immediately after chronic drug self-administration (cocaine), at early drug withdrawal (1 day abstinent; cocaine and heroin) and after extended withdrawal (3 weeks abstinent; cocaine). In addition, we investigated the influences of exposure to a cocaine-associated context on regulation of phosphoprotein status in withdrawal. This last approach is both essential and clinically relevant, since exposure to an environmental context associated with cocaine induces both drug-seeking behavior (Badiani and Robinson 2004) and neurobiological consequences (Lu et al. 2005) (Mattson et al. 2005). Finally, we determined whether addiction-related differences in cocaine self-administration would be related to differential sensitivity in functional D_1 and D_2 receptor responses, since alterations in protein phosphorylation could both 1) reflect changes in dopamine receptor sensitivity with pharmacological challenge and 2) contribute to these receptor changes through compensatory adaptations.

CHAPTER 2

Region-Specific Tolerance to Cocaine-Regulated cAMP-Dependent Protein Phosphorylation Following Chronic Self-Administration

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Introduction

Chronic cocaine self-administration leads to neurobiological changes that contribute to either tolerance or sensitization to cocaine's behavioral effects depending on the duration of cocaine exposure and the behavioral measure employed. For example, while sensitization is commonly reported for the psychomotor and incentive motivational effects following chronic cocaine self-administration (Sutton et al. 2000b), these changes are often accompanied by tolerance–like increases in drug consumption (Emmett-Oglesby et al. 1993). However, whether such tolerance or sensitization is reflected in the biochemical response to cocaine following chronic self-administration is unknown.

The acute biochemical response to cocaine involves increases in both cAMP-dependent and cAMP-independent protein phosphorylation via convergent dopamine D_1 and NMDA glutamate receptor signaling in striatal regions (Nishi et al. 2000). Cocaine-induced phosphorylation of the a serine 845 residue of the GluR1 AMPA receptor subunit is specifically mediated by cAMP-dependent protein kinase (PKA), but this effect remains constant with repeated intraperitoneal injections (Mattson et al. 2005). In contrast, cocaine-induced phosphorylation of extracellular signal-regulated kinase (ERK) and cyclic AMP response element binding protein (CREB) are enhanced by repeated injections, apparently through cAMP-independent mechanisms (Mattson et al. 2005). Acute cocaine exposure also increases ERK phosphorylation in other dopamine terminal regions including the prefrontal cortex, amygdala and hippocampus (Valjent et al. 2004), but whether repeated exposure would alter these acute responses remains unknown.

Thus, either increases or decreases in cocaine-regulated protein phosphorylation could contribute to the behavioral phenomena of sensitization and tolerance, respectively. Moreover, cocaine could differentially regulate protein phosphorylation when intravenous injections are delivered in a reinforcing context with self-administration when compared to passive experimenter-administered cocaine.

In this study, we measured PKA-dependent phosphorylation of GluR1 AMPA (Ser 845) and NR1 (Ser 897) NMDA receptors in multiple dopamine terminal regions immediately after 4 hrs of intravenous cocaine self-administration in rats. We compared PKA-mediated GluR1 phosphorylation with GluR1 phosphorylation mediated by protein kinase C (PKC) and CaMKII (Ser 831). We also measured cocaine-induced ERK phosphorylation (Thr 183/Tyr 185) mediated by ERK kinase (MEK), and downstream CREB phosphorylation at the Ser 133 residue that is regulated by both ERK and PKA in addition to multiple other kinases. Phosphorylation of these post-synaptic proteins was compared with pre-synaptic regulation of PKA-specific phosphorylation of the dopamine synthesizing enzyme tyrosine hydroxylase (TH) on the serine 40 residue, and synapsin phosphorylation (Ser 9) at a site regulated by both PKA and calcium calmodulin kinase I (CaMKI). We compared the ability of intravenous cocaine to regulate these phosphoproteins before and after chronic administration to determine whether differential tolerance or sensitization developed in PKA-dependent and independent pathways. Finally, we compared cocaine-induced protein phosphorylation in self-administering animals with animals receiving cocaine passively by yoked injection to identify potential

reinforcement-related regulation. The functional implications of these findings are discussed in relation to synaptic and network level neurotransmission.

Materials and methods

Subjects and surgery

Male, Sprague-Dawley rats initially weighing 275-325g (Charles River, Kingston, RI, USA) were individually housed in a climate-controlled environment (21° C) on a 12-hr light-dark cycle (lights on at 7:00 AM) in according to the National Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals*. Animals initially were maintained on a restricted diet at 85% original body weight and all were trained to press a lever for 45 mg sucrose pellets in operant chambers (Med Associates, Georgia, VT) on a fixed-ratio 1 (FR1) reinforcement schedule until acquisition criteria were achieved (100 pellets self-administered for 3 consecutive days). This procedure was used to facilitate subsequent acquisition of cocaine self-administration. Following acquisition of lever press behavior, animals were fed ad libitum prior to surgical implantation of a chronic indwelling intrajugular catheter as described previously (Edwards *et al*., 2006). Catheters were flushed daily with 0.2 ml of heparinized (20 IU/ml) bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml) to prevent clotting and antibiotic ointment was applied daily to the catheter exit wound to prevent infection.

Cocaine self-administration procedures in rats

Following a 1 week recovery period, animals were trained to perform a single lever press response (FR1) to self-administer intravenous injections of cocaine hydrochloride (500 μg/kg/50 μl injection) or saline delivered over 2.5 sec in daily 4-hr sessions for 3 weeks (6 days/week) in operant test chambers as described (Edwards et al. 2006). During each injection, a cue light above the active lever was illuminated, and the house light was extinguished, followed by an additional 12.5 sec time-out (TO) period when the both lights were extinguished and lever press responses had no programmed consequence. Chronic yoked cocaine animals received passive non-contingent cocaine injections in an identical temporal pattern as their cocaine self-administering partners. Another study group received yoked injections of saline on all but the last session when they received cocaine injections for the first time (acute yoke). Age- and group-matched untreated controls remained in their home cages but were handled daily.

Measurement of protein phosphorylation in brain tissue homogenates

Immediately after completion of the last test session, self-administering and yoked animals were gently placed in a plexiglass restrainer and euthanized by microwave irradiation aimed at the head region (5 kW, 1.5 s, Murimachi Kikai Co., LTD. Tokyo, Japan). Rat brains were rapidly dissected and regional tissue samples were obtained with 12-16 gauge punches from chilled coronal brain slices (1.5 mm thick). Tissue samples were immediately homogenized by sonication in lysis buffer (320 mM sucrose, 5 nM

HEPES, 50 mM NaF, 1 mM EGTA, 1 mM 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, and stored at –80°C until determination of protein concentrations by the Lowry method. Samples of 20 μg protein were subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide ($pGluR_1^{S845}$, $pGluR_1^{S831}$, pERK, and $pCREB^{S133}$) or 7.5% acrylamide (pNR_1^{S897}, $pSynapsin^{S9}$, and pTH^{S40}) with a Tris/Glycine/SDS buffer (Bio-Rad, Hercules, CA), followed by electrophoretic transfer to PVDF membranes (polyvinylidene; Amersham, Piscataway, NJ). Membranes were blocked overnight in 5% nonfat milk at 4° C, and incubated in primary antibody for $pGluR_1^{S845}$ (1:2500; Chemicon, Temecula, CA), $pGluR_1^{S831}$ and pNR_1^{S897} (1:2500 and 1:7500; Upstate, Charlottesville, VA), pERK, $pCREB^{S133}$, $pSynapsin^{S9}$ and pTH^{S40} (1:7500, 1:2500, 1:20K and 1:1500; Cell Signaling, Danvers, MA) for 24 hrs at 4° C. Membranes were washed and labeled with species-specific peroxidase-conjugated secondary (1:10,000; BioRad) for 1 hr at 25° C. Following chemiluminescence detection (ECL plus; Amersham), blots were stripped and reprobed for total protein levels of $GluR_1$, NR_1, CREB and TH (1:10K, 1:7500 and 1:2500; 1:200K; Chemicon), or ERK (both 42 and 44 kD forms) and synapsin (1:7500 and 1:5000; Cell Signaling) as internal standards for phosphoprotein levels. Immunoreactivity was quantified by densitometry (Scion Image) under conditions linear over at least a 3-fold concentration range.

Data analysis

Densitized values were expressed a percentage of the mean for untreated control values (5-6/blot) to normalize data across blots. Phosphoprotein levels, each corrected for individual total protein levels, were compared by 1-factor ANOVA followed by Fishers Least Significant Difference (FLSD) posthoc comparisons among groups, or planned Student's t-tests comparisons with untreated controls.

Results

Figure 2.1A illustrates the saline and cocaine and treatment regimens for the four experimental groups used in the analysis of protein phosphorylation levels. Saline self-administering animals (Saline SA) served as a procedural control for surgery, handling and testing immediately prior to sacrifice. Protein phosphorylation in animals that received passive yoked intravenous cocaine injections throughout 18 days of training (Chronic YK) were compared to animals that received yoked cocaine injections only once in the final test session (Acute YK) to identify the development of tolerance or sensitization in the biochemical response to intravenous cocaine. Chronic cocaine self-administering (Cocaine SA) animals were compared to their Chronic YK partners to identify potential reinforcement-related differences in cocaine-induced protein phosphorylation. All changes in protein phosphorylation were compared to untreated controls that remained in their home cages throughout testing.

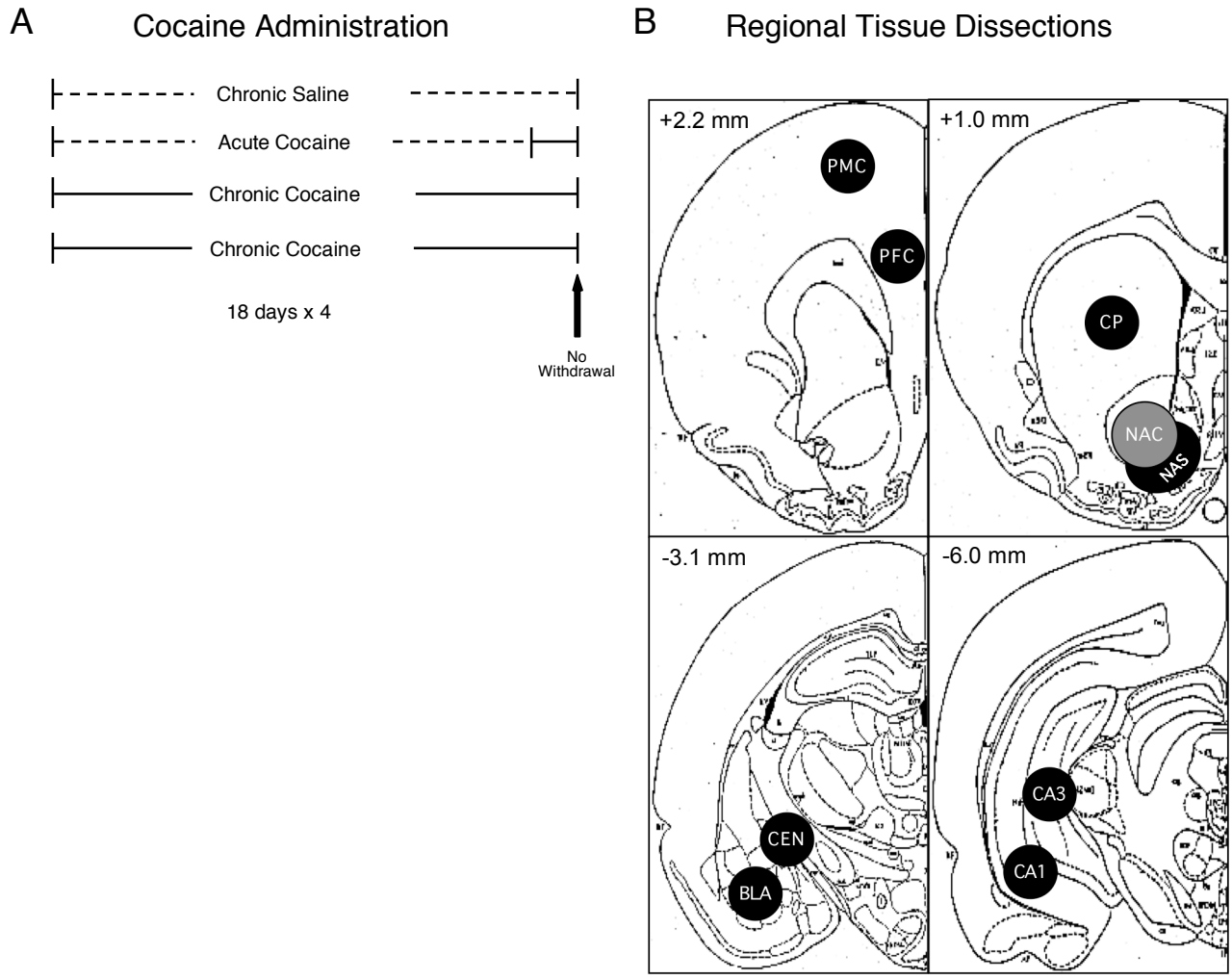


Figure 2.1
(**A**) Timeline depicting treatment regimens for cocaine administration in yoked (YK) and self-administering (SA) animals. Rats self-administered cocaine (500 μg/kg/50 μl injection) or saline in daily 4-hr sessions over 18 days (6 days/week). Chronic YK animals received an identical number of non-contingent cocaine injections in a temporal pattern determined by their self-administering partners. Acute YK animals received yoked saline injections every test day except the last session when they received an identical number of cocaine injections as Chronic YK and Cocaine SA animals. All animals were euthanized by microwave fixation immediately after their last test session. (**B**) Schematic representation of regional brain samples collected with 12-16 gauge punches from 1.5 mm thick coronal slices. Anterior-posterior coordinates represent posterior sides of brain slices (Paxinos & Watson, 1998).

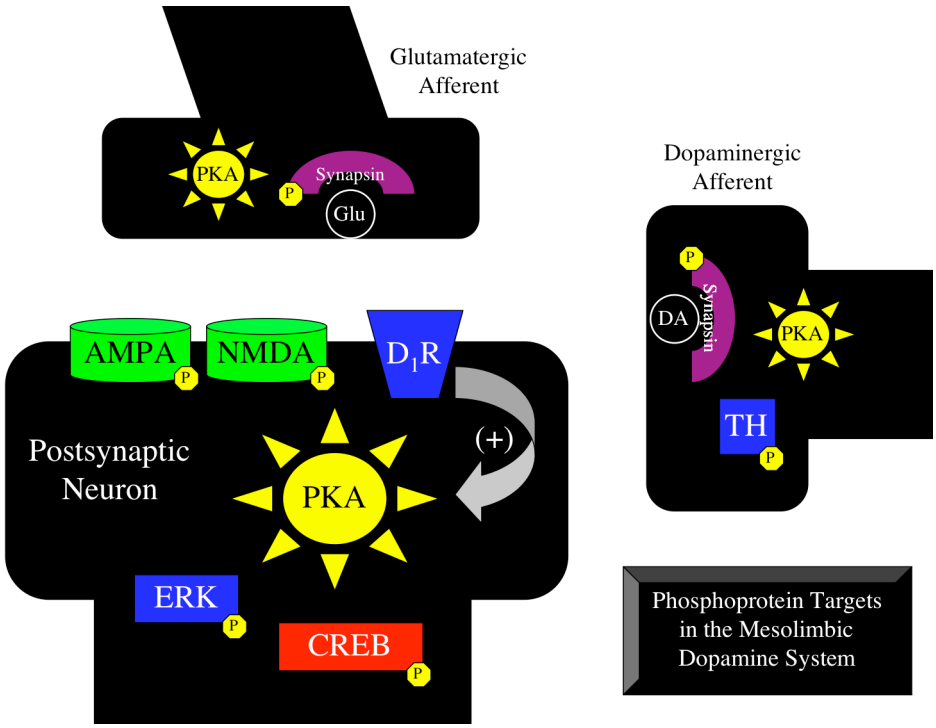
Animals trained to self-administer cocaine developed stable self-administration by the 3rd week of training, with mean daily cocaine intake (days 13-18) ranging from 44.8 mg/kg to 50.2 mg/kg in both chronic SA and chronic YK groups. On the final training day (day 18) cocaine intake ranged from 25.5 mg/kg to 56.5 mg/kg among individual self-administering rats and their Acute and Chronic YK partners. Tissue samples were collected from animals euthanized by microwave fixation immediately following the final 4 hr test session according to anatomical regions depicted in Figure 2.1B. Microwave fixation was used to preserve *in vivo* protein phosphorylation status and avoid rapid alterations typically observed following dissection of un-fixed tissue (O'Callaghan and Sriram 2004). Protein phosphorylation status was determined by site-specific immunolabeling for various PKA, PKC, CaMK (I and II) and MEK substrates listed in Table 2.1. All of the observed changes involved regulation in phosphorylation states since there was no significant regulation in the total amount of these phosphoproteins in any brain region studied immediately following acute or chronic cocaine administration.

Tolerance to PKC/CaMKII-, but not PKA-, mediated phosphorylation in prefrontal (PFC)

In the PFC, PKA-mediated $GluR_1^{S845}$ ($F_{4,52} = 11.018$, $p < 0.001$) and NR_1^{S897} ($F_{4,52} = 6.452$, $p < 0.001$) phosphorylation was significantly increased by > 50% immediately following 4 hrs of intravenous cocaine administration in all 3 cocaine groups, but not in Saline SA animals, indicating a specific effect of cocaine unrelated to surgical and other

Table 2.1 Protein Phosphorylation Sites Investigated

Protein	Phosphorylation Site	Kinase(s)	References
$GluR_1$	Ser^{845}	PKA	Roche et al., 1996; Banke et al., 2000
$GluR_1$	Ser^{831}	PKC/CamKII	Roche et al., 1996; Mammen et al., 1997
NR_1	Ser^{897}	PKA	Tingley et al., 1997; Westphal et al., 1999
ERK	Thr^{183}/Tyr^{185}	MEK	Payne et al., 1991; Adams et al., 2002
CREB	Ser^{133}	Several*	Yamamoto et al., 1988; Mayr & Montminy, 2001*
Synapsin I	Ser^{9}	PKA/CamKI	Huttner et al., 1979; Czernik et al., 1987
TH	Ser^{40}	PKA	Wu et al., 1992; Daubner et al., 1992



testing procedures (Fig. 2.2A). Smaller but significant increases in PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation also were found in all 3 cocaine exposed groups ($F_{4,52}$ = 7.308, $p < 0.001$), but the increase was reduced from 28% in Acute YK animals to 13% after chronic cocaine in both YK and SA groups, indicating the development of tolerance with repeated cocaine administration. In contrast, pre-synaptic regulation of PKA/CaMKI-mediated $synapsin^{S9}$ phosphorylation was not altered by cocaine, indicating a specific cocaine effect on post-synaptic protein phosphorylation in the PFC. A prominent 55-86% increase in ERK phosphorylation was produced in all cocaine exposed groups ($F_{4,52}$ = 8.219, $p < 0.001$), but a similar 66% increase also occurred in Saline SA animals, indicating that ERK phosphorylation in this brain region was induced by acute handling and testing procedures rather than cocaine. Similarly, $CREB^{S133}$ phosphorylation was increased by 34-49% in Acute and Chronic YK groups (T_{28} = 2.217-3.044, p = 0.005 – 0.036), but this increase also was seen in the Saline SA group. Thus, regulation of both ERK and $CREB^{S133}$ phosphorylation in the PFC were not related to cocaine exposure.

Similar increases in protein phosphorylation were found in the premotor cortex (PMC; Fig. 2.2B), except that tolerance to cocaine-induced PKC/CaMKII-mediated phosphorylation of $GluR_1^{S831}$ failed to develop with chronic cocaine administration, and $CREB^{S133}$ phosphorylation failed to increase in any study group. Thus, 4 hrs of intravenous cocaine administration increased $GluR_1^{S845}$ phosphorylation by > 50% ($F_{4,53}$ = 10.586. $p < 0.001$), and $GluR_1^{S831}$ phosphorylation by > 25% (T_{26} = 2.107-2.775, p = 0.01 – 0.045), while NR_1^{S897} phosphorylation increased less (22-38%) than in the PFC ($F_{4,52}$ =

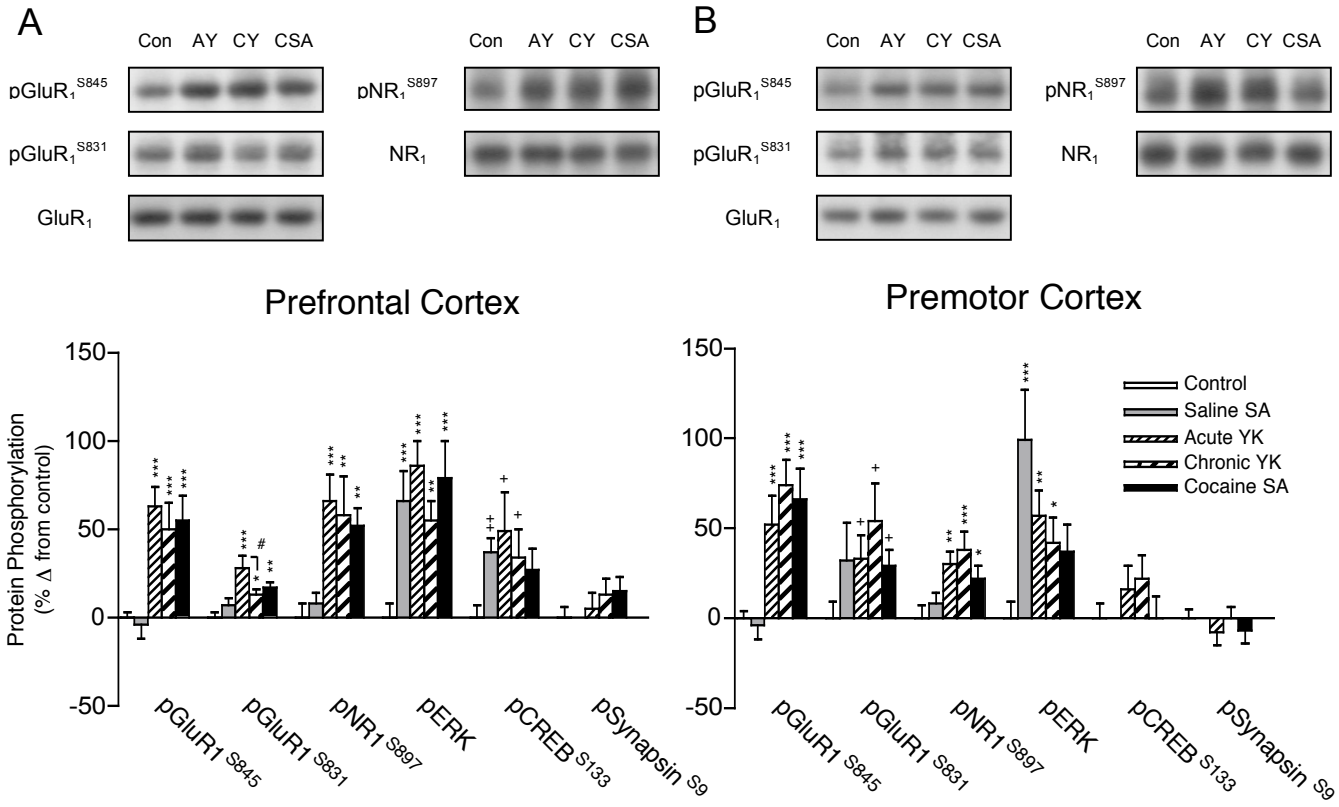


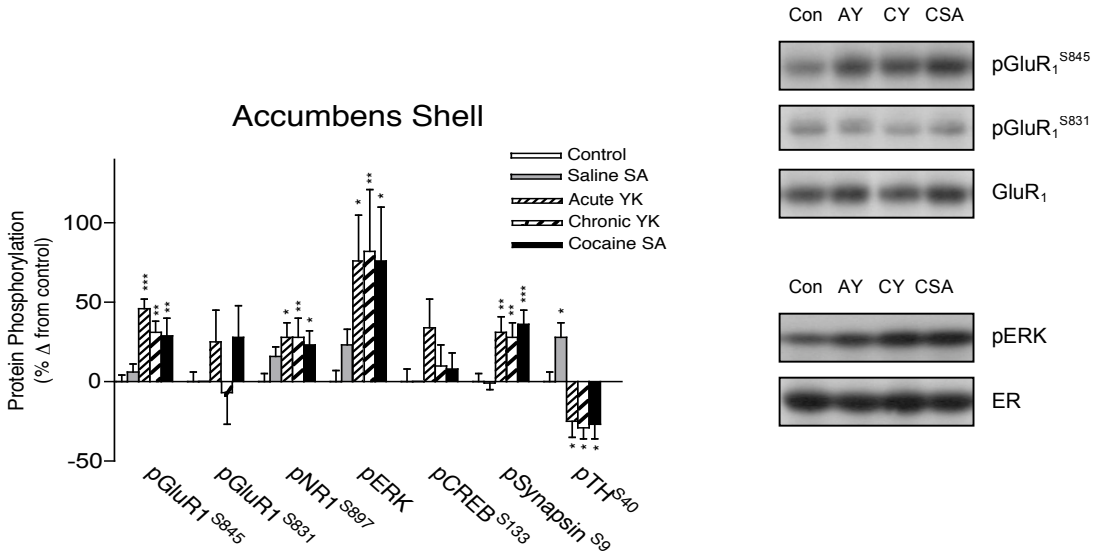
Figure 2.2
Cocaine-induced protein phosphorylation in the prefrontal (PFC) and premotor (PMC) cortex. (**A**) In the PFC, tolerance develops to cocaine-induced PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation, but not PKA-mediated $GluR_1^{S845}$ or NR_1^{S897} phosphorylation with chronic intravenous administration. ERK phosphorylation increases with either saline or cocaine self-administration (SA). (**B**) In the PMC, tolerance to cocaine-induced $GluR_1^{S831}$ phosphorylation fails to develop. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n = 8-11/group). Symbols indicate values differ from controls ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), or from acute YK cocaine ($^{\#}p < 0.05$) by Fisher's LSD tests. Differs from controls by planned Student's t-test comparisons ($^{+}p < 0.05$, $^{++}p < 0.01$). Representative blots for phosphorylated and total $GluR_1$and NR_1protein are shown for control (Con), acute yoke (AY), chronic yoke (CY), and chronic cocaine self-administering (CSA) animals.

4.261, $p = 0.005$). ERK phosphorylation was significantly elevated in both Acute and Chronic YK groups, but also in the Saline SA group similar to results in the PFC ($F_{4,53} = 6.090$, $p < 0.001$). Together, these data suggest that cocaine-specific regulation involves both GluR1 and NR1 phosphorylation in both limbic (PFC) and motor (PMC) prefrontal cortical areas, but that tolerance to PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation develops only in limbic prefrontal cortex.

