EXPLORING SEX DIFFERENCES IN PSYCHOSTIMULANT-INDUCED

ACTIVATION OF THE TAIL OF THE VENTRAL TEGMENTAL AREA

By

TORRY SCOTT DENNIS

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Abstract

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Torry Scott Dennis, PhD

The University of Texas at Arlington, 2014

Supervising Professor: Linda Perrotti, PhD.

It is well established that women and men respond differently to drugs of abuse. Women begin use at an earlier age, progress through the stages of addiction more quickly, and are more vulnerable to relapse than men. Through the support of both human and animal literature, it is clear that one of the driving factors mediating this difference is ovarian hormones. Increases in the ovarian hormone estradiol have been linked to potentiated acquisition of cocaine administration, increased responding for cocaine in the maintenance of addiction, and increased cue-reactivity in female rats. Recent research has begun to characterize the primary reward circuit in female rats and women, but extended structures associated with the modulation of this circuit have not received as much attention. A newly characterized brain region, called the tail of the ventral tegmental area (tVTA), has been proposed to act as a "brake" on the dopamine system. While the unique function of this area has received

attention in male rats, no such work has been conducted to investigate its function in female rats. The goal of the current project was to explore the recruitment of this area in a female population in response to cocaine administration and to determine the extent to which estradiol modulates this recruitment. Specifically, we wanted to determine the degree to which female rats express the same prominent cluster of FosB/DeltaFosB-expressing neurons that characterize the tVTA in male rats following cocaine administration, characterize the cytoarchitecture of these FosB/DeltaFosB-expressing neurons in female animals by identifying the distribution of FosB/DeltaFosB induction in both GABAergic and dopaminergic cells, and to identify any fluctuations in the degree of FosB/DeltaFosB expression in response to the presence or absence of estradiol replacement in ovariectomized female rats. Overall, we found that (1) female rats do express FosB/DeltaFosB in the tVTA following cocaine administration, (2) FosB/DeltaFosB expression in the tVTA is overwhelmingly expressed in GABAergic cells and almost completely absent in dopaminergic cells regardless of sex or treatment with estradiol, (3) acute pretreatment with estradiol does not meaningfully increase the recruitment of the tVTA when compared to acute peanut oil pretreated female animals, and (4) extended estradiol access significantly increases tVTA recruitment in response to cocaine administration when compared to both acute peanut oil and acute estradiol treated female animals. Taken all together, these data suggest that

estradiol meaningfully regulates the recruitment of the tVTA in response to cocaine administration, and that this recruitment is more strongly regulated by the genomic effects of estradiol.

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Chapter 1

Tail of the Ventral Tegmental Area

<u>1.1 Introduction</u>

Addictive drugs have the ability to highjack the behavior of an individual. The ability of these drugs to do so relies on their capacity to strongly activate the reward circuit of the brain. This circuit begins in the ventral tegmental area (VTA) and sends dopaminergic projections throughout the limbic system and the cortex (Koob & Volkow, 2009). Drugs of abuse, such as cocaine, activate dopaminergic signaling in this pathway acutely and alter dopaminergic signaling with repeated use, making this system crucial for the acquisition and maintenance of behaviors associated with addiction (Nestler, 2005). Recently, a GABAergic cell population in the posterior VTA, called the tail of the VTA (tVTA), has been identified as having a large modulatory role in the regulation of this mesocorticolimbic signaling. Specifically, it has substantial projections to the dopaminergic neurons within the VTA and is proposed to act as a "brake" on the dopamine system (Barrot et al., 2012). Since its discovery, the tVTA has been implicated in a wide array of behaviors, including motor control (Jhou, Fields, Baxter, Saper, & Holland, 2009a),

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prediction error, and responses to aversive and appetitive stimuli (Hong, Jhou, Smith, Saleem, & Hikosaka, 2011). Recent research has sought to identify the role of the tVTA within the context of drug addiction and as a modulator of motivated behavior (Jhou et al., 2013; Kaufling et al., 2010b; Perrotti et al., 2005).

1.2 Neuroanatomy of the tVTA

The tVTA, also commonly referred to as the rostromedial tegmental nucleus (RMTg) (Jhou, Fields, Baxter, Saper, & Holland, 2009a), specifically refers to a group of GABAergic neurons in the posterior VTA that exert a strong inhibitory influence on dopaminergic function (Barrot et al., 2012; Jhou, Geisler, Marinelli, Degarmo, & Zahm, 2009b). The ability of the tVTA to exert this influence lies in its efferent projections. Major projections that have been identified include the VTA and the pars compacta of the substantia nigra (areas that have been linked to addiction, motivation, and motor activation) (see Figure 1.1) (Barrot et al., 2012; Bourdy & Barrot, 2012; Jhou et al., 2013; Jhou, Geisler, Marinelli, Degarmo, & Zahm, 2009b; Kaufling, Veinante, Pawlowski, Freund-Mercier, & Barrot, 2010a). Additional studies supporting this inhibitory connection from the tVTA report that direct electrical stimulation of the tVTA results in a 50% decrease in dopaminergic firing in the VTA (Lecca, Melis, Luchicchi, Muntoni, & Pistis, 2012), and tVTA inhibition results in a dopaminergic excitation (Jalabert et al., 2011).

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Just as important are the afferent connections. Although the tVTA receives afferent fibers from a number of different locations (medial prefrontal cortex, cingulate cortex, preoptic area, lateral hypothalamus, superior colliculus, periaqueductal gray, dorsal raphe, lateral tegmental nucleus), a great majority of the research conducted has focused on the inputs received from the lateral habenula (Barrot et al., 2012; Bourdy & Barrot, 2012; Geisler & Zahm, 2005; Kaufling, Veinante, Pawlowski, Freund-Mercier, & Barrot, 2009). Part of the reason for this is that substantial glutamatergic projections from the lateral habenula innervate GABAergic cells in the tVTA (Brinschwitz et al., 2010; Gonçalves, Sego, & Metzger, 2012; Hong et al., 2011; Jhou, Fields, Baxter, Saper, & Holland, 2009a; Jhou, Geisler, Marinelli, Degarmo, & Zahm, 2009b; Kaufling et al., 2009; Omelchenko, Bell, & Sesack, 2009; Stamatakis & Stuber, 2012). In a rodent population, stimulation of the lateral habenula results in increased activity of the tVTA (Jalabert et al., 2011; Lecca et al., 2011). Additionally, research in nonhuman primates has established that tVTA neurons receive excitatory inputs from the lateral habenula and electrical stimulation of this area results in a decrease in the firing rate of dopaminergic neurons in the VTA (Hong et al., 2011).
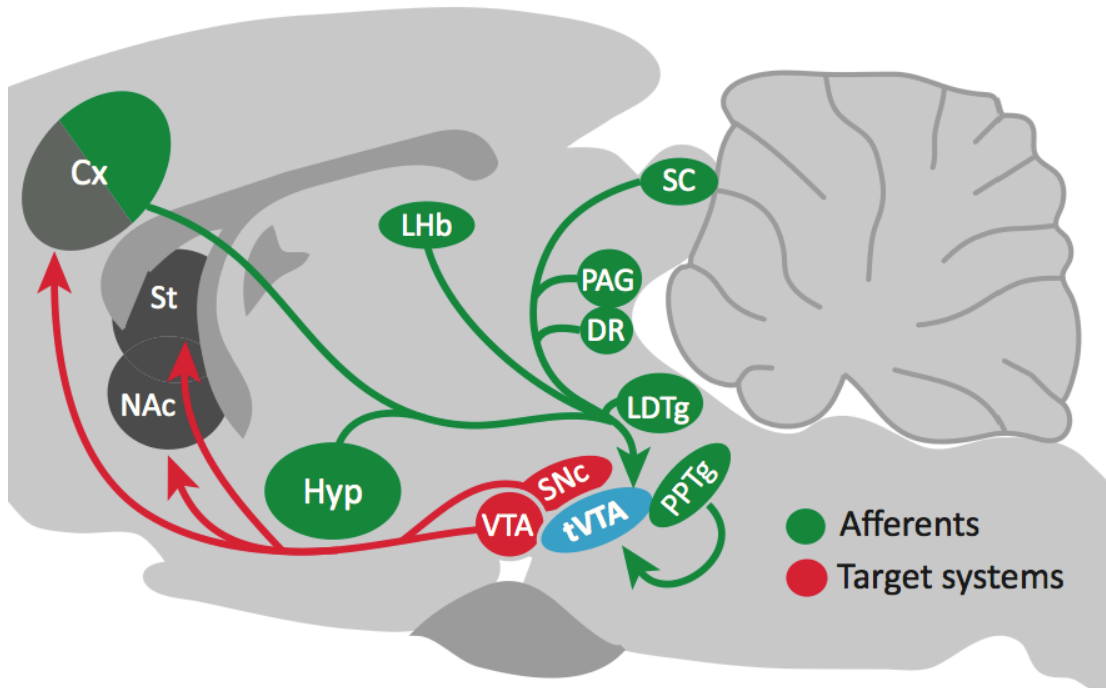
Figure 1.1 Major afferent and efferent connections of the tVTA

Reprinted without permission from (Bourdy & Barrot, 2012)

1.3 Potential Role in Reward Prediction Error

One theory to consider is that the tVTA is a potential point of convergence for the modulation of dopaminergic signaling in negative reward prediction error (Barrot et al., 2012; Bourdy & Barrot, 2012; Hong et al., 2011). Reward prediction error refers to a type of reinforcement-based learning that occurs when the outcome of a "reward event" is either more positive or more negative than expected. The "prediction error" refers specifically to the difference between the expected and the actual outcome and can be positive (outcome is better than expected) or negative (outcome is worse than

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expected) (Schultz, Dayan, & Montague, 1997; Steinberg et al., 2013). Dopamine signaling has long been implicated in this process. When a reward presentation is unpredicted or the effect of a reward is greater than expected (positive reward prediction error), an increase in dopaminergic VTA activity is observed (Hollerman & Schultz, 1998; Mirenowicz & Schultz, 1994). No change in VTA firing is observed if there is no difference between expectation and outcome, but an inhibition occurs if the predicted reward is omitted (Hollerman & Schultz, 1998).

