Molecular mechanisms of estradiol-mediated sex differences in cocaine reward

Determining the molecular events underlying the role of estradiol in mediating sex differences in cocaine reward.

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This work is dedicated to all the strong women role models in my life.

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Abstract

Research has demonstrated that women rapidly progress from recreational cocaine use to dependence, consume greater quantities of cocaine, experience more positive subjective effects of cocaine and have higher incidences of relapse during abstinence. These effects have been well replicated in animal models of cocaine addiction and indicate an enhanced sensitivity and therefore, vulnerability of females to cocaine addiction. This increased vulnerability of women and female animals to cocaine addiction has been attributed to enhanced cocaine reward. Furthermore, it has been demonstrated that the female hormone estradiol (E2) is a key mediator of the aforementioned effects of cocaine in women and female animals. For example, higher levels of E2 correspond to enhanced rewarding effects of cocaine, therefore increased cocaine consumption and higher incidences of relapse. However, studies identifying the influence of E2 on cocaine-associated reward and its underlying neurobiological mechanisms are severely lacking. Preliminary evidence from our laboratory demonstrated that female animals have increased sensitivity to cocaine-conditioned reward – measured by conditioned place preference (CPP) – and this increased sensitivity is mediated by increased circulating levels of E2 when animals are getting conditioned to cocaine reward. In the present study we have further explored the influence of increased levels of E2 during cocaine conditioning in females and demonstrate that, indeed, E2 mediates cocaine-conditioned reward by potentiating cocaine-context associations. Additionally, we observed that E2-mediated increases in cocaine-CPP were associated with increased activation of ERK1/2 and mTOR proteins in the mesolimbic reward pathway. To experimentally assess the involvement of ERK1/2 and mTOR in E2-mediated enhanced cocaine-CPP, we systemically inhibited ERK1/2 and mTOR activity during cocaine-conditioning. Systemic inhibition of ERK1/2 phosphorylation during conditioning inhibited cocaine-CPP in females whereas mTOR inhibition during conditioning did not. In conclusion, we have established that E2 enhanced cocaine-conditioned reward by potentiating cocaine-context associations formed during the cocaine-conditioning. ERK1/2 activation during cocaine-conditioning is necessary for the potentiation of cocaine-conditioned reward by E2. While, mTOR activation during cocaine-conditioning does not influence E2-mediated enhancement of cocaine-conditioned reward. This is the first study assessing the interaction between E2, ERK1/2 and mTOR signaling, and E2-mediated cocaine conditioned reward.

Supervising Professor: Dr. Linda I. Perrotti

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Chapter 1 Background and Significance

1.1 Introduction.

Drug addiction or substance use disorder is a chronic, relapsing, neuropsychiatric illness characterized by a loss of control over intake, persistent drug craving during abstinence, and high motivation to take the drug (Becker & Chartoff, 2019). In 2018, approximately 2.2 million people reported regular use of cocaine and 1 million individuals met criteria for cocaine use disorder in the United States (Bose et al., 2018), making cocaine use disorder a serious public health concern. Although, overall more men use and are addicted to cocaine than women, women exhibit a more rapid progression from initial cocaine use to dependence than men (Becker & Hu, 2008). Women also report experiencing enhanced positive subjective effects (feelings of euphoria) of cocaine than men (Becker & Chartoff, 2019; Quiñones-Jenab, 2006; Rubinow & Schmidt, 2019). This enhancement of cocaine-induced affect (due to enhanced cocaine reward) is speculated to be the reason women progress more rapidly through the stages of addiction than men (Becker & Hu, 2008). This further translates into higher levels of cocaine craving during abstinence leading to relapse. Indeed, during periods of abstinence, women report experiencing higher levels of cocaine craving and relapse rates than men (Becker & Chartoff, 2019). Lastly, during bouts of relapse women take larger amounts of cocaine than men (Chen & Kandel, 2002; Gallop et al., 2007; Ignjatova & Raleva, 2009; Robbins et al., 1999). Collectively, these data indicate that women may be more severely affected by cocaine use than men and therefore represent a particularly vulnerable population.

Like humans, female rodents escalate self-administration of cocaine and progress to addictive behaviors more rapidly than males (Becker & Chartoff, 2019). Females rodents also demonstrate higher motivation to consume cocaine than their male counterparts (Becker & Chartoff, 2019). In addition, females consume larger quantities of cocaine and experience increased rewarding and reinforcing effects of cocaine compared to their male counterparts (Becker & Koob, 2016). Post abstinence, female rodents exhibit a heightened addictive phenotype, in that they consume greater amounts of cocaine and demonstrate greater dysregulation of cocaine intake when given free access to it than males (Lynch & Taylor, 2004). Female rodents demonstrate prolonged extinction times, as compared to males; a phenotype which is indicative of heightened sensitivity and craving for cocaine (Fuchs et al., 2005; Kosten & Zhang, 2008; Lynch & Taylor, 2005). Females also demonstrate increased responsivity towards

cocaine taking under stress than males indicating a higher intensity of negative physical and psychological withdrawal symptoms and therefore, higher relapse probability (Fox & Sinha, 2009). Lastly, female rodents more readily reinstate cocaine use after a period of abstinence in the absence of any reinforcing cues when compared to males (Anker & Carroll, 2010; Buffalari et al., 2012).

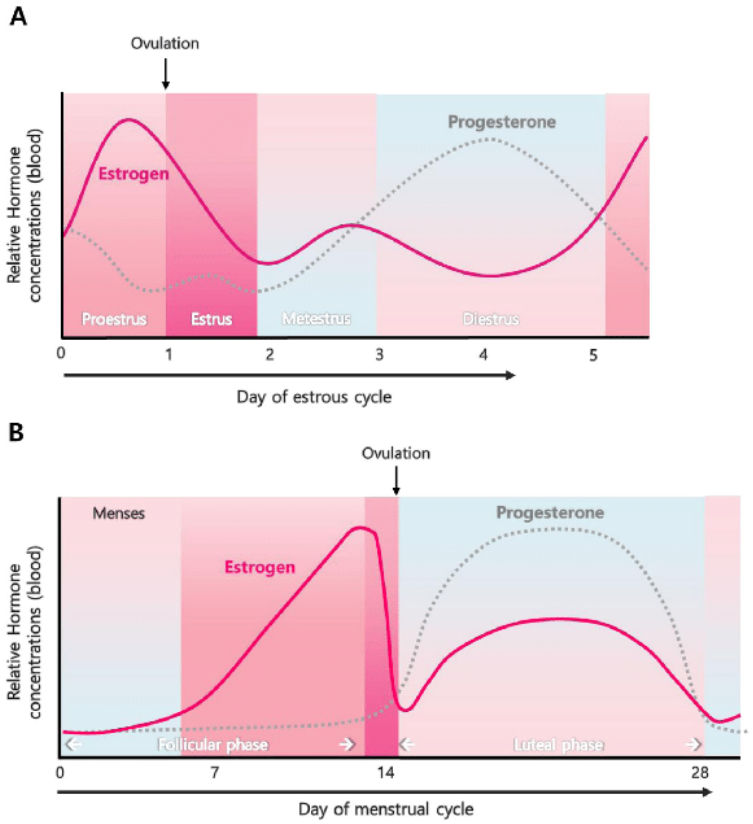
Taken all together, these data demonstrate sex differences in preclinical rodent models of cocaine addiction and recapitulate and extend findings from the clinical literature. Specifically, women and female rodents are decisively more vulnerable to developing an addictive phenotype after exposure to cocaine than men or male rodents, possibly due to enhanced rewarding and positive affective effects of cocaine.

1.2 Sex hormones and cocaine addiction.

In females, the phase of the menstrual/estrous cycle and the release of reproductive hormones associated with each phase, influences synaptic transmission, sex-specific motivated behaviors, as well as motivation related to drug-taking and addiction-related behaviors (Kokane & Perrotti, 2020). Therefore, it is essential that the hormonal conditions of women be taken into consideration when discussing sex differences in cocaine addiction.

The 28-day human menstrual cycle consists of follicular, periovulatory, and luteal phases. During the follicular phase (10-12 days), estradiol (E2) is secreted from the ovary as the follicle develops and circulating concentrations of E2 increase daily. The next phase is the peri-ovulatory phase (2-4 days) during which a rapid increase in E2 triggers the release of luteinizing hormone from the pituitary that induces ovulation. The last phase is the luteal phase (10-12 days) which is characterized by the release of relatively high concentrations of both E2 and progesterone from the remains of the follicle that is retained by the ovary (corpus luteum). Menstruation occurs at the end of the luteal phase (unless pregnancy occurs). During menstruation hormone levels are at their lowest points, indicating the beginning of the next cycle (Becker et al., 2005). Rats and mice have a 4-5 day estrous cycle (EC) comprised of phases that function similarly to the phases in the human menstrual cycle. The rat/mouse follicular phase (2-3 days) is called diestrus. A periovulatory phase, called proestrus, occurs during the day of E2 and progesterone surges. The estrus phase of the cycle occurs on the day following the E2 and progesterone surges; this is when the female rodent ovulates and is sexually receptive (See **Figure 1**).

Figure 1. *Hormonal changes in rodent estrous cycle and female menstrual cycle. Comparable changes occur in circulating estrogen and progesterone hormones in rat EC (A) and human menstrual cycle (B) (reprinted with permission from* Hong & Choi, 2018*).*



Phase of the EC has been shown to influence an animal's motivation to self-administer cocaine (Roberts et al., 1989); cocaine-self administration is highest during proestrus and estrus and lowest during diestrus (Feltenstein et al., 2011). In other words, female rats consume greater amounts of cocaine when circulating levels of E2 are high and cocaine consumption is reduced at times when E2 levels are lower. Further support of the influence of circulating ovarian hormones in differences in the reinforcing properties of drugs comes from self-administration paradigms using ovariectomized (OVX)-hormone-treated rodents. These experiments have consistently demonstrated a role for E2 in enhancing the responsivity to cocaine. More specifically, OVX alone decreases the rate of acquisition of cocaine self-administration and reinstatement of previously extinguished cocaine-seeking behavior. E2 administration to OVX animals restores acquisition of cocaine self-administration

rates as well as reinstatement of cocaine-seeking behavior to levels comparable with those of intact female rodents (Frye, 2007; Hu et al., 2004; Larson et al., 2005; Lynch et al., 2001, 2010). Furthermore, this effect was specific for female rodents since there was no effect of E2 replacement on cocaine self-administration behaviors of male rodents (Jackson et al., 2006).

1.3 Involvement of the mesolimbic reward pathway in cocaine addiction.

Cocaine addiction is a complex neuropsychological disorder involving different areas of the brain and different neurotransmitter systems. Rich literature from rodent studies has replicated different addiction-related behaviors in animal models of drug abuse, including cocaine, using different behavioral paradigms. All these studies have implicated the mesolimbic reward pathway to be the primary target of drugs of abuse including cocaine. Evolutionarily, the mesolimbic reward pathway is necessary for organisms to engage in behaviors that are reinforcing and to motivate actions that produce rewarding feelings of pleasure (Gardner, 2011). This pathway is comprised of midbrain dopamine (DA) neurons that arise in the ventral tegmental area (VTA) and substantia nigra, and project to forebrain regions (striatum, prefrontal cortex, hippocampus and amygdala - **Figure 2**) (Kokane & Perrotti, 2020; Wise, 2004). It modulates information flow through the limbic system to regulate and/or promote behaviors related to survival and perpetuation of the species (i.e. feeding, drinking, mating, maternal and paternal behaviors, and social interaction) (Gardner, 2011; White & Milner, 1992). Furthermore, activation of this pathway has been observed during drug consumption, formation of drug-cue associations, drug memories, cue-induced reinstatement of drug use and relapse. It has been demonstrated that chronic drug use produces persistent enhanced activation of this pathway which results in long-term structural and functional changes (Nestler, 2016).

The striatum, generally speaking, gathers inputs from the neocortex, and then sends projections to other nuclei of the basal ganglia which ultimately reach cortical areas implicated in motor planning and execution. The striatum is a structure that is highly conserved across species and critically important for broad range of cognitive, sensorimotor, and limbic-related functions. The striatum is anatomically divided into the ventral striatum (nucleus accumbens; NAc) and dorsal striatum (a.k.a. caudate putamen; DS) (Voorn et al., 2004; Yin et al., 2004). The dorsal striatum can be subdivided into dorsomedial striatum (DMS), which is vital for goal-directed learning, and the dorsolateral striatum (DLS) implicated in stimulus-response learning (Yin et al., 2005, 2006). Compulsive

responding to drug-associated cues is established when the DS is engaged by the intrastriatal loops between the NAc and DS (Figure 2). Thereafter, exposure to drug-associated cues induces activation of the DS without the presence of the drug. Such activation of DS neurons may induce craving for the drug and has been proposed to be the initiator of drug seeking and relapse (Koob & Volkow, 2016). The NAc is a heterogeneous structure and is subdivided into shell and core components which are chemoarchitecturally and functionally distinct (Di Chiara, 1999; Groenewegen et al., 1999; Zahm & Brog, 1992). The central area of the NAc, called the NAc core, is distinct and is surrounded medially, ventrally, and laterally by the NAc shell. Anatomically, the NAc core/shell subdivisions can be differentiated in terms of the input they receive from prefrontal cortical regions and their output projections – The NAc core receives the majority of its prefrontal input from the prelimbic region of the cortex and lateral orbitofrontal cortex and projects out predominantly to the substantia nigra (Ikemoto, 2007; Zahm & Brog, 1992); the NAc shell receives its cortical input from infralimbic cortex and medial lateral orbitofrontal cortex (Ebrahimi et al., 1992; Wright & Groenewegen, 1996) and sends out projections to the pallidum and the VTA (Heimer et al., 1991). The NAc core is critically involved in the development and expression of addiction-related behaviors (Koob & Volkow, 2010) and is recruited in Pavlovian conditioning (Koós & Tepper, 1999; Sunsay & Rebec, 2014). For example, typically, NAc core interacts with brain regions associated with motor circuitry, thus coordinating drug-induced behavioral output, while the NAc shell interacts with limbic and autonomic brain regions, indicating significant regulation of reward, emotional, and visceral responses to drug-associated stimuli (Heimer & Alheid, 1991; Zahm & Brog, 1992). As such, the NAc shell is suspected to mediate the reinforcing properties of novelty, feeding behavior, rewarding substances and stimuli (interoceptive and external cues) which induce drug relapse, while the core seems to play a role in spatial learning, conditioned responses, responses to motivational stimuli, and impulsive choices. Together, the NAc core and shell control the enactment and reinforcement of conditioned behaviors through interaction with reward circuitry (Meredith et al., 2008). In this way, it is generally accepted that the NAc shell is more involved in shorter-term aspects of addiction, for instance reward; whereas, the NAc core plays a role in longer lasting reward-directed behaviors (Ito et al., 2004; Meredith et al., 2008).

Along with projections to the striatum, the VTA sends DA projections to the medial prefrontal cortex (mPFC) These DA projections activate the glutamatergic systems of the prefrontal cortex. This causes the prefrontal cortex to attribute salience to neutral stimuli causing them to be associated with subjective effects of the drug (Koob &

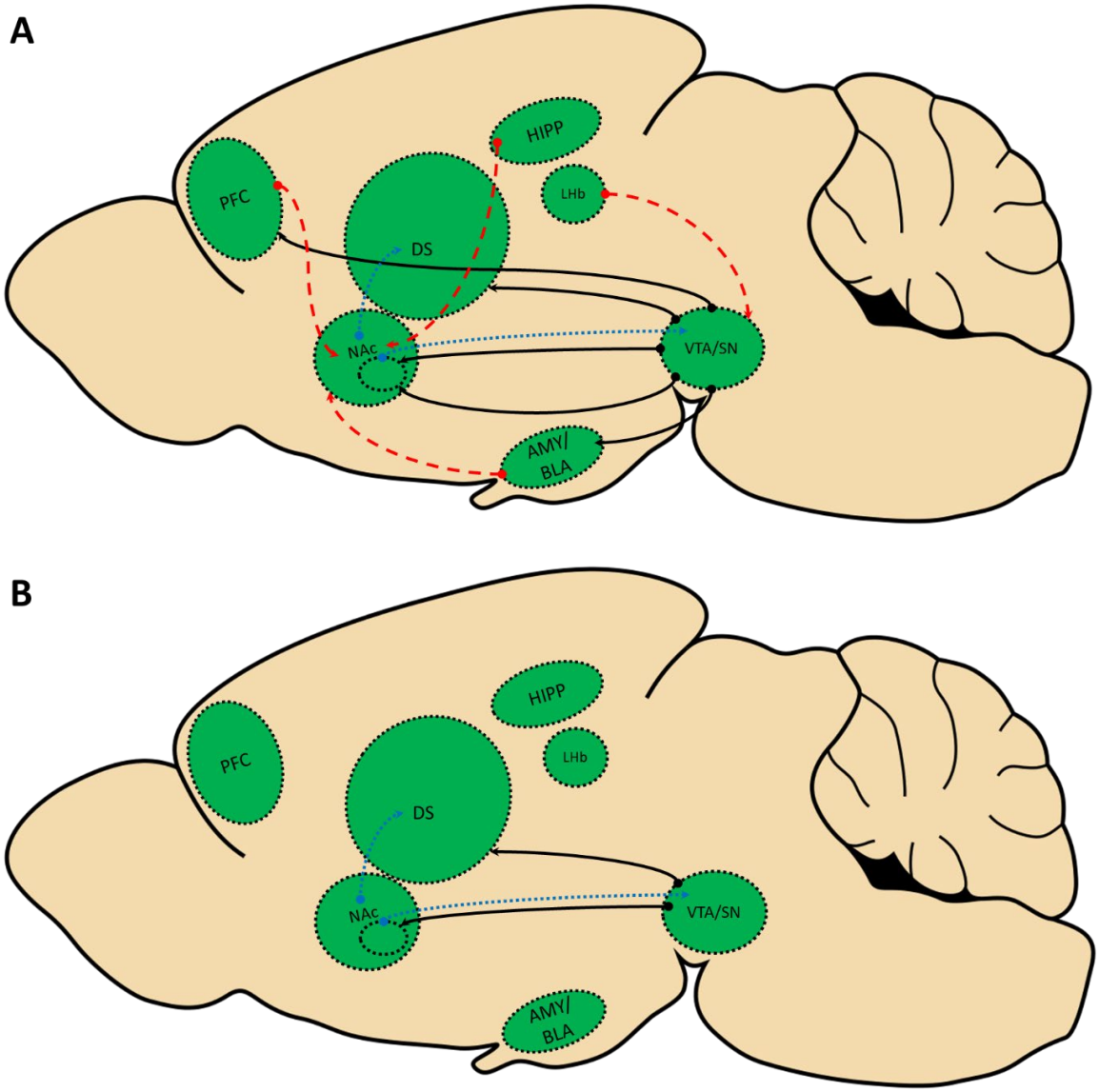
Volkow, 2016). Protracted abstinence from drugs of abuse leads to over activation of the glutamatergic systems that induce strong craving-like responses via glutamatergic activation of the NAc , thus indicative of this circuit's importance in cravings associated with drugs of abuse. Drug-induced reinstatement also involves glutamatergic projections to the NAc that modulate DA release within the NAc (**Figure 2**) (Koob & Volkow, 2016).

The hippocampus has been implicated in the formation of drug-context memories, drug-cue associations, and reconsolidation of drug memories. In addition, the hippocampus has been implicated in reinstatement of drug-taking behavior leading to relapse via cue and contextual triggers (Kutlu & Gould, 2016). These behaviors are mediated by the glutamatergic projections sent from the hippocampus to the NAc (See **Figure 2**).

Finally, the basolateral amygdala (BLA) plays a critical role in the response to natural reward and drug-associated cues. Anatomically, the BLA receives DA inputs from the VTA and provides outputs to neurons in the NAc (Koob, 2009; Koob & Volkow, 2016). Because the BLA serves as an interface between VTA DAergic inputs and outputs to the PFC and NAc, it is well positioned to sub serve associative memory functions (Floresco et al., 2001; Floresco et al., 1998; Koob & Volkow, 2016). Through the convergence of DAergic inputs with sensory-associative information, BLA neurons encode emotionally salient memories (Chang & Grace, 2014). Increases in activity in adjacent central nucleus of the amygdala (CeA) are associated with the anxiety-like effects of acute withdrawal and the increased drug intake associated with dependence (Koob & Le Moal, 2001).

Figure 2. *Mesolimbic Reward Pathway.*

Panel (A) shows a schematic of the mesolimbic reward pathway and panel (B) shows pathway in cocaine conditioned reward. Dotted circle within the NAc shows NAc core and the remaining part of the NAc constitutes NAc shell. Dopaminergic projections arising from the VTA are shown in black. The PFC, hippocampus and amygdala send out glutamatergic projections to the NAc (in red dashed lines). The inhibitory GABAergic MSN projections from NAc are shown in blue dotted lines.