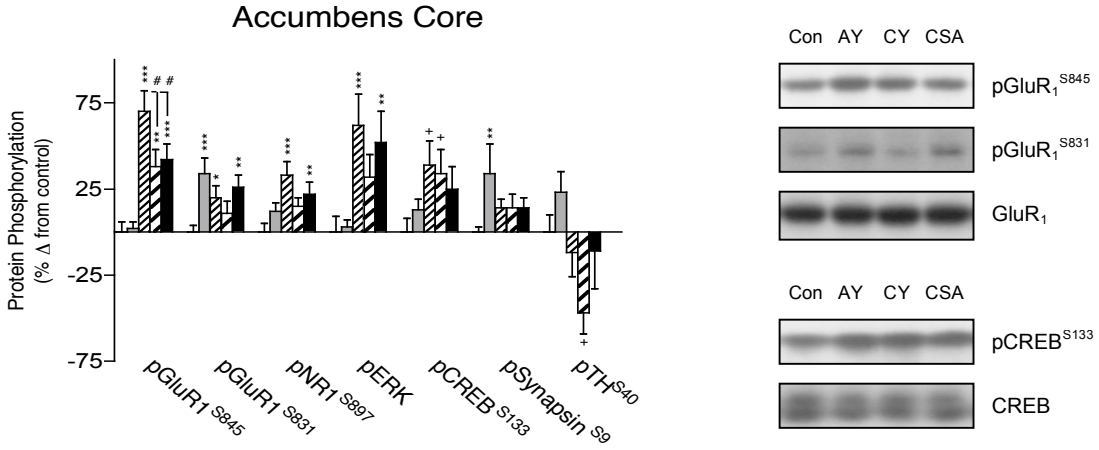
Tolerance to PKA-, but not PKC/CaMKII- or MEK-, mediated phosphorylation in striatal brain regions

In contrast to the PFC, tolerance to PKA-mediated $GluR_1^{S845}$ phosphorylation, but not PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation, developed in striatal brain regions following chronic cocaine administration in both YK and SA groups when compared to Acute YK cocaine administration (Fig. 2.3). Thus, $GluR_1^{S845}$ phosphorylation increased by 46% in the nucleus accumbens shell (NAS; $F_{4,63} = 8.015$, $p < 0.001$), 70% in the nucleus accumbens core (NAC; $F_{4,60} = 11.668$, $p < 0.001$), and 104% in the caudate putamen (CPU; $F_{4,53} = 11.996$, $p < 0.001$) after 4 hrs of intravenous cocaine administration in the Acute YK group. Cocaine-regulation of $GluR_1^{S845}$ phosphorylation was reduced to 38% in the NAC and to 46% in the CPU following chronic administration in both YK and SA groups. Tolerance to cocaine effects was less evident in the NAS where cocaine produced smaller increases with acute cocaine administration. Interestingly, PKA-mediated increases in NR_1^{S897} phosphorylation remained relatively

A


Accumbens Shell
Control
Saline SA
Acute YK
Chronic YK
Cocaine SA
Protein Phosphorylation
(% Δ from control)
100
50
0
-50
pGluR1 S845
pGluR1 S831
pNR1 S897
pERK
pCREB S133
pSynapsin S9
pTHS40
Con AY CY CSA
pGluR1S845
pGluR1S831
GluR1
Con AY CY CSA
pERK
ER

B


Accumbens Core
Protein Phosphorylation
(% Δ from control)
75
25
-25
-75
pGluR1 S845
pGluR1 S831
pNR1 S897
pERK
pCREB S133
pSynapsin S9
pTHS40
Con AY CY CSA
pGluR1S845
pGluR1S831
GluR1
Con AY CY CSA
pCREBS133
CREB

C

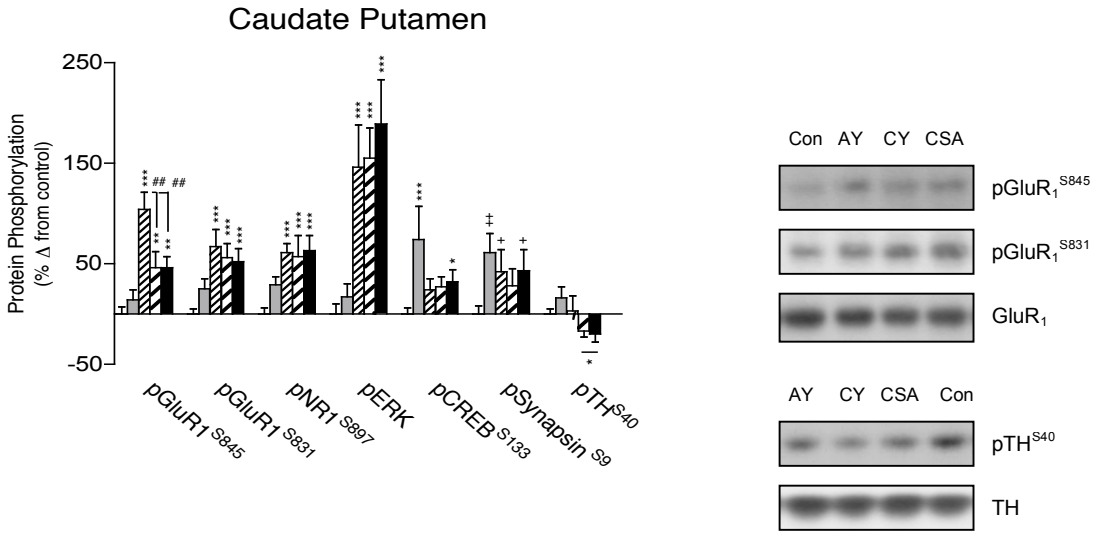

Caudate Putamen
Protein Phosphorylation
(% Δ from control)
250
150
50
-50
pGluR1 S845
pGluR1 S831
pNR1 S897
pERK
pCREB S133
pSynapsin S9
pTHS40
Con AY CY CSA
pGluR1S845
pGluR1S831
GluR1
AY CY CSA Con
pTHS40
TH

Figure 2.3
Cocaine-induced protein phosphorylation in striatal subregions. (**A**) In the nucleus accumbens shell, cocaine increases PKA-mediated $GluR_1^{S845}$ and NR_1^{S897} phosphorylation, while decreasing PKA-mediated TH^{S40} phosphorylation, but does not significantly increase PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation. (**B**) In the nucleus accumbens core, tolerance develops to cocaine-induced PKA-mediated $GluR_1^{S845}$, but not NR_1^{S887}, phosphorylation with chronic intravenous administration, while cocaine-induced $CREB^{S133}$ phosphorylation is produced by yoked (YK) but not self-administered (SA) cocaine. (**C**) In the caudate-putamen, tolerance also develops to cocaine-induced PKA-mediated $GluR_1^{S845}$ phosphorylation, but not PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation, and cocaine reduces TH^{S40} phosphorylation only after chronic administration. Cocaine-induced ERK phosphorylation remains constant in all striatal regions with acute and chronic administration. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n = 8-13/group). Symbols indicate values differ from controls ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), or from acute YK cocaine ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$) by Fisher's LSD tests (differs from controls by planned Student's t-test comparisons, $^{+}p < 0.05$, $^{++}p < 0.01$). Representative blots for phosphorylated and total $GluR_1$, TH, ERK and CREB protein are shown for control (Con), acute yoke (AY), chronic yoke (CY), and chronic cocaine self-administering (CSA) animals.

unchanged with acute or chronic cocaine administration, increasing by 23-28% in the NAS ($F_{4,49} = 2.930$, $p = 0.030$), 15-33% in the NAC ($F_{4,48} = 4.353$, $p = 0.004$), and 57-63% in the CPU ($F_{4,53} = 6.771$, $p < 0.001$). These results suggest that regulation of $GluR_1^{S845}$ and NR_1^{S897} phosphorylation may occur in distinct post-synaptic compartments through local interactions with PKA. In contrast to PKA, cocaine-specific increases in PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation (52-67%) were limited to the CPU ($F_{4,53} = 7.114$, $p < 0.001$), since increases in the NAC ($F_{4,58} = 4.254$, $p = 0.004$) were found in both cocaine and saline SA groups.

Contrary to increases in PKA-mediated phosphorylation of post-synaptic proteins, 4 hrs of intravenous cocaine administration decreased PKA-mediated TH^{S40} phosphorylation by 25-29% in dopamine terminals in the NAS ($F_{4,49} = 7.697$, $p < 0.001$), while increasing PKA/CaMKI-mediated $synapsin^{S9}$ phosphorylation ($F_{4,50} = 6.546$, $p < 0.001$), but there was no change in this response following chronic administration (Fig. 2.3A). In the CPU, minor decreases in TH^{S40} phosphorylation were found only after chronic cocaine administration in pooled YK and SA groups ($F_{3,54} = 3.359$, $p = 0.025$), and increases in $synapsin^{S9}$ phosphorylation were not specifically related to cocaine administration.

In striatal regions, MEK regulation of ERK phosphorylation differed from cortical regions, since it was specifically induced by cocaine and not by Saline SA and associated procedural effects. ERK phosphorylation increased 76-82% in the NAS ($F_{4,51} = 2.984$, $p = 0.027$), 32-62% in the NAC ($F_{4,60} = 4.311$, $p = 0.004$), and marked 146-189% increases

in the CPU that were greater than any other brain region examined ($F_{4,53} = 10.072$, $p < 0.001$). There was no change in the magnitude of ERK phosphorylation following chronic cocaine administration. In contrast, cocaine-induced $CREB^{S133}$ phosphorylation was virtually absent in the NAS, and a 34-39% increase in the NAC was limited to Acute or Chronic YK cocaine administration ($T_{29\text{-}30} = 2.329\text{-}2.668$, $p = 0.012–0.027$), since significant increases were not found in Chronic SA animals. In the CPU, a significant 32% increase in $CREB^{S133}$ phosphorylation was found in Chronic SA animals, but an even larger 74% increase was found in Saline SA animals indicating an effect of the testing procedures rather than cocaine exposure ($F_{4,52} = 4.238$, $p = 0.005$). These data suggest that PKA and MEK activity do not necessarily translate into an increase in $CREB^{S133}$ phosphorylation status after 4 hrs of cocaine administration.

Tolerance to PKA-, but not PKC/CaMKII- or MEK-, mediated phosphorylation in basolateral (BLA) and centromedial (CEN) amygdala

In both BLA and CEN amygdala subregions, tolerance developed to cocaine-induced PKA-mediated $GluR_1^{S845}$ phosphorylation following chronic administration (Fig. 2.4), similar to changes in striatal regions. Thus, $GluR_1^{S845}$ phosphorylation in the BLA was reduced from 64% in the Acute YK group to 39% in Chronic YK and SA groups ($F_{4,60} = 13.4416$, $p < 0.001$). In the CEN, $GluR_1^{S845}$ phosphorylation was reduced from 38% in the Acute YK group to 20% in Chronic YK and SA groups ($F_{4,60} = 4.721$, $p = 0.002$). Cocaine increased PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation in the BLA by 51-65% in all groups ($F_{4,61} = 5.108$, $p = 0.001$), but no significant increases in $GluR_1^{S831}$

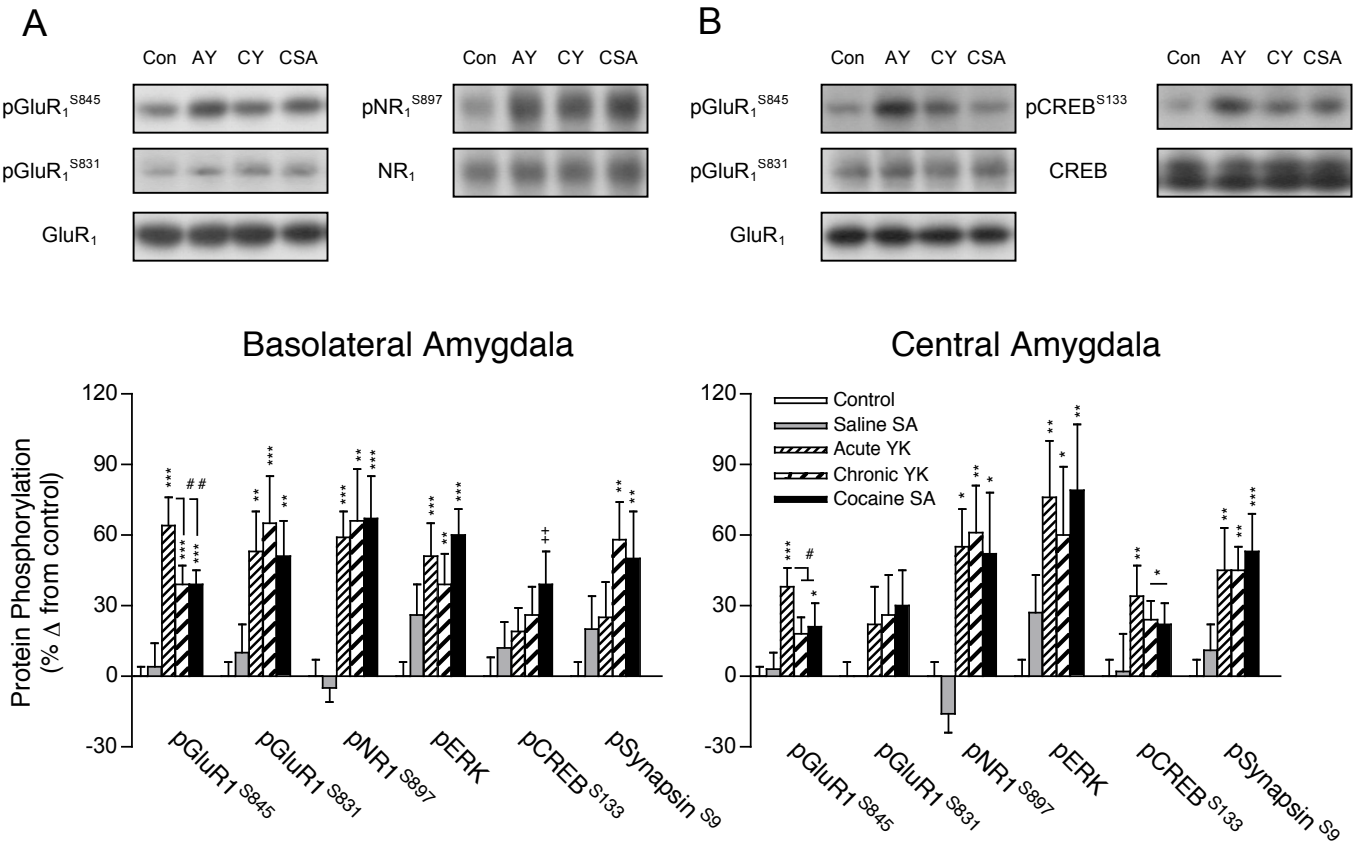


Figure 2.4
Cocaine-induced protein phosphorylation in amygdala subregions. (**A**) In the basolateral nucleus, tolerance develops to cocaine-induced PKA-mediated $GluR_1^{S845}$, but not PKC/CaMKII-mediated $GluR_1^{S831}$ or PKA-mediated NR_1^{S897} phosphorylation, following chronic intravenous administration. (**B**) In the central nucleus, tolerance also develops to cocaine-induced PKA-mediated $pGluR_1^{S845}$, but not NR_1^{S887} phosphorylation. Cocaine-induced ERK phosphorylation remains constant with acute and chronic administration in both amygdala subregions. Only the amygdala subregion show increases in $CREB^{S133}$ phosphorylation following 4 hrs of cocaine (and not saline) self-administration. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n = 8-12/group). Symbols indicate values differ from controls ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), or from acute YK cocaine ($^{\#}p < 0.05$) by Fisher's LSD tests. Differs from controls by planned Student's t-test comparisons ($^{++}p < 0.01$). Representative blots for phosphorylated and total $GluR_1$, NR_1 and CREB protein are shown for control (Con), acute yoke (AY), chronic yoke (CY), and chronic cocaine self-administering (CSA) animals.

phosphorylation were detected in the CEN. PKA-mediated increases in NR_1^{S897} phosphorylation were relatively larger in the amygdala than in the NAS or NAC, increasing by 59-67% in the BLA ($F_{4,61} = 6.750$, $p < 0.001$) and 52-61% in the CEN ($F_{4,59} = 4.771$, $p = 0.002$), but tolerance failed to develop with chronic cocaine in YK and SA groups similar to results in striatal regions.

In the BLA, cocaine increased ERK phosphorylation (39-60%) in all cocaine-exposed groups ($F_{4,59} = 5.584$, $p = 0.001$), but a 39% increase in $CREB^{S133}$ phosphorylation was found only in the Chronic SA group ($T_{32} = 2.731$, $p = 0.010$). Thus, regulation of $CREB^{S133}$ phosphorylation in the BLA is enhanced by reinforcement-related influences. In the CEN, cocaine increased ERK phosphorylation by 60-79% ($F_{4,60} = 3.565$, $p = 0.011$), and this effect was accompanied by 22-34% increases in $CREB^{S133}$ phosphorylation in all cocaine exposed groups ($F_{3,61} = 3.218$, $p = 0.029$). $Synapsin^{S9}$ phosphorylation in pre-synaptic BLA terminals increased by 50-58% with chronic, but not acute, cocaine administration in both YK and SA groups ($F_{4,61} = 3.553$, $p = 0.011$), while 45-53% increases were found in all cocaine exposed groups in the CEN ($F_{4,60} = 4.398$, $p = 0.003$). Cocaine-induced $synapsin^{S9}$ phosphorylation was greater in amygdala than in striatal regions. All cocaine-induced protein phosphorylation in the amygdala was related specifically to cocaine administration, since there were no significant changes in the Saline SA group relative to untreated controls.

Sensitization in cocaine-induced $synapsin^{S9}$ phosphorylation in the CA3 hippocampal subregion

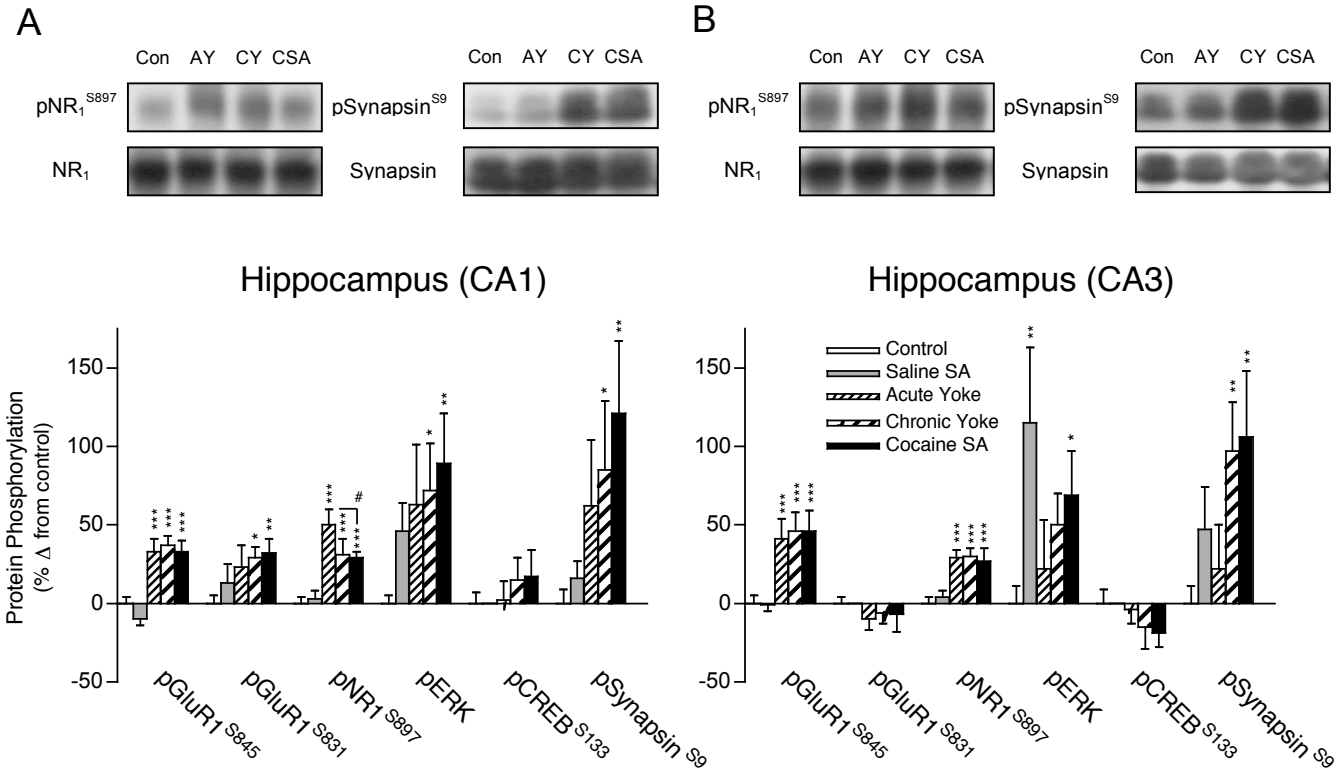


Figure 2.5
Cocaine-induced protein phosphorylation in hippocampal subregions. (**A**) In the CA1 subregion, tolerance develops to cocaine-induced PKA-mediated NR_1^{S897} phosphorylation, but not PKA-mediated $GluR_1^{S845}$ phosphorylation, following chronic intravenous administration. (**B**) In the CA3, tolerance fails to develop to cocaine-induced PKA-mediated NR_1^{S897} or $GluR_1^{S845}$ phosphorylation. In both subregions, sensitization develops to cocaine-induced synapsin ^{S9} phosphorylation after chronic administration. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n = 8-11/group). Symbols indicate values differ from controls ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), or from acute yoked cocaine ($^{\#}p < 0.05$) by Fisher's LSD tests. Representative blots for phosphorylated and total NR_1 and synapsin are shown for control (Con), acute yoke (AY), chronic yoke (CY), and chronic cocaine self-administering (CSA) animals.

The largest increases in PKA/CaMKI-mediated synapsin^{S9} phosphorylation were found in the CA1 and CA3 subregions of the hippocampus, but only with chronic and not acute cocaine administration (Fig. 2.5). In the CA1 subregion, synapsin^{S9} phosphorylation increased by 85-121% in Chronic YK and SA groups ($F_{4,52} = 2.860$, $p = 0.032$), while synapsin^{S9} phosphorylation increased by 97-106% in the CA3 subregion ($F_{4,52} = 3.271$, $p = 0.018$). These results indicate that sensitization develops in cocaine-induced synapsin^{S9} phosphorylation in pre-synaptic terminals with repeated administration. Similarly, ERK phosphorylation in the CA1 significantly increased (72-89%) only in chronic YK and SA groups ($F_{4,52} = 2.933$, $p = 0.029$). In the CA3, a 69% increase in ERK phosphorylation in Chronic SA animals was paralleled by a large 115% increase in the Saline SA group ($F_{4,51} = 2.965$, $p = 0.028$), potentially reflecting exposure to response-contingent injection cues in both Saline and Cocaine SA groups. CREB^{S133} phosphorylation failed to increase in either CA1 or CA3 hippocampal subregions.

While chronic cocaine administration produced tolerance to PKA-mediated $GluR_1^{S845}$ phosphorylation in striatal and amygdala subregions, tolerance developed to PKA-mediated NR_1^{S897} phosphorylation in the hippocampal CA1 subregion, where a 50% increase in the Acute YK group was reduced to only a 29% increase in Chronic SA animals ($F_{4,52} = 10.803$, $p < 0.001$). In contrast, PKA-mediated $GluR_1^{S845}$ phosphorylation was similar (33-37%) in all cocaine-exposed groups ($F_{4,52} = 13.537$, $p < 0.001$). In the CA3 subregion, both NR_1^{S897} ($F_{4,51} = 8.111$, $p < 0.001$) and $GluR_1^{S845}$ ($F_{4,52} = 6.253$; $p < 0.001$) phosphorylation were increased to similar levels by acute and chronic cocaine administration, with increases of 27-30% and 41-46% in these subunits, respectively.

Thus, in addition to striatal and amygdala regions, tolerance developed to cocaine-induced PKA-mediated phosphorylation in the CA1 subregion of the hippocampus, but the effect involved NR_1^{S897} instead of $GluR_1^{S845}$ phosphorylation, further supporting the notion of highly localized PKA regulation in post-synaptic membranes. PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation increased by 23-32% specifically in the CA1, and not CA3, subregion ($F_{4,52} = 3.060$, $p = 0.024$), and achieved significance only with chronic administration in YK and SA groups.

Reinforcement-related regulation of protein phosphorylation in self-administering animals

Certain changes in protein phosphorylation were significantly correlated with the number of cocaine injections and specifically in Chronic SA animals (Fig. 2.6). Thus, PKA-mediated $GluR_1^{S845}$ phosphorylation in the CPU and BLA correlated positively with individual levels of cocaine intake in Chronic SA, but not Chronic YK animals (Table 2.2), despite the development of substantial tolerance to cocaine-induced $GluR_1^{S845}$ phosphorylation in both groups. Interestingly, in the NAC, $GluR_1^{S845}$ phosphorylation was negatively correlated with cocaine intake in self-administering animals (Fig 2.6). Thus, animals that voluntarily consume greater amounts of cocaine apparently develop even greater tolerance to cocaine-induced $GluR_1^{S845}$ phosphorylation in the NAC, while simultaneously developing less tolerance to cocaine-induced $GluR_1^{S845}$ phosphorylation in the CPU and BLA, than animals with lower preferred levels of cocaine intake. In

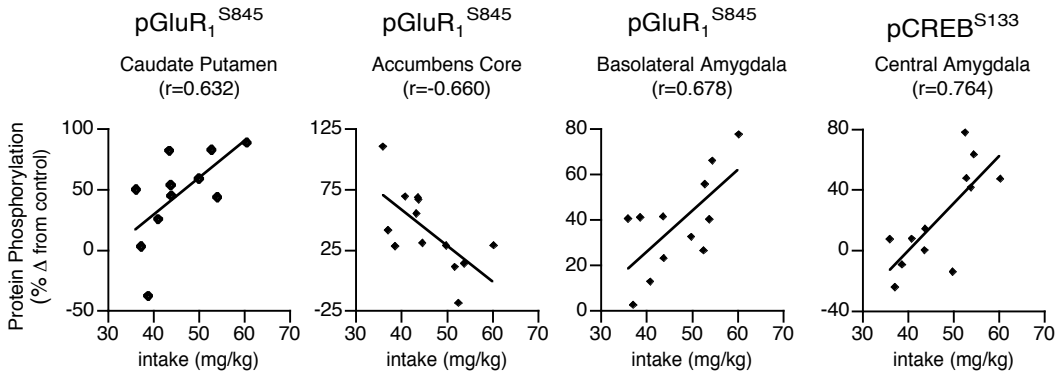


Figure 2.6
Correlation of protein phosphorylation with individual levels of cocaine intake averaged over the last 6 sessions in self-administering animals. Linear regression analysis found positive correlations for $GluR_1^{S845}$ phosphorylation in the caudate putamen ($p = 0.036$) and basolateral amygdala ($p = 0.016$), and $pCREB^{S133}$ phosphorylation in the central amygdala ($p = 0.004$) with increasing cocaine intake. A negative correlation for $GluR_1^{S845}$ phosphorylation in the nucleus accumbens core ($p = 0.014$) may reflect greater tolerance with higher daily cocaine intake in self-administering animals. Correlations are related to cocaine reinforcement since they were not found in chronic YK cocaine animals (see Tables 2 and 3).

addition, cocaine-induced $CREB^{S133}$ phosphorylation in the CEN was positively correlated with cocaine intake in self-administering animals (Fig. 2.6). These positive correlations likely reflect dose-dependent increases in cocaine-induced protein phosphorylation. However, since positive correlations only occur in Chronic SA and not Chronic YK groups, they are related specifically to the behavioral context of cocaine reinforcement.

There were other significant negative correlations that occurred specifically in Chronic YK and not Chronic SA groups (Table 2.3). PKC/CaMKII-mediated $GluR_1^{S831}$ phosphorylation was negatively correlated with the number of cocaine injections in the CA1, and PKA-mediated NR_1^{S897} phosphorylation was negatively correlated with the number of cocaine injections in the CA3, despite an overall lack of tolerance in this response when compared to Acute YK cocaine administration. $CREB^{S133}$ phosphorylation also was negatively correlated with the number of cocaine injections in the NAS of Chronic YK animals. These negative dose-related correlations suggest that tolerance may develop to cocaine-induced regulation of these phosphoproteins with higher but not lower cocaine doses. In contrast, the number of Acute YK cocaine injections were negatively correlated with $GluR_1^{S845}$ and $CREB^{S133}$ phosphorylation in the NAC, and ERK phosphorylation in the NAS and CEN, suggesting that within-session tachyphylaxis rapidly develops with more cocaine injections over the 4 hrs of acute exposure. However, this dose-related loss in cocaine-induced protein phosphorylation was no longer evident with chronic YK administration with or without the development of tolerance. Finally, the number of cocaine injections was negatively correlated with

Table 2.2 Positive Correlations of Cocaine Intake and ΔProtein Phosphorylation

	$pGluR_1^{S845}$		**$pCREB^{S133}$**
Intake Group	CPU	BLA	CEN
Acute Yoke	r = -0.304(10)	r = -0.448(11)	r = 0.008(11)
Chronic Yoke	r = -0.103(10)	r = -0.103(11)	r = -0.182(11)
Cocaine SA	r = 0.632*(11)	r = 0.678*(12)	r = 0.764**(12)

Asterisks indicate *p<0.05, **p<0.01.

Table 2.3 Negative Correlations of Cocaine Intake and ΔProtein Phosphorylation

	$pGluR_1^{S845}$		**$pGluR_1^{S831}$**	**pNR_1^{S897}**
Intake Group	PMC	NAC	CA1	CA3
Acute Yoke	r = -0.665*(10)	r = -0.771**(11)	r = -0.238(9)	r = -0.398(9)
Chronic Yoke	r = -0.758*(10)	r = -0.131(12)	r = -0.642*(10)	r = -0.738*(10)
Cocaine SA	r = -0.057(11)	r = -0.660*(13)	r = 0.012(11)	r = -0.175(11)

	pERK		**$pCREB^{S133}$**	
Intake Group	NAS	CEN	NAS	NAC
Acute Yoke	r = -0.713*(9)	r = -0.608*(11)	r = -0.467(12)	r = -0.760**(12)
Chronic Yoke	r = -0.248(11)	r = -0.338(12)	r = -0.777**(12)	r = -0.394(11)
Cocaine SA	r = -0.357(10)	r = -0.240(12)	r = -0.175(13)	r = -0.339(12)

Asterisks indicate *p<0.05, **p<0.01.

$GluR_1^{S845}$ phosphorylation in the PMC with either Acute or Chronic YK administration, but such tachyphylaxis was completely abolished in Chronic SA animals, despite no overall group differences in cocaine-induced $GluR_1^{S845}$ phosphorylation.

Discussion

In this study, we measured changes in protein phosphorylation status immediately following 4 hrs of intravenous cocaine self-administration in animals with chronic self-administration experience, and compared these results to animals that received an identical number and temporal pattern of cocaine injections via passive yoked administration. We compared the response following chronic yoked cocaine injections to the response induced by acute yoked injections to identify the development of tolerance or sensitization in cocaine regulation of protein phosphorylation. Acute exposure to 4 hrs of passive intravenous cocaine injections produced widespread but differential changes in both pre- and post-synaptic protein phosphorylation in several terminal regions of the mesolimbic dopamine system. Cocaine increased PKA-mediated phosphorylation of the AMPA glutamate receptor subunit $GluR_1$ at serine 845 in every brain region studied. The greatest increase in $GluR_1^{S845}$ phosphorylation was found in the CPU (104%), while the smallest was found in the hippocampal CA1 region (33%) with acute cocaine administration. Other phosphoproteins were differentially regulated across brain regions.