There is evidence that the lateral habenula serves as a major source of input into this system for negative reward prediction error (Matsumoto & Hikosaka, 2007). The lateral habenula exerts an indirect inhibitory effect on the VTA. Activation of the lateral habenula stimulates the tVTA, which increases its inhibitory hold on the VTA (Hong et al., 2011). In support of this, tVTA activity is increased during reward omission (particularly reward omission that is rare or surprising), which corresponds with a decrease in VTA dopamine cell activity (Jhou, Fields, Baxter, Saper, & Holland, 2009a).

Studies in primates reveal that cells in the lateral habenula become active when exposed to cues that predict that no reward will be given and inhibited by cues that predict presence of reward. In the same study, simultaneous recordings were gathered from dopaminergic VTA cells whose

action appeared to directly oppose that of the lateral habenula (Matsumoto &

Hikosaka, 2007). Additional primate studies confirmed that conditioned stimuli

that have been previously paired with both the absence of reward and the

presence of punishment strongly activate the lateral habenula. It was also

found that this set of neurons are inhibited when receiving unpredicted reward

(liquid reward) and activated by an unconditioned stimulus of the punishment

(air puff in the face) (Matsumoto & Hikosaka, 2009).

Taken all together, inputs from the lateral habenula contribute

meaningfully to negative reward prediction error. It is important, however, to

note that while the lateral habenula has been implicated in these things in a

major way, other afferent projections to the tVTA are not precluded from

involvement. More research is required to fully elucidate the pathways

involved in these mechanisms.

1.4 Recruitment of the tVTA in Response to Drugs of Abuse

A number of studies have investigated the role of the tVTA in addiction

through the administration of drugs of abuse. Psychostimulant administration

reliably recruits this area (as measured by Fos-induction). More specifically,

both acute and chronic self-administration of cocaine induces Fos expression

in the tVTA of rats (Zahm et al., 2010). FosB/DeltaFosB expression in this

brain region is also observed after experimenter-administered acute and

chronic administration of psychostimulants (cocaine and amphetamine) (Kaufling et al., 2010b; Perrotti et al., 2005). Induction of Fos family proteins appears to be selective for psychostimulants (cocaine, amphetamine, methamphetamine), as acute administration of ethanol, ketamine, phencyclidine, and THC as well as both acute and chronic administration of opiates do not result in FosB/DeltaFosB induction in the tVTA (Kaufling et al., 2010b; Perrotti et al., 2005). Interestingly, c-Fos expression is also observed in the lateral habenula after acute cocaine administration (Jhou, Fields, Baxter, Saper, & Holland, 2009a), suggesting that Fos-induction in the tVTA in response to psychostimulant administration may be the result of excitatory input from the lateral habenula. However, recording studies show an inhibition of tVTA firing in response to cocaine administration (Lecca et al., 2011). So why is there Fos induction? A paper by Jhou et al. 2013 shed some light on this question by recording the electrical activity of the lateral habenula in waking rats in response to cocaine administration. They observed what they described as a "biphasic response", in which an initial inhibition of firing was followed by an excitatory reaction. This shift from an inhibitory to an excitatory response of the lateral habenula corresponds to the acute shift from the rewarding to the aversive properties of cocaine (Jhou et al., 2013). The implication of this work is that tVTA activation might perhaps be mediated through a delayed mechanism that is most active during the aversive or dysphoric phases after cocaine consumption. The researchers in this study

provided further evidence of this in behavioral experiments by producing aversive conditioning with infusion of AMPA into the tVTA and abolishing cocaine-induced avoidance behaviors through tVTA lesion (Jhou et al., 2013).

Preliminary studies from our own laboratory have sought to investigate the role of the tVTA through lesion studies in the encoding and expression of conditioned reward as measured through cocaine conditioned place preference. Because the administration of cocaine reliably recruits the tVTA (Kaufling et al., 2010b; Perrotti et al., 2005), and the tVTA is known to have an inhibitory hold on dopaminergic signaling (Lecca et al., 2012), it was hypothesized that lesioning of the tVTA would result in a potentiation of conditioned reward. Preliminary data for animals tested at a 5mg/kg dose of cocaine show that there are no significant differences between control and tVTA-lesioned animals (Figure 1.2). This, however, does not necessarily indicate that there are no differences to be observed in cocaine conditioned place preference. There is evidence that the recruitment of the tVTA in response to acute cocaine administration is dose-dependent. A study by Kaufling et al. 2010 shows that FosB/DeltaFosB expression is very low in rats who received a 5mg/kg acute injection of cocaine when compared to 10mg/kg and 20mg/kg. This suggests that higher doses of cocaine would likely result in a greater recruitment of this area in a conditioned place preference paradigm as well. It would be useful to expand this study into higher doses of cocaine to

detect a potential difference in preference for tVTA-lesioned animals. It is likely that for the cocaine conditioned place preference paradigm specifically, tVTA-lesioned animals at higher doses would display a potentiated preference above controls (due to the lack of rebound inhibition on dopaminergic signaling). These data would give us a richer picture of the involvement of the tVTA in cocaine reward.

Test-Pretest Drug Box Cocaine CPP
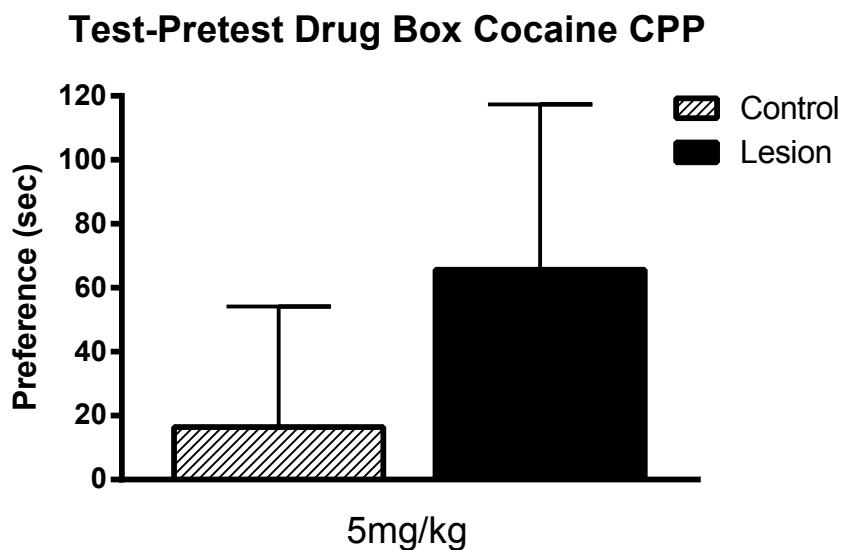

Figure 1.2 Cocaine CPP in tVTA-lesioned animals

Preferences Scores for control and tVTA-lesioned rats in cocaine conditioned place preference. No statistical differences were detected.

The putative role of the tVTA in morphine reward is different than its function with psychostimulants. The tVTA densely expresses μ-opioid receptors (Jhou, Geisler, Marinelli, Degarmo, & Zahm, 2009b) and the

activation of these receptors by opioids (such as morphine) decreases the activity of this area (Jalabert et al., 2011). This μ-opioid receptor-mediated inhibition of the tVTA allows for the disinhibition of dopaminergic firing from the VTA. This process has been proposed as the mechanism through which morphine exerts its rewarding effects (Bourdy & Barrot, 2012). Additional lesion studies from our laboratory have sought to further investigate the role of the tVTA in morphine reward. According to the working model of morphine action in the tVTA, lesioning this area should decrease or abolish place preference for morphine. Consistent with this idea, preliminary data from our laboratory suggests that at 1.25mg/kg of morphine, tVTA lesion does eliminate preference for the morphine-paired chamber (Figure 1.3). We do not, however, observe the same reduction in preference for the tVTA-lesioned animals at the 5mg/kg morphine dose. There are a few explanations for this absence of lesion-induced knock down of preference at this dose. While the inhibition of the tVTA by morphine is primarily responsible for disinhibiting dopaminergic VTA activity, there is evidence for the action of opioids in other reward-related brain regions. For example, infusion of morphine directly into the nucleus accumbens induces conditioned place preference (van der Kooy, Mucha, O'Shaughnessy, & Bucenieks, 1982). Additionally, infusion of mu-opioid receptor agonists into the nucleus accumbens is sufficient to reinstate cocaine self-administration (Simmons & Self, 2009). Although these effects are limited, and clearly not the primary mechanism of reward, it is possible that at higher

doses (and in the absence of tonic tVTA-induced VTA inhibition) morphine action in the nucleus accumbens was sufficient to induce a preference. An alternate explanation could be that lesions did not cover the entire tVTA, and that some residual function was expressed at the higher dose of morphine. More studies must be conducted to verify these hypotheses as possible mechanisms.

Test-Pretest Drug Box Morphine CPP
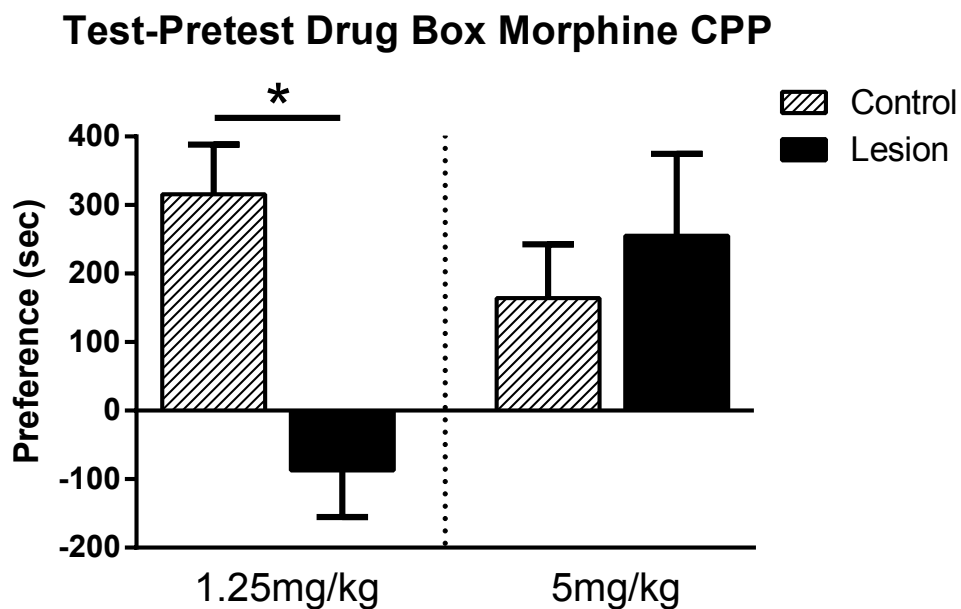
Figure 1.3 Morphine CPP in tVTA-lesioned animals

Preferences Scores for control and tVTA-lesioned rats in morphine conditioned place preference. Asterisk indicates significant difference (p<0.05) between control and lesion groups.

