Role of DARPP-32 pathway in cocaine-induced behavioral sex differences

By

Luyi Zhou

A dissertation submitted to the Graduate Faculty in Biology

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy, The City University of New York

2009

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

_____ Professor Laurel Eckhardt ___

_____ _____

Date Executive Officer

_____ Professor Vanya Quinones-Jenab __

_____ _____

Date Chair of Examining Committee

Vanya Quinones-Jenab, Ph.D.

Maria Figueiredo-Pereira, Ph.D.

Shirzad Jenab, Ph.D.

Ann Ho, Ph.D.

Yan Zhou, Ph.D.

Scott Russo, Ph.D.

Supervisory Committee

The City University of New York

Abstract

Role of DARPP-32 pathway in cocaine-induced behavioral sex differences

by

Luyi Zhou

Advisor: Professor Vanya Quinones-Jenab

Accumulating evidence suggests that behavioral responses to cocaine are sexually dimorphic: female rats are more sensitive to cocaine. Female rats exhibit exaggerated and more robust locomotor responses to cocaine than do males. Females also more quickly develop cocaine-induced conditioned place preference (CPP) and behavior sensitization with lower doses, and more readily acquire cocaine self-administration. However, the underling mechanisms for this behavioral sex differences remain unknown. The dopamine- and cAMP-regulated phosphoprotein (DARPP-32) signaling pathway has been shown to mediate intracellular responses to acute and chronic cocaine in male rodents. The purpose of this proposal is to assess the role of DARPP-32 pathways in cocaine-induced behavioral sex differences in male and female rats. We hypothesize that acute and chronic cocaine activates DARPP-32 signaling in a sexual dimorphism way. To test this hypothesis, in the acute cocaine experiment, rats received saline or cocaine (30mg/kg), while in the chronic cocaine experiment, rats received saline, acute cocaine or chronic cocaine (15mg/kg), Protein levels of the DARPP-32 signaling proteins in the nucleus accumbens (NAc) and caudate putamen (CPu) were measured via western blot. Locomotor and stereotyped activities were also measured in the chronic cocaine experiment. In the acute cocaine experiment, female rats had heightened basal levels of DARPP-32 cascade; while male rats exhibited higher induction of the cascade after acute

cocaine administration. However, these sex differences were mainly observed in the NAc, not CPu. In the chronic cocaine experiment, female rats developed behavior sensitization and tolerance to cocaine earlier than males. In male rats, the heightened DARPP-32 signaling were mostly found in the NAc when behavior sensitization was observed; while in female rats, increased signaling were mostly found in the CPu when behavior tolerance was observed. In addition, the protein levels of several DARPP-32 signaling proteins, including ΔFosB, FosB, Cdk5 and p35, correlated with the behavior activities. Taken together, these results suggest that DARPP-32 signaling pathway is altered in a sexual dimorphic way after acute and chronic cocaine treatment, and it may play a critical role for the sex differences at the behavior level.

Acknowledgements

I would especially like to thank my advisor, Professor Vanya Quinones-Jenab, and my co-advisor, Dr. Shirzad Jenab, for their intellectual guidance, and support. I thank you for your patience and cooperation on the difficult days of my journey to a Ph.D. In addition, you always gave me lots of freedom allowing me to explore new ideas. I also thank my supervisory committee, Dr. Maria Figueiredo-Pereira, Dr. Ann Ho, Dr. Yan Zhou and Dr. Scott Russo for their guidance and input in this project.

I would also like to thank all of my colleagues of our laboratory. Without their contributions and help I would not have been able to complete my dissertation work.

Finally, I would like to thank my parents, my mother Minfang Chen and my father Dahua Zhou, for everything they have done for me. Especially my mother, her encouragement and unconditional support allowed me to overcome the adversity and obstacles of my life.

I dedicate this dissertation to Dr. Wei-Lun Sun. As my husband and colleague, your support, love and the existence of my life made it possible for me to complete personal and professional goals. Thank you with all my heart.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

I. Background

A. Sex difference in cocaine abuse

Cocaine is an active alkaloid extracted from the leaf of the Erythroxylon coca, which grows primarily in Bolivia, Peru, Ecuador, and Columbia (Madras et al., 2005). There are basically two chemical forms of cocaine: the hydrochloride salt, once dissolved, can be taken intravenously or intranasally; and the smokable "freebase" (Madras et al., 2005). The use of cocaine as fatigue resist is a long-term tradition for the people of Columbia, and the history of medical use of cocaine according to its anesthetic and psychoactive effects started around middle 1800s (Platt, 1997). It wasn't long before the addictive properties of cocaine became apparent by the late 1890s, and the use of cocaine was finally banned by the Harrison Narcotic Act in 1914 (Platt, 1997). According to the U.S. Substance Abuse and Mental Health Services Administration study (SAMHSA, 2004), 14.2% of the population aged 12 and older in the United States report lifetime use of cocaine, and 33% of the 2 million Americans who currently used cocaine in the past month are women. There seems to be no sex differences in the progression to intense drug use following the initial use, so the uneven distribution of cocaine use between the sexes may be due to the fact that males are more likely to have an initial opportunity to use drugs (Van Etten et al., 1999).

Recent clinical and preclinical studies indicate sex differences in response to cocaine administration in humans. For example, cocaine exposure results in men achieving a faster "high" and higher peak of plasma cocaine levels, and report more episodes of euphoria than women (Lukas et al., 1996). However, cocaine causes a greater

level of “nervousness” in women than in men (Kosten et al., 1996) and women report a greater level of drug craving upon introduction of cocaine cues than men (Robbins et al., 1999). This may suggest that women are more sensitive to cocaine than men. In fact, women begin using cocaine and enter treatment at earlier ages than men (Griffin et al., 1989), and have a more severe cocaine use at intake than men (Kosten et al., 1993).

Sex differences in response to acute and chronic cocaine administration are studied in rodents. After acute cocaine treatment, female rats show higher locomotor and stereotyped activities than males (Haaren and Meyer, 1991; Sell et al., 2000; Walker et al., 2001; Chin et al., 2002; Festa et al., 2004). Females also show a prolonged time of response after a single cocaine injection (Festa et al., 2003). Male rats require higher doses of cocaine to exhibit similar locomotor response than those in female rats (Festa et al., 2004). Moreover, female rats learn the rewarding effect of cocaine through conditioned place preference (CPP) with lower doses treatment and shorter time when compared to male rats (Russo et al., 2003). Similarly, female rats show cocaine self-administration faster and at a lower dose (Lynch et al., 1999). Unlike acute cocaine administration, sex differences in the development and expression of behavioral sensitization or tolerance (definitions defined in section C) have not been well-studied. Regardless of the administration paradigm, chronic cocaine administration consistently produces a greater sensitization of the locomotor response in females, and sensitization is achieved with a lower dose of cocaine (Van Haaren and Meyer, 1991; Chin et al., 2002). However, no sex differences in stereotyped behavior are observed after chronic cocaine treatment (Chin et al., 2002). Taken together, it is suggested that female rats have augmented response to cocaine regardless of the paradigm, and they are more sensitive to

the behavioral effects of cocaine than male rats. It is suggested that female rats are more sensitive to the behavioral effects of cocaine than male rats. However, what are the mechanism regulating this sexual dimorphic pattern is not well understood.

B. Cocaine-induced behavioral responses

After acute cocaine administration, enhanced locomotor behaviors, such as ambulatory (horizontal) and rearing (vertical) activities are observed in a dose dependent manner (Zubrycki et al., 1990). With increasing dose of cocaine, stereotyped behavior (including sniffing, head waves, forepaw treading and more sluggish behavior) are also dose-dependently induced (Blanchard et al., 1998). However, different changes in behavior are observed after chronic cocaine administration. Either tolerance or sensitization is developed with repeated administration of cocaine. Behavioral sensitization refers to the enhancement of a behavioral response after repeated exposure to psychostimulants that is greater than a previous acute exposure (Kalivas and Weber, 1988). Behavior sensitization to cocaine is intensely studies in male rats, as it may serve as a good model for neuroadaptations involved in cocaine addiction and craving (Robinson and Berridge, 1993; Wise and Bozarth, 1981). Behavior tolerance refers to the decrease in the cocaine-induced locomotion after repeated exposure to psychostimulants than a previous acute exposure (King et al., 1999). However, the specific changes found after chronic treatment with cocaine are very much dependent on the route and schedule of drug administration, as well as choose of control. Repeated intermittent administration (for example, i.p. injection) of cocaine develops sensitized locomotion, while continuous administration (for example, osmotic pump) develops tolerance in locomotor responses

(Reith et al., 1987; Hope et al., 2005). Compare to the sensitization paradigm, higher-dose and/or longer-duration of cocaine administration is required to induce behavior tolerance in locomotor activities (King et al., 2004). In rodents, sensitized locomotion can last for up to 4 month (Henry et al., 1995; Shuster et al., 1977), while tolerance lasts much shorter, up to 7 days (King et al., 1992).

C. Dopamine synthesis and reward circuit in the brain

The neurotransmitter dopamine plays a central role in drug reward and abuse study. As shown in Fig. 1, dopamine synthesis originates from tyrosine (Nagatsu et al., 1964). There are two steps involved. In the first rate-limiting step, L-tyrosine is converted into L-DOPA by the enzyme tyrosine hydroxylase (TH) (Moore and Dominic, 1971). L-DOPA is subsequently converted to dopamine by L-amino acid decarboxylase. Upon synthesis, dopamine is packaged into vesicles and transported to the terminals for storage (Winkler et al., 1987). The release of dopamine is trigged by the influx of Ca2+ due to membrane depolarization. Once released into the synaptic cleft, dopamine is immediately uptaken into the terminals via the dopamine transporters (DAT), and converted to dihydoxyphenylacetic acid (DOPAC) by intraneuronal monoamine oxidase (MAO) (Horn, 1990; Krueger, 1990).

The mesocorticalimbic dopamine pathway is the major circuit mediating drug reward (Fig. 2). It originates from the ventral tegmental area (VTA), and projects to several areas in the forebrain including the frontal cortex, nucleus accumbens (NAc), dorsal striatum/caudate putamen (CPu), olfactory tubercle, hippocampus and amygdale (Koob, 1992; Nestler and Aghajanian, 1997; Hummel and Unterwald, 2002). Lesions of

the pathway disrupt the behavior response to psychostimulant drugs. For example, 6-OHDA lesions of CPu and NAc abolishes the psychostimulant-induced stereotyped and locomotor behavior respectively (Kelly et al., 1975; Kelly and Iversen, 1975). Ibotenic acid lesion of PFc disrupts the development and expression of behavior sensitization in response to chronic psychostimulants exposures (Li and Wolf, 1997; Pierce et al., 1998; Li et al., 1999). The striatum (NAc and CPu) is the major neuroanatomical target for midbrain dopaminergic neurons. More than 90% of the neurons there are the medium spiny GABAergic neurons, which project to various areas responsible for cocaine-induced behavior (Freund et al., 1984). The direct pathway projects to the internal segment of the globus pallidus and substatia nigra pars reticulata via, and the indirect pathway relays through the external segment of the globus pallidus and the subthalmic nucleus (Albin et al., 1989).

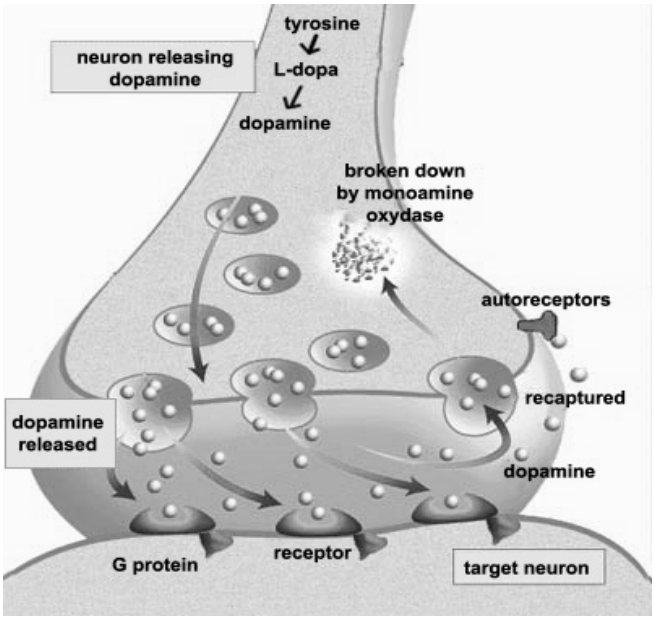

Figure 1: Dopamine synthesis and release. Adapted from The brain from top to bottom. http://www.thebrain.mcgill.ca

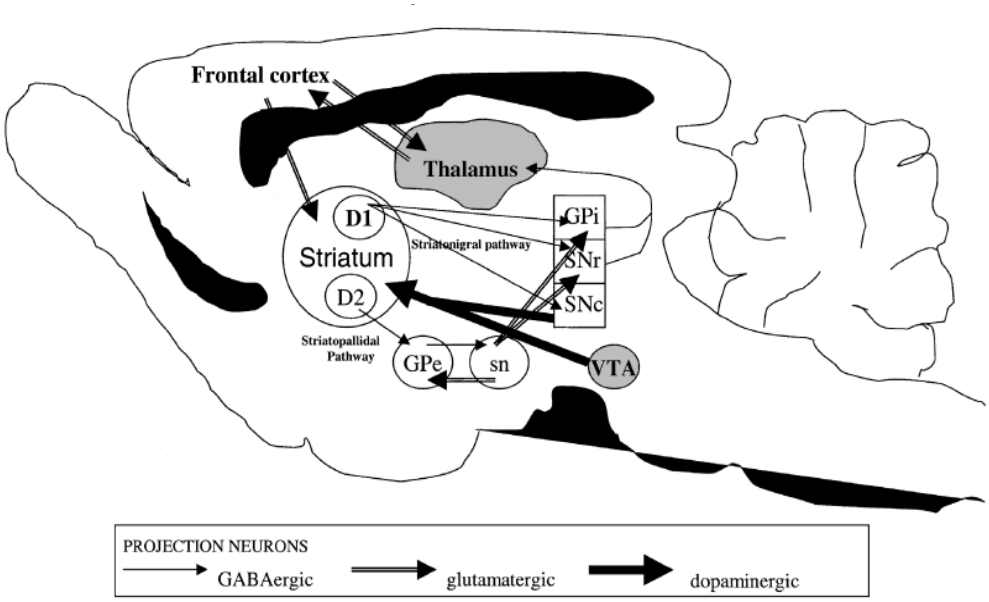

Figure 2: The reward circuit. Dopamine projections form the ventral tegmental area (VTA) to the frontal cortex, nucleus accumbens (NAc), dorsal striatum/caudate putamen (CPu) are of primary interest in studies of drug abuse. Adapted from Hummel and Unterwald, 2002.

D. Cocaine's effects on dopamine transmission

Almost all addictive drugs increase the extracellular dopamine by one way or another and prolong the activation of postsynaptic neuron (Koob, 1992). Cocaine directly binds to DAT with high affinity and prevents the reuptake of dopamine (Heikkila et al., 1975), so the dopamine concentration in the synapse and the dopamine transmission are elevated (Fig. 3). In vivo studies show that there is an enhanced dopamine release from accumbal and striatal tissue slices in rats sensitized to psychostimulants, such as cocaine (Castaneda et al., 1988; Kolta et al., 1985; Robinson and Becker, 1982; Yamada et al., 1988). In vitro microdialysis in the striatum, NAc and medial prefrontal cortex reveals an immediate increase in the extracellular dopamine levels within the mesolimbic system after acute cocaine administration (Carboni et al., 1989; Church et al., 1987; Maisonneuve and Kreek, 1994; Maisonneuve et al., 1995). Chronic cocaine exposure

potentiates mesolimbic dopamine concentration by attenuating the GABA-mediated inhibition of VTA neurons, which in turn increase dopamine release (Bonci and Williams, 1996). The enhancement of dopamine transmission in the mesolimbic system appears to mediate the locomotor sensitization and rewarding of cocaine.

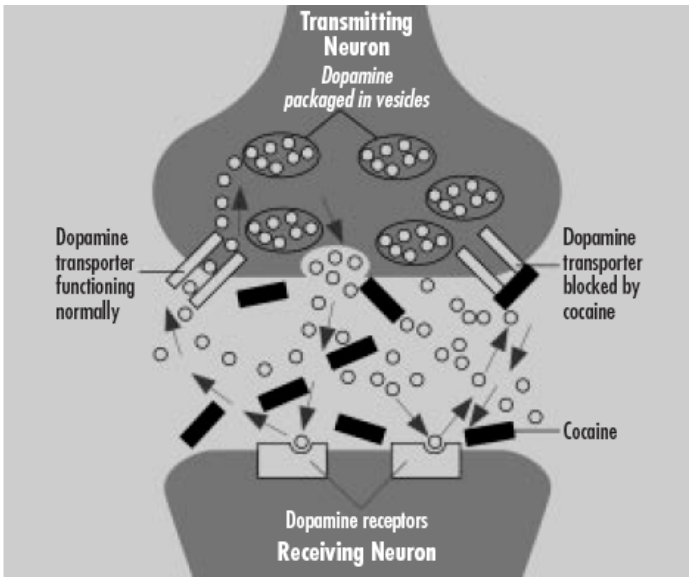

Figure 3: Cocaine blocks the reuptake of dopamine by binding to the dopamine transporter (DAT), and increases the synaptic dopamine level. Adapted from NIDA Research Report - Cocaine Abuse and Addiction (web report, 2004). http://www.drugabuse.gov.

E. Role of dopamine receptors in cocaine-induced behavioral response

There are two classes of dopamine receptors: D1 (D1 and D5 receptors) and D2 (D2, D3 and D4 receptors) (Sibley and Monsma, 1992). All are seven transmembrane G protein-coupled receptors (Missale et al., 1998). D1 dopamine receptors are predominantly distributed in the direct pathway, while D2 receptors mostly in the indirect pathway, but they are not exclusive (Gerfen et al., 1990). There are also D2 autoreceptors on dopamine nerve terminals themselves (Surmeier et al., 1996).

Many of the receptor-mediated effects can be clearly dissociated while others can not, which suggests a modulation or interactive role for these two receptors in cocaine-potentiated dopamine neurotransmission. D1 and D2 dopamine receptors play different roles in the behavioral activity induced by acute and chronic cocaine administration. Rats pretreated with D1 receptor antagonist (SCH 23390) or D2 receptor antagonist (raclopride) show an attenuated ambulatory, rearing and stereotyped behavior response to acute-cocaine administration (Cabib et al., 1991; Ushijima et al., 1995). Pretreatment with D1 receptor agonist (SK&F38393) increases rearing response to acute-cocaine treatment, but has slightly effect on stereotyped behavior (Ushijima et al., 1995). Pretreatment with D2 receptor agonist (quinpirole and bromocriptine) inhibits cocaine-stimulated ambulatory and rearing response, but induces typical stereotypy such as sniffing, licking, and gnawing (Ushijima et al., 1995). Conversely, D1 and D2 antagonists differentially affect the chronic cocaine administration, which produces behavior sensitization. Dopamine D1 receptor antagonist (SCH 23390) blocks the expression of chronic cocaine-induced behavior sensitization, while D2 antagonist (eticlopride) has no effect (Ushijima et al., 1995; McCreary and Marsden, 1993). These findings suggest the different role of the two receptors: D1 receptor may be more important in cocaine-induced locomotor behavior, while D2 receptor may be more important in cocaine-induced stereotyped behavior.

II. Intracellular mechanisms

A. DARPP-32 signaling transduction after cocaine administration

As shown in Fig. 4, upon the exposure of acute cocaine, D1 dopamine receptors are activated by the concentrated synaptic dopamine, which in turn causes the coupling of stimulatory G protein (Gs or Golf) to the receptors (Sibley and Monsma, 1992). The coupling activates the adenylyl cyclase, which converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Increase in the production of cAMP enhances the activity of protein kinase A (PKA), which phosphorylates various proteins (Edelman et al., 1987; Mello et al., 1989). One of the aim proteins is DARPP-32 (dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein, 32 kDa), which was initially found as a main target for dopamine-activated adenylyl cyclase in striatum (Walaas et al., 1983). DARPP-32 is phosphorylated at the threonine 34 site (P-Thr34-DARPP-32) by PKA, and it becomes an inhibitor of protein phosphatase-1 (PP-1), which is a major serinine/threonine protein phosphatase (Hemmings et al., 1984; Nishi et al., 1997; Nishi et al., 2000; Svenningsson et al., 2004; Nairn and Shenolikar, 1992). P-Thr34-DARPP-32, by inhibiting PP-1, increases the phosphorylation of various neurotransmitter receptors, voltage-gated ion channels, and transcription factors, such as CREB (cAMP-responsive element binding protein) (Blank et al., 1997; Snyder et al., 1998; Yan et al., 1999; Strack et al., 1997; Bito et al., 1996). On the contrary, P-Thr34-DARPP-32 is dephosphorylated by PP-2B, a Ca2+/calmodulin-dependent protein phosphatase (Goto et al., 1986; King et al., 1984). PP-2B activity is increased by Ca2+ influx when glutamate binds to NMDA or AMPA receptors (Halpain et al., 1990). PKA and PP-2B together balance the phosphorylation of DARPP-32, and finally regulate and fine-tune the phosphorylation state of PP1 target proteins.

Unlike D1 receptor, activation of D2 class receptors inhibits adenylyl cyclase activity by coupling to Gi proteins (Sibley and Monsma, 1992), in turn, inhibits the formation of cAMP, and as a result, P-Thr34-DARPP-32 is dephosphorylated into DARPP-32 (Nishi et al., 1997; Lindskog et al., 1999).

Chronic cocaine exposure increases the PKA dependent expression of the Fos family transcription factor, ΔFosB (Hope et al., 1994; Kelz and Nestler, 2000). As transcriptional regulated target genes of ΔFosB, Cdk5 (Cyclin-dependent kinase 5) and p35 expression are both elevated (Bibb et al., 2001; Ishizuka et al., 1995). Cdk5 is a neuronal serine/threonine protein kinase, which is regulated through the association with its activators, such as p35 (Tsai et al., 1994; Zheng et al., 1998). Cdk5 phosphorylates DARPP-32 at Thr-75 site (P-Thr75-DARPP-32), which diminishes the phosphorylation at Thr-34 site. Moreover, the phosphorylation of Thr-75 by Cdk5 transforms DARPP-32 form a PP-1 inhibitor into a strong inhibitor of PKA (Bibb et al., 1999). By inhibiting PKA, Cdk5 finally attenuates the D1/PKA/P-Thr34-DARPP-32/PP-1 signaling, and dampens locomotor behavior to chronic cocaine and develops cocaine addiction. Like the dephosphorylation at the Thr34 site, the P-Thr75-DARPP-32 is also dephosphorylated by PP2A (protein phosphotase-2A), whose activation is PKA-dependent (Nishi et al., 2000). Interestingly, an acute dose of cocaine raises the P-Thr34-DARPP-32 levels by PKA, and at the same time reduces the phosphorylation at the Thr75 site via the PP-2A pathway (Nishi et al., 2000).

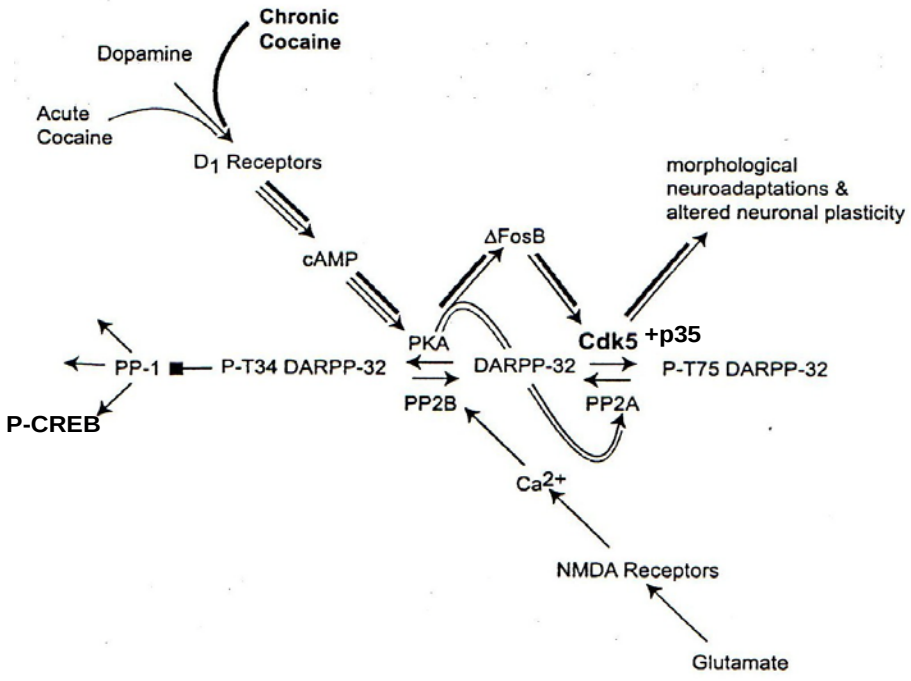

Figure 4: DARPP-32 signaling transduction pathway upon cocaine exposure. Thin line depicts the signaling machinery associated with acute cocaine exposure. Heavy line details cellular pathways targeted by chronic cocaine exposure. Adapted from Benavides and Bibb, 2004.

B. Role of DARPP-32 pathway in cocaine addiction

The highest levels of DARPP-32 are found in medium spiny neurons in CPu, NAc, olfactory tubercle, bed nucleus of stria terminalis, and parts of amygdaloid complex (Ouimet et al., 1984), which have a high correlation with the drug reward circuit. This suggests that DARPP-32 may play an important role in drug addiction.

Studies on the DARPP-32 pathway activation upon cocaine administration into wild-type animals are limited. Acute cocaine injection elevates the P-Thr34-DARPP-32 and suppressed P-Thr75-DARPP-32 protein levels in striatum and mPFc at 30 min after cocaine treatment (Nishi et al., 2000; Rauggi et al., 2005; Takahashi et al., 2005; Synder et al., 2000). However, cocaine administration does not change the expression of

DARPP-32 at 30 min time point (Nishi et al., 2000). Acute cocaine administration increases the expression of c-fos in NAc and CPu (Takahashi et al., 2005; Torres and Rivier, 1994). The increased phosphorylation of CREB on the Ser133 site in striatum indicates the inhibition of PP-1 activity (Takahashi et al., 2005). No changes in Cdk5 and p35 protein levels somehow prove that their activation is not induced by acute but chronic cocaine administration (Takahashi et al., 2005). After chronic cocaine administration, the protein level of P-Thr75-DARPP-32 increases, accompanied by a decrease in the P-Thr34-DARPP-32 in both NAc and CPu (Bibb et al., 2001; Scheggi et al., 2004). Different results have been found in the protein level of DARPP-32, either increase or no change is observed (Bibb et al., 2001; Hu et al., 2005). Increases in DARPP-32 protein levels may result from the increases in the P-Thr34-DARPP-32 protein levels in NAc (Hu et al., 2005). Repeated cocaine administration leads to increasing or decreasing activity of PKA, which depends on whether intense or moderate doses are used (Terwilliger et al., 1991; Crawford et al., 2004). The expression of ΔFosB is also elevated in striatum after chronic cocaine administration. Increases in p35 and Cdk5 protein levels are also observed (Bibb et al., 2001; Scheggi et al., 2004; Fienberg et al., 2006; Hiroi et al., 1999; Liu et al., 2003). Chronic-cocaine administration does not change the PP1 protein levels, but attenuates PP-2B protein levels in NAc. This is likely related to a significant decrease in Ca2+ influx (Hu et al., 2005). These changes in the DARPP-32 pathway is accompanied by the enhanced locomotor activity after acute cocaine treatment or behavioral sensitization after chronic cocaine administration, which indicates that DARPP-32 pathway is in rolled in the short- and long-term behavioral actions of cocaine.

DARPP-32 KO mice show attenuated locomotor response after single cocaine administration (Fienberg et al., 2006) and cocaine-induced conditioned place preference (CPP) (Zachariou et al., 2006). However these mice exhibit heightened behavioral sensitization in response to repeated cocaine injections due to blunted ΔFosB in CPu induced by cocaine as compared to wild-type mice (Fienberg et al., 2006; Hiroi et al., 1999). Mice with point mutation in phosphorylation sites of DARPP-32 also show specific behavioral alternations after cocaine administration. Thr34-Ala-DARPP-32 mice show attenuated cocaine-induced CPP, lower acute cocaine-induced locomotor activity and higher behavioral sensitization as well as required prolonged time to acquired cocaine self-administration compared to wild-type mice (Zachariou et al., 2006; Zhang et al., 2006; Valjent et al., 2005). The lower behavioral activity of Thr34-Ala-DARPP-32 mice in response to cocaine is related to attenuated extracellular dopamine levels in CPu and immediate early genes in NAc induced by either acute or chronic cocaine administration in relative to wild-type mice (Zachariou et al., 2006; Zhang et al., 2006). In contrast, Thr75-Ala-DARPP-32 mice show normal cocaine-induced CPP, cocaine self-administration and acute cocaine-induced hyperlocomotor but exhibit higher behavioral sensitization in response to repeated cocaine administration (Zachariou et al., 2006; Zhang et al., 2006). Taken together, these studies suggest that the phosphorylation at Thr34 and Thr75 site of DARPP-32 is critical for short-term and long-term cocaine-induced behavioral alternations, respectively.

Studies of the role of DARPP-32 pathway in sex differences are quite limited. So far, only two recent studies found sexual dimorphic responses to cocaine in this signaling pathway. Nazarian et al. (2008) demonstrated that PKA protein levels in the NAc were

overall higher in females than males, and Lynch et al. (2007) showed that protein levels of DARPP-32 phosphorylated at the PKA site were higher in females than males. Taken together, these findings suggest that sex differences in the DA-PKA signaling regulation may contribute to sex difference in the initiation and development of rewarding properties to cocaine.

III. Influence of hormone

A. Role of estrogen and progesterone on the behavioral response to cocaine

Endogenous and exogenous estrogen and progesterone affect the cocaine-induced behavioral response. Acute cocaine administration induces lowest behavior response during diestrus, but higher in estrus (Sell et al., 2000; Quinones-Jenab et al., 1999). Cocaine-induced locomotor activity is attenuated in ovariectomized (OVX) female rats (Walker et al., 2001; Chin et al., 2002), but augmented in estrogen or estrogen + progesterone replacement treated rats (Sell et al., 2002).

Unlike the acute cocaine studies, the effect of estrous cycle on chronic-cocaine induced behavior is not consistent. More consistent results are found in OVX and hormone replacement experiments. Chronic cocaine administrations fail to produce locomotor sensitization after 14 days of treatment in OVX females (Chin et al., 2002). However, estrogen and estrogen + progesterone replacement in OVX female rats enhance the behavioral sensitization of chronic cocaine administration (Sell et al., 2002; Sircar and Kim, 1999).

B. Role of estrogen and progesterone in intracellular signal transduction after cocaine administration

As mentioned above, estrogen and progesterone affect the cocaine-induced behavior response, but how they affect the intracellular signal transduction responsible for the behavior changes are not clear yet. Recently findings show that estrodial replacement increases mRNA levels of D1 in the hypothalamus, and D2 in the midbrain (Zhou et al., 2002). Furthermore, OVX female rats have lower D1-sensitive adenylate cyclase activity in striatum (Kumakura et al., 1979), while the estradiol treatment to striatal culture enhances its activity and inhibits the activity of D2-sensitive adenylate cyclase (Maus et al., 1989). Enhanced DARPP-32 phosphorylation at the Thr-34 site in the medial preoptic nucleus, bed nucleus of the stria terminalis, paraventricular nucleus, and the ventromedial nucleus of the hypothalamus is observed after the estrodial treatment (Auger et al., 2001). Taken those findings together, estrogen is involved in the modulation of dopaminergic intracellular signal transduction in various parts of hypothalamus.

Progesterone and dopamine enhance the activation of cAMP and PKA, and increases Thr-34 DARRP-32 phosphorylation in the hypothalamus (Mani et al., 2000). However, unlike the dopamine-induced increase in the dopamine transduction system, the progesterone-induced increase is not D1 receptor mediated, as the increase in the dopaminergic signal transduction is not affected by the application of D1 antagonist (SCH 23390) (Mani et al., 2000). These findings mentioned above suggest that estrogen and progesterone play a modulatory role in the intracellular dopaminergic signal transduction. However, stronger evidences from more important regions, such as NAc

and CPu are needed to further demonstrate the involvement of estrogen and progesterone in the signal transduction mechanism reacting to cocaine exposure.

IV. Significance of work

As mentioned above, better understanding the mechanism of the differences in cocaine response in males and females is critical. Although the important role of DARPP-32 pathway in alteration of cocaine-induced behaviors has been well studied in male mice/rats, no studies have addressed its role in behavioral sex differences. Understanding the cellular mechanism by which sex differences are produced may help developing more effective therapeutic methods to cocaine addiction.

The overall hypothesis is that a heightened basal and cocaine response in females contribute to the exaggerated response in females. Our working hypothesis is divided into three levels. Firstly, it is predicted that there is either a basal protein level difference or an activation time course difference in the DARPP-32 pathway after acute cocaine administration, which may explain the behavior difference. Secondly, we predict that the development of behavior sensitization and tolerance may be different between males and females. Female rats are more sensitive to cocaine's psychomotor effect, so they may develop sensitization and tolerance with fewer cocaine injections. Lastly, it is predicted that the adaptation in the DARRP-32 pathway upon different duration of cocaine treatment may be sex different, which may contribute to the differences in behavior development. Detecting sex differences at these three levels will increase our understanding of to what extent does the DARPP-32 pathway contribute to the cocaine-

induced sex differences, in turn better understanding of how cocaine's actions differ between males and females.