Region-specific tolerance to cocaine-induced $GluR_1$ and NR_1 phosphorylation following chronic self-administration

Substantial tolerance developed to cocaine-induced PKA-mediated $GluR_1^{S845}$ phosphorylation in the NAC, CPU, BLA, and CEN, with 39-56% reductions in the acute response following chronic administration. In the NAC, the level of tolerance to PKA-mediated $GluR_1^{S845}$ phosphorylation was specifically associated with higher levels of stabilized cocaine intake self-administering animals, a behavioral index thought to reflect more addicted biological states (Ahmed and Koob 1998). The development of greater tolerance to cocaine-induced $GluR_1^{S845}$ phosphorylation in these animals could be related to the fact that they self-administered a higher amount of cocaine over the course of testing. Conversely, it is possible that tolerance to cocaine's biochemical response caused these animals to self-administer greater amounts of cocaine. Indeed, chronic cocaine self-administration has been shown to reduce D_1 receptor-stimulated adenylate cyclase activity (Graziella De Montis et al. 1998b), and experimental blockade of D_1 receptors in either nucleus accumbens or amygdala (but not CPU) causes a similar escalation in cocaine intake (Caine et al. 1995). In addition, reduced D_1 receptor coupling to adenylate cyclase could account for a reduction in cocaine actions on PKA-mediated $GluR_1^{S845}$ phosphorylation following chronic administration. Chronic cocaine self-administration up-regulates PKA levels in the nucleus accumbens (Lu et al. 2003), and PKA up-regulation could account for increased basal $GluR_1^{S845}$ phosphorylation in the NAS and CPU after 1 day WD (see Chapter 4). It is possible that PKA up-regulation would act reciprocally to phosphorylate and down-regulate D_1 signaling pathways as

shown *in vitro* (Gardner et al. 2001), leading to reduced dopamine-mediated $GluR_1^{S845}$ phosphorylation by cocaine. In this regard, sustained (but not acute) activation of PKA in the NAC causes a tolerance-like escalation in cocaine intake in self-administering animals (Self et al. 1998), similar to the effect of D1 receptor blockade and consistent with negative feedback regulation.

In contrast to the NAC, cocaine-induced $GluR_1^{S845}$ phosphorylation in the CPU and BLA increased with the number of cocaine injections self-administered among individual rats, despite the overall development of substantial tolerance to this response compared to acute cocaine administration. These dose-related increases in $GluR_1^{S845}$ phosphorylation were not found in their Chronic YK partners that received an identical pattern and number of cocaine injections throughout testing, although the Chronic YK group developed similar tolerance as the Chronic SA group. Thus, both positive and negative correlations in $GluR_1^{S845}$ phosphorylation in the NAC, CPU and BLA were specifically related to cocaine reinforcement and not chronic cocaine exposure. These correlations also could reflect the fact that self-administering animals were allowed to titrate their preferred amount of cocaine intake, whereas yoked animals had no control over the level of cocaine intake.

PKA-mediated $GluR_1^{S845}$ phosphorylation potentiates excitatory currents mediated by AMPA receptors (Roche et al. 1996; Banke et al. 2000), and promotes AMPA receptor insertion in synaptic membranes ultimately leading to an enhancement of synaptic plasticity (Esteban et al. 2003; Mangiavacchi and Wolf 2004). Tolerance to cocaine-induced $GluR_1^{S845}$ phosphorylation would reduce the impact of cocaine on

excitatory input to striatal and amygdala neurons during self-administration, a view supported by findings that chronic cocaine self-administration reduces neuronal excitability in the nucleus accumbens (Schramm-Sapyta *et al*., 2006). This reduction is reflected by reduced AMPA-mediated currents in accumbens neurons that may preclude further weakening with protocols to induce long-term depression (LTD) in cocaine-exposed animals (Martin et al. 2006). Since LTD is associated with reduced PKA-mediated $GluR_1^{S845}$ phosphorylation (Song and Huganir 2002), and trafficking of PKA away from post-synaptic membranes in the hippocampus (Smith et al. 2006), a reduction in cocaine's ability to induce $GluR_1^{S845}$ phosphorylation could reflect similar LTD-associated PKA trafficking in striatal and amygdala regions. Furthermore, a weakened cocaine effect on $GluR_1^{S845}$ phosphorylation following chronic administration could facilitate sensitization to cocaine's psychomotor and incentive motivational effects, since our preliminary results suggest that down-regulating endogenous GluR1 function in nucleus accumbens neurons induces cocaine sensitization and promotes cocaine-seeking behavior (Bachtell et al. 2005). Conversely, over-expression of GluR1 attenuates cocaine-conditioned place preference, cocaine sensitization, and cocaine-seeking behavior (Kelz et al. 1999; Sutton et al. 2003; Bachtell et al. 2005).

In addition to PKA-mediated $GluR_1^{S845}$ phosphorylation, cocaine also increased PKC /CaMKII-mediated $GluR_1^{S831}$ phosphorylation, but this response was restricted to 5 of 9 regions studied, with greatest increases in the CPU (52-67%) and BLA (51-65%), and also in both cortical subregions and the hippocampal CA1 subregion. Cocaine-induced $GluR_1^{S831}$ phosphorylation also would facilitate excitatory currents mediated by

AMPA receptors (Derkach et al. 1999), and could reflect a cocaine-induced elevation in intracellular calcium. However, tolerance to cocaine-induced $GluR_1^{S831}$ phosphorylation developed only in the PFC, representing a 54% reduction from acute cocaine administration, but failed to develop in regions displaying tolerance to PKA-mediated $GluR_1^{S845}$ phosphorylation. This effect could be related to hypofrontality and other changes in the physiological response to cocaine that developed in the PFC after chronic self-administration (Kalivas et al. 2005), and was not found in the non-limbic PMC cortical region. There was no significant cocaine-related $GluR_1^{S831}$ phosphorylation in the shell subregion of the nucleus accumbens, the CEN amygdala subregion, or the CA3 subregion of the hippocampus.

Furthermore, tolerance to PKA-mediated phosphorylation of the NR_1^{S897} NMDA glutamate receptor subunit failed to develop in striatal and amygdala brain regions that exhibited tolerance to PKA-mediated $GluR_1^{S845}$ phosphorylation with chronic cocaine administration. Given that $GluR_1$ and NR_1 subunits co-localize in excitatory post-synaptic membranes, these findings could reflect a high degree of compartmentalization that differentially regulates PKA access to $GluR_1$ and NR_1 following chronic cocaine. The greatest increase in NR_1^{S897} phosphorylation occurred in the BLA (59-67%) and CEN (52-61%) amygdala subregions, but also substantial increases in the CPU, with less regulation in all other regions studied. However, tolerance to PKA-mediated NR_1^{S897} phosphorylation did develop in the CA1 hippocampal region after chronic self-administration, reflecting a 42% reduction in phosphorylated NR_1^{S897} from acute cocaine administration. PKA-mediated phosphorylation of the serine 897 residue on NR_1

enhances NMDA receptor-mediated currents (Westphal et al. 1999), and so tolerance to cocaine-induced NR_1^{S897} phosphorylation could lessen the increase in excitability in the CA1 region of the hippocampus during self-administration, similar to reduced phosphorylation of $GluR_1^{S845}$ in striatal regions.

Dissociation of cocaine-induced ERK and CREB^{S133} phosphorylation in the NAS and hippocampal subregions

ERK is activated via dual phosphorylation of threonine 183 and tyrosine 185 by MEK (Payne et al. 1991), ultimately involving convergence of multiple and complex signaling cascades. ERK phosphorylation was significantly increased in all regions studied following acute and chronic cocaine administration, but similar or greater ERK phosphorylation was found in animals self-administering saline in both PFC and PMC and in CA3 hippocampal subregions, indicating an effect of testing unrelated to cocaine exposure in these brain areas. There was no evidence for tolerance to cocaine-induced ERK phosphorylation following chronic administration in any brain region studied. These findings agree with previous studies showing ip administration of cocaine and other drugs of abuse acutely increases ERK phosphorylation in striatal and amygdala regions through dopamine-dependent mechanisms (Valjent et al. 2004). However, a recent study found an enhancement in cocaine-induced ERK phosphorylation following repeated ip cocaine injections in the nucleus accumbens, and relatively weak increases (<50%) in the CPU (Mattson et al. 2005). In our study, greater increases in striatal ERK phosphorylation are likely due to pronged (4 hr) exposure to multiple intravenous cocaine

injections with either acute or chronic cocaine administration. ERK activity has been implicated in the formation, retrieval and reconsolidation of memory in cortical and hippocampal regions (Hasegawa 2000). Thus, regulation of ERK activity during both acute and chronic cocaine administration could be involved in the induction and reconsolidation of critical associations between cocaine, the cocaine-related environment, and the act of lever-pressing in self-administering animals. ERK activity in the CEN also is necessary for enhancement (incubation) of cocaine-seeking behavior in withdrawal from self-administration (Lu et al. 2005), suggesting that ERK plays a role in the recall of cocaine-related memories and their influence on motivated behavior.

Many long-term consequences of PKA and ERK activity are mediated through activation of the transcription factor CREB via phosphorylation of serine 133 leading to recruitment of CREB binding protein and transcriptional activation (Yamamoto et al. 1988). The functional effects of CREB-mediated transcription can depend on the specific brain region where CREB activity increases (Carlezon et al. 2005). We found that 4 hrs of acute intravenous cocaine administration increased $CREB^{S133}$ phosphorylation only in the NAC (39%) and CEN (34%). Tolerance to cocaine-induced $CREB^{S133}$ phosphorylation was found in the CEN, reflecting a 36% reduction in the response to acute cocaine. In chronic self-administering animals, cocaine-induced $CREB^{S133}$ phosphorylation also increased in the BLA, but was not significantly increased in the NAC. No other brain regions showed cocaine-related increases in $CREB^{S133}$ phosphorylation, since increases in the PFC and CPU were similar or greater in animals self-administering saline.

The lack of cocaine-induced CREB^{S133} phosphorylation with either acute or chronic administration in the hippocampal subregions and NAS, in particular, is interesting since a previous study found large (>150%) increases in nucleus accumbens CREB^{S133} phosphorylation that peak 20 min after an ip injection following repeated daily exposure (Mattson et al. 2005). These latter findings may suggest a rapid desensitization or tachyphylaxis to cocaine-induced CREB^{S133} phosphorylation during ongoing intravenous cocaine administration. Indeed, the dissociation of substantial ERK phosphorylation in the absence of detectable CREB phosphorylation in the NAS and other brain regions would support this notion. Furthermore, we found a negative correlation between the number of yoked cocaine injections and the level of CREB^{S133} phosphorylation in the NAC (Acute YK) and NAS (Chronic YK), indicating that cocaine–induced CREB^{S133} phosphorylation remained elevated with the administration of fewer cocaine injections (Table 2.3). Conversely, CREB^{S133} phosphorylation in the CEN amygdala subregion correlated positively with the number of cocaine injections, but only in animals self-administering cocaine (Table 2.2 and Fig. 2.6). Reasons for these differential responses to cocaine injections are unknown, but could reflect regional differences in the density of dopaminergic innervation. In any event, CREB activation in the CEN could play a role in the formation and consolidation of cocaine-associated memories as discussed above.

Pre-synaptic regulation of TH and synapsin I phosphorylation following acute and chronic cocaine administration

In striatal regions with dense dopaminergic innervation, we measured cocaine regulation of TH phosphorylation in pre-synaptic dopaminergic terminals. Dopamine synthesis is increased by PKA-mediate TH phosphorylation at serine 40 (Daubner et al. 1992), but acute ip cocaine administration reduces TH^{S40} phosphorylation in striatal regions (Jedynak et al. 2002), presumably through dopaminergic action at presynaptic D_2 autoreceptors. In the NAS, we found that TH^{S40} phosphorylation was consistently reduced by about 25% after 4 hrs of intravenous cocaine administration in either acute or chronic cocaine groups, whereas decreases in TH^{S40} phosphorylation in the NAC (yoked only) and CPU were only found after chronic cocaine administration, indicating sensitization in this response in regions with relatively less dopaminergic innervation (Heimer et al. 1997). Cocaine-induced decreases in TH^{S40} phosphorylation would provide inhibitory feedback regulation of dopamine synthesis, but the development of sensitization in this response with chronic administration in the NAC and CPU could also reflect sensitization in cocaine-induced dopamine release and D_2 autoreceptor activation (Kalivas and Duffy 1993). Interestingly, TH^{S40} phosphorylation increased in the NAS in animals self-administering saline, opposite to decreases in cocaine-injected animals. There was no change in the total amount of TH in the NAS following chronic cocaine self-administration, consistent with our previous report where decreases in TH required 1 week of withdrawal to develop after chronic cocaine self-administration (Schmidt et al. 2001).

In contrast to decreases in TH^{S40} phosphorylation, 4 hrs of intravenous cocaine increased pre-synaptic phosphorylation of synapsin I in the NAS and CEN regions of the

extended amygdala with either acute or chronic administration. Phosphorylation of synapsin I at serine 9 by either PKA or CaMKI causes its dissociation from synaptic vesicles and ultimately promotes neurotransmitter release (Czernik et al. 1987). In addition, the synapsin family proteins are involved in facilitating cocaine-induced increases in dopamine levels in the mesolimbic dopamine system (Venton et al. 2006). Cocaine regulation of synapsin^{S9} phosphorylation in the striatum was specifically induced in the NAS, since increases in the NAC and CPU also occurred in animals self-administering saline. In the BLA, CA1 and CA3 regions, cocaine-induced synapsin^{S9} phosphorylation was increased after chronic, but not acute, administration, potentially reflecting a sensitization in cocaine-induced neurotransmitter release in these regions. Interestingly, there was no effect of cocaine administration on synapsin^{S9} phosphorylation in neocortical regions, potentially related to ability of cocaine to produce widespread cortical inhibition in humans (London et al. 1999).

In summary, we found that chronic intravenous cocaine administration results in region- and substrate-specific tolerance to cAMP/PKA-mediated phosphorylation. Tolerance to PKA-mediated $GluR_1$ phosphorylation was found in striatal and amygdala subregions, whereas tolerance to NR_1 phosphorylation was found in the CA1 subregion of hippocampus. The acute effects of cocaine produced negative dose-related correlations in $GluR_1^{S845}$, ERK, and CREB ^{S133} phosphorylation in many regions, suggesting rapid desensitization in this response within a 4 hr test session. Following chronic cocaine administration, passively delivered yoked injections produced negative dose-related correlations in the phosphorylation of $GluR_1^{S831}$, NR_1^{S897}, and CREB ^{S133} in

other regions, suggesting more rapid desensitization in the biochemical response with repeated exposure. Such desensitization also could be related to stressful aspects of uncontrollable yoked cocaine injections, since they were not found in animals self-administering cocaine. In contrast, all of the positive dose-related correlations with protein phosphorylation were found specifically in self-administering animals, including $GluR_1^{S845}$ in the CPU and BLA, and $CREB^{S133}$ in the CEN. Thus, these positive correlations were related to behavioral reinforcement rather than the amount of cocaine consumed, since they were not correlated in animals receiving cocaine by chronic yoked injections. There was no evidence for the development of tolerance in cocaine regulation of TH^{S40} phosphorylation, and cocaine-induced $synapsin^{S9}$ phosphorylation markedly increased with chronic administration in the BLA and hippocampus, suggesting that tolerance and sensitization to cocaine's biochemical response differentially develop in pre- and post-synaptic neuronal compartments. These differential region- and substrate-specific adaptations to cocaine-induced protein phosphorylation illustrate the complexity of cellular responses in mesolimbic dopamine terminal regions that could underlie the development of cocaine addiction.

CHAPTER 3

Phosphorylation of GluR1, ERK and CREB

Following Spontaneous Withdrawal from Chronic Heroin Self-Administration

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Introduction

The reinforcing effects of opiate drugs like heroin are mediated by opiate receptors in the ventral tegmental area (Bozarth and Wise 1981), nucleus accumbens (Olds 1982), and hippocampus (Stevens et al. 1991) through both dopamine-dependent and –independent mechanisms. Following chronic heroin self-administration, neuroadaptations in mesolimbic dopamine neurons and their target regions are thought to play a central role in the establishment and maintenance of opiate addiction (Self and Nestler 1995; Nestler and Aghajanian 1997). Given that opiate receptors are coupled to inhibitory G protein that reduce cyclic AMP formation (Childers 1991), compensatory up-regulation in PKA levels has been reported in the nucleus accumbens following chronic heroin self-administration (Self et al. 1995). However, the functional consequences of this PKA up-regulation on cyclic AMP/PKA-dependent protein phosphorylation in heroin WD has not been studied. Increased PKA activity in the nucleus accumbens can cause escalation in drug or alcohol intake (Self et al. 1998; Wand et al. 2001) and the aversive aspects of opiate withdrawal (WD) (Valverde et al. 1996). These latter aversive motivational effects could promote heroin self-administration in dependent subjects through alleviation of dysphoria and negative reinforcement (Hutcheson et al. 2001).

In addition to PKA-mediated protein phosphorylation, naltrexone-precipitated WD from chronic experimenter-administered morphine administration was shown to induce substantial ERK phosphorylation in cortex and striatum, but no changes were found after 24 hrs of spontaneous WD (Asensio et al. 2006). Double phosphorylation of ERK at Thr 183 and Tyr 185 is a reliable index of ERK activity (Rossmondo et al. 1992),

and leads to activity-dependent gene regulation (Sweatt 2004; Thomas and Huganir 2004). Furthermore, many of the long-term consequences of both PKA and ERK activity could involve downstream activation of the transcription factor CREB via phosphorylation of Ser 133 leading to recruitment of CREB binding protein and transcriptional activation (Yamamoto et al. 1988). In this regard, naltrexone-precipitated WD from continuous morphine administration induces CREB activation in the nucleus accumbens and amygdala, but decreases CREB activity in the ventral tegmental area (Shaw-Lutchman et al. 2002). Whether a similar CREB regulation occurs during spontaneous WD from chronic intravenous heroin self-administration on an intermittent schedule approximating human drug use patterns is unknown.

In this study, we measured PKA-specific phosphorylation of $GluR_1$ AMPA receptor subunits (Ser 845) as an *in vivo* marker of functional increases in PKA activity after 24 hrs of spontaneous WD from chronic heroin self-administration in daily 6 hr sessions. We compared PKA-dependent $GluR_1$ phosphorylation with ERK phosphorylation mediated by ERK kinase in multiple limbic and non-limbic brain nuclei. In striatal regions, we also measured $CREB^{S133}$ phosphorylation regulated by both PKA and ERK activity. We compared $CREB^{S133}$ phosphorylation with $GluR_1$ and ERK phosphorylation, since ERK signaling can play a crucial downstream role in PKA-mediated $CREB^{S133}$ phosphorylation in striatal regions (Grewal et al. 2000; Zanassi et al. 2001). We tracked the development of protein phosphorylation changes from 12 to 24 hrs of heroin WD, and determined whether naltrexone administration would exacerbate changes after 24 hrs. The functional consequence of $GluR_1$, ERK and $CREB^{S133}$

phosphorylation on the behavioral manifestation of opiate dependence is discussed in relation to intracellular signaling and regional neurotransmission.

Materials and methods

Subjects and surgery

Individually housed, male, Sprague-Dawley rats initially weighing 275-325g (Charles River, Kingston, RI, USA) were maintained in a climate-controlled environment (21° C) on a 12-hr light-dark cycle (lights on at 7:00 AM) in according to the National Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals*. Prior to HSA, animals were trained to lever-press for 45 mg sucrose pellets while maintained on a restricted diet at 85% original body weight. Sucrose self-administration was conducted in ventilated operant chambers (Med Associates, Georgia, VT) on a fixed-ratio 1 (FR1) reinforcement schedule until acquisition criteria were achieved (100 pellets self-administered for 3 consecutive days) to facilitate subsequent acquisition of heroin self-administration. Following acquisition of lever-press behavior, animals were fed ad libitum prior to surgical implantation of a chronic indwelling intrajugular catheter as described previously (Edwards et al. 2007). Catheters were flushed daily with 0.2 ml of heparinized (20 IU/ml) bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml) to prevent clotting and antibiotic ointment was applied daily to the catheter exit wound to prevent infection.

Heroin self-administration procedures in rats

Following a 1 week recovery from surgical catheterization, animals were allowed to acquire HSA by performing a single lever press response (FR1) to receive intravenous injections of heroin (60 μg/kg/100 μl injection) delivered over 5 sec in daily 6-hr sessions for 18 days in the operant test chambers. During each injection, the house light was turned off and a cue light above the active lever was illuminated, followed by an additional 10 sec time-out (TO) period when the both lights were off and lever responding produced no programmed consequence. Untreated controls for baseline protein phosphorylation comparison were matched by age and batch to HSA animals, but remained in their home cages and were handled daily throughout the testing procedure to habituate potential stress responses prior to euthanization. An additional group of home cage controls was injected with naltrexone hydrochloride (1 mg/kg) 1 hr prior to sacrifice to determine potential effects in non-dependent animals.

Measurement of protein phosphorylation in brain tissue homogenates

Twelve or 24 hrs after completion of the last HSA test session, animals were gently placed in a plexiglass restrainer and euthanized by microwave irradiation aimed at the head region (5 kW, 1.5 s, Murimachi Kikai Co., LTD. Tokyo, Japan). For determination of spontaneous and naltrexone-precipitated WD effects, animals withdrawn for 24 hrs were split into two groups that received were injected 1 hr prior to sacrifice with either naltrexone (1 mg/kg) or saline (1 ml/kg) following habituation of the injection procedure with 3 daily saline injections. Rat brains were rapidly dissected and regional tissue samples collected with 12-16 gauge punches from chilled coronal brain slices (1.5 mm

thick). Tissue samples were immediately homogenized by sonication in lysis buffer (320 mM sucrose, 5 nM HEPES, 50 mM NaF, 1 mM EGTA, 1 mM EDTA, 1% SDS, with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails I and II diluted 1:100; Sigma, St. Louis, MO, USA), boiled for 5 min, and stored at –80°C until determination of protein concentrations by the Lowry method. Samples of 20 μg protein were subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels using a Tris/Glycine/SDS buffer (Bio-Rad, Hercules, CA, USA), followed by electrophoretic transfer to PVDF membranes (polyvinylidene; Amersham, Piscataway, NJ, USA). Membranes were blocked overnight in 5% nonfat milk at 4° C, and incubated in primary antibody for $pGluR_1^{S845}$ (1:2500; Chemicon, Temecula, CA, USA), pERK (1:7500; Cell Signaling, Danvers, MA, USA), or $pCREB^{S133}$ (1:2500; Cell Signaling) for 24 hrs at 4° C. Membranes were washed and labeled with species-specific peroxidase-conjugated secondary (1:10,000; BioRad) for 1 hr at 25° C. Following chemiluminescence detection (ECL plus; Amersham), blots were stripped and reprobed for total protein levels of $GluR_1$ (1:10K; Chemicon), ERK (1:7500; Cell Signaling), or CREB (1:1500; Chemicon) as internal standards for phosphoprotein levels. Regional levels of phosphorylated and total $GluR_1$, ERK, and CREB were compared with β-tubulin (1:250K; Chemicon). Immunoreactivity was quantified by densitometry (Scion Image) under conditions linear over at least a 3-fold concentration range.

Data analysis

Mean daily heroin self-administration was compared by 2-factor ANOVA (study group x day). Since there were no differences in heroin self-administration between study groups, these data were pooled, and heroin intake in the first 3 days was compared to last 3 days by paired t-tests to determine the degree of escalation in heroin self-administration throughout testing. Densitized phosphorylated and total protein levels were expressed as a percentage of the mean for untreated control values (6-8/blot) to normalize data across blots. Phosphoproteins were corrected for individual total protein levels, and were compared by 1-factor ANOVA followed by Fishers Least Significant Difference (FLSD) posthoc comparisons among groups. Both phosphorylated and total $GluR_1$ levels were regulated in the VTA, and were analyzed separately in addition to phospho/total protein ratios.

Results

Animals (n=8-12/group) self-administered heroin (60 μg/kg/100 μl infusion) over 18 consecutive 6 hr sessions conducted during the dark cycle. As shown in Fig. 3.1a, protein phosphorylation was measured at both 12 and 24 hrs after the last self-administration session to track the development of changes in protein phosphorylation during spontaneous WD. The ability of a 1 hr pretreatment with naltrexone (1 mg/kg) to augment WD-induced regulation after 24 hrs was compared to spontaneous WD (saline pretreatment), and to naltrexone pretreatment in drug-naïve animals. Protein phosphorylation in all experimental groups was compared to untreated controls that remained in their home cages throughout testing. Animals trained to self-administer

heroin developed stable self-administration by the 3rd week of training, with mean daily (days 13-18) heroin intake averaging 2.9 ± 0.4 mg/kg (12 hr WD group), 3.4 ± 0.4 mg/kg (24 hr WD group), and 3.1 ± 0.5mg/kg (24 hr WD + NTX group). Taken together, self-administering animals demonstrated a mild but significant escalation of heroin intake between the first three (2.7 ± 0.1) and last three (3.3 ± 0.2) self-administration sessions ($T_{181} = 2.939$, $p = 0.0037$).

Tissue punches were collected from animals euthanized by microwave fixation according to anatomical regions depicted in Fig. 3.1b. Microwave fixation was used to preserve *in vivo* protein phosphorylation status and avoid rapid alterations typically observed following dissection of un-fixed tissue (O'Callaghan and Sriram 2004). Except where specifically noted, all of the observed changes involved regulation in phosphorylation states since there was no regulation in the total amount of these proteins in all brain regions except the ventral tegmental area.