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1.3.1 Sex differences and estrogen regulation of mesolimbic reward pathway.

Sex differences have been observed both in the organization and functioning of the brain areas of the mesolimbic reward pathway (Kokane & Perrotti, 2020). These differences are present in the neuronal populations, receptor expression and functioning of these receptors within different areas of the reward pathway. Additionally, there is a difference in the influence of ovarian hormonal levels on the expression and activity of the neurons within the mesolimbic reward pathway (Kokane & Perrotti, 2020).

Sex and levels of ovarian hormones influence DA neurons in the VTA (Gillies & McArthur, 2010; Johnson et al., 2010; Morissette et al., 2008). Female rodents have a significantly greater proportion of DA neurons in the VTA compared to their male counterparts (Kritzer & Creutz, 2008). In addition, sex differences in the shape and volume of the VTA as well as in the distribution and size of DA cell populations have been identified (McArthur et al., 2007). The activity of VTA-DA neurons appears to be sensitive to circulating levels of E2. For example, basal firing rates of DA neurons of the VTA vary during the different phases of the rodent estrous cycle: DA firing rates are highest in estrus, lowest in proestrus, and intermediate in diestrus (Zhang et al., 2008). Moreover, E2 replacement to OVX rats influences firing rate, spontaneous activity, DA release, DA transporter activity, and overall responsiveness of striatal neurons to DA (Calipari et al., 2017; Zhang et al., 2008). Overall, the ability of E2 to influence activity of VTA-DA neurons strongly suggests the involvement of locally expressed estrogen receptors (Creutz & Kritzer, 2002; Milner et al., 2010; Shughrue et al., 1996).

The GABAergic medium spiny neuron (MSN) is the predominant striatal neuron type (~95%) (Gerfen & Surmeier, 2011). The major target of the VTA-DA projections is striatal GABAergic MSNs. The striatum of male rats contains about 10% more D1 DA receptors than that of intact female or OVX rats, but no sex difference in the number or binding characteristics of striatal D2 DA receptors (Hruska & Silbergeld, 1980; Lévesque & Di Paolo, 1988), however one experiment reported female rats had fewer D2 receptors than males (Miller, 1983). Interestingly, E2 rapidly downregulates D2 DA receptor binding in the striatum of females (Bazzett & Becker, 1994). Sex differences and E2 sensitivity of striatal DA response to synaptic activity are well documented (Yoest et al., 2018). For example, administration of E2 to OVX rats increases DA release, turnover, and DA uptake (Becker & Beer, 1986; Becker & Ramirez, 1981; Di Paolo, 1994). In addition, E2 acutely increases DA receptor density and DA binding (Di Paolo et al., 1985; Lévesque & Di Paolo, 1989; Di Paolo, 1994; Shieh & Yang, 2008). Evidence

from studies using intact rodents add to the above studies and clearly demonstrate sex differences in baseline DA activity and stimulated DA activity in the striatum (Becker et al., 2012; Becker & Hu, 2008). More specifically, female rats exhibit greater basal concentrations of DA and stimulated DA concentrations in the striatum compared to those of males (Castner et al., 1993; Walker et al., 1999). The ratio of levels of striatal DOPAC/DA (a measure of neurotransmitter turnover) are highest during the proestrus stage of the estrous cycle as compared to the other stages of the cycle, suggesting a greater magnitude of DA turnover when circulating levels of E2 are high (Xiao & Becker, 1994).

Robust sex differences and hormone sensitivity in the NAc core are well documented (Becker & Hu, 2008; Yoest et al., 2019). Reports of sex differences/hormone sensitivity in NAc shell are less robust and/or more variable compared to core and likely depend upon interactions with other environmental influences (Brancato et al., 2017; Forlano & Woolley, 2010). The sex differences in the NAc core appear to be mediated primarily via influences on excitatory synaptic and electrophysiological properties of NAc neurons and striatal terminals (Dorris et al., 2015; Mermelstein et al., 1996; Wissman et al., 2011). In addition, sexual dimorphisms in synaptic properties and dendritic spine density of GABAergic MSNs in the NAc core have also been identified. In females, GABAergic MSNs of NAc core have anatomically larger spines and higher dendritic spine density than in males (Forlano & Woolley, 2010) and the frequency of mEPSCs in the core is higher in females than males (Wissman et al., 2011). In both the core and shell there is no evidence for sex differences in the magnitude of DA projections (Forlano & Woolley, 2010; Wissman et al., 2012). Other neuroanatomical attributes such as MSN soma size, cellular density and gross region volume have not been found to be sexually dimorphic (Meitzen et al., 2011; Wong et al., 2016). Electrophysiological properties of GABAergic MSNs in the core change across the EC (Proaño et al., 2018). For example, during diestrus, the excitatory synaptic input onto these MSNs decreases in magnitude, while intrinsic excitability increases. In other words, mESPC frequency and amplitude are decreased during diestrus compared to other estrous cycle phases, while properties such as action potential rheobase, threshold, input resistance, and resting membrane potential change to increase cellular excitability (Proaño et al., 2018). During proestrus and estrus excitatory synaptic input increases and intrinsic excitability decreases. Frequency and amplitude of mEPSCs are also increased compared to diestrus phase (Proaño et al., 2018). These findings demonstrate the likelihood that higher and lower levels of E2 differentially regulate the electrophysiological properties of GABAergic MSNs of the

NAc core. Based on these data, it can be inferred that lower levels of E2 during diestrus prepares the GABAergic MSNs towards tonic activation while higher levels of E2 during proestrus and estrus induces tonic release of GABA.

Taken all together, this evidence demonstrates that the organization and functioning of the mesolimbic reward pathway is influenced by E2, and that cyclical changes in E2 which are observed in females across the estrous cycle would continually modulate the functioning of this pathway. This would further imply that activation of the mesolimbic reward pathway by cocaine and other drugs of abuse would also be influenced by cyclical changes in E2 levels.

1.4 Molecular mechanisms associated with cocaine reward.

At a neurotransmitter level, cocaine exerts its psychomotor stimulant effects by increasing extracellular DA levels by binding to the DA transporter (DAT) in the striatum; a membrane protein located on dendrites of DAergic neurons, responsible for the reuptake of DA from the synaptic cleft (Mortensen & Amara, 2003; Zhu & Reith, 2008). Through this inhibition of DA reuptake, cocaine increases synaptic DA levels thereby potentiating activation at postsynaptic DA receptors. Cocaine produces a buildup of DA wherever the brain has DA transporters. However, the psychoactive and addictive effects of cocaine are generated by the drug's ability to produce a buildup of DA in mesolimbic reward structures (Hyman et al., 2006; Kalivas & McFarland, 2003; Nestler, 2001). It has been demonstrated that cocaine produces persistent enhanced activation of the mesolimbic reward pathway resulting in long-term structural and functional changes within neurons of the mesolimbic reward pathway. These neuroadaptive changes include enhancement in neurotransmitter release, increased synaptic plasticity and dendritic arborization within areas of the mesolimbic reward pathway (Koob & Volkow, 2016). The increased neurotransmission (GABAergic, dopaminergic and glutamatergic) activates downstream molecular mechanisms within these areas.

The neuroadaptive changes discussed above are caused by long-lasting changes occurring in downstream molecular signaling pathways. Studies conducted in male rodents have demonstrated that acute cocaine exposure increases extracellular signal-regulated kinase (ERK) activity in the VTA, NAc, BLA and mPFC (Radwanska et al., 2005; Valjent et al., 2004, 2005). This increase in phosphorylated ERK (pERK) expression is associated with increased phosphorylation of its downstream targets including CREB and the NR2B subunit of the NMDA receptor (Sun et al., 2016). CREB drives generation of silent synapses which are critical to the synaptic development

associated with enhanced drug seeking underlying addiction (Eagle et al., 2019). A series of studies have also implicated the PI3K-Akt/mTOR signaling pathway in cocaine-induced neuroadaptations in the mesolimbic reward pathway (Neasta et al., 2014; Ucha et al., 2020). The key effector protein within this pathway which drives cocaine-induced neuroadaptations is mammalian target of rapamycin (mTOR) protein (Neasta et al., 2014). mTOR stimulates the translation of mRNA of key proteins that cause the neuroadaptations in the mesolimbic reward pathway. These neuroadaptations further give rise to cocaine-associated addictive behaviors.

DA receptor activation and phosphorylation of the NR2B subunit of the NMDA receptors in the NAc induces ERK1/2 activation. This process has been observed in cocaine-associated behavioral sensitization, reward, dependence and addiction (Cahill et al., 2014; Garcia Pardo, 2016; Iñiguez et al., 2010). Cocaine-CPP expression increases phosphorylated ERK1/2 in the NAc, amygdala and PFC (Chen & Xu, 2010; Kong et al., 2011). CREB is a transcription factor regulating the expression of several genes involved in synaptic plasticity, neuroadaptations dendritic arborization and have been implicated in the development of cocaine addiction-related behaviors. CREB gets phosphorylated by ERK1/2 signaling pathway. Increased phosphorylation of CREB in the NAc, blunts the rewarding effects of cocaine thereby driving cocaine self-administration (Barrot et al., 2002; Carlezon et al., 1998; Larson et al., 2011). Through empirical evidence, it has been proposed that increased activity of CREB leads to increased excitability of NAc MSNs via increased expression and synaptic transmission of NMDA receptors. This may in turn induce a negative feedback loop that blunts rewarding effects of cocaine and eventually drives escalation (Dong et al., 2006). Phosphorylation of CREB also drives formation of new dendritic spines by increasing the expression of NMDARs but not AMPARs leading to the formation of silent synapses (Murphy & Segal, 1997; Segal & Murphy, 1998). Studies have indicated formation of “silent synapses” to be critical to the enhancement of cocaine seeking (Huang et al., 2015). ΔFosB is induced in the D1-type MSNs by chronic exposure to cocaine and has been proposed to be the molecular switch for addiction (Nestler, 2008; Perrotti et al., 2008). Its expression is regulated by cocaine and CREB within NAc MSNs and increases cocaine reward and self-administration (Eagle et al., 2019; Robison et al., 2013; Vialou et al., 2012). It is also necessary and sufficient for cocaine-induced dendritic spine formation through increase in the expression of silent synapses in the D1-type MSNs of NAc. Conversely, it decreases their expression in D2-type MSNs of the NAc thereby enhancing rewarding effects of cocaine (Grueter et al., 2013). It has been shown to be critical in mediating the effects of cocaine on NAc’s ability to integrate

glutamatergic inputs from the hippocampus, mPFC and amygdala (Eagle et al., 2019). Taken together, these studies indicate activation of ERK1/2 via DAergic and glutamatergic activation of D1 DA receptor and NMDA receptor respectively leads to the phosphorylation of CREB. Phosphorylated CREB independently and through the regulation of ΔFosB activates the neuroadaptations that drive cocaine addiction.

PI3K including its downstream targets Akt, glycogen-synthase kinase-3 (GSK3), mTOR and S6 kinase play a key role in cocaine-induced neuroadaptations. Activation of PI3K-Akt/mTOR signaling pathway in the NAc has been demonstrated to be critically involved in the expression of cocaine-conditioned place preference (Bailey et al., 2012). PI3K-Akt/mTOR signaling pathway in the hippocampus and the PFC are also involved in the reinstatement of cocaine-CPP, withdrawal from cocaine SA, cue-induced reinstatement of cocaine (James et al., 2014; Shi et al., 2014; Wang et al., 2010). In the NAc, mPFC and VTA of male rats, acute systemic administration of cocaine resulted in and an enhancement in the phosphorylation level of the ribosomal protein S6, a downstream effector protein of mTOR, within 1 hour. Importantly, this increase was completely abolished by a systemic pre-treatment with the selective mTORC1 inhibitor, rapamycin (Wu et al., 2011). Another study demonstrated that the activation of mTORC1 appears to be transient, at least in the NAc, as S6 was not phosphorylated 24 hours following acute administration of cocaine (Bailey et al., 2012). Activation of NMDA receptors, an important modulator of cocaine-dependent behaviors, triggers mTOR signaling in the presence of cocaine-paired cues (Wang et al., 2010). Furthermore, focal inhibition of mTORC1 within the NAc core suppressed cue-induced reinstatement of cocaine seeking (Wang et al., 2010).

Taken together, it is evident that ERK1/2 and mTOR play a significant role in cocaine-related behaviors. Acute administration of cocaine induces a rapid activation of both ERK1/2 and mTOR in different areas of the mesolimbic reward pathway. Activation of both ERK1/2 and mTORC1 in the mesolimbic reward pathway promotes neuroadaptations that underlie certain behavioral alterations induced by repeated cocaine exposure (Neasta et al., 2014). Therefore, molecular mechanisms of cocaine addiction predominantly involve ERK1/2 and PI3K-Akt/mTOR signaling pathways.

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1.4.1 Estradiol regulation of molecular mechanisms associated with cocaine addiction.

Sex differences in behavioral effects of cocaine are the result of underlying neurobiological changes brought about by the interactions between cocaine and ovarian hormones within the reward pathway (Kokane & Perrotti, 2020). Although specific neurobiological mechanisms remain to be fully elucidated, there exist a plethora of studies associating a combined effect of cocaine and E2 on VTA DAergic neurotransmission and striatal GABAergic, DAergic, and glutamatergic neurotransmission towards the behavioral differences seen between sexes in cocaine addiction (Kokane & Perrotti, 2020). However, studies elucidating the molecular events associated with the influence of E2 on cocaine-conditioned reward are lacking.

E2 and DA systems interact to modulate striatal function and resultant behavior (Becker, 1990; Becker & Beer, 1986; Bitar et al., 1991; Di Paolo et al., 1985). For example, E2 increases striatal DA release and turnover (Becker et al., 1984; Becker & Beer, 1986; Becker & Ramirez, 1981; Thérése Di Paolo et al., 1985) and density of striatal DA uptake sites (Morissette et al., 1990). Post-synaptically, E2 increases striatal D1 receptors, while decreasing high-affinity D2, and increasing low-affinity D2 binding (Di Paolo et al., 1985; Lévesque & Di Paolo, 1988, 1989; Shieh & Yang, 2008). E2 promotes the sensitivity of VTA-DA neurons to cocaine (Zhang et al., 2008) which, in turn, enhances cocaine-stimulated striatal DA release (Febo et al., 2003; Peris et al., 1991). Therefore, the potentiating effects of E2 on striatal DA activity are, in part, responsible for the sex and hormone-related differences in subjective and physiological responses to cocaine (Becker & Ramirez, 1981; Becker & Rudick, 1999; Quiñones-Jenab, 2006; Walker et al., 2001). The effect of E2 in the female striatum is also mediated by E2 receptors on GABAergic MSNs that enhance DA release via disinhibition of local dopaminergic terminals (Grove-Strawser et al., 2010; Mermelstein, 2009; Mermelstein et al., 1996; Schultz et al., 2009). These effects of E2 on cocaine self-administration and CPP may be due to the ability of E2 to act on mesolimbic DA system to regulate reward and motivation, through the ability of E2 to increase cocaine-stimulated DA release in NAc (Tobiansky et al., 2016) and alter signaling pathways and gene expression in striatum (Grove-Strawser et al., 2010; Le Saux et al., 2006; Peterson et al., 2015, 2016).

Interactions between E2 and cocaine have been demonstrated in that E2 enhances the sensitivity of the DAergic neurons of the VTA to cocaine and increases cocaine-induced DA release in the striatum (Peris et al., 1991; Zhang et al., 2008). A recent study by Calipari et al. (2017) demonstrated that female mice in estrus showed

increased cocaine-induced DA release compared to female mice in diestrus or male mice (Calipari et al., 2017). Indeed, these neurobiological effects resulted from greater firing rate of VTA-DA projections and DA release in the NAc of female mice in estrus (Calipari et al., 2017; Zhang et al., 2008). Moreover, these estrus females also displayed increased phosphorylation of DAT protein. Taken together, it can be hypothesized that higher levels of circulating E2 increase DA levels in the NAc which is due to increased inhibition of DAT activity under the influence of cocaine (Calipari et al., 2017). All of the aforementioned E2-mediated effects on neurotransmitter systems of the reward pathway lead to changes in downstream signaling cascades (Kokane & Perrotti, 2020).

Sex differences in baseline and cocaine-induced activation of ERK1/2 signaling pathway in the NAc have been documented. Males and females have different basal and cocaine-induced levels of pERK1/2, ΔFosB, and pCREB in the NAc (Nygard et al., 2013). Studies have also shown E2 regulation of cAMP and PKA pathways which are upstream modulators of ERK1/2 activity. For example, the activity of various intracellular signaling cascades fluctuates with the estrous cycle in the NAc of cocaine-treated and intact female rats and saline controls (Weiner et al., 2009). Attempts to clarify the specific role of E2 on these intracellular signaling cascades have used OVX females. The results of one study show that OVX E2 treated females demonstrate E2-induced initiation of PKA cascades and CREB protein phosphorylation via activation of G-protein dependent cell signaling cascades (Hammes & Levin, 2007). The downstream effects of these results likely contribute to the structural sexual dimorphisms seen in dendritic morphology and spine density (Forlano & Woolley, 2010; Wissman et al., 2011, 2012).

E2 has acute, rapid (nongenomic) effects, which are initiated via binding at plasma associated membrane estrogen receptors (mER) (Boulware et al., 2005; Mermelstein & Micevych, 2008; Micevych & Mermelstein, 2008). Signaling at mERs activates G-protein dependent cell signaling cascades, including PKA and ERK1/2 (Björnström & Sjöberg, 2005; Dhandapani & Brann, 2002; Rønnekleiv et al., 2007; Vasudevan & Pfaff, 2007). These signaling cascades are similar to those initiated by DA at D1 receptors. In fact, evidence for the role for mERs in mediating the rapid effects of E2 stems from its effects on CREB phosphorylation (pCREB). ERα and ERβ antagonists mimic the effects of E2 while the mER antagonist ICI 182,780 blocks the rapid effects of E2 on pCREB. In this way, E2 activates both mER and D1 receptor G-protein-dependent cell signaling cascades including activation of the MAPK pathway, and phosphorylation of CREB (Hammes & Levin, 2007).

Molecular mechanisms of estradiol-mediated sex differences in cocaine reward

Adult female rats exclusively express membrane-bound ERs (GPER1, membrane associated ERα and ERβ) in MSNs of the DS and NAc, but express few or no nuclear ERs (Almey et al., 2012, 2015; Grove-Strawser et al., 2010; Mermelstein et al., 1996; Schultz et al., 2009). Membrane ERs are expressed on axon terminals, somas, and dendritic spines (Almey et al., 2012, 2015, 2016). Functionally, the activation of MSN membrane ERs have been shown to increase sensitivity to drugs of abuse in females (Eisinger et al., 2018) and change dendritic spine morphology and density in the NAc (Peterson et al., 2015). Previous work has established that application of E2 rapidly increases DA (Becker, 1990; Pasqualini et al., 1996) and decreases GABA production (Hu et al., 2006) in the NAc and DS which suggests that E2 may indirectly influence DA signaling by first releasing inhibition of GABAergic signaling, and perhaps also directly upon DA-producing regions (such as the VTA). In striatal MSNs, E2, acting through membrane-associated ERα and ERβ receptors coupled to mGluRs modulates phosphorylation of the transcription factor CREB (Grove-Strawser et al., 2010; Mermelstein et al., 1996).

Similar to the DS, E2 also rapidly modulates glutamate signaling in NAc core and these effects are sex-specific and bidirectional (Krentzel et al., 2019). The mechanism whereby E2 enhances drug-induced plasticity is via interactions with mGluRs. Specifically, E2 activates mGluR5 signaling in the NAc core, which in turn, leads to alterations in dendritic structure (Grove-Strawser et al., 2010; Martinez et al., 2014; Peterson et al., 2015). These alterations induce neuroadaptations which are long-lasting and cause long-term enhanced activation of the mesolimbic reward pathway. Since this occurs under the chronic influence of drugs of abuse, for the activation of the reward pathway post neuroadaptive changes, DA released due to “naturally” rewarding stimuli is insufficient. Hence, normal rewarding stimuli become less rewarding and full activation of the reward pathway requires drug consumption. Thus, increasing the reward value of drugs eventually leading to addiction and dependence.