V. Specific aims

Specific aim 1: To determine if sex differences exist in the basal and acute cocaine-induced activation of intracellular signal transduction mechanisms.

Specific aim 2: To determine if sex differences exist in the development of chronic cocaine-induced behavior sensitization and tolerance.

Specific aim 3: To determine if sex differences exist in the chronic cocaine-induced activation of intracellular signal transduction mechanisms, and whether the intracellular response correlates with the behavior response.

Chapter 2: Basal and acute cocaine-induced sex differences in DARPP-32-mediated signaling pathway

I. Introduction

As more attention is paid to sex and hormonal effects on drug abuse, it is becoming apparent that men and women react differently to cocaine. More women initiate cocaine use at a younger age than men (NSDUH, 2007). Women also take shorter time to develop cocaine abuse than men (Ridenour et al., 2005). Women experience more nervousness and report higher ratings of "feel good" after intermittent intranasal administration of cocaine (Kosten et al., 1996; McCance-Katz et al., 2005). These data suggest that women are more sensitive than men to the additive properties of cocaine. However, those sex differences are limited to women in their luteal phase, when the subjective effects of cocaine were lower compared to the follicular phase (Sofuoglu et al., 1999; Evans et al., 2002; Evans and Foltin, 2006). Other clinical studies have revealed conflicting findings of no sex or menstrual cycle differences, which may result from different dose and route of cocaine administration (Mendelson et al., 1999; Collins et al., 2007). However, Lukas et al. (1996) even found that women take longer to feel cocaine's subjective effects, report less euphoria and dysphoria than men.

Similar to humans, female rodents also show exaggerated and more robust locomotor responses to cocaine than do males (Van Haaren and Meyer, 1991; Schindler and Carmona, 2002; Harrod et al., 2005). Females also more quickly develop cocaine-induced conditioned place preference (CPP) and behavior sensitization with lower doses, and more readily acquire cocaine self-administration (Russo et al., 2003; Lynch and Carroll, 1999; Van Haaren and Meyer, 1991; Chin et al., 2002; Hu et al., 2004; Jackson et al., 2006). Taken together, clinical and rodent studies suggest that sex-specific

differences exist at all stages of the cocaine abuse process, including induction, maintenance, and relapse.

Sex differences in the mesocorticolimbic dopamine (DA) system, a regulator of cocaine's psychomotor and rewarding effects (Koob, 1992; Hyman and Malenka, 2001), have also been demonstrated (Festa et al., 2004; Festa et al., 2006; Walker et al., 2006). Cocaine reduces dopamine type 1 (D1) receptor binding levels in the striatum of male but not female rats (Festa et al., 2006). Furthermore, D1 receptor antagonists block cocaine-induced CPP and locomotor responses with different efficacies between sexes: D1 receptor antagonists inhibit cocaine's effects with a lower dose range in female than in male rats (Nazarian et al., 2004; Festa et al., 2006). After cocaine administration, accumbal DA release is higher in females than in males (Walker et al., 2006). Additionally, whereas cocaine decreases dihydroxyphenylacetic acid (DOPAC) to DA turnover ratios in the nucleus accumbens (NAc) of male rats, in female rats it significantly reduces total levels of DA, DOPAC, and homovanillic acid metabolites (Festa et al., 2004). In female rats, the nigrostriatal DA neurotransmission is more tightly regulated by autoreceptor and transporter mechanisms than in male rats, a difference that may be related to the greater autoreceptor control of DA activity in females (Walker et al., 2006). These sexual dimorphic patterns in DA system activation after cocaine treatment strongly suggest sex difference in cocaine-induced dopamine-protein kinase A (PKA)-mediated signaling pathway responses. Indeed, two recent studies found sexual dimorphic responses to cocaine in this signaling pathway. Nazarian et al. (2009) demonstrated that PKA protein levels in the NAc were overall higher in females than males, and Lynch et al. (2007) showed that protein levels of dopamine- and cAMP-

regulated phosphoprotein of Mr 32 kDa (DARPP-32) phosphorylated at the PKA site were higher in females than males. Taken together, these findings suggest that sex differences in the DA-PKA signaling regulation in the NAc may contribute to sex difference in the initiation and development of rewarding properties to cocaine.

The DARPP-32 signaling pathway has been shown to mediate intracellular responses to DA. Phosphorylation of DARPP-32 at the Thr34 site (P-Thr34-DARPP-32) is required for cocaine actions in the striatum (Zachariou et al., 2006); i.e., acute cocaine administration increases phosphorylation of DARPP-32 at the Thr34 site (Nishi et al., 2000; Greengard, 2001). Moreover, in DARPP-32 knockout mice and mice with site mutations of P-Thr34-DARPP-32, typical cocaine-induced locomotor activities are attenuated as compared with activities in control mice (Valjent et al., 2005; Fienberg et al., 2006; Zachariou et al., 2006). On one hand, P-Thr34-DARPP-32 is a potent inhibitor of protein phosphatase-1 (PP-1), which is a serine/threonine protein phosphatase (Svenningsson et al., 2005). On the other hand, P-Thr34-DARPP-32 is dephosphorylated by protein phosphatase-2B (PP-2B), a $^{+}$/calmodulin-dependent protein phosphatase (King et al., 1984; Goto et al., 1986). Although no studies to date have determined whether PP-1 and PP-2B are altered after acute cocaine administration, in male rats it has been shown that repeated cocaine administration decreases PP-2B but not PP-1 protein levels in the NAc (Hu et al., 2005).

It is yet to be determined whether acute cocaine administration alters the DA-mediated intracellular cascades in a sexually dimorphic pattern. Because females not only have more locomotor responses to acute cocaine treatment but also develop CPP and self-administration faster than males, we hypothesized that the cocaine-induced DARPP-32

signaling pathway responses are heightened in females as compared with males. To test this hypothesis, we made side-by-side comparisons between male and female rats of DARPP-32, P-Thr34-DARPP-32, PP-1, and PP-2B protein levels in the NAc and CPu (areas known to regulate cocaine's behavioral and reward responses (Nestler, 2001)) after saline or acute cocaine treatment.

II. Methods

Animals: 60-day-old male and female Fischer rats (Charles River, Raleigh, NC) were individually housed in Plexiglas chambers (20 × 20 × 41 cm) layered with beta chips. Rats were given free access to food and water and maintained on a 12-hour light/dark cycle (lights on at 9:00 a.m.). All rats were weighed, handled, and intraperitoneally (i.p.) injected daily with saline for 5 consecutive days prior to testing, except for Naïve rats which were never touched until the day of sacrifice. A total of four rats per group were used for each sex or time-course comparison after saline or drug treatment.

Repeated vaginal lavage attenuates cocaine-induced activity, abolishes estrous cycle effects, and establishes CPP in female rats, thus possibly increasing DA-mediated responses (Walker et al., 2002). Therefore, as noted by Walker et al. (2002), the use of lavaged female rats could result in inaccurate behavioral responses when making side-by-side comparisons with male rats. For this reason, females were randomly assigned to experimental groups without regard to their estrous cycle. Animal care and use was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD) and approved by the Hunter Institution Animal Care Use Committee.

Materials: Cocaine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). DARPP-32 antibody was purchased from Cell Signaling Technologies (Beverly, MA). P-Thr34-DARPP-32, PP-1α (catalytic subunit of PP-1), CaN-A (calcineurin A, catalytic subunit of PP-2B), and CaN-B (calcineurin B, regulatory subunit of PP-2B) antibodies were purchased from Sigma (St Louis, MO). α-tubulin antibody was purchased from Santa Cruz Technologies (Santa Cruz, CA). All appropriate secondary antibodies were purchased from Amersham Pharmacia (Piscataway, NJ).

Cocaine administration and protein measurements: Cocaine solutions were prepared daily by dissolving the drug in physiological saline (0.9%). On the day of testing, rats were injected (i.p.) with saline or cocaine (30 mg/kg) and sacrificed 5, 15, 30, 45, or 90 minutes later. After decapitation (following a brief 20-second exposure to), their brains were removed, flash frozen in 2-methylbutane (-40° C), and stored at -80° C until used.

The NAc and CPu were dissected from coronal sections (1-mm thick) using a matrix (ASI Instruments; Warren, MI). Tissue was homogenized using a Polytron handheld homogenizer (Kinematica; Luzern, Switzerland) in homogenizing buffer [HEPES 7.9 (20 mM), KCl (10 mM), EDTA (1 mM), NP40 (0.2%), glycerol (10%), NaCl (200 mM), pepstatin, leupeptin, DTT (1 M), aprotenin, PMSF (100 mM), NaF (50 mM), and (1 mM)]. Total protein content was determined with use of a Bradford kit (Bio-Rad Laboratories; Hercules, CA).

Western blot analysis: Protein samples (40 μg) were boiled in Lammeli buffer containing 5% β-mercaptoethanol and loaded onto 10% SDS-PAGE. Gels were

electrophoresed, transferred to nitrocellulose membranes, and blocked for 60 minutes with 5% non-fat dry milk in tris-buffer-saline-tween (TBST, pH = 7.4) at room temperature. Membranes were probed overnight at 4°C, with P-Thr34-DARPP-32 (1:2000), PP-1α (1:4000), CaN-A (1:15000), CaN-B (1:5000), and DARPP-32 (1:1000). After three washes with TBST, membranes were then incubated with their appropriate secondary antibody (1:1000) for 60 minutes at room temperature, followed by three more washes with TBST. Antibody binding was detected using an enhanced chemiluminescence kit (Amersham Pharmacia; Piscataway, NJ). Resulting films were scanned and quantified with a computer densitometer and Image Quant Program (Molecular Dynamics; Sunnyvale, CA). To compare sex differences in the protein levels, samples from saline-treated male and female rats were loaded onto the same gel. Within each sex, to determine the time course of changes in protein levels after treatment, all saline-treated samples (5 min-90 min), or cocaine-treated (5 min-90 min) samples with saline control (5 min) were run on the same gel. A total of three sets of gels were run for each determinant. To normalize band intensity to protein levels, membranes were re-probed with α-tubulin antibody (1:1000).

Statistical analysis: All protein levels were first expressed as a ratio to α-tubulin levels. Data were presented as mean ± SEM. Student's t-tests were used to determine sex differences. For comparison between sexes in cocaine-treated rats, percentage changes of protein levels between the cocaine-treated group and the average of protein levels in saline controls of the same sex were used. One-way ANOVAs followed by post-hoc LSD

analysis were used to determine differences during the time course. Statistical significance was considered to be $p < 0.05$ for all analyses.

III. Results

A. Sex differences in intracellular signal transduction in naïve rats

In both NAc and CPu, no significant sex differences in protein levels of DARPP-32, P-Thr34-DARPP-32, PP-1α, CaN-A or CaN-B were observed in naïve male and female rats (Fig. 5).

B. Sex differences in protein levels in saline-treated rats

In NAc, 5 minutes after saline administration, females had significantly higher protein levels of DARPP-32 and P-Thr34-DARPP-32 than males ($t=13.765$, $p<0.001$; and $t=2.921$, $p<0.05$, respectively; Fig. 6A and 6B, and Table 1). Thirty minutes after saline administration, there were higher protein levels of PP-1α in females than males ($t=2.720$, $p<0.05$; Table 1). At both 5 and 15 minutes after saline treatment, female rats had significantly higher CaN-A protein levels than males ($t=10.580$, $p<0.001$; $t=2.463$, $p<0.05$, respectively; Fig. 6D and Table 1). However, CaN-B protein levels were significantly higher in females than males only at 5 minutes after saline treatment ($t=8.250$, $p<0.001$; Fig. 6E and Table 1). Within each sex, no statistically significant differences were observed 5 to 90 minutes after saline administration.

In CPu, 5 minutes after saline treatment, female rats exhibited significant higher protein levels of P-Thr34-DARPP-32 and PP-1α when compared to males ($t=2.858$, $p<0.05$; and $t=3.090$, $p<0.05$, respectively; Fig. 6G and 6H). DARPP-32, CaN-A and

CaN-B protein levels did not statistically deferrer between male and female rats (t=0.633, p=0.55; t=2.157, p=0.074; and t=2.167, p=0.073, respectively; Fig. 6F, 6I and 6J).

C. Sex differences in intracellular signal transduction mechanisms after acute cocaine administration

In the NAc of male rats, higher P-Thr34-DARPP-32 protein levels were observed 30 minutes after cocaine treatment when compared with saline controls; however, the difference failed to reach statistical significance (p=0.058; Fig. 7B). In male rats, CaN-A protein levels were significantly higher at 30 minutes after cocaine administration than at other time points ($p<0.001$ for all comparisons; Fig. 7D). CaN-A protein levels were also significantly higher 90 minutes after cocaine treatment as compared to saline controls ($p<0.02$; Fig. 7D). Furthermore, CaN-B protein levels were significantly higher 15 minutes after drug treatment when compared with saline controls ($p<0.05$; Fig. 7E).

In the NAc of female rats, 45 minutes after cocaine administration, DARPP-32 protein levels were lower than 15 minutes after cocaine administration, and P-Thr34-DARPP-32 protein levels were decreased compared to saline controls ($p<0.05$ for both comparisons; Fig. 7F and 7G, respectively). Thirty minutes after cocaine administration, PP-1α protein levels were significantly higher than at any other time point after cocaine treatment ($p<0.05$ for all comparisons; Fig. 7H). Forty-five minutes after cocaine administration, CaN-A protein levels were significantly lower than saline controls and significantly lower than 5 minutes after cocaine treatment ($p<0.05$ for both comparisons; Fig. 7I).

In NAc, between-sex comparisons reveal that while in male rats the percentage change of proteins in the DARPP-32 pathway increased across time, in females it

decreased. Thus, males had a significantly higher percentage change in both DARPP-32 and P-Thr34-DARPP-32 30 to 90 minutes after cocaine administration than female rats ($p<0.05$ for all comparisons; Table 2). Similarly, sex differences were observed in the magnitude and duration of PP-1α, CaN-A and CaN-B. Specifically, male rats had higher induction of PP-1α 15 and 45 minutes after cocaine treatment than female rats ($p<0.05$ for both comparisons; Table 2). Male rats also had higher induction of CaN-A 30 to 90 minutes after cocaine administration than female rats ($p<0.05$ for all comparisons; Table 2). However, male rats had a significantly greater percentage change in CaN-B at 30 and 45 minutes after cocaine treatment as compared with female rats ($p<0.05$ for both comparisons; Table 2).

As shown in Figure 8, overall, in the CPu of both males and females, acute cocaine administration did not significantly alter protein levels of DARPP-32, P-Thr34-DARPP-32, CaN-A, or CaN-B through time.

Between-sex comparisons reveal that while in male rats the percentage change of proteins in the DARPP-32 pathway increased across time, in females it decreased. Thus, males had a significantly higher P-Thr34-DARPP-32 protein induction 30 minutes after cocaine administration than female rats ($p<0.05$; Table 3). Similarly, sex differences were observed in the magnitude and duration of CaN-A and CaN-B. Specifically, 45 minutes after cocaine administration, male rats had higher induction of CaN-A; while CaN-B protein induction was higher in males 15 and 45 minutes after cocaine treatment ($p<0.05$ for all comparisons; Table 3).

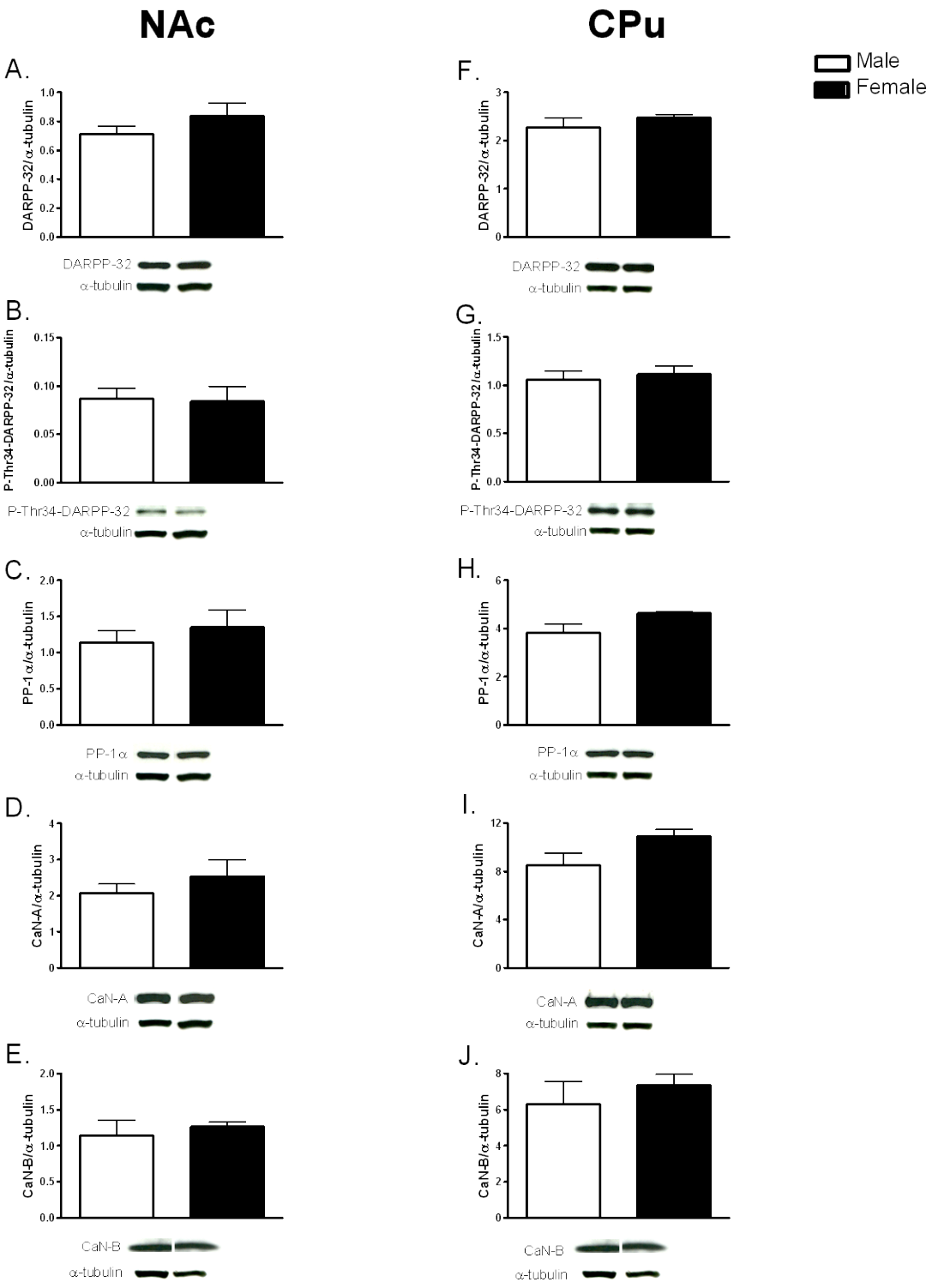

Figure 5: Western blot analysis of DARPP-32 (A, F), P-Thr34-DARPP-32 (B, G), PP-1α (C, H), CaN-A (D, I), and CaN-B (E, J) in the NAc (A-E) and CPu (F-J) of naïve animals. Data presented as mean protein levels normalized to α-tubulin (±S.E.M).

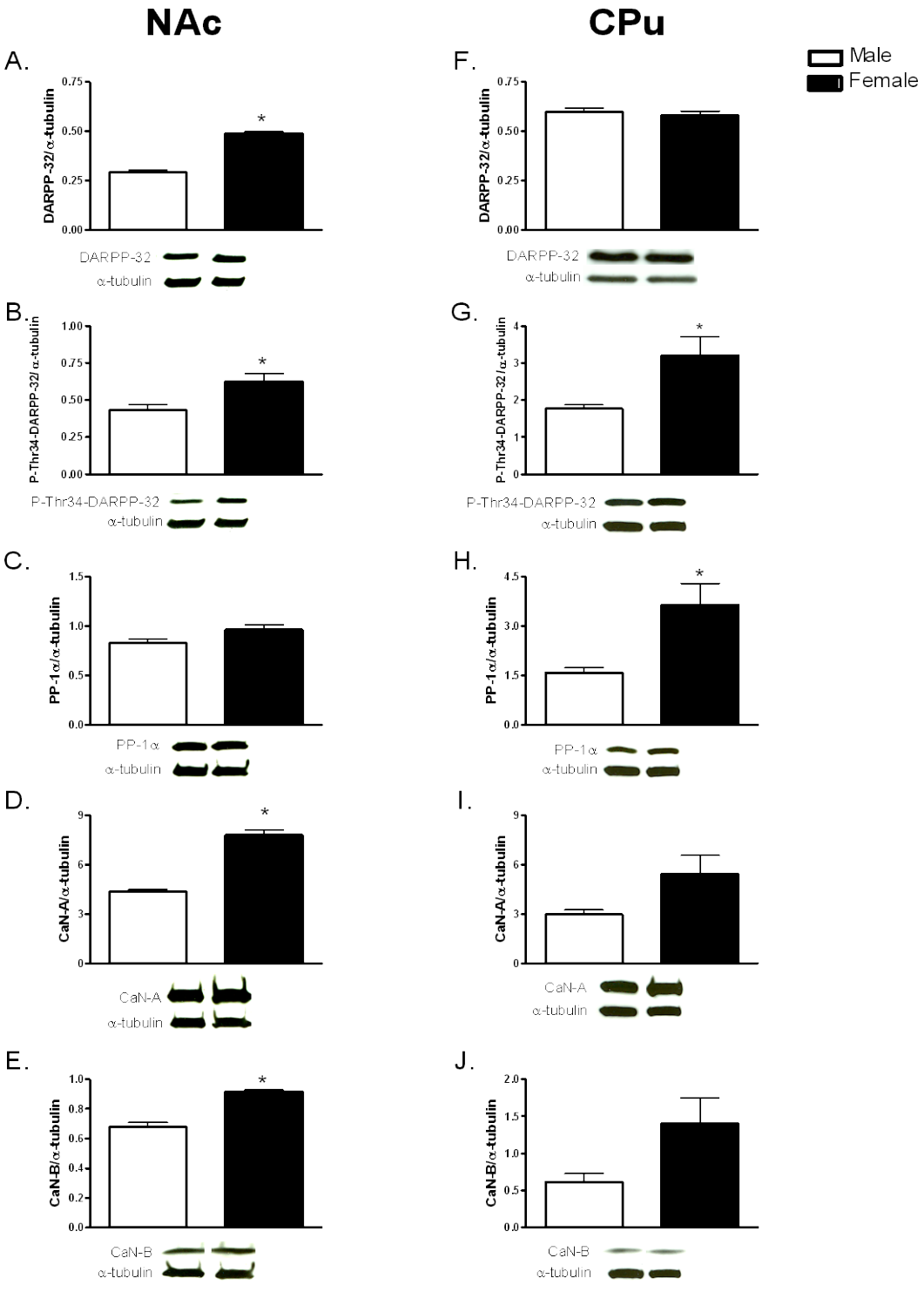

Figure 6: Western blot analysis of DARPP-32 (A, F), P-Thr34-DARPP-32 (B, G), PP-1α (C, H), CaN-A (D, I), and CaN-B (E, J) in the NAc (A-E) and CPu (F-J) of saline-treated animals. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). * Represents statistical gender difference ($p<0.05$). (n=4 per group)

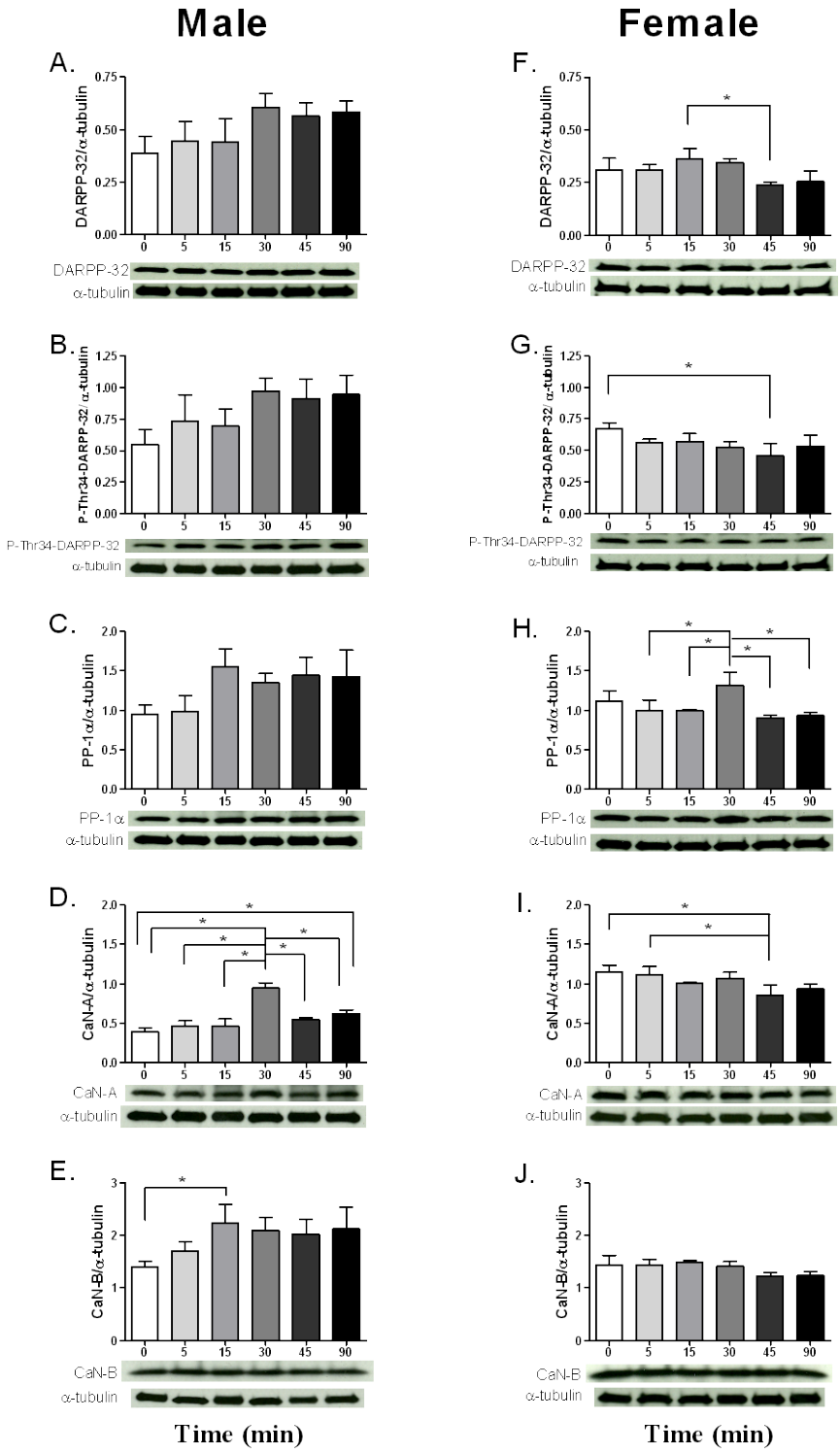

Figure 7: Western blot analysis of DARPP-32 (A, F), P-Thr34-DARPP-32 (B, G), PP-1α (C, H), CaN-A (D, I), and CaN-B (E, J) in the NAc of male (A-E) and female (F-J) rats after acute cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). * Represents statistical difference ($p<0.05$). (n=4 per group)

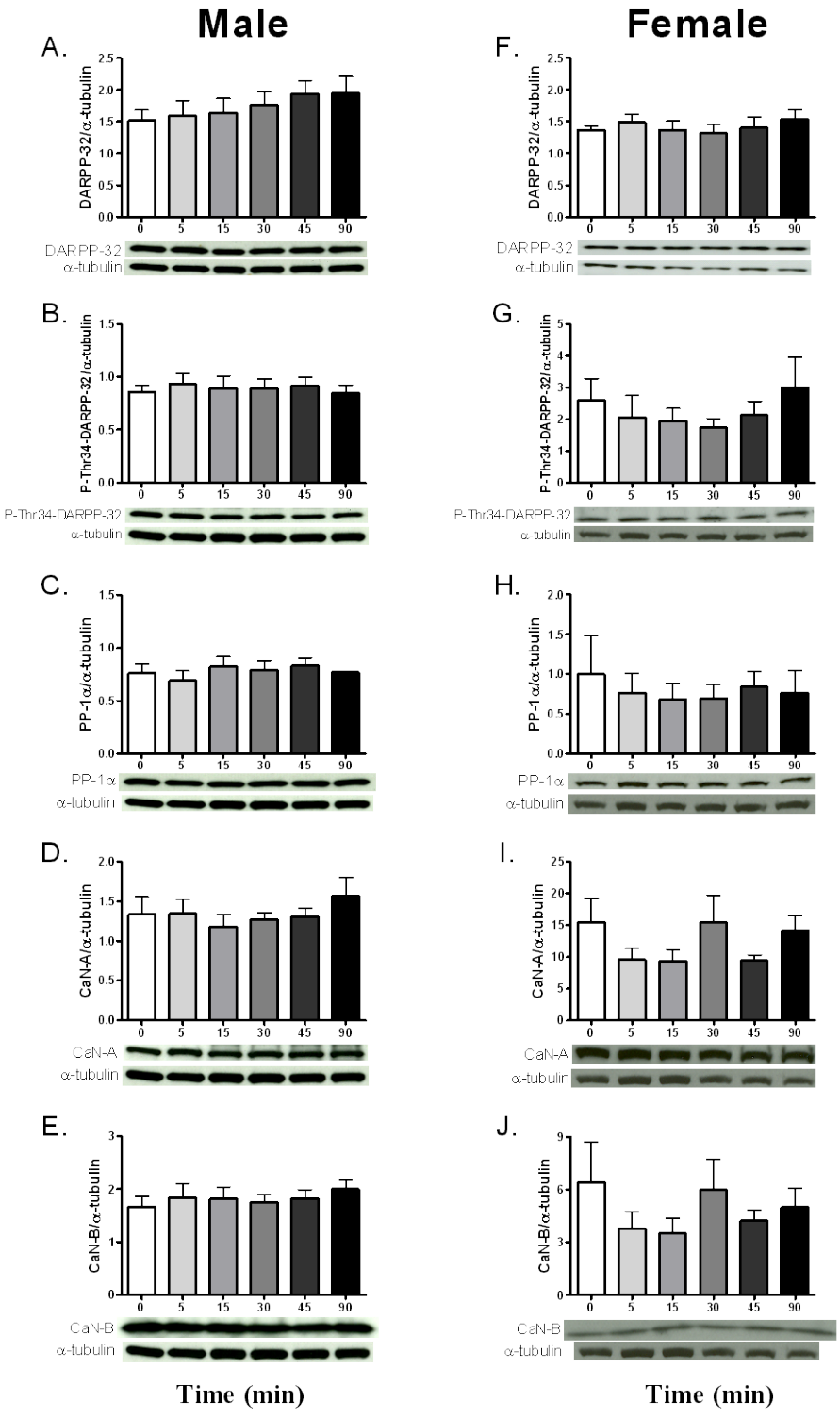

Figure 8: Western blot analysis of DARPP-32 (A, F), P-Thr34-DARPP-32 (B, G), PP-1α (C, H), CaN-A (D, I), and CaN-B (E, J) in the CPu of male (A-E) and female (F-J) rats after acute cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). (n=4 per group)

Table 1. Western blot analysis of protein levels of DARPP-32 signaling proteins in NAc of saline-treated animals.

Time Point	5 min		15 min		30 min		45 min		90 min	
Protein	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
DARPP-32	0.30 ± 0.01	**0.49 ± 0.01***	1.80 ± 0.38	1.76± 0.22	1.63 ± 0.34	1.68 ± 0.30	1.84 ± 0.23	1.88 ± 0.22	2.13 ± 0.37	1.62 ± 0.24
P-Thr34-DARPP-32	0.43 ± 0.04	**0.62 ± 0.05***	2.18 ± 0.43	2.08 ± 0.32	1.86 ± 0.14	2.33 ± 0.51	2.00 ± 0.25	1.85 ± 0.24	2.30 ± 0.43	1.53 ± 0.20
PP-1α	0.83 ± 0.04	0.97 ± 0.05	3.27 ± 0.75	4.14 ± 0.36	3.00 ± 0.30	**3.91 ± 0.15***	1.94 ± 0.15	1.95 ± 0.21	1.94 ± 0.25	1.63 ± 0.19
CaN-A	4.38 ± 0.10	**7.81 ± 0.31***	5.90 ± 0.75	**8.56 ± 0.78***	5.16 ± 0.65	7.18 ± 0.73	3.51 ± 0.14	4.36 ± 0.62	3.60 ± 0.23	3.81 ± 0.27
CaN-B	0.68 ± 0.03	**0.92 ± 0.01***	7.28 ± 0.76	9.03 ± 1.02	6.17 ± 0.52	8.18 ± 1.35	6.36 ± 0.86	6.58 ± 1.34	6.69 ± 0.96	6.26 ± 1.09

Data are presented as the mean protein levels normalized with α-tubulin ± SEM.
*Represents statistically significant differences between sexes. (N = 4 per group)

Table 2. Percentage change of protein levels of DARPP-32 signaling proteins in NAc of cocaine-treated animals.