Regional comparison of basal protein phosphorylation in drug-naïve animals

In order to provide a relative comparison of basal phosphoprotein levels across brain regions in untreated controls, 30 μg protein samples from each brain region were subjected to Western blot analysis of phosphorylated $GluR_1^{S845}$, ERK, and $CREB^{S133}$, followed by re-probing for corresponding total protein levels. Relative to the prefrontal cortex, $GluR_1^{S845}$ phosphorylation was ~50% lower in the caudate putamen, despite similar total amounts of $GluR_1$ in these regions (Fig. 3.2a). Similarly, $GluR_1^{S845}$ phosphorylation was substantially reduced in both the ventral tegmental area and

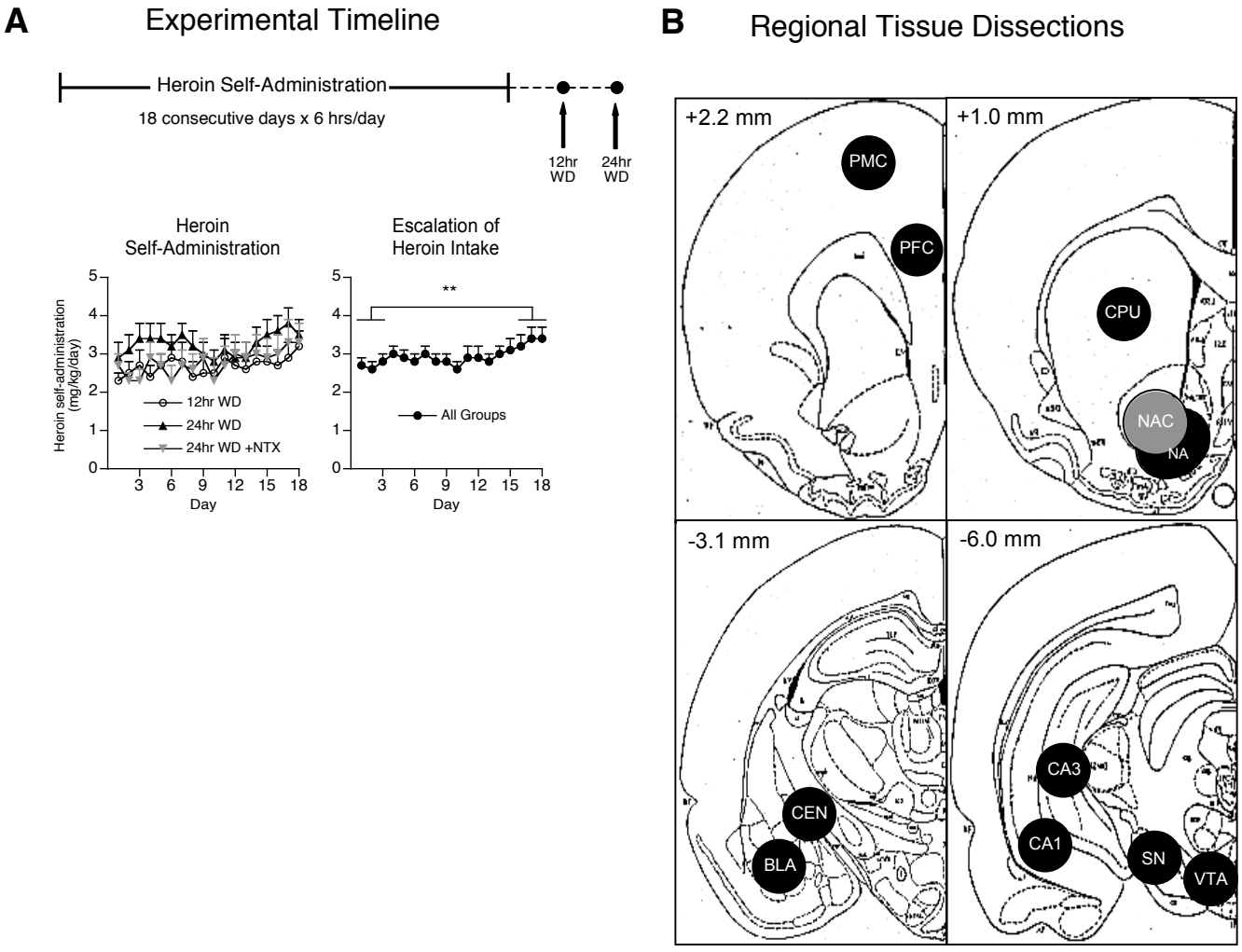
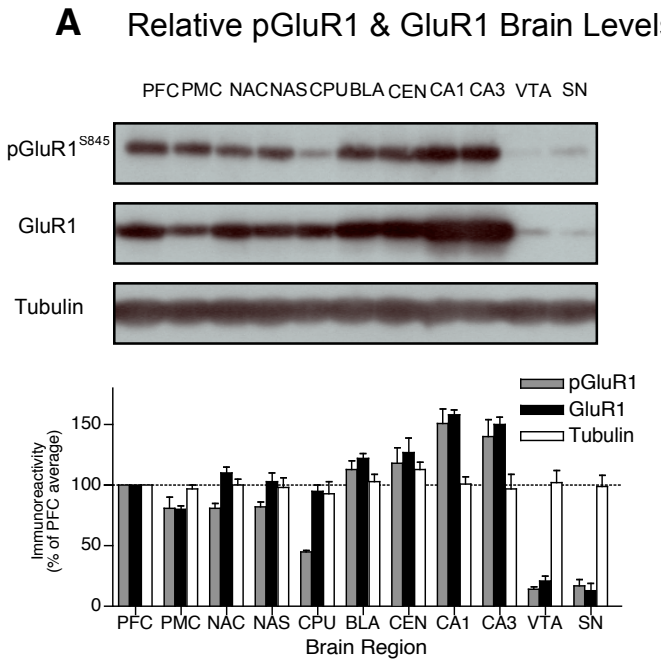


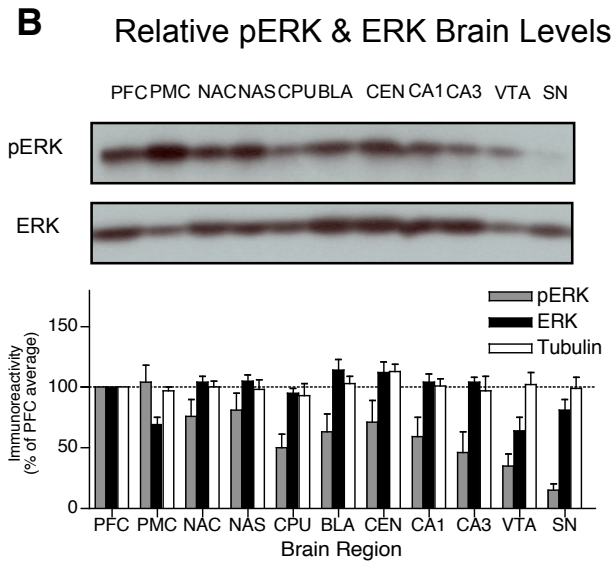
Figure 3.1
(A) Experimental timeline. Rats self-administered heroin (60μg/kg/50μl injection) over 18 consecutive 6-hr sessions during the dark cycle. Animals were sacrificed at either 12 or 24 hrs after their last session (12hr WD and 24hr WD group, respectively). Animals in the 24hr WD were injected with naltrexone (1mg/kg, 24hr WD +NTX group) or saline (1ml/kg) and sacrificed 1-hr later. Experimental groups did not differ in average heroin intake, but together exhibited a mild but significant escalation of heroin intake. Asterisks indicate significant difference between first three and last three days intake by Student's t-test (**$p < 0.01$). (B) Schematic representation of regional brain samples collected with 12-16 gauge punches from 1.5mm thick coronal slices. Anterior-posterior coordinates represent posterior sides of brain slices (Paxinos & Watson, 1998). Premotor cortex (PMC), prefrontal cortex (PFC), caudate putamen (CPU), nucleus accumbens core (NAC), nucleus accumbens shell (NAS), basolateral amygdala nucleus (BLA), central amygdala nucleus (CEN), substantia nigra (SN, ventral tegmental area (VTA).

substantia nigra (>80%) regions compared to prefrontal cortex. However, in both of these regions, reductions in basal $GluR_1^{S845}$ phosphorylation was paralleled by markedly reduced amounts of $GluR_1$ protein. In contrast, ~50% greater basal $GluR_1^{S845}$ phosphorylation and total $GluR_1$ levels were found in both CA1 and CA3 hippocampal subregions. Basal ERK phosphorylation was reduced by 50% or more in the caudate putamen, CA3, ventral tegmental area and substantia nigra, while total ERK protein was decreased only in the premotor cortex and ventral tegmental area relative to prefrontal cortex (Fig. 3.2b). In striatal subregions, neither $CREB^{S133}$ phosphorylation nor total amounts of CREB protein substantially differed between nucleus accumbens core, shell, and caudate putamen (Fig. 3.2c). Thus, only basal $GluR_1^{S845}$ and ERK phosphorylation in the caudate putamen was substantially reduced independent of total protein levels, and these changes were not paralleled by reduced $CREB^{S133}$ phosphorylation. β-tubulin levels were similar across all brain regions (Fig. 3.2a).

Dissociation of WD-induced $GluR_1^{S845}$ and ERK phosphorylation in prefrontal but not premotor cortex

In the prefrontal cortex, ERK phosphorylation increased by > 50% after 24 hrs, but not 12 hrs, WD from chronic heroin self-administration when compared to untreated controls ($F_{4,49} = 4.595$, $p = 0.003$), and there were no changes in PKA-mediated $GluR_1^{S845}$ phosphorylation (Fig. 3.3a). In contrast, in non-limbic premotor cortex, both $GluR_1^{S845}$ ($F_{4,36} = 6.925$, $p < 0.001$) and ERK ($F_{4,49} = 3.679$, $p = 0.013$) phosphorylation increased


A
Relative pGluR1 & GluR1 Brain Levels
PFC PMC NAC NAS CPU BLA CEN CA1 CA3 VTA SN
pGluR1S845
GluR1
Tubulin
pGluR1
GluR1
Tubulin
Immunoreactivity (% of PFC average)
150
100
50
0
PFC PMC NAC NAS CPU BLA CEN CA1 CA3 VTA SN
Brain Region


B
Relative pERK & ERK Brain Levels
PFC PMC NAC NAS CPU BLA CEN CA1 CA3 VTA SN
pERK
ERK
pERK
ERK
Tubulin
Immunoreactivity (% of PFC average)
150
100
50
0
PFC PMC NAC NAS CPU BLA CEN CA1 CA3 VTA SN
Brain Region

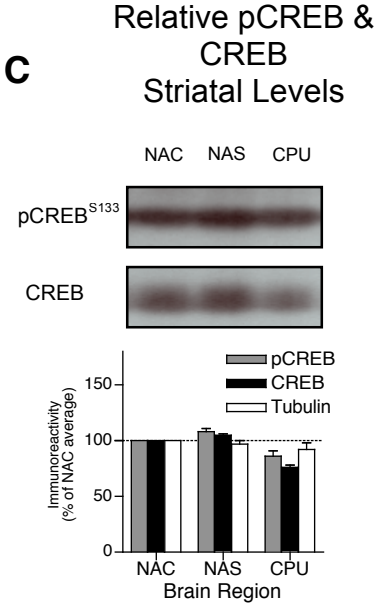

C
Relative pCREB & CREB Striatal Levels
NAC NAS CPU
pCREBS133
CREB
pCREB
CREB
Tubulin
Immunoreactivity (% of NAC average)
150
100
50
0
NAC NAS CPU
Brain Region

Figure 3.2
Brainwide levels of phosphoproteins and proteins analyzed in this study (n=3). (A) Relative to basal PFC levels, $pGluR_1^{S845}$ levels were reduced by ~50% in the CPU and >90% in the VTA and SN, while greater $pGluR_1^{S845}$ levels were found in the CA1 and CA3 hippocampal subregions. Regional $GluR_1$ levels largely mirrored those of $pGluR_1^{S845}$, with the exception of the CPU, where reductions in phosphoprotein level was not due to lower basal $GluR_1$. Tubulin levels were similar in all regions studied. (B) Relative to basal PFC levels, pERK levels were lower in more posterior brain regions, especially in the VTA and SN. Total ERK levels were largely consistent between regions, with lower levels observed in the PMC and VTA. (C) Basal $pCREB^{S133}$ and CREB levels were equal across striatal regions.

after 24 hrs spontaneous WD by 36% and 41%, respectively (Fig. 3.3b). The lack of changes after 12 hrs indicates that increased protein phosphorylation after 24 hrs is related to spontaneous WD and not chronic heroin self-administration, per se. Naltrexone administration failed to augment WD-induced protein phosphorylation in cortical subregions.

Differential WD-induced regulation of $GluR_1^{S845}$, ERK and $CREB^{S133}$ phosphorylation in striatal subregions

PKA-mediated $GluR_1^{S845}$ phosphorylation increased by 27% in the nucleus accumbens shell ($F_{4,51} = 6.299$, $p < 0.001$), but not nucleus accumbens core or caudate putamen striatal subregions after 24 hrs spontaneous WD from chronic heroin self-administration (Fig. 3.4a-c). In contrast, spontaneous withdrawal failed to substantially increase ERK phosphorylation in the nucleus accumbens, but naltrexone administration increased ERK phosphorylation in the shell subregion by 48% ($F_{4,51} = 3.537$, $p = 0.013$) and core subregion by 66% ($F_{4,51} = 4.461$, $p = 0.004$), without increasing $GluR_1^{S845}$ or $CREB^{S133}$ phosphorylation. Since naltrexone failed to alter ERK phosphorylation in drug-naïve animals, these changes suggest that significant opiate receptor tone exists during spontaneous 24 hr WD in the core and shell, or that naltrexone effects in other brain regions produce systems level interactions that influence ERK phosphorylation in the nucleus accumbens (see below). A mild but similar increase in ERK phosphorylation (33-42%) was found in the caudate putamen after 24 hr WD in spontaneous and

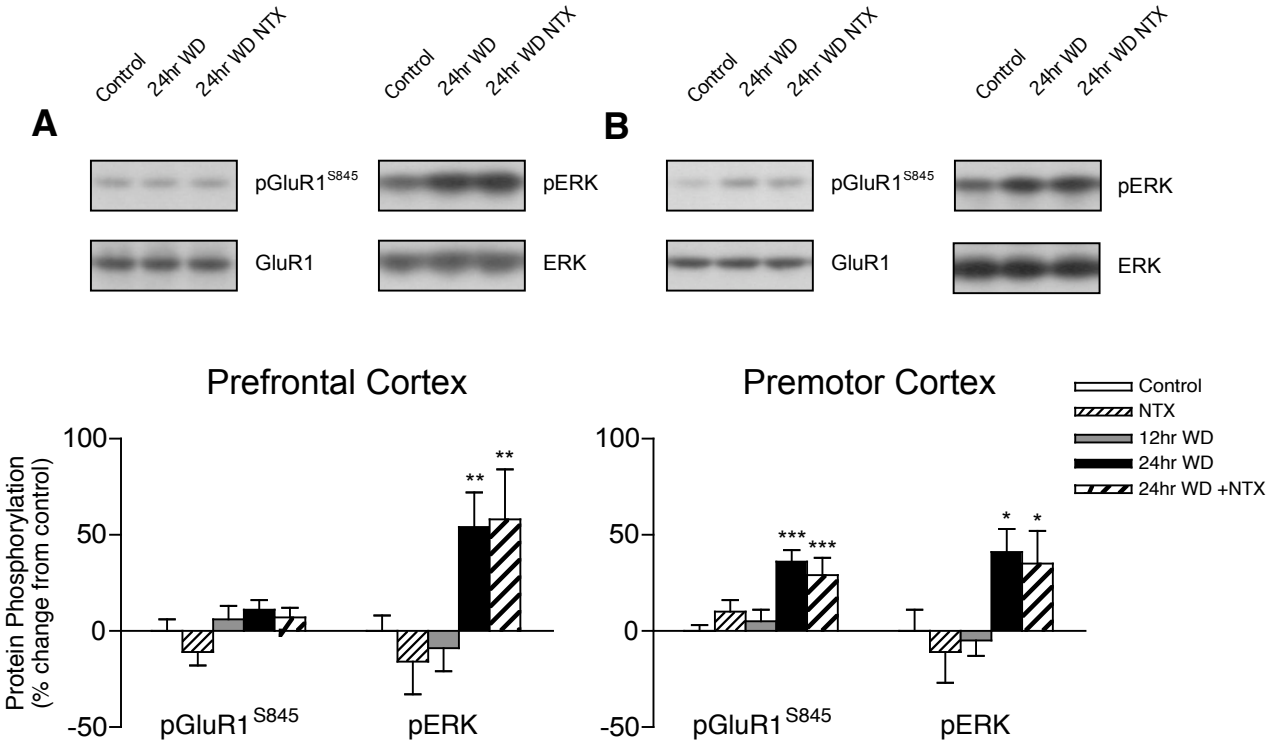


Figure 3.3
Protein phosphorylation in the prefrontal (PFC) and premotor (PMC) cortex after WD from heroin self-administration (HSA). (A) In the PFC, ERK phosphorylation is increased at 24 hrs WD from HSA. In contrast, no increase in $GluR_1^{S845}$ is seen at either WD time point. (B) In the PMC, both $pGluR_1^{S845}$ and pERK levels are elevated at 24 hrs WD. No changes in phosphorylation of either substrate is seen following naltrexone administration or after 12 hrs WD from HSA. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n=8-11/group). Symbols indicate values differ from controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) by Fisher's LSD tests. Representative blots for phosphorylated and total $GluR_1$ and ERK protein are shown for control, 24hr WD, and 24hr WD +NTX animals.

naltrexone WD groups ($F_{3,50} = 2.823$, $p = 0.048$; pooled 24 hr WD groups). Similarly, downstream $CREB^{S133}$ phosphorylation increased by 34-52% in the caudate putamen after 24 hrs WD from heroin self-administration with or without naltrexone pretreatment ($F_{4,51} = 4.738$, $p = 0.003$), but $CREB^{S133}$ phosphorylation failed to increase in nucleus accumbens. Thus, while increased ERK and CREB phosphorylation occur together in the caudate putamen, neither PKA-mediated phosphorylation of $GluR_1^{S845}$ or phosphorylated (activated) ERK translate into increased $CREB^{S133}$ phosphorylation in nucleus accumbens core and shell subregions after WD from heroin self-administration. There was no regulation of protein phosphorylation after 12 hrs spontaneous WD, indicating that increased phosphorylation after 24 hrs was induced specifically by WD from chronic heroin self-administration.

WD time determines differential $GluR_1^{S845}$ and ERK phosphorylation in amygdala subregions

In the basolateral amygdala nucleus, $GluR_1^{S845}$ phosphorylation increased by 31% after 24 but not 12 hrs spontaneous WD from heroin self-administration (Fig. 3.5a), and naltrexone administration enhanced phosphorylation to 48% ($F_{4,43} = 7.529$, $p < 0.001$). In contrast, in the central amygdala nucleus, $GluR_1^{S845}$ phosphorylation increased by 41% after only 12 hrs WD ($F_{4,46} = 4.289$, $p = 0.005$), an effect that dissipated by 24 hrs WD unless naltrexone was administered (Fig. 3.5b). Therefore, increased PKA-mediated phosphorylation apparently occurs earlier in central relative to basolateral amygdala

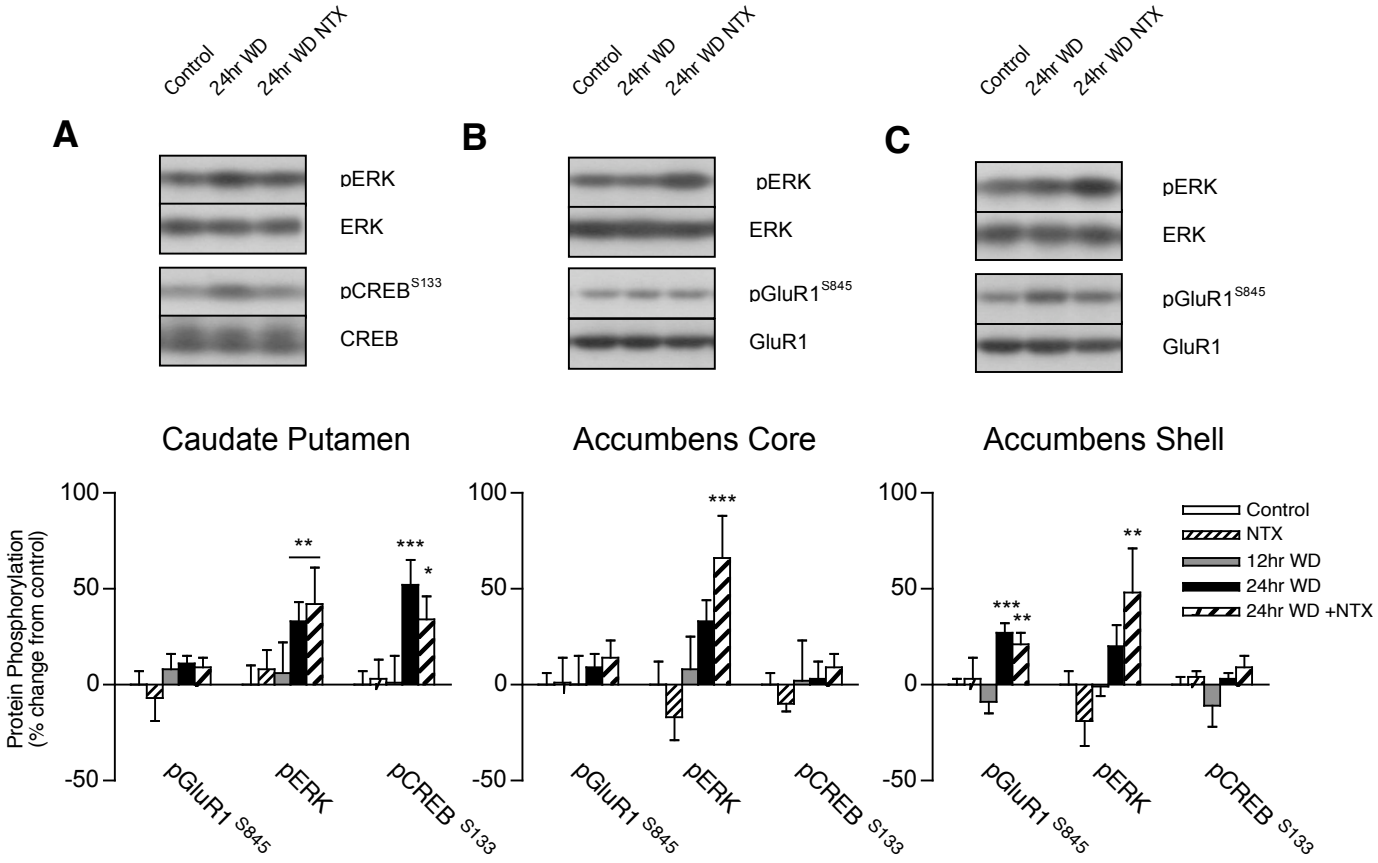


Figure 3.4
Protein phosphorylation in the striatum after WD from HSA. (A) In the caudate putamen (CPU), both ERK and CREB^{S133} phosphorylation are increased at 24 hrs WD. However, no change in GluR$_1$^{S845} phosphorylation is seen in this region. (B) In the nucleus accumbens core (NAC), ERK phosphorylation is significantly increased only after naltrexone-precipitated WD. (C) In the nucleus accumbens shell (NAS), ERK phosphorylation is significantly increased only after naltrexone-precipitated WD, while GluR$_1$^{S845} phosphorylation is increased in both 24 hr WD groups. No changes in phosphorylation of either substrate is seen following naltrexone administration or after 12 hrs WD from HSA. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n=8-11/group). Symbols indicate values differ from controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) by Fisher's LSD tests. Representative blots for phosphorylated and total GluR$_1$, ERK, and CREB protein are shown for control, 24hr WD, and 24hr WD +NTX animals.

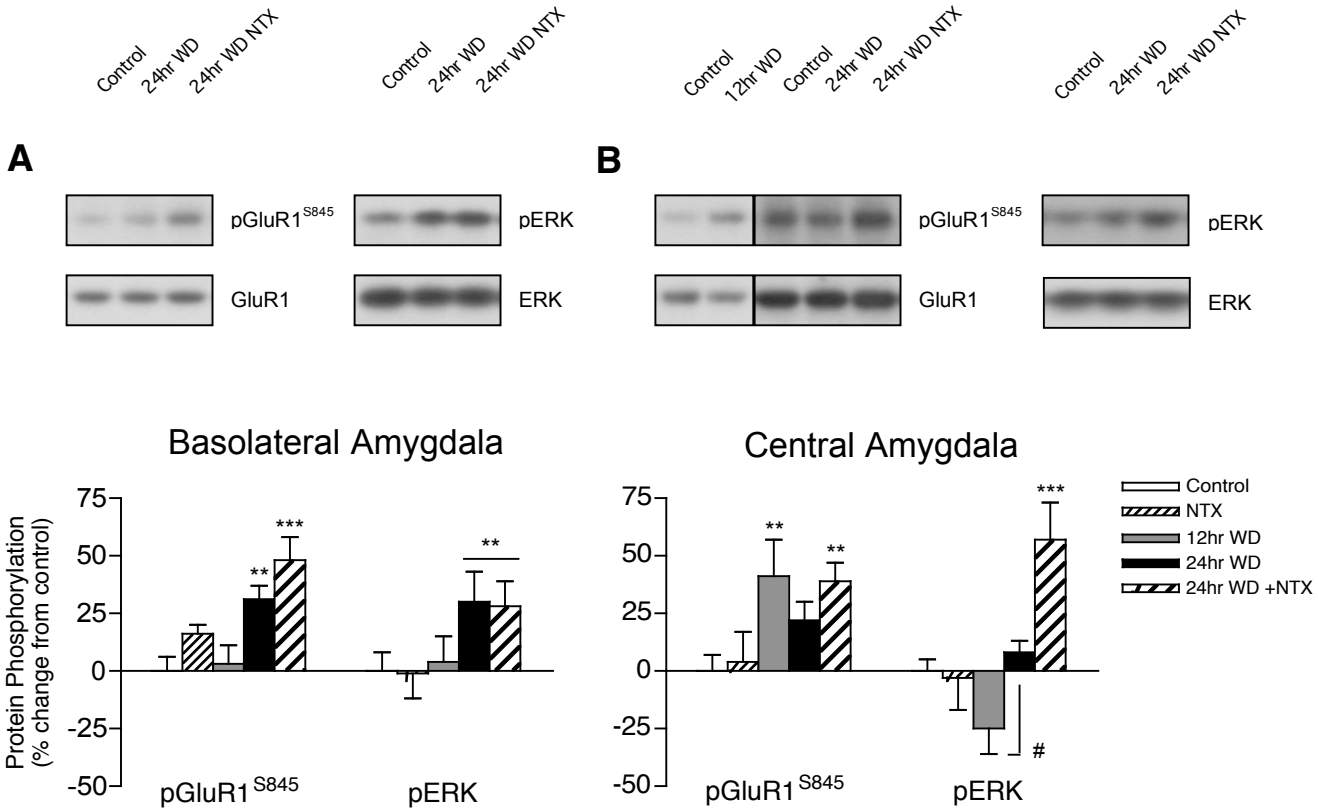


Figure 3.5
Protein phosphorylation in amygdala subregions after WD from HSA. (A) In the basolateral nucleus, both $GluR_1^{S845}$ and ERK phosphorylation are increased in both 24 hrs WD groups, but not at 12 hrs WD. (B) In the central nucleus, $GluR_1^{S845}$ phosphorylation is increased after 12 hrs WD from HSA, but not 24 hrs WD except by NTX-precipitated WD. Trending decreases in ERK phosphorylation at 12 hrs WD are reversed by 24 hrs WD, while pERK is significantly increased at after NTX-precipitated WD 24 hrs after HSA. No changes in phosphorylation of either substrate is seen following naltrexone administration. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n=8-11/group). Symbols indicate values differ from controls (**$p < 0.01$, ***$p < 0.001$) or from 12hr WD group (#$p < 0.05$) by Fisher's LSD tests. Representative blots for phosphorylated and total $GluR_1$ and ERK protein are shown for control, 12hr WD, 24hr WD, and 24hr WD +NTX animals.

nuclei during spontaneous withdrawal from chronic heroin self-administration. Similarly, naltrexone administration after 24 hrs WD markedly induced a 57% increase in ERK phosphorylation in the central amygdala nucleus ($F_{4,46} = 8.191$, $p < 0.001$), but there were no increases in spontaneous WD, and phosphorylated ERK levels actually decreased earlier after 12 hrs WD. A mild ~29% increase in ERK phosphorylation occurred after 24 hrs WD in the basolateral amygdala nucleus regardless of naltrexone administration ($F_{3,46} = 2.803$, $p = 0.05$; pooled 24 hr WD groups), but there were no changes after 12 hrs WD. Thus, increases in $GluR_1^{S845}$ and ERK phosphorylation are dissociated in central but not basolateral amygdala nucleus in spontaneous WD.

Both $GluR_1^{S845}$ and ERK phosphorylation are increased by spontaneous WD in CA1 and CA3 hippocampal subregions

$GluR_1^{S845}$ phosphorylation increased by 26% in the CA1, and 57% in the CA3 ($F_{4,35} = 7.806$, $p < 0.001$), after 24 hrs spontaneous WD from chronic heroin self-administration ($F_{4,35} = 4.757$, $p = 0.004$), and neither effect was augmented by naltrexone pretreatment (Fig. 3.6a-b). More prominent ERK phosphorylation was found after 24 hrs spontaneous WD in the CA1 subregion, with increases of 39% that further increased to 67% with naltrexone pretreatment ($F_{4,35} = 6.217$, $p = 0.001$). In the CA3 subregion, ERK phosphorylation increased similarly by 89-98% with or without naltrexone pretreatment ($F_{4,35} = 6.875$, $p < 0.000$). No changes in protein phosphorylation were detected after 12 hrs spontaneous WD from in either hippocampal subregion, indicating that increased

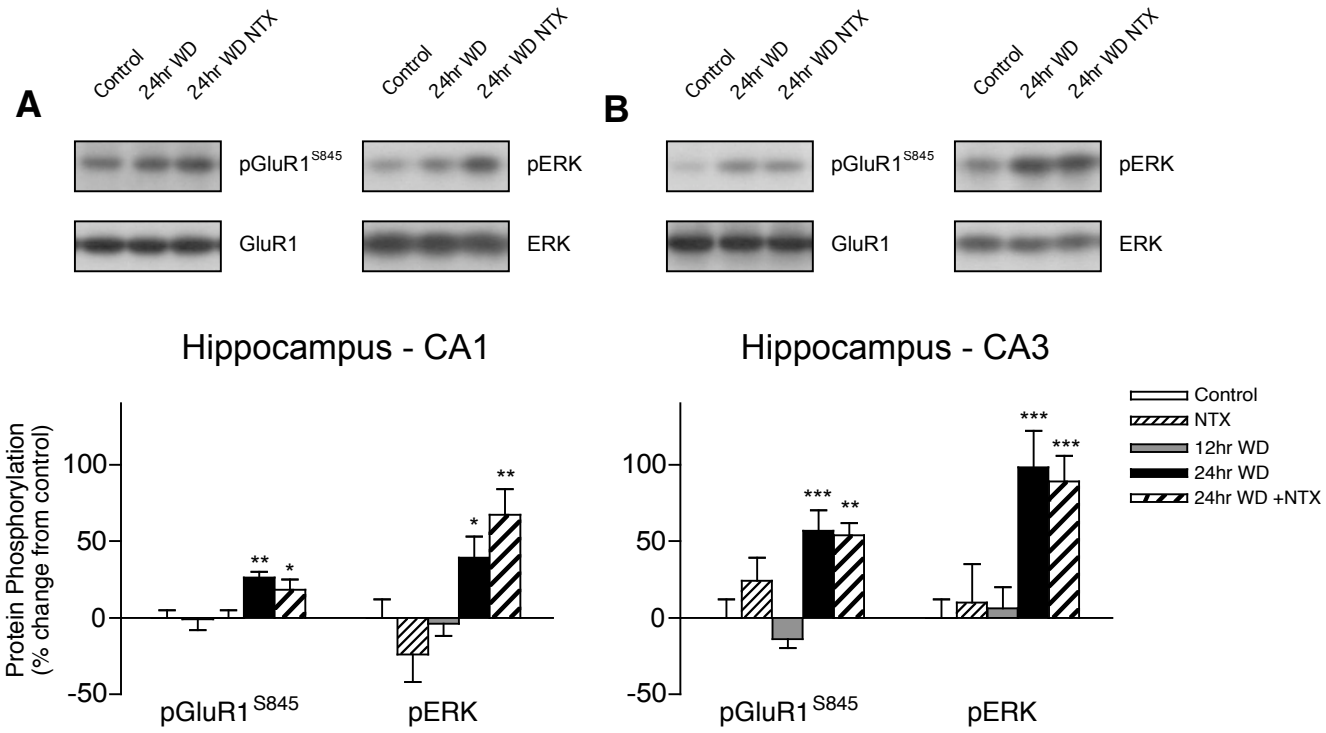


Figure 3.6
Protein phosphorylation in hippocampal subregions after WD from HSA. (A) In the CA1 subregion, both $GluR_1^{S845}$ and ERK phosphorylation are increased in both 24 hrs WD groups. (B) In the CA3 subregion, both $GluR_1^{S845}$ and ERK phosphorylation are increased in both 24 hrs WD groups to a greater extent vs. CA1. No changes in phosphorylation of either substrate is seen following naltrexone administration or after 12 hrs WD from HSA. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n=8-11/group). Symbols indicate values differ from controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) by Fisher's LSD tests. Representative blots for phosphorylated and total $GluR_1$ and ERK protein are shown for control, 24hr WD, and 24hr WD +NTX animals.