1.5 Potential Role of the tVTA in Opponent Process

Given what is known about the location, connections, and function of the tVTA, it is interesting to posit that it is an integral part of the neurocircuitry responsible for the opponent process theory of addiction. Briefly, the opponent process theory as it relates to addiction is composed of two processes: the a-process, and the b-process. When an individual first starts using drugs, they experience a large positive affective component or "high" (the a-process), followed by a relatively small negative affective component (the b-process). As drug use continues, the positive affective a-process diminishes (tolerance occurs), and the negative b-process increases in magnitude (see Figure 1.4) (Koob & Le Moal, 2008). The drug seeking behavior effectively shifts from one marked by positive reinforcement to one marked by negative reinforcement. In other words, the individual no longer takes drugs primarily to feel good, but rather does so to feel less bad. The Fos induction observed in both acute and chronic psychostimulant administration could reflect the initiation of plasticity and a subsequent sensitization of the tVTA in response to these drugs that contributes to a lasting b-process. It is, therefore, possible that the tVTA is a crucial part of the anti-reward system that contributes to tolerance and negative reinforcement in cocaine addiction.
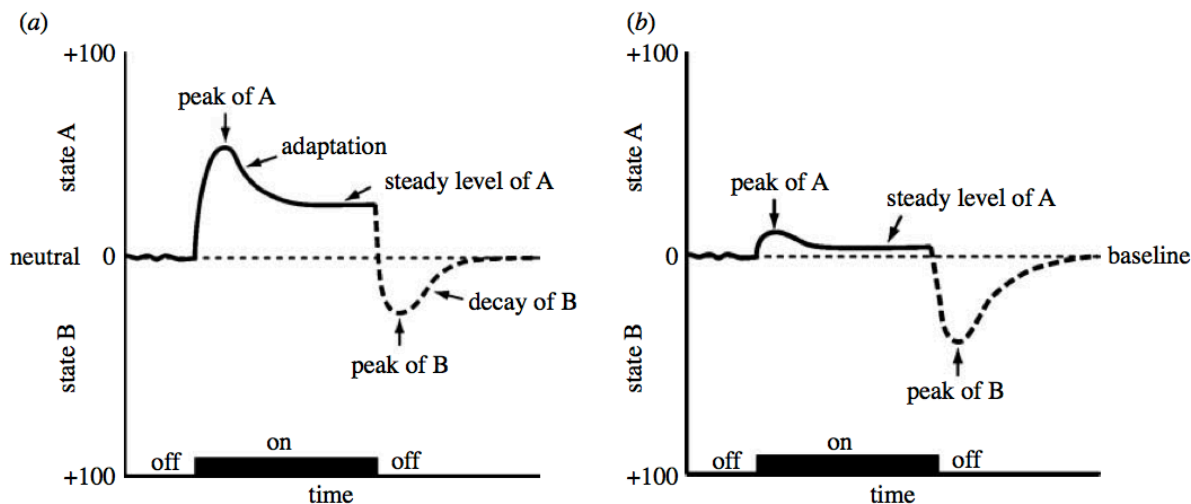
Figure 1.4 The opponent process theory of affective dynamics

Reprinted without permission from (Koob & Le Moal, 2008). (a) Representation of the a-process and b-process when drug administration initially begins. State A refers to the positive affective component of addictive drugs and state B refers to the aversive component. In this stage the a-process is large, and the b-process is relatively small. (b) Representation of the a-process and b-process after continued drug use. The positive affective component is no longer as large, yet the negative b-process is greater in magnitude and longer lasting.

Despite the lack of Fos induction in response to addictive non-psychostimulant drugs, it would be interesting to see if a baseline tonic increase in tVTA firing is observed in electrophysiological studies following acute or chronic administration of all addictive drugs. If this is the case, the tVTA might represent a universal mechanism through which lasting changes in the "b-process" occurs. It is important that more research be conducted to confirm the role of the tVTA in the opponent process theory and to expand its theoretical function in this regard beyond psychostimulants.

1.6 Expanding the Literature

The level of investigation of the neuroanatomy and the function of the tVTA reflect that of an emerging field. As such, not much opportunity has arisen to investigate sex differences apparent in this structure. In fact, every experiment referenced thus far has been conducted exclusively in male animals. Sex differences in patterns of addiction are well established and must be considered to get a full picture of the disease. Therefore, it is of utmost importance that the basic aspects of this structure be characterized in female rats and the role of gonadal hormones considered. The next section will describe sex differences in cocaine addiction and discuss the importance of extending the characterization of the tVTA in a female sample.

Chapter 2

Role of Sex and Ovarian Hormones in Addiction

2.1 Differences Between Men and Women in Addiction

For a long time, it was assumed that addiction was primarily a problem of men. As a consequence, much of the research on the etiology and treatment of addiction was conducted on and for a male population. Over the past few decades, awareness of the problem of addiction in women has increased. As a result, a modest but growing field devoted to characterizing sex differences has emerged (Evans, 2007). From this research we have discovered that women and men do respond to drugs of abuse in distinctly different ways. With cocaine, for example, women begin use at an earlier age, escalate use in a shorter period of time, and progress through the stages of addiction more quickly (Becker & Hu, 2008; Gawin, 1989; Lejuez, Bornovalova, Reynolds, Daughters, & Curtin, 2007; Lynch, Roth, & Carroll, 2002). One study reports that when cocaine-dependent individuals were exposed to cues associated with cocaine use, women reported a higher subjective craving than men (Robbins, Ehrman, Childress, & O'Brien, 1999). Another study found that women are more likely to suffer depression during periods of abstinence and are more vulnerable to relapse than men (Griffin et

al., 1989). It is likely that these differences are due, in part, to the modulatory influence of the ovarian hormones (Evans, 2007). To this point, women report greater subjective responses to cocaine in the follicular phase (marked by rising levels of estrogen and low levels of progesterone) than in the luteal phase (when estrogen levels are moderate, but there is a spike in progesterone) (Evans & Foltin, 2006; Evans, Haney, & Foltin, 2002; Sofuoglu, Dudish-Poulsen, Nelson, Pentel, & Hatsukami, 1999). These differences have also been observed in studies using animal models that have allowed us to more fully elucidate the specific mechanisms at work concerning sex differences and the role of ovarian hormones in addiction.

2.2 Sex Differences in Animal Models of Addiction

Research performed in a human population has been confirmed and expanded upon by experiments in the animal literature. Sex differences are observed in multiple phases of addiction. In self-administration studies, female rats acquired self-administration more quickly than male rats, self-administered cocaine more rapidly, and showed a more rapid escalation of intake (Hu, Crombag, Robinson, & Becker, 2004; Lynch, 2008; Lynch & Carroll, 1999; Roth & Carroll, 2004). Female rats also responded more vigorously to a priming dose after acute and extended periods of extinction when compared to males (Lynch & Carroll, 2000; Lynch & Taylor, 2004). In conditioned place preference studies, female rats develop preference at lower

doses (Russo et al., 2003) and display a greater reinstatement preference after forced abstinence (compared to male rats) to the previously cocaine-paired chamber when given a priming dose of cocaine (Bobzean, Dennis, Addison, & Perrotti, 2010). While these overall differences in sex have been observed, there is also evidence in the animal literature that ovarian hormones regulate addiction behaviors.

Estradiol specifically appears to act as a facilitator of addiction behaviors. For example, when female rats are ovariectomized, those who receive estradiol replacement also acquire cocaine self-administration much more rapidly than those who do not and respond more over the acquisition period (Hu et al., 2004; Hu & Becker, 2008; Jackson, Robinson, & Becker, 2006; Lynch, Roth, Mickelberg, & Carroll, 2001). Under a chronic cocaine self-administration paradigm, intact cycling female rats displayed greater responding when in the proestrus phase of the estrous cycle (marked by peak estradiol levels) (Feltenstein, Byrd, Henderson, & See, 2009). In addition, female rats will work harder to obtain a single infusion of cocaine than males, and respond more when circulating levels of estradiol are high (Lynch, 2008).

2.3 Modulation of Dopaminergic Signaling by Estradiol.

The modulatory influence of estradiol on addiction behaviors certainly has its source in its effect on the cell and the resulting changes within the

reward and motivation neural circuits. Estrogen receptors are densely expressed throughout the mesocorticolimbic dopamine system and have been shown to modulate the function of this system (Creutz & Kritzer, 2002; McEwen, 2002). A number of studies have attempted to directly determine the effect of estradiol on this system. For example, acute estradiol administration has been shown to increase dopamine turnover (Di Paolo, Rouillard, & Bédard, 1985), down-regulate D2 dopamine receptors (Bazzett & Becker, 1994), and increase the density of dopamine uptake sites in the striatum (Morissette, Biron, & Di Paolo, 1990). In response to drugs of abuse, estradiol administration has also been shown to increase amphetamine- (Becker, 1990; Becker & Beer, 1986) and cocaine-induced dopamine release in the striatum (Peris, Decambre, Coleman-Hardee, & Simpkins, 1991). Additionally, repeated cocaine administration decreased striatal D2/D3 receptors in ovariectomized estradiol replaced rats (Febo et al., 2003). There is also evidence that estradiol modulates the activity of the midbrain dopamine system. Electrophysiological recording studies report an increase in burst firing patterns in the VTA in response to estradiol administration (Zhang, Yang, Yang, Jin, & Zhen, 2008), suggesting that estradiol exposure promotes dopaminergic function.

It is clear that changes in ovarian hormones over the estrous cycle and estradiol replacement have a profound impact on both baseline dopaminergic

function and the modulation of dopamine systems in response to psychostimulant administration. It is likely that the regulation of these systems by estradiol represent an underlying mechanism through which the behavioral sex differences in addiction occur.