E2 stimulation of mERs rapidly stimulates ERK1/2-dependent pCREB, and peripheral administration of E2 initiates MAP kinase and ERK1/2 signaling pathways (Dewing et al., 2007, 2008; Kelly & Rønnekleiv, 2008; Mhyre & Dorsa, 2006) and decreases L-type calcium channel-mediated CREB activity (Boulware et al., 2005, 2007; Gu et al., 1996; Wakino et al., 2005; Zhou et al., 1996). Inhibition of either MEK or PKC significantly inhibits E2-mediated DA efflux, while inhibiting PI3 kinase or PKA does not affect E2-mediated DA efflux (Alyea & Watson, 2009). E2 induction of TH involves membrane-initiated E2 signaling, rapid activation of dual PKA/ERK1/2 signaling pathways, leading to pCREB activity (Maharjan et al., 2010), while the mER antagonist ICI 182,780 blocks the rapid effects of

E2 on pCREB. Thus, mER mediated activation of these intracellular signaling cascades influences the activity of a variety of transcription factors which likely contribute to gene transcription independently of nuclear ER (Mhyre & Dorsa, 2006).

Although extensive research has attributed several signaling molecules and transcription factors in different behaviors and processes associated with cocaine addiction, sex differences studies on cocaine-conditioned reward and involvement of E2 on these is severely limited. However, based on the above discussion, E2 and cocaine separately or together, affect all of the aforementioned signaling molecules/transcription factors.

Chapter 2 Support for scientific premise

2.1 Rationale.

Continued drug use, drug-seeking and relapse to former patterns of drug use during abstinence is heavily dependent on learned associations between the drug and environmental cues and/or contexts as well as the individual's physical and emotional reactivity to these stimuli (O'Brien et al., 1990; Robbins et al., 1999). Cue reactivity and exposure to cues are important factors in continued cocaine use and relapse to drug use/abuse. Clinical literature demonstrates that encountering cues previously associated with drug use is a common trigger for relapse (Becker & Hu, 2008; Bose et al., 2018). Clinical studies examining real-time reports of cue exposure, mood, cocaine craving and use demonstrate that drug use is frequently associated with cue exposure than with craving, and that exposure to drug-associated conditioned stimuli increases desire to use drugs in drug-dependent individuals. Investigations into gender differences in reactivity to cocaine cues have produced inconsistent results - some studies report greater cue reactivity in women, greater reactivity in men, or no differences (Becker & Chartoff, 2019; Becker & Koob, 2016; Hu et al., 2004). Among factors that may explain inconsistencies is the fact that these studies failed to account for menstrual cycle when measurements were taken. Neuroimaging studies indicate that menstrual cycle period is an important factor to consider, as women demonstrate greater neural activation to reward stimuli during the follicular versus the luteal phase of the cycle (Bobzean et al., 2014; Koob & Volkow, 2016) and greater brain reactivity to conditioned cocaine cues in female than in male cocaine abusers even though the self-reported craving responses did not differ (Bobzean et al., 2014; Koob & Volkow, 2016).

Vulnerability of females to cocaine-associated cues and contexts is an important underlying factor in the sex-differences seen in cocaine addiction (Robbins et al., 1999). Because females are more sensitive to cocaine-conditioned stimuli than males, it is likely that the molecular neuroadaptations, which occur during the development of conditioned associations between cocaine and environmental cues, are different and potentially more robust and/or resilient in females. The CPP paradigm is used to determine the conditioned rewarding effects of drugs in rodents because the contextual (environmental) cues used within the paradigm acquire secondary appetitive properties when paired with a rewarding stimulus (i.e. drug of abuse) (Bardo & Bevins, 2000; Tzschentke, 2007). Additionally, the cues/context itself acquires rewarding properties that are directly associated with the subjective rewarding effects of the drug which are experienced by each individual animal that is run through this paradigm.

Molecular mechanisms of estradiol-mediated sex differences in cocaine reward

Sex differences in the rewarding properties of cocaine have been demonstrated using this paradigm by our group and others (Bobzean et al., 2010; Kokane et al., 2019; Segarra et al., 2014). Specifically, female rats demonstrate acquisition of conditioned place preference to lower doses of cocaine compared to males (Russo et al., 2003; Zakharova et al., 2009). This indicates that at lower doses females experience an increase in the magnitude of the rewarding effects of cocaine when compared to males (Russo et al., 2003; Zakharova et al., 2009). However, at higher doses, cocaine place preference is similar between males and females. This suggests that female's sensitivity to cocaine's effects is dose dependent. In addition, reinstatement of extinguished cocaine-induced CPP is more pronounced in female rats (Bobzean et al., 2010). Circulating levels of E2 influence the magnitude of CPP, however these effects vary according to the dose and length and time course of hormone treatment. We and others have reported that OVX female rats demonstrate lower scores CPP compared to intact females (Kokane et al., 2019; Russo et al., 2003). In a series of experiments in which chronic continual administration of E2 was given throughout the duration of the CPP paradigm, E2 treatment alone did not influence CPP scores when compared to untreated animals (Russo et al., 2003). Taken together it is evident that E2 influences cocaine-CPP and that it depends on the pattern/time course when E2 and cocaine are both elevated in females.

There is a surprising gap in our current understanding of the molecular mechanisms underlying E2-dependent changes in the neuronal excitability and synaptic efficacy mediating motivated behaviors. To date, no studies exist examining the effects on molecular mechanisms resulting from the interactions between cocaine, conditioned reward, and E2. Thus, the main aim of this study was to establish the influence of E2 during the formation of associations between the subjective, rewarding effects of cocaine and cocaine-conditioned reward. Additionally, we wanted to identify and establish the role of ERK1/2 and mTOR activity within different nodes of the mesolimbic reward pathway on E2-mediated enhanced cocaine conditioned reward in females. The studies conducted herein are novel as they characterize the effects of E2 on cocaine-environment associations formed during the development of cocaine-CPP. Additionally, we have demonstrated the involvement of the two key molecular substrates implicated in cocaine conditioned reward – ERK1/2 and mTOR (discussed above) and examined the functional interplay between E2 and these molecules in the development of cocaine-environment associations.

Chapter 3 Results

3.1 Experiment 1: Sex differences in cocaine-conditioned reward are influenced by high levels of E2 during cocaine conditioning.

These experiments were conducted to confirm and extend our previous reports of sex differences in cocaine conditioned reward (Bobzean et al., 2010) and set the stage for inquiry into the mechanisms by which fluctuations in ovarian hormones may mediate these sex differences. Here we present data demonstrating dose-dependent sex differences in cocaine-conditioned reward in intact male versus female Long Evans rats. Furthermore, we also demonstrate cocaine-conditioned reward in intact female Long Evans rats to be dependent on increased E2 levels during cocaine conditioning.

3.1.1 Sex differences in cocaine-conditioned reward.

Intact male and female rats were conditioned with 0, 5, 10, or 15 mg/kg doses of cocaine and then tested for cocaine CPP (see General Methods). Overall, our findings demonstrate robust dose-dependent sex differences in cocaine-conditioned reward.

CPP scores for intact male and female rats conditioned are shown in **Figure 3**. A 2 (Sex: Male, Female) X 4 (Dose: 0, 5,10,15 mg/kg cocaine) X 2 (Repeated measures factor: CPP score-Saline, CPP score-Cocaine) three-way mixed ANOVA revealed significant differences between and within the groups for cocaine dose, sex and CPP score. Sex of the animal had a significant effect on CPP score, $F(1, 80) = 4.921$, $p = 0.029$, ${}_p\eta^2 = 0.058$. Additionally, cocaine dose had a significant effect on CPP score, $F(3, 80) = 13.751$, $p < 0.001$, ${}_p\eta^2 = 0.340$. A significant three-way interaction effect was also observed between sex, cocaine dose and CPP score, $F(3, 80) = 2.958$, $p = 0.037$, ${}_p\eta^2 = 0.100$. Post-hoc comparisons employing Bonferroni correction for cocaine dose revealed that males acquired CPP at two (10 mg/kg: $M = 141.394$, $SE = 43.877$, $p = 0.002$ and 15 mg/kg: $M = 181.575$, $SE = 40.361$, $p < 0.001$) of the three doses tested, while females acquired CPP at all three doses (5 mg/kg: $M = 220.439$, $SE = 38.892$, $p < 0.001$, 10 mg/kg: $M = 307.488$, $SE = 40.361$, $p < 0.001$, and 15 mg/kg: $M = 168.474$, $SE = 51.450$, $p = 0.002$).

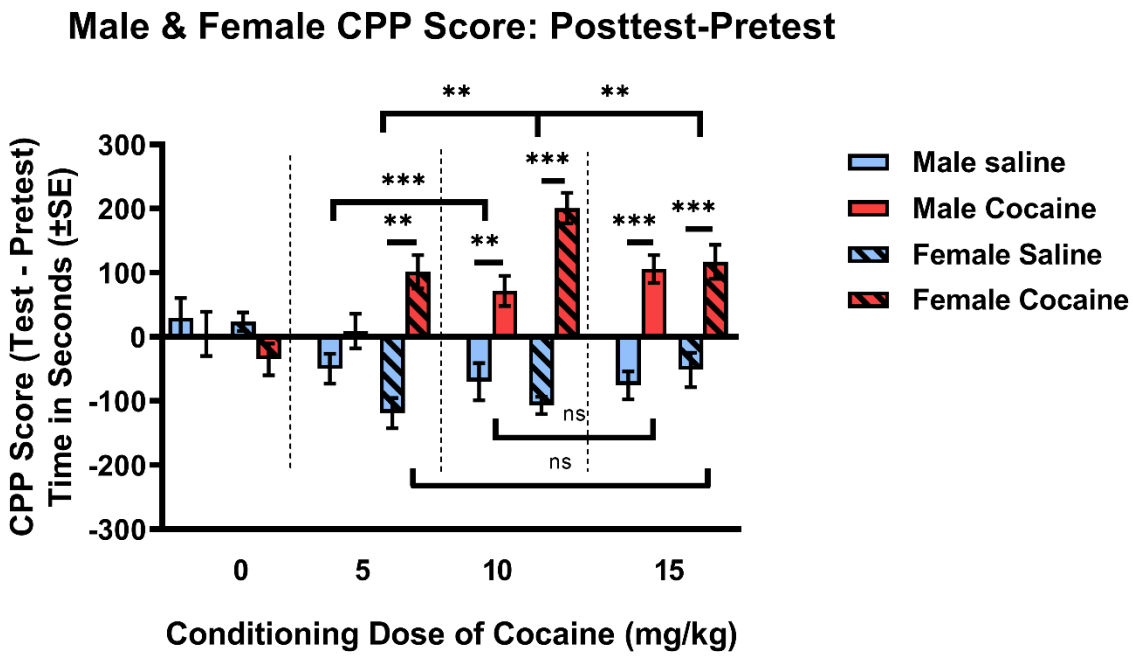
The influence of sex on acquisition of CPP was apparent at the 5 and 10 mg/kg dose. At 5 mg/kg dose females spent significantly more time in the cocaine-paired chamber than males during the test phase ($M = 92.461$, $SE = 38.396$, $p = 0.046$). Males did not acquire cocaine-CPP at this dose. At 10 mg/kg dose, both males and females

spent significantly more time in the cocaine-paired chamber during test phase with a significant sex difference, however, CPP score for females was significantly higher than males at this dose (M = 129.151, SE = 35.491, p < 0.001). At 15 mg/kg does, no significant sex-differences in the time spent in cocaine-paired chamber were observed between males and females (M = 10.995, SE = 38.929, p = 0.778).

The overall trend for cocaine-CPP at different doses of cocaine was also significantly different between males and females. Females showed a gradient in the cocaine place preference at different doses of cocaine such that at lower doses (5 mg/kg: M = 220.439, SE = 38.892, p < 0.001 and 10 mg/kg: M = 307.488, SE = 40.361, p < 0.001) cocaine place preference was greater while at higher dose (i.e. 15 mg/kg: M = 168.474, SE = 51.450, p = 0.002), cocaine place preference declined. Additionally, CPP scores for females differed at different doses of cocaine (See **Figure 3** and **Table 1**). Males only acquired cocaine place preference beginning at 10 mg/kg dose (M = 141.394, SE = 43.877, p = 0.002) and this effect did not change as cocaine dose was increased (at 15 mg/kg: M = 181.575, SE = 40.361, p < 0.001) (See **Figure 3** and **Table 1**). This also demonstrated that the 10 mg/kg dose of cocaine can be considered as a threshold dose for cocaine-CPP for both males and females and can be used to further characterize and delineate the molecular signaling mechanisms associated with sex-differences in cocaine-CPP.

These behavioral data demonstrate robust dose-dependent sex differences in cocaine place preference/cocaine-conditioned reward. Interestingly, females appeared to be sensitive to the differences in cocaine dose whereas males did not. These data indicated that females formed a stronger association to the cocaine-paired environment at lower doses, thus making females significantly more vulnerable to cocaine conditioned reward.

Figure 3. *Dose dependent sex differences in cocaine conditioned reward as demonstrated by cocaine-CPP. Robust sex differences were observed in cocaine conditioned reward between males and females. Red and blue bars represent CPP scores in cocaine- and saline-paired chambers respectively. Solid bars represent CPP scores for males and patterned bars represent CPP scores for females. Males showed acquisition of cocaine-CPP at 10 and 15 mg/kg dose of cocaine while females showed cocaine-CPP at 5, 10 and 15 mg/kg doses of cocaine. Additionally, the trend in cocaine-CPP differs between males and females. In males, cocaine-CPP creased with increase in cocaine dose whereas in females, cocaine-CPP increased at 5 and 10 mg/kg does of cocaine but decreased at the 15 mg/kg dose (***p < 0.001; **p < 0.01).*



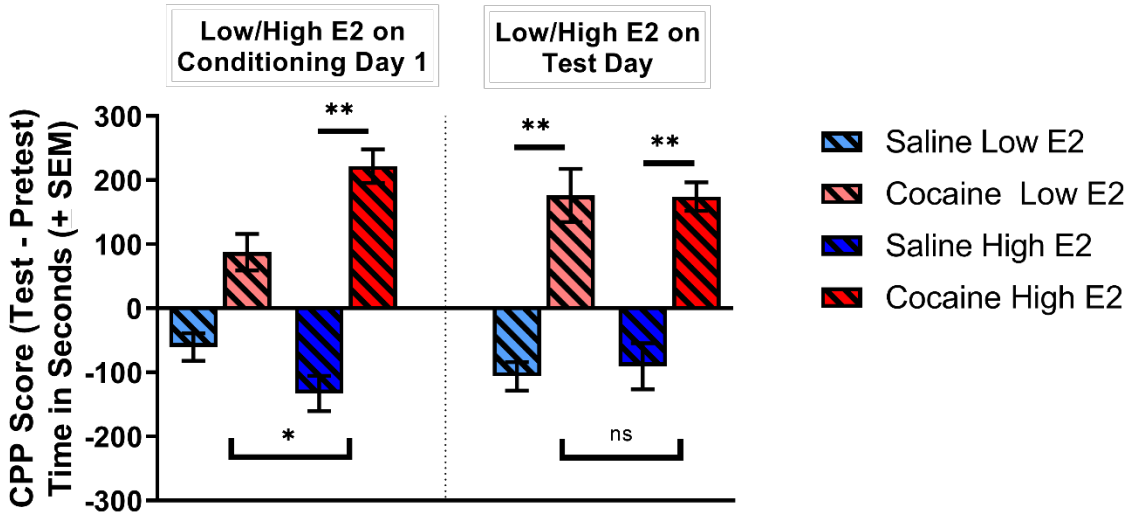
3.1.2 EC stage affects cocaine conditioned reward.

To test the hypothesis that estrous cycle stage influences associations for cocaine-paired stimuli, we reanalyzed the *female* data from the previous experiment *by estrous cycle.* Vaginal lavage testing was used to determine EC stage of female *during experimentation*. Acquisition of cocaine-CPP at 10 mg/kg dose of cocaine was compared between intact female Long Evans rats that were in proestrus/estrus (high levels of E2) and those that were in metestrus/diestrus (low levels of E2) on the first day of cocaine conditioning to those that were in proestrus/estrus and those that were in metestrus/diestrus on the test day (**Figure 4**). These analyses were conducted to determine the effect of high versus low levels E2 on cocaine-CPP during the conditioning versus test phases of cocaine-CPP. Results from this analysis demonstrated E2-dependent differences in the acquisition of cocaine-CPP.

Two separate repeated-measures ANOVAs were performed to assess effect of EC stage (2 levels: High E2 and Low E2) on CPP score during cocaine conditioning or test day. Analyses revealed significant differences between and within the groups for EC stage and CPP score for cocaine conditioning, $F(1, 11) = 8.045$, $p = 0.016$, ${}_p\eta^2 = 0.422$, but not for test day, $F(1, 10) = 049$, $p = 0.829$, ${}_p\eta^2 = 0.005$. Post-hoc comparisons employing Bonferroni correction for CPP score demonstrated that intact female Long Evans rats with high E2 levels on the first day of cocaine conditioning spent significantly more time in cocaine-paired compartment on test day as compared to those with low E2 levels ($M_{Cocaine} = 133.965$, $SE_{Cocaine} = 44.262$, $p = 0.012$). Alternatively, post-hoc comparisons employing Bonferroni correction for CPP score demonstrated that intact female Long Evans rats with high E2 levels on the test day did not spend significantly more time in cocaine-paired compartment as compared to those with low E2 levels on test day ($M_{Cocaine} = 1.937$, $SE_{Cocaine} = 43.572$, $p = 0.965$). This indicated that acquisition of cocaine-CPP is dependent on levels of E2 when cocaine-cue associations are formed during conditioning but not on acquisition of cocaine-CPP *per se* (See **Figure 4**).

Therefore, it was concluded that the enhanced sensitivity to the cocaine-conditioned reward in females maybe because of higher levels of E2 during the formation of associations between the rewarding effects of cocaine and the contextual environment. In other words, E2-mediated formation of cocaine-context associations drove the sensitivity for cocaine-conditioned reward in female animals.

Figure 4. *Effect on cocaine-CPP in females is dependent on E2 levels during cocaine conditioning. High levels of E2 during cocaine conditioning enhanced cocaine-CPP. High or low levels of E2 during test did not affect the magnitude of cocaine-CPP. Light-colored blue and red bars indicate CPP scores in saline- and cocaine-paired compartments respectively in female animals with low levels of E2. Bright-colored blue and red bars indicate CPP scores in saline- and cocaine-paired compartments respectively in female animals with high levels of E2. Graph has been split between female animals that received 10 mg/kg cocaine during conditioning and had high or low levels of E2 during conditioning or on test day of cocaine-CPP (**p < 0.01).*



3.2 Experiment 2: Acute pretreatment with E2 prior to cocaine conditioning enhances cocaine CPP.

To directly test the hypothesis that increased levels of E2 during the conditioning phase of CPP is responsible for augmented CPP scores in females and females during estrus/proestrus, we experimentally manipulated E2 levels during acquisition of cocaine-CPP. Here, we demonstrate that experimentally increasing E2 levels during cocaine-conditioning enhances cocaine-CPP in ovariectomized (OVX) female rats indicating that elevations in levels of E2 during cocaine conditioning influences the expression of cocaine-CPP. To begin to understand the molecular mechanisms underlying the influence of E2 on the enhancement of CPP, we measured levels of ERK1/2 and mTOR protein activation in the VTA, NAc and dorsal striatum. Overall, our findings indicate that sex differences in cocaine-conditioned reward are due, in part, to the influence of E2 on cocaine-cue

associations, and these E2-mediated sex differences are likely manifested in intracellular signaling events (specifically in the ERK1/2 and mTOR signaling pathways) in the VTA, NAc and dorsal striatum.

3.2.1 E2 pretreatment during cocaine conditioning enhanced cocaine-CPP.

To determine the influence of E2 on cocaine-cue associations, we systemically injected OVX female Long Evans rats with 5 µg of estradiol benzoate (EB) in peanut oil (PO) 30 mins before cocaine conditioning session. We included three treatment conditions for EB – 1. OVX *rats* that did not receive EB (OVX); 2. OVX *rats* that received 5 µg of EB on first day of cocaine conditioning (OVX+1/3EB); and 3. OVX *rats* that received 5 µg of EB on all three days of cocaine conditioning (OVX+3/3 EB). Additionally, we also included a group of intact females (Intact). The Intact group did not receive EB and was used to compare the effects of different EB treatment conditions on cocaine-CPP and their underlying neurobiological correlates. We selected the 10 mg/kg dose of cocaine for these experiments based on the findings from our previous experiments. Furthermore, a separate set of control animals (Intact controls, OVX controls, OVX+1/3EB controls, OVX+3/3 EB controls) was also included. The role of these animals was to assess the effect of different EB treatment conditions alone on cocaine-CPP and its underlying neurobiological correlates. These animals received the same treatment of EB but received 0 mg/kg cocaine/0.1 mL/kg 0.9% (w/v) saline during cocaine conditioning.