$GluR_1^{S845}$ and ERK phosphorylation were produced specifically by heroin WD rather than chronic heroin exposure itself.

Up-regulation in both phosphorylated and total GluR1 levels in the ventral tegmental area in spontaneous heroin WD

In the VTA, $GluR_1^{S845}$ phosphorylation increased by 35%, after 12 hrs spontaneous WD from heroin self-administration ($F_{4,45} = 5.666$, $p = 0.001$). However, after 24 hrs spontaneous WD, both $GluR_1^{S845}$ phosphorylation ($F_{2,32} = 6.548$, $p = 0.004$) and total GluR1 protein levels ($F_{1,33} = 5.666$, $p = 0.006$; pooled 24 hr WD groups) increased by 47% and 40% (Fig. 3.7a, left panel), respectively, resulting in no change in $GluR_1^{S845}$/GluR1 protein ratios (Fig. 3.7a, right panel). Thus, spontaneous WD caused an increase in PKA-mediated $GluR_1^{S845}$ phosphorylation, followed by an up-regulation in the amount of GluR1, and this effect was specifically found in the ventral tegmental area (mesolimbic), and not adjacent substantia nigra tissue (Fig. 3.7b). While naltrexone pretreatment failed to augment $GluR_1^{S845}$ phosphorylation in the ventral tegmental area after chronic heroin self-administration, it actually decreased basal $GluR_1^{S845}$ phosphorylation by 27% in drug-naïve animals, and decreased ERK phosphorylation by 24-30% in both ventral tegmental area ($F_{4,45} = 4.703$, $p = 0.003$) and substantia nigra ($F_{4,46} = 6.467$, $p < 0.000$). These results indicate that naltrexone pretreatment increases $GluR_1^{S845}$ and ERK phosphorylation in heroin self-administering animals relative to phosphorylation decreases in drug-naive animals. While ERK phosphorylation failed to

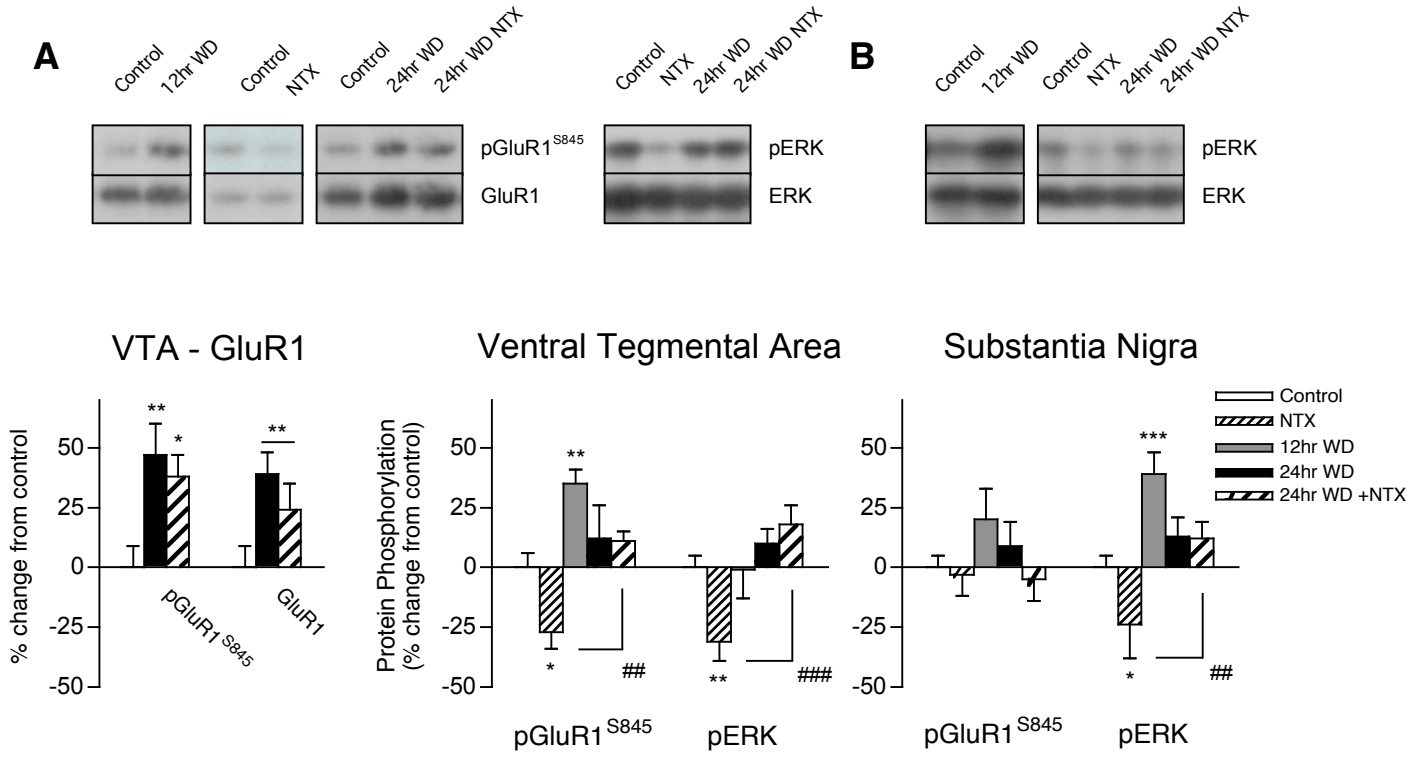


Figure 3.7
Protein phosphorylation in ventral tegmental area (VTA) and substantia nigra (SN) after WD from HSA. (A) In the VTA, naltrexone-induced decreases in $GluR_1^{S845}$ and ERK phosphorylation are reversed by 24 hrs WD from HSA. 12 hrs WD from HSA produces increases in $GluR_1^{S845}$ phosphorylation, while at 24 hrs WD both $pGluR_1^{S845}$ and total $GluR_1$ are increased. (B) In the SN, naltrexone-induced decreases in ERK phosphorylation are reversed by 24 hrs WD from HSA, while 12 hrs WD produces increases in pERK. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n=8-11/group). Symbols indicate values differ from controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) or from 12hr WD group (##$p < 0.01$, ###$p < 0.001$) by Fisher's LSD tests. Representative blots for phosphorylated and total $GluR_1$ and ERK protein are shown for control, NTX, 12hr WD, 24hr WD, and 24hr WD +NTX animals.

increase in spontaneous WD in the ventral tegmental area, ERK increased 39% in the substantia nigra after 12 hrs, but not 24 hrs, spontaneous WD.

Discussion

In this study, we measured changes in protein phosphorylation status after 12 and 24 hrs of spontaneous WD from chronic intravenous heroin self-administration in daily 6 hr sessions. Heroin itself has an estimated plasma half-life of only 2-8 min in both humans and rodents (Way et al. 1960; Inturissi et al. 1984). After absorption into tissue, heroin is metabolized to morphine, which is then converted to morphine-3-glucuronide (an inactive compound) and morphine-6-glucuronide, a potent opioid agonist (Milne et al. 1966; Ulens et al. 2001). Repeated exposure to heroin increases the rate of morphine-6-glucuronide formation, and elevated morphine-6-glucuronide levels persist for up to 24 hrs after heroin administration (Antonilli et al., 2003). Therefore, we also administered the opiate receptor antagonist naltrexone after 24 hrs WD to determine whether changes in protein phosphorylation would intensify. Most increases in protein phosphorylation were evident after 24 but not 12 hrs, indicating an acute response to heroin withdrawal rather than an up-regulation produced by chronic heroin use. In contrast, $GluR_1^{S845}$ and ERK phosphorylation increased after only 12 hrs WD in dopamine cell body regions of the ventral tegmental area and substantia nigra and this effect diminished after 24 hrs,

potentially reflecting the continued presence of morphine-6-glucuronide that would disinhibit dopamine neuron activity via well-characterized opioid inhibition of GABAergic interneurons (Johnson and North 1992). In the central amygdala nucleus, early 12 hr WD also increased $GluR_1^{S845}$ phosphorylation, but this effect may reflect enhanced sensitivity to declining opiate receptor tone in WD, since phosphorylation increases persisted after 24 hrs with naltrexone administration.

Chronic opiate use leads to a dysregulation of cellular and synaptic function in mesolimbic regions that regulate incentive motivation, as well as responses to stress (Robinson and Berridge 1993; De Vries and Shippenberg 2002). Together these changes are believed to progress and exacerbate the behavioral manifestation of opiate WD. Chronic heroin self-administration up-regulates molecular components of the cyclic AMP signaling pathway in the nucleus accumbens (Self et al. 1995), and continuous morphine exposure also up-regulates this pathway in the amygdala (Terwilliger at al. 1991). Such up-regulation in PKA levels could lead to rebound activation of cyclic AMP-PKA-mediated $GluR_1^{S845}$ phosphorylation in the nucleus accumbens shell and both basolateral and central amygdala nuclei in our study. Similar changes could underlie PKA-mediated $GluR_1^{S845}$ phosphorylation in premotor cortex and both CA1 and CA3 hippocampal subregions after 24 hrs WD from heroin self-administration. Consistent with this notion, $GluR_1^{S845}$ phosphorylation failed to increase after 24 hrs WD in the prefrontal cortex, caudate putamen, ventral tegmental area (independent from total GluR1) and substantia nigra, regions where chronic heroin self-administration and/or continuous morphine exposure fails to up-regulate adenylate cyclase or PKA levels (Duman et al. 1998; Nestler

and Tallman 1988; Terwilliger et al. 1991; Self et al. 1995). In contrast, these previous studies failed to find up-regulation in adenylate cyclase or PKA levels in whole hippocampus, possibly suggesting that increased $GluR_1^{S845}$ phosphorylation in CA1 and CA3 after 24 hrs WD reflects increased neurotransmitter release and activation of receptors coupled to stimulatory G proteins, rather than up-regulation in the cyclic AMP-PKA pathway.

We compared PKA-mediated $GluR_1^{S845}$ phosphorylation with ERK phosphorylation that could reflect neuronal activation in heroin WD. In many regions, WD from heroin self-administration induced both $GluR_1^{S845}$ and ERK phosphorylation, suggesting a link between the two signaling pathways. Indeed, many forms of synaptic plasticity induced by behavioral training involve interaction between PKA and ERK signaling (English and Sweatt 1997; Selcher et al. 1999; Winder et al. 1999; Patterson et al. 2001). WD-induced changes were found predominately at the later, 24 hr WD time point. It is of note that this WD time point corresponds to the development of conditioned place aversion after chronic opiate administration (Bechara et al. 1995).

Frontal Cortex

Long-term exposure to drugs of abuse has been shown to blunt neural processing in prefrontal cortical regions, leading to an inability to maintain inhibitory control over maladaptive behaviors, including drug seeking in WD (Jentsch and Taylor 1999). Furthermore, PFC activity can be inversely related to activity in subcortical dopamine systems (Jackson et al. 2001; Meyer-Lindenberg et al. 2002), leading some investigators to dichotomize mesolimbic and mesocortical dopamine function. Consistent with these

observations, and in contrast to most regions investigated in this study, we found no changes in PFC $GluR_1^{S845}$ phosphorylation at any time after heroin self-administration (Figure 3.3). In contrast, ERK phosphorylation was increased after 24 hrs WD from HSA, suggesting a non-PKA dependent activation of MEK. Indeed, both spontaneous and NTX-precipitated WD (48 hrs) from chronic morphine has been shown to increase levels of protein kinase C (PKC) in the frontal cortex (Ventayol et al. 1997; Escriba and Garcia-Sevilla 1999) and this could lead to activation of MEK-ERK signaling through stimulation of Raf-1. Also at the later WD time point, both $pGluR_1$ and pERK were increased in the premotor cortex, a non-limbic region that mediates dopamine's effects on cognitive motor functions via D1 receptor activation (Sawaguchi 2000).

Striatum/Accumbens

The ventral striatum/nucleus accumbens receives dense dopaminergic input from the ventral tegmental area (VTA) and is a major neural substrate for opiate reward (Vaccarino et al. 1985; Wise 1989) as well as for negative reinforcement during opiate WD (Koob et al. 1992). In comparison, dorsal regions of the striatum obtain dopaminergic innervation from the substantia nigra, and may mediate the transition to habitual and compulsive drug use seen after chronic drug self-administration (Gerdeman et al. 2003; Everitt and Robbins 2005). Chronic opiate administration leads to differential regulation of dopamine levels in the striatum depending on route of exposure. Thus, chronic morphine pelleting procedures result in a reduction of electrically-evoked dopamine release that lasts for weeks into WD, while intermittent opiate exposure causes a WD time-dependent recovery in evoked dopamine release (Tjon et al. 1994), with

subsequent sensitization upon further opiate exposure (Diana et al. 1999). Part of this initial deficit may be due to structural neuronal damage caused by chronic opiate treatment (Sklair-Tavron et al. 1996), while hyper-sensitivity of DA neurons in WD may mediate reinstatement of drug-seeking behavior (Vanderschuren and Kalivas 2000), especially after opiate priming (Stewart and Wise 1992). These and other effects of striatal dopamine are mediated largely via postsynaptic D_1 and D_2 dopamine receptors. Nucleus accumbens D_1 receptors are critical for conditioned responses that develop to environmental cues predicting opiate administration (Shippenberg and Elmer 1998). Additionally, inhibition of cAMP signaling by D_2 dopamine receptor stimulation in nucleus accumbens attenuates the somatic signs of opiate WD (Harris and Aston-Jones 1994), while D_2 antagonism exacerbates this state (Funada and Shippenberg 1996), consistent with a role for increased PKA-mediated phosphorylation in WD behaviors.

A recent study (Chartoff et al. 2006) found that precipitated WD from continuous morphine treatment increased striatal pCREB levels, more so in the dorsal striatum. In our study, heroin self-administration increased CREB phosphorylation exclusively in the dorsal striatum, possibly contributing to habitual behavior that might come to predominate in addicted, self-administering animals (Everitt and Robbins 2005). Chartoff et al. also found similar increases in CREB phosphorylation with administration of the dysphoric agent lithium chloride (Shippenberg et al. 1988) in the NAc, but not caudate, further suggesting a role for CREB in the NAc shell in gating generalized responses to emotional stimuli (Barrot et al. 2002). Indeed, CREB overexpression in the ventral striatum produces a negative affective state (Pliakas et al. 2001), an effect thought

mediated in part by CREB-mediated regulation of the endogenous opioid dynorphin (Carlezon et al. 1998).

In comparison, our findings resonate with a previous study (Muller and Unterwald 2005) that found increases in ΔFosB (a downstream product of CREB activation) exclusively in the caudate putamen, but not nucleus accumbens, after chronic heroin administration. In contrast, Muller et al. found that chronic morphine injections induced ΔFosB in both dorsal and ventral striatum. Since heroin is rapidly metabolized to morphine *in vivo*, it is widely believed that morphine is responsible for the pharmacological actions of heroin (Way et al. 1960), and one would expect similar biochemical effects between the two drugs. Notably, however, the heroin metabolite 6-monoacetylmorphine possesses higher efficacy than morphine at mu-opioid receptors (Selley et al. 2001). This and other evidence (Lange et al. 1980) that distinct pharmacological profiles exist for these two drugs, and the route and temporal pattern of daily intermittent intravenous self-administration, may account for the distinct patterns of pCREB and ΔFosB regulation by heroin vs. morphine.

As a functional readout of CREB activation, striatal CRE (cAMP response element) activity was previously examined after continuous opiate adminstration (Shaw-Lutchman et al. 2002). It should be noted that Shaw-Lutchman et al. were unable to detect striatal CRE activity with intermittent opiate injections (a model that more closely resembles daily heroin self-administration). In that study, CRE-mediated transcription was moderately increased throughout the striatum during spontaneous opiate WD. Moreover, this effect was greatly enhanced in both nucleus accumbens core and shell, but

not caudate, in animals undergoing NTX-precipitated WD. In a similar fashion, our study found significant increases in accumbens pERK (a potential activator of CREB and thus CRE activation) only after NTX-precipitated WD (Figure 3.4). Chartoff et al. (2006) also found no change in $pGluR_1$ levels in either the caudate or whole accumbens, while our data distinguished an increase in $pGluR_1$ exclusively in the NAc shell (Figure 3.4), a subregion of the accumbens thought to be sensitive to stressful states such as behavioral extinction (Sutton et al. 2003).

Amygdala

The basolateral amygdala (BLA) plays an important role in conditioned reinforcement and heroin-seeking behavior (Fuchs and See 2002), while the central nucleus (CEN) receives excitatory projections from the BLA and processes the establishment, maintenance, and expression of emotional memories (Pitkanen et al. 1997). The central amygdala is also associated with the expression of dysphoria that occurs during early phases of opiate WD (Koob 1999). At the molecular level, augmented PKA signaling within these regions enhances appetitive learning processes (Jentsch et al. 2002), although it's important to note that the treatments used in this study might increase MEK signaling as well.

A recent study found increased AMPA $GluR_1$ receptor labeling on plasma membrane of dendrites in BLA, but not CEN, in rats self-administering morphine (Glass et al. 2005). Trafficking of $GluR_1$ receptors has been shown to be dependent on PKA phosphorylation of $GluR_1$ at Ser 845 (Esteban et al. 2003). Because the animals in the

Glass et al. study were sacrificed immediately after their last self-administration session, no WD occurred, and this may have precluded any observation of effects in the CEN. The present study focused on WD time-dependent changes in protein phosphorylation in these regions, and found distinct temporal and regional profiles of $GluR_1^{S845}$ regulation (Figure 3.5). In the BLA, $GluR_1$ (and ERK) phosphorylation was increased at 24 hrs, but

not 12 hrs, WD. In contrast, CEN $pGluR_1^{S845}$ was increased at 12 hrs WD, but not significantly increased at the later time point unless WD was precipitated by naltrexone. The visualization of these early WD effects would seem to confirm the CEN's sensitive role in opiate WD. Interestingly, a trending decrease in ERK phosphorylation at 12 hrs WD was reversed with 24 hrs WD, further suggesting an earlier sensitivity of CEN signaling in opiate WD. Finally, there was a robust increase in CEN ERK phosphorylation only with naltrexone-precipitated WD. This finding corresponds to Shaw-Lutchman et al.'s (2002) finding that CRE activity (potentially driven by ERK phosphorylation of CREB) was induced under similar conditions, predominately in the CEN. Interestingly, that study also found that CEN CRE activity during precipitated WD was prominent in CRF containing neurons in the, a result that could promote enhancement of CRF transmission (Spengler et al. 1992) and augmentation of WD symptoms (Heinrichs et al. 1995).

Hippocampus

Hippocampal circuitry is essential to spatial learning, and is thought to restore and distribute drug-associated information throughout the mesolimbic system (Nestler

2001a), leading to facilitation of relapse after dopaminergic stimulation of this region (Vorel et al. 2001). Hippocampal CA1 LTP is reduced during WD from chronic opiate treatment (Pu et al. 2002), leading to impaired learning of the Morris water maze test. Moreover, this LTP deficiency can be restored either by re-exposure to morphine or a PKA inhibitor, suggesting that an up-regulated cAMP system contributes to this defect. Chronic opiate exposure, including heroin self-administration, also inhibits adult hippocampal neurogenesis (Eisch et al. 2000), and this effect is hypothesized to mediate the cognitive deficits observed in heroin users. We found that 24 hrs WD from HSA increased CA1 $GluR_1^{845}$ phosphorylation (Figure 3.6), an adaptation that would also preclude hippocampal LTP. Moreover, in the spontaneous WD group, the increase in $pGluR_1$ positively correlated with average preferred levels of heroin intake ($r = 0.800$, $p = 0.017$). Increased $GluR_1$ phosphorylation by PKA is also known to mediate its trafficking to hippocampal membranes (Gao et al. 2006). In this regard, our data corroborates that of Moron et al. (Moron et al. 2007), who found that chronic morphine leads to decreases in the levels of hippocampal $GluR_1$ associated with clathrin, and increases in synaptosomal $GluR_1$. Interestingly, the largest WD-induced increases in both $GluR_1^{S845}$ and ERK phosphorylation was found in the CA3 subregion of the hippocampus. Changes in both CA1 and CA3 $GluR_1$ phosphorylation are mirrored by corresponding, larger increases in ERK phosphorylation at 24 hrs WD, suggesting a coupling of these two kinase pathways. In fact, it has been demonstrated that Rap1 shunts cAMP signaling to a specific membrane-associated pool of ERK in the hippocampus to regulate synaptic plasticity and spatial memory (Morozov et al. 2003).

Midbrain

Naltrexone (NTX) is a non-selective opiate receptor antagonist used to acutely precipiate opiate WD symptoms by blocking extant opiate binding to receptors throughout the limbic system (Stinus et al. 1990). In heroin-naïve animals, NTX can also block a tonic inhibitory effect exerted by endogenous opioid peptides acting on opioid receptors. Although only a single dose and time point were used in this study, it would appear that there exists sufficient endogenous opiate tone in the midbrain (VTA and SN) to influence protein phosphorylation status such that, when midbrain opioid receptors are inhibited, regional GABAergic interneurons can reduce protein phosphorylation in dopaminergic (DA) neurons (Figure 3.7). These DA neurons contain primarily metabotropic $GABA_B$ receptors (Margeta-Mitrovic et al. 1999), which would likely decrease both PKA and MEK phosphorylation. This effect on mesolimbic DA neurons does not seem to translate into phosphorylation changes in dopaminergic projection areas. Additionally, this effect of NTX is absent in animals withdrawn from heroin self-administration, a finding in line with prior evidence suggesting differential midbrain signaling in opiate-naïve vs. opiate-dependent animals (for review, see Bechara et al. 1998). Opiates produce reward through a dopamine-independent neural system in the drug-naïve state, but after chronic opiate exposure (and adequate WD time), VTA-mediated opiate reward switches to a dopamine-dependent system (Nader and van der Kooy 1997). The behavioral and biochemical evidence of this switch was manifested only after 21 hrs of opiate WD (Laviolette et al. 2004), and could in part account for many of the WD time-dependent mesolimbic phosphorylation changes observed in the current study.

In contrast to limbic terminal regions, no increases in $pGluR_1^{S845}$ or pERK ratios were found at 24 hrs WD in the midbrain. This could be due to enhanced presynaptic inhibition of the excitatory inputs to VTA dopamine cells caused by opiate WD (Manzoni and Williams 1999), and corresponding blunting of mesolimbic dopamine output observed in early WD. In addition, a recent study (Georges et al. 2006) found that, although chronic morphine increased firing of VTA DA neurons, this effect disappears after 24 hrs WD.

Our study also agrees with Fitzgerald et al. (Fitzgerald et al. 1996), which found that repeated, intermittent morphine injections increased total $GluR_1$ levels in the VTA, an adaptation that may contribute to the induction of opiate sensitization (Carlezon et al. 1997). In extension of that data, we also observed a concomitant increase in phosphorylated $GluR_1$, suggesting that this adaptation is functional at the membrane.

Together, differential WD-time dependent patterns of protein phosphorylation in distinct midbrain, limbic, and cortical regions may exacerbate various aspects of heroin WD. These findings also lend support to the notion that PKA phosphorylation and trafficking of $GluR_1$ at excitatory synapses throughout the brain could contribute to the development of drug addiction (Malenka 2003).

CHAPTER 4

Time-Dependent Increases in Striatal Protein Phosphorylation after Re-Exposure to a Cocaine-Paired Environment

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Introduction

Relapse to drug-seeking is a defining hallmark of the addicted state, and can persist for several months into withdrawal (O'Brien 1997). An enhancement of drug craving underlies the potential for relapse in humans, and may be exacerbated by sensitization to cocaine-associated cues (Ehrman et al. 1992; Satel et al. 1995). It has further been hypothesized that subjective feelings of craving are even withdrawal time-dependent in humans (Gawin and Kleber 1986). Importantly, these craving states can be modeled in rodents with a history of cocaine self-administration via presentation of cocaine-paired cues after a period of forced withdrawal (Davis and Smith 1976; Stewart et al. 1984). In this case, an animal's non-reinforced lever-pressing behavior is indicated as a measure of drug craving and relapse. Use of this model has lead to the discovery that relapse induced by exposure to cocaine-associated cues does indeed increase over time (Tran-Nguyen et al. 1998; Grimm et al. 2001).

Neuroadaptations in mesolimbic dopamine reward circuitry may underlie or potentiate persistent drug seeking in withdrawal (Self and Nestler 1998; Nestler 2001; Kalivas 2004). Chronic exposure to cocaine is known to produce an up-regulation of cAMP-PKA signaling pathway components in several mesolimbic regions (including nucleus accumbens) (Terwilliger et al. 1991; Striplin and Kalivas 1993; Freeman et al. 2001; Lu et al. 2003), although it is unknown whether this adjustment leads to stabilized changes in PKA-mediated phosphorylation in long-term withdrawal, or alters phosphorylation induced by states of craving and relapse. Likewise, the MEK/ERK pathway is also activated in nucleus accumbens after acute and chronic cocaine

administration (Valjent et al. 2000; Mattson et al. 2005), although whether these changes persist into withdrawal is also undetermined. Moreover, cocaine could differentially regulate protein phosphorylation when intravenous injections are delivered in a reinforcing context with self-administration when compared to passively administered cocaine.

In this study, we measured PKA-dependent phosphorylation of GluR1 AMPA (Ser 845) receptors as well as ERK phosphorylation (Thr 183/Tyr 185) mediated by ERK kinase (MEK) in the striatum (nucleus accumbens and caudate putamen) during early (1 d) or late (3 wks) withdrawal from chronic cocaine self-administration in rats. Additional groups of animals were re-exposed to their individual cocaine administration chambers for 1 hr at each withdrawal time point in order to measure context-induced changes in protein phosphorylation associated with relapse to drug-seeking. Finally, we compared protein phosphorylation in self-administering animals with animals receiving cocaine passively by yoked injection to identify potential reinforcement-related regulation in cocaine withdrawal. The potential for withdrawal- and context-induced protein phosphorylation in maintaining cocaine addiction by exacerbating the potential for drug seeking in withdrawal is discussed.

Materials and methods

Subjects and surgery

Male, Sprague-Dawley rats initially weighing 275-325g (Charles River, Kingston, RI, USA) were individually housed in a climate-controlled environment (21° C) on a 12-hr

light-dark cycle (lights on at 7:00 AM) in according to the National Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals*. Animals initially were maintained on a restricted diet at 85% original body weight and all were trained to press a lever for 45 mg sucrose pellets in operant chambers (Med Associates, Georgia, VT) on a fixed-ratio 1 (FR1) reinforcement schedule until acquisition criteria were achieved (100 pellets self-administered for 3 consecutive days). This procedure was used to facilitate subsequent acquisition of cocaine self-administration. Following acquisition of lever press behavior, animals were fed ad libitum prior to surgical implantation of a chronic indwelling intrajugular catheter as described previously (Edwards *et al*., 2006). Catheters were flushed daily with 0.2 ml of heparinized (20 IU/ml) bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml) to prevent clotting and antibiotic ointment was applied daily to the catheter exit wound to prevent infection.

Cocaine self-administration procedures in rats

Following a 1 week recovery period, animals were trained to perform a single lever press response (FR1) to self-administer intravenous injections of cocaine hydrochloride (500 μg/kg/50 μl injection) or saline delivered over 2.5 sec in daily 4-hr sessions for 3 weeks (6 days/week) in operant test chambers as described (Edwards et al. 2006). During each injection, a cue light above the active lever was illuminated, and the house light was extinguished, followed by an additional 12.5 sec time-out (TO) period when both lights were extinguished and lever press responses had no programmed consequence. Chronic yoked cocaine animals received passive non-contingent cocaine injections in an identical

temporal pattern as their cocaine self-administering partners. Age- and group-matched untreated controls, in addition to animals in cocaine withdrawal, remained in their home cages but were handled daily.

Measurement of protein phosphorylation in brain tissue homogenates

One day (early withdrawal) or three weeks (late withdrawal) after their last training session, self-administering and yoked animals were gently placed in a plexiglass restrainer and euthanized by microwave irradiation aimed at the head region (5 kW, 1.5 s, Murimachi Kikai Co., LTD. Tokyo, Japan). Additional groups of self-administering and yoked animals at identical withdrawal time points were placed back into their respective operant test chambers for 1 hr before euthanasia. During this hour, drug-paired lever responses resulted in exactly the same environmental consequences described above (i.e. serving as a conditioned reinforcer), but with no cocaine infusion. Following euthanasia, rat brains were rapidly dissected and regional tissue samples were obtained with 12-16 gauge punches from chilled coronal brain slices (1.5 mm thick). Tissue samples were immediately homogenized by sonication in lysis buffer (320 mM sucrose, 5 nM HEPES, 50 mM NaF, 1 mM EGTA, 1 mM 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, and stored at –80°C until determination of protein concentrations by the Lowry method. Samples of 20 μg protein were subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide with a Tris/Glycine/SDS buffer (Bio-Rad, Hercules, CA), followed by electrophoretic transfer to PVDF membranes (polyvinylidene; Amersham, Piscataway, NJ). Membranes were blocked overnight in 5% nonfat milk at

4° C, and incubated in primary antibody for $pGluR_1^{S845}$ (1:2500; Chemicon, Temecula, CA) or pERK (1:7500; Cell Signaling, Danvers, MA) for 24 hrs at 4° C. Membranes were washed and labeled with species-specific peroxidase-conjugated secondary (1:10,000; BioRad) for 1 hr at 25° C. Following chemiluminescence detection (ECL plus; Amersham), blots were stripped and reprobed for total protein levels of $GluR_1$ (1:10K; Chemicon), or ERK (both 42 and 44 kD forms) (1:7500; Cell Signaling) as internal standards for phosphoprotein levels. Immunoreactivity was quantified by densitometry (Scion Image) under conditions linear over at least a 3-fold concentration range.