2.4 Project Goals

While studies have investigated the recruitment of the tVTA in response to psychostimulant administration, no research has directly measured differences between males and females in this area, nor have hormonal influences on the activation of this area in response to drugs of abuse been explored. Given the apparent differences that are observed between men and women in drug abuse, the corroborating evidence from the animal literature, and the varying effects of estradiol on both the dopamine system and on behavior, it is of scientific interest to characterize potential sex differences and the influence of estradiol administration in the tVTA in response to psychostimulant administration.

The overall aim of the current project was to characterize the tVTA in female rats, and to explore the modulatory role of estradiol on FosB/DeltaFosB induction in this area in response to acute cocaine administration. Specifically, the goals were to (1) determine the degree to which female rats express the same prominent cluster of FosB/DeltaFosB-expressing neurons that

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characterize the tVTA in male rats following cocaine administration, (2) characterize the cytoarchitecture of these FosB/DeltaFosB-expressing neurons in female animals by identifying the distribution of FosB/DeltaFosB induction in both GABAergic and dopaminergic cells, and (3) to identify any fluctuations in the degree of FosB/DeltaFosB expression in response to the presence or absence of estradiol replacement in ovariectomized female rats.

Chapter 3

Methods

3.1 Animals

Adult female and male Long Evans rats were used for this study. Rats were housed 3-4 per cage. Food and water were supplied *ad libitum*. Animals were kept on a 12-hour reverse light/dark cycle with the dark cycle starting at 7:00am. All portions of the experiment were conducted during the dark cycle.

3.2 Ovariectomy

Female rats underwent ovariectomies (OVX) to control for the influence of gonadal hormones on the activation of the tVTA. Female rats were anaesthetized with a 2-3% isoflurane-oxygen vapor mixture. Both flanks of the rat were shaved and povidone-iodine was applied to the skin prior to incision. A 10mm incision was made into the skin just below the ribs, exposing the muscle. A 5mm incision was made in the muscle and the ovary pulled through the incision. A hemostat was used to clamp the oviduct just proximal to the ovary and a ligature was tied below the hemostat. The ovary was then removed with scissors and the hemostat released. The incision in the muscle was sutured shut and the skin was rejoined with surgical wound clips. This

entire procedure was repeated on the contralateral side. After completion of the surgery, rats were taken off of the gas anesthetic and lidocaine ointment was immediately applied to the incision in the skin. Rats were allowed to recover in individual cages and were recombined with their cage mates 4-5 days after the surgery.

3.3 Vaginal Lavage Testing

Four to five days after the ovariectomy surgery, female rats underwent daily vaginal lavage testing for at least 8 days (the length of two full estrous cycles) to confirm that estrous cycling had ceased. Vaginal secretions were collected by inserting a pipet tip into the vagina of the rat and infusing and extracting 40μl of 0.9% saline. Secretion samples were placed on a slide and observed under a light microscope at 20X magnification. The phase of the cycle (estrus, metestrus, diestrus, proestrus) was determined by the type (cornified cells, nucleated epithelial cells, and leukocytes) and distribution of cells present. Successful ovariectomy resulted in a cessation of cycling, and a consistent distribution of cells each day. *Note*: To control for experimenter contact, male rats were handled on a daily basis for a minimum of 8 days before acute cocaine administration.

3.4 Acute Cocaine Administration

After eight days of vaginal lavage testing (for female subjects), rats underwent hormone replacement and acute drug administration. Female rats were pretreated with a subcutaneous injection of either 0.1ml peanut oil (vehicle) or 5μg of 17β-Estradiol 3 benzoate (dissolved in 0.1ml peanut oil) and placed back into their home cage. Thirty minutes following injections of peanut oil or estradiol, rats were given either a 15mg/kg intraperitoneal injection of cocaine hydrochloride or saline and placed back into their home cage (Figure 3.1). Cocaine and saline injections were administered at a volume of 1ml/kg. Rats were allowed to experience the effects of the drug in the home cage. Three hours after the initial cocaine administration, rats were anesthetized with a 1ml intraperitoneal 400mg/ml dose of chloral hydrate in preparation for a transcardial perfusion. The procedure for male rats was identical to that of the female rats with the exception that male rats did not receive estradiol injections (only 0.1ml peanut oil). A breakdown of the conditions is shown in Table 3.1.

Estradiol or Peanut Oil Inj $\xrightarrow{\text{30 mins}}$ Cocaine or Saline Inj $\xrightarrow{\text{3 hrs}}$ Sacrifice

Figure 3.1 Timeline of treatment for primary groups

Table 3.1 Breakdown of the conditions for the primary groups

Primary Groups
Female: OVX + Acute Peanut Oil Vehicle: Cocaine Treated (15mg/kg)
Female: OVX + Acute Peanut Oil Vehicle: Saline Treated
Female: OVX + Acute Estradiol Benzoate: Cocaine Treated (15mg/kg)
Female: OVX + Acute Estradiol Benzoate: Saline Treated
Male: Peanut Oil Vehicle: Cocaine Treated (15mg/kg)
Male: Peanut Oil Vehicle: Saline Treated (15mg/kg)

3.5 Alternative Timeline for Acute Cocaine Administration

In order to investigate the potential role of "genomic" estrogen signaling in the cocaine-induced expression of FosB/DeltaFosB in the tVTA, we produced an additional group of ovariectomized female rats that followed an alternative timeline of estradiol administration. These rats received a 5μg dose of 17β-Estradiol 3 benzoate (dissolved in 0.1ml peanut oil) both 48 and 24 hours before an acute 15mg/kg cocaine injection (but not 30 minutes before) (Figure 3.2). After the cocaine injection, the procedure was identical to the timeline described in the "primary" experiment. This additional group is indicated in Table 3.2.

Estradiol Inj $\xrightarrow{\text{24 hrs}}$ Estradiol Inj $\xrightarrow{\text{24 hrs}}$ Cocaine Inj $\xrightarrow{\text{3 hrs}}$ Sacrifice
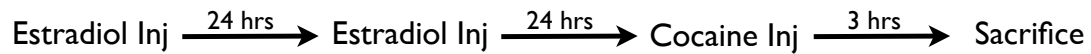
Figure 3.2 Timeline of treatment for alternative group

Table 3.2 Breakdown of the condition for the extended group

Extended Preliminary Group
Female: OVX + Extended Estradiol Benzoate: Cocaine Treated (15mg/kg)

3.6 Perfusion

Once unconscious (from chloral hydrate), rats were transcardially perfused with 100ml PBS and 200ml of 4% paraformaldehyde dissolved in PBS. Upon completion, brains were extracted and stored in a 4% paraformaldehyde PBS solution for 24 hours and then transferred to 20% glycerol in PBS for a minimum of 48 hours before sectioning. This entire process fixes proteins in place throughout the brain for later examination using immunohistochemistry and immunofluorescence.

3.7 Brain Sectioning

Brains were coronally sliced (40μm thick) on a freezing microtome. The tVTA was serially sectioned into five different tubes in order have a full representation of the brain in multiple tubes (which allows for multiple

immunohistochemical and immunofluorescence probes on the same animal). Free-floating sections were stored in a solution of 0.01% sodium azide dissolved in PBS to prevent microbial growth.

3.8 Immunohistochemistry for FosB/DeltaFosB

Immunohistochemistry for FosB/DeltaFosB was used to detect the recruitment of the tVTA in response to cocaine administration, and to investigate the potential differences between male and female rats, in addition to the potential modulatory role of estradiol in this recruitment. Slices of the tVTA were separated into new tubes and pre-washed with PBS (3 x 10 minutes). Tissue was then washed in 3% hydrogen peroxide to destroy endogenous peroxidases, and subsequently washed in PBS (3 x 10 minutes) and incubated in a blocking solution for an hour (0.5% Triton X, 5% donkey serum in PBS). Tissue was then incubated for three days at 4 degrees celsius in an anti-FosB/DeltaFosB primary antibody solution (Santa Cruz, Cat# SC-48; dilution of 1:2000 with 0.5% Triton X and 1% donkey serum in PBS). Next, tissue was washed in PBS (3 x 10 minutes) followed by a two hour incubation in biotin-conjugated donkey anti-rabbit secondary antibody solution (Jackson Immuno, cat# 711-065-152; dilution of 1:200 with 0.5% triton X and 1% donkey serum in PBS) at room temperature. Tissue was washed with PBS (3 x 10 minutes) and incubated for 90 minutes in avidin/biotinylated enzyme complex (Vector VECTASTAIN ABC Kit, cat# PK-6100; dilution 1:400 in PBS)

and subsequently washed in PBS (2 x 10 minutes) and Tris-HCl (0.5M, pH 7.5; 3 x 10 minutes). FosB/DeltaFosB staining was visualized through the application of a DAB peroxidase substrate kit (Vector, SK-4100) for two minutes. The DAB reaction was stopped by PBS washes (3 x 10 minutes). Tissue was serially mounted on Fisherbrand Superfrost Plus slides and air dried. Tissue was dehydrated in graded ethanols and cleared with Fisherbrand Citrisolv. Slides were cover slipped using DPX as the mounting medium. Sections between -6.04 and -7.04 from bregma (representing the range of the tVTA) were hand counted under a light microscope. All slides were coded prior to counting to prevent bias and only decoded after all data was collected.