A 2 (Cocaine Dose: 0, 10 mg/kg) X 4 (EB treatment: Intact, OVX, OVX+1/3 EB, OVX+3/3 EB) X 2 (Repeated measures factor - CPP Score: Saline CPP Score, Cocaine CPP Score) three-way mixed ANOVA revealed significant differences between and within the groups for dose, EB treatment and CPP score. Specifically, CPP score was significantly different between the two doses of cocaine, $F(1, 76) = 21.987$, $p < 0.001$, ${}_p\eta^2 = 0.224$. CPP score also differed significantly across EB treatment conditions, $F(3, 76) = 3.432$, $p = 0.021$, ${}_p\eta^2 = 0.119$. There was a significant interaction between cocaine dose, EB treatment and CPP scores, $F(1, 76) = 5.689$, $p = 0.001$, ${}_p\eta^2 = 0.183$. Post-hoc comparisons employing Bonferroni correction for CPP scores demonstrated that at 10 mg/kg dose of cocaine, all animals spent significantly more time in the cocaine-paired compartment than saline-paired compartment on test day (Intact females: $M = 148.034$, $SE = 47.638$, $p = 0.003$; OVX females: $M = 63.549$, $SE = 31.509$, $p = 0.048$; OVX+1/3 EB: $M = 122.967$, $SE = 39.856$, $p = 0.003$; OVX+3/3EB: $M = 314.483$, $SE = 42.012$, $p < 0.001$). Time spent in either saline-paired or cocaine-paired compartments did not differ for all control animal groups. This demonstrated that all animals that were conditioned with 10 mg/kg of cocaine, regardless of EB

treatment condition, developed cocaine-CPP. Furthermore, post-hoc comparisons employing Bonferroni correction for different EB treatment conditions demonstrated significantly different CPP scores. Specifically, OVX females that received EB pretreatment before cocaine conditioning on all three days of conditioning had significantly greater CPP scores than animals in OVX (M = 138.119, SE = 31.365, $p < 0.001$) and OVX+1/3EB (M = 105.798, SE = 34.587, $p = 0.018$) groups (See **Figure 5**). There were no other significant differences in CPP scores. These data demonstrated that EB treatment during conditioning potentiated acquisition of cocaine-CPP at 10 mg/kg dose of cocaine. Elevated EB levels throughout conditioning and not just the first day of conditioning are essential for E2-mediated enhancement of cocaine-CPP. Interestingly, lack of E2 during conditioning (as in the case of OVX females), suppressed cocaine-CPP. Therefore, EB treatment prior to cocaine conditioning potentiated the association between cocaine reward and the contextual environment allowing for an enhanced cocaine-conditioned reward.

Taken together, these data corroborate and extended our findings from previous experiments, in that decrease in E2 levels during cocaine conditioning attenuated cocaine-CPP while increased levels of E2 potentiated it. This demonstrated that E2 influenced cocaine-context associations during conditioning and elaborated its importance in mediating cocaine reward in females.

Figure 5. *Differences in cocaine-CPP in intact, OVX and OVX females pretreated with EB.*

Panel (A) shows CPP scores of experimental females that received 10 mg/kg of cocaine during conditioning. Panel (B) shows CPP scores of control females. Control females received the same treatment of E2 as experimental females and received 0 mg/kg of cocaine during conditioning. Patterned blue and red bars represent CPP scores in saline- and cocaine-paired compartments respectively. (A) E2 treatment prior to cocaine conditioning on all three days (OVX+3/3EB group) enhanced cocaine-CPP. Elimination of endogenous E2 by OVX attenuated cocaine-CPP (OVX group). E2 treatment prior to first day of cocaine conditioning (OVX+1/3EB group) did not enhance cocaine-CPP. (B) Control females did not develop cocaine-CPP.

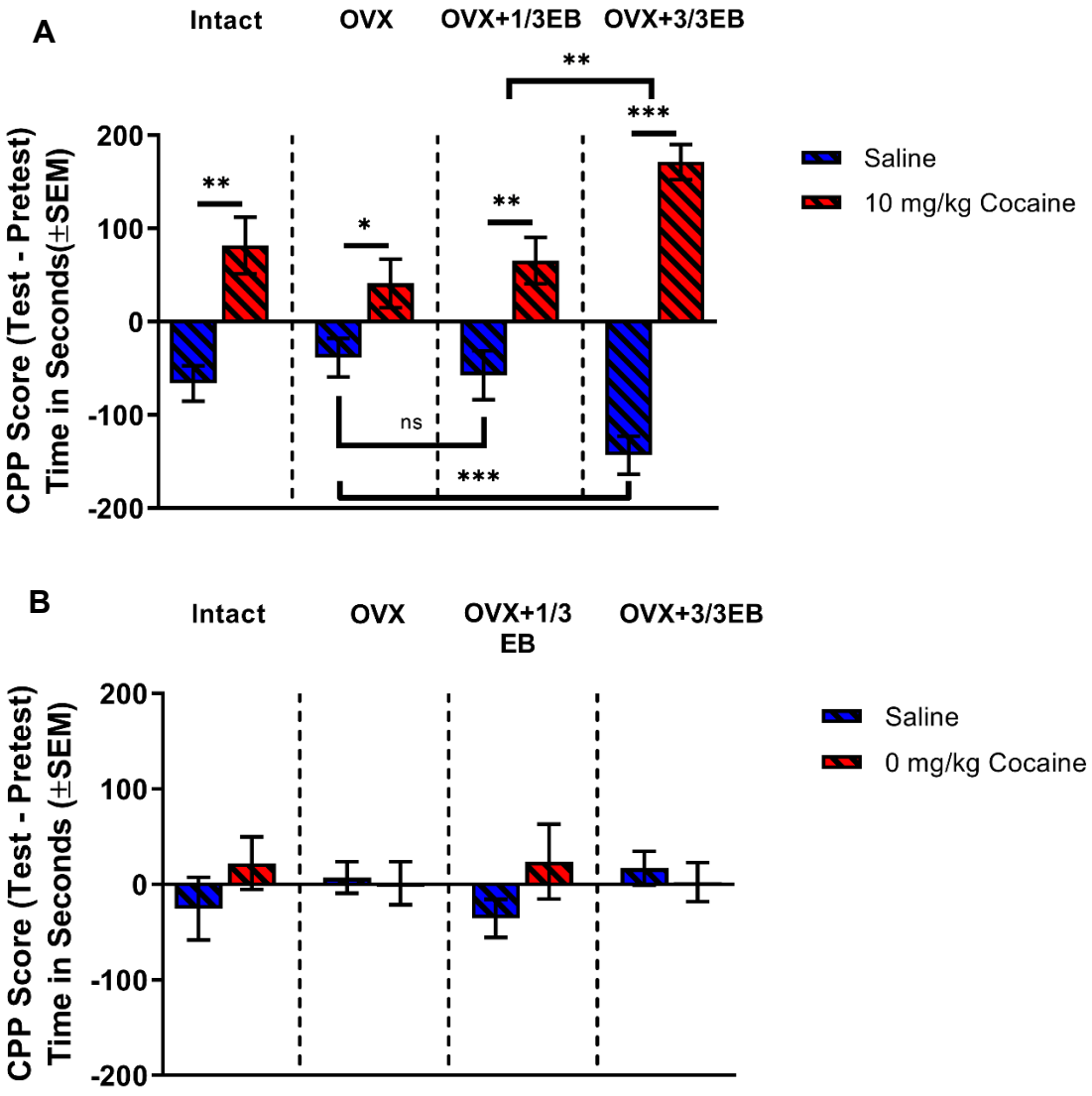


Table 1. *Mean differences in the CPP scores of OVX+3/3EB treatment condition and all other groups.*

Cocaine Dose				Mean difference	*SE*	*p*-value
0 mg/kg	Saline	OVX+3/3EB	Intact	42.47	29.762	ns
			OVX	18.971	24.901	ns
			OVX+1/3EB	52.557	32.603	ns
	Cocaine	OVX+3/3EB	Intact	-19.928	34.359	ns
			OVX	-6.121	37.638	ns
			OVX+1/3EB	-21.537	34.359	ns
10 mg/kg	Saline	OVX+3/3EB	Intact	-76.969	32.86	ns
			OVX	-112.906	27.169	0.001
			OVX+1/3EB	-85.718	29.96	0.033
	Cocaine	OVX+3/3EB	Intact	89.48	37.936	ns
			OVX	138.119	31.365	< 0.001
			OVX+1/3EB	105.798	34.587	0.018

Molecular mechanisms of estradiol-mediated sex differences in cocaine reward

3.2.2 Molecular correlates of estrogen-mediated enhanced cocaine-CPP.

Previous studies have shown that acute and chronic cocaine administration induces ERK phosphorylation, and ERK is implicated in the acquisition of cocaine-induced CPP and cocaine-associated environments (Calipari et al., 2017; Russo et al., 2003; Zakharova et al., 2009). Several published studies, conducted in male animals, have implicated mTOR activation in the VTA and NAc upon exposure to cocaine (Neasta et al., 2014). Neuroadaptations induced by repeated cocaine exposure and underlying behavioral alterations induced by cocaine require activation of both ERK1/2 and mTOR (Lu et al., 2006; Miller & Marshall, 2005; Wang et al., 2010). Taken together, these data indicate involvement of ERK1/2 and mTOR signaling pathways in cocaine addiction. However, these studies were limited by the inclusion only male animals.

Therefore, in order to assess if E2-mediated effects on cocaine conditioned reward in females were effectuated by activation of ERK1/2 and mTOR signaling pathways, we probed VTA, NAc core, NAc shell and DS tissue for phosphorylated ERK1/2 (pERK1/2) and phosphorylated mTOR (pmTOR) expression. Immediately after completion of cocaine-CPP test, animals were rapidly decapitated and tissue from VTA, NAC core, NAc shell and DS was isolated using tissue punches. Total protein was extracted from the tissue material. Western blotting was used to determine expression of pERK 1/2 and pmTOR. A total of six groups were included in this experiment: Intact females, OVX females that did not receive EB during conditioning, OVX females that received EB on all three days of conditioning and their respective control groups that received 0 mg/kg cocaine during conditioning.

To assess the effect of E2 treatment during conditioning on the expression and activation of mTOR protein, a 2 (Cocaine dose: 0 mg/kg and 10 mg/kg) X 3 (EB treatment condition: Intact females, OVX females and OVX+3/3 EB females) X 4(Repeated measures factor - Brain region: VTA, DS, NAc core and NAc shell) was conducted. Analyses revealed significant differences between and within groups for all EB treatment conditions in mTOR phosphorylation within the brain regions, $F(6, 40) = 6.350$, $p < 0.001$, ${}_p\eta^2 = 0.488$. There was also a significant interaction between brain region, cocaine dose and EB treatment condition, $F(6, 40) = 6.512$, $p < 0.001$, ${}_p\eta^2 = 0.494$. Post-hoc comparisons employing Bonferroni correction were conducted to assess individual group differences in mTOR activity. Females treated with EB during conditioning had significantly greater activity of mTOR in the VTA and NAc core compared to control females, intact females and OVX females. OVX females that did not receive EB during conditioning had significantly lower levels of mTOR activity in the VTA, NAc core and NAc shell compared

to EB treated females and intact females (See **Figure 6** and ***Table 2***). Taken together, this demonstrated that elimination of endogenous E2 lowered the activity of mTOR in the VTA, NAc core and NAc shell. EB treatment during conditioning enhanced the activity of mTOR in the VTA and NAc core. Additionally, these effects on mTOR activity were correlated to differences in after EB treatment cocaine-CPP. Furthermore, there were no significant effects on mTOR activity within the VTA, DS, NAc core or NAc shell of the control animals. This indicated that EB does not affect mTOR activity independently. From these data, it can be inferred that EB-mediated enhanced cocaine-CPP maybe occurring via the activation of mTOR in the VTA and NAc core.

To assess the effect of E2 treatment during conditioning on the expression and activation of ERK1 protein, a 2 (Cocaine dose: 0 mg/kg and 10 mg/kg) X 3 (EB treatment condition: Intact females, OVX females and OVX+3/3 EB females) X 4(Repeated measures factor - Brain region: VTA, DS, NAc core and NAc shell) was conducted. Analyses revealed significant differences in ERK1 activity between and within groups for all EB treatment conditions across the brain regions, $F(6, 34) = 9.813$, $p < 0.001$, ${}_p\eta^2 = 0.634$. A significant effect of cocaine dose on ERK1 activity across different brain regions was also observed, $F(3, 17) = 13.711$, $p < 0.001$, ${}_p\eta^2 = 0.708$. Additionally, there was also a significant interaction effect on ERK1 activity between brain region, cocaine dose and EB treatment condition, $F(6, 34) = 7.850$, $p < 0.001$, ${}_p\eta^2 = 0.581$. Post-hoc comparisons employing Bonferroni correction were conducted to assess individual group differences in ERK1 activity. Females treated with EB during conditioning had significantly greater activity of ERK1 in the VTA, DS and NAc shell compared to control females, intact females and OVX females. OVX females that did not receive EB during conditioning had significantly lower levels of ERK1 activity in the VTA, DS, NAc core and NAc shell compared to EB-treated females and intact females (See **Figure 7** and **Table 3**). Taken together, this demonstrated that elimination of endogenous E2 lowered the activity of ERK1 in the VTA, DS, NAc core and NAc shell. EB treatment during conditioning enhanced the activity of ERK1 in the VTA, DS and NAc shell. Furthermore, the increase in ERK1 activity after EB treatment was significantly greater than that seen in intact females. Additionally, these effects on ERK1 activity were correlated to differences in cocaine-CPP after EB treatment. Furthermore, there were no significant effects on ERK1 activity within the VTA, DS, NAc core or NAc shell of the control animals. This indicated that EB does not affect ERK1 activity independently. From these data, it can be inferred that EB-mediated enhanced cocaine-CPP maybe occurring via the activation of ERK1 in the VTA, DS and NAc shell.

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To assess the effect of E2 treatment during conditioning on the expression and activation of ERK2 protein, a 2 (Cocaine dose: 0 mg/kg and 10 mg/kg) X 3 (EB treatment condition: Intact females, OVX females and OVX+3/3 EB females) X 4(Repeated measures factor - Brain region: VTA, DS, NAc core and NAc shell) was conducted. Analyses revealed significant differences in ERK2 activity between and within groups for all EB treatment conditions across the brain regions, $F(6, 36) = 12.103$, $p < 0.001$, ${}_p\eta^2 = 0.669$. A significant effect of cocaine dose on ERK2 activity across different brain regions was also observed, $F(3, 17) = 93.260$, $p < 0.001$, ${}_p\eta^2 = 0.943$. Additionally, there was also a significant interaction effect on ERK2 activity between brain region, cocaine dose and EB treatment condition, $F(6, 36) = 5.739$, $p < 0.001$, ${}_p\eta^2 = 0.489$. Post-hoc comparisons employing Bonferroni correction were conducted to assess individual group differences in ERK2 activity. Females treated with EB during conditioning had significantly greater activity of ERK2 in the VTA, DS and NAc core and NAc shell compared to control females, intact females and OVX females. OVX females that did not receive EB during conditioning had significantly lower levels of ERK1 activity in the VTA, DS, NAc core and NAc shell compared to EB-treated females. However, they only had significantly lower levels of ERK2 activity in DS compared to intact females (See **Figure 7** and **Table 4**). Taken together, this demonstrated that elimination of endogenous E2 lowered the activity of ERK2 in the DS. EB treatment during conditioning enhanced the activity of ERK2 in the VTA, DS and NAc core and NAc shell. Furthermore, the increase in ERK2 activity across all brain regions after EB treatment was significantly greater than that seen in intact females. These effects on ERK2 activity were related to differences in cocaine-CPP after EB treatment. Furthermore, there were no significant effects on ERK2 activity within the VTA, DS, NAc core or NAc shell of the control animals. This indicated that EB does not affect ERK2 activity independently. From these data, it can be inferred that EB-mediated enhanced cocaine-CPP maybe occurring via the activation of ERK2 in the VTA, DS, NAc core and NAc shell.

These findings demonstrated that E2-mediated effects on cocaine conditioned reward in female animals employed ERK1/2 and mTOR signaling pathways. Moreover, the activity of mTOR was greater in the VTA and NAc core of OVX females that were treated with EB during conditioning compared to intact and OVX females. ERK1/2 activity was greater in OVX females treated with EB during conditioning than intact and OVX females in all brain regions. Furthermore, lack of endogenous E2 inhibited the activity of both mTOR and ERK1/2. This indicated that both, E2-mediated enhancement of cocaine-conditioned reward and decrease in cocaine conditioned reward due

to lack of endogenous E2 involve both ERK1/2 and mTOR signaling. Since these effects of E2 on mTOR and ERK1/2 signaling were correlative, we decided to experimentally assess the involvement of mTOR and ERK1/2 signaling in E2-mediated enhanced cocaine-conditioned reward by systemically inhibiting their activity.

Table 2. *Mean and SEM of mTOR activity in different areas of the brain across different EB treatment conditions.*

Cocaine Dose	Treatment	Brain Region	Mean	Std. Error
0 mg/kg	Intact	VTA	105.840	15.622
		DS	33.446	14.367
		NAc Core	14.980	23.916
		NAc Shell	8.270	8.498
	OVX	VTA	59.242	15.622
		DS	69.797	14.367
		NAc Core	74.528	23.916
		NAc Shell	31.130	8.498
	OVX+3/3EB	VTA	18.995	15.622
		DS	29.496	14.367
		NAc Core	39.058	23.916
		NAc Shell	25.295	8.498
10 mg/kg	Intact	VTA	115.445	12.755
		DS	61.701	11.731
		NAc Core	44.937	19.527
		NAc Shell	80.936	6.939
	OVX	VTA	53.871	13.972
		DS	75.611	12.851
		NAc Core	22.047	21.391
		NAc Shell	65.952	7.601
	OVX+3/3EB	VTA	147.071	13.972
		DS	61.380	12.851
		NAc Core	196.724	21.391
		NAc Shell	38.106	7.601

Figure 6. *Changes in mTOR activity across different EB treatment conditions.*

*mTOR activity changed across different EB treatment conditions. Individual plots represent mTOR activity in different areas of the mesolimbic reward pathway. Individual bars represent the percent change in pmTOR expression compared to mTOR expression between control (patterned light orange bars) and experimental (patterned dark orange bars) conditions.***p < 0.001, **p < 0.01.*

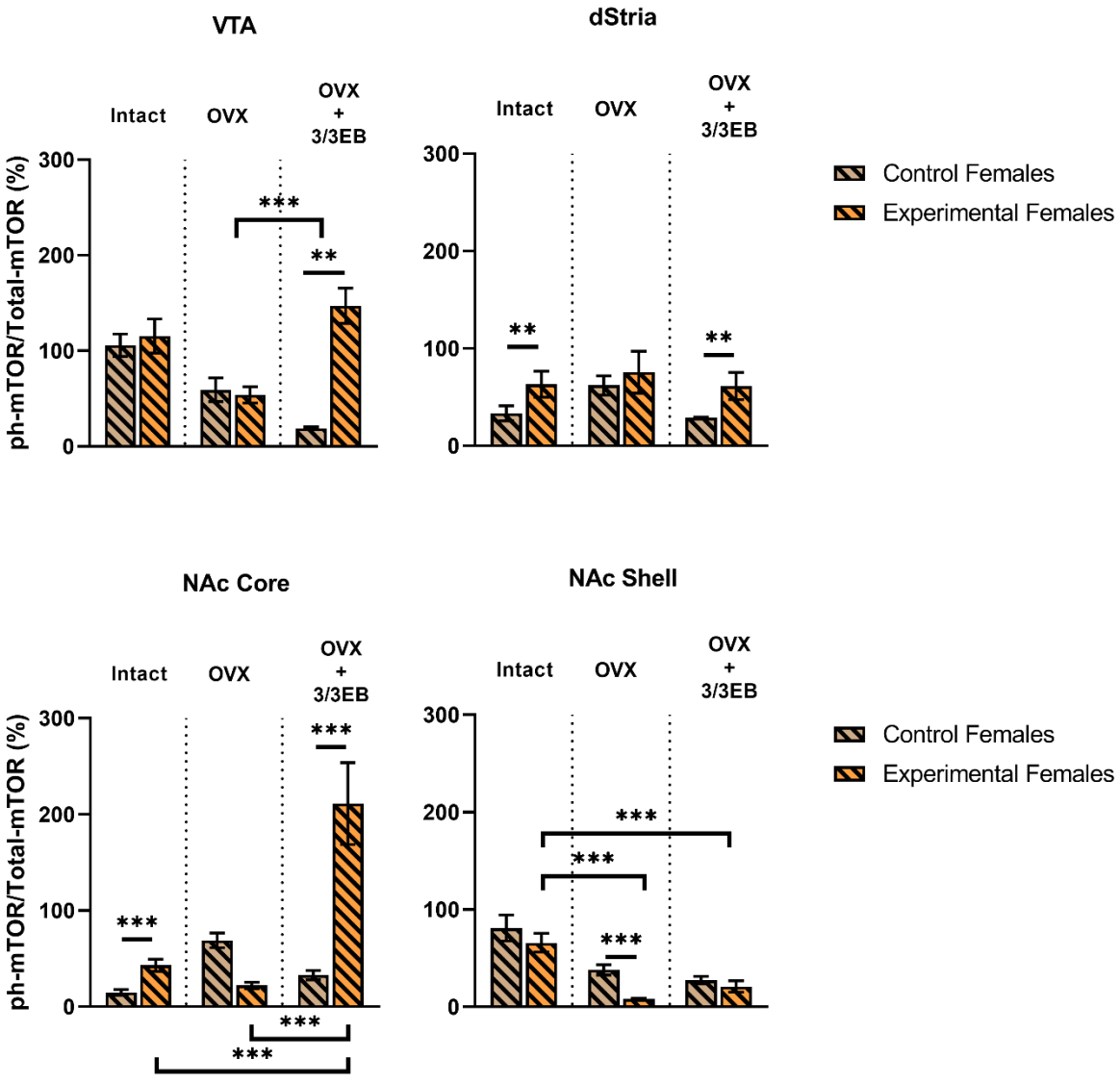


Table 3. *Mean and SEM of ERK1 activity in different areas of the brain across different EB treatment conditions.*