Time Point	5 min		15 min		30 min		45 min		90 min	
Protein	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
DARPP-32	15.7 ± 23.9	0.2 ± 7.8	14.4 ± 28.4	16.3 ± 16.2	57.3 ± 16.8	**10.3 ± 7.1***	46.3 ± 16.0	**-23.5 ± 4.4***	51.6 ± 13.6	**-18.0 ± 16.0***
P-Thr34-DARPP-32	33.4 ± 37.7	-15.8 ± 4.2	25.8 ± 24.3	-14.5 ± 8.9	76.6 ± 18.0	**21.3 ± 6.5***	65.6 ± 27.2	**-31.8 ± 15.1***	71.6 ± 28.0	**-19.9 ± 12.5***
PP-1α	3.8 ± 21.1	-10.7 ± 12.0	63.5 ± 23.5	**-11.0 ± 2.0***	42.6 ± 11.5	18.5 ± 14.3	52.0 ± 24.2	**-18.8 ± 2.4***	50.2 ± 35.5	-16.1 ± 3.4
CaN-A	19.6 ± 15.8	-3.5 ± 9.0	18.3 ± 22.5	-12.5 ± 0.9	140.2 ± 17.7	**-7.7 ± 6.1***	38.9 ± 7.4	**-25.3 ± 10.9***	58.8 ± 12.0	**-19.0 ± 5.7***
CaN-B	22.1 ± 12.4	0.0 ± 7.5	60.4 ± 25.0	3.0 ± 2.6	50.0 ± 17.7	**1.9 ± 6.2***	44.7 ± 19.9	**-14.9 ± 4.7***	52.2 ± 29.4	-14.4 ± 5.9

Data are presented as percentage change of protein levels ± SEM as compared to 5 min saline controls of the same sex. * Represents statistically significant differences between sexes. (N = 4 per group)

Table 3. Percentage change of protein levels of DARPP-32 signaling proteins in CPu of cocaine-treated animals.

Time Point	5 min		15 min		30 min		45 min		90 min	
Protein	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
DARPP-32	5.1 ± 15.5	8.3 ± 9.3	7.8 ± 14.9	0.0 ± 10.0	16.4 ± 13.3	-3.8 ± 10.5	27.7 ± 13.4	2.3 ± 11.9	28.4 ± 17.3	12.4 ± 10.8
P-Thr34-DARPP-32	8.9 ± 11.1	-20.8 ± 26.5	4.0 ± 13.6	-25.6 ± 15.5	3.6 ± 10.3	**-33.2 ± 10.7***	6.8 ± 9.3	-17.9 ± 16.6	-1.5 ± 9.1	15.4 ± 35.9
PP-1α	-8.9 ± 11.5	-23.6 ± 24.7	8.5 ± 11.9	-31.3 ± 19.8	4.0 ± 11.4	-30.4 ± 17.8	9.8 ± 9.0	-16.0 ± 18.9	0.8 ± 0.9	-23.5 ± 27.4
CaN-A	1.0 ± 13.0	-38.1 ± 11.8	-11.6 ± 11.1	-39.8 ± 12.1	-4.9 ± 6.4	0.1 ± 27.7	-1.9 ± 7.5	**-38.9 ± 5.8***	17.7 ± 17.0	-8.1 ± 15.0
CaN-B	10.6 ± 16.1	-41.0 ± 15.0	9.2 ± 13.4	**-45.5 ± 13.7***	5.5 ± 8.1	-6.8 ± 26.9	9.4 ± 10.2	**-34.3 ± 9.7***	20.4 ± 10.0	-22.0 ± 16.2

Data are presented as percentage change of protein levels ± SEM as compared to 5 min saline controls of the same sex. * Represents statistically significant differences between sexes. (N = 4 per group)

IV. Discussion

Results presented here are novel in that they demonstrate basal sex differences and a sexually dimorphic pattern of activation after acute cocaine administration in the DARPP-32 intracellular cascades. These results extend current knowledge by showing that the known basal and cocaine-induced sex differences at the release, receptor (number and specificity), and reuptake levels in the DA system also include differences at the DA-mediated second messenger intracellular responses.

Female rats had higher basal protein levels in most DARPP-32 pathway components. Lynch et al. (2007) have also shown that female Sprague Dawley rats have higher basal levels of phosphorylated DARPP-32. Thus our results, which are consistent with their observations, demonstrate that the DARPP-32 cascade is heightened in female rats as compared with males. Basal sex differences in the DARPP-32 cascade suggest three postulates. First, because female rats have higher accumbal dopaminergic tone than males (Becker, 1999; Walker et al., 2006), PKA may be activated at higher levels by high basal DA levels, and in turn, this high level of PKA activation increases the phosphorylation of DARPP-32 at the Thr34 site (Nishi et al., 2000). Indeed, Nazarian et al. (2009) have demonstrated that female rats have higher basal protein levels of PKA in NAc than male rats. This finding is further supported by previous reports of higher basal dopamine release and uptake in female rats (Walker et al., 2000), which may underlie the higher basal levels of P-Thr34-DARPP-32 mediated by D1/PKA activation in females than males. The second postulate is that the differential efficiency of D1 receptors between sexes (Schindler and Carmona, 2002; Festa et al., 2006) may also have an impact on the sex differences in P-Thr34-DARPP-32 protein levels. Because females are

more sensitive to D1 agonists, less ligand may be needed to increase the activation of the D1/PKA/DARPP-32 cascade in females than in males. Thirdly, estrogen increases the basal extracellular concentration of DA in the striatum (Xiao and Becker, 1994). Furthermore, enhanced DARPP-32 phosphorylation at the Thr-34 site after the estradiol treatment has also been observed in the medial preoptic nucleus, bed nucleus of the stria terminalis, paraventricular nucleus, and the ventromedial nucleus of the hypothalamus (Auger et al., 2001). Therefore, females' higher estrogen levels may contribute to the basal sex differences in P-Thr34-DARPP-32 protein levels. Further experiments are necessary to determine whether one or all these postulates contribute to the sexual dimorphic pattern of DARPP-32-mediated proteins. Still unknown is the extent to which the higher basal levels of DARPP-32 phosphorylation at the Thr34 site in female rats contribute to the sexual dimorphism. However, we postulate that females' robust and prolonged motor responses after acute cocaine administration are in part mediated by their having initial higher basal levels of P-Thr34-DARPP-32.

P-Thr34-DARPP-32 inhibits PP-1 activity (Svenningsson et al., 2005). Thus, higher basal protein levels of PP-1 in females may reflect a disinhibitory effect caused by higher P-Thr34-DARPP-32 protein levels. In addition, PP-1 protein levels are also part of PKA regulation (Surmeier et al., 1995). Thus, higher PKA activation in females (either by the higher dopamine tone (Becker, 1999; Walker et al., 2006) or higher PKA protein levels (Nazarian et al., 2009)) may also counterbalance the inhibitory effect of P-Thr34-DARPP-32 in PP-1, which in turn indirectly elevates PP-1 protein levels. However, further study is needed to determine the extent to which the higher basal levels of P-Thr34-DARPP-32 in female rats contribute to the sexual dimorphism in PP-1 protein

levels. To further confirm our finding and address the differences between the levels of protein expression and the levels of protein activity, future studies measuring the phosphatase activity of PP-1 in male and female rats are warranted.

PP-2B protein levels were also found to be higher in saline-treated female rats than in males. Since PP-2B dephosphorylates P-Thr34-DARPP-32 (King et al., 1984; Goto et al., 1986), the elevated PP-2B protein levels may represent a positive feedback mechanism to counteract the higher basal protein levels of P-Thr34-DARPP-32 observed in the females. Alternatively, recent evidence has also shown that the activation of PP-2B is necessary to maintain the dopamine D1-agonist-stimulated G-protein activation in the forebrain cortical tissue (Adlersberg et al., 2004). Thus, the higher PP-2B protein levels may further contribute to higher DARPP-32-mediated signaling activation in females.

In the basolateral amygdala (BLA), female rats have higher DA outflow after acute stress than do their male counterparts (Mitsushima et al., 2006). Stress-induced activation of the BLA also increases the DA extracellular level in the NAc (Floresco et al., 1998; Howland et al., 2002), possibly through BLA excitatory projections into the NAc (Kelley et al., 1982; Wright et al., 1996; Mulder et al., 1998). In addition, females have higher DA levels in the striatum than males after acute stress. Furthermore, in the prefrontal cortex (PFc, an area known to regulate cocaine reward (Kalivas et al., 2005)), enhanced dopaminergic activities by acute stress were observed in male rats only (Dalla et al., 2008). It was postulated that the increased cortical DA function in turn inhibits the dopaminergic activities in the NAc (Ventura et al., 2002). However, in females, due to their higher basal dopaminergic activities in the PFc, it is possible that acute stress can not further increase cortical DA function, resulting in failure to inhibit the high

dopaminergic activities in the NAc (Dalla et al., 2008). This dysregulation of DA-mediated responses in the PFc-NAc pathway contributes to the female's liability in adapting to acute stress. Given that saline administrations may be an acute stressor, the sexual dimorphic pattern of the DARPP-32 cascade may reflect sex differences in administration-induced stress-mediated responses, such as DA releases and dopaminergic activities. Indeed, because such differences were observed for the most part soon after the saline administration, in addition, no sex differences were observed in naïve rats, these data further support this postulate. Recently, it has been postulated that sex differences in stress-mediated responses may contribute to the known sex differences in the pattern of cocaine abuse (see review in Becker et al., 2007). Thus, stress-induction of the DARPP-32 cascade may have an impact on cocaine-induced regulation in this cascade.

After cocaine treatment, sex differences in the pattern of DARPP-32 activation were observed. Although striatal P-Thr34-DARPP-32 has been reported to be increased after acute cocaine administration, most of these studies are done on male mice (Nishi et al., 2000; Valjent et al., 2005; Zachariou et al., 2006). Indeed, only Rauggi et al. (2005) reported an induction of P-Thr34-DARPP-32 occurring 30 minutes after cocaine treatment in the NAc of male rats; no studies were done in the rats' CPu. The pattern of P-Thr34-DARPP-32 protein level changes reported here is similar to that observed by Rauggi et al. (2005). Since their study and ours use different cocaine doses, a dose response study may be needed to further clarify these differences between the two studies. In female rats, cocaine administration decreased the protein levels of DARPP-32, P-Thr34-DARPP-32 in NAc and not changed in CPu. Because females' higher basal protein levels are already at "ceiling" levels, it is possible that cocaine can not further

increase these protein levels. As a protein that dephosphorylates P-Thr34-DARPP-32, the subsequent changes in PP-2B protein levels in both males and females may counteract the changes of P-Thr34-DARPP-32 protein levels after cocaine treatment. However, in female rats, PP-1 protein levels in the NAc were increased after acute cocaine administration, whereas no significant changes were observed in the CPu. Unlike the higher basal levels in the CPu, the basal protein levels of PP-1 in the NAc of females did not start at high "ceiling" levels, which made possible a further increase by acute cocaine treatment.

After cocaine treatment, higher induction in protein levels was observed in males than females. Indeed, in male rats the protein levels of DARPP-32 signaling proteins were increased after cocaine administration, but the overall levels of these proteins were decreased in females. This difference further demonstrated a sexual dimorphic pattern in the profile of this pathway after cocaine administration. However, the contributions to sex differences of short-term changes in kinase activities, which are independent of transcriptional changes, are yet to be determined.

When experience takes the form of exposure to drug abuse, alterations in DARPP-32 function and activation appear to cause tolerance and dependence, an adaptation commonly associated with the development and maintenance of rewarding behaviors (Bibb et al., 2001). Sex specific differences exist at all stages of the cocaine-abuse progress wherein females are more sensitive during different phases. Since both Lynch et al. (2007) and Zachariou et al. (2006) demonstrated that dopamine-related protein changes may not always be concordant with cocaine-reinforcing effects, a study is needed to determine to what extent heightened basal responses in females and a sexual

dimorphic pattern of DARPP-32-pathway contribute to the sex differences in the regulation of central nervous system plasticity that induce drug dependence. However, taken together, our findings suggest that females may have a different profile of elevated D1-mediated intracellular second messenger transduction, which in turn may underlie their higher rewarding sensitivity and locomotor behavior. An important issue not studied here but by a most recent study in our lab is the effect of gonadal hormones and their fluctuation on the DARPP-32 cascade. It is feasible that because DA release and reuptake is affected by estrogen and progesterone circulatory levels, the DARPP-32 pathway will be equally affected. Further studies need to be done to address this important issue, which is highly relevant to women's health.

Chapter 3: Sex differences in chronic cocaine-induced behavioral and DARPP-32 signaling

I. Introduction

In 2007, 35% of the 5.7 million Americans aged 12 or older used cocaine in the past year were women (NSDUH, 2008). Recently, more attention is paid to sex and hormonal effects on drugs of abuse, due to different reactions to cocaine between genders. For instance, more women begin using cocaine at a younger age than men (NSDUH, 2007). Women also more quickly develop from casual use to cocaine abuse than men (Ridenour et al., 2005). In addition, women are more likely to relapse trigged by stress or depression, and report shorter abstinence periods than do men (Kosten et al., 1993; Robbins et al., 1999; Van Etten and Anthony, 2001). After intermittent intranasal administration of cocaine, women experience more nervousness and report higher ratings of "feel good" (Kosten et al., 1996; McCance-Katz et al., 2005). Thus, women are more sensitive to the addictive properties of cocaine than men. However, those sex differences are limited to women in their luteal phase, when the subjective effects of cocaine were lower compared to the follicular phase (Sofuoglu et al., 1999; Evans et al., 2002; Evans and Foltin, 2006). Other clinical studies have revealed conflicting findings of no sex or menstrual cycle differences, which may result from different dose and route of cocaine administration (Mendelson et al., 1999; Collins et al., 2007). However, Lukas et al. (1996) even found that women take longer to feel cocaine's subjective effects, report less euphoria and dysphoria than men.

Similar to human, sex differences in response to cocaine also exist in animal models of cocaine addiction. Female rodents exhibit exaggerated and more longer-lasting

locomotor responses to cocaine than do males (Van Haaren and Meyer, 1991; Schindler and Carmona, 2002; Harrod et al., 2005). Females also more quickly develop behavior sensitization with lower doses of cocaine (Chin et al., 2002; Hu et al., 2004; Jackson et al., 2006). Sex differences also exist in the development of cocaine reward and reinforcement responses. For example, female rats are more rapidly to acquire cocaine self-administration, and develop cocaine-induced conditioned place preference (CPP) with lower doses and fewer conditioning sessions than do males (Lynch and Carroll, 1999; Van Haaren and Meyer, 1991; Russo et al., 2003).

In reward-associated areas including the nucleus accumbens (NAc) and caudate-putamen (CPu), the dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein, 32 kDa (DARPP-32) signaling pathway is involved in the intracellular transduction in response to dopamine. Depending on the site of phosphorylation, DARPP-32 can act either as a phosphatase inhibitor or as a kinase inhibitor. By binding on D1 dopamine receptors, extracellular dopamine, activates PKA. PKA exert two effects on DARPP-32 cascade. On one hand, it phosphorylates DARPP-32 at the threonine 34 site (P-Thr34-DARPP-32) as an inhibitor of a major serinine/threonine protein phosphatase -- protein phosphatase-1 (PP-1). P-Thr34-DARPP-32, by inhibiting PP-1, increases the phosphorylation of various neurotransmitter receptors, voltage-gated ion channels, and transcription factors, such as cAMP-responsive element binding protein (CREB) (Blank et al., 1997; Snyder et al., 1998; Yan et al., 1999; Strack et al., 1997; Bito et al., 1996). On the other hand, the concomitant activation of PKA enhances the expression of immediate-early genes, such as Fos, ΔFosB (a truncated isoform of FosB) and Jun (Koob, 1996; Kelz et al, 1999). Cdk5, a downstream target gene of ΔFosB, when

associated with a regulatory subunit, p35, it could phosphorylate DARPP-32 at Thr-75 site (P-Thr75-DARPP-32) (Maccioni et al, 2001; Bibb et al, 2001). P-Thr75-DARPP-32 is a strong inhibitor of PKA and attenuates the D1/PKA/P-Thr34-DARPP-32/PP-1 signaling (Bibb et al., 1999). On the contrary, there is also dephosphorylation mechanism to balance the phosphorylation of DARPP-32. For example, P-Thr34-DARPP-32 is dephosphorylated by PP-2B, a Ca2+/calmodulin-dependent protein phosphatase (Goto et al., 1986; King et al., 1984); while the P-Thr75-DARPP-32 is also dephosphorylated by PP2A (protein phosphotase-2A) in a PKA-dependent manner (Nishi et al., 2000).

The DARPP-32 cascade plays a critical role in mediating cocaine-induced behavioral changes. Acute cocaine administration elevates the P-Thr34-DARPP-32 and suppresses P-Thr75-DARPP-32 protein levels in the striatum of male rodents (Nishi et al., 2000; Rauggi et al., 2005; Takahashi et al., 2005; Synder et al., 2000). After chronic cocaine administration, the protein level of P-Thr75-DARPP-32 increases, accompanied by a decrease in the P-Thr34-DARPP-32 in both NAc and CPu of male rats (Bibb et al., 2001; Scheggi et al., 2004). Moreover, DARPP-32 knockout mice and mice with site mutations of P-Thr34-DARPP-32 show blunted acute cocaine-induced locomotor activity (Fienberg et al., 2006) and attenuated cocaine-induced conditioned place preference (CPP), but have heightened behavioral sensitization in response to repeated cocaine exposure (Zachariou et al., 2002). In contrast, mice with site mutations of P-Thr75-DARPP-32 show normal acute cocaine-induced hyper-locomotor response and cocaine-induced CPP, but exhibit reduced behavioral sensitization in response to repeated cocaine administration (Zachariou et al., 2006; Zhang et al., 2006). Taken together, these studies suggest that DARPP-32 is critical for short-term and long-term cocaine-induced

behavioral alternations. Chronic cocaine administration also enhances CREB phosphorylation and ΔFosB protein levels in the striatum (Walters and Blendy 2001; Fienberg et al., 1998; Hiroi et al., 1999). However, the induction of these two proteins exert opposite effects on drug response in that phosphorylated CREB seems to mediate tolerance to cocaine rewarding effect; while ΔFosB mediates cocaine-induced behavioral sensitization (for review, see Nestler, 2004). On the other hand, FosB shows reducing induction after repeated drug exposure, and it does not seem to correlate to behavior changes in male mice (Nestler et al., 2001). In striatum, chronic treatment with cocaine increases in Cdk5 and p35 protein levels (Bibb et al., 2001; Scheggi et al., 2004). Increased activation of Cdk5 has been implicated in mediating drug induced tolerant locomotor responses (Bibb et al., 2001; Taylor et al., 2007).

Although the role of DARPP-32 pathway in cocaine induced behavior responses has been well studied in male rodents, its role in cocaine-induced behavioral sex-differences is still unclear. Recently, our laboratory and others demonstrated sexual dimorphic responses to acute-cocaine treatment in DARPP-32-mediated signaling. In the NAc, Zhou et al. (2009) demonstrated that female rats had higher basal protein levels of DARPP-32 and P-Thr34-DARPP-32, but male rats exhibited higher activation of these proteins after acute cocaine administration. Lynch et al. (2007) also showed that through self-administration, female rats had higher protein levels of P-Thr34-DARPP-32 in the NAc and CPu than males. Nazarian et al. (2009) demonstrated females have higher basal and cocaine-induced PKA protein levels in the NAc than males, but the male rats showed longer-lasting induction of p-CREB protein levels after acute cocaine regimen. Together, these findings suggest that sex differences in the DARPP-32 signaling

regulation may also contribute to sex difference in the development of rewarding properties to cocaine. However, to date, no studies have considered the relationship between protein levels within the DARPP-32 cascade and behavior activities in respond to cocaine treatment. The aim of this present study was to determine whether chronic cocaine administration alters the DARPP-32 intracellular cascades in a sexually dimorphic pattern, and whether the alternation pattern is correlated with their behavior responses.

II. Methods

Animals: 60-day-old male and female Fischer rats (Charles River, Raleigh, NC) were individually housed in Plexiglas chambers (20 × 20 × 41 cm) layered with beta chips. Rats were given free access to food and water and maintained on a 12-hour light/dark cycle (lights on at 9:00 a.m.). All rats were weighed, handled for 7 days prior to testing. Animals were randomly assigned to experimental groups. Animal care and use was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD), and approved by the Hunter Institution Animal Care Use Committee.

Drug and administration: Cocaine hydrochloride was purchased from Sigma chemical Co. (St. Louis, MO). Cocaine solutions were prepared daily by dissolving in physiological saline (0.9%). Throughout the study, all injections were administered in each rat's home cage. For the chronic administration paradigm, daily saline or cocaine (15mg/kg) was injected (i.p.) into male and female rats from saline, acute or chronic

groups, and with different days of administration (2, 5 and 14 days). In the saline group, all animals received daily saline injection; in the acute group, saline was injected all the times, except for the last testing day, in which a single cocaine injection was given; in the chronic group, all rodents were administered by daily cocaine.

Behavior measurements: Behavior measurements were carried on in the home cage for each rat for 60 min immediately after drug treatment. Both locomotor and stereotyped behaviors were recoded. For locomotor behavior, a Cage Rack Photobeam Activity System from San Diego Instruments I (San Diego, CA) was used to monitor the ambulatory and rearing activities. The system consists of two frames placed around the rat's home cage. Ambulatory activity was determined by total counts of two consecutive photobeam interruptions in the lower frame. Rearing activity was represented as total counts of vertical motions detected by the upper frame. Total locomotor activity represents the sum of all counts in the horizontal frame. Stereotyped behavior was videotaped for 45 seconds each at 15, 30, and 45 minutes after administration. The videotapes were analyzed by three trained observers, who were blind to the animal's treatment conditions. The Creese and Iversen rating scale (1974) was used for the rating of the cocaine-induced stereotyped behavior.

Calculation for locomotor counts differences: To summarize the effect of repeated exposure to cocaine administration on locomotor behavior sensitization or tolerance in male and female rats, the differences of locomotor counts between acute and chronic groups were used as an indicator. Briefly, ambulatory, rearing or total locomotor counts

of individual rats in the chronic-cocaine treated group were subtracted from the average locomotor counts of the correspondent acute-cocaine treated rats.

Brain tissue dissection and protein preparation: After decapitation (following a brief 20 s exposure to), rat brains were removed, flash frozen in 2-methylbutane (-40° C), and stored at -80° C until used. The areas of CPu and NAc were dissected out from coronal sections (2mm thick) and homogenized using a Polytron handheld homogenizer (Kinematica, Luzern, Switzerland) in a Lysis buffer [(50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.02% sodium azide) containing protease inhibitors mixture (pepstatin, leupeptin, 1M DTT, aproteinin, 100 mM PMSF, 50mM NaF, and 1 mM)]. Total protein content was determined using a Bradford kit from Bio-Rad Laboratories (Hercules, CA).

Antibodies: DARPP-32 antibody and antibody recognizing both FosB and ΔFosB was purchased from Cell Signaling Technologies (Beverly, MA). P-Thr34-DARPP-32, P-Thr75-DARPP-32, PP1α, CaN-A and CaN-B antibodies were purchased from Sigma (St Louis, MO). PP-2Ab, PP-2Ac, Cdk5, p35 and α-tubulin antibodies were purchased from Santa Cruz Technologies (Santa Cruz, CA). PKAc (catalytic subunit of PKA) antibody was purchased from BD Biosciences (San Jose, CA). p-CREB antibody was purchased from Millipore (Billerica, MA). All appropriate secondary antibodies were purchased from Amersham Pharmacia (Piscataway, NJ).

Western blot analysis: Protein samples were boiled in Lammeli buffer containing 1% β-mercaptoethanol and loaded onto SDS-PAGE. Gels were electrophoresed, transferred to nitrocellulose membranes, and blocked for 30 min with 5% non-fat dry milk in tris-buffer-saline-tween (TBST) at room temperature. Membranes were then probed with primary antibodies overnight at 4°C. After three washes with TBST, membranes were incubated with their appropriate secondary antibody for 60 min at room temperature, followed by three more washes with TBST. For normalization of protein levels, all membranes were re-probed with α-tubulin antibody followed with the secondary antibody. Antibody binding was detected using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia, Piscataway, NJ). Films were then quantified using a computer densitometer and Image Quant Program (Molecular Dynamics).

Statistical analysis of data: Western blot data were presented as specific protein levels normalized to α-tubulin levels. Stereotypic data were analyzed by calculating the area under the curve (AUC) for the activity score plotted against time. Two-way analysis of variance (ANOVAs) followed by Post Hoc tests was used for behavior and protein level analysis. Pearson Correlation Coefficient was used for correlation between behavior activities and protein levels. Determination of statistically significant differences was considered at the 0.05 probability level ($p < 0.05$).

III. Results

A. Sex differences in behavioral responses to chronic cocaine administration

1. Sex difference in cocaine-induced ambulatory activity

As shown in Fig. 9, two-way ANOVA showed significant main effect of treatment in all durations [2 days: $F_{(2,50)}$=24.890, p<0.001; 5 days: $F_{(2,54)}$=25.904, p<0.001; and 14 days: $F_{(2,53)}$=30.485, p<0.001]; significant main effect of gender after all treatments [2 days: $F_{(1,50)}$=5.333, p<0.05; 5 days: $F_{(1,54)}$=11.195, p<0.01; and 14 days: $F_{(1,53)}$=12.818, p<0.01]. Significant main effect of Treatment × Gender interaction after 2, and 14 days of administration was also shown [2 days: $F_{(2,50)}$=3.815, p<0.05; and 14 days: $F_{(2,53)}$=5.527, p<0.01, respectively]. However, no main effect of interaction between treatment and gender was observed after 5 days of drug administration.

Overall, regardless of gender and treatment duration, both acute and chronic cocaine treatment significantly increased ambulatory activity as compared with their saline controls (p<0.05 for all comparisons; Fig. 9). In male rats, ambulatory activity was significantly higher after 5 days of chronic cocaine treatment when compared to acute-cocaine treated group, indicating the development of behavior sensitization (p<0.01; Fig. 9B). However, no difference between acute and chronic cocaine treated groups was found after 2 or 14 days of treatment (Fig. 9A and 9C). In females, chronic cocaine treatment also produced sensitized higher ambulatory activities than acute treatment after 2 days of administration (p<0.01; Fig. 9A). However, female rats receiving chronic cocaine administration for 14 days demonstrated significantly lower ambulatory activity than those receiving acute cocaine administration, showing behavior tolerance (p<0.001; Fig. 9C). No differences between acute and chronic cocaine treated groups were observed after 5 days of administration (Fig. 9B).

Females revealed higher ambulatory activity than males in the chronic cocaine groups after 2 days of administration (p<0.05; Fig. 9A); in the acute-cocaine groups after

5 days of administration ($p<0.01$;Fig. 9B); and in both saline and acute-cocaine groups after 14 days of administration ($p<0.05$ and $p<0.01$, respectively; Fig. 9C).

2. Sex difference in cocaine-induced rearing activity

As shown in Fig. 10, two-way ANOVA showed significant main effect of treatment in all treatment durations [2 days: $F_{(2,50)}=42.684$, $p<0.001$; 5 days: $F_{(2,54)}=35.628$, $p<0.001$; and 14 days: $F_{(2,53)}=32.317$, $p<0.001$]. Significant main effect of gender was shown after 2 and 14 days of administration, and a marginal significant effect was obtained after 5 days of administration [2-days: $F_{(1,50)}=16.588$, $p<0.001$; 14-days: $F_{(1,53)}=10.594$, $p<0.01$; and 5-days: $F_{(1,54)}=3.799$, $p=0.056$]. Two-way ANOVA also showed significant main effect of Treatment × Gender interaction after 2 and 14 days of administration, but not after 5 days of administration [2 days: $F_{(2,50)}=7.460$, $p<0.01$; and 14 days: $F_{(2,53)}=3.867$, $p<0.05$].

In both male and female rats, acute- and chronic-cocaine treatment significantly increased rearing activity as compared with their saline controls regardless of treatment length ($p<0.05$ for all comparisons; Fig. 10). However, no differences between the acute and chronic cocaine treated groups were observed in male rats. In females, after 2 days of drug administration, rearing activity was significantly higher in chronic cocaine treated group when compared with acute cocaine treated group, suggesting the establish of sensitization ($p<0.01$; Fig. 10A). However, 5 and 14 days of chronic cocaine administration induced behavior tolerance, that lower rearing activity when compared to their correspondent acute cocaine treatment ($p<0.02$ and $p<0.001$; Fig. 10B and 10C).

In comparison with males, female rats revealed higher rearing activity in the chronic-cocaine groups after 2 days of administration ($p<0.001$; Fig. 10A); in acute-cocaine groups after 5 and 14 days of administration ($p<0.05$ and $p<0.01$, respectively; Fig. 10B and 10C).

3. Sex difference in cocaine-induced total locomotor activity

As seen in Fig. 11, two-way ANOVA showed significant main effect of treatment in all durations [2 days: $F_{(2,50)}=35.873$, $p<0.001$; 5 days: $F_{(2,54)}=41.365$, $p<0.001$; and 14 days: $F_{(2,53)}=42.184$, $p<0.001$]; significant main effect of gender after all treatments [2-days: $F_{(1,50)}=10.195$, $p<0.01$; 5-days: $F_{(1,54)}=24.359$, $p<0.001$; and 14-days: $F_{(1,53)}=14.913$, $p<0.001$]. Significant main effect of Treatment × Gender interaction after 2 and 14 days of administration was also observed [2 days: $F_{(2,50)}=4.109$, $p<0.05$; 5 days: $F_{(2,54)}=3.329$, $p<0.05$; and 14 days: $F_{(2,53)}=7.408$, $p<0.01$].

Regardless of gender and drug treatment duration, both acute and chronic cocaine treatment significantly increased total locomotor activity as compared with their saline controls ($p<0.05$ for all comparisons; Fig. 11). In male rats, after 5 days of chronic cocaine treatment induced sensitized total locomotor activity when compared to acute-cocaine treated group ($p<0.01$; Fig. 11B). No difference between acute and chronic cocaine-treated groups was found after 2 or 14 days of treatment (Fig. 11A and 11C). In females, after 2 days of administration, chronic cocaine treatment also result in behavior sensitization in total locomotor activities as compared to acute treatment ($p<0.01$; Fig. 11A). However, female rats receiving chronic cocaine administration for 14 days demonstrated significantly lower locomotor activity than those receiving acute cocaine administration, indicating the development of behavior tolerance ($p<0.001$; Fig. 11C). No

differences between acute and chronic cocaine treated groups were observed after 5 days of administration (Fig. 11B).

Female rats revealed higher total locomotor activity than males in both saline and chronic-cocaine groups after 2 days of administration ($p<0.05$ and $p<0.01$, respectively; Fig. 11A); in both acute- and chronic-cocaine groups after 5 days of administration ($p<0.01$ and $p<0.02$, respectively; Fig. 11B); and in both saline and acute-cocaine groups after 14 days of administration ($p<0.05$ and $p<0.01$, respectively; Fig. 11C).

4. Sex difference in locomotor activity counts differences between acute and chronic groups

Further analysis of the locomotor activity differences between acute and chronic cocaine treated groups was shown in Figure 12. After 2 days of cocaine administration, female rats developed sensitized ambulatory, rearing and total locomotor activities ($p<0.05$ for all comparisons; Fig. 12A). After 5 days of cocaine administration, male rats developed ambulatory and total locomotor sensitization, while females developed tolerance in rearing activities ($p<0.05$ for all comparisons; Fig. 12B). After 14 days of cocaine administrations, female rats developed behavior tolerance in ambulatory, rearing and total locomotor activities ($p<0.05$ for all comparisons; Fig.12C).

5. Sex differences in cocaine-induced stereotypic behavior

As shown in Fig. 13, two-way ANOVA showed significant main effect of treatment in all durations [2 days: $F_{(2,58)}=26.440$, $p<0.001$; 5 days: $F_{(2,58)}=39.699$, $p<0.001$; and 14 days: $F_{(2,58)}=51.919$, $p<0.001$]; significant main effect of gender after all

treatments [2-days: $F_{(1,58)}$=11.585, p<0.001; 5-days: $F_{(1,58)}$=10.631, p<0.01; and 14-days: $F_{(1,58)}$=21.246, p<0.001]. No significant main effect of Treatment × Gender interaction was observed throughout the treatment length.

In general, both acute and chronic cocaine treatment significantly increased the stereotypic behavior in male and female rats regardless the length of treatment (P<0.001 for all comparisons; Fig. 13). Differences between male and female rats were also observed along the treatment duration. After 2 days of chronic cocaine treatment, higher stereotypic behavior was observed in females when compared to males (p<0.02, Fig. 13A). After 5 days of treatment, both saline and acute cocaine treatment stimulated higher stereotypic activities in females when compared to male rats (p<0.05, for both comparisons, Fig. 13B). After 14 days of treatment, female rats exhibited higher stereotypic behavior 15 min after acute- or chronic-cocaine treatment (p<0.001 and p<0.05, respectively; Fig. 13C).

B. Sex differences in DARPP-32 pathway signaling after chronic cocaine treatment

1. DARPP-32 signaling Sex differences in NAc

In the NAc of male rats, although P-Thr34-DARPP-32 protein levels were higher after 5 days of treatment than after 14 days, main effect of day failed to reach statistical significance, (p<0.05; $F_{(2,18)}$=3.189, p=0.065; respectively; Fig. 14B). However, the protein levels of DARPP-32 and P-Thr75-DARPP-32 did not significantly change along time (Fig. 14A and 14C). In female rats, no significant changes in the protein levels of DARPP-32, P-Thr34-DARPP-32 or P-Thr75-DARPP-32 were observed (Fig. 14D-F).