3.9 Double Labeling IHC for FosB/DeltaFosB and TH

A subset of brains from animals treated with cocaine (n=3 per group) underwent double labeling immunohistochemistry for FosB/DeltaFosB and tyrosine hydroxylase (TH). This tissue was serially stained, starting with FosB/DeltaFosB immunohistochemistry and followed by TH immunohistochemistry. The procedure for the FosB/DeltaFosB portion of the stain was identical to that of the single-labeled FosB/DeltaFosB stain described in the previous paragraph. Once the tissue was stained for FosB/DeltaFosB and washed with PBS (3 x 10 minutes), it was incubated in a blocking solution (0.5% Triton X, 5% donkey serum) for one hour. The tissue was then incubated in an anti-TH antibody solution (Millipore, cat# AB1542;

dilution of 1:40,000 with 0.5% triton X and 1% serum in PBS) at room temperature overnight. Next, tissue was washed in PBS (3 x 10 minutes) and incubated for 90 minutes in a biotin-conjugated donkey anti-sheep secondary antibody solution (Jackson Immuno, cat# 713-065-147; dilution of 1:200 with 0.5% triton X and 1% donkey serum in PBS). Tissue was once again washed in PBS (3 x 10 minutes) and incubated in avidin/biotinylated enzyme complex (Vector VECTASTAIN ABC Kit, cat# PK-6100; dilution 1:400 in PBS). After washes in PBS (2 x 10 minutes) and Tris-HCl (0.5M, pH 7.5; 3 x 10 minutes), TH staining was visualized through the application of an SG peroxidase substrate kit (Vector, SK-4700) for 90 seconds. The SG reaction was stopped through a five minute wash in distilled water, followed by washes in PBS (3 x 10 minutes). Tissue was serially mounted on Fisherbrand Superfrost Plus slides and air dried. Tissue was dehydrated in graded ethanols and cleared with Fisherbrand Citrisolv. Slides were cover slipped using DPX as the mounting medium. Sections between -6.04 and -7.04 from bregma (representing the range of the tVTA) were hand counted. TH-positive and TH-negative FosB/DeltaFosB-expressing cells were counted. Every slide was coded prior to counting to prevent bias and only decoded after all data was collected.

3.10 Double Labeling IF for FosB/DeltaFosB and GAD

To confirm that FosB/DeltaFosB expression between male and female rats was consistently embedded within a GABAergic cell population in the tVTA, a subset of brains from cocaine-treated animals (n=3 per group) were double-labeled for FosB/DeltaFosB and Glutamic Acid Decarboxylase (GAD). Slices of the tVTA were separated out into a new tube and pre-washed in PBS (3 x 10 minutes), followed by a one-hour incubation in a blocking solution (0.5% triton X and 3% donkey serum). Next, an anti-FosB/DeltaFosB antibody (Santa Cruz SC-48; diution 1:200) and an anti-GAD antibody (Millipore MAB5406; dilution of 1:500) were co-incubated with 0.5% Tween 20 and 3% donkey serum in PBS for 20 hours. After washes in PBS (3 x 10 minutes), the tissue was co-incubated in a Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson Immuno 711-165-1152; 1:400) and a DyLight 488-conjugated donkey anti-mouse secondary antibody (Jackson Immuno 715-485-150; 1:400) in PBS for four hours. The tissue was then washed in PBS (3 x 10 minutes) and subsequently mounted on Fisherbrand Superfrost Plus slides and air dried. Tissue was dehydrated in graded ethanols and cleared with Fisherbrand Citrisolv. Slides were cover-slipped using Vectashield (Vector, H-1000) to reduce photobleaching. Sections were examined under a confocal laser-scanning microscope to determine the colocalization of FosB/DeltaFosB-positive nuclei embedded within a GAD-positive cell structure. Every slide was coded prior to counting to prevent bias and only

decoded after all data was collected.

Chapter 4

Results

4.1 Confirming Cessation of Estrous Cycle

All female animals in the current experiment underwent ovariectomy. In order to confirm that the ovariectomy was successful, each rat underwent vaginal lavage testing over eight days to confirm that cessation of cycling had occurred. If the animal continued to cycle over this period, they were thrown out of the experiment. Fortunately, all female rats displayed a cessation of cycling, indicating that all ovariectomies were successful. The picture below (Figure 4.1) depicts a representative sample taken from an ovariectomized female rat.
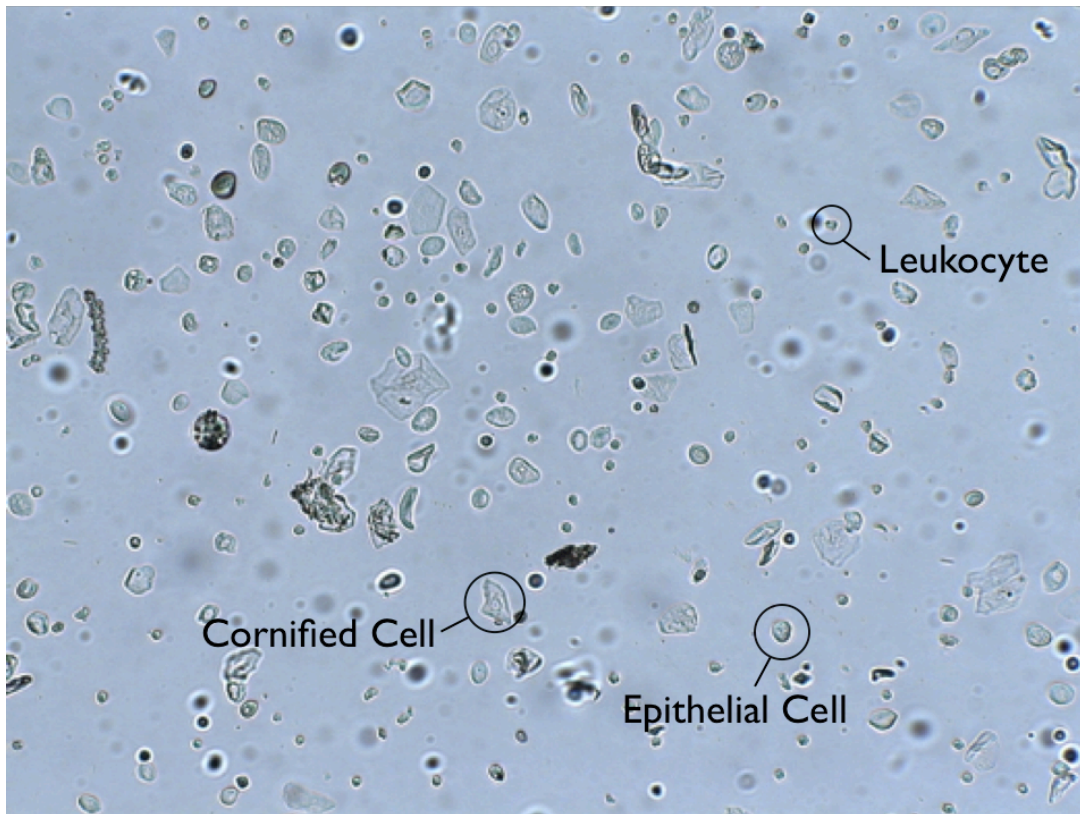
Figure 4.1 Estrous cycle sample from ovariectomized rat

Picture represents a typical sample taken from an ovareiectomized rat. Samples typically exhibit relatively equal distribution of leukocytes, cornified cells, and epithelial cells indicative of the metestrus phase of the estrous cycle (which is characterized by low levels of estradiol and low progesterone).

4.2 Statistics for FosB/DeltaFosB Expression

A priori independent t-tests were performed to compare each of the cocaine-treated groups with the appropriate saline control group. Cocaine-treated groups displayed significantly more FosB/DeltaFosB-positive cells in the tVTA when compared to the matched saline control in female peanut oil treated groups [t(11) = 5.86, p<0.001], female estradiol treated groups [t(11) =

6.92, p<0.001], and male groups [t(12) = 7.14, p<0.001] (see Figure 4.3). A picture comparing saline to cocaine-treated animals can be seen in Figure 4.2.

A priori independent t-tests were also performed to compare the effect of pretreatment regimen on cocaine-treated animals. There were no significant differences between the estradiol pretreated female cocaine animals when compared to the peanut oil pretreated female cocaine animals [t(12) = 0.73, p=*ns*] or to the peanut oil pretreated male cocaine animals [t(12) = 1.22, p=*ns*]. There were, however, marginally significant differences between peanut oil pretreated female cocaine animals and male cocaine animals [t(12) = 1.95, p=0.075].
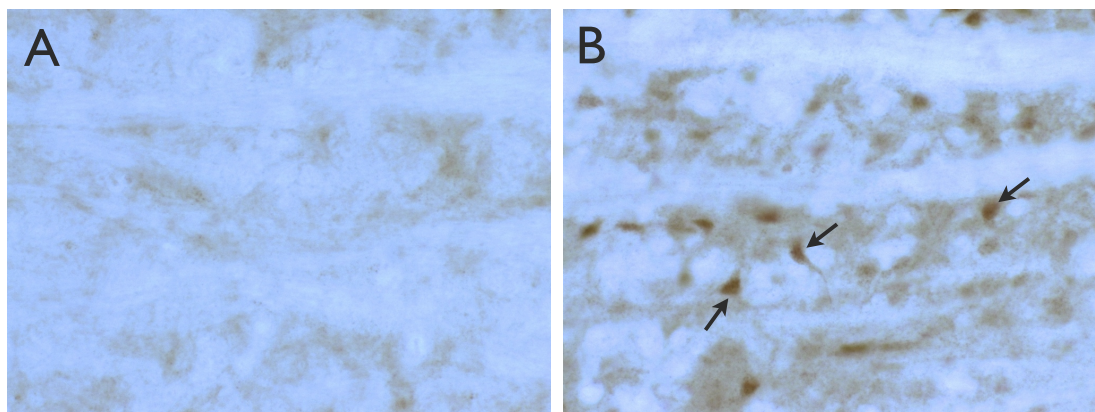
Figure 4.2 Representative pictures of FosB/DeltaFosB expression.

Image "A" shows lack of FosB/DeltaFosB expression in the tVTA of a saline-treated animal. Image "B" shows FosB/DeltaFosB expression in the tVTA of cocaine-treated animals. Black arrows indicate representative FosB/DeltaFosB expression. Picture taken at 40X magnification.
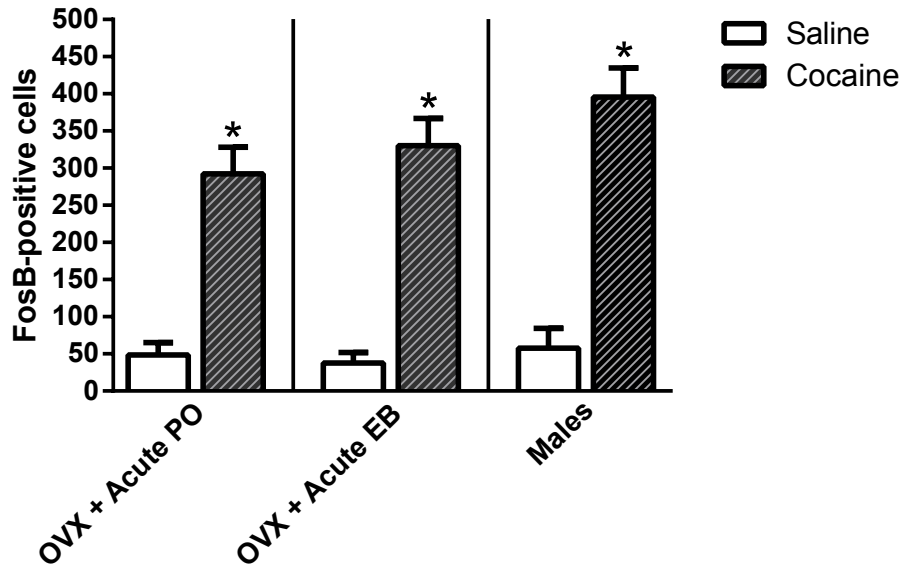
Figure 4.3 FosB/DeltaFosB expression in the tVTA

Results of FosB/DeltaFosB induction in the tVTA. * Indicates significant
difference from saline control group. n=6-8 per group.