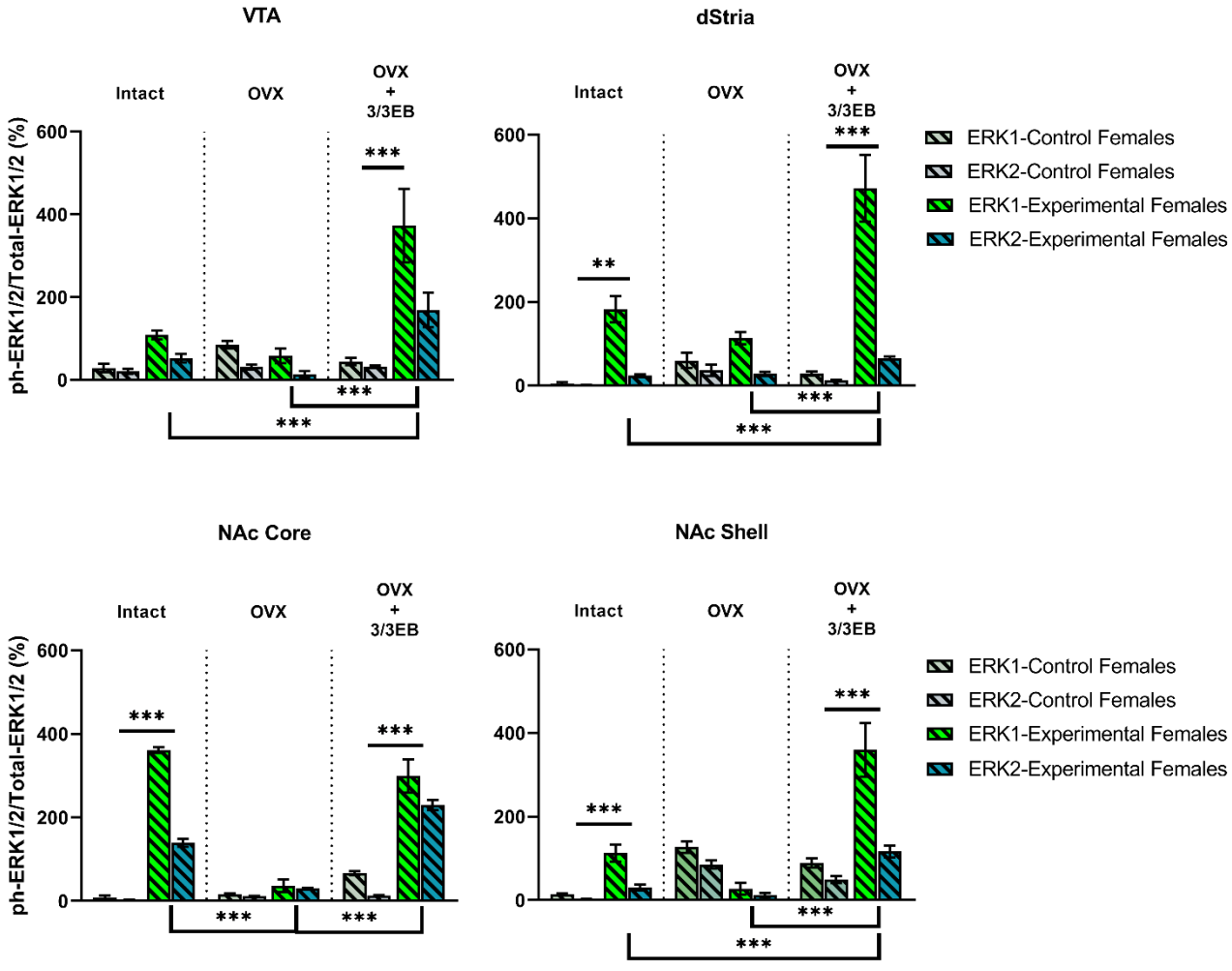
Cocaine Dose	Treatment	Brain Region	Mean	Std. Error
0 mg/kg	Intact	VTA	28.877	41.658
		DS	4.363	39.019
		NAc Core	8.880	19.155
		NAc Shell	14.019	31.361
	OVX	VTA	85.383	46.575
		DS	59.987	43.625
		NAc Core	16.291	21.416
		NAc Shell	126.727	35.063
	OVX+3/3EB	VTA	44.371	46.575
		DS	28.619	43.625
		NAc Core	66.144	21.416
		NAc Shell	88.901	35.063
10 mg/kg	Intact	VTA	108.779	46.575
		DS	182.669	43.625
		NAc Core	361.353	21.416
		NAc Shell	112.808	35.063
	OVX	VTA	58.285	53.780
		DS	113.179	50.374
		NAc Core	36.564	24.729
		NAc Shell	27.368	40.487
	OVX+3/3EB	VTA	372.715	41.658
		DS	472.127	39.019
		NAc Core	299.151	19.155
		NAc Shell	359.626	31.361

Table 4. *Mean and SEM of ERK2 activity in different areas of the brain across different EB treatment conditions.*

Cocaine Dose	Treatment	Brain Region	Mean	Std. Error
0 mg/kg	Intact	VTA	20.709	14.070
		DS	0.694	6.064
		NAc Core	2.709	6.896
		NAc Shell	3.384	8.587
	OVX	VTA	31.451	14.070
		DS	36.322	6.064
		NAc Core	11.179	6.896
		NAc Shell	85.261	8.587
	OVX+3/3EB	VTA	31.963	12.585
		DS	12.232	5.423
		NAc Core	13.023	6.168
		NAc Shell	49.400	7.681
10 mg/kg	Intact	VTA	52.544	11.488
		DS	23.510	4.951
		NAc Core	139.203	5.630
		NAc Shell	29.996	7.011
	OVX	VTA	13.939	16.247
		DS	27.845	7.002
		NAc Core	29.660	7.962
		NAc Shell	9.393	9.916
	OVX+3/3EB	VTA	169.463	16.247
		DS	65.282	7.002
		NAc Core	229.995	7.962
		NAc Shell	116.579	9.916

Figure 7. *Changes in ERK1/2 activity across different EB treatment conditions.*

*ERK1/2 activity changed across different EB treatment conditions. ERK1/2 activity was greatest in the OVX females treated with E2 during conditioning. Individual plots represent ERK1/2 activity in different areas of the mesolimbic reward pathway. Individual bars represent the percent change in pERK1 and ERK2 expression compared to ERK1 and 2 expression respectively. Patterned light green and blue bars represent ERK1 and ERK2 activity respectively in females belonging to the control condition. Patterned dark green and blue bars represent ERK1 and ERK2 activity respectively in females belonging to the experimental condition.***p < 0.001, **p < 0.01.*



3.3 Experiment 3: Effect of inhibiting ERK1/2 and mTOR activation on E2-mediated enhanced cocaine-conditioned reward.

In these experiments we tested the hypothesis that E2 influences cocaine-induced alterations in activity of ERK1/2 and mTOR signaling pathways in the VTA, DS, and NAc during formation of cocaine-context associations, which ultimately affects the acquisition of cocaine conditioned reward In order to establish a causal relationship between cocaine-conditioned reward, E2 and ERK1/2 and mTOR signaling pathways, we systemically administered inhibitors of ERK1/2 and mTOR phosphorylation, individually and in combination, to systematically delineate the involvement of these signaling pathways in E2-mediated potentiation of cocaine-conditioned reward.

3.3.1 ERK1/2 inactivation negated E2-mediated cocaine-conditioned reward in females.

To test the hypothesis that systemic inhibition of ERK1/2 prior to cocaine-conditioning will inhibit E2-mediated enhanced cocaine conditioned reward, a group of OVX females was injected with 25 mg/kg of SL327 and 5 µg of EB 1 h and 30 min prior to each cocaine conditioning session respectively. A control group of OVX females that did not receive SL327 pretreatment was included. All animals received 10 mg/kg cocaine during cocaine conditioning. Cocaine-CPP scores were used to assess behavioral effects of inhibition of ERK1/2 phosphorylation by SL327.

A 4 (Inhibitor pretreatment: Vehicle, OVX+3/3EB+SL327, OVX+3/3EB+Rapamycin, OVX+3/3EB+SL327+Rapamycin) X 2(Repeated measures factor – CPP score: Saline CPP score, Cocaine CPP score) two-way mixed ANOVA was conducted to assess the effect of inhibitor pretreatment on cocaine-CPP. These statistical analyses revealed significant differences between and within the groups for SL327 pretreatment treatment and CPP score. Specifically, inhibitor pretreatment had significant effect on cocaine-CPP score, $F(3, 16) = 4.599$, $p = 0.017$, ${}_p\eta^2 = 0.463$. Post-hoc comparisons employing Bonferroni correction for CPP score demonstrated that SL327 pretreated OVX+3/3EB female animals spent significantly less time ($M = 219.557$, $SE = 44.467$, $p < 0.001$) in cocaine-paired compartment during test than vehicle pretreated OVX+3/3EB female animals ($M = 58.286$, $SE = 48.711$, $p = 0.249$). This demonstrated that while vehicle pretreated female animals developed cocaine-CPP, SL327 pretreatment failed to produce cocaine-CPP. Therefore, blocking ERK1/2 activation prior to cocaine-conditioning in animals administered with E2 during conditioning blocked the acquisition of cocaine-CPP. This indicated that

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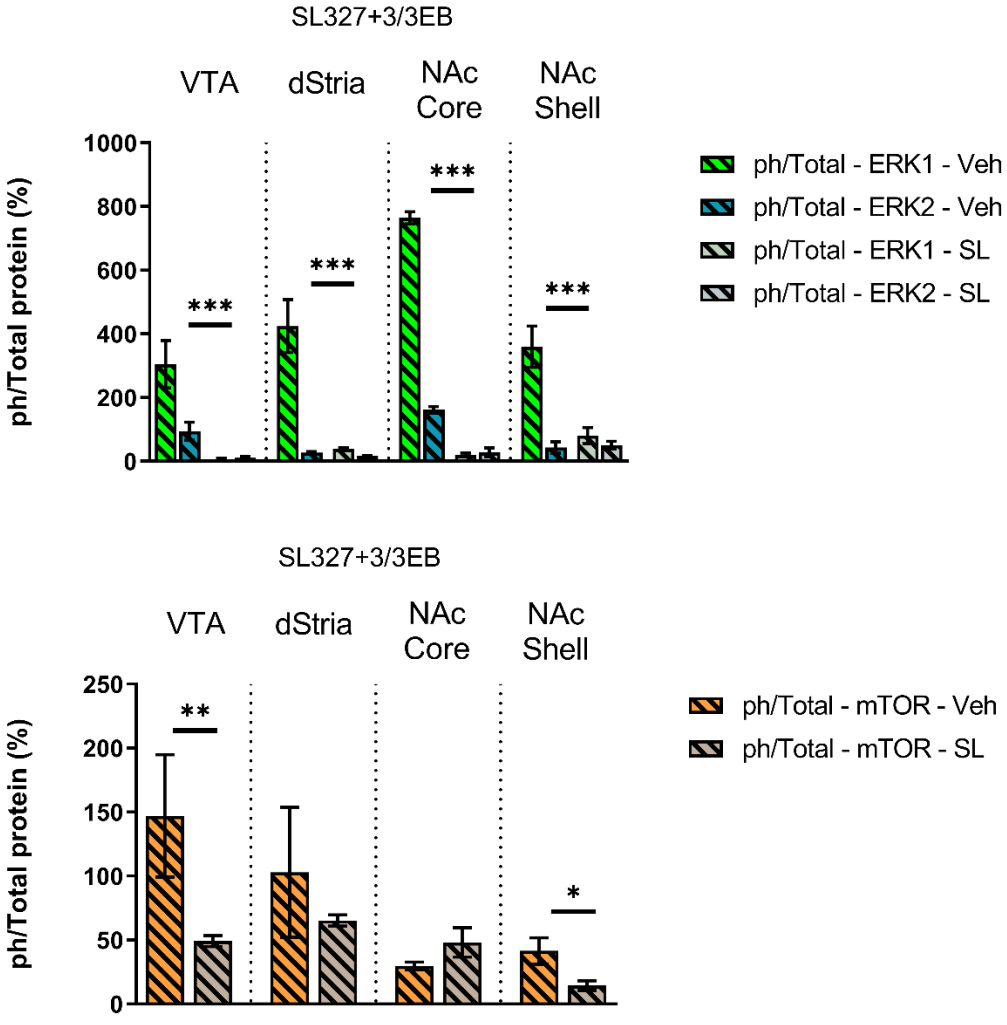
ERK1/2 activation is necessary during cocaine-conditioning for E2-mediated enhanced acquisition of cocaine-CPP in female animals (See **Figure 11.**).

After completion of cocaine-CPP test, inhibition of ERK1/2 phosphorylation by SL327 at the protein level was assessed by probing pERK1/2 protein expression in the VTA, DS, NAc core and NAc shell. Furthermore, to determine if activation of ERK1/2 is necessary for mTOR activation, pmTOR protein expression was also probed in this group of animals. A 4 (Inhibitor treatment condition: Vehicle, SL327, Rapamycin and SL327+Rapamycin) X 4 (Repeated measures factor – Brain region: VTA, DS, NAc core and NAc shell) two-way repeated measure ANOVA revealed significant between and within groups differences on ERK1 activation, $F(9, 36) = 2.444$, $p = 0.028$, ${}_p\eta^2 = 0.379$. Post-hoc comparisons employing Bonferroni correction demonstrated that ERK 1 activity was significantly lower in the VTA, DS, NAc core and NAc shell compared to vehicle treated female animals (See **Figure 8** and **Table 5**). This indicated that SL327 successfully inhibited ERK1 activity in VTA, DS, NAc core and NAc shell. Additionally, it also demonstrated that inhibition of ERK1 activity during cocaine-conditioning was responsible for the inhibition of E2-mediated enhanced cocaine-conditioned reward. A 4 (Inhibitor treatment condition: Vehicle, SL327, Rapamycin and SL327+Rapamycin) X 4 (Repeated measures factor – Brain region: VTA, DS, NAc core and NAc shell) two-way repeated measure ANOVA revealed significant between and within groups differences on ERK2 activation, $F(9, 39) = 3.881$, $p = 0.001$, ${}_p\eta^2 = 0.472$. Post-hoc comparisons employing Bonferroni correction demonstrated that ERK 2 activity was significantly lower in the NAc core compared to vehicle treated female animals. This indicated that SL327 only inhibited ERK2 activity in NAc core (See **Figure 8** and **Table 6**). Additionally, it also demonstrated that inhibition of ERK2 activity in the NAc core during cocaine-conditioning was responsible for the inhibition of E2-mediated enhanced cocaine-conditioned reward.

We also probed for pmTOR expression and activation in animals that were treated with SL327 during conditioning to assess the possibility of ERK1/2 inhibition affecting mTOR activity. A 4 (Inhibitor treatment condition: Vehicle, SL327, Rapamycin and SL327+Rapamycin) X 4 (Repeated measures factor – Brain region: VTA, DS, NAc core and NAc shell) two-way repeated measure ANOVA revealed marginal significant differences between and within groups on mTOR activation, $F(9, 39) = 2.141$, $p = 0.049$, ${}_p\eta^2 = 0.331$. Post-hoc comparisons employing Bonferroni correction demonstrated that mTOR activity was significantly lower in the VTA and the NAc shell compared to vehicle treated female animals (See **Figure 8** and **Table 7**). This indicated that SL327 inhibited mTOR

activity in VTA and NAc shell. Additionally, it also demonstrated that inhibition of mTOR activity in the VTA and NAc shell during cocaine-conditioning maybe associated with inhibition of E2-mediated enhanced cocaine-conditioned reward.

Figure 8. *Changes in activity of ERK1/2 and mTOR caused by SL327 pretreatment during cocaine conditioning. Individual plots represent the decrease in activity of ERK1/2 and mTOR in different brain areas of the mesolimbic reward pathway of OVX females pretreated with SL327 during cocaine conditioning. Individual bars represent the percent change in pERK1, pERK2 and pmTOR expression compared to ERK1, ERK 2 and mTOR expression respectively. Patterned light green, light blue and light orange bars represent ERK1, ERK2 and mTOR activity respectively in females pretreated with SL327. Patterned dark green, dark blue and dark orange bars represent ERK1, ERK2, and mTOR activity respectively in females pretreated with vehicle control.***p < 0.001, **p < 0.01, *p < 0.05.*



3.3.2 Inhibition of mTOR activity had no effect on E2-mediated cocaine-conditioned reward in females.

Our previous data demonstrated that pmTOR expression was increased in the VTA, NAc core and NAc shell of OVX females that were treated with EB during conditioning, indicative of the activation of mTOR signaling pathway in E2-mediated enhanced cocaine conditioned reward. We hypothesized that while mTOR activation is necessary for expression of cocaine-CPP in males (Bailey et al., 2012), in females it was involved in EB-mediated strengthening of cocaine-context associations during cocaine conditioning. Therefore, to test this hypothesis, a group of OVX females was systemically injected with 5 mg/kg of rapamycin and 5 µg of EB 1 h and 30 min prior to each cocaine conditioning session respectively. Rapamycin is a specific allosteric-inhibitor of mTOR and binds to it to induce a conformational change resulting in a non-functional mTOR (Neasta et al., 2014). A control group of OVX females that did not receive Rapamycin pretreatment was included. All animals received 10 mg/kg cocaine during cocaine conditioning. Cocaine-CPP scores were used to assess behavioral effects of inhibition of ERK1/2 phosphorylation by SL327.

