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The Role of Bdnf in 17β-Estradiol-Induced Facilitation of Extinction of Cocaine Seeking

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THE ROLE OF BDNF IN 17β-ESTRADIOL-INDUCED FACILITATION OF EXTINCTION OF COCAINE SEEKING

by

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ABSTRACT

THE ROLE OF BDNF IN 17β-ESTRADIOL-INDUCED FACILITATION OF EXTINCTION OF COCAINE SEEKING

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Females are more susceptible to drug dependence than males, and these differences in addictive behaviors are mediated, in large part, by 17β-estradiol (E_2). E_2 enhances memory in a variety of behavioral paradigms and may act to enhance the formation of drug-related memories. Interestingly, due to its mnemonic effects, E_2 promotes drug seeking, but also may aid in extinguishing drug seeking behaviors. Treatment of addiction is modeled through extinction, and the infralimbic medial prefrontal cortex (IL-mPFC) is responsible for inhibiting drug seeking after extinction. However, the cellular mechanisms by which E_2 facilitates extinction remain unknown. Previous work suggests that the memory-enhancing effects of E_2 are mediated by brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin-related kinase B (TrkB). Thus, we hypothesized that E_2-facilitated extinction is dependent upon BDNF/TrkB signaling. To identify this mechanism, we used the conditioned place preference paradigm (CPP) in which rats are trained to associate one chamber, but not another, with cocaine. Following conditioning, ovariectomized rats were systemically administered E_2 prior to each extinction trial. Furthermore, IL-mPFC infusions of a Trk receptor antagonist did not impair, but facilitated

extinction. Systemic injections of a TrkB receptor antagonist and E_2 did impair extinction, whereas injections of vehicle and E_2 facilitated extinction. The results of this experiment are in agreement with our hypothesis, however, an E_2-facilitated extinction was not consistently observed in this study. In order to determine the cellular mechanism by which E_2 increases intrinsic excitability in IL-mPFC pyramidal neurons, we performed patch-clamp electrophysiology while bath-applying Trk receptor antagonists. Our results revealed that blockade of Trk receptors attenuated E_2-induced increase in intrinsic excitability. These results indicate that E_2 potentiates intrinsic excitability via BDNF/TrkB signaling in IL-mPFC pyramidal neurons. This study demonstrates that optimizing E_2 levels and/or augmenting BDNF function could enhance therapeutic interventions in alleviating learning and memory disorders such as drug addiction.

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INTRODUCTION

Sex differences in addiction

In 2014, The National Survey on Drug Use and Health (NSDUH) reported that approximately 1.5 million Americans aged 12 and older currently used cocaine. The prevalence of addiction is reported to be greater in men compared to women (55.1% vs 44% overall, 18.1% vs 11.4% for cocaine respectively, SAMSHA 2010), therefore, most treatment programs and research on drug abuse focus on the male population. However, cocaine dependence is greater in females than males at a younger age (Kandel, Chen, Warner, Kessler, & Grant 1997; Weiss, Martinez-Raga, Griffin, Greenfield, & Hufford, 1997; Chen & Kandel, 2002), women progress more rapidly from casual drug use to dependence (McCance-Katz, Carroll, & Rounsaville, 1999; Westermeyer & Boedicker, 2000), they engage in binge-like patterns of abuse (Becker & Hu, 2008; Brady & Randall, 1999; Lynch, Roth, & Carroll, 2002), and tend to have greater lifetime use compared to men (Lejuez, Bornovalova, Reynold, Daughters, & Curtin, 2007). In terms of relapse, women have shorter periods of abstinence and are more susceptible to relapse following stressful life events or depression (McKay, Rutherford, Cacciola, Kabasakalian-McKay, & Alterman 1996; Elman, Karlsgodt, & Gastfriend, 2001; Back, Brady, Jackson, Salstrom, & Zinzow, 2005). Psychosocial factors may contribute to gender differences in cocaine-related behaviors, however, preclinical studies suggest that biological factors may also play a significant role in sex differences in cocaine seeking.