The results from the preliminary experiment with extended pretreatment
with estradiol (48 and 24 hours prior to cocaine administration) results in a
greater induction of FosB/DeltaFosB in the tVTA when compared to both the
acute estradiol pretreated [t(12) = 10.95, p<0.01] and acute peanut oil
pretreated [t(13) = 3.209, p<0.01] OVX female cocaine groups, and suggests
that changes in the induction of FosB/DeltaFosB in this area are preferentially
dependent on genomic estrogen signaling rather than non-genomic signaling.
Data from this group has been embedded into the original figure for the sake
of comparison (see Figure 4.4).

tVTA Expression of FosB/DeltaFosB
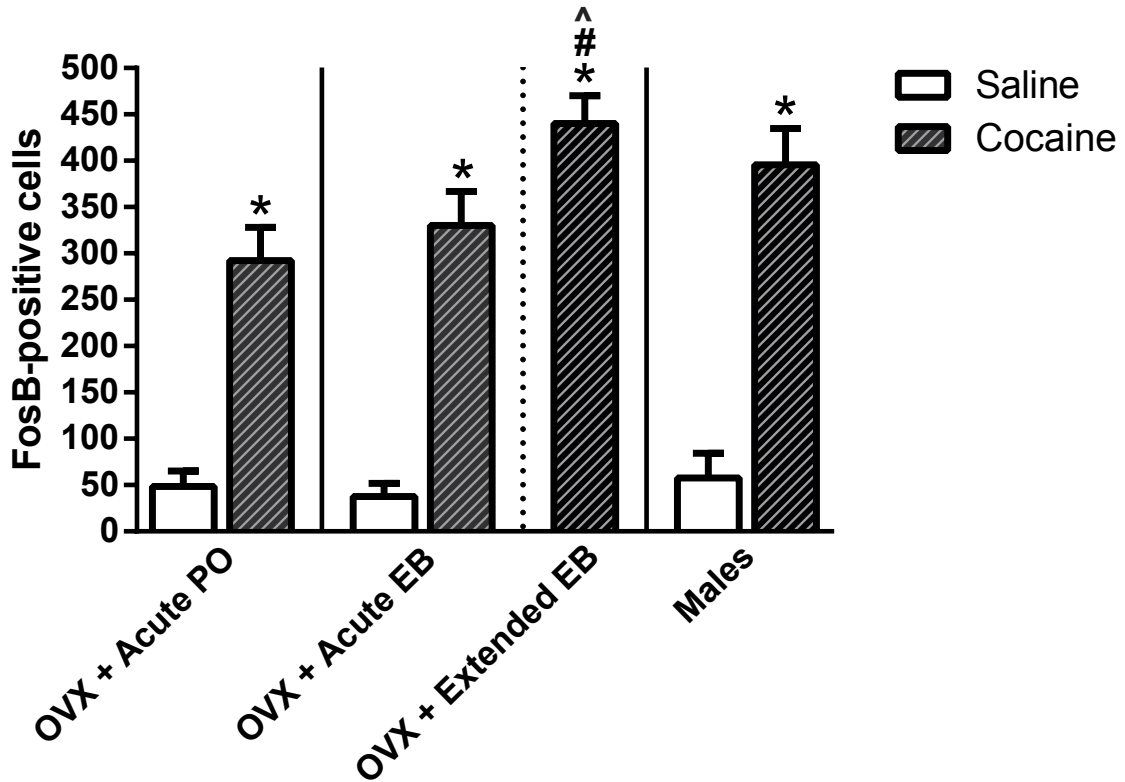
Figure 4.4 Effect of Extended Estradiol on tVTA

Results including the preliminary extended estradiol pretreatment group.
* Indicates significant difference from saline control group. # indicates
significant difference from OVX Acute PO pretreated cocaine group. ˆ
indicates significant difference from OVX acute EB pretreated cocaine group.
n=6-8 per group.

4.3 Results for FosB/DeltaFosB & GAD Double Label

To investigate the extent to which FosB/DeltaFosB expression under

the current paradigm is colocalized within a GABAergic cell population in the

tVTA, FosB/DeltaFosB-positive cells were determined to be embedded within either a GAD+ or GAD- cell body (see Figure 4.5). To compare groups, a ratio score was created to represent the proportion of GAD+ FosB/DeltaFosB-expressing cells to total FosB/DeltaFosB expression. A priori independent t-tests indicate no significant differences comparing male cocaine-treated to both the peanut oil pretreated female cocaine group [$t(4) = 0.74$, p=ns] or the estradiol pretreated female cocaine group [$t(4) = 1.4$, p=ns]. Additionally, no significant differences were observed between peanut oil pretreated female cocaine animals and estradiol pretreated female cocaine animals [$t(4) = 1.62$, p=ns] (see Table 4.1).

Table 4.1 Summary FosB/DeltaFosB and GAD double labeling

FosB+ Cells	GAD+ (Mean)	GAD- (Mean)	% GAD +
OVX + Acute PO Cocaine	273.33	19.00	93.50%
OVX + Acute EB Cocaine	355.67	21.00	94.42%
Males Cocaine	319.67	27.67	92.03%
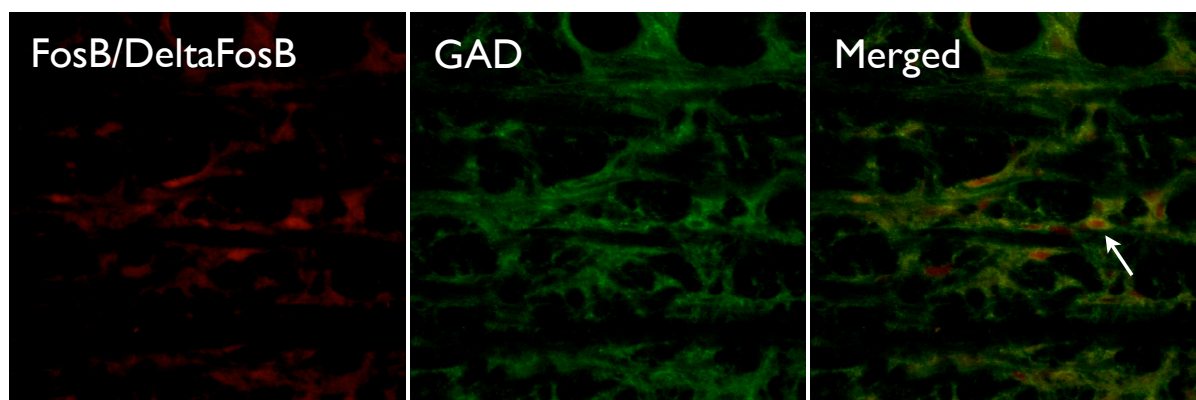
Figure 4.5 Representative FosB/DeltaFosB GAD double label

White arrow indicates example of colocalized expression. Picture taken at 40X magnification.

4.4 Results for FosB/DeltaFosB & TH Double Label

Similarly, to investigate the extent to which FosB/DeltaFosB expression under the current paradigm is embedded within a dopaminergic cell population in the tVTA, FosB/DeltaFosB-positive cells were determined to be embedded within either a TH+ or TH- cell body (see Figure 4.6). To compare groups, a ratio score was created to represent the proportion of TH- FosB/DeltaFosB-expressing cells to total FosB/DeltaFosB expression. A priori independent t-tests indicate no significant differences comparing male cocaine-treated to both the peanut oil pretreated female cocaine group [t(4) = 2.37, p=*ns*] or the estradiol pretreated female cocaine group [t(4) = 1.40, p=*ns*]. Additionally no significant differences were observed between peanut oil pretreated female cocaine animals and estradiol pretreated female cocaine animals [t(4) = 1.62, p=*ns*] (see Table 4.2).

Table 4.2 Summary FosB/DeltaFosB and TH double labeling

FosB+ Cells	TH+ (Mean)	TH- (Mean)	% TH -
OVX + Acute PO Cocaine	1.00	355.00	99.72%
OVX + Acute EB Cocaine	0.66	357.33	99.81%
Males Cocaine	0.33	396.00	99.92%
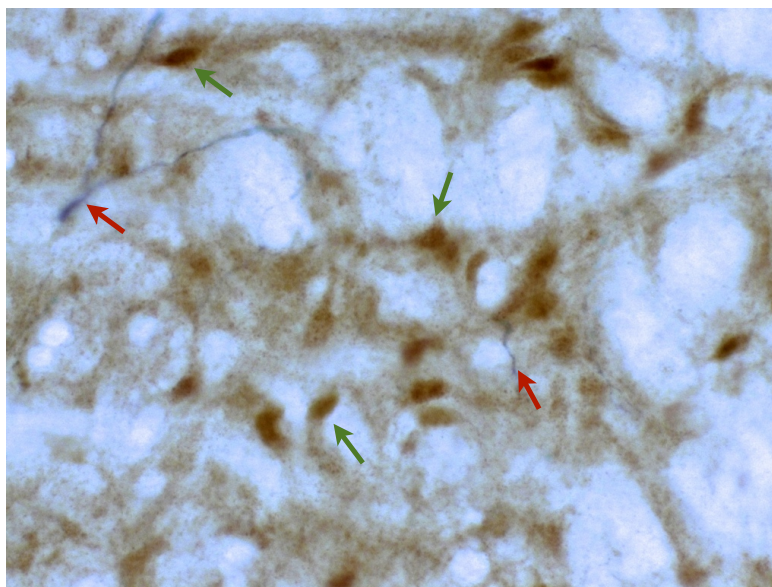
Figure 4.6 Representative FosB/DeltaFosB TH double label

Representative FosB/DeltaFosB staining is marked by green arrows. Representative TH fibers are marked by red arrows. Picture taken at 40X magnification.