As shown in Fig. 15C, in male rats, main day effect on PP-2Ab protein levels

were observed, that 14 days of treatment significantly decreased PP-2Ab protein levels than 2 or 5 days ($F_{(2,18)}$=5.247, $p<0.02$; $p<0.01$ and $p<0.05$, respectively). In addition, when compared to 14 days of saline treatment, acute and 14-day chronic cocaine treatment significantly lowered protein levels of PP-2Ab in male NAc ($p<0.05$ and $p<0.02$, respectively, Fig. 15C). However, PP-2Ac protein levels did not alter in male rats (Fig. 15D). In female rats, no significant changes in either PP-2Ab or PP-2Ac protein levels were observed in female rats (Fig. 15G and H). No significant changes in CaN-A or CaN-B protein levels were observed in either sex (Fig. 15A-B and 15E-F).

In male rats, there were main day effect on PKAc and p-CREB protein levels ($F_{(2,18)}$=3.839, $p<0.05$; $F_{(2,18)}$=19.722, $p<0.001$, respectively; Fig. 16A and C). Specifically, PKAc protein levels were higher after 5 days of treatment than 2 days ($p<0.02$; Fig. 16A). p-CREB protein levels were also elevated after 5 days, as compared to 2 and 14 days ($p<0.001$ for both comparisons; Fig. 16C). In addition, both acute and chronic cocaine treatment increased p-CREB protein levels in male rats when compared to saline treatment for 5 days ($p<0.001$ and $p<0.02$; Fig. 16C). PP-1α protein levels did not change in the NAc of males (Fig. 16B). In females, significant main effect of day were also observed in p-CREB protein levels, indicating that the p-CREB protein level was lower after 2 days of treatment than 5 or 14 days ($F_{(2,18)}$=4.673, $p<0.05$; $p<0.05$ and $p<0.01$, respectively; Fig. 16F). However, no significant changes were observed in PKAc and PP-1α protein levels (Fig. 16D and 16E).

In males, as shown in Fig. 17A and B, both FosB and ΔFosB protein levels were higher after 5 days than 14 days of treatment ($p<0.02$ and $p<0.01$). However, main day effect were only observed in ΔFosB, but failed to reach statistical significance in FosB

($F_{(2,18)}$=4.412, p<0.05; $F_{(2,18)}$=3.449, p=0.054, respectively). Five-day cocaine treatment also increased ΔFosB protein levels than saline treatment for 5 days (p<0.05; Fig. 17B). Changes in Cdk5 and p35 protein levels were also similar, that they were both elevated after 5 days of treatment when compared with 2 and 14 days, and day effected reached significance in both proteins (Cdk5: $F_{(2,18)}$=6.037, p<0.01; p<0.01 and p<0.05; p35: $F_{(2,18)}$=12.175, p<0.001; p<0.001 for both comparisons; Fig. 17C and D). In the NAc of female rats, main day effect were observed in both FosB and ΔFosB ($F_{(2,18)}$=3.956, p<0.05; $F_{(2,18)}$=3.881, p<0.05, respectively; Fig. 17E and F). However, the protein level changes along time were different -- after 5 days of treatment, FosB protein levels were lower, while ΔFosB protein levels were higher, when compared with their protein levels after treatment for 2 days (p<0.02 for both comparisons; Fig. 17E and F). In addition, regardless of treatment length, both acute and chronic treatment elevated FosB protein levels compared to their controls, except for 14-day cocaine treatment (p<0.05 for all comparisons; Fig. 17E). Five- and 14-day cocaine treatment also increased ΔFosB protein levels when compared with saline (p<0.05 for both comparisons; Fig. 17E). No significant changes were observed in Cdk5 or p35 protein levels in the NAc of female rats (Fig. 16G and 17H).

2. DARPP-32 signaling sex differences in CPu

In males, no significant changes were observed in the protein levels of DARPP-32, P-Thr34-DARPP-32 or P-Thr75-DARPP-32 (Fig. 18A-C). In female rats, 14 days of treatment increased DARPP-32 and P-Thr34-DARPP-32 protein levels when compared with 2 days of treatment (p<0.02 for both comparisons; Fig. 18D and 18E). However,

significant main day effect were observed in DARPP-32 protein levels only, it failed to reach statistic significance in P-Thr34-DARPP-32 protein levels ($F_{(2,18)}$=4.109, $p<0.05$; and $F_{(2,18)}$=3.415, p=0.055, respectively; Fig. 18E and 18F). No significant protein level changes were observed in P-Thr75-DARPP-32 (Fig. 18F).

In male rats, no changes were observed in the protein levels of CaN-A, CaN-B, PP-2Ab or PP-2Ac (Fig. 19A-D). In females, two-way ANOVA showed significant main effect of treatment, main effect of day, and main effect of Treatment × Day interaction in CaN-A protein levels ($F_{(1,18)}$=4.971, $p<0.05$; $F_{(2,18)}$=7.105, $p<0.01$; $F_{(2,18)}$=3.682, $p<0.05$, respectively; Fig. 19E). Post-hoc tests revealed higher protein levels after 14-day chronic cocaine treatment, when compared with its correspondent saline and acute cocaine treatment, as well as to chronic cocaine treatment for 2 and 5 days ($p<0.02$ for all comparisons). However, CaN-B protein levels did not change along time (Fig. 19F). Similar to males, the PP-2Ab and PP-2Ac did not change along time in the CPu of female rats (Fig. 19G and H).

In male rats, main day effect were observed in PP-1α protein levels, that 14 days of treatment increased protein levels compared to 2 and 5 days of treatment ($F_{(2,18)}$=5.661, $p<0.02$; $p<0.01$ and $p<0.05$, respectively; Fig. 20B). Compared to its saline control, 2-day chronic cocaine treatment decreased PP-1α protein levels in the CPu of male rats ($p<0.05$; Fig. 20B). However, no significant changes were observed in the protein levels of PKAc and p-CREB (Fig. 20A and C). In females, main effect of day was observed in PKAc protein levels, that PKAc protein levels were higher after 14 days of treatment than 2 and 5 days ($F_{(2,18)}$=7.340, $p<0.01$; $p<0.05$ and $p<0.001$, respectively; Fig. 20D). As shown in Fig. 20E, both main effect of treatment and day were observed in PP-1α protein levels

($F_{(1,18)}$=10.962, p<0.01; $F_{(2,18)}$=8.925, p<0.01, respectively). Specifically, chronic cocaine treatment elevated PP-1α protein levels higher than acute cocaine treatment. Also, PP-1α protein levels were higher after 14 days of treatment than 2 or 5 days of treatment (p<0.001 and p<0.01). In addition, 14-day chronic cocaine treatment also elevated PP-1α protein levels compared to its saline control (p<0.05). Two-way ANOVA showed significant main effect of treatment in female p-CREB protein levels, that chronic cocaine significantly increased p-CREB protein levels as compared to acute cocaine treatment ($F_{(1,18)}$=5.418, p<0.05; Fig. 20F). Main effect of day failed to reach statistical significance, although p-CREB protein levels were higher after 14 days of treatment than 5 days ($F_{(2,18)}$=3.337, p=0.059; p<0.05, respectively; Fig. 20F).

In male rats, there was a main day effect for FosB and ΔFosB ($F_{(2,18)}$=4.368, p<0.05; $F_{(2,18)}$=5.754, p<0.02, respectively; Fig. 21A and B). The protein levels of FosB and ΔFosB were both higher after 5 days of treatment than 2 days (p<0.01 for both comparisons). In addition, 14-day chronic cocaine treatment increased the protein levels of FosB when compared to saline controls (p<0.05). However, 2-day chronic cocaine treatment decreased ΔFosB protein levels than its saline controls (p<0.05). Although the main day effect failed to reach statistical significance, the protein levels of Cdk5 after 14 days treatment were higher when compared to 2 days of treatment ($F_{(2,18)}$=3.538, p=0.051; p<0.05, respectively; Fig. 21C). However, no changes were observed in p35 protein levels (Fig. 21D). In the CPu of female rats, main effect of day was observed in FosB protein levels, that its protein levels were higher after 2 days of treatment than 5 days ($F_{(2,18)}$=3.734, p<0.05; p<0.02, respectively; Fig. 21E). 2-day chronic cocaine treatment and acute cocaine treatment on day 5 both significantly increased FosB protein levels as

compare to their saline controls ($p<0.05$ for both comparisons). On the contrary, no significant day effect was observed in ΔFosB protein levels. Instead, there was significant main effect of treatment that chronic cocaine significantly increased ΔFosB protein levels when compared with acute cocaine treatment ($F_{(1,18)}=6.185$, $p<0.05$; Fig. 21F). Five- and 14-day chronic cocaine treatment also significantly elevated ΔFosB protein levels than their saline controls ($p<0.02$ and $p<0.05$). In females, Cdk5 protein levels were higher after 14 days of treatment than 2 or 5 days, and main day effect also reached statistical significance ($p<0.05$ and $p<0.001$; $F_{(2,18)}=7.255$, $p<0.01$, respectively; Fig. 21G). Two-way ANOVA showed significant main effect of treatment, as well as main effect of Treatment × Day interaction in p35 protein levels ($F_{(1,18)}=8.931$, $p<0.01$; $F_{(2,18)}=11.049$, $p<0.001$, respectively; Fig. 21H). Post-hoc tests revealed higher protein levels after 14-day chronic cocaine treatment, when compared with its correspondent acute cocaine treatment, as well as to chronic cocaine treatment for 2 and 5 days ($p<0.05$ for all comparisons). In addition, acute cocaine treatment on day 5 and 14 significantly lowered p35 protein levels than acute cocaine treatment on day 2; acute cocaine on day 14 administration also lowered p35 protein levels compared to its saline controls ($p<0.02$ for all comparisons).

C. Correlation between behavior activity and DARPP-32 cascade protein levels

In male rats, as shown in Fig. 22, the ΔFosB protein levels in CPu positively correlate with the stereotypic counts of the males ($r=0.351$, $p<0.05$).

In female rats, the FosB protein levels in the NAc positively correlate with ambulatory, rearing, total locomotor and stereotypic counts ($r=0.489$, $p<0.01$; $r=0.445$,

p<0.01; r=0.500, p<0.01; and r=0.504, p<0.01, respectively; Fig. 23 A-D). Similarly, the FosB protein levels in the CPu also positively correlate with these behaviors (ambulatory: r=0.480, p<0.01; rearing: r=0.552, p<0.001; total: r=0.513, p<0.001; and stereotypic: r=0.532, p<0.001, respectively; Fig. 24A-D). On the other hand, the Cdk5 protein levels in the CPu of female rats negatively correlate with locomotor and stereotypic activities (ambulatory: r= -0.361, p<0.05; rearing: r= -0.435, p<0.01; total: r= -0.389, p<0.02; and stereotypic: r= -0.409, p<0.02, respectively; Fig. 25A-D), while p35 protein levels in CPu negatively correlate with locomotor activities only (ambulatory: r= -0.409, p<0.02; rearing: r= -0.391, p<0.02; total: r= -0.366, p<0.05; and stereotypic: r= -0.338, p<0.05, respectively; Fig. 26A-D).

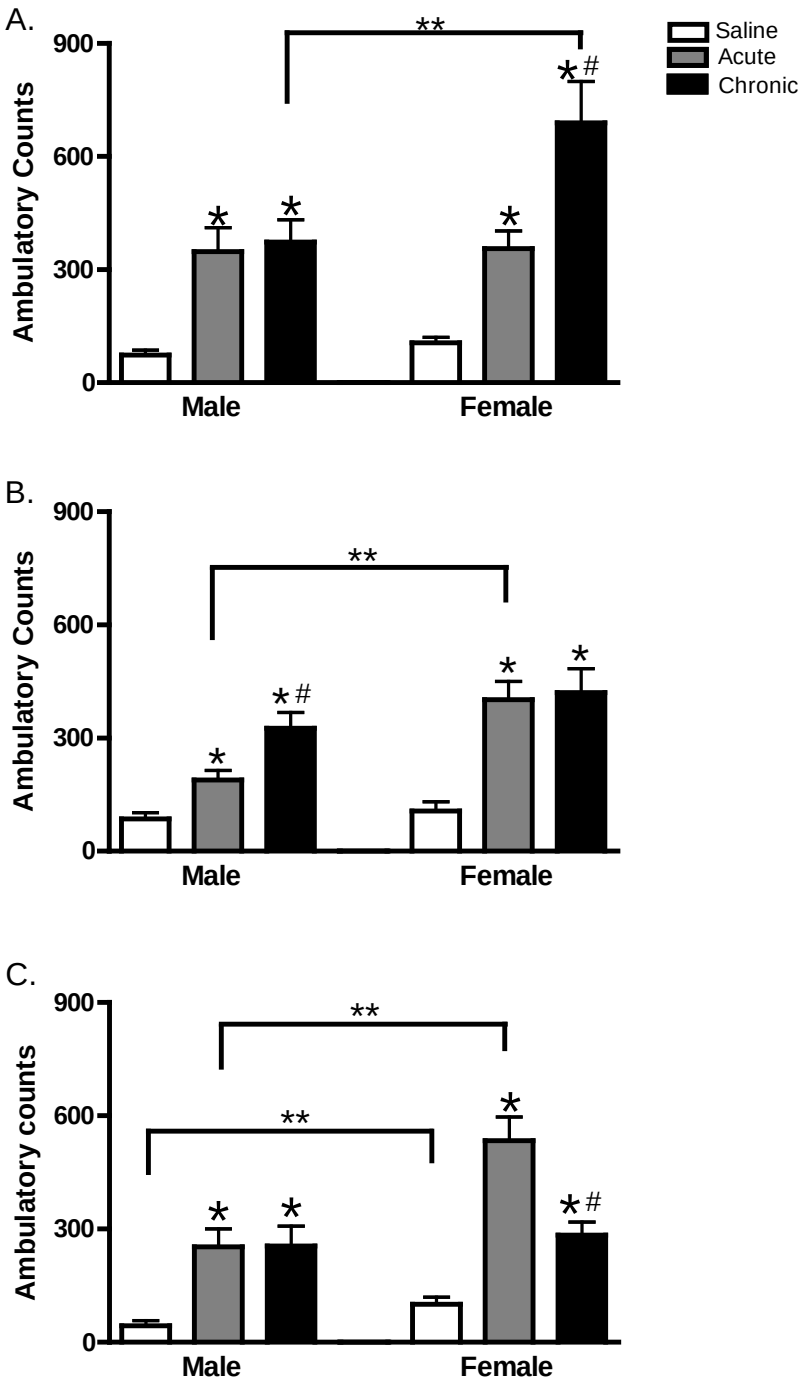

Figure 9: Ambulatory activities after 2 (A), 5 (B), and 14 (C) days. Data presented as mean±S.E.M of the sum of ambulatory counts 1 hour after treatment. * Represents significant differences to saline groups. # Represents significant differences between acute and chronic groups. ** Represents significant differences between male and female rats for the same treatment groups ($p<0.05$). (n=9-11 per group)

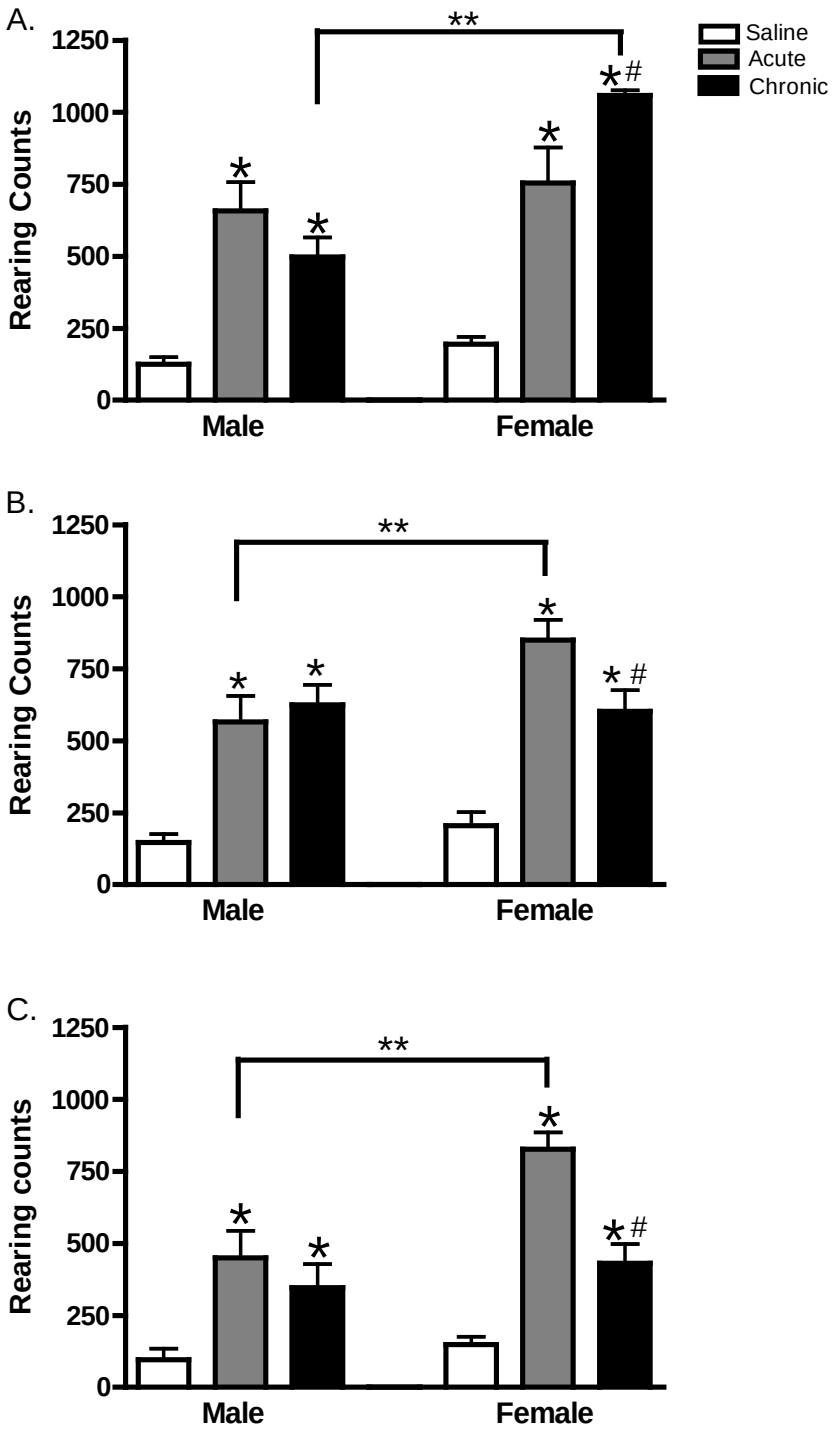

Figure 10: Rearing activities after 2 (A), 5 (B), and 14 (C) days. Data presented as mean±S.E.M of the sum of rearing counts 1 hour after treatment. * Represents significant differences to saline groups. # Represents significant differences between acute and chronic groups. ** Represents significant differences between male and female rats for the same treatment groups ($p<0.05$). (n=9-11 per group)

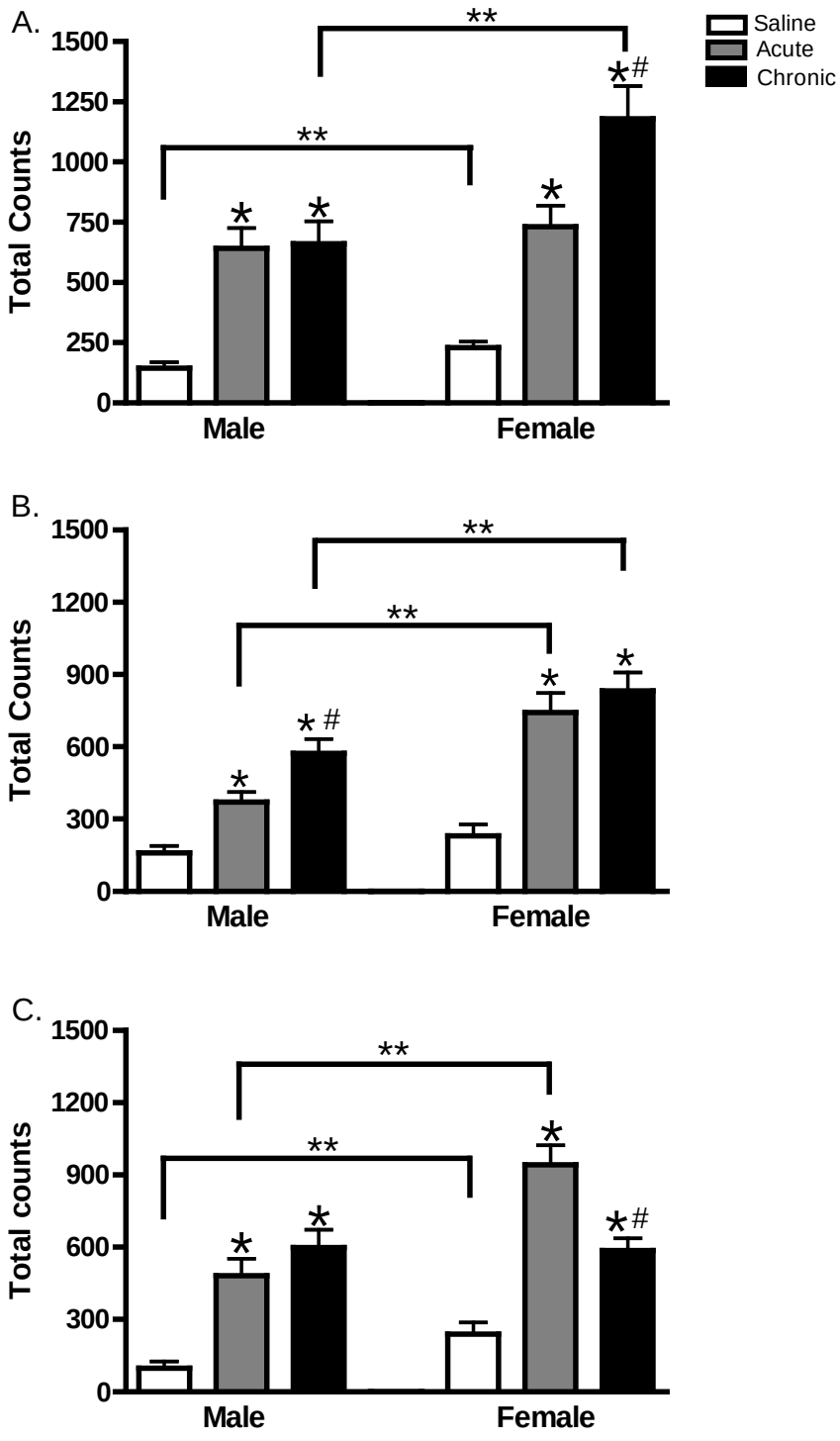

Figure 11: Total locomotor activities after 2 (A), 5 (B), and 14 (C) days. Data presented as mean±S.E.M of the sum of total locomotor counts 1 hour after treatment. * Represents significant differences to saline groups. # Represents significant differences between acute and chronic groups. ** Represents significant differences between male and female rats for the same treatment groups ($p<0.05$). (n=9-11 per group)

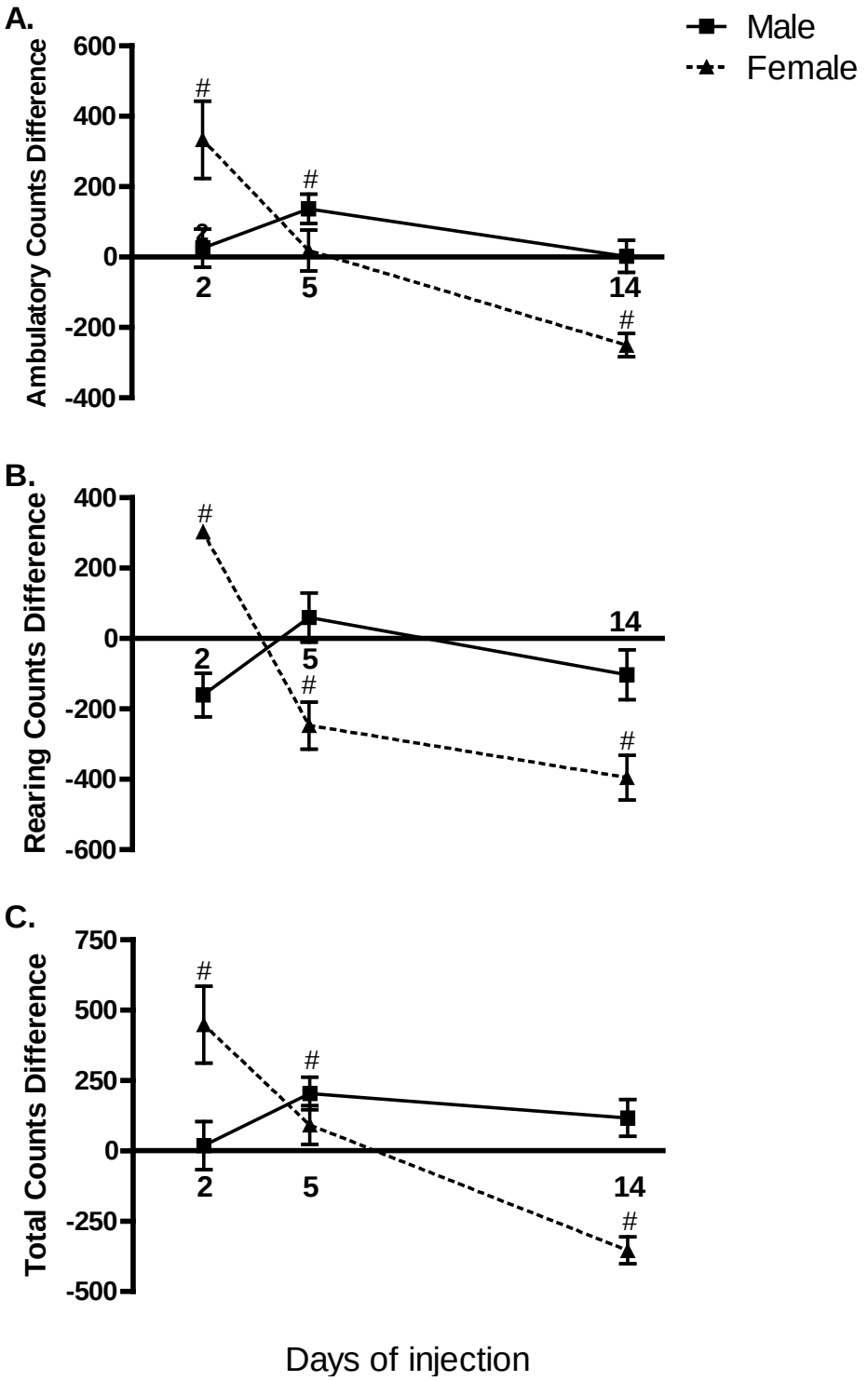

Figure 12: Ambulatory (A), rearing (B), and total locomotor (C) counts differences between acute and chronic cocaine treated groups after 2, 5, and 14 days. Data presented as mean±S.E.M of individual chronic counts – average acute counts. # Represents significant difference between acute and chronic at $p<0.05$ level. (n=9-11 per group)

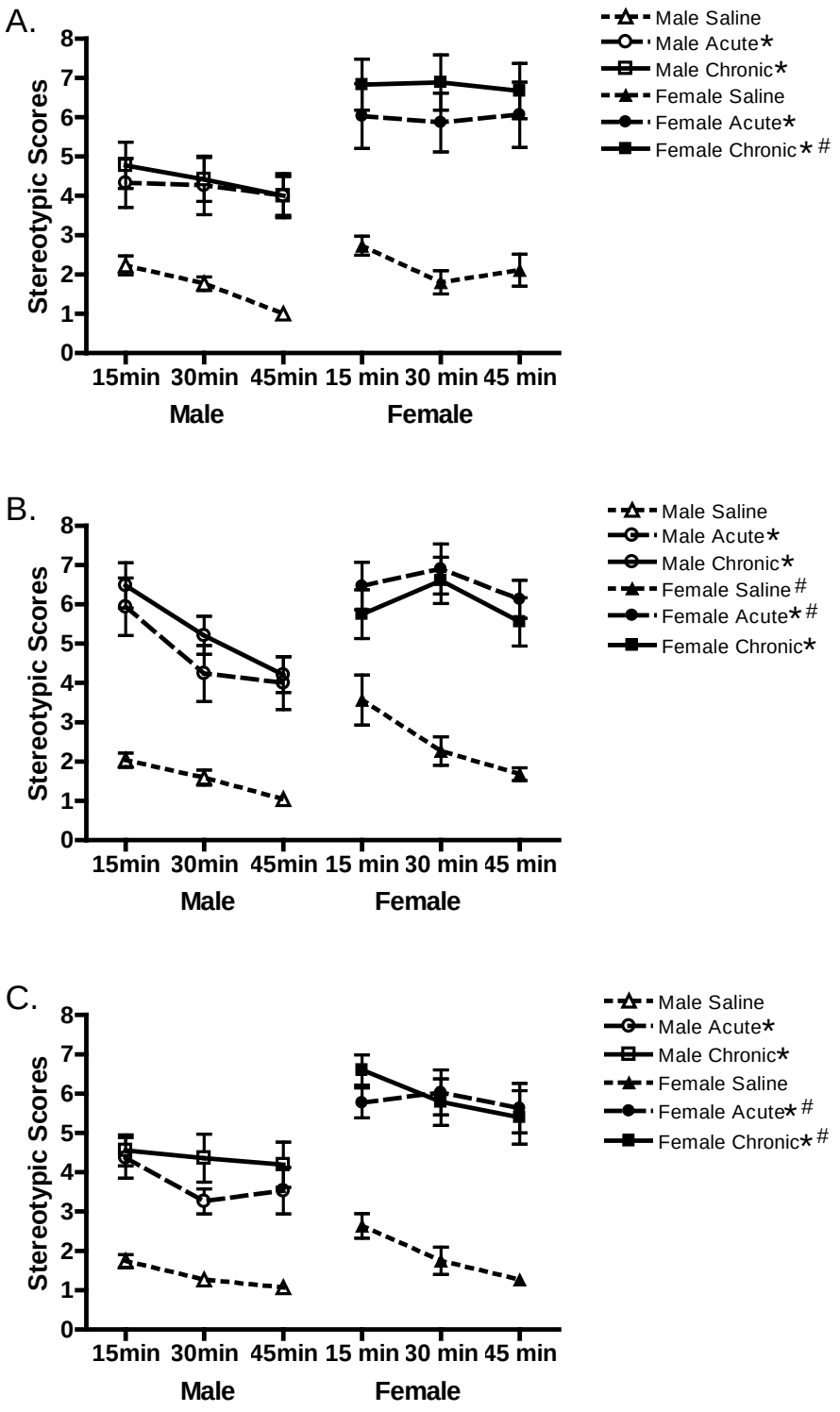

Figure 13: Stereotypic activities after 2 (A), 5 (B), and 14 (C) days. Data presented as mean±S.E.M of the stereotypic scores 15, 30 and 45 minutes after treatment. * Represents significant differences to saline group of the same sex. # Represents significant differences between male and female rats for the same treatment. (n=9-11 per group)

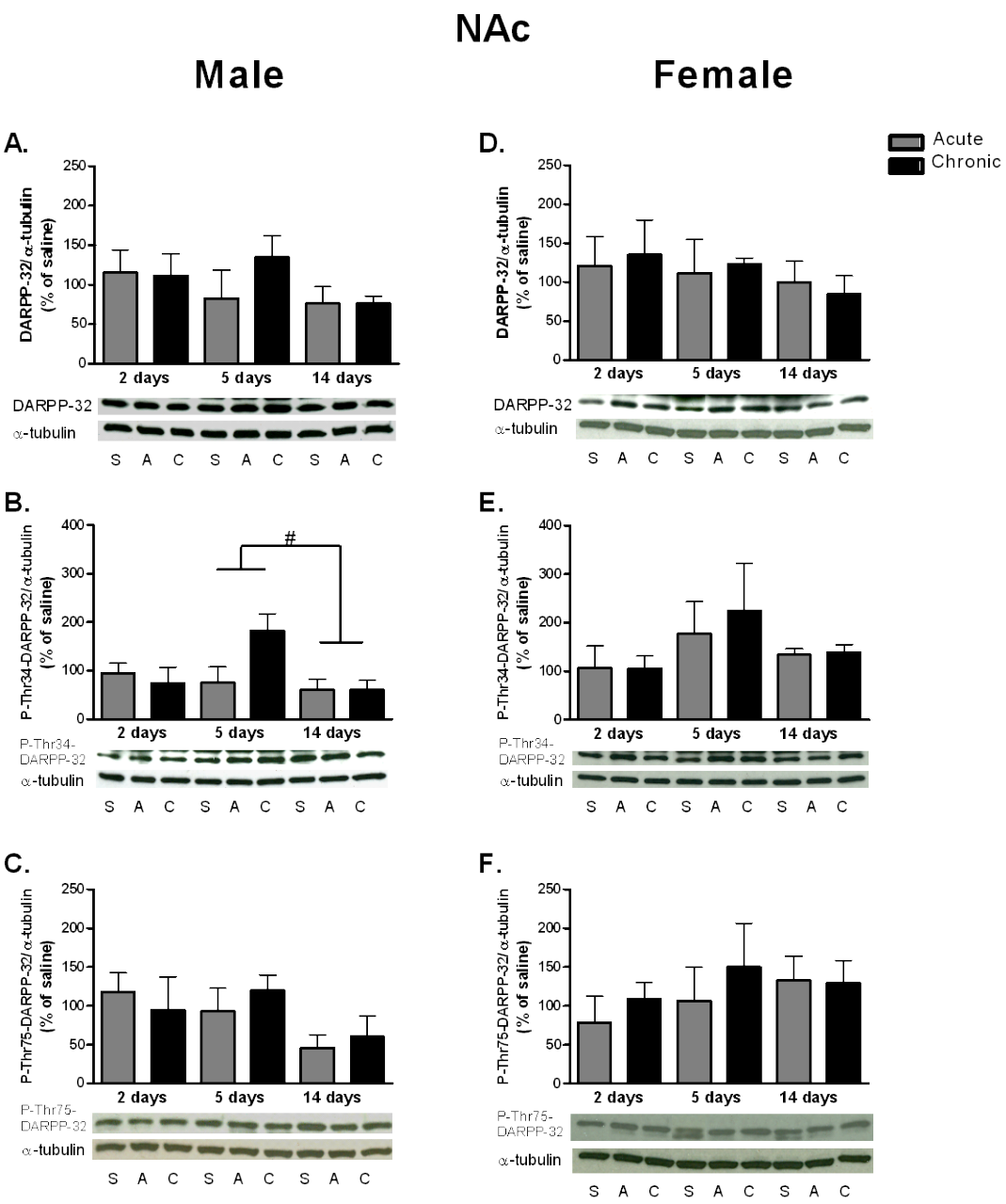

Figure 14: Western blot analysis of DARPP-32 (A, D), P-Thr34-DARPP-32 (B, E) and P-Thr75-DARPP-32 (C, F) in the NAc of male (A-C) and female (D-F) rats after acute and chronic cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). # Represents significant difference between different lengths of treatment ($p<0.05$). (n=4 per group)