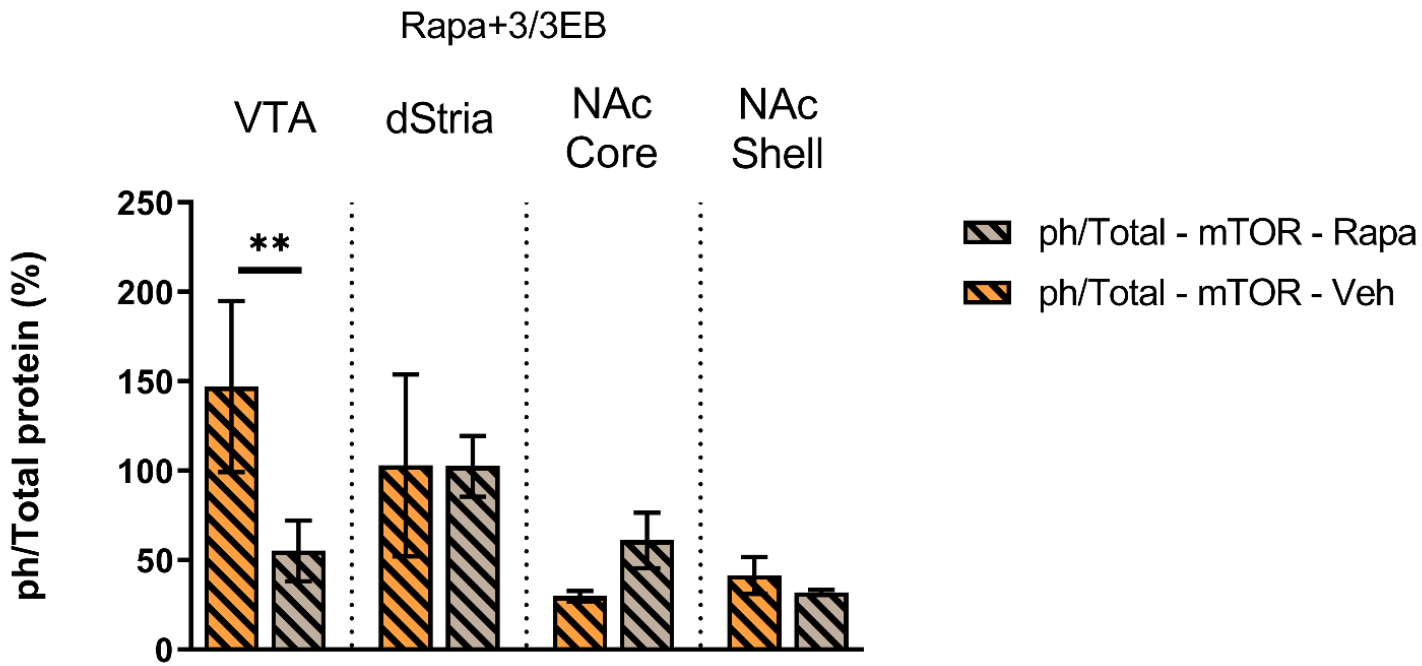
A 4 (Inhibitor pretreatment: Vehicle, OVX+3/3EB+SL327, OVX+3/3EB+Rapamycin, OVX+3/3EB+SL327+Rapamycin) X 2(Repeated measures factor – CPP score: Saline CPP score, Cocaine CPP score) two-way mixed ANOVA was conducted to assess the effect of inhibitor pretreatment on cocaine-CPP. These statistical analyses revealed significant differences between and within the groups for Rapamycin pretreatment treatment and CPP score. Specifically, inhibitor pretreatment had significant effect on cocaine-CPP score, $F(3, 16) = 4.599$, $p = 0.017$, ${}_p\eta^2 = 0.463$. However, post-hoc comparisons employing Bonferroni correction for CPP score demonstrated that Rapamycin pretreated OVX+3/3EB female animals did not spend significantly less time ($M = 285.752$, $SE = 48.711$, $p < 0.001$) in cocaine-paired compartment during test than vehicle pretreated OVX+3/3EB female animals ($M = 219.557$, $SE = 44.467$, $p < 0.001$). This demonstrated that Rapamycin pretreatment was not able to block cocaine-CPP. Therefore, blocking mTOR activation prior to cocaine-conditioning in females administered with E2 during conditioning did not affect the acquisition of cocaine-CPP. This indicated that mTOR activation was not necessary during cocaine-conditioning for E2-mediated enhanced acquisition of cocaine-CPP in female animals (See **Figure 11**).

Inhibiting mTOR activation did not block E2-mediated enhanced cocaine-conditioned reward. To assess the efficacy of rapamycin at a molecular level, we probed expression and activation of pmTOR in the VTA, DS, NAc

core and NAc shell in OVX females treated with rapamycin and EB during cocaine-conditioning. A 4 (Inhibitor treatment condition: Vehicle, SL327, Rapamycin and SL327+Rapamycin) X 4 (Repeated measures factor – Brain region: VTA, DS, NAc core and NAc shell) two-way repeated measure ANOVA revealed marginal significant differences between and within groups on mTOR activation, $F(9, 39) = 2.141$, $p = 0.049$, ${}_p\eta^2 = 0.331$. Post-hoc comparisons employing Bonferroni correction demonstrated that mTOR activity was significantly lower in the VTA alone. This indicated that rapamycin inhibited mTOR activity in VTA but not in the DS, NAC core or NAc shell (See **Figure 9** and **Table 7**). Additionally, it also demonstrated that inhibition of mTOR activity in the VTA but not the DS, NAc core or NAc shell during cocaine-conditioning is not associated with inhibition of E2-mediated enhanced cocaine-conditioned reward.

Figure 9. *Changes in activity of mTOR caused by Rapamycin pretreatment during cocaine conditioning.*

*OVX females pretreated with rapamycin during cocaine conditioning only showed decreased mTOR activity in the VTA. Individual bars represent the percent change in pmTOR expression compared to mTOR expression. Patterned light orange bars represent mTOR activity in females pretreated with SL327. Patterned dark orange bars represent mTOR activity in females pretreated with vehicle control.**p < 0.01.*



3.3.3 Combined inhibition of both ERK1/2 and mTOR activation inhibited E2-mediated cocaine conditioned reward in females.

Several studies suggest that ERK1/2 activation maybe necessary for cocaine-mediated induction of mTOR signaling (Neasta et al., 2014; Ucha et al., 2020). Cocaine-associated neuroadaptations in the various areas of mesolimbic reward pathway require the activation of both ERK1/2 and mTOR (Neasta et al., 2014). Additionally, there is concomitant increase in the activation of both ERK1/2 and mTOR after exposure to a cocaine-paired cues (Neasta et al., 2014; Ucha et al., 2020). Furthermore, there exists a strong cross-talk between the ERK1/2 and mTOR signaling pathways (Mendoza et al., 2011). In order to determine if E2-mediated potentiation of the cocaine conditioned reward also required the combined activation of ERK1/2 and mTOR in females, we systemically administer both SL327 (25 mg/kg) and rapamycin (5 mg/kg), and 5 µg of EB 1 h and 30 min prior to each cocaine conditioning session respectively to OVX females. A control group of OVX females that did not receive SL327 pretreatment was included. All animals received 10 mg/kg cocaine during cocaine conditioning. Cocaine-CPP scores were used to assess behavioral effects of the combined inhibition of ERK1/2 and mTOR phosphorylation by SL327 and Rapamycin.

A 4 (Inhibitor pretreatment: Vehicle, OVX+3/3EB+SL327, OVX+3/3EB+Rapamycin, OVX+3/3EB+SL327+Rapamycin) X 2(Repeated measures factor – CPP score: Saline CPP score, Cocaine CPP score) two-way mixed ANOVA was conducted to assess the effect of inhibitor pretreatment on cocaine-CPP. These statistical analyses revealed significant differences between and within the groups for SL327+Rapamycin pretreatment treatment and CPP score. Specifically, inhibitor pretreatment had significant effect on cocaine-CPP score, $F(3, 16) = 4.599$, $p = 0.017$, ${}_p\eta^2 = 0.463$. Post-hoc comparisons employing Bonferroni correction for CPP score demonstrated that SL327+Rapamycin pretreated OVX+3/3EB female animals spent significantly less time ($M = 99.963$, $SE = 54.461$, $p = 0.085$) in cocaine-paired compartment during test than vehicle pretreated OVX+3/3EB female animals ($M = 219.557$, $SE = 44.467$, $p < 0.001$). This demonstrated that while vehicle pretreated female animals developed cocaine-CPP, SL327+Rapamycin pretreatment failed to produce cocaine-CPP. Therefore, blocking ERK1/2 and mTOR activation prior to cocaine-conditioning in animals administered with E2 during conditioning blocked the acquisition of cocaine-CPP. Considering this data in combination with previously presented effects of the individual inhibition of ERK1/2 and mTOR activation, indicated that this effect may be due

to the inhibition of ERK1/2 activation alone. However, data from these animals does indeed corroborate further the importance of ERK1/2 activation during cocaine-conditioning for E2-mediated enhanced acquisition of cocaine-CPP. In female animals (See **Figure 11**).

To assess the effect of the inhibitor on the phosphorylation levels of ERK1/2 and mTOR, we analyzed the phosphorylated protein expression levels. Post completion of cocaine-CPP test, animals were rapidly decapitated and brain tissue from the VTA, DS, NAc core and NAC shell was isolated. Protein extracted from these brain regions was assessed for relative pERK1/2 and pmTOR expression. A 4 (Inhibitor treatment condition: Vehicle, SL327, Rapamycin and SL327+Rapamycin) X 4 (Repeated measures factor – Brain region: VTA, DS, NAc core and NAc shell) two-way repeated measure ANOVA revealed significant between and within groups differences on ERK1 activation, $F(9, 36) = 2.444$, $p = 0.028$, ${}_p\eta^2 = 0.379$. Post-hoc comparisons employing Bonferroni correction demonstrated that ERK 1 activity was significantly lower in the VTA, DS, NAc core and NAc shell compared to vehicle treated female animals (See **Figure 10** and **Table 5**). This indicated that SL327+rapamycin successfully inhibited ERK1 activity in VTA, DS, NAc core and NAc shell. Additionally, it also demonstrated that inhibition of ERK1 activity during cocaine-conditioning was responsible for the inhibition of E2-mediated enhanced cocaine-conditioned reward in these animals. A 4 (Inhibitor treatment condition: Vehicle, SL327, Rapamycin and SL327+Rapamycin) X 4 (Repeated measures factor – Brain region: VTA, DS, NAc core and NAc shell) two-way repeated measure ANOVA revealed significant between and within groups differences on ERK2 activation, $F(9, 39) = 3.881$, $p = 0.001$, ${}_p\eta^2 = 0.472$. Post-hoc comparisons employing Bonferroni correction demonstrated that ERK 2 activity was significantly lower in the NAc core compared to vehicle treated female animals. This indicated that SL327+rapamycin only inhibited ERK2 activity in NAc core (See **Figure 10** and **Table 6**). Additionally, it also demonstrated that inhibition of ERK2 activity in the NAc core during cocaine-conditioning was responsible for the inhibition of E2-mediated enhanced cocaine-conditioned reward. Since these results were like those seen in animals that were treated with SL327 alone, it seems likely that they were driven by SL327 and not rapamycin.

To assess if there was a combined effect of ERK1/2 and mTOR inhibition, a 4 (Inhibitor treatment condition: Vehicle, SL327, Rapamycin and SL327+Rapamycin) X 4 (Repeated measures factor – Brain region: VTA, DS, NAc core and NAc shell) two-way repeated measure ANOVA was conducted. Analyses revealed marginal significant differences between and within groups on mTOR activation, $F(9, 39) = 2.141$, $p = 0.049$, ${}_p\eta^2 = 0.331$. Post-hoc

comparisons employing Bonferroni correction demonstrated that combined treatment with SL327 and rapamycin did not affect mTOR activity in the VTA compared to vehicle treated female animals. However, mTOR activity in the NAc shell was significantly lower in this group compared to vehicle treated female animals (See **Figure 10** and **Table 7**). This indicated that SL327 and rapamycin inhibited mTOR activity in the NAc shell but not the VTA. Additionally, it also demonstrated that inhibition of mTOR activity in the NAc shell but not the VTA during cocaine-conditioning maybe associated with inhibition of E2-mediated enhanced cocaine-conditioned reward.

Figure 10. *Changes in activity of ERK1, ERK 2 and mTOR caused by combined pretreatment with SL327 and Rapamycin during cocaine conditioning.*

*Individual plots represent the decrease in activity of ERK1/2 and mTOR in different brain areas of the mesolimbic reward pathway of OVX females pretreated with a combination of SL327 and rapamycin during cocaine conditioning. Individual bars represent the percent change in pERK1, pERK2 and pmTOR expression compared to ERK1, ERK 2 and mTOR expression respectively. Patterned light green, light blue and light orange bars represent ERK1, ERK2 and mTOR activity respectively in females pretreated with SL327. Patterned dark green, dark blue and dark orange bars represent ERK1, ERK2, and mTOR activity respectively in females pretreated with vehicle control.***p < 0.001, *p < 0.05.*

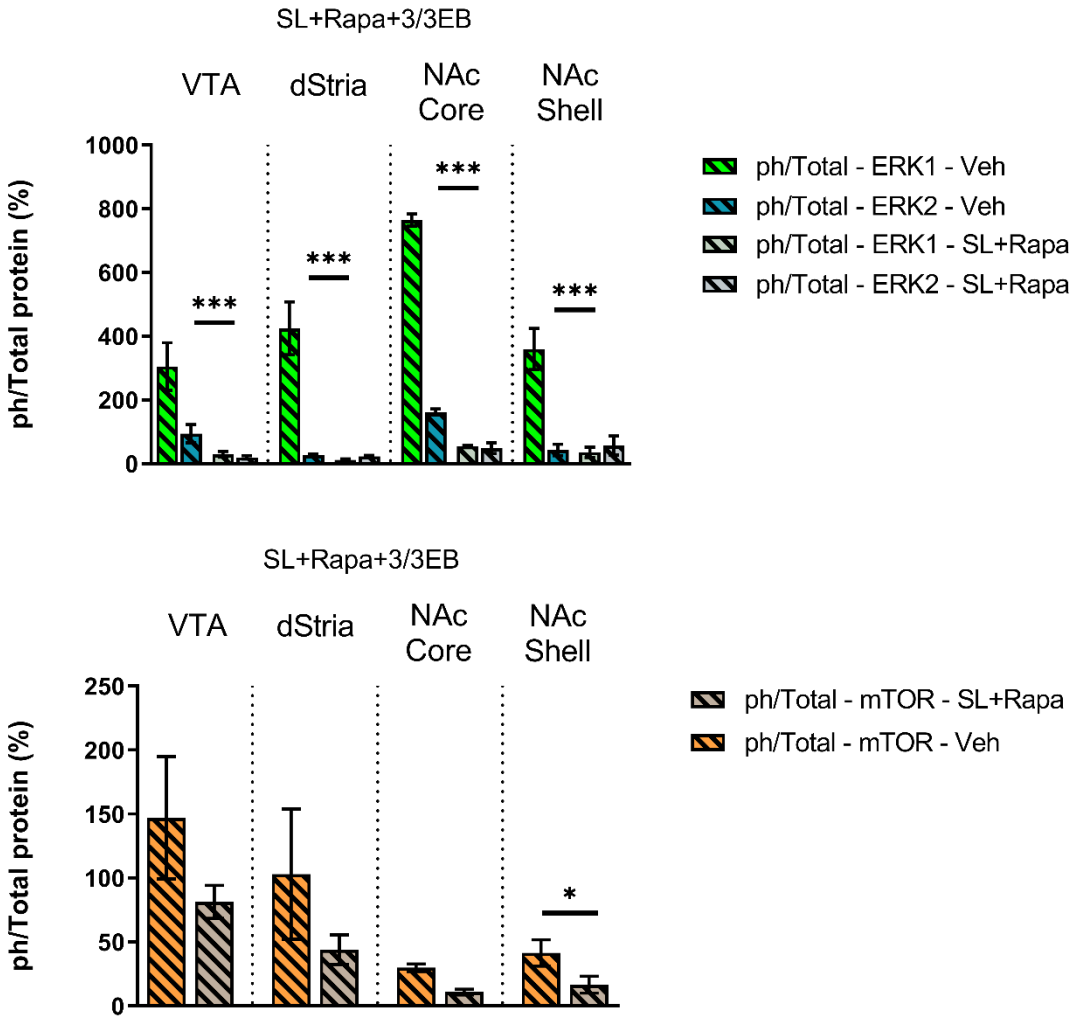


Figure 11. *Effect of inhibitor pretreatment on E2-mediated enhanced cocaine-conditioned reward.*

Blue and red bars indicate saline and cocaine CPP scores respectively. CPP scores from animals in different inhibitor pretreatment conditions are represented using different plots (Vehicle pretreated animals – Veh, SL327 pretreated animals – SL327, Rapamycin pretreated animals – Rapamycin and animals pretreated with both SL327 and rapamycin – SL327+Rapamycin). Only Veh and Rapamycin pretreated groups acquired E2-mediated cocaine-conditioned reward. SL327 and SL327+Rapamycin animals did not acquire E2-mediated cocaine-CPP. Data demonstrated that SL327 is effective in blocking E2-mediated cocaine-CPP and hence is required for the E2-mediated enhancement of cocaine-CPP.

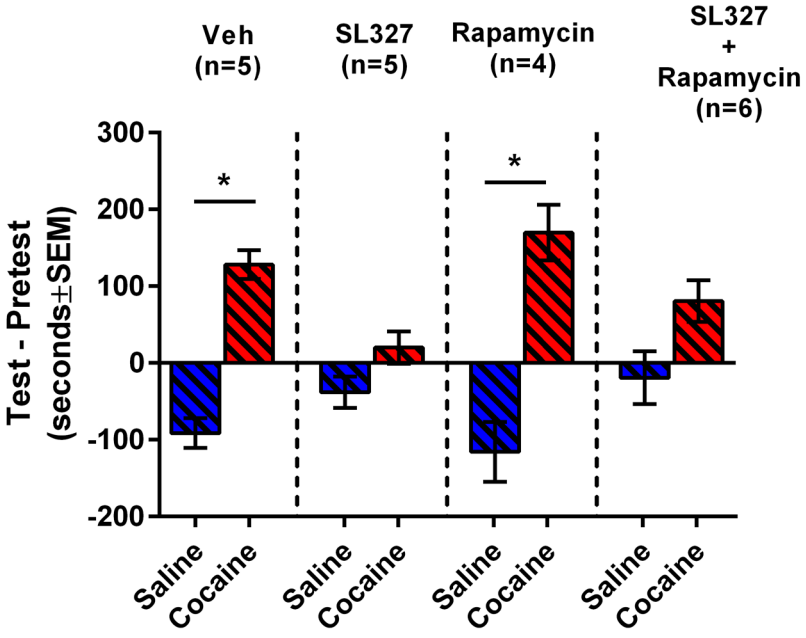


Table 5. *Mean and SEM of ERK1 activity in different areas of the brain across different inhibitor conditions.*

Treatment	Brain Region	Mean	Std. Error
Vehicle	VTA	305.250	41.372
	DS	424.838	41.514
	NAc Core	764.805	11.140
	NAc Shell	314.588	33.685
SL327	VTA	7.168	41.372
	DS	39.120	41.514
	NAc Core	20.570	11.140
	NAc Shell	80.897	33.685
Rapa	VTA	91.758	41.372
	DS	3.202	41.514
	NAc Core	51.175	11.140
	NAc Shell	71.120	33.685
SL327+Rapa	VTA	29.898	41.372
	DS	12.073	41.514
	NAc Core	55.490	11.140
	NAc Shell	35.915	33.685

Table 6. *Mean and SEM of ERK2 activity in different areas of the brain across different inhibitor conditions.*

Treatment	Brain Region	Mean	Std. Error
Vehicle	VTA	94.618	21.629
	DS	26.876	2.860
	NAc Core	161.938	14.175
	NAc Shell	43.384	21.569
SL327	VTA	11.411	21.629
	DS	17.086	2.860
	NAc Core	28.322	14.175
	NAc Shell	51.213	21.569
Rapa	VTA	128.594	21.629
	DS	1.633	2.860
	NAc Core	51.175	14.175
	NAc Shell	71.120	21.569
SL327+Rapa	VTA	19.968	19.345
	DS	22.639	2.558
	NAc Core	49.081	12.679
	NAc Shell	57.042	19.292

Table 7. *Mean and SEM of mTOR activity in different areas of the brain across different inhibitor conditions.*

Treatment	Brain Region	Mean	Std. Error
Vehicle	VTA	147.071	16.323
	DS	102.957	16.484
	NAc Core	29.916	9.388
	NAc Shell	41.504	5.111
SL327	VTA	49.205	16.323
	DS	65.279	16.484
	NAc Core	48.216	9.388
	NAc Shell	14.544	5.111
Rapa	VTA	55.255	16.323
	DS	102.573	16.484
	NAc Core	61.110	9.388
	NAc Shell	33.373	5.111
SL327+Rapa	VTA	81.387	14.600
	DS	44.099	14.744
	NAc Core	10.861	8.397
	NAc Shell	16.598	4.571

Chapter 4 General Methods

All procedures for the experiments described in the previous section were be approved by the University of Texas at Arlington Institutional Animal Care and Use Committee and in accordance with the National Institute of Health (NIH) Guide for Care and Use of Laboratory Animals.