Chapter 5

Discussion

5.1 Recapitulation of the Results

The purpose of the current study was multifold. First, we were interested in investigating the extent to which female rats show the same FosB/DeltaFosB expression in the tVTA in response to acute cocaine administration that has previously been observed in a male population. Our results indicate that female rats do, in fact, display this cocaine-induced expression of FosB/DeltaFosB. Additionally, marginal sex differences were observed with acute peanut oil pretreated male cocaine animals expressing more FosB/DeltaFosB in the tVTA when compared to acute peanut oil pretreated female cocaine animals. We were also interested in looking at the cytoarchitecture of FosB/DeltaFosB-expressing cells in the tVTA between male and female groups. Our data indicate that, regardless of sex or pretreatment, cocaine-induced FosB/DeltaFosB is overwhelmingly expressed in GABAergic cells and almost completely absent in dopaminergic cells within the tVTA. These data are consistent with previous literature in male rats (Kaufling et al., 2010b). Lastly, we wanted to investigate the extent to which estradiol replacement in an ovariectomized female population would modulate

the cocaine-induced recruitment of the tVTA as measure by FosB/DeltaFosB. Our data indicate that there is no significant effect for acute (30 minutes before) pretreatment with estradiol (when compared to peanut oil) between the cocaine-treated female groups.   However, pretreatment with extended estradiol significantly increases the recruitment of the tVTA when compared to acute peanut oil and acute estradiol treated cocaine female animals.

5.2 Differences in the Recruitment of the tVTA Between Sexes

There are marginal sex differences in the recruitment of the tVTA (as measured by FosB/DeltaFosB induction), with non-estradiol replaced female rats displaying lower recruitment of this area when compared to the male group. Given the proposed role of the tVTA, it is interesting to posit that female rats display decreases in the activation of the tVTA, allowing for a potentiated response by VTA neurons and/or the reduction of an aversive signal during acute withdrawal. In other words, female rats may be less sensitive to the aversive properties of cocaine, therefore increasing its overall appetitive value (although there are certainly alternative explanations). It is worth noting, however, that the role of Fos induction in the tVTA in stress/aversion is a bit mixed. Foot shock and shock-predictive cues induce c-Fos (Jhou, Fields, Baxter, Saper, & Holland, 2009a) in the tVTA, whereas chronic restraint stress does not induce DeltaFosB in this area (Perrotti et al., 2005). To further explore the extent to which the observed sex difference reflects sensitivity to

cocaine-induced aversion, a potential future experiment could incorporate both male and female animals into a cocaine place aversion experiment.

While no experiments have explicitly looked at cocaine-induced aversion in ovariectomized female rats, we do observe differences in reward and addiction behaviors in this population. Under a conditioned place preference paradigm, ovariectomized female rats with no hormone replacement show lower preference for a previously cocaine-paired chamber when compared to an intact female group (Russo et al., 2003). In self-administration studies, ovariectomy decreases acquisition and cocaine-primed reinstatement, yet estradiol replacement reverses these deficits (Frye, 2007; Larson, Roth, Anker, & Carroll, 2005; Lynch et al., 2001). These data indicate that a lack of estradiol in ovariectomized groups results in a dampening of reward and addiction behaviors. In the current experiment, we see a lower recruitment of the tVTA in a non-estradiol-replaced ovariectomized group, which would indicate less inhibitory action on dopamine systems in response to cocaine administration. However, because ovariectomy is shown to decrease reward, it is likely that the lower level of FosB/DeltaFosB expression in the tVTA observed in the ovariectomized cocaine-treated group was not the result of increased reward and decreased aversion, but rather the result of decreased reward and a corresponding weak anti-reward recruitment by the tVTA. The weak recruitment of the tVTA in ovariectomized animals in

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response to cocaine is substantiated by electrophysiological evidence in the VTA. When no estradiol replacement is provided, cocaine administration has no appreciable effect on VTA firing. However, when animals receive estradiol replacement prior to cocaine administration, a gradual decrease in VTA activity is observed (Zhang et al., 2008). If this decrease in VTA firing is dependent on the tVTA, this suggests that recruitment of the tVTA in response to cocaine administration may be estradiol-dependent in a female population.

Based on the current data, the previous explanation of weak reward induction and a corresponding weak anti-reward response is incomplete. If the lower cocaine-induced expression of FosB/DeltaFosB in the tVTA in acute peanut oil pretreated ovariectomized animals is actually the result of a weak reward response, then estradiol replacement (which is known to facilitate dopaminergic signaling and reward) would likely result in an increased recruitment of the tVTA. However, there is no significant increase in FosB/DeltaFosB induction with acute estradiol pretreatment when compared to acute peanut oil pretreatment prior to cocaine administration. A potential explanation for this could be found in examining mechanisms of estradiol action. Estrogen signaling occurs through two different primary mechanisms, which can be differentially activated through manipulation of the timeline of estradiol administration. The timeline of the primary experiment (pretreatment with estradiol 30 minutes before cocaine administration) preferentially

activates "non-genomic" estrogen signaling. Activation of this mechanism allows for rapid changes in cell function that occur in a matter of seconds to minutes (Micevych & Mermelstein, 2008; Morissette et al., 2008). These changes occur through estradiol-activated protein-protein interactions between membrane-bound estrogen receptors (ERα and ERβ) and metabotropic g-protein coupled glutamate receptors (mGluR1, mGluR2, mGluR3, and mGluR5) (Mermelstein & Micevych, 2008). However, it is also clear that estradiol administration can have longer lasting effects within the addiction neurocircuitry. These effects are mediated through a second type of estrogen signaling called "genomic" signaling. This refers to the binding and activation of estrogen receptors within the cytosol (see Figure 5.1). Under this model, once cytoplasmic estrogen receptors are activated by estradiol, dimerization and translocation to the nucleus occurs, where it can alter DNA transcription (Nilsson et al., 2001). The onset of the genomic effects of estrogen signaling are typically slower, but longer lasting (Morissette et al., 2008). Therefore, it was of interest to the current experiment to determine if estradiol administration given on a timeline to activate "genomic" signaling would meaningfully modulate the cocaine-mediated induction of FosB/DeltaFosB in the tVTA. This was the rationale for the inclusion of an extended estradiol pretreatment group.
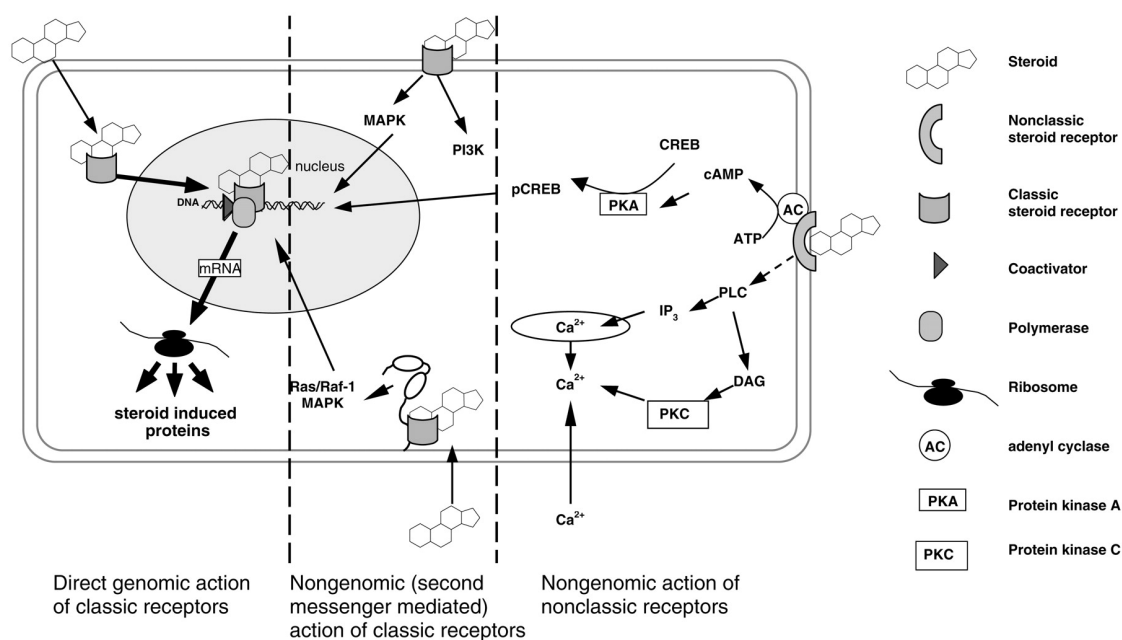
Figure 5.1 Genomic and non genomic actions in steroid signaling

Reprinted without permission from (Losel et al., 2003). The changes resulting from acute estradiol treatment are mediated through the non genomic mechanism. The changes resulting from extended estradiol treatment are mediated through genomic mechanisms.

The results from our preliminary data suggest that extended pretreatment with estradiol (48 and 24 hours prior to cocaine administration) results in a greater recruitment of the tVTA (as measured by FosB/DeltaFosB induction) when compared to both the acute estradiol pretreated and acute peanut oil pretreated female cocaine groups. These results suggest that the genomic effects of estradiol enhance the cocaine-induced recruitment of the tVTA in a female population. These data are consistent with the previously discussed experiment investigating the response of VTA neurons to pretreatment of estradiol prior to cocaine administration (Zhang et al., 2008).