Data analysis

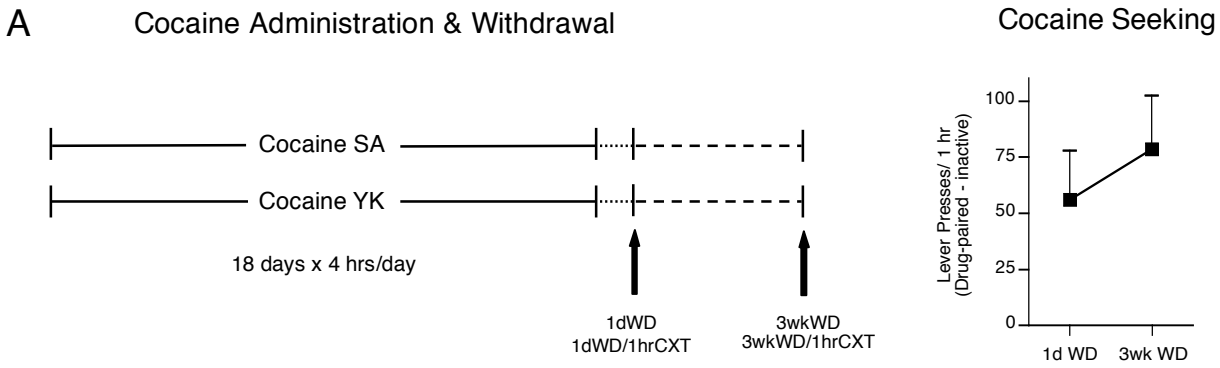
Densitized values were expressed a percentage of the mean for untreated control values (5-6/blot) to normalize data across blots. Phosphoprotein levels, each corrected for individual total protein levels, were compared by 1-factor ANOVA followed by Fishers Least Significant Difference (FLSD) posthoc comparisons among groups, or planned Student's t-tests comparisons with untreated controls.

Results

Figure 4.1A illustrates the cocaine treatment regimens for the four experimental groups used in the analysis of protein phosphorylation levels. Chronic cocaine self-administering (Cocaine SA) animals were compared to partnered animals that received passive yoked intravenous cocaine injections throughout 18 days of training (Cocaine

YK) to identify potential reinforcement-related differences in both cocaine withdrawal- and context-induced protein phosphorylation. All changes in protein phosphorylation were compared to untreated controls that remained in their home cages throughout testing. Animals trained to self-administer cocaine developed stable self-administration by the 3rd week of training, with mean daily cocaine intake (days 13-18) ranging from 46.0 mg/kg to 49.7 mg/kg in both cocaine SA and cocaine YK groups. In addition to comparing alterations in basal protein phosphorylation, between early (1 d) and late (3 wks) cocaine withdrawal, additional groups of animals were re-exposed to their individual cocaine-paired training chambers for one hour at these WD time points immediately prior to euthanasia. Drug-paired and inactive lever presses were recorded and subtracted to obtain a measure of cocaine-seeking behavior at both WD time points. As shown in Figure 4.1A, we observed a modest time-dependent increase in cocaine seeking (averaging 56.1 lever presses at 1 d WD and 78.5 lever presses at 3 wks WD), consistent with other studies utilizing animals with similar levels of cocaine exposure.

Tissue samples were collected from animals euthanized by microwave fixation after 1 d or 3 wks cocaine withdrawal according to anatomical regions depicted in Figure 4.1B. Microwave fixation was used to preserve *in vivo* protein phosphorylation status and avoid rapid alterations typically observed following dissection of un-fixed tissue



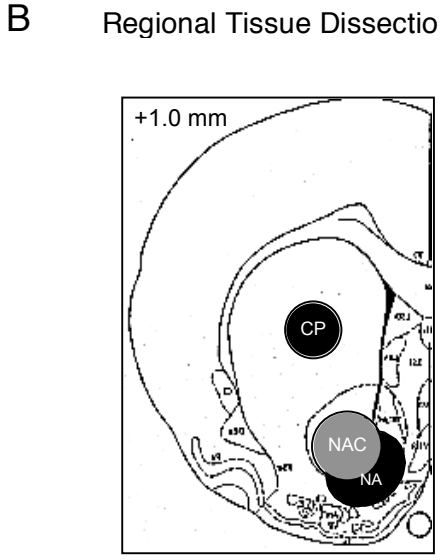


Figure 4.1
(**A**) Timeline depicting cocaine treatment regimens and withdrawal times from cocaine administration in yoked (Cocaine YK) and self-administering (Cocaine SA) animals. Rats self-administered cocaine (500 μg/kg/50 μl injection) or saline in daily 4-hr sessions over 18 days (6 days/week). Chronic YK animals received an identical number of non-contingent cocaine injections in a temporal pattern determined by their self-administering partners. Animals were euthanized by microwave fixation after early (1 d) and late (3 wks) cocaine withdrawal. Also at these time points, additional groups of animals were re-exposed to their individual cocaine-paired administration chambers for one hour prior to euthanasia, whereby a modest time-dependent increase in cocaine seeking was observed. (**B**) Schematic representation of regional brain samples collected with 12-16 gauge punches from 1.5 mm thick coronal slices. Anterior-posterior coordinates represent posterior sides of brain slices (Paxinos & Watson, 1998).

(O'Callaghan and Sriram 2004). All of the observed changes involved regulation in phosphorylation states since there was no significant regulation in the total amount of these phosphoproteins in any brain region studied.

Increased PKA-mediated $GluR_1^{S845}$ phosphorylation in accumbens shell after both early and late cocaine withdrawal

In the nucleus accumbens shell (NAS), PKA-mediated $GluR_1^{S845}$ phosphorylation (Figure 4.2A) was significantly increased by 19% at 1d WD ($F_{2,17} = 3.479$, $p = 0.05$) in both Cocaine SA and Cocaine YK groups, indicating a general effect of chronic cocaine administration to increase NAS PKA activity in early withdrawal. Interestingly, this effect was not seen in animals re-exposed to the cocaine-associated context at this time point. By 3wks WD, p $GluR_1^{S845}$ levels remained elevated (20%, $T_{22} = 2.526$, $p = 0.019$) exclusively in self-administering animals, suggesting a reinforcement-related maintenance of enhanced PKA activity into late withdrawal. Moreover, this effect was similar in animals re-exposed to the cocaine-associated context (18% increase, $T_{28} = 2.566$, $p = 0.016$).

Context-associated increases in NAS ERK phosphorylation after long-term cocaine withdrawal

In contrast to PKA-mediated $GluR_1^{S845}$ phosphorylation, MEK-mediated ERK phosphorylation was not increased at either 1 d or 3 wks WD (Figure 4.2b), indicating

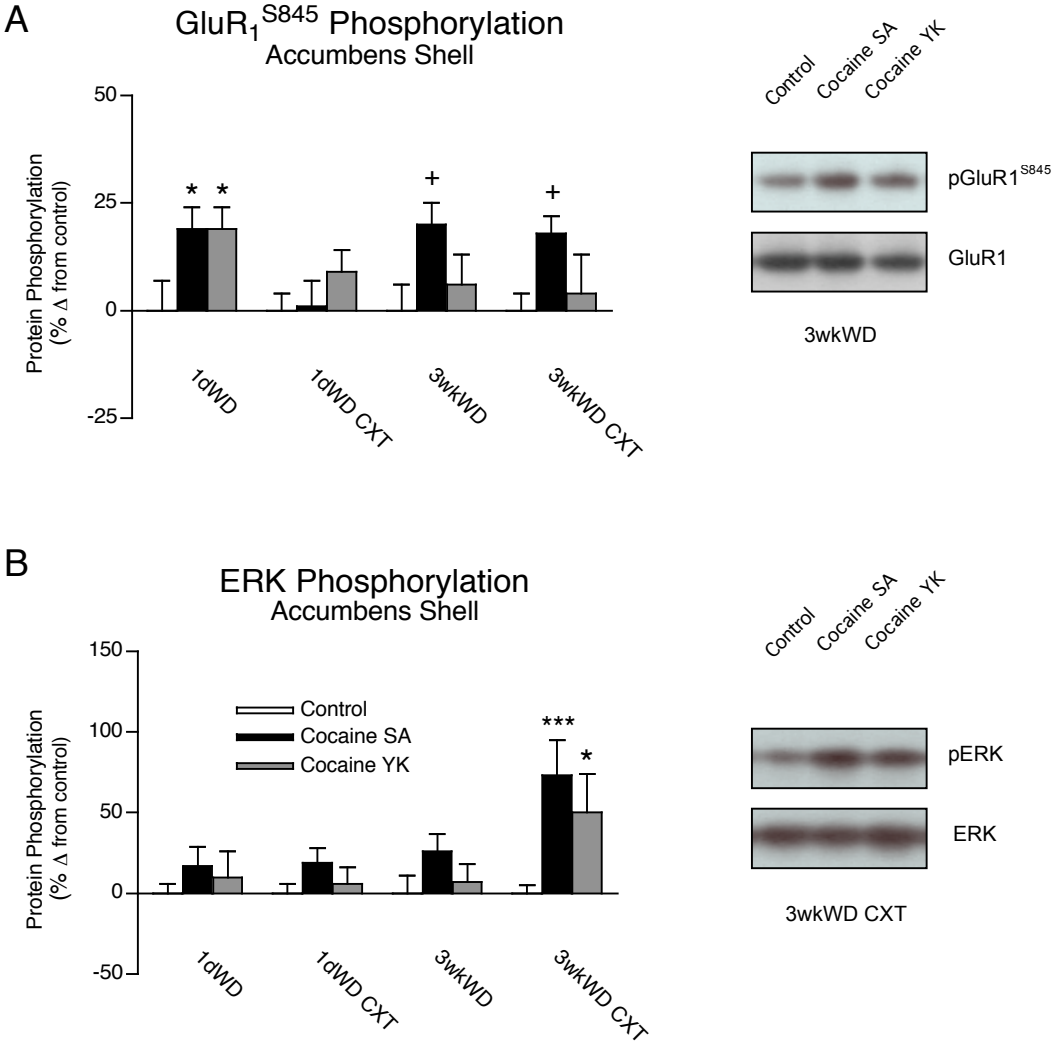


Figure 4.2
Protein phosphorylation in the accumbens shell (NAS) during withdrawal from active vs. passive cocaine administration. (**A**) PKA-mediated $GluR_1^{S845}$ phosphorylation is increased following 1 d WD from both yoked and self-administered cocaine, while after 3 wks WD, this effect is observed exclusively in self-administering animals. (**B**) MEK-mediated ERK phosphorylation is increased in both SA and YK groups after re-exposure to cocaine administration chambers at 3 wks WD. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n = 7-11/group). Symbols indicate values differ from controls ($^*p < 0.05$, $^{***}p < 0.001$) by Fisher's LSD tests, or differ from controls ($^+p < 0.05$) by planned Student's t-test comparisons. Representative blots for phosphorylated and total $GluR_1$ and ERK protein are shown for control (Control), cocaine yoke (Cocaine YK), and cocaine self-administering (Cocaine SA) animals.

that up-regulated NAS PKA signaling in both early and late cocaine WD does not generalize to the MEK/ERK kinase pathway. However, despite a lack of basal regulation at 3wks WD, re-exposure to the cocaine-associated context at this time point robustly increased ERK phosphorylation ($F_{2,36} = 7.89$, $p = 0.001$) by 73% and 50% in the Cocaine SA and Cocaine YK groups, respectively. Exposure to the cocaine-associated context had no effect at the early 1 d WD time point.

Reinforcement-related increases in accumbens core (NAC) protein phosphorylation after re-exposure to self-administration context in long-term cocaine withdrawal

In contrast to the NAS, basal $pGluR_1^{S845}$ in the NAC is not changed at either early or late withdrawal times (Figure 4.3a). However, despite an absence of regulation at 3wks WD, re-exposure to the cocaine-associated context at this time point increased PKA-mediated phosphorylation of NAC GluR1 by 31% ($F_{2,37} = 3.317$, $p = 0.047$) in cocaine self-administering animals. Importantly, this change was not seen in animals that had previously received passive cocaine injections (Cocaine YK group), implying a reinforcement-specific neuroadaptation in PKA signaling at this time point. In a similar fashion, ERK phosphorylation was also increased (68%, $F_{2,37} = 5.729$, $p = 0.007$) exclusively in the Cocaine SA group upon context re-exposure at 3wks withdrawal (Figure 4.3b).

Reinforcement-related increases in caudate putamen (CPU) $GluR_1^{S845}$ phosphorylation in early cocaine withdrawal

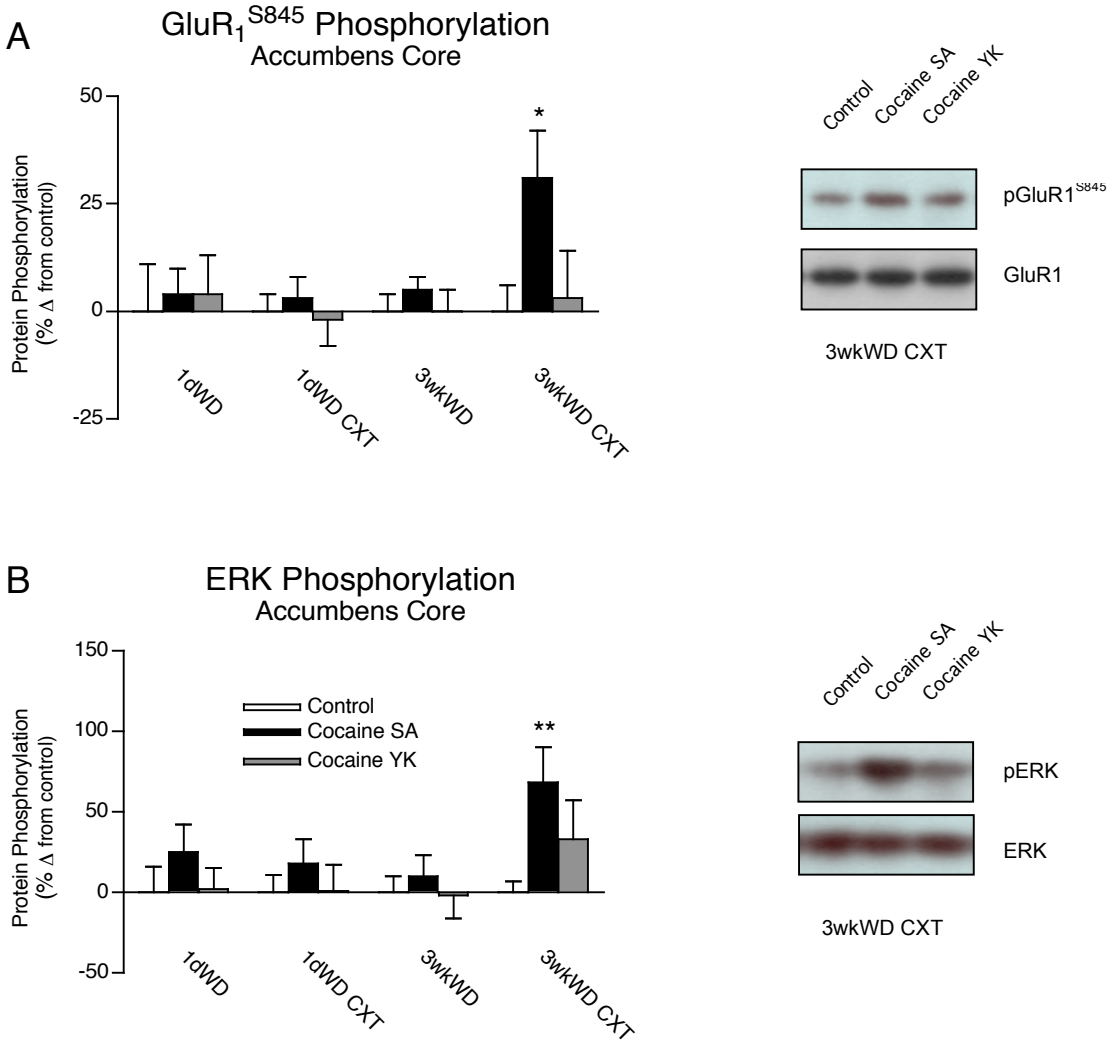


Figure 4.3
Protein phosphorylation in the accumbens core (NAC) during withdrawal from active vs. passive cocaine administration. (**A**) PKA-mediated $GluR_1^{S845}$ phosphorylation is increased exclusively in self-administering animals (Cocaine SA group) after re-exposure to cocaine administration chambers at 3 wks WD. (**B**) MEK-mediated ERK phosphorylation is also increased exclusively in self-administering animals (Cocaine SA group) after re-exposure to cocaine administration chambers at 3 wks WD. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n = 7-11/group). Symbols indicate values differ from controls (*$p < 0.05$, **$p < 0.01$) by Fisher's LSD tests. Representative blots for phosphorylated and total $GluR_1$ and ERK protein are shown for control (Control), cocaine yoke (Cocaine YK), and cocaine self-administering (Cocaine SA) animals.

Similar to the NAS, basal CPU $GluR_1^{S845}$ phosphorylation is also increased at 1 d WD ($F_{2,33} = 3.79$, $p = 0.03$), but only significantly in the Cocaine SA group (23%, p = 0.01) (Figure 4.4a). Furthermore, a similar 24% reinforcement-related increase in PKA signaling was still observed after re-exposing self-administering animals to the cocaine-paired context at 1 d WD ($F_{2,28} = 5.024$, $p = 0.014$) indicating no further increases in basal phosphorylation by exposure to the context. After 3wks WD from cocaine administration, $pGluR_1^{S845}$ levels remain increased by 19% when both Cocaine SA and YK groups are pooled ($F_{1,17} = 5.676$, $p = 0.029$). In a similar fashion to the early WD time point, re-exposure to the cocaine-paired context failed to modulate basal increases, as CPU $GluR_1^{S845}$ phosphorylation remained increased by 21% in pooled Cocaine SA and YK groups ($F_{1,37}= 5.666$, $p = 0.023$) after re-exposure.

Context-associated increases in CPU ERK phosphorylation after long-term cocaine withdrawal

Similar to $pGluR_1^{S845}$ regulation, MEK-mediated ERK phosphorylation is also increased exclusively in Cocaine SA animals at 1d WD (36%, $T_{22} = 2.347$, $p = 0.028$) (Figure 4.4b). However, despite the absence of pERK regulation at 3 wks WD, re-exposure to the cocaine-associated context at this time point greatly enhanced ERK phosphorylation ($F_{2,36} = 8.284$, $p = 0.001$) by 97% and 62% in the Cocaine SA and Cocaine YK groups, respectively, and effect no found with context exposure after 1 d WD.

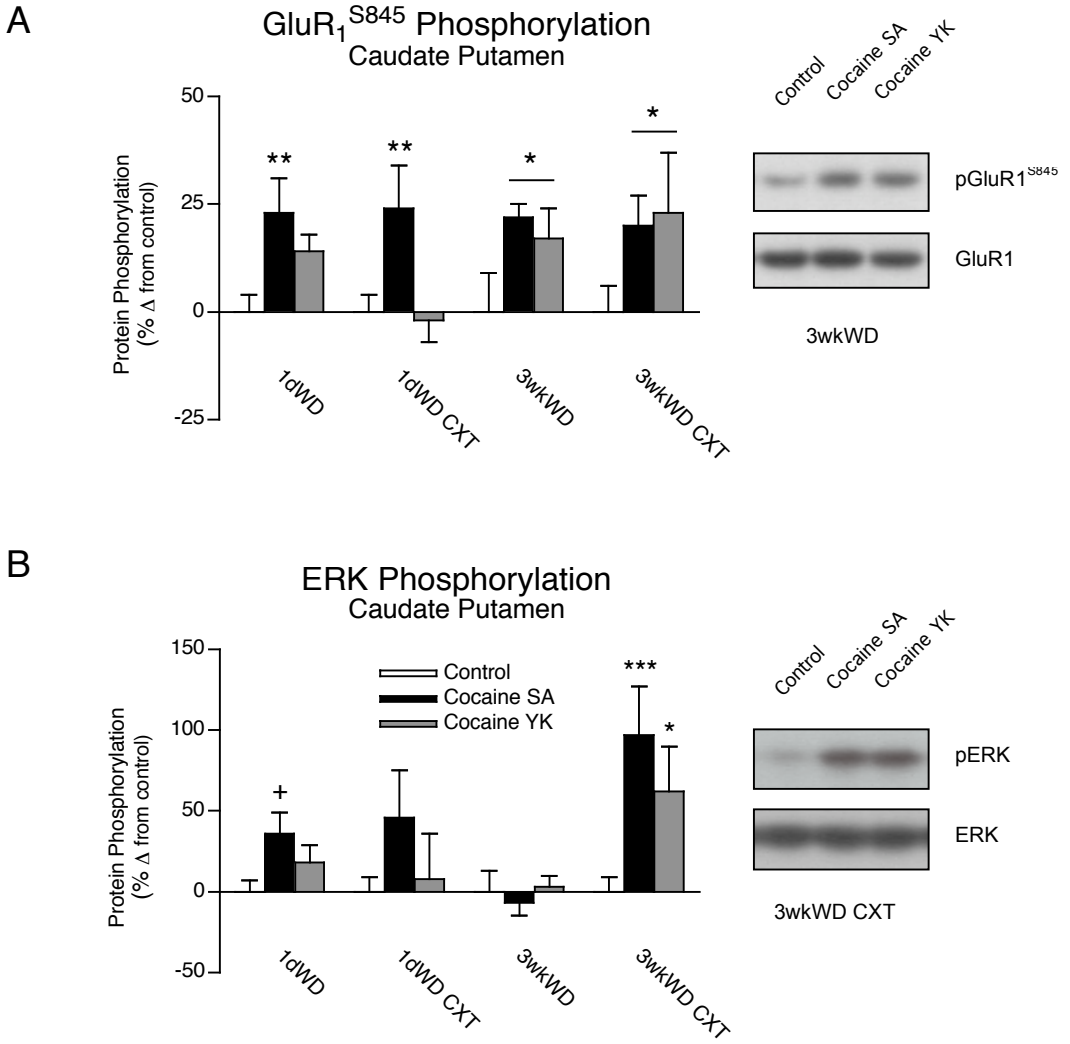


Figure 4.4
Protein phosphorylation in the caudate putamen (CPU) during withdrawal from active vs. passive cocaine administration. (**A**) PKA-mediated $GluR_1^{S845}$ phosphorylation is increased exclusively in self-administering animals (Cocaine SA group) at 1 d WD, while $pGluR_1^{S845}$ levels are elevated in pooled SA and YK groups at 3 wks WD. (**B**) MEK-mediated ERK phosphorylation is also increased exclusively in self-administering animals (Cocaine SA group) at 1 d WD, while pERK levels are increased in both SA and YK groups after re-exposure to cocaine administration chambers at 3 wks WD. Data reflect the mean ± SEM of phosphorylated/total protein ratios expressed as a percentage of untreated controls (n = 7-11/group). Symbols indicate values differ from controls ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) by Fisher's LSD tests or differ from controls ($^+p < 0.05$) by planned Student's t-test comparisons. Representative blots for phosphorylated and total $GluR_1$ and ERK protein are shown for control (Control), cocaine yoke (Cocaine YK), and cocaine self-administering (Cocaine SA) animals.

Discussion

In this study, we measured changes in striatal protein phosphorylation status after early (1 d) or late (3 wks) cocaine withdrawal in animals with chronic self-administration experience, and compared these results to animals that received an identical number and temporal pattern of cocaine injections via passive yoked administration. We also compared the response following re-exposure to the cocaine-associated context at both early and late cocaine withdrawal to identify changes associated with relapse induced by exposure to cocaine-paired environmental stimuli.

While drugs of abuse share a common ability to increase dopamine in the nucleus accumbens, which is believed to underlie their powerful reinforcing effects (Di Chiara and Imperato 1988), excitatory glutamatergic afferents to nucleus accumbens neurons are thought to mediate the conditioned reinforcing effects of cocaine cues (Di Ciano and Everitt 2001). In addition to these effects in the ventral striatum, the dorsal striatum (caudate putamen) is also activated during cue-elicited cocaine seeking in rodents (Ito et al. 2002), as well as cue-induced drug craving in humans (Garavan et al. 2000; Volkow et al. 2006).

Incubation of Cocaine Craving

The incubation of craving in cocaine withdrawal is hypothesized to relate to the intensity of previous drug exposure (Lu et al. 2004). Thus, several studies attempting to replicate

this phenomenon have reported either moderate time-dependent changes (Tran-Nguyen et al. 1998; Neisewander et al. 2000; Semenova and Markou 2003), no changes (Di Ciano and Everitt 2002; Deroche-Gamonet et al. 2003; Marinelli et al. 2003), or even decreases (Ciccocioppo et al. 2004) in drug seeking over time. In the present study, animals self-administering cocaine in daily 4-hr sessions for 3 weeks displayed a modest trend for increased drug-seeking behavior between 1 d and 3 wks WD (Figure 4.1A), an observation in line with prior reports.

Nucleus Accumbens

The nucleus accumbens is a heterogeneous structure with two distinct regions, shell (NAS) and core (NAC) (Voorn et al. 1989; Heimer et al. 1991), which contribute in different ways to conditioned and unconditioned processes involved in drug addiction. The NAC is involved in the control of goal-directed behavior by associative processes, as selective lesions of this region disrupt learned Pavlovian influences on appetitive behavior (Parkinson et al. 2000; Hall et al. 2001). The NAS may play a greater role in signaling novelty (Rebec et al. 1997) and mediating the primary reinforcing effects of drugs of abuse (Kalivas and McFarland 2003).

Consistent with previous studies demonstrating an up-regulation in the amount of PKA following chronic cocaine administration (Lu et al. 2003; Terwilliger et al. 1991), we found that basal PKA-mediated $GluR_1^{S845}$ phosphorylation in the nucleus accumbens shell (NAS) is increased at 1d WD in both Cocaine SA and YK groups (Figure 4.2a), indicating a general pharmacological effect of chronic cocaine leading to up-regulation of

PKA signaling, and functional consequences on downstream substrate phosphorylation, by cocaine. Interestingly, this effect was absent in animals re-exposed to the cocaine-paired context at 1 d WD. This finding resonates with a microdialysis study that found diminished NAS dopamine overflow in cocaine-experienced animals when re-exposed to operant chambers under extinction conditions (Neisewander et al. 1996). After three weeks of cocaine withdrawal, $pGluR_1^{S845}$ levels remained elevated only in cocaine self-administering animals, reflecting a reinforcement-related maintenance of increased PKA activity. ERK phosphorylation was not increased at either 1 d or 3 wks WD in the NAS (Figure 4.2b). However, re-exposure to the drug-paired context robustly increased NAS pERK levels solely at the late withdrawal time point in both Cocaine SA and Cocaine YK groups, indicating a general conditioned effect of cocaine-paired environments to increase NAS ERK activity in late withdrawal, regardless of self-administration experience. In comparison, despite a lack of regulation of either $GluR_1^{S845}$ or ERK phosphorylation at either WD time point in the accumbens core (NAC), both of these phosphoproteins were increased after re-exposure to the cocaine administration chambers at 3 wks, but not 1 d, withdrawal exclusively in the NAC of self-administering animals. The absence of effect in yoked cocaine animals is evidence that the NAC may play a specific, reinforcement-related role in the enhanced cocaine seeking observed at late cocaine withdrawal. Moreover, context-induced increases in $GluR_1^{S845}$ and ERK phosphorylation specifically in the NAC is consistent with a role for this region in mediating goal-directed behavior in self-administering animals.

Caudate Putamen

Given the role of the dorsal striatum in establishing stimulus-response habits with prolonged training (Yin et al. 2004), this region may come to regulate the habitual nature of excessive, pathological drug seeking (Robbins and Everitt 1999). In the present study, basal PKA-mediated $GluR_1^{S845}$ phosphorylation was increased at 1 d WD in the caudate putamen (CPU, Figure 4.4a). However, in contrast to effects seen in the NAS, this adaptation was only found in animals that self-administered cocaine, and was maintained after re-exposure to their self-administration context. This reinforcement-related change was accompanied by a concomitant increase in pERK at 1 d WD in the Cocaine SA group. Following 3 wks of withdrawal from cocaine, $GluR_1^{S845}$ phosphorylation was increased in both treatment groups, and this effect was not influenced by re-exposure to the cocaine-paired context. In contrast, CPU ERK phosphorylation was robustly increased in both groups only after context re-exposure at late withdrawal.

Our striatal data complement and extend a recent study (Lu et al. 2005) that implicates ERK signaling in the central nucleus of the amygdala (CEN) in mediating the phenomenon of cocaine craving incubation. Together, the CEN, along with the NAC, have been shown to mediate Pavlovian influences on instrumental behavior (Hall et al. 2001). Similar to time-dependent, context-elicited ERK phosphorylation in the CEN, we report here similar regulation of not only pERK, but also PKA-mediated $GluR_1^{S845}$ phosphorylation in the nucleus acumens core subregion under these conditions. Moreover, we show that this effect in the NAC is not present in animals that received passive cocaine infusions. This latter distinction is important, given that we also found

time-dependent increases in context-induced ERK phosphorylation in both NAS and CPU regardless of whether cocaine was administered in a contingent or non-contingent fashion. Taken together, these differential region- and substrate-specific adaptations to withdrawal- and context-induced protein phosphorylation could underlie the maintenance of cocaine addiction by exacerbating the potential for drug seeking in withdrawal.