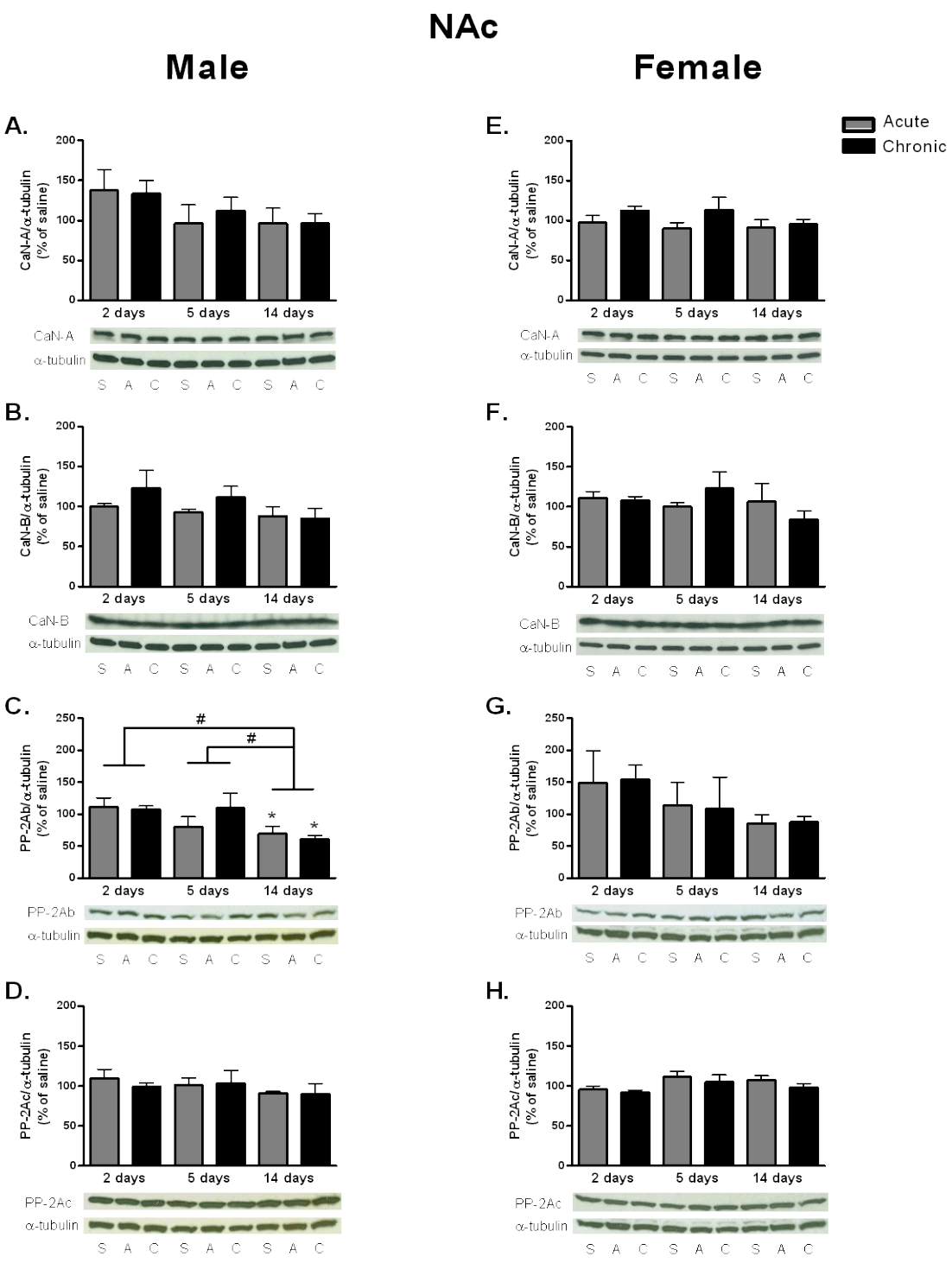

Figure 15: Western blot analysis of CaN-A (A, E), CaN-B (B, F), PP-2Ab (C, G) and PP-2Ac (D, H) in the NAc of male (A-D) and female (E-H) rats after acute and chronic cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). # Represents significant difference between different lengths of treatment. * Represents significant difference to saline control of the same length of treatment ($p<0.05$). (n=4 per group)

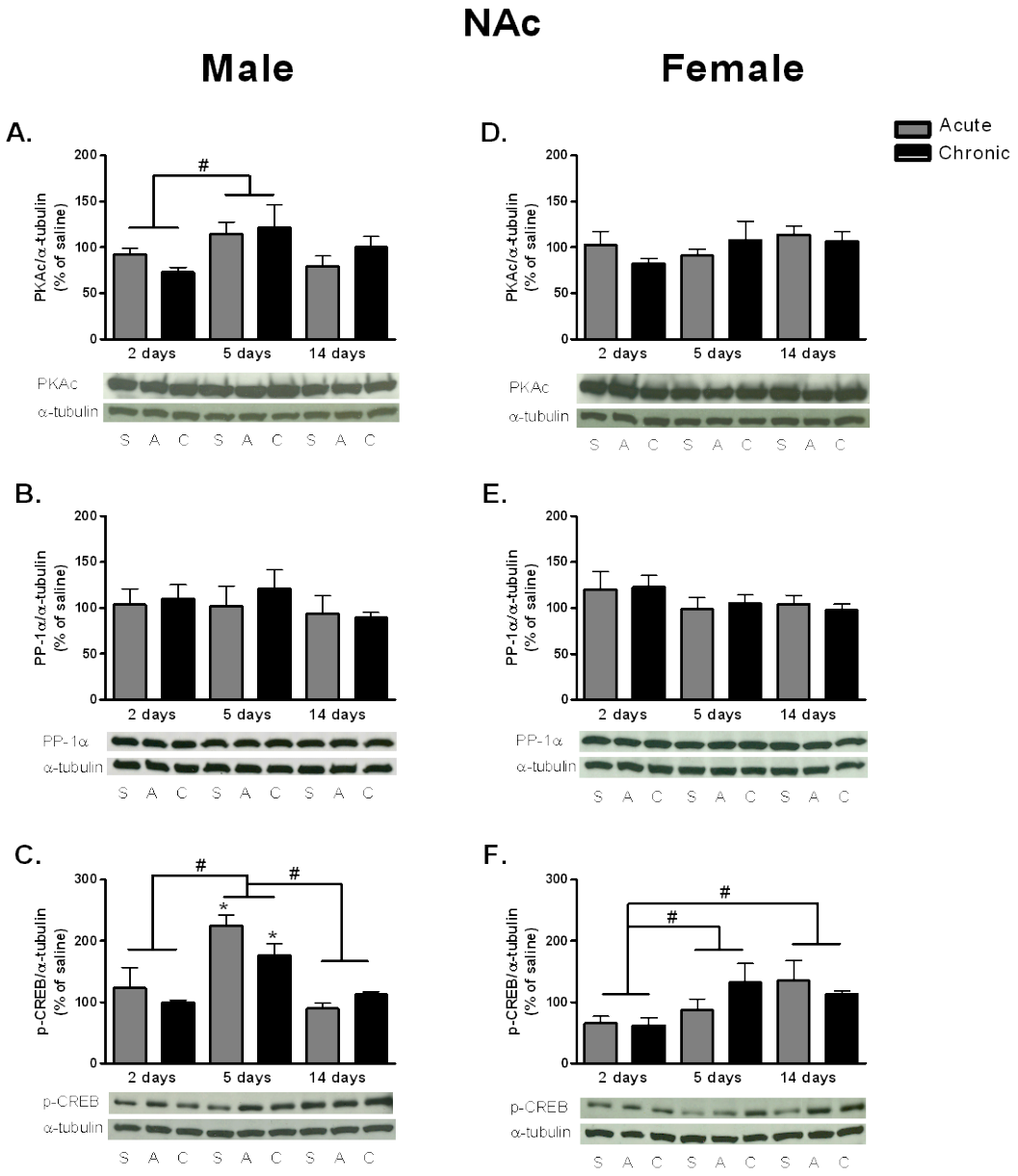

Figure 16: Western blot analysis of PKAc (A, D), PP-1α (B, E) and p-CREB (C, F) in the NAc of male (A-C) and female (D-F) rats after acute and chronic cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). # Represents significant difference between different lengths of treatment. * Represents significant difference to saline control of the same length of treatment ($p<0.05$). (n=4 per group)

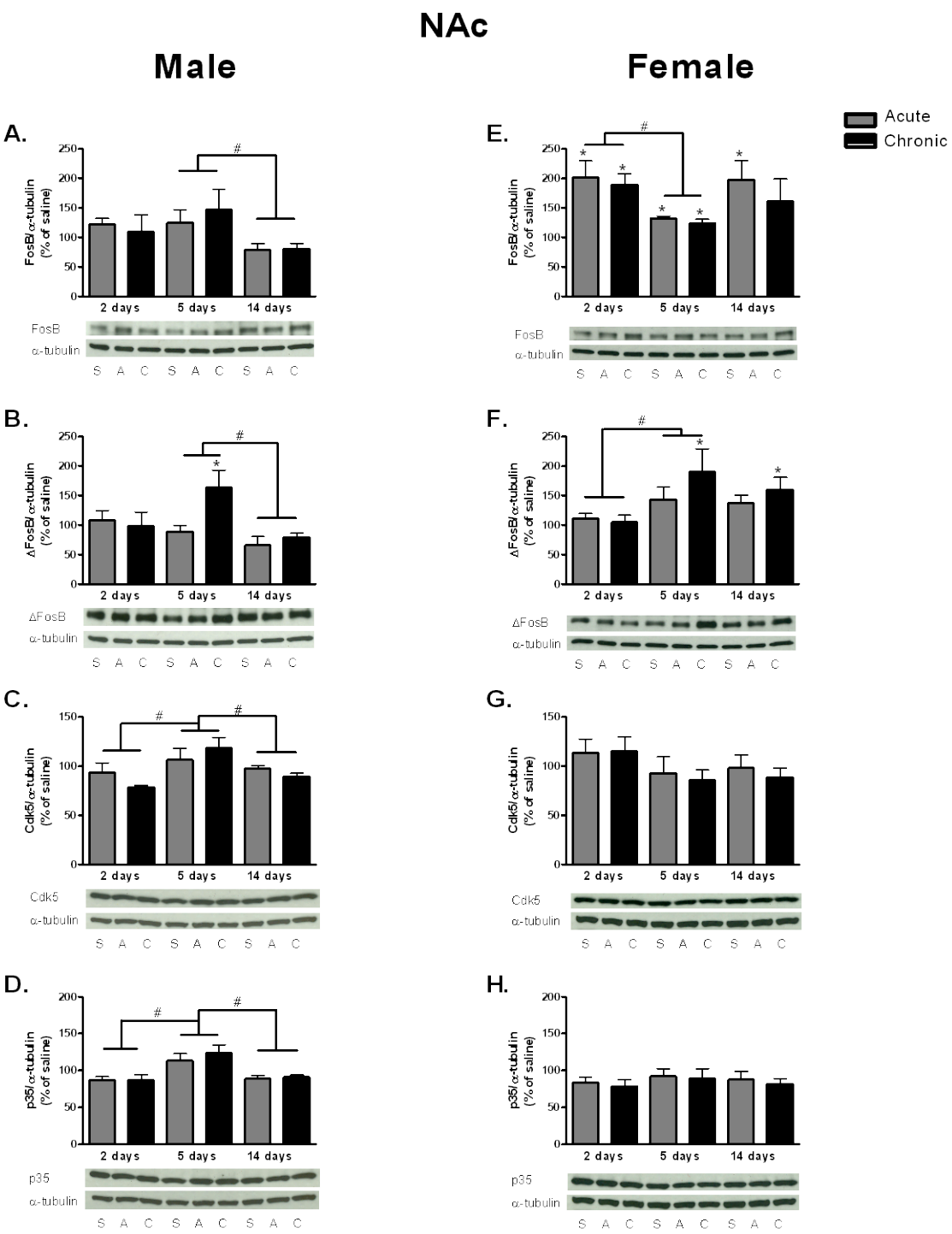

Figure 17: Western blot analysis of FosB (A, E), ΔFosB (B, F), Cdk5 (C, G) and p35 (D, H) in the NAc of male (A-D) and female (E-H) rats after acute and chronic cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). # Represents significant difference between different lengths of treatment. * Represents significant difference to saline control of the same length of treatment ($p<0.05$). (n=4 per group)

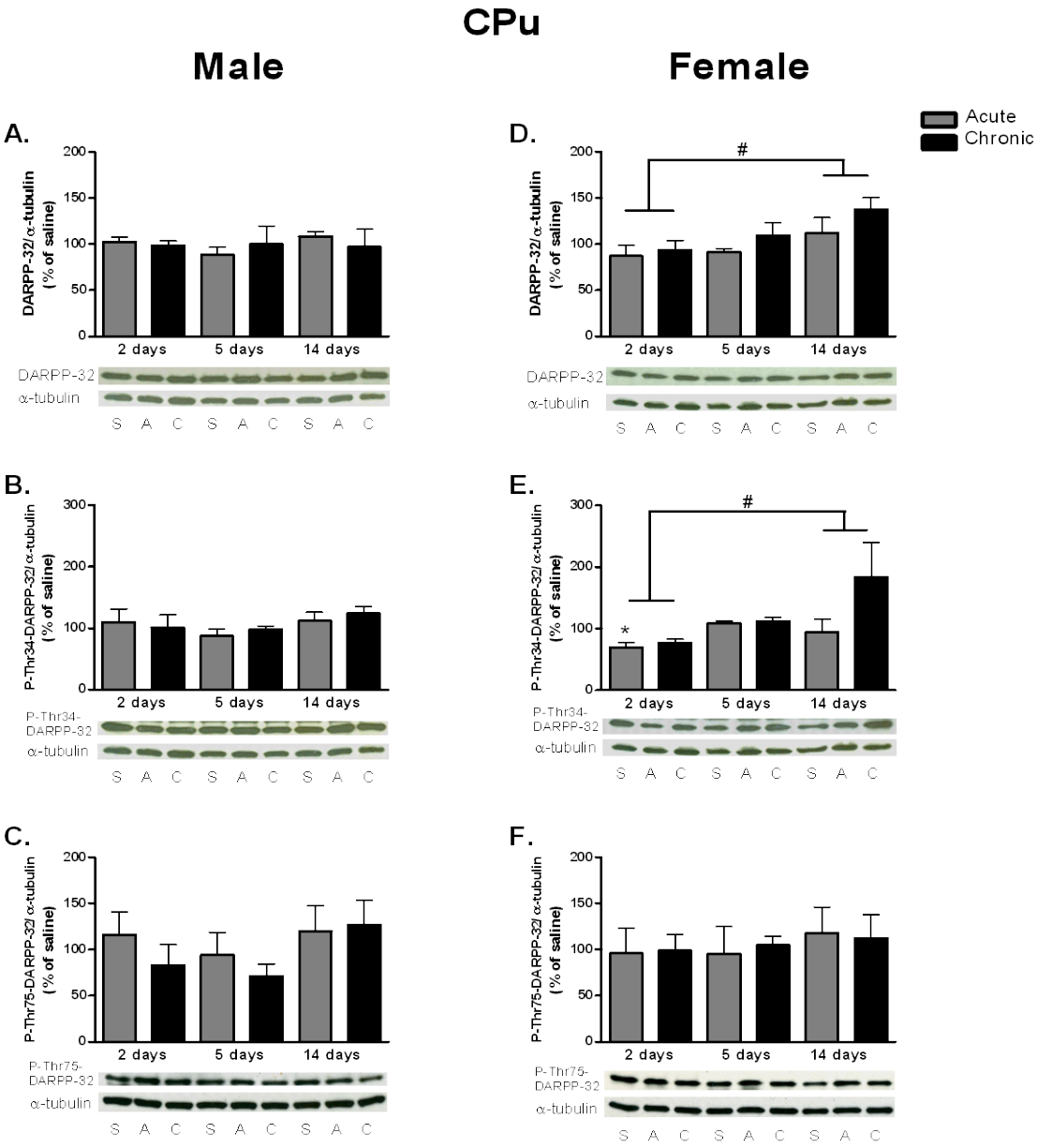

Figure 18: Western blot analysis of DARPP-32 (A, D), P-Thr34-DARPP-32 (B, E) and P-Thr75-DARPP-32 (C, F) in the CPu of male (A-C) and female (D-F) rats after acute and chronic cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). # Represents significant difference between different lengths of treatment. * Represents significant difference to saline control of the same length of treatment ($p<0.05$). (n=4 per group)

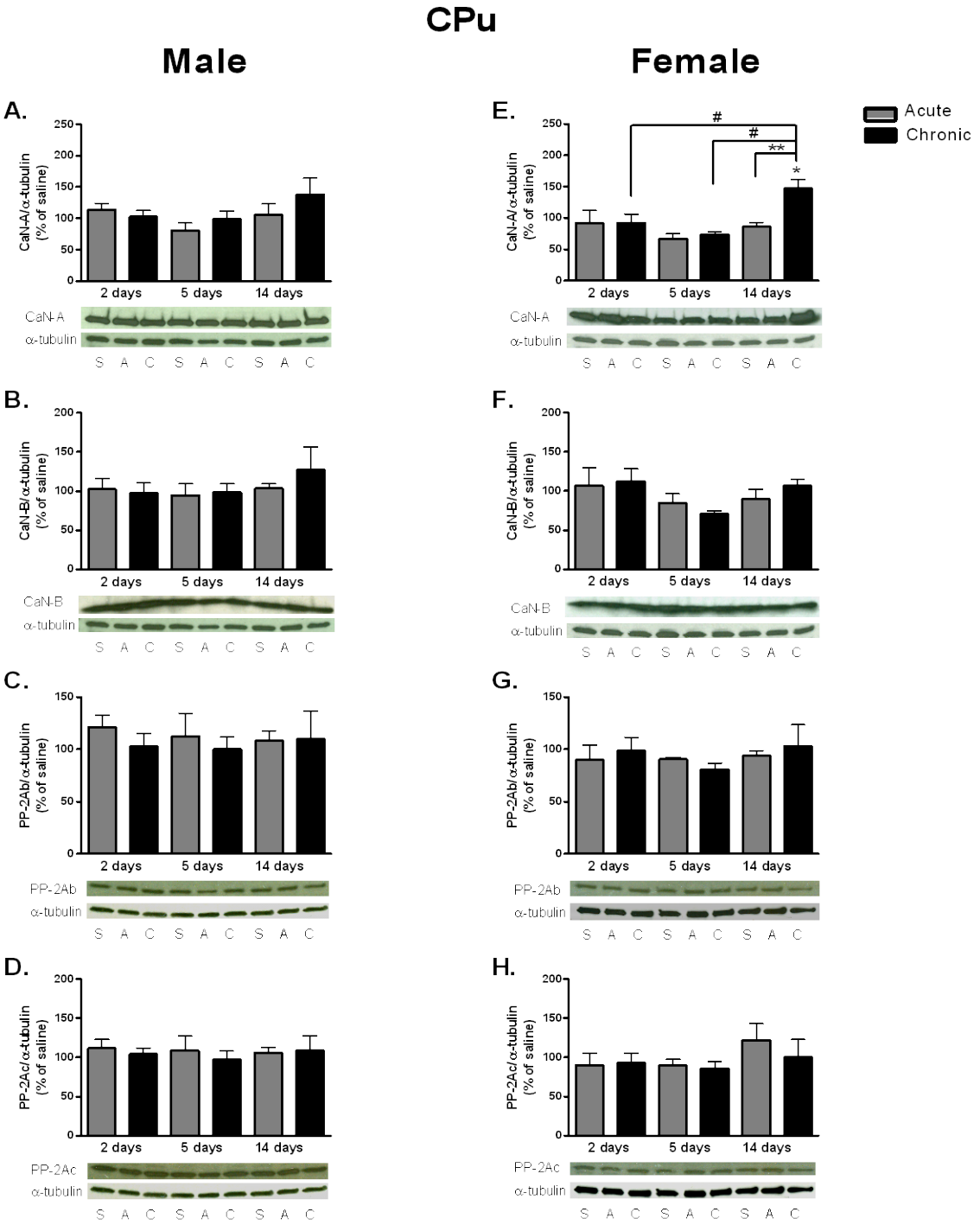

Figure 19: Western blot analysis of CaN-A (A, E), CaN-B (B, F), PP-2Ab (C, G) and PP-2Ac (D, H) in the CPu of male (A-D) and female (E-H) rats after acute and chronic cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). # Represents significant difference between different lengths of treatment. * Represents significant difference to saline control of the same length of treatment. ** Represents significant difference between acute and chronic treatment ($p<0.05$). (n=4 per group)

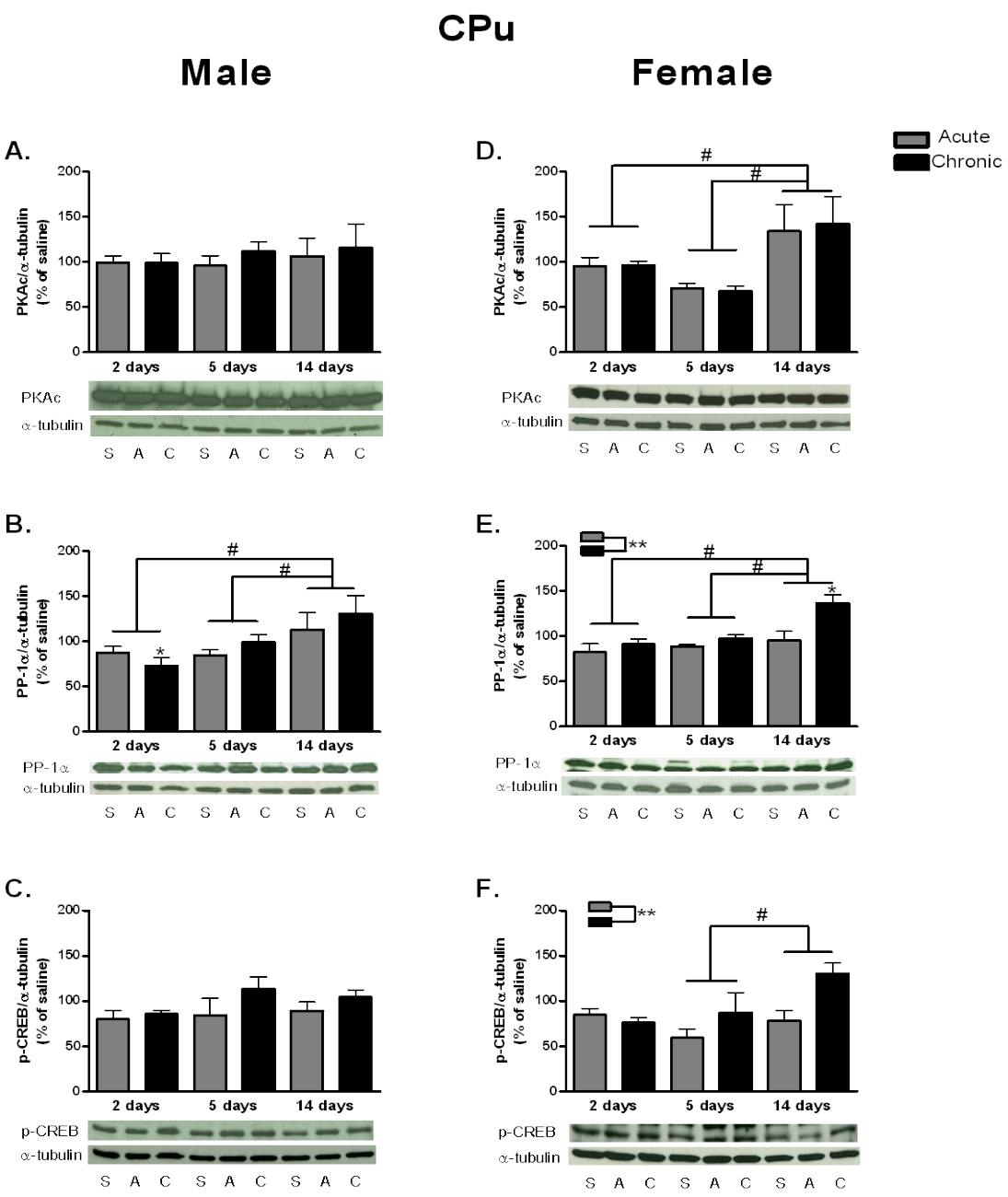

Figure 20: Western blot analysis of PKAc (A, D), PP-1α (B, E) and p-CREB (C, F) in the CPu of male (A-C) and female (D-F) rats after acute and chronic cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). # Represents significant difference between different lengths of treatment. * Represents significant difference to saline control of the same length of treatment. ** Represents significant difference between acute and chronic treatment ($p<0.05$). (n=4 per group)

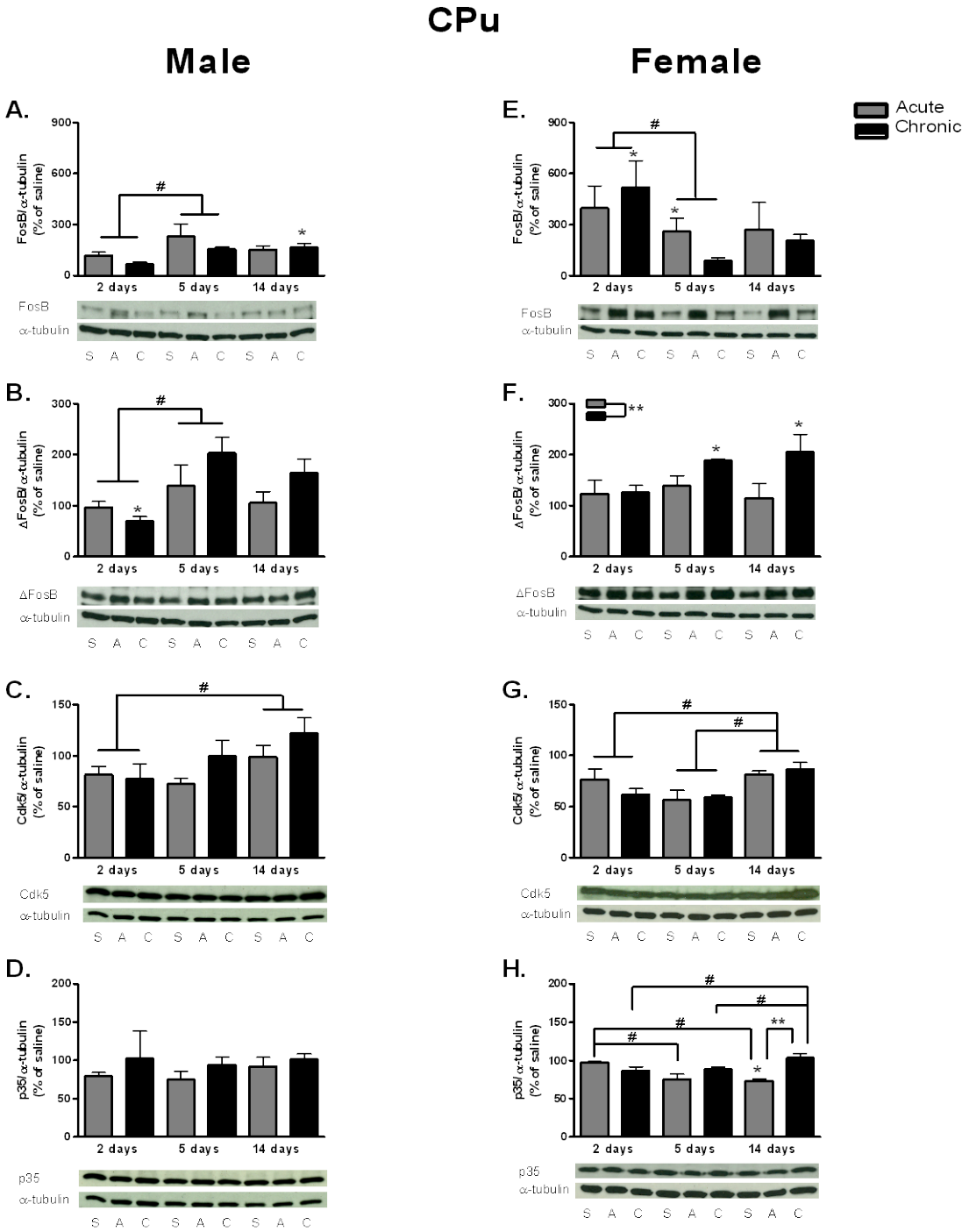

Figure 21: Western blot analysis of FosB (A, E), ΔFosB (B, F), Cdk5 (C, G) and p35 (D, H) in the CPu of male (A-D) and female (E-H) rats after acute and chronic cocaine treatment. Data presented as mean protein levels normalized to α-tubulin (±S.E.M). # Represents significant difference between different lengths of treatment. * Represents significant difference to saline control of the same length of treatment. ** Represents significant difference between acute and chronic treatment ($p<0.05$). (n=4 per group)

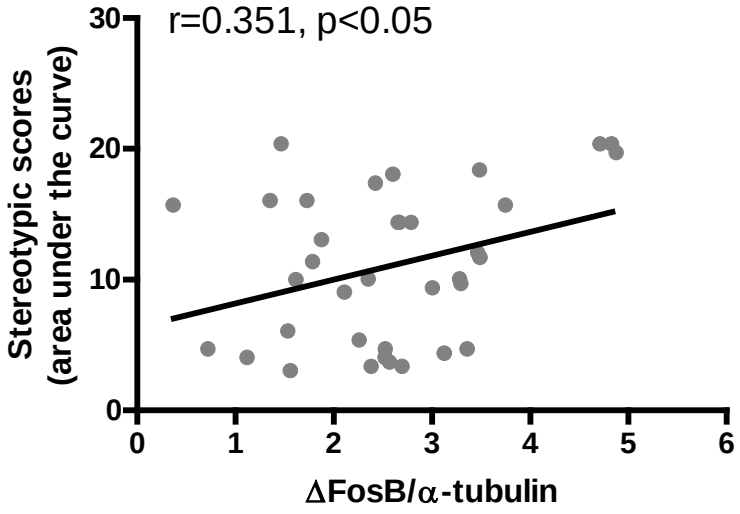

Fig. 22: Correlation between ΔFosB protein levels in the CPu of male rats and stereotypic activities.

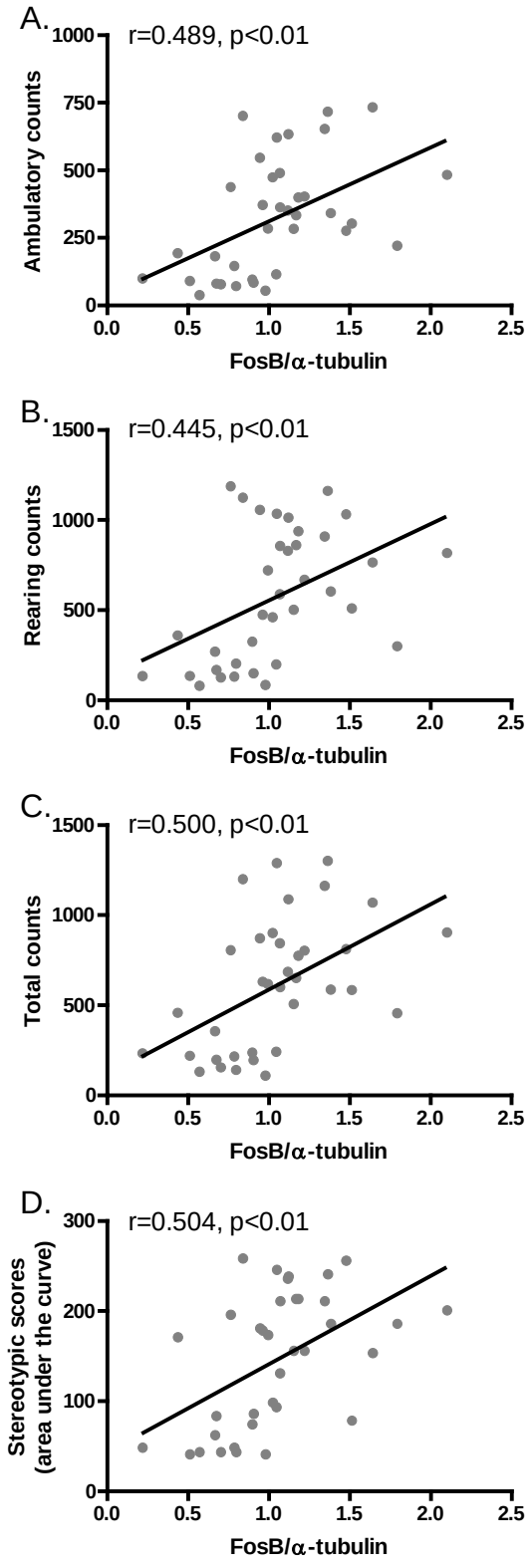

Fig. 23: Correlation between FosB protein levels in the NAc of female rats and ambulatory (A), rearing (B), total locomotor (C) and stereotypic (D) activities.