4.1 Animals.

Adult male and female Long Evans rats were purchased from Charles River Laboratories, Houston, TX. Animals were group housed (three per cage) in the Animal Care Facility at the University of Texas at Arlington. All animals were maintained on a 12:12 reversed dark/light cycle to allow for behavior assessments to be conducted during the dark-phase of the animals' cycle. Upon arrival to our facility, rats were allowed two days of acclimation to the colony room before handling and/or any experimental procedures were started. Food and water were provided *ad libitum*. Depending upon the experiment, animals were randomly assigned to the experimental groups designated in

Table 8. *Animal treatment groups for all experiments.*

	Sex/Groups	OVX	EB pretreatment	Inhibitor pretreatment	Cocaine dose (Sample size)
Experiment 1	Males	NA	NA	NA	0 mg/kg (*n* = 10)
					5 mg/kg (*n* = 8)
					10 mg/kg (*n* = 11)
					15 mg/kg (*n* = 13)
	Females	NA	NA		0 mg/kg (*n* = 11)
					5 mg/kg (*n* = 14)
					10 mg/kg (*n* = 13)
					15 mg/kg (*n* = 8)
Experiment 2	Intact Female	NA	NA	NA	0 mg/kg (*n* = 8)
	OVX Female	OVX	NA		0 mg/kg (*n* = 16)
	OVX+1/3EB Female	OVX	Conditioning Day 1		0 mg/kg (*n* = 6)
	OVX+3/3EB Female	OVX	All 3 conditioning days		0 mg/kg (*n* = 12)
	Intact Female	NA	NA		10 mg/kg (*n* = 7)
	OVX Female	OVX	NA		10 mg/kg (*n* = 16)
	OVX+1/3EB Female	OVX	All 3 conditioning days		10 mg/kg (*n* = 10)
	OVX+3/3EB Female	OVX	All 3 conditioning days		10 mg/kg (*n* = 9)

Experiment 3	Veh	OVX	All 3 conditioning days	NA	10 mg/kg (*n* = 6)
	EB+SL327	OVX	All 3 conditioning days	SL327 (25 mg/kg i.p.)	10 mg/kg (*n* = 5)
	EB+Rapa	OVX	All 3 conditioning days	Rapamycin (5 mg/kg i.p.)	10 mg/kg (*n* = 5)
	EB+SL327+Rapa	OVX	All 3 conditioning days	SL327 (25 mg/kg i.p.)+Rapamycin (5 mg/kg i.p.)	10 mg/kg (*n* = 4)

4.2 Ovariectomization.

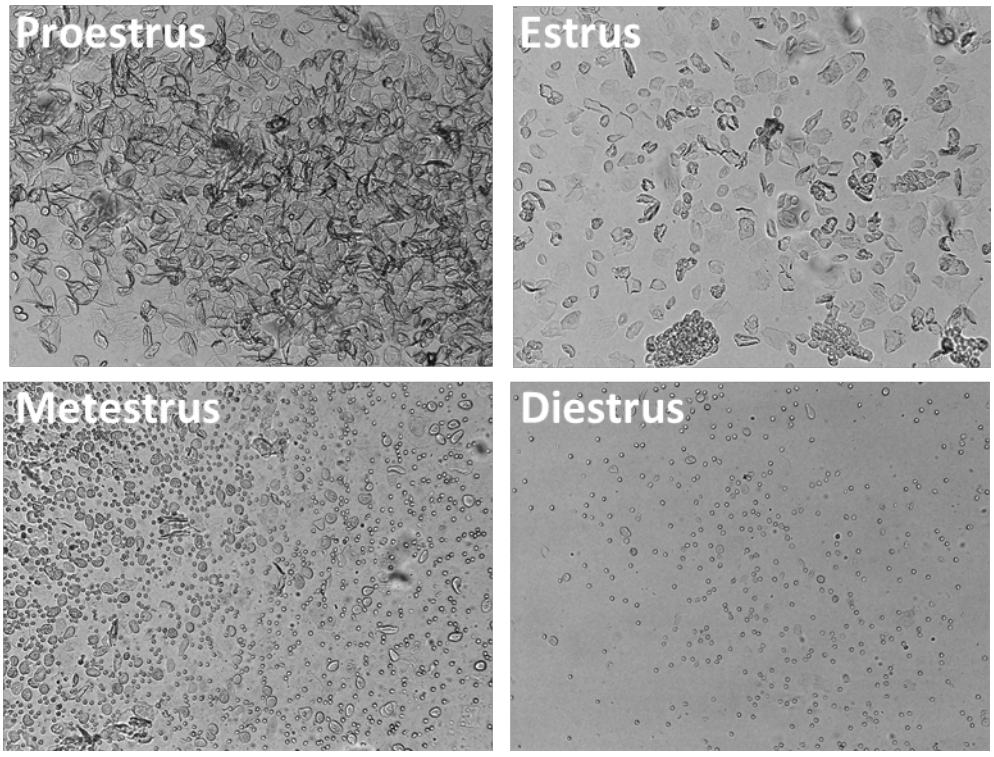
Female rats were anesthetized using 2-3% isoflurane (4% for induction) in an oxygen/nitrous oxide 30%/70% mixture. Animals were placed in prone position on a heating pad. A 4*4 cm area cephally from the iliac crest was shaved and thoroughly disinfected using iodide solution. Rats were covered with a surgery sheet. Two 2-3 cm incisions were made laterally and skin from underlying fascia was bluntly dissected. Another 1 cm incision through the fascia was made. Thereafter, superficial dissection of the fascia was done until abdominal cavity was reached. Using tweezers, the adipose tissue that surrounds the ovary in the abdominal cavity was gently pulled out. Each ovary and surrounding adipose tissue was cut and blood vessels were cauterized using a hot blade. The remaining tissue was put back into the abdominal cavity. Wounds were closed using monofilament sutures and skin was stapled back. Each animal was placed in a heated cage (25-27 °C) for at least 1 h after surgery. Thereafter, animal was returned back to its home cage. All OVX animals were allowed 3-5 days of recovery.

4.3 Determination of EC phases by vaginal lavage testing.

All female animals underwent vaginal lavage testing to track estrous cycle and/or to verify cession of cycling following OVX surgery. Lavage testing for intact females was began after the rats were acclimated to the facility and continued throughout the experiment. Lavage testing for OVX rats began 7 days after OVX surgery and continued for 14 days. The vaginal lavage procedure utilizes the cell composition within the vagina to determine estrous cycle stage. Cell composition within the proximal end of the rat's vaginal opening changes with respect to the stage of estrous cycle. Each stage of the estrous cycle differs in terms of cell composition within this area and has been well characterized to coincide with the hormonal fluctuations. **Figure 10** shows the predominant cell population for each of the four stages of the estrous cycle. Briefly: Proximal end of rat's vagina was flushed with 30-50 µL of 0.9% filtered-sterilized saline solution using a blunt tip and micropipette. Solution containing cells was then recollected and pipetted onto a glass slide. Cells were immediately visualized using Zeiss Axioskop fluorescence phase contrast microscope. Vaginal lavage images were captured using attached camera at 10X and 20X magnification. Cell counts were conducted to determine percentage of each cell type, namely leukocytes, nucleated epithelial cells and cornified epithelial cells. Prior to OVX surgery, this procedure was used to assess normal estrous

cycling of all females. Post OVX, this procedure was used to confirm the cessation of the estrous cycle. Lavages from non-cycling animals lacked nucleated or cornified epithelial cells and may show only leukocytes.

Figure 12. *Representative vaginal lavage smears showing cell composition for rodent estrous cycle stages. Figure shows representative images of vaginal lavage during proestrus, estrus, metestrus and diestrus stages of rodent EC. Proestus is characterized by the presence of cornified epithelial cells. Estrus is characterized by presence of round nucleated epithelial cells. Metestrus is characterized by presence of leukocytes along with cornified and nucleated round epithelial cells. Diestrus is characterized by presence of only leukocytes without epithelial cells.*



4.4 Drugs and administration.

Cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% saline. Intraperitoneal (i.p.) injections of cocaine were administered at a volume of 1 mL/kg, at a dose of 0, 5, 10 or 15 mg/kg. Saline (0.9% wt/vol) was injected i.p. at a volume of 1 mL/kg. A total of 5 µg of EB dissolved in peanut oil (PO) was injected

subcutaneously (s.c.) 30 minutes prior to cocaine conditioning either on conditioning day 1 or all three conditioning days. Control animals received the exact same time course and dose of EB treatment. SL327 (Selleckchem, Houston, TX) was dissolved in a solution containing 5% DMSO, 30% PEG and 10% Tween-80 to a final concentration of 25 mg/mL and injected at a dose of 25 mg/kg i.p. 1 hour prior to cocaine conditioning. The dose of SL327 selected was based on previously published studies (Valjent et al., 2000, 2004, 2005). Rapamycin (Selleckchem, Houston, TX #S1039) was dissolved in a solution containing 5% DMSO, 30% PEG and 10% Tween-80 in DI water to a final concentration of 5 mg/mL and injected at a dose of 5 mg/kg i.p. 1 hour prior to cocaine conditioning. The dose of rapamycin was selected based upon published studies demonstrating inhibition of mTOR blocked cocaine-induced locomotor sensitization in females and the expression of cocaine-CPP in males (Bailey et al., 2012; Wu et al., 2011). Control animals were injected with either 5% DMSO, 30% PEG and 10% Tween-80 in DI water and s.c. with PO to control for any effects of the injections. All drugs, hormone and saline solutions were injected at a volume of 1 mL/kg. Prior to saline conditioning, animals did not receive either hormone or inhibitor. All injections were administered prior to cocaine conditioning. All animals were drug- and hormone-free during pretest and test phases of cocaine-CPP.

4.5 Cocaine conditioned place preference.

CPP was conducted using standard procedures. The equipment used consisted of commercially manufactured, three-chamber apparati (Med Associates, Fairfax, VT). Two contextually distinct chambers (8.25″ W × 8″ H × 11.75″ L) with fifteen infrared photobeam detectors for automated data collection were used for cocaine/vehicle (0.9% saline) conditioning. These chambers were separated from one another by a smaller neutral (gray) chamber (8.25″ W × 8″ H × 4.75″ L) with three infrared photobeam detectors. All compartments were equipped with a 60 W light mounted in the ceiling. One of the larger chambers had black walls and a steel rod floor. The other large chamber had white walls and a wire mesh floor. Within the apparatus, each chamber was separated from the next by guillotine doors.

The CPP procedure comprised of 3 phases: pretest, conditioning, and test phase. To identify any preexisting chamber bias (pretest phase), rats were permitted to explore the entire apparatus for 15-mins prior to conditioning and time spent in each of the three chambers was recorded. Chambers were randomly assigned to

saline or cocaine for all following conditioning sessions. These pairings were kept constant throughout the conditioning phase. On each of the three saline conditioning days, rats received 1ml/kg i.p. injection of 0.9% saline and were confined to the saline-paired chamber for 30-mins. For each of the three cocaine-conditioning days, rats received either inhibitor (SL327 or rapamycin) or EB or 10 mg/kg cocaine hydrochloride (see Table 2). Some OVX female rats received either 25 mg/kg SL327 i.p., 5 mg/kg rapamycin i.p. or both 25 mg/kg SL327 i.p. and 5 mg/kg rapamycin i.p. administered consecutively and 5 µg EB 1 h and 30 mins prior to cocaine administration and placed in the drug-paired chamber for 30-mins. Control groups followed the same treatment schedules and procedures except that they were injected with 0 mg/kg cocaine hydrochloride or 5 µg EB or vehicle solution in which the inhibitors were prepared. On preference test day, after a 5-min habituation period in the middle neutral chamber, rats were given free access to all chambers for 15 minutes under drug-, inhibitor- and hormone-free state. Total time spent in each chamber was recorded. Acquisition of cocaine-CPP was determined using a CPP score. CPP score was calculated as the difference in the time spent in individual chambers between test and pretest phases.

4.6 Brain isolation and tissue punches.

After completion of the test phase of the cocaine-CPP, animals were euthanized by rapid decapitation. Brain extraction from the skull was conducted on ice. Using a brain matrix, whole brain was sliced into a series of 2 mm sections. Appropriate brain areas (VTA, NAc shell, NAc core, and DS) were anatomically identified. Tissue was collected using 0.5 mm and 1 mm tissue punches. Tissue samples were weighed and rapidly frozen using liquid nitrogen until further analyses.

4.7 Western blotting.

Total protein was extracted from VTA, NAc core, NAc shell and DS punches by sonication in radioimmunoprecipitation assay lysis buffer (RIPA lysis buffer; Sigma-Aldrich Co. LLC #R0278) containing a cocktail of protease inhibitors (SIGMAFast Protease inhibitor tablets; Sigma-Aldrich Co. LLC #S8820) and phosphatase inhibitors (Phosphatase inhibitor cocktail 3; Sigma-Aldrich Co. LLC #P0044). Samples were centrifuged at 12,000 g for 20 min at 4 °C. The supernatant containing proteins was separated and used for further analysis.

Total protein quantification was conducted using bicinchoninic acid assay (Pierce BCA protein assay Kit, Thermo Scientific). Protein samples containing 30 µg of total protein were mixed with gel loading Laemmli buffer (1:3; Bio-rad Laboratories, Inc.) and boiled at 95 °C for 5 min. After the samples cooled down to room temperature, they were loaded onto the polyacrylamide gels for separation.

Western blotting procedure was used to determine the expression levels of pERK1/2, ERK1/2, pmTOR, and mTOR. Sodium dodecyl sulfate based polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate out the proteins. The separated proteins were then transferred to polyvinylidene fluoride (PVDF) membrane blot. After transfer, the membrane was blocked by incubating it in freshly prepared TRIS buffered saline (TBS) containing 5% non-fat dry milk and tween-20 (0.01%) for one hour at room temperature. Thereafter, the blot was incubated with either of the following primary antibodies: pERK1/2 (1:1000; CST#9101), ERK1/2 (1:1000; CST#4695), pmTOR (1:1000; CST#2971), or mTOR (1:1000; CST#2983). GAPDH (1:1000; CST#2118) was the house-keeping protein used for normalization. Following overnight incubation with primary antibodies at 4 °C, the blot was then incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (1:3000; Sigma-Aldrich Co. LLC) for an hour at room temperature (RT). Immunoreactive protein bands were detected by enhanced chemiluminescence reagents using ChemiDoc MP Imaging system (Bio-rad Laboratories, Inc.). Images of immunoblots were processed and analyzed using ImageLab software (Bio-rad Laboratories, Inc.). Differences in the normalized expression of pERK1/2 and pmTOR, expression were determined relative to normalized ERK1/2 and mTOR expression respectively to assess changes in the activation of these proteins within the aforementioned brain areas in all groups.

4.8 Statistical analyses.

Statistical analyses were conducted using repeated measures and mixed ANOVAs as described in individual results sections (Results). Pair-wise comparisons employing Bonferroni correction were used to assess individual group differences. Significant main effects and interactions were reported along with significant pair-wise comparisons. P-value of less than 0.05 was considered statistically significant.

Number of animals to be included in each of the experimental groups was determined *a priori* using G-power software. Animal numbers were finalized based on effect size estimates derived from previously published

literature and our own preliminary results. **Table 8** provides details of experimental groups and number of animals included in each group.

Chapter 5 Discussion

The main goal of this study was to determine the influence of E2 on sex differences in the formation of cocaine reward and to identify molecular mechanisms underlying the influence of E2 on the development of associations between cocaine-induced affective state and the contextual environment. It is important to appreciate that, within the realm of addiction, reinforcement and reward are two different phenomena to streamline the inferences from the results of our study. While, there have been multiple studies assessing the involvement of E2 and its underlying neurobiological correlates in cocaine reinforcement (Becker et al., 2012; Becker & Hu, 2008; Becker & Koob, 2016; Hu et al., 2004; Jackson et al., 2006), very few studies have assessed its involvement and the associated molecular mechanisms in the acute rewarding effects of cocaine (Nygard et al., 2013; Russo et al., 2003; Sun et al., 2007; Weiner et al., 2009). Since the aim of this study was to assess cocaine reward, we used the CPP paradigm to specifically assess E2-mediated enhancement of associations developed while learning to associate context with cocaine reward. In this paradigm, when an environment is repeatedly paired with drug administration, it leads to the formation of an association between the environmental context and the rewarding effect of the drug such that the previously neutral environmental context becomes rewarding. This paradigm allows for objective assessment of the conditioned rewarding effects of the drug and thus was used for our study. The assessment of neurobiological changes occurring due to E2-mediated enhanced cocaine reward was limited to VTA, NAc and DS – key nodes of the reward pathway previously shown to be implicated in regulating cocaine reward (Nestler, 2005; Russo & Nestler, 2013). The main findings of the studies conducted herein are 1) E2 enhances cocaine conditioned reward by potentiating the formation of associations between rewarding effects of cocaine and the contextual environment during the cocaine conditioning; 2) ERK1/2 activation during cocaine-conditioning is necessary for the potentiation of cocaine-conditioned reward by E2.

5.1 Sex differences in the conditioned rewarding effects of cocaine are mediated by E2.

Our results demonstrated robust sex differences in cocaine conditioned reward. Female rats showed higher cocaine-CPP than males at lower doses of cocaine (5 and 10 mg/kg) indicating that females were more sensitive to lower conditioning doses of cocaine. At the 15 mg/kg dose of cocaine, while males continued to exhibit the same level of preference regardless of the conditioning dose of cocaine. One explanation for this would be that in females,

the subjective effects of cocaine dissipated rapidly at the highest conditioning dose and were replaced by the negative affective state that follows the initial cocaine “high”. This negative affect perhaps lowered the rewarding effects of cocaine in turn attenuating the acquisition of cocaine-CPP at test. To avoid the influence of this on the assessments of cocaine-CPP, we used the 10 mg/kg dose for all further experiments.

Next, we found that higher sensitivity to the acute rewarding effects of cocaine in females was modulated by estrous cycle stage. We postulated that higher levels of E2 during the proestrus/estrus stages enhanced formation of associations between cocaine and the environmental context was associated with higher cocaine-conditioned reward. We verified this hypothesis by artificially elevating E2 levels in OVX females during cocaine conditioning. E2 elevations during cocaine conditioning enhanced cocaine conditioned reward. This indicated that E2 either – 1. enhanced the rewarding and positive affective effects of cocaine in females or 2. It enhanced the formation of associations between cocaine reward an environmental context. It seems that E2 induces its effect on cocaine reward by enhancing both - the rewarding effects of cocaine and the formation of cocaine-context associations. Both these effects drove the E2-mediated enhancement in the acquisition of cocaine conditioned reward (further discussed in detail in 5.6 Interaction between E2, ERK1/2, mTOR and formation of cocaine-context associations). However, the focus of this study was to identify the influence of E2 on the acute conditioned rewarding effects of cocaine and not the cause of this effect. It would be interesting to understand the influence of E2 on cocaine reward and formation of cocaine-context associations in further studies. Interestingly, elimination of endogenous E2 by OVX significantly attenuated cocaine-conditioned reward. These results directly support the role of E2 in the modulation of cocaine reward. Additionally, these results also indicate that inhibiting E2 may have the therapeutic potential to decrease sensitivity of females to the acute rewarding effects of cocaine.

5.2 Molecular correlates of E2-mediated enhanced cocaine-CPP.

At the molecular level, we found that E2-mediated enhanced cocaine-conditioned reward was associated with increased activation of ERK1/2 and mTOR in different nodes of the mesolimbic reward pathway. Specifically, mTOR activity was higher in the VTA and NAc core while ERK1/2 activity was higher in the VTA, DS and NAc shell of OVX female rats treated with E2 during conditioning compared to intact and OVX female rats that did not receive E2. Furthermore, use of the CPP-paradigm not only demonstrates the involvement of ERK1/2 and mTOR in

cocaine-reward but also in the formation of cocaine-context associations which are heavily involved in cocaine craving during abstinence and relapse (Becker & Chartoff, 2019; Kokane & Perrotti, 2020).

5.2.1 Involvement of ERK1/2 in E2-mediated enhanced cocaine-CPP

Results from our experiments showed that E2 treatment during cocaine conditioning was associated with increased activity of ERK1/2 in the VTA, DS and NAc shell of females. ERK1/2 belongs to a family of mitogen-activated protein kinases (MAPKs) and has been characterized to respond to extracellular stimuli. Upon activation, pERK translocated to the nucleus and leads to the phosphorylation of Elk-1, a ternary complex factor which binds to a serum response element (SRE) binding site and promoting the expression of immediate early genes (IEGs) related to neuroadaptations (Sun et al., 2016).

Several studies conducted in male rodents have heavily implicated ERK1/2 activation in cocaine addiction. Acute administration of cocaine in male rodents increased ERK1/2 activity in the DS, NAc, PFC, BLA, and hippocampus (Girault et al., 2007; Valjent et al., 2000, 2004, 2005). This increased expression of pERK1/2 and its downstream targets including Elk-1, CREB, and IEGs is dependent upon activation of MEK by D1 DA receptor and NMDA receptor in these areas of the mesolimbic reward pathway (Girault et al., 2007; Radwanska et al., 2006; Radwanska et al., 2005; Valjent et al., 2000, 2006, 2010). Induction of silent synapses, a characteristic feature of cocaine-mediated neuroadaptations involved pERK induction in the NAc core in cocaine-sensitized male animals (Boudreau et al., 2007, 2009; Sun et al., 2016). Cocaine challenge after withdrawal from repeated cocaine administration increased pERK expression in the DS and NAc of male animals (Bertran-Gonzalez et al., 2008; Janes et al., 2009; Yoon et al., 2007). Cocaine-CPP experiments conducted in male animals demonstrated that ERK1/2 activation in the VTA was necessary for acquisition of cocaine-CPP (Pan et al., 2011). In males, exposure to the cocaine-paired context during CPP testing increases ERK phosphorylation in the NAc core but not NAc shell (Miller & Marshall, 2005). Moreover, ERK1/2 activation in NAc core accompanied cocaine-induced reinstatement of CPP (Sun et al., 2016) while ERK1/2 activation in NAc core and DS accompanied context-induced reinstatement of CPP (Bertran-Gonzalez et al., 2008). Post cocaine-CPP establishment, re-exposure to cocaine-paired context, induced pERK, pCREB and ΔFosB in the VTA, DS, D1-DA receptor containing neurons of NAc core and hippocampus (Sun et al., 2016). Taken together, these studies implicate the importance of ERK1/2 activation in the VTA and NAc core for the formation of cocaine-context associations. The results of the present study demonstrate,

for the first time, elevations in ERK1/2 activity in VTA, and DS and NAc shell of E2-treated females, indicating the importance of ERK1/2 activation in cocaine-CPP in females. In addition, ERK1/2 activity was increased in DS and NAc shell instead of the NAc core as seen in male animals (Sun et al., 2016). This demonstrated that E2-mediated enhanced acquisition of cocaine-CPP involved different nodes of the reward pathway. Therefore, ERK1/2 activation in the DS and NAc shell but not NAc core would underlie E2-mediated enhanced acquisition of cocaine-CPP in females. Future studies characterizing the differences in the molecular mechanisms underlying involvement of NAc core in males and of NAc shell in females in cocaine conditioned reward need to be conducted to fully understand this effect. One way of doing this would be to administer ERK1/2 inhibitors in either of these areas and assess it effect on cocaine-CPP in males and females.