CHAPTER 5

Addiction-Related Alterations in D_1 and D_2 Dopamine Receptor Behavioral Responses Following Chronic Cocaine Self-Administration

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Introduction

Drug addiction can be modeled in rodents based on phenotypic differences in drug consumption and drug-seeking behavior in an attempt to delineate "addicted" from "non-addicted" biological states (Ahmed and Koob 1998a; Ahmed et al. 2000; Piazza et al. 2000; Sutton et al. 2000a). In one approach, the addicted phenotype is selected from outbred rat populations based on higher preferred levels of drug intake. Previous studies have shown that rats with higher preferred levels of cocaine intake also exhibit a propensity for cocaine-seeking behavior when reinforcement is withheld during self-administration or after a period of withdrawal, thereby encompassing both addictive traits in a single subpopulation (Piazza et al. 2000; Sutton et al. 2000a).

Cocaine taking and seeking behaviors are strongly regulated by D_1 and D_2 classes of dopamine receptors. Systemic pretreatment with either D_1 or D_2 receptor agonists reduces cocaine self-administration in rats, whereas pretreatment with either D_1 or D_2 receptor antagonists increases intake when access to cocaine is relatively unrestricted (Koob et al. 1987; Corrigall and Coen 1991; Caine et al. 1999), suggesting that both receptors provide inhibitory feedback regulation of cocaine intake during self-administration. Relapse to cocaine-seeking behavior also is strongly regulated by both D_1 and D_2 dopamine receptor classes, but here D_1 and D_2 receptors mediate opposing influences. Selective stimulation of D_2 receptors strongly induces, or reinstates, cocaine-seeking behavior after cocaine-seeking responses have been extinguished in withdrawal, whereas selective D_1 receptor stimulation attenuates cocaine's ability to reinstate this

behavior (Wise et al. 1990; Self et al. 1996; De Vries et al. 1999; Alleweireldt et al. 2002). A similar D_1/D_2 dichotomy regulates cocaine seeking in non-human primates (Khroyan et al. 2000), and also may suppress and stimulate craving responses in humans, respectively (Haney et al. 1998; Haney et al. 1999). Together, these studies suggest that D_2 receptors could play a major role in eliciting relapse to cocaine-seeking behavior when environmental stimuli such as cocaine cues or stress activate the mesolimbic dopamine system (Phillips et al. 2003), while D_1 receptor tone may provide inhibitory regulation over cocaine seeking.

The transition from non-addicted to addicted states could reflect differential adaptations in the sensitivity of these D_1 and D_2 receptor-mediated responses, whether sensitization in D_2-mediated responses that trigger cocaine seeking, or tolerance in D_1-mediated responses that inhibit drug seeking. Moreover, higher preferred levels of cocaine intake in addicted animals could reflect a compensatory response to reduced D_1 receptor function. Furthermore, such differences could emerge or be exacerbated during withdrawal from cocaine, since the propensity for cocaine seeking increases in a time-dependent manner from early to late withdrawal times (Tran-Nguyen et al. 1998; Grimm et al. 2001).

In this study, we tracked changes in D_1 and D_2 receptor-mediated locomotor responses from before the onset of cocaine self-administration to early (2-3 days) and late (4 weeks) withdrawal times. We compared these changes with individual differences in preferred levels of cocaine intake to identify correlational, neuroadaptive changes that

could contribute to the development of addiction. Importantly, we also compared the ability of D_1 and D_2 receptor stimulation to modulate relapse to cocaine seeking after a period of withdrawal to identify potential differences associated with a cocaine-addicted phenotype.

Materials and Methods

Subjects. Sixty-four outbred male Sprague-Dawley rats (Charles River, Kingston, NY), weighing 300-325 g on arrival, were individually housed in a climate-controlled environment (21°C) on a 12-hr light-dark cycle (lights on at 7:00 A.M.) in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Animals were allowed free access to lab chow and water, except during initial lever-press training for sucrose pellets and during initial acquisition of cocaine self-administration (see below).

Surgery. Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) supplemented with atropine sulfate (0.10 mg, s.c.), prior to surgical implantation of a chronic indwelling intravenous catheter. The catheters consisted of Silastic tubing (0.02" i.d. x 0.037" o.d.; Green Rubber, Woburn, MA) treated with tridodecylmethyl ammonium chloride (TDMAC) heparin (Polysciences Inc., Warrington, PA). Each catheter was secured at the jugular vein with Mersiline surgical mesh (General Medical, New Haven, CT), and passed subcutaneously to exit the back through 22 gauge cannula (Plastics One, Roanoke, VA) embedded in dental cement on a 1" Marlex surgical mesh base (Bard Inc., Cranston, RI). Following surgery, animals received a prophylactic injection of penicillin

(60,000IU/0.2 ml, i.m.), and antibiotic ointment was applied daily to the exit wound. Catheters were flushed daily with 0.2 ml of heparinized (20 IU/ml) bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml) to prevent clotting and curb infection.

Locomotor and self-administration apparati. Locomotor activity was recorded in the dark in circular test chambers with a 12 cm wide runway, equipped with four pairs of photocells located at 90-degree intervals around the 1.95 m perimeter. The operant test chambers (Med Associates, East Fairfield, VT) used for self-administration, extinction, and reinstatement testing were contextually distinct from the locomotor test chambers, and located in a different room. Each chamber was enclosed in a ventilated, sound-attenuating box and was equipped with an injection pump assembly consisting of a Razel Model A injection pump (Stamford, CT) and a 10-ml glass syringe connected to a fluid swivel (Instech, Plymouth, PA) with Teflon® tubing. Tygon® tubing connected the swivel to the animal's catheter exit port and was enclosed by a metal spring secured to Teflon® threads on the catheter assembly. Each operant chamber contained two response levers (4 cm x 2 cm), located 2 cm off the floor; during self-administration testing, a 20 g lever-press response at the active lever delivered an intravenous cocaine injection, and produced no programmed consequence at the inactive lever. Each cocaine injection was delivered over 2.5 s in a 50-ul volume. During the injection period, a cue light (above the lever) was illuminated and the house light was extinguished, followed by an additional 12.5 s time-out interval when the house lights remained off and responding at the active lever had no programmed consequences. The illumination of the house light signaled the end of the 15 s injection/time-out interval.

Serial testing procedures. To facilitate acquisition of cocaine self-administration, animals were maintained on a restricted diet (15g of food/day) to prevent weight gain and trained to press the active lever for 45-mg sucrose pellets until they reached acquisition criterion (100 correct responses) on three successive days. All animals reached criterion in each of three daily sessions, with the first session (overnight) lasting approximately 12hrs and the last two sessions lasting less than 20min. Animals were fed *ad libitum* at least 1 day prior to surgical catheterization and allowed to recover for 1 week before the onset of testing. All rats subsequently underwent an identical sequence of serial testing in locomotor and self-administration procedures beginning always at 8:00 A.M. (see Figure 5.1). On the first 2 days, locomotor pre-tests were conducted with either the D_1 agonist SKF 81297 or the D_2 agonist quinpirole (saline, 0.1, 0.3, 1.0 mg/kg/ml, s.c.) in counterbalanced order in a within-session (6 hr) dose-response procedure. In this procedure, rats were habituated to the testing environment for 2 hrs, and then removed from the locomotor apparatus and given an injection of saline, replaced in the test chamber and locomotor activity was recorded for 1 hr. Rats were removed again and injected hourly with ascending doses of either the D_1 or D_2 agonist for the final 3 hours of testing, and locomotor activity following each dose was recorded.

Self-administration testing commenced 3 days after the second locomotor pre-test, and rats were allowed to acquire cocaine self-administration (0.5 mg/kg/injection, i.v.) on a fixed-ratio 1: time-out 15 sec reinforcement schedule in daily 4 hr test sessions for 3 weeks (6 days/week). Animals were maintained at a constant body weight during the

first week of cocaine self-administration to facilitate acquisition, but fed *ad libitum* for the remainder of testing. Catheter patency was verified on non-experimental days by intravenous infusion of the short-acting barbiturate sodium methohexital (0.1 mg/0.1ml); a positive test was indicated by rapid onset of brief anesthesia. Under these conditions, 40 of 40 rats acquired cocaine self-administration, as indicated by self-administering > 50 cocaine injections in each of the final 6 days of acquisition training. Another 5 rats lost catheter patency during training; these rats were excluded from the analysis. Following the 3-week acquisition phase, animals were tested in a between-session self-administration dose-response procedure with decreasing cocaine doses available each day for five days (1.0, 0.3, 0.1, 0.03 and 0 mg/kg, i.v.). After determination of self-administration dose-response curves, a second locomotor test was conducted with the D_1 and D_2 agonists identical to the initial test and corresponding to days 2-3 of early cocaine withdrawal (including the final 0 mg/kg dose in self-administration testing).

On days 11-15 of cocaine withdrawal, animals returned to the operant test chambers for extinction testing in the absence of cocaine reinforcement for 4 hrs/day. Responses at the drug-paired lever were recorded but had no programmed consequence. During the following week (days 18-25 of cocaine withdrawal), the ability of the D_2 agonist quinpirole (saline, 0.3, 1.0, and 3.0 mg/kg, s.c.) to reinstate cocaine-seeking behavior, and the ability of the D_1 agonist SKF 81297 (saline, 1.0 and 3.0 mg/kg, s.c.) to attenuate reinstatement of cocaine seeking induced by a cocaine priming injection (15 mg/kg, i.p.), was assessed under extinction conditions. Reinstatement test sessions were 4 hrs in duration; the first three hours served to extinguish residual responding to low

levels prior to pharmacological challenge with cocaine or quinpirole (SKF 81297 or saline was given as a pre-treatment 30 min prior to cocaine challenge). Both drug-paired and inactive lever responses were recorded during the final 1 hr of the test session immediately following challenge with cocaine or quinpirole. The order of dose presentation was counterbalanced for the D_1 and D_2 agonists across test days, except for the high dose of quinpirole that was always tested last due to potential sensitizing influences. After reinstatement testing, a third and final locomotor test was conducted with the D_1 and D_2 agonists over 2 days in late cocaine withdrawal (days 28-29) in a manner identical to earlier tests (the order of drug testing for each animal was maintained throughout the experiment).

A second experiment was conducted under identical serial testing conditions except that the non-selective dopamine receptor agonist apomorphine (saline, 0.1, 0.3 and 1.0 mg/kg/ml, s.c.) and the non-dopaminergic NMDA glutamate receptor antagonist MK-801 (saline, 0.05, 0.10, and 0.15 mg/kg/ml, s.c.) were used as positive controls in locomotor and reinstatement challenge experiments instead of the D_1 and D_2 agonists. In this experiment 24 out of 25 rats met acquisition criterion, and were included in the final analysis. Both drugs were tested for their locomotor activating effects, whereas only MK-801 was tested in reinstatement since pilot data found that apomorphine was generally ineffective as a reinstating stimulus, consistent with a previous study (De Vries et al. 1999).

Measurement of brain cocaine levels. Seven days after completion of all behavioral studies, 12 self-administering animals were given an i.v. cocaine challenge (2 mg/kg) and

sacrificed by decapitation exactly 10 min later. Whole brains were dissected, rapidly frozen in isopentane, and stored at –80 degrees C until testing. On the day of assay, brains were homogenized in deionized water (1:4 by brain weight). One ml of the 1:4 diluted homogenate (0.25 gram tissue) from each brain was assayed as follows: 200 ng of deuterated cocaine was added as an internal standard, and the pH was adjusted to 9.3 with ammonium chloride buffer. Cocaine was extracted into 4 ml of n-butyl chloride, and subsequently back extracted into 0.5 ml of 0.1 N sulfuric acid. The pH was adjusted back to 9.3 with ammonium chloride buffer and the cocaine was extracted into 2 ml of n-butyl chloride and dried. The residue was reconstituted with 30 ul of n-butyl chloride. After calibration with a standard curve, the extracts were quantified on an Agilent 5973N GC-MS in selected ion mode, and results corrected for deuterated cocaine recovery.

Post-hoc analysis of low and high cocaine intake subpopulations. Animals in the first (n = 40) and second (n = 24) experiment were grouped into low and high cocaine intake subgroups determined by the median of average cocaine intake over the final 6 of 18 test sessions during acquisition training in each experiment. Cumulative locomotor dose-response data for each test drug (not including saline) were analyzed by 2-factor ANOVA with repeated measures on test (pre-test, early withdrawal, late withdrawal). Self-administration and locomotor dose-response curves, active/inactive lever response ratios, and extinction and reinstatement data were analyzed separately by 2-factor ANOVA with repeated measures on dose/test session (D_1 and D_2 challenge in reinstatement were analyzed separately). Normalized reinstatement data were analyzed by non-parametric Kruskal-Wallis tests. Significant interactive effects were followed by tests for simple

effects between low and high subgroups at each dose/test session. Brain cocaine levels and extinction latencies were compared by Student's t-tests. In addition, 11 pre-determined variables were compared by linear regression analysis, and by stepwise multiple regression analysis using cocaine intake as the dependent variable.

Drugs. SKF 81297 ((+/-)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide), quinpirole ((4a*R-trans*)-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-1*H*-pyrazolo[3,4-g] quinoline hydrochloride), apomorphine (6-Methyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinoline-10,11-diol), and MK-801 (5H-dibenzo[a,d]cyclohepten-5,10-imine, 10,11-dihydro-5-methyl-, (5S)-) were purchased from Sigma (St. Louis, MO). All drugs were dissolved in a physiological saline vehicle. Ascorbic acid was added to apomorphine test vehicles (0.1%) to prevent oxidation of the drug. Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Research Triangle Park, NC), and was dissolved in sterile-filtered physiological saline.

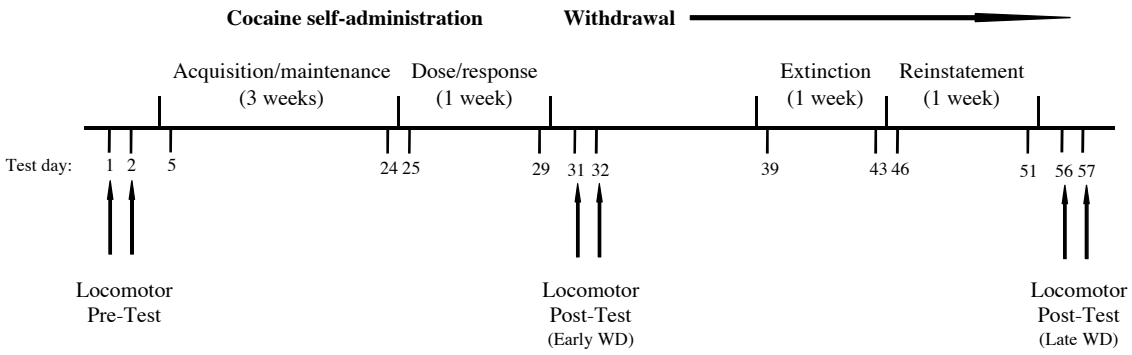

Figure 5.1
Serial behavioral testing procedure.

Results

Animals self-administering cocaine (0.5 mg/kg/injection in 18 daily 4-hr sessions) were divided into low and high intake groups (n = 20/group) based on a median split of the average cocaine intake for their last 6 days of self-administration training (Fig. 5.2A). Low and high intake groups averaged 42.5 ± 2.1 and 54.7 ± 3.6 mg/kg/day of cocaine intake, respectively, in days 13-18 of training. Self-administration data in this acquisition phase were not statistically analyzed since they reflect the independent variable used to define study groups. Active and inactive lever response ratios were calculated for each training day, with no significant group effect found ($F_{1,38} = 1.598$, $p = 0.214$), demonstrating that discrimination learning was similar between the groups. Cocaine self-administration produces an inverted U-shaped dose-response curve on low fixed-ratio schedules, spanning dose thresholds for maintaining self-administration, and a descending limb where increasing the injection dose prolongs the duration of cocaine effects, resulting in fewer self-injections over time. Cocaine self-administration dose-response curves were determined across 6 test sessions (Fig. 5.2B). High intake animals exhibited a vertical shift in peak self-administration rates when compared to the low intake group (main effect of group, $F_{1,38} = 15.775$, $p < 0.001$), indicative of greater cocaine reinforcement in these animals, and consistent with phenotypic differences observed in a previous study (Piazza et al. 2000). High intake animals also self-administered substantially greater amounts of cocaine on the descending limb of the curve (main effect across 4 doses, $F_{1,38} = 42.118$, $p < 0.001$), an effect that is more apparent when dose-response data are transformed into dose-intake data based on the

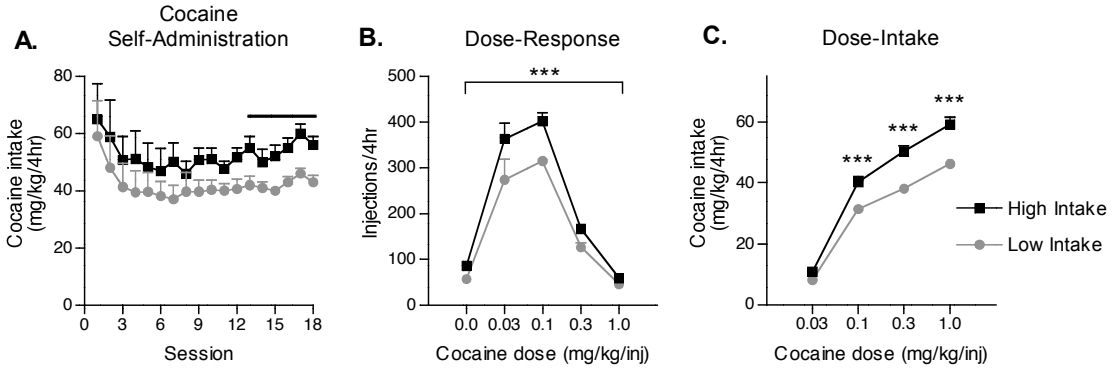

Figure 5.2
Cocaine self-administration in 40 outbred Sprague-Dawley rats. (**A**) Low and high intake groups (n = 20/group) were determined by the median of average daily cocaine self-administration (0.5 mg/kg/injection) during the last 6 of 18 4-hr training sessions (solid line). (**B**) High intake animals exhibit an upward shift in peak self-administration rates, and a rightward shift in the descending limb of the self-administration dose-response curve. (**C**) Cocaine self-administration data converted to dose-intake curves shows that high intake animals take greater daily amounts of cocaine at doses on the descending limb of the dose-response curve shown in panel B. Data reflect the mean ± SEM for low and high intake groups in each test session. Asterisks indicate a main effect of group by ANOVA (***$p < 0.001$).

total amount of cocaine consumed in the 4 hr test sessions (Fig. 5.2C). This difference in preferred levels of cocaine intake was exacerbated by increasing the injection dose available for self-administration (group x dose interaction, $F_{3,114} = 7.076$, $p < 0.001$).

Following an 11-day withdrawal period, animals returned to the self-administration test chambers, and resistance to extinction of cocaine-seeking behavior was determined by the number of non-reinforced drug-paired lever responses recorded in daily 4-hour test sessions for 5 days. Figure 5.3A shows that high intake animals engaged in greater drug-paired lever responding than low intake animals (main effect of group, $F_{1,38} = 8.368$, $p = 0.006$), but responding declined to equivalent levels by the final extinction test session (group x test day interaction, $F_{4,152} = 9.784$, $p < 0.001$). Thus, after a period of withdrawal, cocaine seeking elicited by initial exposure to the cocaine-paired environmental context was two fold greater in high than in low intake animals. Moreover, the latency to extinguish self-administration behavior, defined as the number of test sessions required to achieve < 60 lever presses in 4 hours, was also significantly longer in the high than in low intake groups ($p = 0.039$). Taken together, high intake animals exhibited differences in cocaine self-administration and cocaine-seeking behavior that are consistent with addiction-related changes reported in previous studies.

Given that D_1 and D_2 receptors mediate opposite modulation of cocaine-seeking behavior in reinstatement tests, we determined whether the ability of D_1 and D_2 receptors to modulate this behavior would differ in low and high intake groups. Figure 5.4A shows that high intake animals were less sensitive, or refractory, to the ability of the full

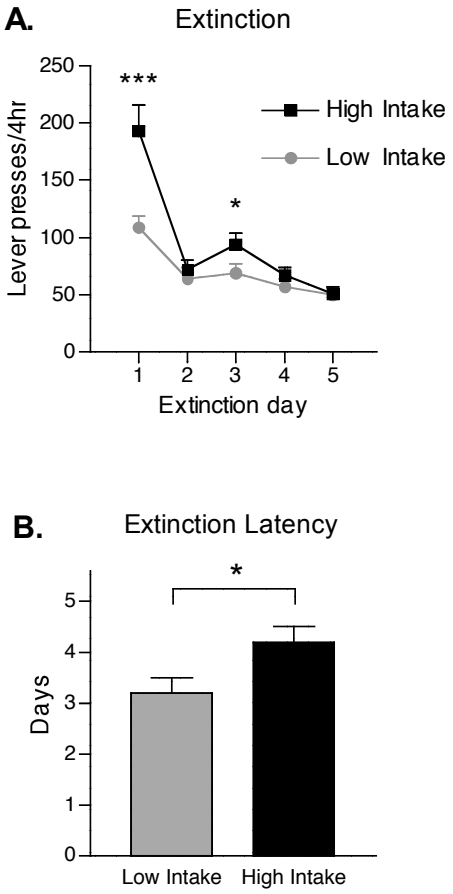

Figure 5.3
Extinction of cocaine self-administration in low and high intake animals. (**A**) High intake animals exhibit 2-fold greater non-reinforced, drug-paired lever responses during the initial 4-hr extinction test when compared to low intake animals. (**B**) High intake animals also show greater resistance to extinction of cocaine self-administration compared to low intake animals, achieving extinction criteria (< 60 drug-paired lever responses/session) with longer latencies (n = 20 group). Data reflect the mean ± SEM for low and high intake groups. Asterisks indicate that high differ from low intake animals by tests for simple effects following significant group by test session interaction or by t-test for extinction latencies (*$p < 0.05$, ***$p < 0.001$).

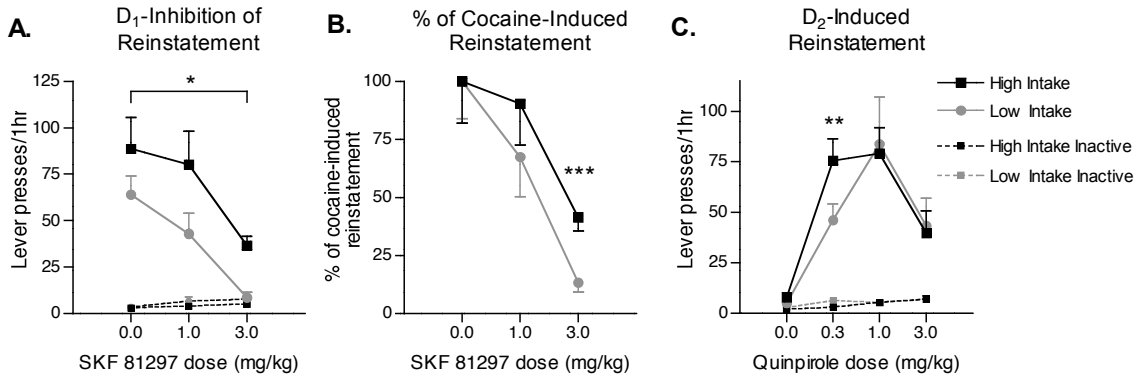

Figure 5.4

Differential sensitivity to opposite modulation of cocaine-seeking behavior by D_1 and D_2 receptor agonists in low and high intake animals. (**A**) Reduced ability of the D_1 receptor agonist SKF 81297 (given as a 30 min pretreatment) to inhibit cocaine seeking elicited by an i.p. priming injection of cocaine (15 mg/kg) in high relative to low intake animals (n = 20/group). (**B**) Resistance to D_1–mediated inhibition of reinstatement in high intake animals after normalization of data shown in A to individual baselines (100%) for cocaine-primed reinstatement (saline pretreatment). (**C**) Conversely, high intake animals are more sensitive to the ability of the D_2 receptor agonist quinpirole to induce cocaine-seeking behavior. Data reflect the mean ± SEM for low and high intake groups. Astericks indicate (A) *$p < 0.05$; group effect, (B) ***$p < 0.001$ by non-parametric Mann-Whitney U test, (C) **$p < 0.01$ by test for simple effects.

efficacy D_1 agonist SKF 81297 to attenuate drug-paired lever responding elicited by a cocaine priming injection in reinstatement tests that were conducted from days 18-25 of withdrawal from cocaine self-administration (main effect of group, $F_{1,38} = 6.926$, $p = 0.012$). Although D_1 agonist treatment dose-dependently attenuated cocaine seeking in both groups (main effect of dose, $F_{2,76} = 14.783$, $p < 0.001$), high intake animals showed a trend for greater cocaine-induced seeking behavior in the absence of D_1 agonist treatment (control condition), and so the attenuation of cocaine seeking by D_1 agonist treatments was normalized to cocaine alone (100%). Despite normalization to within-group baselines, high intake animals displayed subsensitivity to D_1 receptor-mediated inhibition of reinstatement at the highest dose tested (Fig. 5.4B, $U = 343$, $p < 0.001$).

Fig. 5.4C shows that treatment with the full efficacy D_2 receptor agonist quinpirole dose-dependently reinstated cocaine seeking, resulting in an inverted U-shaped dose-response curve, (main effect of dose, $F_{3,114} = 20.406$, $p < 0.001$). In contrast to D_1 receptor-mediated attenuation of cocaine seeking, high intake animals were more sensitive to reinstatement of cocaine seeking induced by a low dose of quinpirole compared to the low intake group (dose x group interaction on ascending limb, $F_{1,38} = 7.208$, $p = 0.011$), although there were no differences in response to priming with the saline vehicle. A lower dose of quinpirole (0.1 mg/kg) was tested in a small population of animals (n=4/group), with no group differences observed. Means of lever pressing in the 1 hr period preceding each reinstatement stimulus ranged from 2.8 ± 1.4 to 6.1 ± 3.4 for low intake animals and from 4.3 ± 1.3 to 7.7 ± 2.4 for high intake animals. Thus,

high intake animals exhibit opposing alterations in sensitivity to D_1 and D_2 receptor regulation of cocaine-seeking behavior when compared to low intake animals. However, neither the maximal reinstating efficacy of quinpirole, or responding on the descending limb of the curve, differed between the two groups. Inactive lever responding failed to differ between low and high intake animals in reinstatement experiments, with mean responding ranging from 2.1 ± 0.8 to 8.1 ± 1.9 lever presses in low intake animals, and 2.6 ± 0.9 to 5.7 ± 2.1 in high intake animals, across all treatment conditions.