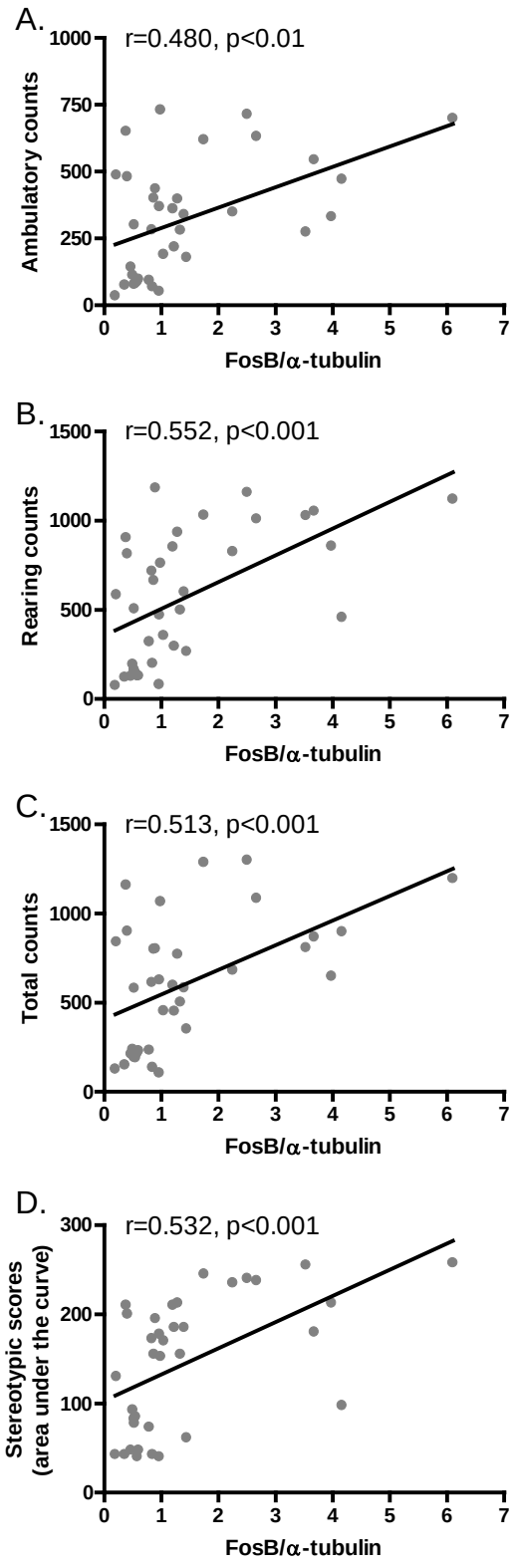

Fig. 24: Correlation between FosB protein levels in the CPu of female rats and ambulatory (A), rearing (B), total locomotor (C) and stereotypic (D) activities.

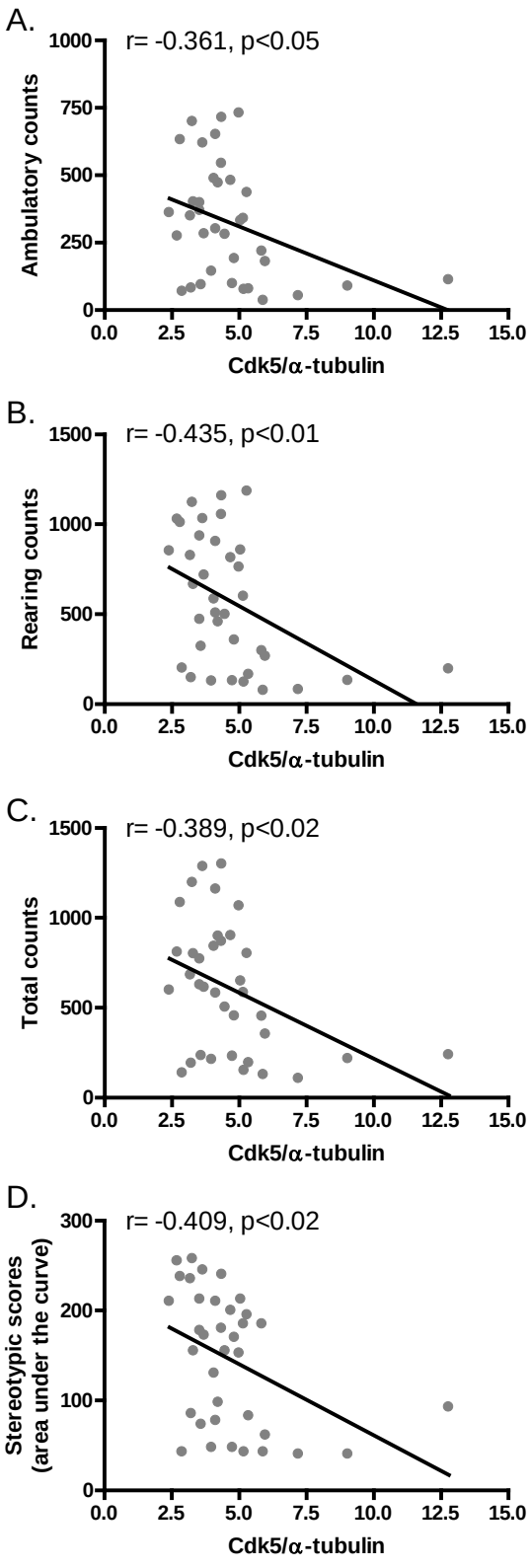

Fig. 25: Correlation between Cdk5 protein levels in the CPu of female rats and ambulatory (A), rearing (B), total locomotor (C) and stereotypic (D) activities.

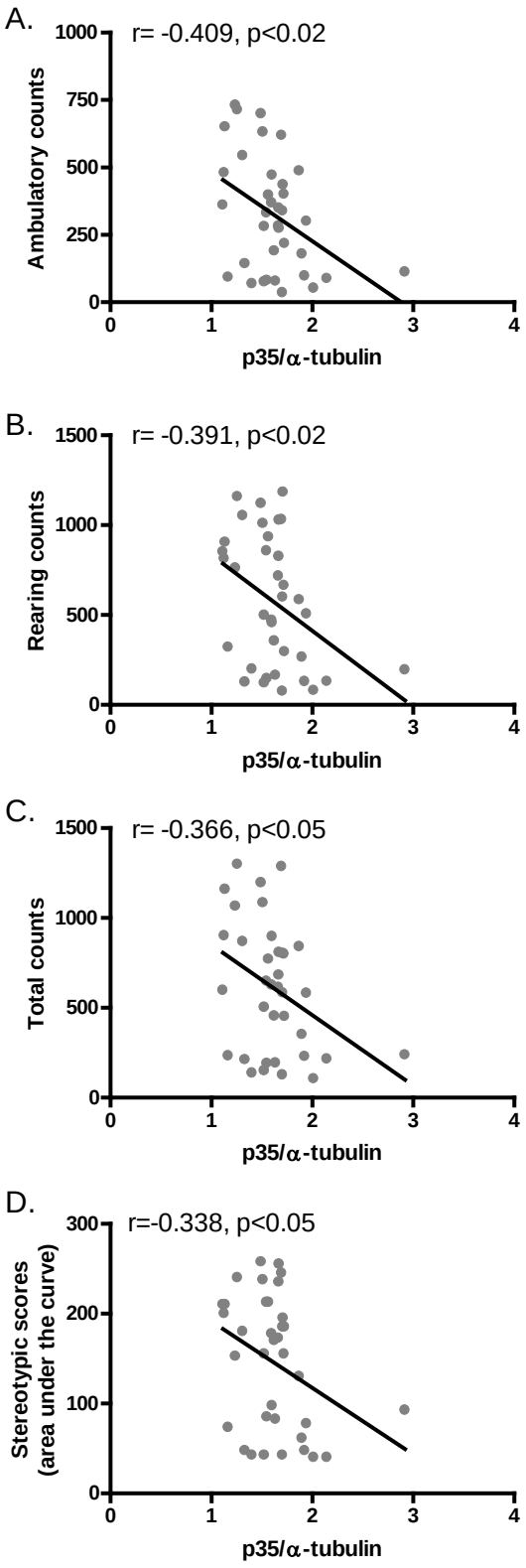

Fig. 26: Correlation between p35 protein levels in the CPu of female rats and ambulatory (A), rearing (B), total locomotor (C) and stereotypic (D) activities.

IV. Discussion

A. Sex differences in behavioral response to chronic cocaine treatment

In a consistent with previous findings from our group and others demonstrating that acute cocaine treatment increased behavioral responses in female rats (Chin et al., 2002; Festa et al., 2004; Sell et al., 2000; Van Haaren and Meyer, 1991; Walker et al., 2001), we also showed that the locomotor activity was higher in female rats when compared to their male counterparts. After chronic cocaine administration, in agreement with Chin et al. (2002) and Becker et al. (1999), we observed that female rats developed locomotor sensitization to cocaine more rapidly and more robust than male rats. In line with our previous findings (Festa et al., 2004; 2006), although acute cocaine induced stereotypic behavior in both male and female rats, no significant sex difference was observed. On the other hand, a sex dimorphic stereotypic behavior was observed with repeated exposure to cocaine. However, in comparison to acute cocaine groups, chronic cocaine could not further potentiate stereotypic behavior changes in either sex. Thus, repeated cocaine induced behavioral sensitization may not be revealed via stereotypic behavior rating, a categorical rating system. Alternatively, environment novelty has been implicated in the development of psychostimulant-induced behavioral sensitization (review in Robinson et al, 1998). Since the same home cage was used in measuring the behavior alternations in the present study, it may partially account for the lacking of difference in stereotypic behavior between acute and chronic cocaine administration within sex.

Interestingly, for the first time, we further demonstrated that female but not male rats developed locomotor tolerance after prolonged cocaine regimen. In male rats, previous studies indicated that intermittent cocaine administration (e.g. once injection per

day) frequently induces sensitization to locomotor effects (Churchill et al., 1999; Henry and White 1991, 1995; Sorg et al., 1993). In contrast, locomotor tolerance response can be achieved via continuous cocaine administration (e.g. minipumps; King et al., 1992; Martin-Iverson and Burger 1995; Kunko et al., 1998; Izenwasser and French et al., 2002; Reith et al., 1987). These two different behavioral responses are associated with changes of dopamine receptors occupancy, in that sensitization induces more dopamine receptors occupancy and tolerance results in the decrease of dopamine receptors occupancy (Martin-Iverson and Burger 1995). Behaviorally, studies have shown that female rats are more sensitive to dopamine D1 receptor antagonism in response to cocaine (Festa et al., 2006; Schindler and Carmona 2002; Nazarian et al., 2005). Previously, we demonstrated that female rats had more dopamine D1 receptor occupancy in the CPu, which may contribute to higher locomotor activity after acute cocaine administration (Festa et al., 2006). In addition, after chronic cocaine administration (14 days, binge injection), a recent study showed that female rats may have higher δ-opioid receptor membrane expression in the NAc core (Ambrose-Lanci et al., 2008). Due to the effect of dopamine D1 receptor activation in mediating δ-opioid receptor desensitization (Unterwald et al., 1993; Unterwald and Cuntapay 2000), it is plausible that a blunted dopamine D1 receptor activity and/or occupancy may contribute to the elevated δ-opioid receptor membrane expression in female rats. Collectively, when compared to the male, the left-shift of locomotor change in female rats may related to higher dopamine D1 receptor sensitivity leading to earlier behavioral augmentation/sensitization in response to acute and chronic cocaine treatment; the reduction in dopamine D1 activation may underlie the locomotor tolerance induced by prolonged cocaine administration.

Compared to the horizontal movement measured by ambulatory and total locomotor activities, the vertical movement indicated by rearing activities exhibited a tendency to develop tolerance. In male rats, sensitization was absent in rearing behavior; while in females, tolerance had been developed as early as 5 days. These differences may result from the fact that horizontal and vertical movements are incompatible to each other, as the horizontal and vertical movement could not be exhibited at the same time. From our results, it is possible that when chronic cocaine treatments induce more movements in the horizontal direction, the movement in the vertical direction tend to decrease.

B. Sex differences in DARPP-32 cascade after chronic cocaine treatment

1. Sex differences in DARPP-32 and its phosphorylations

Recently, we have observed sex difference in DARPP-32 in response to acute cocaine, indicating that male rats had lower basal level but higher induction of DARPP-32 when compared to females (Zhou et al., 2009). Here sex differences were also observed in chronic cocaine-induced DARPP-32 and its phosphorylations.

In the NAc of male rats, P-Thr34-DARPP-32 protein levels were increased after 5 days of treatment, when behavior sensitization was observed in male rats. However, P-Thr75-DARPP-32 protein levels did not change along time of treatment. It is contradict to the previous findings suggesting that chronic cocaine treatment decreased P-Thr34-DARPP-32 and increased P-Thr75-DARPP-32, respectively, in striatum of behavior sensitized male rats (Bibb et al., 2001; Scheggi et al., 2004 and 2007). The discrepancy may be due to different sensitization protocols: in their experiments all animals were received a withdraw/washout period before a cocaine challenge, while in our experiment,

rats were sacrificed 1 hour after last injection. It is possible that the length of abstinence period may affect the phosphorylation level of DARPP-32. Indeed, Scheggi et al. (2007) demonstrated that with two washout lengths, 10 days and 3 month, the decrease in P-Thr34-DARPP-32 and increase in P-Thr75-DARPP-32 induced by chronic cocaine treatment were only observed after 10 days washout. Moreover, using cocaine self-administration model, Lynch et al. (2007) demonstrated that the P-Thr34-DARPP-32 protein level in the NAc of male rats was increased after 0-day abstinence, but its elevated phosphorylation level was decreased to the control level after 10-day abstinence period. Therefore, our experiment aimed to measure the DARPP-32 phosphorylation right after chronic cocaine administration, which is similar to the increase of P-Thr34-DARPP-32 with 0-day abstinence as a result of cocaine self-administration. Another possible reason for the contradictory findings may be due to the cell type-specific regulation for DARPP-32 phosphorylation. There are two subpopulations of striatal cells – striatonigral neurons mainly express D1-like receptors, and striatopallidal neurons highly express D2-like receptors (Gerfen et al., 1990). D1-like receptors are coupled to Gs/olf proteins, which promote the formation of cAMP and the PKA activation; on the other hand, D2-like receptors are coupled to Gi proteins, which negatively regulate the formation of cAMP and the activity of PKA (Stoof and Kebabian, 1981). Previous study demonstrated that, after the exposure of cocaine, the PKA-mediated increases of P-Thr34 DARPP-32 and decreases of P-Thr75 DARPP-32 were mainly restricted in the striatonigral neurons, while an opposite phosphorylation pattern on DARPP-32 was observed in striatopallidal neurons (Bateup et al., 2008). In the present study, we did not differentiate specific neurons, thus, the “net” effect of DARPP-32 in both types of

neurons was measured via our western blot analysis. In addition, distinct ways for tissue dissection (e.g. tissue punching and gross dissection by hand) may occur among studies leading to different ratio of these two types of neurons; in turn, the mixed total effect detected by western blot may also show different results.

In female rats, both DARPP-32 and P-Thr34-DARPP-32 protein levels increased in the CPu after 14 days, when behavior tolerance was observed in female rats. Similar to the result of NAc, P-Thr75-DARPP-32 protein levels in the CPu did not change. These results suggested that compared to P-Thr75-DARPP-32, P-Thr34-DARPP-32 may be more critical for chronic cocaine-induced behavior changes. Previous studies indicated that DARPP-32 knockout mice and mice with site mutations at Thr34 show heightened behavioral sensitization in response to repeated cocaine injections (Zachariou et al., 2006). It suggests that DARPP-32 and its phosphorylated Thr-34 isoform have potentially inhibitory effect on behavioral response to chronic cocaine treatment. Therefore, in female rats, increases of DARPP-32 and P-Thr34-DARPP-32 protein levels may lead to the behavior tolerance in response to chronic cocaine exposure. On the other hand, in Thr75 site mutation mice, chronic cocaine-induced locomotor sensitization was unaffected or absent (Valjent et al., 2005; Zachariou et al., 2006). Thus, the functional significance of P-Thr75-DARPP-32 in cocaine-induced behavior changes should be further determined.

No treatment effect was observed in either sex. However, acute cocaine treatment on Day 2 resulted in decreases of P-Thr34-DARPP-32 protein levels in the CPu of female rats when compared to saline control. Cocaine-induced DARPP-32 phosphorylation changes were generally observed at 30min in male rodents or 45min in female rats after

cocaine treatment, and gradually returned to baseline (Takahashi et al., 2005; Rauggi et al., 2005; Nishi et al., 2000; Synder et al., 2000; Zhou et al., 2009). In the present study, we measured protein levels 60min after last treatment which may partially account for the failure to detect any cocaine effect on DARPP-32 phosphorylation. However, the higher basal protein levels and delayed induction of P-Thr34-DARPP-32 in females (Zhou et al., 2009) potentially make the decrease of P-Thr34-DARPP-32 still detectable. Nevertheless, a time-course designed study is necessary to elucidate the change in the DARPP-32 phosphorylation in response to both acute and chronic cocaine administration.

2. Sex differences in PP-2B/PP-2A regulation

PP-2B, a Ca2+/calmodulin-dependent serine/threonine protein phosphatase, dephosphorylates P-Thr34-DARPP-32. Although an increase of P-Thr34-DARPP-32 was observed in the NAc of male rats, we did not find similar changes in PP-2B protein levels. Previous studies have demonstrated that after chronic cocaine treatment, PP-2B protein levels were decreased in the NAc of male rats (Hu et al., 2005) possibly as a result of decreases in $^{+}$ influx and intracellular released $^{+}$ (Zhang et al, 2002; Hu et al, 2004). It is possible that the decrease in PP-2B was only observed in the particulate fractions, but not in the soluble fractions (Hu et al., 2005). In addition, the immunoreactivity of PP-2B is higher in the soluble fractions than that in the particulate fractions (Lin et al., 2002). Since we measured the overall protein level of PP-2B and did not separate our samples into different fractions, it is plausible that the effect of chronic cocaine-induced PP-2B changes in the particulate fractions was masked. Thus, measurements in different fractions are needed to evaluate PP-2B protein levels changes

in response to cocaine. On the other hand, in female rats, the CaN-A protein levels in the CPu were increased after 14 days of cocaine administration, with parallel increases in both DARPP-32 and P-Thr34-DARPP-32 protein levels. CaN-A is the catalytic subunit of PP-2B and its increase may indicate higher activation of PP-2B after chronic cocaine treatment. The increased PP-2B protein levels may represent a positive feedback mechanism to counteract the higher protein levels of P-Thr34-DARPP-32 in the females after chronic cocaine treatment. We previously demonstrated that when compared to male rats, females have higher basal PP-2B protein levels, and delayed protein induction in response to cocaine (Zhou et al., 2009). Thus, it may partially explain the detectable PP-2B changes in females only.

PP-2A is the predominant serine-threonine protein phosphotase mediating the dephosphorylation of P-Thr75-DARPP-32 (Nishi et al., 2000). Previous study demonstrated that, after chronic methamphetamine, a decrease in PP-2A protein levels was observed in ventral striatum of male rats (Chen and Chen, 2005). In our experiment, we further showed that, after 14 days of cocaine administration, the PP-2A regulatory B subunit protein levels were decrease in the NAc of male rats with a similar trend of P-Thr75 DARPP-32 reduction. This finding may suggest a positive feedback mechanism in P-Thr75-DARPP-32 dephosphorylation by PP-2A. However, although to a lesser extent, other protein phosphatase including PP-1 and PP-2C also dephosphorylates DARPP-32 at Thr75 site (Nishi et al., 2000), which may underlie the marginal decrease of P-Thr75 DARPP-32 in the current study. In female rats, after chronic cocaine treatment, we did not observe any changes in PP-2A protein levels due to the unaltered P-Thr75 DARPP-32 protein levels. Previous study showed that PKA mediates the activity of PP-2A (Usui et

al., 1988). Several possible explanations may underlie the inconsistency in PKA and PP-2A protein level changes in females. First, although PKA could increase the activity of PP-2A, it may not be reflected on its protein levels. Second, compared to males, female rats have higher basal PKA-mediated signaling and proteins expression (Zhou et al., 2009; Nazarian et al., 2009). It is plausible that the higher PKA protein levels in females lead to an augmentation in the PP-2A protein expression under physiological condition. Thus, additional cocaine administration cannot further potentiate its expression. Third, previous studies demonstrate that PKA stimulates the activity of PP-2A by phosphorylating its B' regulatory subunit (Usui et al., 1988 and 1998). Therefore, in addition to the B subunits of PP-2A examined in current study, it is worthy to further measure B' regulatory subunit protein levels to evaluate the effect of PKA on PP-2A activation.

3. Sex differences in PKA regulation and downstream PP-1/p-CREB

PKA phosphorylates DARPP-32 at Thr34 site. In line with an increase of P-Thr-34 DARPP-32, herein, we also demonstrated that the PKA protein levels were increased after repeated cocaine exposure. Specifically, in male rats, PKA protein levels in the NAc were increased after 5 days administration, while no change was observed in the CPu. In contrast, in females, no change was observed in the NAc, while increases of PKA protein levels were found in the CPu after 14 days regimen. Recently, our laboratory has shown that female rats have higher basal and cocaine-induced PKA protein expression in the NAc but not in the CPu (Nazarian et al., 2009). Due to the heightened basal and induction of PKA in the NAc of females, a "ceiling effect" may make it difficult to further

accumulate PKA protein levels along days of cocaine treatment. However, in males, because the PKA protein levels were initially at a relatively low status, an increase of PKA expression can be further elevated by cocaine in the NAc. On the other hand, in the CPu, the PKA basal protein levels were similar between male and female rats. Although the chronic cocaine-induced sex dimorphic induction pattern in the CPu is still unknown, it is possible different brain regions and underlying PKA protein expression may contribute to the behavioral sex difference in response to cocaine (See discussion in part C).

In consistent with previous findings indicating that chronic cocaine had no effect on PP-1 protein levels in the NAc of male rats (Hu et al., 2005), we did not find any alternations of PP-1 protein expression after chronic cocaine treatment. It is well established that the PP-1 activity was inhibited by P-Thr34 DARPP-32 (Nishi et al., 1997). However, it has been documented that PP-1 could be activated by PKA in striatal neurons (Surmeier et al, 1995). Collectively, these findings may suggest that the PP-1 activity and/or protein levels expression was determined by the inhibitory effect of P-Thr-34 DARPP-32 and the activational effect of PKA. Thus, after 5 days of cocaine administration, the elevation of both P-Thr34 DARPP-32 and PKA may counteract to each other resulting in unaltered PP-1 protein levels in the NAc of male rats. Interestingly, in the CPu of female rats, although both P-Thr34-DARPP-32 and PKA were increased after 14 days of treatment, an argumentation on PP-1 protein levels was still observed. It is possible that, after prolonged cocaine administration, the activation effect of PKA is stronger than the inhibitory effect of P-Thr34-DARPP-32 in female rats. However, it should be cautious that the protein level alteration does not always accompany activity

changes. For instance, in the CPu of male rats, with no changes in P-Thr34-DARPP-32 or PKA protein levels, an increase of PP-1 protein levels was still obtained after 14 days of treatment. A similar finding was observed in p-CREB protein levels. Previous study has indicated that PKA and PP-1 mediate the CREB phosphorylation and dephosphorylation, respectively (Carlezon et al., 2005). Therefore, changes in p-CREB expression revealed the net effect of both PP-1 and PKA. In male rats, the change of p-CREB protein level was accompanied by alteration in PKA. In the CPu of female rats, the heightened p-CREB protein levels after 14 days were as a result of higher PKA and PP-1 protein levels. However, in the NAc of females, the increased p-CREB protein levels along time did not correlate with PKA or PP-1 protein levels, but may correlate with the activity of PKA and PP-1. Additional studies measuring the activation instead of protein levels were required to support the postulation.

Study in male rats demonstrated that the activation of PKA is related to behavioral sensitization but not tolerance in response to repeated exposure of cocaine (Hope et al., 2005). On the other hand, p-CREB has been revealed as a neuroadaptation phenomenon to limit behavioral sensitization in response to cocaine (Carlezon et al., 2005). For example, studies have suggested that the greater activation of p-CREB is associated with blunted psychomotor and rewarding effects in response to chronic cocaine (Carlezon et al., 1998; Pliaks et al., 2001). CREB mutant mice showed heightened cocaine-induced place conditioned preference and locomotor sensitization (Walters and Blendy 2001). In addition, previous study in our lab showed that regardless of cocaine treatment paradigm, the females have higher PKA protein levels in the NAc when compared to the males, but males have longer-induction of p-CREB protein levels

after cocaine (Nazarian et al., 2009). Thus, after chronic cocaine administration, the elevated PKA and persistently increased p-CREB in striatum may result in the rapid development of behavioral sensitization and tolerance in female rats. Similarly, after 5 days regime with the exhibition of behavioral sensitization, an upregulation of PKA-CREB signaling was evident in the NAc of male rats. These finding suggest that, the activation of CREB signaling is important in representing a negative mechanism to counteract the cocaine-induced behavioral sensitization through PKA activation. However, unlike in female rats, no further changes in PKA and p-CREB protein levels were observed in the male after 14 days of cocaine treatment, which may explain the their inability to develop behavioral tolerance.

4. Sex differences in ΔFosB/Cdk5 regulation

Cocaine causes a long-term neuroadaptation through the regulation of several gene expressions. Among them, Fos-related family has been implicated in this process underlying the behavioral alternation in response to cocaine. For instance, in male rats, the initial cocaine exposure leads to FosB induction in the striatum, whereas prolong cocaine administration results in tolerance in its induction (Hope et al., 1992; Chen et al., 1995). FosB mutant mice exhibited higher psychomotor and rewarding effects in response to cocaine administration (Hiroi et al., 1997). In the present study, a day/time course effect was observed in FosB protein levels in male rats: an initial augmentation of FosB followed by an induction of tolerance after 14 days cocaine treatment. On the 5th day, male rats with repeated cocaine administration also exhibited behavioral sensitization, thus, the increasing protein levels of FosB may contribute to their

behavioral alternation. On the other hand, the FosB protein levels in females were transiently increased after 2 days of cocaine injection and became tolerant subsequently indicating a left-shit activation pattern when compared to the male. Further analysis demonstrated a positive correlation between behavioral changes and FosB protein levels. Taken together, these finding indicated that FosB induction represent as a critical mediator associated with cocaine-induced behavioral sensitization in both sexes. In the comparison of male, the differential FosB protein induction profile in female rats may underline behavioral sensitization and tolerance with repeated cocaine administration.

After chronic cocaine injection or self-administration, a sustained accumulation of ΔFosB has been found in various brain regions including the striatum (Hope et al., 1994; Nye et al., 1995; Moratalla et al., 1996; Pich et al., 1997; Lee et al., 2006; Perrotti et al., 2008). Behaviorally, overexpression of ΔFosB in mice enhances locomotor responses and the sensitivity to rewarding properties of cocaine (Kelz et al., 1999; Colby et al., 2003). In male rats, when compared to other treatment days, the ΔFosB protein levels were transiently increased after 5 days of cocaine administration. However, in our males' CPu and NAc extracts, we did not obtain a sustained elevation of ΔFosB after 14 days of cocaine treatment. The discrepancy may be due to different injection schedules (e.g. twice daily injection) or higher dose of cocaine (e.g. 30 mg/kg of cocaine) used in previous studies. In contrast, ΔFosB protein level expression in female rats was accumulated across two weeks cocaine injection in both CPu and NAc. Previous study demonstrated that, after cocaine administration, the dopamine release in the nucleus accumbens is higher in females than in males (Walker et al., 2006). When compared to male rats, female rats demonstrated higher sensitivity in response to dopamine D1

receptor antagonism (Schindler and Carmona, 2002; Nazarian et al., 2004; Festa et al., 2006). A sex dimorphic PKA protein levels was also documented (Nazarian et al., 2009; present result). Therefore, it is plausible that the heightened dopamine/D1-mediated signaling leads to sustained ΔFosB protein expression in females after repeated cocaine exposure.

Cdk5 has been identified as a down stream target of ΔFosB. For example, overexpression of ΔFosB leads to elevated Cdk5 expression (Kelz et al., 1999; Bibb et al., 2001). After repeated cocaine administration, an increase of Cdk5 activity and protein expression as well as its co-activator, p35, has been found (Scheggi et al., 2004; Lu et al., 2003; Bibb et al., 2001; Kumar et al., 2005). Herein, in male NAc after 5 days of cocaine injection, we demonstrated that the Cdk5 and p35 protein levels were accumulated with exhibition of behavioral sensitization. Both pharmacological and genetic inhibition of Cdk5 activity in the NAc of rodents resulted in a super-sensitivity to locomotor and rewarding effects of repeated cocaine exposure (Bibb et al., 2001; Benavides et al., 2007). Thus, the increase of Cdk5 and p35 protein levels in the NAc may represent as a positive feedback mechanism to counteract the dopamine-mediated signaling underlying behavioral sensitization. However, in a sex and region dependent manner, repeated cocaine administration induced Cdk5 and p35in the females' CPu but not the NAc. Further analysis showed that the Cdk5 expression in the female CPu is negatively correlated to behavior alternations, indicating that Cdk5 protein expression may underlie the behavioral alternation (e.g. sensitization and tolerance) in the female. In contrast, previous study by Lynch (2007) showed no sex differences in Cdk5 after chronic cocaine regimen. The discrepant result may be due to methodology difference (cocaine self-

administration vs. passive administration by experimenters), since the failure to induce Cdk5 after cocaine self-administration has been reported (Seiwell et al., 2007). Although the reason underlying the region specific protein expression induced by cocaine between sexes is still unknown, in terms of both behavioral and biochemical alternation, it seems that neuronal activity transition from NAc to CPu may contribute to the development of cocaine-induced sexual dimorphism (see section C).

C. Regional specific sex differences

The striatum is a heterogeneous structure in mediating the transitional progress of drug addiction. Hypothesis has suggested that repeated exposure to psychostimulants may result in a shift from mesocorticolimbic to striatal control behavior (Everitt et al., 2001; Everitt and Wolf 2002). For example, the NAc is critical to the initial reinforcing and locomotor effects of psychostimulants (Hurd et al., 1989; Di Chiara and Imperato 1988; Robledo et al., 1992). On the other hand, the CPu has been implicated to compulsive drug seeking behavior as a habit-like learning process (Everitt and Wolf 2002; Packard and Knowlton 2002; White and McDonald 2002). In rodents and primates, cocaine self-administration studies indicated that the neuronal activation and dopamine release in the CPu parallel with cue-induced cocaine seeking behavior (Ito et al., 2002; Porrino et al., 2004; Fuchs et al., 2006). Additionally, clinical studies revealed that cue-induced craving is correlated to the neuronal activation in the CPu (Garavan et al., 2000; Volkow at al., 2006). The generalibility from self-administration to drug-induced sensitization/tolerance is unknown. However, both experimental paradigms may share similar properties. For example, behaviorally, rodents with cocaine self-administration

history exhibited behavioral sensitization in response to subsequent challenge (Hooks et al., 1994; Phillips and Di Ciano 1996). In addition, behavior sensitized rats demonstrated enhanced drug self-administration including cocaine (Horger et al., 1990; Mendrek et al., 1998; Lorrain et al., 2000). Thus, in a certain level, it is likely that similar neuronal circuits may underlie both self-administration and behavioral sensitization paradigms. Furthermore, similar to self-administration, drug-induced behavior sensitization has been proposed as an associative learning process. Robinson and Berridge (1993, 2003) postulated an incentive-sensitization theory of drug addiction which suggested that repeated drug administration not only results in behavioral sensitization, it also could sensitize the neuronal circuits to assign incentive salience to drug or drug-related cue. The incentive salience could induce intense "wanting" behavior similar to the drug-seeking behavior in self-administration paradigm. On this basis, it is possible that the neuronal activity shift from NAc to CPu may also occur in the behavior sensitization paradigm as in drug self-administration. Moreover, if tolerance is the endpoint of drug-induced sensitization design, we boldly to suggest that the CPu may mediate the behavioral tolerance as its role in the drug-seeking behavior of self-administration model.