Activity of VTA dopamine neurons in ovariectomized female rats is relatively unchanged across time in response to escalating doses of i.v. cocaine administration. However, when ovariectomized female rats are estradiol replaced both 25 hours and 1 hour before escalating doses of cocaine are administered, a gradual cocaine-induced decrease in dopaminergic VTA firing is observed (Zhang et al., 2008). The timeline of estradiol administration in this experiment (both 25 and 1 hour prior to cocaine administration) would activate "genomic" estrogen signaling mechanisms (in addition to "non-genomic). In our data, when ovariectomized animals are estradiol replaced (under the extended paradigm), a greater recruitment of the tVTA is observed (via FosB/DeltaFosB induction), which would in theory correspond with the estradiol-dependent decrease in VTA activity in response to cocaine administration observed in the Zhang 2008 article. However, because experiments in the Zhang 2008 article did not separate out estradiol administration to detect differences in genomic from non-genomic signaling, it is not possible to concretely say which is responsible for the observed effect. Based on our data, however, it is likely that a greater reduction in VTA firing would be observed in response to the timeline activating the genomic effects of estrogen signaling.

5.3 Incorporating the tVTA into Observed Sex Differences in Addiction.

In addiction, it has been proposed that female rats (and women) experience greater initial reward (a-process), which more strongly recruits the anti-reward system (b-process), when compared to a male population (O'Dell & Torres, 2014). Under the model of opponent process, as drug use continues this a-process diminishes and a slower and longer lasting b-process develops. These continued changes result in a shift from positive reinforcement to negative reinforcement (Koob & Le Moal, 2008). After this shift occurs, individuals begin to seek out and consume drugs to alleviate the negative state induced by the sensitized anti-reward system. Evidence exists for a greater sensitivity to reward (Russo et al., 2003), a greater escalation of drug intake (Becker & Hu, 2008; Roth & Carroll, 2004), and a potential vulnerability to enhanced anti-reward recruitment (Becker, Perry, & Westenbroek, 2012) in a female population. These ideas are consistent with the literature, indicating that women experience a "telescoping effect", which describes a process in which they transition through the phases of addiction more quickly (Haas & Peters, 2000). It is possible that some of these sex differences could be explained, in part, by differential recruitment of the tVTA. Our study is the first to investigate this as a possibility.

Caution must be taken in inserting the observed differences from our work into the theoretical framework of enhanced reward and anti-reward recruitment in a female population. Because the current experiment had no behavioral component and because the differences observed are in response to acute administration of cocaine, any explanation of meaning attached to behavior is an extrapolation. However, it is likely that the observed differences have implications within this framework. Our results indicate that ovariectomy with no hormone replacement attenuates cocaine-induced FosB/DeltaFosB expression in the tVTA when compared to the male group. As discussed previously, this attenuation is likely the result of weak reward activation and a corresponding weak anti-reward recruitment. Because estradiol replacement in ovariectomized animals is shown to facilitate dopamine signaling and reward (Lynch, 2008; Peris et al., 1991), it was expected that acute pretreatment of estradiol would result in a corresponding increase in the recruitment of the tVTA. Our results do not support this notion. However, extended treatment with estradiol meant to preferentially activate "genomic" estrogen signaling resulted in a much greater recruitment of the tVTA. If we accept the premise that activation of the tVTA is a component of anti-reward recruitment, then these data suggest that in a female population, genomic estrogen signaling may be more important in the anti-reward response than non-genomic estrogen signaling.

5.4 Limitations and Future Directions

A number of future studies could clarify and potentially solidify some of the ideas presented throughout this document. First, we need to expand on the preliminary results from the extended estradiol group. Although we do see differences between this group and no estradiol replacement, the necessary controls have not yet been put into place. This would include the production of additional groups displayed in Table 5.1. It is unlikely that extended pretreatment with peanut oil for both the saline and cocaine treated groups would have any appreciable effect on FosB/DeltaFosB induction in the tVTA. However, the inclusion of an extended estradiol pretreatment group for saline is crucial. This would allow us to determine if extended estradiol pretreatment results in a meaningful induction of FosB/DeltaFosB in the absence of cocaine. If there is an induction of FosB/DeltaFosB in the tVTA with extended estradiol treatment (without cocaine), then the observed differences in the preliminary extended estradiol pretreatment group with cocaine could be the result of a compound effect. If there is no appreciable induction of FosB/DeltaFosB with extended estradiol pretreated (without cocaine), then the differences observed in the preliminary data are likely the effect of estradiol-mediated changes in the dopamine system.

Table 5.1 Proposed groups to fill out current preliminary data

Proposed Additional Control Groups
Female: OVX + Extended Peanut Oil Vehicle: Cocaine Treated (15mg/kg)
Female: OVX + Extended Peanut Oil Vehicle: Saline Treated
Female: OVX + Extended Estradiol Benzoate: Saline Treated

Moving beyond acute administration

While the acute administration of cocaine in the current experiment allowed us to detect a modulatory influence of extended estradiol (although not acute estradiol), the addiction process consists of more than the first experience with a given drug of abuse. Neuroadaptive changes in the dopamine system take place with repeated exposure to cocaine (Lack, Jones, & Roberts, 2008; Lee, Parish, Tomas, & Horne, 2011; Weiss, Markou, Lorang, & Koob, 1992). Investigating the influence of ovarian hormones under a more chronic regimen of non-contingent cocaine administration or eventually into contingent consumption and withdrawal would allow for a more complete characterization of this system that occurs over time and with repeated use. To confirm the results that we obtained from the acute cocaine groups, we should also incorporate both acute and extended estradiol pretreatment groups under a more chronic cocaine administration regimen. This would allow us to determine if the genomic effects observed with acute cocaine

meaningfully contribute to the changes that occur with repeated cocaine exposure.

Cautions about using ovariectomized animals

It is also important that caution be taken in comparing males to ovariectomized (as opposed to intact) female rats. While ovariectomy allows for the control of fluctuation in ovarian hormones, it does not mirror a completely natural state. By the time that these animals are sacrificed, they have effectively been in an estradiol- and progesterone-deprived state for approximately two weeks. Studies suggest that estradiol is protective of dopamine neurons in the midbrain. Ovariectomy results in a decrease in VTA and substantia nigra dopamine cells (a loss that is prevented by estradiol treatment) (Johnson et al., 2010). Because habenular activation is known to be dopamine-dependent (Jhou et al., 2013), a potential reduction in dopamine cells could dampen the recruitment of this area in response to initial cocaine administration. In addition to the previous explanation, it is possible that the observed lower FosB/DeltaFosB expression in the tVTA of ovariectomized rats following acute cocaine administration may be accentuated by this potential deprivation. It is important that future studies employ the use of freely-cycling intact female rats to understand how natural fluctuations in ovarian hormones affect the expression of Fos-induction following cocaine administration.

Cautions about the timeline of Fos expression

There are a few considerations that need to be taken into account concerning the timeline of the experiment. First, the sacrifice time point in the current experiment was three hours after cocaine administration. This time point was based on previous literature observing peak cocaine-induced FosB/DeltaFosB expression in the tVTA after 3 hours in a male population (Kaufling et al., 2010b). To ensure that the observed differences between male and female groups are not the result of a sex-dependent difference in the timeline of FosB/DeltaFosB expression, it might be useful to run a study confirming the timeline in which peak FosB/DeltaFosB expression is observed in the tVTA in a female population. This would tell us if the observed differences in our current data are primarily the effect of the experimental manipulation, or perhaps the partial result of differences in the timeline of peak expression of FosB/DeltaFosB between sexes.

In addition to the use of molecular markers in the current study, it may also be useful to employ electrophysiological techniques to improve the temporal resolution of the measurement of the tVTA recruitment in response to cocaine administration. As observed in Jhou 2013, initial administration of cocaine acts on the lateral habenula in a biphasic manner. It is likely that tVTA activation follows a similar pattern. It would be useful to understand exactly when the recruitment of this area is observed in response to cocaine

administration and to determine if long-term changes in firing patterns are observed after chronic cocaine exposure.

Location of estrogen receptors

Moreover, future studies must focus on how exactly estrogen is influencing the tVTA. One possibility is that estrogen is modulating the activity of major afferents to the tVTA, therefore altering its activity and inhibitory hold on the dopamine system (indirect). The second potential mechanism is that estradiol is interacting specifically with estrogen receptors in cells within the tVTA (direct). It is also possible that direct and indirect pathways could act in sympathy or in opposing manners. A first step would be to identify the presence of estrogen receptors in both the tVTA and in the afferents to the tVTA. It might also be useful to administer agonists that are selective for either ERα or ERβ to determine the extent to which the observed effects are receptor-specific.

5.5 Conclusion

Overall, we found that (1) female rats do express FosB/DeltaFosB in the tVTA following cocaine administration, (2) FosB/DeltaFosB expression in the tVTA is overwhelmingly expressed in GABAergic cells and almost completely absent in dopaminergic cells regardless of sex or treatment with estradiol, (3) acute pretreatment with estradiol does not meaningfully increase the

recruitment of the tVTA when compared to acute peanut oil pretreated female animals, and (4) extended estradiol pretreatment significantly increases tVTA recruitment in response to cocaine administration when compared to both acute peanut oil and acute estradiol treated female animals. Taken all together, these data suggest that estradiol in ovariectomized rats meaningfully regulates the recruitment of the tVTA in response to cocaine administration, and that this regulation is likely mediated through the genomic effects of estrogen signaling. Future studies incorporating intact female animals and a longer time course of cocaine administration must be performed to further characterize the role of ovarian hormones in the recruitment of the tVTA.

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Biographical Information

Dr. Torry Dennis is primarily interested in the investigation of the mechanisms underlying addiction and drug seeking behaviors. Under the umbrella of addiction interests include (1) sex differences and the influence of ovarian hormones, (2) involvement of memory systems and memory reconsolidation, (3) involvement of the tVTA/RMTg, and (4) the interaction and overlap between stress and addiction pathways in the brain.

During his undergraduate career, he joined a laboratory that studied the effects of stress on immune function and disease progression. While there, he was immersed in an environment that taught him a great deal about the neuroscience of learning, memory, stress, and addiction. In 2008 he graduated from Texas A&M University with a Bachelors of Science in Psychology and minors in Business and Neuroscience. In 2008 he joined the Health Psychology and Neuroscience program at the University of Texas at Arlington under the mentorship of Dr. Linda Perrotti. Here he received a more focused training in the fundamentals of addiction neuroscience. In 2014 Dr. Dennis graduated with his PhD from the Department of Psychology at the University of Texas at Arlington. After graduation, Dr. Dennis will continue his research in addiction neuroscience as a postdoctoral research fellow at the Medical University of South Carolina under the direction of Dr. Jacqueline McGinty.