Reinstatement of pre-conditioned behaviors cannot be determined prior to conditioning (self-administration), and so we utilized the unconditioned psychomotor response to dopamine receptor challenge to track the development of changes in sensitivity to D_1 or D_2 receptor stimulation from before the onset of cocaine self-administration to early and late withdrawal times. Figure 5.5A shows cumulative horizontal locomotor responses to D_1 and D_2 receptor challenge in a within-session dose-response determination conducted before and after cocaine self-administration. There was no difference in D_1 and D_2 receptor-mediated locomotor responses between low and high intake groups before cocaine self-administration, and in early cocaine withdrawal (2-3 days), but a significant divergence in D_1 and D_2 receptor responsiveness emerged at the late withdrawal time (28-29 days). Thus, neither group developed significant sensitization to D_1 receptor challenge after early withdrawal from chronic cocaine self-administration, but low intake animals ultimately developed ~50% sensitization to D_1 receptor challenge at late cocaine withdrawal, and showed a significantly greater

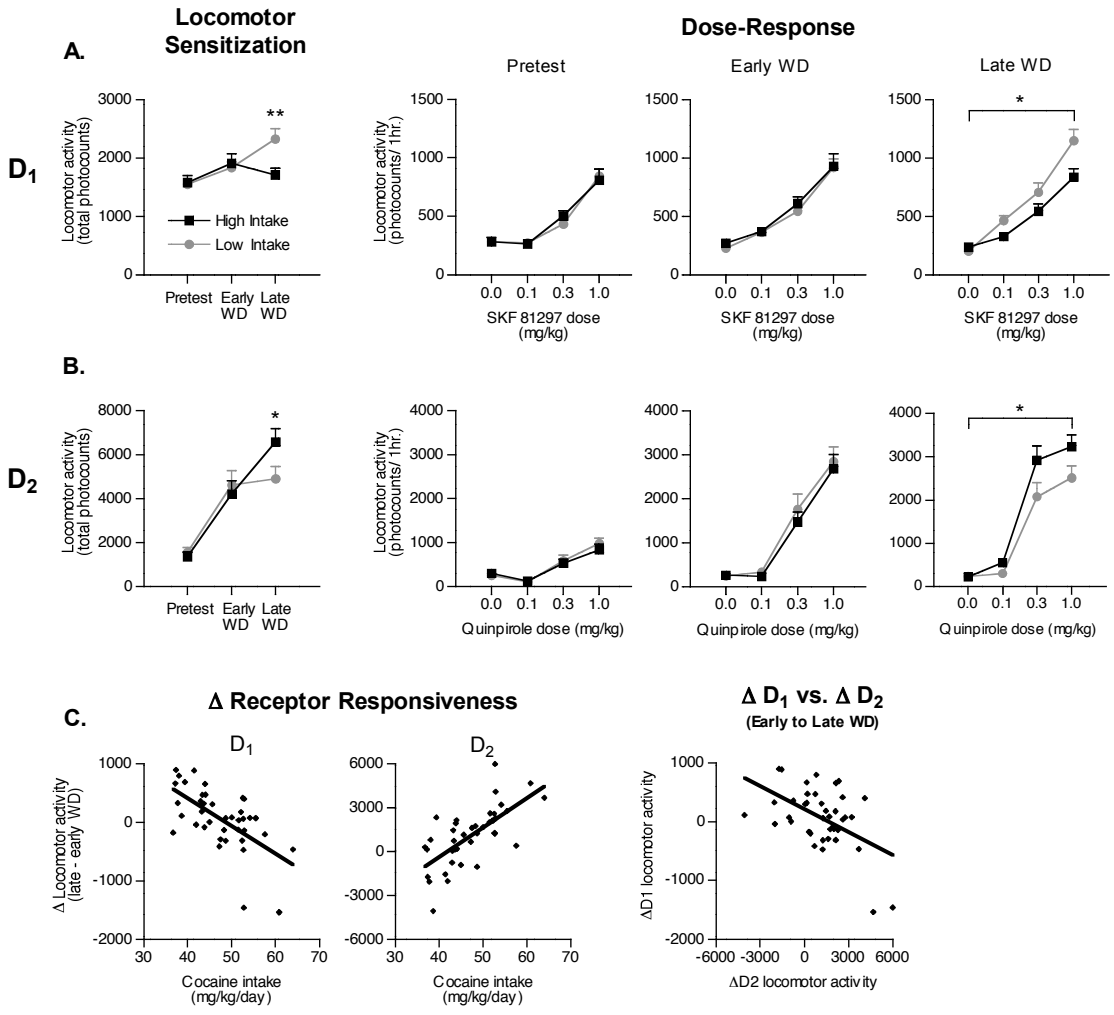

Figure 5.5 Differential development of D_1 and D_2 receptor sensitization in low and high intake animals during withdrawal from chronic cocaine self-administration. (**A**) Low intake animals develop D_1 receptor sensitization, whereas high intake animals develop D_2 receptor sensitization (N = 20 group). Data reflect cumulative locomotor dose-response tests (0.1-1.0 mg/kg) with the D_1 agonist SKF 81297 and the D_2 agonist quinpirole before self-administration and at early (2-3 days) and late (28-29 days) withdrawal times. (**B**) Locomotor dose-response data for low and high intake animals challenged with the D_1 and D_2 agonists at late withdrawal. (**C**) Average daily cocaine intake during the last 6 days of acquisition training is negatively correlated with the change in D_1 responsiveness from early to late withdrawal ($r = -0.507$, $p = 0.001$), but positively correlated with the change in D_2 responsiveness ($r = 0.686$, $p < 0.001$). (**D**) The development of D_1 sensitization is negatively correlated with the development of D_2 sensitization from early to late withdrawal ($r = -0.412$, $p = 0.001$). Data in A and B reflect the mean ± SEM for low and high intake groups. Asterisks indicate that high differ from low intake animals by main group effect or tests for simple effects following significant group by dose interaction (*$p < 0.05$, **$p < 0.01$).

response than high intake animals that failed to sensitize to D_1 challenge (group x time interaction, $F_{2,76} = 8.026$, $p = 0.001$). Conversely, although both groups developed a profound D_2 receptor sensitization (~400%) when challenged early after cocaine self-administration, this D_2 receptor sensitization intensified from early to late withdrawal only in high, but not in low, intake animals, resulting in a 6 fold increase when compared to pre-self-administration baselines (group x time interaction, $F_{2,76} = 6.670$, $p = 0.002$).

Figure 5.5B shows locomotor dose-response curves for D_1 and D_2 agonists in low and high intake groups challenged at the late withdrawal time. While neither group differed in their locomotor response to saline, low intake animals were more responsive to all doses of the D_1 agonist SKF 81297 compared to high intake animals (main effect of group, $F_{1,38} = 6.631$, $p = 0.014$). Conversely, high intake animals were more responsive to all doses of the D_2 agonist quinpirole compared to low intake animals (main effect of group, $F_{1,38} = 5.599$, $p = 0.023$). Thus, differential sensitization in D_1 and D_2 receptor responses developed in low and high intake rats as a consequence of cocaine self-administration, and these changes paralleled differences found in reinstatement tests.

We also compared a series of behavioral measures reflecting these differences by linear regression analysis of continuous data in the entire population of 40 self-administering rats (Table 5.1). Individual levels of cocaine intake during the final 6 days of self-administration training were negatively correlated with the change in D_1-stimulated locomotion from early to late withdrawal ($r = -0.507$, $p = 0.001$), but positively correlated with the change in D_2-stimulated locomotion ($r = 0.686$, $p < 0.001$),

Table 5.1. Correlation of individual self-administration, extinction, reinstatement and locomotor responses

						Reinstatement Stimulus				Locomotor Sensitization	
Variable	Intake Average	Cocaine SA 1.0 mg/kg	Cocaine SA 0.1 mg/kg	Extinction Day 1	Extinction Latency	Cocaine RSTMT	Cocaine + D1 (low)	Cocaine + D1 (high)	D2 RSTMT (low)	ΔD1 (late-early WD)	ΔD2 (late-early WD)
Intake Average	-	**0.807*** **	**0.635*** **	**0.450** **	0.172	0.163	0.232	**0.559*** **	0.154	**-0.507*** **	**0.686*** **
Cocaine SA - 1.0 mg/kg	-	-	**0.556*** **	0.282	0.128	0.247	0.250	**0.415** **	0.228	**-0.420** **	**0.677*** **
Cocaine SA - 0.1 mg/kg	-	-	-	**0.441** **	0.067	**0.341***	**0.557*** **	**0.571*** **	0.379	-0.301	**0.420***
Extinction Day1	-	-	-	-	**0.532*** **	**0.371***	**0.377***	**0.346***	0.204	**-0.325***	**0.389***
Extinction Latency	-	-	-	-	-	0.074	0.170	0.098	**0.387***	-0.148	0.183
Cocaine RSTMT	-	-	-	-	-	-	0.304	0.149	0.316	-0.059	0.159
Cocaine RSTMT + D1 (low)	-	-	-	-	-	-	-	**0.399***	0.216	-0.098	0.176
Cocaine RSTMT + D1(high)	-	-	-	-	-	-	-	-	**0.441***	-0.282	**0.436** **
D2 RSTMT (low)	-	-	-	-	-	-	-	-	-	-0.045	0.194
ΔD1 (late-early WD)	-	-	-	-	-	-	-	-	-	-	**-0.412** **
ΔD2 (late-early WD)	-	-	-	-	-	-	-	-	-	-	-

Correlation coefficients (Pearson's Product Moment) for comparisons of eleven test variables. Correlation coefficients with *p* values <0.05 are indicated in bold (n = 40 rats).
Asterisks indicate *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001. Abbreviations: SA, self-administration; RSTMT, reinstatement stimulus; WD, cocaine withdrawal.

and these relationships are illustrated graphically in Fig. 5.5C. As shown in Fig. 5.5D, the capacity of animals to develop locomotor sensitization to the D_1 agonist was negatively correlated with their capacity to develop further sensitization to the D_2 agonist ($r = -0.412$, $p < 0.01$), indicating that the two phenomena may be mutually exclusive (Fig. 5.5D). Similarly, resistance to D_1-mediated inhibition of cocaine seeking (high dose) was positively correlated with D_2-mediated reinstatement of cocaine seeking (low dose). Resistance to D_1-mediated inhibition of cocaine seeking (high dose) also correlated positively with levels of cocaine intake, and with the amount of cocaine seeking measured during the initial extinction test session (Table 5.1), consistent with differences exhibited by low and high intake groups. Other differences between low and high intake groups, such as reinstatement induced by the D_2 agonist (low dose), failed to correlate significantly with cocaine intake, possibly due to greater within-subject variance in these behavioral measures. Finally, stepwise multiple regression analyses using average cocaine intake as the dependent variable found 5 predictors of cocaine intake (adjusted $r^2 = 0.715$, $p < 0.001$), including extinction latency, the change in either D_1 or D_2 receptor-mediated locomotor responses from early to late withdrawal, peak cocaine self-administration rates in dose-response testing (0.1 mg/kg/injection), and cocaine intake at the highest dose tested on the descending limb of the dose-response curve (1.0 mg/kg/injection).

To determine whether preferred levels of cocaine intake would relate to responses produced by simultaneous D_1/D_2 dopamine receptor activation, or whether differences

would generalize to similar behavior induced by non-dopaminergic pharmacological challenge, a separate group of low and high intake animals (n = 12/group) was tested with the mixed D_1/D_2 dopamine receptor agonist apomorphine and the NMDA glutamate receptor antagonist MK-801 in locomotor and reinstatement tests (Fig. 5.6). High intake animals in this experiment exhibited similar upward shifts in self-administration dose-response (main effect of group, $F_{1,22} = 24.150$, $p < 0.001$) and dose-intake (main effect of group, $F_{1,22} = 36.709$, $p < 0.001$) curves when compared to low intake animals. High intake animals also exhibited greater drug-paired lever responding in the initial extinction test session (group x test day interaction, $F_{4,88} = 5.719$, $p < 0.001$), and a longer latency to extinguish self-administration behavior ($p = 0.025$), thereby replicating earlier results. However, there were no significant group differences in the ability of the NMDA antagonist MK-801 to reinstate cocaine seeking, even at a low dose where sensitivity to the D_2 agonist quinpirole differed. In addition, MK-801-mediated locomotor responses failed to differ between low and high intake groups, despite a profound sensitization to MK-801 following chronic cocaine self-administration, similar to the degree of sensitization to D_2 agonist challenge. There also were no group differences in the locomotor response to the mixed D_1/D_2 agonist apomorphine, and cumulative dose-response curves in cocaine withdrawal were similar to pre-self-administration baselines. Brain cocaine levels were determined 10 min following an intravenous cocaine injection (2 mg/kg) in 12 rats that completed the second experiment. At this time, about 50% of cocaine is metabolized based on a previous study in rats (Piazza, et al 2000).

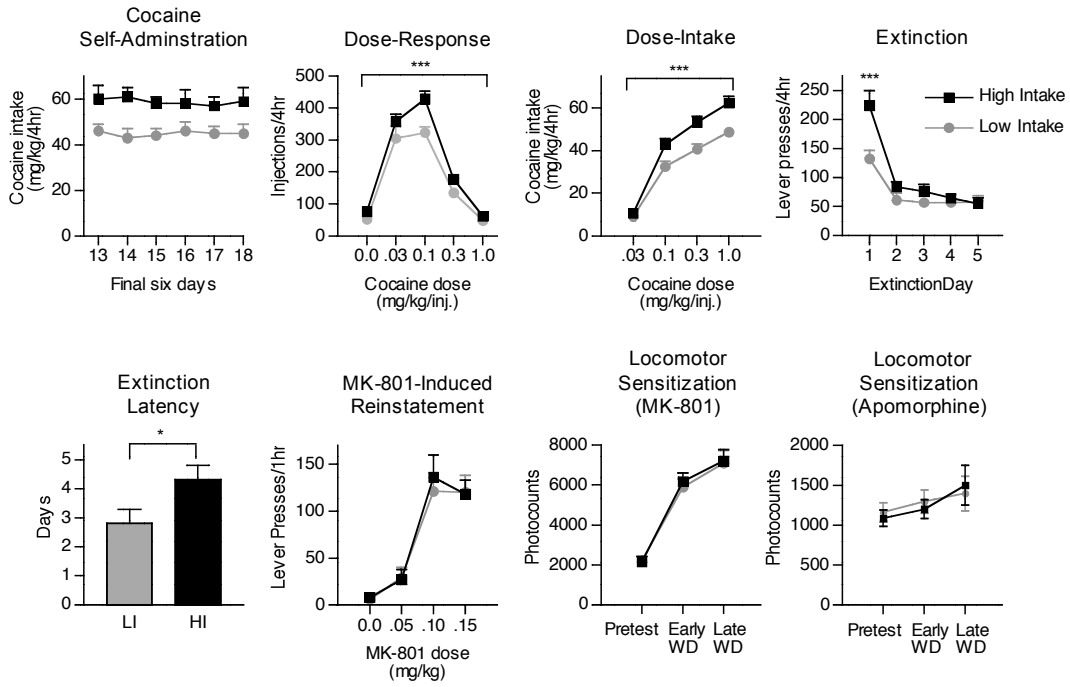

Figure 5.6
Lack of differential responsiveness in low and high intake animals following non-dopaminergic challenge with the NMDA glutamate receptor antagonist MK-801, or generalized D_1/D_2 receptor activation with apomorphine, in locomotor sensitization and reinstatement tests (n = 12 group). Low intake (LI) and high intake (HI) animals in this experiment exhibited differences in cocaine self-administration dose-response, dose-intake, and extinction responding similar to the previous experiment. Asterisks indicate that high differ from low intake animals by main group effect, tests for simple effects following significant group by dose interaction, or by t-test for extinction latencies (*$p < 0.05$, ***$p < 0.001$).

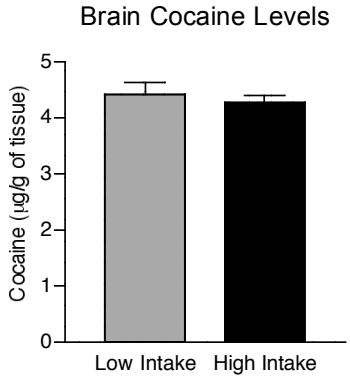

Figure 5.7 Brain cocaine levels are similar in low and high intake animals. Brain tissue was collected 10 min after a 2 mg/kg intravenous cocaine injection and cocaine levels were analyzed by gas chromatography/mass spectrophotometry methods. Low and high intake groups (n = 6/group) averaged 43.7 ± 3.3 and 59.5 ± 4.1 mg/kg/day of cocaine intake, respectively, over the final 6 days of self-administration training.

Low and high intake groups (n=6/group) for this determination averaged 43.7 ± 3.3 and 59.5 ± 4.1 mg/kg/day of cocaine intake, respectively, over the final 6 days of self-administration training. Figure 5.7 shows that brain cocaine levels were indistinguishable in low and high intake groups, averaging about 4 ug/g of brain tissue. These results indicate that preferred levels of cocaine intake do not originate from individual differences in cocaine metabolism or other factors influencing brain cocaine concentrations.

Discussion

The study shows that within an outbred rat population, animals with higher preferred (self-regulated) levels of cocaine intake become less sensitive to D_1, but more sensitive to D_2, receptor-mediated responses when compared to animals with lower preferred levels of cocaine intake, and after an extended period of withdrawal from chronic cocaine self-administration. High intake animals also exhibited an increase in peak self-administration rates, and greater cocaine intake when self-administering higher doses on the descending limb of the dose-response curve. Increases in peak self-administration rates are thought to reflect enhanced motivation for cocaine (reinforcing efficacy), a view supported by the fact that high intake animals exert greater effort to maintain self-administration when response requirements are increased (Piazza et al. 2000). In contrast, increased self-administration when cocaine is freely available reflects a reduction in inhibitory feedback regulation of drug intake, because reducing the unit dose/injection produces a similar increase in self-administration rates. Similar

phenotypic differences in the dose-response for cocaine self-administration are produced by prolonging daily access to the drug, as animals are thought to transit from non-addicted to addicted states (Ahmed and Koob 1998). These differences cannot be explained by differential metabolism or bioavailability of cocaine in the present study, since brain cocaine levels were identical 10 min after cocaine challenge. In addition, high intake animals exhibited greater cocaine-seeking behavior when reinforcement was withheld, and longer extinction latencies were predictive of higher cocaine intake by multiple regression analysis. These findings support the relationship between higher cocaine intake and a propensity for drug- and cue-induced relapse to cocaine seeking using reinstatement procedures in other studies (Sutton et al. 2000a), and suggest that animals with higher preferred levels of cocaine intake encompass changes in drug–taking and –seeking behaviors indicative of a cocaine-addicted phenotype (Self 2004). However, we found no evidence for escalation in 4 hr test sessions, consistent with other studies showing escalation with longer access, and in Wistar rather than Sprague-Dawley rats (Ahmed and Koob, 1998).

There were no differences in the locomotor response to D_1 and D_2 receptor challenge before the onset of cocaine self-administration or initially after cocaine withdrawal, indicating that differential sensitivity in these responses is not due to pre-existing individual differences, but emerges as a consequence of chronic cocaine self-administration and longer withdrawal times. Differential sensitivity to D_1 and D_2 receptor regulation of cocaine seeking paralleled changes in locomotor responsiveness after 4 weeks of withdrawal, despite the fact that D_1 and D_2 receptors mediate opposite

effects on cocaine-seeking behavior and directionally similar effects on locomotor behavior. Thus, high intake animals were less sensitive to the ability of D_1 receptor agonist SKF 81297 to inhibit cocaine seeking induced by a cocaine priming injection, and sensitivity to this response also was negatively correlated with cocaine intake across all animals. This difference probably reflects the development of sensitization to D_1 responses in low intake animals, rather than tolerance in high intake animals, since low intake animals developed sensitized locomotor responses relative to pre-self-administration baselines. Indeed, the emergence of D_1 receptor-mediated locomotor sensitization in withdrawal was negatively correlated with, and predictive of, prior cocaine intake during self-administration. Therefore, D_1 receptor sensitization could reflect a neuroadaptation to cocaine that protects against a propensity for relapse in withdrawal.

Changes in D_1 receptor sensitivity are probably not caused by D_1 receptor up-regulation, since other studies have found a down-regulation of striatal D_1 receptor binding and D_1-stimulated adenylate cyclase activity in the nucleus accumbens of rats and non-human primates following chronic cocaine self-administration (Graziella De Montis et al. 1998a; Moore et al. 1998a). In addition, escalating cocaine intake has been associated with a decreased capacity for rate-increasing effects of a mixed D_1/D_2 dopamine receptor antagonist, and increased sensitivity to the rate suppressing effects of higher doses, suggesting a decrease in the amount of dopamine receptors available for blockade (Ahmed and Koob 2004). Thus, in both individual difference and extended access models of cocaine-addicted rats, animals with higher preferred levels of cocaine

intake exhibit evidence for reduced dopamine receptor function when compared to low intake animals. Our results suggest this effect may be attributable entirely to lower D_1 receptor function, since both low and high intake animals developed a profound sensitization to D_2 receptor-mediated responses after chronic cocaine self-administration. Moreover, lower D_1 receptor responsiveness could underlie higher cocaine intake in animals with prolonged daily access, since blockade of D_1 receptors produces similar increases in cocaine self-administration (Koob et al. 1987; Maldonado et al. 1993).

However, other studies have reported increases in D_1 receptor binding in several striatal regions 1 day following a shorter period of cocaine self-administration in monkeys (Nader et al. 2002), and functional increases in D_1 receptor-mediated neurophysiological responses that develop several days after withdrawal from passive cocaine administration in rats (Henry and White 1991). This latter finding could relate to sensitization of D_1–mediated behavioral responses in low intake animals, since this effect also required a longer withdrawal period to develop. In addition, levels of cocaine exposure that produce supersensitive D_1-mediated neurophysiological responses are more similar to levels in low than high intake animals in our study.

In contrast to D_1 receptors, stimulation of D_2 receptors induces rather than inhibits relapse to cocaine-seeking behavior (Self et al. 1996; De Vries et al. 1999). We found that high intake animals were more sensitive to the ability of D_2 receptor stimulation with quinpirole to trigger cocaine-seeking responses at the drug-paired lever. This effect was paralleled by enhanced locomotor responses to D_2 receptor stimulation in

late withdrawal, despite the fact that low intake animals also developed substantial locomotor sensitization to D_2 receptor challenge. The development of further sensitization to D_2 receptor responses from early to late withdrawal was positively correlated with, and predictive of, higher preferred levels of cocaine intake across all animals, and negatively correlated with the capacity to develop sensitization to D_1 receptor challenge. Thus, the capacity to develop D_1 receptor sensitization within individual rats could occlude the capacity to develop further D_2 sensitization. It is important to note that locomotor testing was conducted in an environment distinct from the cocaine-self-administration test chambers, and so changes in D_1 and D_2 receptor responsiveness occurred in the absence of cocaine-conditioned locomotor effects.

Although cocaine self-administration produced functional increases in D_2 receptor activity, several studies have found reduced D_2 receptor binding in striatal and other brain regions following chronic psychostimulant use in monkeys and humans (Volkow et al. 1990; Volkow et al. 1993; Moore et al. 1998b; Volkow et al. 2001; Nader et al. 2002; Martinez et al. 2004), and binge cocaine administration in rats (Maggos et al. 1998). Given that prominent D_2 receptor sensitization develops in both low and high intake groups, and this effect is substantiated by a previous study comparing cocaine to saline self-administration (De Vries et al. 2002), it is possible that D_2 receptor sensitization reflects intracellular alterations in D_2 receptor coupling, or an indirect enhancement via perturbations in neural input to D_2-containing neurons, rather than an increase in D_2 receptor surface expression. Further investigation is needed to determine

whether these changes are reflected in differential sensitivity to dopamine receptor-regulated biochemical or electrophysiological responses.

In a separate experiment, low and high intake animals were challenged with the mixed D_1/D_2 receptor agonist apomorphine and the non-competitive NMDA glutamate receptor antagonist MK-801 in identical locomotor and reinstatement (MK-801 only) tests. The psychomotor and reinstating effects of MK-801 are thought to occur independent of dopamine release and dopamine receptor activation (De Vries et al. 1998). Similar to D_2 receptor challenge, animals in both low and high intake groups displayed robust locomotor sensitization to MK-801 challenge following chronic cocaine self-administration. However, neither group differed in this response over the course of testing, nor were there any differences in the ability of the NMDA antagonist to reinstate cocaine seeking behavior, despite similar phenotypic differences in cocaine self-administration and extinction responding in low and high intake animals. These findings indicate that differential sensitivity to D_2 receptor challenge in low and high intake groups is not due to generalized changes in mechanisms regulating sensitization and reinstatement, but instead reflect a specific enhancement of D_2 receptor responsiveness in high intake animals. Furthermore, there was virtually no sensitization to generalized dopamine receptor activation with the mixed D_1/D_2 receptor agonist apomorphine following chronic cocaine self-administration, similar to D_1 receptor challenge, but also no emergent differences in sensitivity between low and high intake groups in withdrawal. Thus, differential alterations in post-synaptic D_1 and D_2 receptor responsiveness are revealed only when each receptor is stimulated independently, possibly due to competing

D_1 and D_2 receptor effects in low and high intake animals. Given that high intake animals tended to show greater cocaine-induced reinstatement of cocaine seeking, the lack of differences with apomorphine challenge may be related to direct rather than indirect mixed dopamine receptor stimulation. In addition, these data agree with previous findings that the ability of drugs to reinstate cocaine-seeking behavior is related to their ability cross-sensitize with cocaine (De Vries et al. 1999).

Addiction-related changes in cocaine self-administration dose-response curves may reflect increased motivation for drugs (incentive sensitization), but reduced pharmacological impact of drugs on reward processes (tolerance) leading to compensatory increases in drug intake (Emmett-Oglesby et al. 1993; Piazza et al. 2000). Our results suggest that such changes could result in enhancing D_2 and reducing D_1 receptor responsiveness, respectively. Furthermore, the emergence of these features from early to late withdrawal parallels time-dependent increases in cocaine-seeking behaviors that have been shown to persist from weeks to months following chronic cocaine self-administration (Tran-Nguyen et al. 1998; Grimm et al. 2001). Emergence of increased D_2 receptor function is particularly troublesome, since it could exacerbate the ability of conditioned stimuli (cues) and stressful situations, both of which increase mesolimbic dopamine release in rats and humans (Rouge-Pont et al. 1998; Phillips et al. 2003; Pruessner et al. 2004), to facilitate relapse to cocaine use despite efforts to abstain. While our results suggest that reduced D_1 receptor function could exacerbate this situation, they also suggest that restoring the balance between functional D_1 and D_2 receptor responses,

whether through increasing D_1, decreasing D_2, or both should be considered as a potential therapeutic approach for reversing alterations associated with cocaine addiction.

CHAPTER 6

Conclusion

Our hypothesis suggested that drug-addicted animals are less sensitive to D_1 receptor-mediated reward, and escalate their drug intake to compensate for the reduced D_1 tone. In contrast, they may be more sensitive to D_2 receptor-induced drug seeking, reflecting an enhanced propensity for craving and relapse. Furthermore, our hypothesis predicted that hypothetical alterations in D_1 and D_2 receptor responses might come as a consequence of drug-induced neuroadaptations in D_1 and D_2 signaling pathways. Specifically, up-regulation of the cAMP/PKA pathway in addiction may preclude D_1 responses and sensitize D_2 responses through reciprocal negative and positive feedback pathways, respectively.

In accordance with this hypothesis, chronic cocaine self-administration induces tolerance to the ability of cocaine to increase PKA-mediated phosphorylation in striatal, amygdala, and hippocampal regions (Chapter 2), which could reflect either enhanced D_2 dopamine receptor signaling (greater inhibition of PKA) or reduced D_1 signaling (less activation of PKA) caused by excessive drug intake. In comparison, recent studies in our lab have found that experimental up-regulation of NAC cAMP/PKA signaling (via intra-NAC cholera toxin infusions) enhances D2, and attenuates D1, behavioral responses (Whisler et al. 2003) along with increasing PKA-dependent protein phosphorylation. Thus, up-regulation of PKA signaling after chronic cocaine provides a sufficient intracellular mechanism to explain altered dopamine receptor responsiveness in cocaine

addiction. It would be of great interest to extend these cholera toxin studies to amygdala and hippocampal regions to investigate the potential role of dopamine signaling in these areas in facilitating drug addiction (particularly in terms of drug-paired contextual effects on mediating relapse to drug-seeking).

Commensurate with the observed changes in protein phosphorylation status (Chapter 2), we found a corresponding altered responsiveness to dopamine receptor regulation of addictive behavior in outbred animals (Chapter 5), whereby the addicted phenotype was indeed characterized by decreased D_1, and increased D_2, dopamine receptor responsiveness during cocaine withdrawal. Tolerance to D_1 receptor-mediated inhibition of relapse could contribute to escalating drug intake, while increases in post-synaptic D_2 receptor-mediated behaviors in the addicted phenotype could facilitate incentive sensitization as withdrawal time progresses. Although not measured here, it should also be noted that increased D_2 receptor function could also engender heightened sensitivity to conditioned stimuli and stressful environments that are known to promote dopamine release and trigger relapse. In comparison, increases in PKA phosphorylation that persist into heroin and cocaine withdrawal (Chapter 3 & 4), particularly in the nucleus accumbens shell, also suggest that the potential for altered dopamine receptor signaling is enduring. Moreover, increases in PKA phosphorylation induced by exposure to the cocaine-paired environmental context are found after late, but not early, WD in the nucleus accumbens core, suggesting a time-dependent increase in the sensitivity of NAC neurons to activity-dependent protein phosphorylation. Alternatively, there may be greater afferent input to these neurons during context exposure at later WD times. Thus,

addiction-related alterations in D_1 and D_2 dopamine receptor responses may ultimately involve complex reciprocal interactions between adaptations in PKA signaling pathways that subsequently influence D_1 and D_2 receptor signaling during withdrawal and upon exposure to drug-paired environments. An attractive extension of these data would be to directly measure alterations in sensitivity to D_1 and D_2 dopamine receptor-regulation of protein phosphorylation after chronic drug self-administration. One caveat of this approach is that D_1 and D_2 dopamine receptor responsiveness may itself be context-dependent (or confounded by pre-existing and/or context-dependent changes in protein phosphorylation status). Further elucidation of chronic cocaine's effects on the differential physiology of striatal D_1 and D_2 dopamine receptor-containing neurons is also needed, and may be heavily dependent on specific changes in protein phosphorylation status in selective cell types. With the striking bi-directional regulation of cocaine seeking by D_1 and D_2 dopamine receptors, it will also be important to find additional signaling mechanisms (beyond PKA) that are oppositely regulated by these receptors. Finally, given the ability of D_1 receptor agonism to curb drug-seeking behavior across several species, it would seem of vital importance to develop and test D_1 agonist compounds in clinical trials involving abstinent addicts. Most important in this regard is the ability to curb excessive craving for elicit drug use in dependent individuals, without affecting the desire for natural (food, sex) rewards. Ultimately, elucidation of how biological reward systems are differentially regulated in states of controlled vs. excessive drug use will lead to a better understanding of the transition to drug addiction and more effective therapies for this devastating disease.

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VITAE

Lamar Scott Edwards was born December 28, 1976 in Abilene, Texas, son of Ross Sterling Edwards and Barbara Sue Edwards. After graduating from Wall High School in Wall, TX in 1995, Scott attended The University of Texas at Austin. Here, Scott completed the degree of Bachelor of Science in Biochemistry in 2000. The same year Scott entered the Neuroscience Graduate Program at Yale University, and subsequently transferred with David Self's lab to The University of Texas Southwestern Medical Center. Scott married Kimberly Nina Whisler on March 3, 2007, and the couple will be moving to San Diego, CA in the summer of 2007.