Indeed, in this experiment, we did find a neuronal activity shift from NAc to CPu. As summarized in Table 4 and Fig. 27, in male rats, most of the changes in protein levels in the DARPP-32 signaling proteins were observed in the NAc after 5 days of treatment. Specifically, male rats exhibited higher protein levels of P-Thr34-DARPP-32, PKAc, p-CREB, FosB, ΔFosB, Cdk5 and p35 in NAc at day 5. As summarized in Table 5 and Fig. 28, in female rats, most of the changes in DARPP-32 cascade were observed in the CPu after 14 days of treatment. In that, female rats exhibited higher protein levels of DARPP-

32, P-Thr34-DARPP-32, CaN-A, PKAc, PP-1α, p-CREB, Cdk5 and p35 in CPu at day 14. Based on these data, we postulate a transition model illustrated in Fig. 29. In this model, we propose that the DARPP-32 cascade activity in NAc is more critical to the initial locomotor effects (i.e. sensitization) of cocaine. However, as the exposure of cocaine prolongs, there is a transition from NAc to CPu, that DARPP-32 cascade activation in CPu is more crucial for the locomotor effects (i.e. tolerance) of prolonged cocaine treatment. Since male rats develop sensitization and tolerance slower, we were able to observe their initial activation of DARPP-32 cascade in the NAc, but the activation in CPu is possibly beyond our observation time. In the case of female rats, they develop behavior sensitization and tolerance very fast. It is possible that the initial activation of DARPP-32 cascade in the NAc may be too brief to be observed, but the activity in the CPu happened within our observation time. This model is further supported by our experiment of acute cocaine treatment. The changes in the DARPP-32 cascade after acute cocaine are observed in the NAc only, not in the CPu, which further demonstrate the importance of NAc in the initial phase of cocaine response. However, future experiments using shorter and longer treatment are necessary to observe the DARPP-32 cascade activation in the NAc of females and the CPu of male rats are necessary to further support this model.

Table 4. Protein levels changes of DARPP-32 signaling proteins between different lengths of cocaine treatment in male rats.

	NAc			CPu		
	2 Days	5 Days	14 Days	2 Days	5 Days	14 Days
DARPP-32						
P-Thr34-DARPP-32		↑				
P-Thr75-DARPP-32						
CaN-A						
CaN-B						
PP-2Ab			↓			
PP-2Ac						
PKAc		↑				
PP-1α						↑
p-CREB		↑				
FosB		↑			↑	
ΔFosB		↑			↑	
Cdk5		↑				↑
p35		↑				

Table 5. Protein levels changes of DARPP-32 signaling proteins between different lengths of cocaine treatment in female rats.

	NAc			CPu		
	2 Days	5 Days	14 Days	2 Days	5 Days	14 Days
DARPP-32						↑
P-Thr34-DARPP-32						↑
P-Thr75-DARPP-32						
CaN-A						↑
CaN-B						
PP-2Ab						
PP-2Ac						
PKAc						↑
PP-1α						↑
p-CREB	↓					↑
FosB	↑			↑		
ΔFosB	↓					
Cdk5						↑
p35						↑

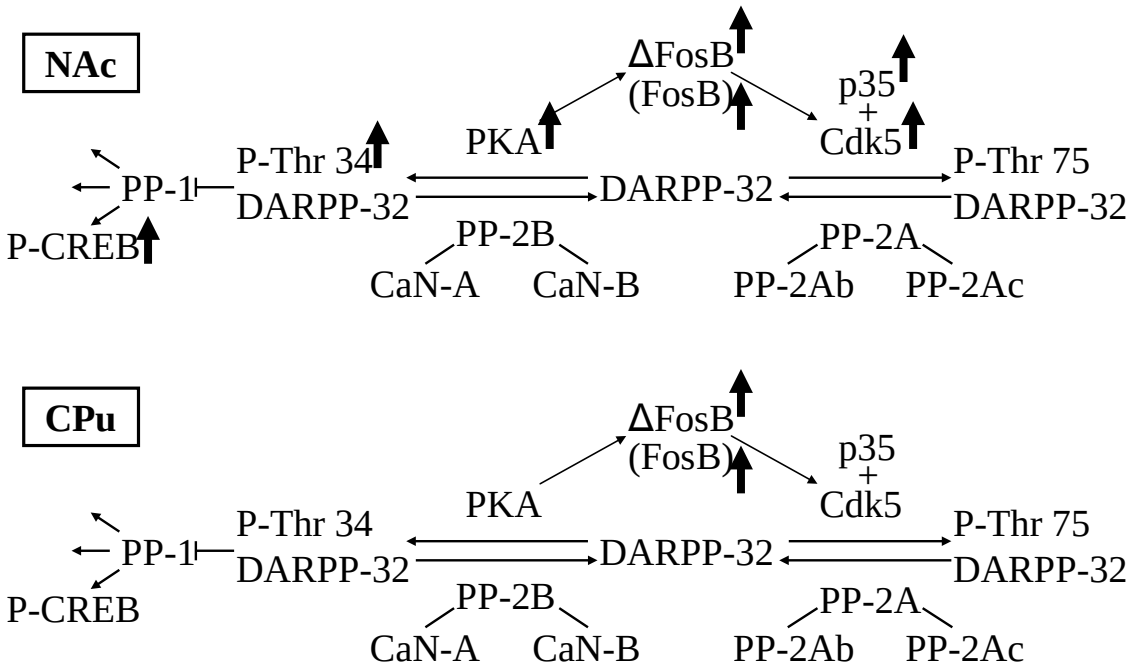

Figure 27: DARPP-32 cascade protein level changes in the male rats after 5 days of cocaine treatment.

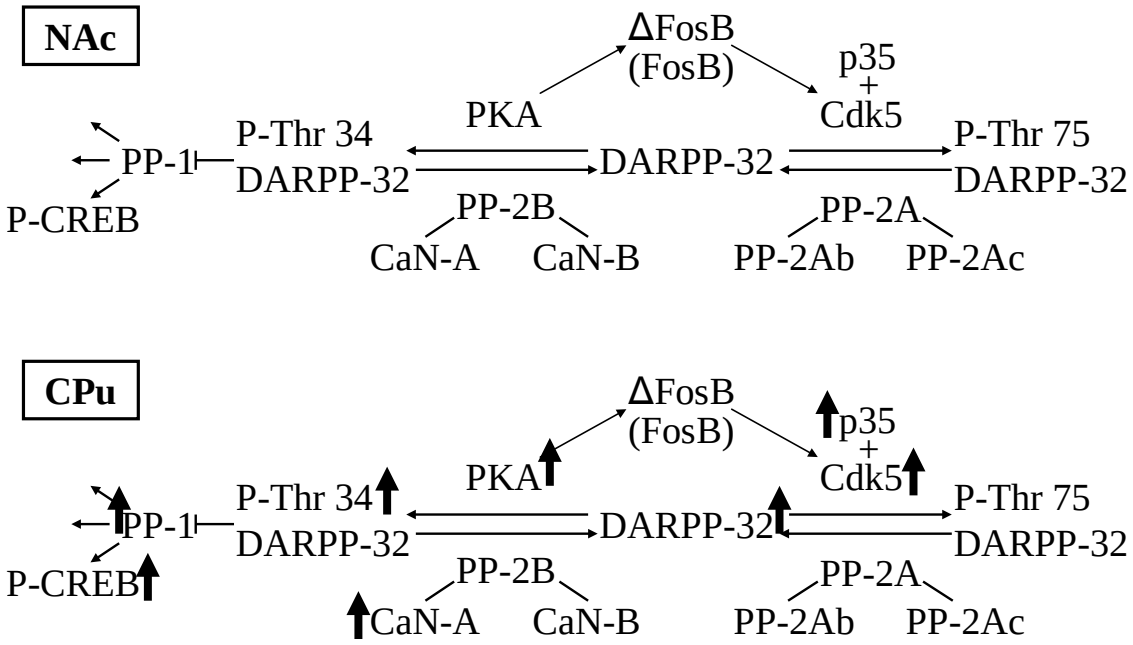

Figure 28: DARPP-32 cascade protein level changes in female rats after 14 days of cocaine treatment.

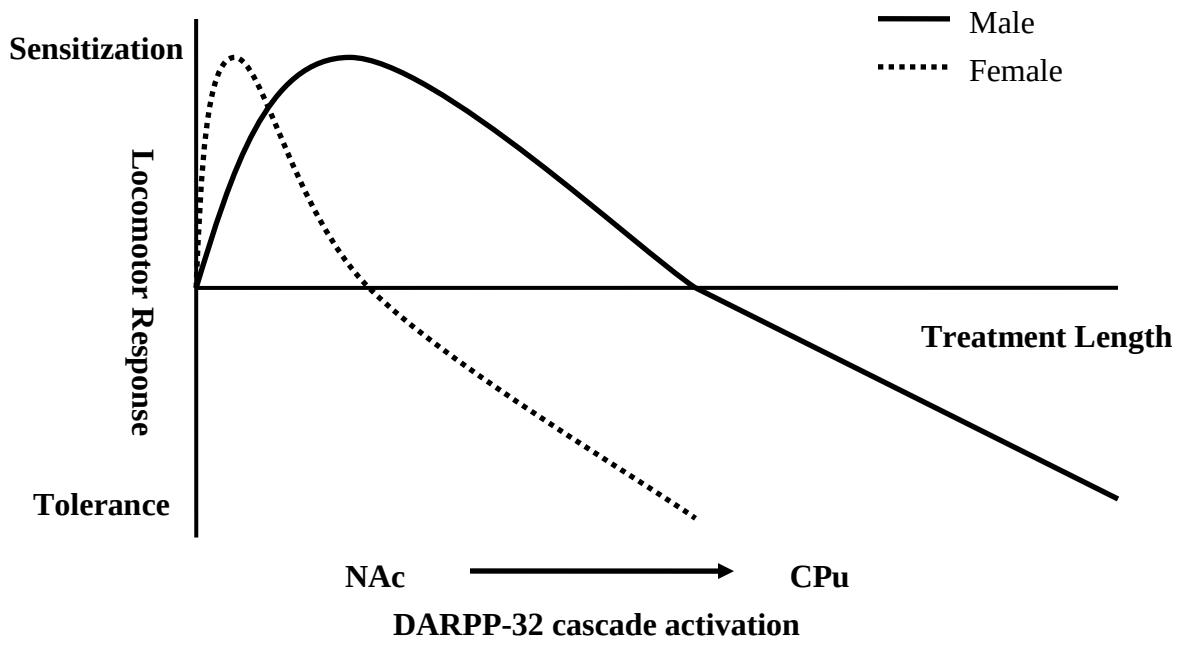

Figure 29: Conceptual model of DARPP-32 cascade activation transition from NAc to CPu, accompanied by the locomotor response changes to chronic cocaine.

Chapter 4: Conclusion

I. Summary of results

In the acute cocaine experiment, female rats have higher intrinsic basal levels of the DARPP-32 cascade when compared to males. After acute cocaine treatment, males have increased higher induction of the cascade, while females have decreased lower induction of the cascade as a result of the "ceiling effect" of their high basal level. These sex differences were mainly observed in the NAc, not CPu.

In the chronic cocaine experiment, female rats exhibited higher locomotor and stereotyped response to cocaine than males. Female rats also developed locomotor sensitization and tolerance to chronic cocaine treatment earlier than males. No sensitization or tolerance was observed in stereotyped activities in either male or female rats. In male rats, the DARPP-32 signaling was heightened mostly in the NAc when behavior sensitization was observed; while in female rats, increased DARPP-32 signaling was found in the CPu when behavior tolerance was observed. In addition, the protein levels of several DARPP-32 signaling proteins, including FosB, Cdk5, p35 and ΔFosB, correlated with the behavior activities. However, the correlation was mostly found in females rats, with an exception of ΔFosB, whose levels in the CPu of male rats correlated with stereotypic activities.

II. Significance of results

The focus of this dissertation was to investigate the role of DARPP-32 cascade in acute and chronic cocaine-induced behavioral sex differences. Overall, the findings of the present experiments have extended more information and understanding about the

underling intracellular mechanisms for sex differences in cocaine action. Previous studies demonstrated the importance of DARPP-32 in mediating the behavioral and rewarding effect of cocaine. For instance, DARPP-32 knockout mice showed attenuated locomotor response after acute cocaine, but had higher locomotor sensitization in response to chronic cocaine (Fienberg et al., 1998; Hiroi et al., 1999). In addition, mice with P-Thr34-DARPP-32 site mutation exhibited lower acute cocaine-induced locomotor response, cocaine-induced conditioned place preference, higher behavioral sensitization, and less sensitivity in response to cocaine self-administration (Zachariou et al., 2006; Zhang et al., 2006). On the other hand, in Thr75 site mutation mice, chronic cocaine-induced locomotor sensitization was unaffected or absent (Valjent et al., 2005; Zachariou et al., 2006). Although the role of DARPP-32 in cocaine action had been well studied in male rodents, its role in cocaine induced sex differences remained unclear. Only Lynch et al. (2007) showed that protein levels of P-Th34-DARPP-32 were higher in cocaine self-administrated females than males. The dissertation tried to filling the blanks by studying the changes in protein levels of DARPP-32 signaling proteins in the NAc and CPu of male and female rats after acute and chronic cocaine treatment.

The first experiment was in order to address the sexual dimorphic changes in DARPP-32 cascade in respond to acute cocaine. It revealed several new findings. First, no sex differences were observed in protein levels of DARPP-32, P-Thr34-DARPP-32, PP-1α, CaN-A and CaN-B in the NAc and CPu of naïve male and female rats. Second, after saline treatment, higher protein levels of DARPP-32, P-Thr34-DARPP-32, CaN-A and CaN-B were observed in the NAc of females when compared to males; while higher P-Thr34-DARPP-32 and PP-1α protein levels were observed in the CPu of female rats.

However, in the NAc, most of the higher inductions in females only existed 5 min after the treatment, except for CaN-A, which was still higher 15 min after treatment. Another exception was PP-1α, which did not show heightened expression in females after 5 min, but changed back into higher 30 min after treatment. Unfortunately, because of the loss of female CPu samples, we were unable to compare the basal sex differences beyond 5 min. Third, in the NAc, after a single cocaine (30 mg/kg) treatment, male rats exhibited increased expression of P-Thr34-DARPP-32 (marginal significant) and CaN-A at 30 min, as well as CaN-B at 15 min; while female rats exhibited decreased expression of DARPP-32, P-Thr34-DARPP-32 and CaN-A at 45 min, but increased PP-1α at 30 min in the NAc. However, no changes were observed in CPu of either males or females. Fourth, male rats exhibited higher percentage changes of DARPP-32, P-Thr34-DARPP-32, PP-1α, CaN-A and CaN-B in the NAc at various time points when compared to females; while in the CPu of males, similar higher percentage changes were only observed in P-Thr34-DARPP-32, CaN-A and CaN-B, and also at fewer time points.

Based on those findings, we postulated that the heightened DARPP-32 cascade in female rats may be responsible for their higher behavior responses to acute cocaine. First, female rats had higher basal protein levels of DARPP-32 signaling proteins in striatum than males. The higher basal levels of P-Thr34-DARPP-32 may result from the higher basal dopamine release and uptake, higher accumbal dopaminergic tone, high level of PKA activation, or higher basal protein levels of PKA in female rats (Walker et al., 2000; Becker, 1999; Walker et al., 2006; Nishi et al., 2000; Nazarian et al., 2009). The higher PP-1α and PP-2B basal protein levels reflected a disinhibitory effect or a positive feedback mechanism caused by the heightened basal P-Thr34-DARPP-32. However,

these sex differences were not intrinsic, as no sex differences observed in naïve rats, but rather stress-induced. It has been demonstrated that females have higher DA levels in the striatum and higher dopaminergic activities in the NAc than males after acute stress (Dalla et al., 2008). Given that saline administrations may be an acute stressor, the sexual dimorphic pattern of the DARPP-32 cascade may reflect sex differences in administration-induced stress-mediated responses, such as DA releases and dopaminergic activities. In addition, such differences were observed for the most part soon after the saline administration, which further supported this postulate. Second, male rats exhibited higher increased activation of DARPP-32 cascade, while female rats had lower decreased induction after acute cocaine. These changes did not necessary mean that females had lower protein levels than males after cocaine, as female rats started with much higher basal levels. The inability of increase in most DARPP-32 signaling proteins actually resulted from the higher basal levels, which exerted a "ceiling effect" on females. The increase of PP-1α in the NAc of females after cocaine treatment further supported the postulation, as the basal levels of PP-1α did not differ between sexes. Third, most of sex differences in basal protein levels and acute cocaine induced protein level changes were observed only in the NAc but not in the CPu. It suggested that compared to CPu, NAc may be more important for the observed sex differences in acute cocaine induced locomotor activities in the same rats used in this experiment (Nazarian et al., 2009). Further support came from that lesions of CPu and NAc abolishes the psychostimulant-induced stereotypic and locomotor behavior respectively (Kelly et al., 1975; Kelly and Iversen, 1975).

The second experiment aimed to address the sexual dimorphic in the development of behavior sensitization and tolerance, as well as in DARPP-32 cascade changes in respond to chronic cocaine. First, in general, both acute and chronic cocaine treatment increased the locomotor and stereotypic activities in male and female rats, and the activities in females were usually higher than the males with same treatment. In addition, in female rats, chronic cocaine increased locomotor activities after 2 days of treatment, but decreased locomotor activities after 14 days of treatment than acute cocaine; while in males, similar increased locomotor activities in chronic cocaine treated rats were observed after 5 days of treatment, and no decrease was observed within treatment length. On the other hand, no differences in stereotypic activities were observed between acute and chronic cocaine treatment in either sexes. Second, treatment effects were only observed in a few proteins in the CPu of females, in that chronic cocaine increased higher protein levels of PP-1α, p-CREB and ΔFosB when compared to acute cocaine. Most protein level changes in the DARPP-32 signaling proteins were day effect, and happened in different areas and at different treatment lengths in males and females. Specifically, in male rats, heightened expression of proteins in DARPP-32 cascade were mostly observed in the NAc after 5 days of treatment, including P-Thr34-DARPP-32, PKAc, p-CREB, FosB, ΔFosB, Cdk5 and p35; while in female rats, heightened expression of DARPP-32 signaling proteins were mostly observed in the CPu after 14 days of treatment, including DARPP-32, P-Thr34-DARPP-32, CaN-A, PKAc, PP-1α, p-CREB, Cdk5 and p35. Third, in male rats, ΔFosB protein levels in the CPu positively correlated with the stereotypic counts. In females, the correlation between protein levels and behavior activities more found in more proteins and more behavior categories. Specifically, FosB protein levels in

both NAc and CPu positively correlated with locomotor and stereotypic counts; while in CPu, Cdk5 protein levels negatively correlated with locomotor and stereotypic activities, and p35 protein levels negatively correlated with locomotor activities only.

From these findings, we revealed that female rats developed behavior sensitization and tolerance earlier than males, and postulated that the differences in speed may result from the sex differences in the DARPP-32 cascade in response to chronic cocaine treatment. First, lacking of differences in stereotypic after acute and chronic cocaine treatment suggest that stereotypic behavior rating, a categorical rating system, may not be a good index for behavior sensitization or tolerance development in home cage. It has been demonstrated that environment novelty is implicated in the development of psychostimulant-induced behavioral sensitization (review in Robinson et al, 1998). Second, compared to males, there was a left-shift locomotor change in female rats, that female rats developed locomotor behavior sensitization and tolerance earlier than males. It has been demonstrated that sensitization induced more dopamine receptors occupancy and tolerance resulted in the decrease of dopamine receptors occupancy (Martin-Iverson and Burger 1995). So the left-shift in females may related to higher dopamine D1 receptor sensitivity leading to earlier behavioral augmentation/sensitization in response to acute and chronic cocaine treatment; the reduction in dopamine D1 activation may underlie the locomotor tolerance induced by prolong cocaine treatment (Festa et al., 2006; Schindler and Carmona 2002; Nazarian et al., 2005). Third, male rats exhibited heightened DARPP-32 signaling in the NAc after 5 days of treatment; while female rats exhibited heightened DARPP-32 signaling in the CPu after 14 days of treatment. The regional and timely sex differences in DARPP-32 signaling were related to the sex

differences in the behavior response. We postulated that there is a DARPP-32 cascade activation transition from NAc to CPu as the chronic cocaine treatment prolongs. And the DARPP-32 cascade activation in NAc is more critical to the initial locomotor effects (i.e. sensitization) of cocaine, while its activation in CPu is more crucial for the locomotor effects (i.e. tolerance) of prolonged cocaine treatment. Since the female rats develop behavior sensitization and tolerance faster than males, their initial DARPP-32 activation in NAc may be too brief for observation, but the CPu activation was observed. In males, the DARPP-32 activation in CPu may be way beyond our treatment length, and we were only able to observe the activation in NAc. However, this model needs further completion from future experiments to observe the activation in female NAc and male CPu missed in this experiment. Fourth, we demonstrated for the first time that the protein levels of ΔFosB in male CPu, and FosB in female NAc and CPu positively correlated with their behavior response to cocaine; while the protein levels of Cdk5 and p35 in female CPu negatively correlated with their behavior response. Those proteins had been demonstrated to mediate behavior sensitization or tolerance (Nestler, 2004; Bibb et al., 2001; Taylor et al., 2007); however, we were able to further quantify the relationship between certain protein levels with behavior activity counts. We related the molecular response with behavior response. This may be good indexed to predict the changes for each other. However, most of the correlations are in female rats only, further studies are necessary to understand the reason for this sex specification.

III. Future directions

In this dissertation, we have demonstrated the importance of sex difference in DARPP-32 cascade to the sexual dimorphism in behavioral response to acute and chronic cocaine. However, to what degree does the DARPP-32 cascade involved need further demonstration, as there are several drawbacks in the current study that need further improvement. First, current western blot could not differentiate effect of cocaine on the striatonigral neurons (mainly express D1-like receptors, and activates PKA/DARPP-32/PP-1) and striatopallidal neurons (highly express D2-like receptors, and inhibits PKA/DARPP-32/PP-1 (Gerfen et al., 1992). We actually measured the total effect on a mixture of two types of neurons. Transgenic mice that allow the analysis of DARPP-32 signaling selectively in striatonigral and striatopallidal neurons would help to further understand the role of DARPP-32 pathway in cocaine action. Second, we only measured the protein levels of the DARPP-32 signaling proteins. However, the changes in protein levels do not always correlate with the protein activities. Since most of the proteins in the DARPP-32 cascade either inhibit or activate the activation of the downstream protein, future studies measuring the activity of DARPP-32 signaling proteins may reveal more accurate changes in the pathway after cocaine treatment. Third, we did not measure the mRNA level. It is possible that the increased protein levels were as a result of new protein synthesis. Furthermore, mRNA level changes faster than protein level. It is very likely that when we couldn't detect protein level changes (i.e. at 60 min), the changes in its mRNA level are already detectable. Translational alterations associated to sex differences in cocaine responses in the DARPP-32 cascade may impact transcription rate—thus protein levels—and/or protein degradation rates. Thus, experiments measuring mRNA levels are necessary to reveal more accurate molecular response to

cocaine. Fourth, for the chronic cocaine paradigm, a different time point of sacrifice is necessary. In the current study, treatment effects on most DARPP-32 signaling proteins were absent. This may result from the longer behavior measurement time. In the acute experiment, we find that the protein level changes usually happen earlier than 60 min. It is possible that the heightened protein levels induced by cocaine have returned to baseline by the time of our measurement. Also, it is impossible for so many proteins to change simultaneously at a certain time point. Checkup at another time point (for example. 30 min) is necessary to further understand the changes of DARPP-32 cascade along time. Fifth, within our treatment length, we did not observe locomotor tolerance in male rats. This may caused by their slow development speed. So future studies extending the treatment length are necessary to check the DARPP-32 cascade changes and study its role in male rats in tolerance. Sixth, an important issue not studied here is the effect of ovarian hormones on DARPP-32 regulation. Future studies measuring the estrogen and progesterone levels in the blood collected at sacrifice should be done to differentiate the stage of estrous cycle of female rats in study, and further analyze the behavior and intracellular response to cocaine of female rats at different estrous cycle. Another way is using OVX female rats with different hormone replacement, which will help elucidate how ovarian hormones modulate of intracellular DARPP-32 signal transduction pathways. Last, we did not run statistical corrections for multiple correlation analysis. However, all significant results were seen mostly only in the CPu of females. Thus, is unlikely that these results were due to random errors in statistical analysis. However, in the future Bonferroni corrections should be run to further exclude the possibility of random errors associated with multiple statistical analyses.

The clinical implications of our data suggest that women's higher response to addictive drugs and quicker development into drug addiction may result from their heightened and earlier molecular response. However, sex differences are limited to women in their luteal phase, when the subjective effects of cocaine were lower compared to the follicular phase (Sofuoglu et al., 1999; Evans et al., 2002; Evans and Foltin, 2006). Other clinical studies have revealed conflicting findings of no sex or menstrual cycle differences, which may result from different dose and route of cocaine administration (Mendelson et al., 1999; Collins et al., 2007). Further investigations into the influence of hormones, dose response and administration route on cocaine-induced intracellular mechanisms will help understanding the biological basis for sex differences in cocaine addiction and developing sex-specific treatments for cocaine addition.

References

Adlersberg M, Hsiung SC, Glickstein SB, Liu KP, Tamir H, Schmauss C (2004) Regulation of dopamine D-receptor activation in vivo by protein phosphatase 2B (calcineurin). J Neurochem 90: 865-873.

Albin RL, Young AB, Penney JB (1989) The functional anatomy of basal ganglia disorders. Trends Neurosci 12: 366-175.

Ambrose-Lanci LM, Peiris NB, Unterwald EM, Van Bockstaele EJ (2008) Cocaine withdrawal-induced trafficking of delta-opioid receptors in rat nucleus accumbens. Brain Res 1210: 92-102.

Auger AP, Meredith JM, Snyder GL, Blaustein JD (2001) Oestradiol increases phosphorylation of a dopamine- and cyclic AMP-regulated phosphoprotein (DARPP-32) in female rat brain. J Neuroendocrinol 13: 761-768.

Bateup HS, Svenningsson P, Kuroiwa M, Gong S, Nishi A, Heintz N, Greengard P (2008) Cell type-specific regulation of DARPP-32 phosphorylation by psychostimulant and antipsychotic drugs. Nat Neurosci 11: 932-9.

Becker JB (1999) Gender differences in dopaminergic function in striatum and nucleus accumbens. Pharmacol Biochem Behav 64: 803-812.

Becker JB, Monteggia L, Perrot-Sinal T, Romeo RD, Taylor JR, Yehuda R, Bale T (2007) Stress and disease: is being female a predisposing factor? J Neurosci 27: 11851-11855.

Benavides DR, Quinn JJ, Zhong P, Hawasli AH, DiLeone RJ, Kansy JW, Olausson P, Yan Z, Taylor JR, Bibb JA (2007) Cdk5 modulates cocaine reward, motivation, and striatal neuron excitability. J Neurosci 27: 12967-12976.

Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL, Yan Z, Sagawa ZK, Ouimet CC, Nairn AC, Nestler EJ, Greengard P (2001) Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. Nature 410: 376-380.

Bibb JA, Snyder GL, Nishi A, Yan Z, Meijer L, Fienberg AA, Tsai LH, Kwon YT, Girault JA, Czernik AJ, Huganir RL, Hemmings HC, Nairn AC, Greegard P (1999) Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. Nature 402: 669-671.

Bito H, Deisseroth K, Tsien RW (1996) CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87: 1203-1214.

Blanchard RJ, Hebert MA, Dulloog L, Kaawaloa N, Nishimura O, Blanchard DC (1998) Acute cocaine effects on stereotype and defense: an ethoexperimental approach. Neurosci Biobehav Rev 23: 179-188.

Blank T, Nijholt I, Teichert U, Kugler H, Behrsing H, Fienberg AA, Greengard P, Spiess J (1997) The phosphoprotein DARPP-32 mediates cAMP-dependent potentiation of striatal N-methyl-D-aspartate responses. Proc Natl Acad Sci USA 94: 14859-14864.

Bonci A, Williams JT (1996) A common mechanism mediates long term changes in synaptic transmission after chronic cocaine and morphine. Neuron 16: 631-639.

Cabib S, Castellano C, Cestari V, Filibeck U, Puglisi-Allegra S (1991) D1 and D2 receptor antagonists differently affect cocaine-induced locomotor hyperactivity in the mouse. Psychopharmacology 105: 335-339.

Carboni E, Imperato A, Perezzani L, DiChiara G (1989) Amphetamine, cocaine phencyclidine and nomifensin increase extracellular dopamine concentration preferentially in the nucleus accumbens of freely moving rats. Neuroscience 28: 653-661.

Carlezon WA Jr, Duman RS, Nestler EJ (2005) The many faces of CREB. Trends Neurosci 28: 436-445.

Carlezon WA Jr, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ (1998) Regulation of cocaine reward by CREB. Science 282: 2272-2275.

Castaneda E, Becker JB, Robinson TE (1988) The long-term effects of repeated amphetamine treatment in vivo on amphetamine, KCl and electrical stimulation evoked striatal dopamine release in vitro. Life Sci 42: 2447-2456.

Chen PC, Chen JC (2005) Enhanced Cdk5 activity and p35 translocation in the ventral striatum of acute and chronic methamphetamine-treated rats. Neuropsychopharmacology 30: 538-549.

Chen J, Nye HE, Kelz MB, Hiroi N, Nakabeppu Y, Hope BT, Nestler EJ (1995) Regulation of delta FosB and FosB-like proteins by electroconvulsive seizure and cocaine treatments. Mol Pharmacol 48: 880-889.

Chin J, Sternin O, Wu HB, Burrell S, Lu D, Jenab S, Perrotti LI, Quinones-Jenab S (2002) Endogenous gonadal hormones modulate behavioral and neurochemical responses to acute and chronic cocaine administration. Brain Res 945: 123-130.

Church W, Justice JB, Byrd LD (1987) The long-term effects of repeated amphetamine treatment in vivo on amphetamine, KCl and electrical stimulation evoked striatal dopamine release in vitro. Eur J Pharmacol 139: 345-348.

Churchill L, Swanson CJ, Urbina M, Kalivas PW (1999) Repeated cocaine alters glutamate receptor subunit levels in the nucleus accumbens and ventral tegmental area of rats that develop behavioral sensitization. J Neurochem 72: 2397-2403.

Colby CR, Whisler K, Steffen C, Nestler EJ, Self DW (2003) Striatal cell type-specific overexpression of DeltaFosB enhances incentive for cocaine. J Neurosci 23: 2488-2493.

Collins SL, Evans SM, Foltin RW, Haney M (2007) Intranasal cocaine in humans: effects of sex and menstrual cycle. Pharmacol Biochem Behav 86: 117-124.

Crawford CA, Choi FY, Kohutek JL, Yoshida ST, McDougall SA (2004) Changes in PKA activity and Gs alpha and Golf alpha levels after amphetamine- and cocaine-induced behavioral sensitization. Synapse 51: 241-248.

Dalla C, Antoniou K, Kokras N, Drossopoulou G, Papathanasiou G, Bekris S, Daskas S, Papadopoulou-Daifoti Z (2008) Sex differences in the effects of two stress paradigms on dopaminergic neurotransmission. Physiol Behav 93(3): 595-605.

Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci USA 85: 5274-5278.

Edelman AM, Blumenthal DK, Krebs EG (1987) Protein serine/threonine kinases. Ann Rev Biochem 56: 567-613.

Evans SM, Foltin RW (2006) Exogenous progesterone attenuates the subjective effects of smoked cocaine in women, but not in men. Neuropsychopharmacology 31: 659-674.

Evans SM, Haney M, Foltin RW (2002) The effects of smoked cocaine during the follicular and luteal phases of the menstrual cycle in women. Psychopharmacology 159: 397-406.

Everitt BJ, Dickinson A, Robbins TW (2001) The neuropsychological basis of addictive behaviour. Brain Res Brain Res Rev 36: 129-138.

Everitt BJ, Wolf ME (2002) Psychomotor stimulant addiction: a neural systems perspective. J Neurosci 22: 3312-3320.

Festa ED, Jenab S, Chin J, Gazi FM, Wu HBK, Russo SJ, Quinones-Jenab V (2003) Frequency of cocaine administration affects behavioral and endocrine responses in male and female Fischer rats. Cell Mol Biol 49: 1275-1280.

Festa ED, Jenab S, Weiner J, Nazarian A, Niyomachai T, Russo SJ, Kemen LM, Akhavan A, Wu HB, Quinones-Jenab V (2006) Cocaine-induced sex differences in D1 receptor activation and binding levels after acute cocaine administration. Brain Res Bull 68: 277-284.

Festa ED, Russo SJ, Gazi FM, Niyomchai T, Kemen LM, Lin S-N, Foltz R, Jenab S, Quinones-Jenab V (2004) Sex differences in cocaine-induced behavioral responses, pharmacokinetics, and monoamine levels. Neuropharmacology 46: 672-687.

Fienberg AA, Hiroi A, Mermelstein PG, Song WJ, Snyder GL, Nishi A, Cheramy A, O'Callaghan JP, Miller DB, Cole DG, Corbett R, Haile CN, Cooper DC, Onn SP, Grace AA, Ouimet CC, White FJ, Hyman SE, Surmeier DJ, Girault JA, Nestler EJ, Greengard P

(1998) DARPP-32: regulator of the efficacy of dopaminergic neurotransmission. Science 281: 838-842.

Floresco SB, Yang CR, Phillips AG, Blaha CD (1998) Basolateral amygdala stimulation evokes glutamate receptor-dependent dopamine efflux in the nucleus accumbens of the anaesthetized rat. Eur J Neurosci 10: 1251.

Freund TF, Powell J, Smith AD (1984) Tyrosine hydroxylase immunoractive boutons in synaptic contact with identified striatonigral neurons, with particular reference to dentritic spines. Neuroscience 13: 1189-1215.

Fuchs RA, Branham RK, See RE (2006) Different neural substrates mediate cocaine seeking after abstinence versus extinction training: a critical role for the dorsolateral caudate-putamen. J Neurosci 26: 3584-3588.