5.2.2 Involvement of mTOR in E2-mediated enhanced cocaine-CPP.

We found increased mTOR activation in the VTA and NAc core of females treated with E2 during conditioning. mTOR is important for intracellular communication as well as vital for key cellular processes like protein synthesis, cell metabolism, autophagy and neuronal plasticity. Evidence indicates that the PI3K-Akt/mTOR pathway is regulated by several neurotransmitter systems including glutamatergic (regulation by NMDA and AMPA receptor function) and DAergic (regulation by D1 and D2 receptor function). mTORC1 is involved in the regulation of dendritic arborization and spine morphology (Jaworski et al., 2005; Kumar et al., 2005), and in some forms of synaptic plasticity like late- phase long-term potentiation in the hippocampus (Ucha et al., 2020).

Although lacking, several lines of evidence in male animals have demonstrated changes in mTOR activity or expression after different treatments with cocaine (Ucha et al., 2020). Drug-induced CPP protocols suggest that mTOR is activated in several areas of the mesolimbic reward pathway. In the VTA, cocaine-CPP was accompanied with mGLUR1-dependent increases in pmTOR, p70-S6K, S6 and other downstream effector proteins of mTOR (Yu et al., 2013). Deletion of mTOR in the VTA attenuated cocaine-CPP and was accompanied by decreased AMPA/NMDA receptor ratio, and frequency and amplitude of mEPSCs of VTA DAergic neurons (Liu et al., 2018). Wang et al. (2010) found that exposure of male rodents to a cocaine-paired environment, after establishment of cocaine-CPP, activated mTORC1 signaling pathway focally within the NAc core (Wang et al., 2010). Taken together, these data suggest that mTOR activation in VTA is necessary for acquisition of cocaine-CPP and exposure

to cocaine-paired environment increases mTOR activation in the NAc core. Here, we report that females treated with E2 during cocaine conditioning also demonstrated increased activation of mTOR in the VTA and NAc core indicating that E2-mediates an increase in mTOR activation similar to what has been previously reported in male animals. However, its influence on cocaine-CPP was not the same. To address this deficiency, further studies comparing the activation of mTOR in the presence of E2 between males and females need to be conducted. Regardless of the lack of sex differences, our studies were the first to demonstrate the involvement of mTOR in the VTA and NAc core in E2-mediated enhanced acquisition of cocaine-CPP

5.3 Inhibition of ERK1/2 activity but not mTOR inhibits E2-mediated enhanced acquisition of cocaine-CPP.

SL327 inhibits the phosphorylation of MEK (an upstream regulator of ERK1/2 phosphorylation) preventing the activation of ERK1/2. Inhibiting ERK1/2 activation during formation of cocaine-context associations using SL327 blocked E2-mediated enhanced acquisition of cocaine-CPP. This confirmed the involvement of ERK1/2 in the formation of cocaine-context associations in E2-mediated enhancement of cocaine conditioned reward. Systemic inhibition of ERK1/2 activity significantly reduced pERK1/2 levels in VTA, DS, NAc core and NAc shell and was accompanied by reduction in mTOR activation in the VTA and NAc shell demonstrating regulation of mTOR by ERK1/2 discussed later. Previous studies have demonstrated that induction and expression of cocaine behavioral sensitization can be inhibited by systemic SL327 injection in male animals (Sun et al., 2016). Systemic pretreatment with 50 mg/kg SL327 prevented the acquisition of cocaine-CPP (Valjent et al., 2000) indicating that the formation of cocaine-context associations are dependent on the activation of ERK1/2. Taken together these studies further emphasize the importance of ERK1/2 activation in the acquisition of cocaine-CPP and exemplify the efficacy of systemic SL327 in the inhibition of ERK1/2 activity. However, they raise the question of how ERK1/2 activity affects E2-mediated acquisition of cocaine-conditioned reward. The effect of SL327 pretreatment on cocaine-CPP in our studies maybe occurring via attenuating – 1) the unconditioned rewarding effects of cocaine or 2) the influence of E2 on the unconditioned rewarding effects of cocaine or 3) the E2-mediated formation of learned associations between cocaine and the environmental context. Further experiments controlling for the influence of E2 on the rewarding effects of cocaine and on the formation cocaine-context associations need to be conducted to validate the exact effect of ERK1/2 inhibition on E2-mediated enhanced acquisition of cocaine-CPP.

Molecular mechanisms of estradiol-mediated sex differences in cocaine reward

ERK1/2 regulation of mTOR has been speculated in cocaine addiction (Neasta et al., 2014). Our study alludes to this phenomenon occurring in E2-mediated enhanced acquisition of cocaine conditioned reward. ERK1/2 inhibition by SL327 in OVX females pretreated with E2 during cocaine conditioning inhibited mTOR in the VTA and NAc shell during E2-mediated enhanced acquisition of cocaine conditioned reward. This indicates ERK1/2 regulation of mTOR in E2 enhanced cocaine conditioned reward in females. Several lines of evidence have demonstrated that acute cocaine administration in males, induces a rapid activation of both ERK1/2 and mTOR in limbic structures (Girault et al., 2007; Valjent et al., 2000, 2004; Wu et al., 2011). Neuroadaptations associated with repeated cocaine exposure are promoted by the activation of ERK1/2 and mTOR in different areas of the mesolimbic reward pathway (Bailey et al., 2012; Girault et al., 2007; Lu et al., 2006; Wang et al., 2010; Wu et al., 2011). Activation of ERK1/2 and mTOR in the formation of associations between cocaine reward and environmental context occurs only within the NAc core in male animals (Lu et al., 2006; Miller & Marshall, 2005; Wang et al., 2010). Furthermore, the ERK1/2 and PI3K-Akt/mTOR pathways regulate each other via cross-inhibition and cross-activation. ERK phosphorylation of GAB negatively feeds into Akt/mTOR activation whereas Akt phosphorylation of Raf has the same effect on ErK1/2 signaling. TSC2 and mTORC1 receive inputs from components of both the ERK1/2 and PI3K-Akt/mTOR signaling pathways (Mendoza et al., 2011). In males, blockade of cocaine-CPP by MEK/ERK inhibitor during cocaine conditioning resulted in inhibition of mTOR in the VTA (Pan et al., 2011). This tight connection raises the possibility that activation of ERK1/2 signaling would be essential for the activation of mTOR by cocaine. Future studies assessing the interaction between ERK1/2 and mTOR need to be conducted to outline the role of ERK1/2 regulation of mTOR in E2 enhanced acquisition of cocaine conditioned reward.

5.4 Inhibition of mTOR had no effect on E2-mediated enhanced acquisition of cocaine-CPP.

Rapamycin is a selective allosteric inhibitor of mTOR phosphorylation. Binding of rapamycin to mTOR results in a conformational change in the structure of the mTOR protein inhibiting its phosphorylation (Neasta et al., 2014). Systemic inhibition of mTOR by rapamycin during cocaine conditioning did not affect E2-mediated enhanced acquisition of cocaine conditioned reward in females. However, mTOR activation in the VTA and NAc core was associated with E2-mediated enhanced acquisition of cocaine conditioned reward (See 5.2.2. Involvement of mTOR in E2-mediated enhanced cocaine-CPP). We think that this is possibly due to CPP phase-dependent effects of

mTOR inhibition, in that mTOR activation is necessary for the expression of cocaine-CPP at test but not during cocaine conditioning. This claim is supported by findings in male animals. Systemic rapamycin administration during cocaine conditioning and prior to CPP test did not inhibit acquisition of cocaine-CPP. However, rapamycin administration prior to another cocaine-CPP test conducted 24 hours later successfully inhibited cocaine-CPP (Bailey et al., 2012). Alternatively, another study found that when rapamycin was administered to rats along with cocaine during the acquisition phase, animals did not express locomotor sensitization when tested after two weeks of withdrawal (Wu et al., 2011). Taken together, these studies indicate that mTOR activity possibly affects the retrieval of cocaine-associated memories and not its immediate acute effects. Further studies with rapamycin administration prior to cocaine-CPP test need to be conducted to assess the CPP phase dependent effects of mTOR as well as its involvement in the retrieval of cocaine-associated memories.

Additionally, rapamycin-pretreated female rats had significantly lower levels of pmTOR in the VTA but not in the NAc core compared to vehicle treated female animals. Taken together with the behavioral data, it demonstrates that the influence of mTOR on E2-mediated enhanced acquisition of cocaine conditioned reward – 1) is not dependent on the immediate effects of cocaine on the VTA and 2) occurs via its increased activation in the NAc core. This introduces a possible sex difference , in that while males require increased activation of mTOR in the VTA and NAc core for cocaine-CPP (Neasta et al., 2014; Ucha et al., 2020), in females it is driven only by its activation in NAc core. Future studies controlling for the influence of mTOR on the immediate effects of cocaine in the VTA as it relates to E2-mediated enhanced acquisition of cocaine conditioned reward need to be conducted.

5.5 Combined inhibition of both ERK1/2 and mTOR inhibits E2-mediated enhanced acquisition of cocaine-CPP.

Based on findings from cocaine-CPP studies conducted in male animals, it is evident that ERK1/2 activation regulates mTOR activation in the acquisition and expression of cocaine-CPP in different nodes of the mesolimbic reward pathway (Neasta et al., 2014; Ucha et al., 2020). This tight association between the ERK1/2 and PI3K-Akt/mTOR signaling pathways in cocaine-CPP seems to be reflected in the E2-mediated enhanced acquisition of cocaine-CPP in females since combined inhibition of both ERK1/2 and mTOR activity inhibited it. However, at the protein level, combined inhibition of ERK1/2 and mTOR during cocaine conditioning successfully inhibited ERK1/2 activity in the VTA, DS, NAc core and NAc shell but inhibited mTOR activity only in the NAc shell. This effect was

similar to that seen in female animals that were pretreated with SL327 only, prior to conditioning indicating the possibility that inhibition of ERK1/2 activity alone by SL327 caused the resultant behavioral effects on E2-mediated enhanced acquisition of cocaine conditioned reward. This further demonstrated that inhibition of mTOR activity during cocaine condition may not be involved in E2-mediated enhanced acquisition of cocaine conditioned reward (discussed in 5.4 Inhibition of mTOR had no effect on E2-mediated enhanced acquisition of cocaine-CPP).

5.6 Interaction between E2, ERK1/2, mTOR and formation of cocaine-context associations.

Given the fact that E2 pretreatment during conditioning enhanced cocaine conditioned reward, it seems highly likely that E2 affects the formation of associations between the rewarding effects of cocaine/cocaine-induced affective state and the contextual environment occurring during conditioning. Additionally, E2 has been shown to affect learning and memory via ERK1/2 modulation (Frick, 2015). The influence of E2 on the functioning of the PI3K-Akt/mTOR pathway has been documented in the reconsolidation of memory, albeit not cocaine-associated memories (Fortress et al., 2013; Frick, 2015; Koss et al., 2018). Formation of cocaine-context associations involves a learning component and the acquisition of cocaine-CPP involves a memory component. Therefore, we predict that E2, regulates the activity of ERK1/2 during the formation of learned associations between the rewarding, positive subjective effects of cocaine and the contextual environment and, mTOR in the retrieval of cocaine-context memories during test phase of cocaine-CPP. Involvement of E2 in the formation and retrieval of cocaine-context memories and its influence on ERK1/2 and mTOR signaling need to be addressed in future experiments.

Alternatively, the influence of E2 on ERK1/2 and mTOR signaling in the context of cocaine-CPP can be explained in terms of a cascade effect of E2 on the modulation of mTOR by ERK1/2. E2-mediated ERK1/2 modulation of mTOR has been characterized in the regulation of object recognition and spatial learning and memory (Frick, 2015). E2 directly activates components of the ERK1/2 signaling pathway by binding to mGluR1 receptors during object recognition and spatial learning and memory tasks (Frick, 2015; Koss et al., 2018). During these processes, PI3K and Akt (upstream regulators of mTOR phosphorylation) are also activated by E2 through its interaction with NMDA receptors (Frick, 2015). Interestingly, activation of PI3K has been shown to the activate ERK1/2 (Adams & Sweatt, 2002). Additionally, direct activation of ERK1/2 by E2 and indirectly through the

activation of PI3K leads to the activation of mTOR (Hoeffer & Klann, 2010; Klann & Dever, 2004; Laplante & Sabatini, 2012; Richter & Klann, 2009). Taken together, these studies show that E2 can directly activate both ERK1/2 and mTOR, and ERK1/2 can activate mTOR in the processes associated with learning and memory (See **Figure 13**). Applying this information to our findings suggests that E2 regulation of ERK1/2 during conditioning maybe required for the activation of mTOR during cocaine-CPP test. This total effect is what leads to the enhancement of cocaine conditioned reward. Further studies are needed to establish this phenomenon in E2-mediated enhanced acquisition of cocaine conditioned reward.

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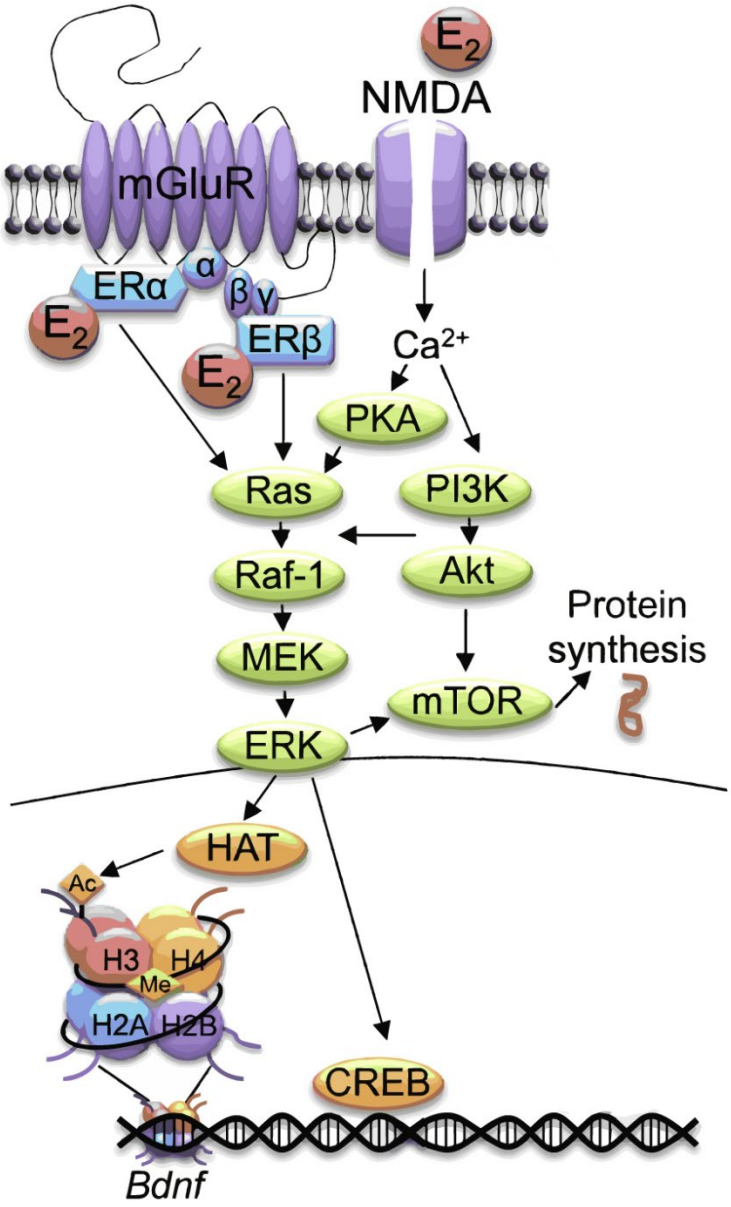
Figure 13. *Involvement of E2 modulation of ERK1/2 and mTOR signaling in hippocampal memory consolidation. ERK1/2 activation by E2 is triggered by interactions between mGluR1 and canonical ERα and ERβ. This E2-mediated activation of ERK1/2 promotes CREB phosphorylation and BDNF synthesis required for hippocampal memory consolidation. PI3K-Akt/mTOR signaling pathway is activated by the interaction between E2 and NMDA receptor. E2-induced phosphorylation of ERK1/2, PI3K and Akt elicits mTOR signaling, promoting local protein synthesis involved in hippocampal memory formation (adapted from Frick, 2015 with permissions).*



Chapter 6 Conclusions

Systematic studies assessing the involvement of E2 during formation of associations between the acute rewarding, positive subjective effects of cocaine, and environmental context are severely lacking. Additionally, studies characterizing the molecular substrates underlying the effects of E2 during the formation of cocaine-context associations are virtually unknown. More importantly, this is the first study demonstrating the role of E2, ERK1/2, and mTOR during formation of cocaine-context associations. Additionally, ours is the first study to assess E2-mediated sex differences in the activity of mTOR in cocaine conditioned reward. We have established the influence of E2 during formation of cocaine-context associations and characterized the role of ERK1/2 and mTOR activity on this effect within significant nodes of the reward pathway during these molecular substrates in females. The novelty of our approach emerges from applying a traditional set of tools to advance the field in terms of understanding the process of addiction from a perspective of conditioned adaptations and sex differences, more specifically, the importance of E2 in mediating the molecular and behavioral adaptations accompanying the process of conditioned drug reward. We have identified a role for E2 in the modulation of intracellular ERK1/2 and mTOR signaling events accompanying cocaine CPP. Furthermore, we have delineated the functional significance of the effects of E2 in these signaling events as they relate to cocaine reward. Elucidating the role of E2 in cocaine-induced intracellular signaling will fill a significant gap in our knowledge regarding the mechanism(s) by which estrogen affects intracellular signaling pathways to indicate the motivational salience of a stimulus. Knowledge of these mechanisms is crucial to our understanding of how fluctuating hormone levels can render females increasingly sensitive to the rewarding effects of cocaine and ultimately, vulnerable to cocaine addiction. This is an important area of study because differences in induction, expression, and/or accumulation of these molecules may lead to discoveries of how to customize treatment and prevention measures toward men, women, post-menopausal women and those on hormone replacement therapy.

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Molecular mechanisms of estradiol-mediated sex differences in cocaine seeking behavior

Biographical Information

Saurabh Sanjeev Kokane was raised in Pune, India. He received his Bachelor's in Biotechnology from Fergusson College, University of Pune, India. He obtained a Master's in Molecular Medicine with specialization in Neuroscience from the University of Sheffield, UK in 2010. For this master's program, he received a Merit award and earned the India Sheffield Scholarship. He gained valuable research experience while working as a research technician at the Sheffield Institute for Translational Neuroscience with his supervisors Dr. Paul Heath and Dr. Janine Kirby. Thereafter, he received a Junior Research Fellowship from the Tata Institute of Fundamental Research, Mumbai, India in May 2011. He worked with Dr. Ullas Kolthur-Seetharam elucidating the role of SIRT1, a histone deacetylase protein in metabolism and ageing. He began his graduate studies at the University of Texas at Arlington in August 2013. He has received a second Master's degree in Experimental Psychology from the University of Texas at Arlington in December 2015 and has obtained a Doctorate in Neuroscience under the mentorship of Dr. Linda Perrotti. His included studying sex differences in cocaine-associated reward and identifying the underlying neurobiological mechanisms associated with these sex differences. His overall research goal is to understand the interplay between neurotransmitter systems of the brain, endocrine mechanisms and the influence of the glial cells in "normal" and "abnormal" behaviors which develop into neurological disorders. Particularly, his interest lies in determining and understanding neurobiological mechanisms which cause these interactions and assessing their role in neurological disorders. Additionally he is also interested in studying the role of destabilizing drug-memories in the context of a therapeutic intervention for addiction disorders.