Garavan H, Pankiewicz J, Bloom A, Cho JK, Sperry L, Ross TJ, Salmeron BJ, Risinger R, Kelley D, Stein EA (2000) Cue-induced cocaine craving: neuroanatomical specificity for drug users and drug stimuli. Am J Psychiatry 157: 1789-1798.

Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ, Sibley DR (1990) D1 and D2 dopamine-regulated gene expression of striatonigral and striatopallidal neurons. Science 250: 1429-1432.

Goto S, Matsukado Y, Mihara Y, Inoue N, Miyamoto E (1986) The distribution of calcineurin in rat brain by light and electron microscopic immunohistochemistry and enzyme-immunoassay. Brain Res 397: 161-172.

Greengard P (2001) The neurobiology of dopamine signaling. Biosci Rep 21: 247-269.

Griffin ML, Weiss RD, Mirin SM, Lange U (1989) A comparison of male and female cocaine abusers. Arch Gen Psychiatry 46: 122-126.

Halpain S, Girault JA, Greengard P (1990) Activation of NMDA receptors induces dephosphorylation of DARPP-32 in rat striatal slices. Nature 343: 369-372.

Harrod SB, Booze RM, Welch M, Browning CE, Mactutus CF (2005) Acute and repeated intravenous cocaine-induced locomotor activity is altered as a function of sex and gonadectomy. Pharmacol Biochem Behav 82(1): 170-181.

Heikkila RE, Orlansky H, Cohen G (1975) Studies on the distinction between uptake inhibition and release of [3H] dopamine in rat brain tissue slices. Biochem Pharmacol 24: 847-852.

Hemmings HC, Greengard P, Tung HY, Cohen P (1984) DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. Nature 310: 503-505.

Henry DJ, White FJ (1991) Repeated cocaine administration causes persistent enhancement of D1 dopamine density within the rat nucleus accumbens. J Pharmacol Exp Ther 258: 882-890.

Henry DJ, White FJ (1995) The persistence of behavioral sensitization to cocaine parallels enhanced inhibition of nucleus neurons. J Neurosci 15: 6287-6299.

Hiroi N, Brown JR, Haile CN, Ye H, Greenberg ME, Nestler EJ (1997) FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects.Proc Natl Acad Sci USA 94: 10397-10402.

Hiroi N, Fienberg AA, Haile CN, Alburges M, Hanson GR, Greegard P, Nestler EJ (1999) Neuronal and behavioural abnormalities in striatal function in DARPP-32-mutant mice. Eur J of Neurosci 11: 1114-1118.

Hooks MS, Duffy P, Striplin C, Kalivas PW (1994) Behavioral and neurochemical sensitization following cocaine self-administration. Psychopharmacology 115: 265-272.

Hope BT, Crombag HS, Jedynak JP, Wise RA (2005) Neuroadaptations of total levels of adenylate cyclase, protein kinase A, tyrosine hydroxylase, cdk5 and neurofilaments in the nucleus accumbens and ventral tegmental area do not correlate with expression of sensitized or tolerant locomotor responses to cocaine. J Neurochem 92: 536-545.

Hope B, Kosofsky B, Hyman SE, Nestler EJ (1992) Regulation of immediate early gene expression and AP-1 binding in the rat nucleus accumbens by chronic cocaine. Proc Natl Acad Sci USA 89: 5764-5768.

Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS, Nestler EJ (1994) Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. Neuron 13: 1235-1244.

Horger BA, Shelton K, Schenk S (1990) Preexposure sensitizes rats to the rewarding effects of cocaine. Pharmacol Biochem Behav 37: 707-711

Horn AS (1990) Dopamine uptake: a review of progress in the last decade. Prog Neurobiol 34: 387-400.

Howland JG, Taepavarapruk P, Phillips AG (2002) Glutamate receptor-dependent modulation of dopamine efflux in the nucleus accumbens by basolateral, but not central, nucleus of the amygdala in rats. J Neurosci 22: 1145.

Hu M, Crombag HS, Robinson TE, Becker JB (2004) Biological basis of sex differences in the propensity to self-administer cocaine. Neuropsychopharmacology 29(1): 81-85.

Hu XT, Ford K, White FJ (2005) Repeated cocaine administration decreases calcineurin (PP2B) but enhances DARPP-32 modulation of sodium currents in rat nucleus accumbens neurons. Neuropsychopharmacology 30: 916-926.

Hummel M, Unterwald EM (2002) D1 dopamine receptor: a putative neurochemical and behavioral link to cocaine action. J Cell Physio 191: 17-27.

Hurd YL, Weiss F, Koob GF, And NE, Ungerstedt U (1989) Cocaine reinforcement and extracellular dopamine overflow in rat nucleus accumbens: an in vivo microdialysis study. Brain Res 498: 199-203.

Hyman SE, Malenka RC (2001) Addiction and the brain: the neurobiology of compulsion and its persistence. Nat Rev Neurosci 2: 695-703.

Ishizuka T, Ino H, Sawa K, Suzuki N, Tatibana M (1995) Promoter region of the mouse cyclin-dependent kinase 5-encoding gene. Gene 166: 267-271.

Ito R, Dalley JW, Robbins TW, Everitt BJ (2002) Dopamine release in the dorsal striatum during cocaine-seeking behavior under the control of a drug-associated cue. J Neurosci 22: 6247-6253.

Izenwasser S, French D (2002) Tolerance and sensitization to locomotor-activating effects of cocaine are mediated via independent mechanisms. Pharmacol Biochem Behav 73: 877-882.

Jackson LR, Robinson TE, Becker JB (2006) Sex differences and hormonal influences on acquisition of cocaine self-administration in rats. Neuropsychopharmacology 31(1): 129-138.

Kalivas PW, Volkow N, Seamans J (2005) Unmanageable motivation in addiction: a pathology in prefrontal-accumbens glutamate transmission. Neuron 45(5): 647-650.

Kalivas PW, Weber B (1988) Amphetamine injection into the ventral mesencephalon sensitizes rats to peripheral amphetamine and cocaine. J Pharmacol Exp Ther 245: 1095-1102.

Kelley AE, Domesick VB, Nauta JH (1982) The amygdalostriatal projection in the rat--an anatomical study by anterograde and retrograde tracing methods. Neuroscience 7: 630.

Kelley PH, Iversen SD (1975) Selective 6-OHDA-induced destruction of mesolimbic dopamine neurons: Abolition of psychostimulant-induced locomotor activity in rats. Eur J Pharmacol 40: 45-56.

Kelly PH, Seviour PW, Iversen SD (1975) Amphetamine and apomorphine responses in rat following 6-OHDA lesions of nucleus accumbens septi and corpus striatum. Brain Res 94: 507-523.

Kelz MB, Chen J, Carlezon WA Jr, Whisler K, Gilden L, Beckmann AM, Steffen C, Zhang YJ, Marotti L, Self DW, Tkatch T, Baranauskas G, Surmeier DJ, Neve RL, Duman RS, Picciotto MR, Nestler EJ (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. Nature 401: 272-276.

Kelz MB, Nestler EJ (2000) ΔFosB: a molecular switch underlying long-term neural plasticity. Curr Poin Neurol 13: 715-720.

King GR, Hillburn C, Pinto G, Konen J (2004) The effects of continuous cocaine dose, treatment, and withdrawal duration on the induction of behavioral tolerance and dopamine autoreceptor function. Pharmacol Biochem Behav 78: 293-300.

King GR, Joyner C, Lee T, Kuhn C, Ellinwood EH Jr. (1992) Intermittent and continuous cocaine administration: residual behavioral states during withdrawal. Pharmacol Biochem Behav 43: 243-248.

King GR, Xiong Z, Douglas S, Ellinwood EH (1999) The effects of continuous cocaine dose on the induction of behavioral tolerance and dopamine autoreceptor function. Eur J Pharmacol 376: 207-215.

King MM, Huang CY, Chock PB, Nairn AC, Hemmings HC, Chan KF, Greengard P (1984) Mammalian brain phosphoproteins as substrates for calcineurin. J Biol Chem 259: 8080-8083.

Kolta MG, Shreve P, De Souza V, Uretsky NJ (1985) Time course of the development of the enhanced behavioral and biochemical responses to amphetamine after pretreatment with amphetamine. Neuropharmacology 24: 823-829.

Koob GF (1992) Drugs of abuse: anatomy, pharmacology, and function of reward pathways. Trends Pharmacol Sci 13: 177-184.

Koob GF (1996) Drug addiction: the yin and yang of hedonic homeostasis. Neuron 16: 893-896.

Kosten TA, Gawin FH, Kosten TR, Rounsaville BJ (1993) Gender differences in cocaine use and treatment response. J Subst Abuse Treat 10: 63-66.

Kosten TR, Kosten TA, McDougle CJ, Hameedi FA, McCance EF, Rosen MI, Oliveto AH, Price LH (1996) Gender differences in response to intranasal cocaine administration to humans. Biol Psychiatry 39: 147-148.

Krueger BK (1990) Kinetics and block of dopamine uptake in synaptosomes from rat caudate nucleus. J Neurochem 55: 260-267.

Kumakura K, Hoffman M, Cocchi D, Trabucci M, Spano PF, Muller EE (1979) Long-term effect of ovariectomy on dopamine-stimulated adenylate cyclase in rat striatum and nucleus accumbens. Psychopharmacology 61: 13-16.

Kumar A, Choi KH, Renthal W, Tsankova NM, Theobald DE, Truong HT, Russo SJ, Laplant Q, Sasaki TS, Whistler KN, Neve RL, Self DW, Nestler EJ (2005) Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. Neuron 48: 303-314.

Kunko PM, French D, Izenwasser S (1998) Alternations in locomotor activity during chronic cocaine administration: effect on dopamine receptors and interaction with opioids. J Pharmacol Exp Ther 285: 277-284.

Lee KW, Kim Y, Kim AM, Helmin K, Nairn AC, Greengard P (2006) Cocaine-induced dendritic spine formation in D1 and D2 dopamine receptor-containing medium spiny neurons in nucleus accumbens. Proc Natl Acad Sci USA 103: 3399-3404.

Li Y, Hu XT, Berney TG, Vartanian AJ, Stine CD, Wolf ME, White FJ (1999) Both glutamate receptor antagonists and prefrontal cortex lesions prevent induction of cocaine sensitization and associated neuroadaptations. Synapse 34: 169-180.

Li Y, Wolf ME (1997) Ibotenic acid lesions of prefrontal cortex do not prevent expression of behavioral sensitization to amphetamine. Behav Brain Res 84: 285-289.

Lin XH, Hashimoto T, Kitamura N, Murakami N, Shirakawa O, Maeda K (2002) Decreased calcineurin and increased phosphothreonine-DARPP-32 in the striatum of rats behaviorally sensitized to methamphetamine. Synapse 44: 181-187.

Lindskog M, Svenningsson P, Fredholm BB, Greengard P, Fisone G (1999) Activation of dopamine D2 receptors decreases DARPP-32 phosphorylation in striatonigral and striatopallidal projection neurons via different mechanisms. Neuroscience 88: 1005-1008.

Liu SJ, Fang ZY, Yang Y, Deng HM, Wang JZ (2003) Alzheimer-like phosphorylation of tau and neurofilament induced by cocaine in vivo. Acta Pharmacol Sin 24: 512-518.

Lorrain DS, Arnold GM, Vezina P (2000) Previous exposure to amphetamine increases incentive to obtain the drug: long-lasting effects revealed by the progressive ratio schedule. Behav Brain Res 107: 9-19.

Lu L, Grimm JW, Shaham Y, Hope BT (2003) Molecular neuroadaptations in the accumbens and ventral tegmental area during the first 90 days of forced abstinence from cocaine self-administration in rats. J Neurochem 85: 1604-1613.

Lukas SE, Sholar MB, Lundahl LH, Lamas X, Kouri E, Wines JD, Kragie L, Mendelson JH (1996) Sex differences in plasma cocaine levels and subjective effects after acute cocaine administration in human volunteers. Psychopharmacology 125: 346-356.

Lynch WJ, Carroll ME (1999) Sex differences in the acquisition of intravenously self-administered cocaine and heroin in rats. Psychopharmacology 144: 77-82.

Lynch WJ, Kiraly DD, Caldarone BJ, Picciotto MR, Taylor JR (2007) Effect of cocaine self-administration on striatal PKA-regulated signaling in male and female rats. Psychopharmacology 191: 263-271.

Maccioni RB, Otth C, Concha II, Munoz JP (2001) The protein kinase Cdk5, structural aspects, roles in neurogenesis and involvement in Alzheimer's pathology. Eur J Biochem 268: 1518-1527.

Madras BK, Colvis CM, Pollock JD, Rutter JL, Shurtleff D, Von Zastrow M (2005) Cell biology of addiction. Cold Spring Harbor Laboratory Press.

Maisonneuve IM, Ho A, Kreek MJ (1995) Chronic administration of a cocaine "binge" alters basal extracellular levels in male rats: an in vivo microdialysys study. J Pharmacol Exp Ther 272: 652-657.

Maisonneuve IM, Kreek MJ (1994) Acute tolerance to the dopamine response induced by a binge pattern of cocaine administration in male rats: an in vivo microdialysis study. J Pharmacol Exp Ther 268: 916-921.

Mani SK, Fienberg AA, O'Callaghan JP, Snyder GL, Allen PB, Dash PK, Moore AN, Mitchell AJ, Bibb J, Greegard P, O'Malley BW (2000) Requirement for DARPP-32 in progesterone-facilitated sexual receptivity in female rats and mice. Science 287: 1053-1056.

Martin-Iverson MT, Burger LY (1995) Behavioral sensitization and tolerance to cocaine and occupation of dopamine receptors by dopamine. Mol Neurobiol 11: 31-46.

Maus M, Bertrand P, Drouva S, Rasolonjanahary R, Kordon C, Glowinski J, Premont P, Enjalbert A (1989) Differential Modulation of D1 and D2 Dopamine-sensitive Adenylate Cyclases by 17Beta-Estradiol in cultured striatal neurons and anterior pituitary cells. J Neurochem 52: 410-418.

McCance-Katz EF, Hart CL, Boyarsky B, Kosten T, Jatlow P (2005) Gender effects following repeated administration of cocaine and alcohol in humans. Subst Use Misuse 40: 511-528.

McCreary AC, Marsden CA (1993) Cocaine-induced behavior: dopamine D1 receptor antagomism by SCH 23390 prevents expression of conditioned sensitization following repeated administration of cocaine. Neuropharmacology 32: 387-391.

Mellon PL, Clegg CH, Correll LA, McKnight GS (1989) Regulation of transcription by cyclic AMP-dependent protein kinase. Proc Natl Acad Sci USA 86: 4887-4891.

Mendelson JH, Mello NK, Sholar MB, Siegel AJ, Kaufman MJ, Levin JM, Renshaw PF, Cohen BM (1999) Cocaine pharmacokinetics in men and in women during the follicular and luteal phases of the menstrual cycle. Neuropsychopharmacology 21: 294-303.

Mendrek A, Blaha CD, Phillips AG (1998) Pre-exposure of rats to amphetamine sensitizes self-administration of this drug under a progressive ratio schedule. Psychopharmacology 135:416-422.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors: from structure to function. Physiol Rev 78: 189-225.

Mitsushima D, Yamada K, Takase K, Funabashi T, Kimura F (2006) Sex differences in the basolateral amygdala: the extracellular levels of serotonin and dopamine, and their response to restraint stress in rats. Eur J Neurosci 24: 3245-3254.

Moore KE, Dominic JA (1971) Tyrosine hydroxylase inhibitors. Fed Proc 30: 859-870.

Moratalla R, Elibol B, Vallejo M, Graybiel AM (1996) Network-level changes in expression of inducible Fos-Jun proteins in the striatum during chronic cocaine treatment and withdrawal. Neuron 17: 147-156.

Mulder AB, Hodenpijl MG, Lopes da Silva FH (1998) Electrophysiology of the hippocampal and amygdaloid projections to the nucleus accumbens of the rat: covergence, segregation, and interaction of inputs. J Neurosci 18: 5102.

Nagatsu T, Levitt M, Udenfriend S (1964) Conversion of L-tyrosine to 3,4-dihydroxyphenylalanine by cell-free preparations of brain and sympathetically innervated tissues. Biochem Biophys Res Commun 14: 543-549.

Nairn AC, Shenolikar S (1992) The role of protein phosphatases in synaptic transmission, plasticity and neuronal development. Curr Opin Neurobiol 2: 296-301.

Nazarian A, Russo SJ, Festa ED, Kraish M, Quinones-Jenab V (2004) The role of D1 and D2 receptors in the cocaine conditioned place preference of male and female rats. Brain Res Bull 63: 295-299.

Nazarian A, Sun WL, Zhou L, Kemen LM, Jenab S, Quinones-Jenab V (2009) Sex differences in basal and cocaine-induced alterations in PKA protein levels in the nucleus accumbens. Psychopharmacology 203: 641-650.

Nestler EJ (2004) Molecular mechanisms of drug addiction. Neuropharmacology 47 suppl 1: 24-32.

Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. Nature Reviews 2: 119-128.

Nestler EJ, Aghajanian GK (1997) Molecular and cellular basis of addiction. Science 278: 58-63.

Nishi A, Bibb JA, Snyder GL, Higashi H, Nairn AC, Greengard P (2000) Amplification of dopaminergic signaling by a positive feedback loop. Proc Natl Acad Sci USA 97: 12840-12845.

Nishi A, Snyder GL, Greengard P (1997) Bidirectional regulation of DARPP-32 phosphorylation by dopamine. Journal of Neuroscience 17: 8147-8155.

NSDUH 2007. Results from the 2006 National Survey on Drug Use and Health. Rockville, MD: Office of Applied Studies.

NSDUH 2008. Results from the 2007 National Survey on Drug Use and Health. Rockville, MD: Office of Applied Studies.

Nye HE, Hope BT, Kelz MB, Iadarola M, Nestler EJ (1995) Pharmacological studies of the regulation of chronic FOS-related antigen induction by cocaine in the striatum and nucleus accumbens. J Pharmacol Exp Ther 275: 1671-1680.

Ouimet CC, Miller PE, Hemmings HC, Walaas SI, Greegard P (1984) DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine innervated brain regions III. J Neurosci 4: 111-124.

Packard MG, Knowlton BJ (2002) Learning and memory functions of the Basal Ganglia. Annu Rev Neurosci 25: 563-593.

Perrotti LI, Weaver RR, Robison B, Renthal W, Maze I, Yazdani S, Elmore RG, Knapp DJ, Selley DE, Martin BR, Sim-Selley L, Bachtell RK, Self DW, Nestler EJ (2008) Distinct patterns of DeltaFosB induction in brain by drugs of abuse. Synapse 62: 358-369.

Phillips AG, Di Ciano P (1996) Behavioral sensitization is induced by intravenous self-administration of cocaine by rats. Psychopharmacology 24: 279-281.

Pich EM, Pagliusi SR, Tessari M, Talabot-Ayer D, Hooft van Huijsduijnen R, Chiamulera C (1997) Common neural substrates for the addictive properties of nicotine and cocaine. Science 275: 83-86.

Pierce RC, Reeder DC, Hicks J, Morgan ZR, Kalivas PW (1998) Ibonetic acid lesions of the dorsal prefrontal cortex disrupt the expression of behavioral sensitization to cocaine. Neuroscience 82: 1103-1114.

Platt JJ (1997) The Problem of cocaine abuse and addiction, in Cocaine addiction: theory, research, and treatment. Harvard University Press: 3-21.

Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA Jr (2001) Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. J Neurosci 21: 7397-7403.

Porrino LJ, Lyons D, Smith HR, Daunais JB, Nader MA (2004) Cocaine self-administration produces a progressive involvement of limbic, association, and sensorimotor striatal domains. J Neurosci 24: 3554-3562.

Quiñones-Jenab V, Ho A, Schlussman SD, Franck J, Kreek MJ (1999) Estrous cycle differences in cocaine-induced stereotypic and locomotor behaviors in Fischer rats. Behav Brain Res 101: 15-20.

Rauggi R, Scheggi S, Cassanelli A, De Montis MG, Tagliamonte A, Gambarana C (2005) The mesolimbic dopaminergic response to novel palatable food consumption increases

dopamine-D1 receptor-mediated signaling with complex modifications of the DARPP-32 phosphorylation pattern. J Neurochem 92: 867-877.

Reith MEA, Benuck M, Lajtha A (1987) Cocaine disposition in the brain after continuous or intermittent treatment and locomotor stimulation in mice. J Pharmacol Exp Ther 243: 281–287.

Ridenour TA, Maldonado-Molina M, Compton WM, Spitznagel EL, Cottler LB (2005) Factors associated with the transition from abuse to dependence among substance abusers: implications for a measure of addictive liability. Drug Alcohol Depend 80:1-14.

Robbins SJ, Ehrman RN, Chidress AR, O'Brian CP (1999) Comparing levels of cocaine cue reactivity in male and female outpatients. Drug Alcohol Depend 53: 223-230.

Robinson TE, Becker JB (1982) Behavioral sensitization is accompanied by an enhancement in amphetamine-stimulated dopamine release from striatal tissue in vitro. Eur J Pharmacol 85: 253-254.

Robinson TE, Berridge KC (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Research Rev 18: 247-291.

Robinson TE, Berridge KC (2003) Addiction. Annu Rev Psychol 54: 25-53.

Robinson TE, Browman KE, Crombag HS, Badiani A (1998) Modulation of the induction or expression of psychostimulant sensitization by the circumstances surrounding drug administration. Neurosci Biobehav Rev 22: 347-354.

Robledo P, Maldonado-Lopez R, Koob GF (1992) Role of dopamine receptors in the nucleus accumbens in the rewarding properties of cocaine. Ann N Y Acad Sci 654: 509-512.

Russo SJ, Jenab S, Fabian SJ, Festa ED, Kemen LM, Quinones-Jenab V (2003) Sex differences in the conditioned rewarding effects of cocaine. Brain Res 970: 214-220.

Scheggi S, Raone A, De Montis MG, Tagliamonte A, Gambarana C (2007) Behavioral expression of cocaine sensitization in rats is accompanied by a distinct pattern of modifications in the PKA/DARPP-32 signaling pathway. J Neurochem 103: 1168-83.

Scheggi S, Rauggi R, Gambarana C, Tagliamonte A, De Montis MG (2004) Dopamine and cyclic AMP-regulated phosphoprotein-32 phosphorylation pattern in cocaine and morphine-sensitized rats. J Neurochem 90: 792-799.

Scheggi S, Rauggi R, Nanni G, Tagliamonte A, Gambarana C (2004) Repeated acetyl-l-carnitine administration increases phospho-Thr34 DARPP-32 levels and antagonizes cocaine-induced increase in Cdk5 and phospho-Thr75 DARPP-32 levels in rat striatum. Eur J Neurosci 19: 1609-1620.

Schindler CW, Carmona GN (2002) Effects of dopamine agonists and antagonists on locomotor activity in male and female rats. Pharmacol Biochem Behav 72: 857-863.

Seiwell AP, Reveron ME, Duvauchelle CL (2007) Increased accumbens Cdk5 expression in rats after short-access to self-administered cocaine, but not after long-access sessions. Neurosci Lett 417: 100-105.

Sell SL, Scalzitti JM, Thomas ML, Cunningham KA (2000) Influence of ovarian hormones and estrous cycle on the behavioral response to cocaine in female rats. J Pharmacol Exp Ther 293: 879-886.

Shuster L, Yu G, Bates A (1977) Sensitization to cocaine stimulation in mice. Psychopharmacology 52: 185-190.

Sibley DR, Monsma FJ (1992) Molecular biology of dopamine receptors. Trends Pharmacol Sci 13: 61-68.

Sircar R, Kim D (1999) Female gonadal hormones differentially modulate cocaine-induced behavioral sensitization in Fischer, Lewis, and Sprague-Dawley rats. J Pharmacol Exp Ther 289: 54-65.

Snyder GL, Fienberg AA, Huganir RL, Greengard P (1998) A dopamine/D1 receptor/protein kinase A/dopamine- and cAMP-regulated phosphoprotein (Mr 32 kDa)/ protein phosphatase-1 pathway regulated dephosphorylation of the NMDA receptor. J Neurosci 18: 10297-10303.

Sofuoglu M, Dudish-Poulsen S, Nelson D, Pentel PR, Hatsukami DK (1999) Sex and menstrual cycle differences in the subjective effects from smoked cocaine in humans. Exp Clin Psychopharmacol 7: 274-283.

Sorg BA, Chen SY, Kalivas PW (1993) Time course of tyrosine hydroxylase expression after behavioral sensitization to cocaine. J Pharmacol Exp Ther 266: 424-430.

Stoof JC, Kebabian JW (1981) Opposing roles for D-1 and D-2 dopamine receptors in efflux of cyclic AMP from rat neostriatum. Nature 294: 366-368.

Strack S, Choi S, Lovinger DM, Colbran RJ (1997) Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. J Biol Chem 272: 13467-13470.

Surmeier DJ, Bargas J, Hemmings HC, Nairn AC, Greengard P (1995) Modulation of calcium currents by a D1 dopaminergic protein kinase/phosphatase cascade in rat neostriatal neurons. Neuron 14: 385-397.

Surmeier DJ, Song WJ, Yan Z (1996) Coordinated expression of dopamine receptors in neostriatum medium spiny neurons. J Neurosci 16: 6579-6591.

Svenningsson P, Nairn AC, Greengard P (2005) DARPP-32 mediates the actions of multiple drugs of abuse. AAPS J 7: E353-360.

Svenningsson P, Nishi A, Fisone G, Girault JA, Nairn AC, Greengard P (2004) DARPP-32: an integrator of neurotransmission. Annu Rev Pharmacol Toxicol 44: 269-296.

Synder GL, Allen PB, Fienberg AA, Valle CG, Huganir RL, Nairn AC, Greengard P (2000) Regulation of phosphorylation of the GluR1 AMPA receptor in the neostriatum by dopamine and psychostimulants in vivo. J Neurosci 20: 4480-4488.

Takahashi S, Ohshima T, Cho A, Sreenath T, Iadarola MJ, Pant HC, Kim Y, Nairn AC, Brady RO, Greengard P, Kulkarni AB (2005) Increased activity of cyclin-dependent kinase 5 leads to attenuation of cocaine-mediated dopamine signaling. Proc Natl Acad Sci USA 102: 1737-1742.

Taylor JR, Lynch WJ, Sanchez H, Olausson P, Nestler EJ, Bibb JA (2007) Inhibition of Cdk5 in the nucleus accumbens enhances the locomotor-activating and incentive-motivational effects of cocaine. Proc Natl Acad Sci USA 104: 4147-4152.

Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM, Nestler EJ (1991) A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res 548: 100-110.

Torres G, Rivier C (1994) Induction of c-fos in rat brain by acute cocaine and fenfluranmine exposure: a comparison study. Brain Res 647: 1-9.

Tsai LH, Delalle I, Caviness VS, Chae T, Harlow E (1994) p35 is a neural-specific regulator subunit of cyclin-dependent kinase 5. Nature 371: 419-423.

Unterwald EM, Cox BM, Kreek MJ, Cote TE, Izenwasser S (1993) Chronic repeated cocaine administration alters basal and opioid-regulated adenylyl cyclase activity. Synapse 15: 33–38.

Unterwald EM, Cuntapay M (2000) Dopamine–opioid interactions in the rat striatum: a modulatory role for dopamine D1 receptors in delta opioid receptor-mediated signal transduction. Neuropharmacology 39: 372–381

Ushijima I, Carino A, Horita A (1995) Involvement of D1 and D2 dopamine systems in the behavioral effects of cocaine in rats. Pharmacol Biochem Behav 52: 737-741.

Usui H, Imazu M, Maeta K, Tsukamoto H, Azuma K, Takeda M (1988) Three distinct forms of type 2A protein phosphatase in human erythrocyte cytosol. J Biol Chem. 263: 3752-3761.

Usui H, Inoue R, Tanabe O, Nishito Y, Shimizu M, Hayashi H, Kagamiyama H, Takeda M (1998) Activation of protein phosphatase 2A by cAMP-dependent protein kinase-catalyzed phosphorylation of the 74-kDa B" (delta) regulatory subunit in vitro and identification of the phosphorylation sites. FEBS Lett 430: 312-316.

Valjent E, Pascoli V, Svenningsson P, Paul S, Herve E, Corvol JC, Stipanovich A, Caboche J, Lombroso PJ, Nairn AC, Greengard P, Herve D, Girault JA (2005) Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. Proc Natl Acad Sci USA 102: 491-496.

Van Etten ML, Anthony JC (2001) Male-female differences in transitions from first drug opportunity to first use: searching for subgroup variation by age, race, region, and urban status. J Womens Health Gend Based Med 10: 797-804.

Van Etten ML, Neumark YD, Anthony JC (1999) Male-female differences in the earliest stages of drug involvement. Addiction 94: 1413-1419.

Van Haaren F, Meyer ME (1991) Sex differences in locomotor activity after acute and chronic cocaine administration. Pharmacol. Biochem. Behav. 39: 923-927.

Ventura R, Cabib S, Puglisi-Allegra S (2002) Genetic susceptibility of mesocortical dopamine to stress determines liability to inhibition of mesoaccumbens dopamine and to behavioral 'despair' in a mouse model of depression. Neuroscience 115(4): 999-1007.

Volkow ND, Wang GJ, Telang F, Fowler JS, Logan J, Childress AR, Jayne M, Ma Y, Wong C (2006) Cocaine cues and dopamine in dorsal striatum: mechanism of craving in cocaine addiction. J Neurosci 26: 6583-6588.

Walaas SI, Aswad DW, Greengard P (1983) A dopamine- and cyclic AMP-regulated phosphoprotein enriched in dopamine innervated brain region. Nature 301: 69-71.

Walker QD, Cabassa J, Hilmar AS, Kuhn CM (2001) Sex differences in cocaine-stimulated motor behavior: Disparate effects of gonadectomy. Neuropsychopharmacol 25: 118-130.

Walker QD, Nelson CJ, Smith D, Kuhn CM (2002) Vaginal lavage attenuates cocaine-stimulated activity and establishes place preference in rats. Pharmacol Biochem Behav 73: 743-752.

Walker QD, Ray R, Kuhn CM (2006) Sex differences in neurochemical effects of dopaminergic drugs in rat striatum. Neuropsychopharmacology 31: 1193-1202.

Walker QD, Rooney MB, Wightman R.M., Kuhn CM (2000) Dopamine release and uptake in are greater in female than male rat striatum as measured by fast cyclic voltammetry. Neuroscience 95: 1061-1070.

Walters CL, Blendy JA (2001) Different requirements for cAMP response element binding protein in positive and negative reinforcing properties of drugs of abuse. J Neurosci 21: 9438-9444.

White NM, McDonald RJ (2002) Multiple parallel memory systems in the brain of the rat. Neurobiol Learn Mem 77: 125-184.

Winkler H, Sietzen M, Schober M (1987) The life cycle of catecholamine-storing vesicles. Ann N Y Acad Sci 493: 3-19.

Wise RA, Bozarth MA (1981) A psychomotor stimulant theory of addiction. Psychol Rev 94: 469-492.

Wright CI, Beijer AVJ, Groenewegen HJ (1996) Basal amygdaloid complex afferents to the rat accumbens are compartmentally organized. J Neurosci 16: 1893.

Xiao L, Becker JB (1994) Quantitative microdialysis determination of extracellular striatal dopamine concentration in male and female rats: effects of estrous cycle and gonadectomy. Neurosci Lett 180: 155-158.

Yamada S, Kojima H, Yokoo H, Tsutsumi T, Takamuki K, Anraku S, Nishi S, Inanaga K (1988) Enhancement of dopamine release from striatal slices of rats that were subchronically treated with methamphetamine. Biol Psychiatry 24: 399-408.

Yan Z, Hsieh-Wilson L, Feng J, Tomizawa K, Allen PB, Fienberg AA, Nairn AC, Greengard P (1999) Protein phosphatase 1 modulation of neostriatal AMPA channels: regulation by DARPP-32 and spinophilin. Nat Neurosci 2: 13-17.

Zachariou V, Benoit-Marand M, Allen PB, Ingrassia P, Fienberg AA, Gonon F, Greegard P, Picciotto MR (2002) Reduction of cocaine place preference in mice lacking the protein phosphatase 1 inhibitors DARPP 32 or Inhibitor 1. Biological Psychiatry 51: 612-620.

Zachariou V, Sgambato-Faure V, Sasaki T, Svenningsson P, Berton O, Fienberg AA, Nairn AC, Greengard P, Nestler EJ (2006) Phosphorylation of DARPP-32 at Threonine-34 is required for cocaine action. Neuropsychopharmacology 31: 555-562.

Zhang Y, Svenningsson P, Picetti R, Schlussman SD, Nairn AC, Ho A, Greengard P, Kreek MJ (2006) Cocaine self-administration in mice is inversely related to phosphorylation at Thr34 (protein kinase A site) and Ser130 (kinase CK1 site) of DARPP-32. J Neurosci 26: 2645-2651.

Zheng M, Leung CL, Liem RK (1998) Region-specific expression of cyclin-dependent kinase 5 (cdk5) and its activators, p35 and p39, in the developing and adult rat central nervous system. J Neurobiol 35: 141-159.

Zhou L, Nazarian A, Sun WL, Jenab S, Quinones-Jenab V (2009) Basal and cocaine-induced sex differences in the DARPP-32-mediated signaling pathway. Psychopharmacology 203: 175-183.

Zhou W, Cunningham KA, Thomas ML (2002) Estrogen regulation of gene expression in the brain: a possible mechanism altering the response to psychostimulants in female rats. Brain Res Mol Brain Res 100: 75-83.

Zubrycki EM, Giordano M, Sanberg PR (1990) The effects of cocaine on multivariate locomotor behavior and defecation. Behav Brain Res 36: 155-159.