Similar to humans, adult female rats are more sensitive to cocaine compared to adult male rats. In the self-administration paradigm, rats are trained to lever press for intravenous infusions of commonly abused drugs. Drug reward is usually presented in conjunction with a light or tone, which becomes a conditioned stimulus (CS+), as an association with a drug reinforcement is

developed (Davis & Smith, 1974; Lynch, Nicholson, Dance, Morgan, & Foley, 2010). Importantly, female rats self-administer cocaine for longer periods and have higher levels of cocaine intake than males (Lynch & Taylor, 2004; Larson, Anker, Gliddon, Fons, & Carroll, 2007; Hu & Becker, 2008; Perry, Westenbroek, & Becker, 2013). Sex differences in addiction have also been observed in the cocaine sensitization paradigm. In this paradigm, repeated exposure to cocaine results in enhanced behavioral responsiveness with subsequent drug administration (Robinson & Becker, 1986; Strakowski, Sax, Setters, & Keck, 1996). Female rats tend to exhibit a greater magnitude of sensitization or rotational behaviors compared to male rats (Hu & Becker, 2003). These sex differences in cocaine seeking have been consistent with findings using another well-established paradigm called the conditioned place preference (CPP) paradigm. In this model, repeated pairings of a drug with a context produces a significant preference for the cocaine-paired environment. Preference is measured by time spent in the drug-paired context compared to time spent in the vehicle-paired context. The preference indicates the association of the environment with the rewarding properties of the drug (Tzschentke, 1998; Tzschentke, 2007). At low doses of cocaine, female rats develop faster associations between the environmental context and cocaine (Russo et al., 2003). Overall, in multiple paradigms, female rats appear to be more vulnerable to the effects of cocaine compared to their male counterparts.

The biological basis for sex-specific differences in cocaine addiction are complex. However, considerable preclinical data suggest that they may be attributed to estrogens (Carroll, Lynch, Roth, Morgan, & Cosgrove, 2004; Lynch et al., 2002; Becker & Hu, 2008). A majority of studies regarding the contribution of estrogens in various behavioral paradigms have been conducted in ovariectomized (OVX) female rodents to provide better experimental control over circulating ovarian hormones through experimenter-administered hormones. For example, in the

self-administration paradigm, OVX female rats treated with the potent estrogen, 17β-estradiol (E_2), facilitated acquisition of cocaine self-administration (Hu, Crombag, Robinson, & Becker, 2004; Roberts, Bennett, & Vickers, 1989; Lynch, Roth, Mickelberg, & Carroll, 2001; Jackson, Robinson, & Becker, 2006). Similarly, OVX rats treated with estradiol benzoate demonstrated significantly higher cocaine-induced locomotor sensitization (Perrotti et al., 2001; Peris, DeCambre, Coleman-Hardee, & Simpkins, 1991; Sircar & Kim, 1999; Hu & Becker, 2003). However, few studies have used the CPP paradigm to investigate the role of estrogens in cocaine-associated learning. Treatment of estradiol benzoate to OVX rats resulted in a significantly higher preference for the cocaine-paired chamber compared to OVX rats that received vehicle treatment (Bobzean, Dennis, & Perrotti, 2014). These data confirm that cocaine has greater abuse liability in females than males, and that E_2 may mediate these effects.

Extinction of drug seeking

One of the major obstacles to overcoming addiction in both sexes is the propensity to relapse. The rewarding properties of drugs become associated with cues, and when these cues are subsequently encountered, they can trigger drug seeking months or years after drug cessation. However, sex differences are observable during the relapse cycle, as women report shorter periods of abstinence between cocaine uses than men (Kosten, Gawin, Kosten, & Rounsaville, 1993; Ignjatova & Raleva, 2008) and are more likely to relapse after stressful or depressive life events compared to men (McKay et al., 1996; Elman et al., 2001; Back et al., 2005). This effect is also observed in female rodents who show greater cocaine-induced reinstatement than male rodents (Lynch & Carroll, 2000; Kippen, 2005; Kerstetter, Aguilar, Parrish, & Kippin, 2008; Anker & Carroll, 2010). One possible approach to treat maladaptive drug-associated memories is through extinction, which is the formation of a new inhibitory memory that results in the

reduction of learned responses to conditioned drug cues (Franken, deHaan, van der Meer, Haffmans, & Hendriks, 1999; Millan, Marchant, & McNally, 2011; Torregrossa & Taylor, 2013). During extinction, an organism learns that a CS, which is the drug-associated cue, no longer predicts the unconditioned stimulus (i.e., the drug; Bouton, Garcia-Gutierrez, Zilski, & Moody, 2006; Eisenberg & Dudai, 2004; Myers, Carlezon, & Davis, 2011). Furthermore, this results in a reduction in response to drug-paired cues and contexts, as well as a decrease in operant responses that were previously reinforced by a drug reward (Chesworth & Corbit, 2015). As mentioned, extinction does not erase memories of conditioned behavior, but rather is new learning which acts to mask or inhibit original learning (Bouton & Todd, 2014). Similar to other forms of memory, the new inhibitory memory is formed in stages. First, the memory is acquired into short-term storage, and then is consolidated into long-term storage. Subsequently, the memory is retrieved, and then is reconsolidated back into long-term storage (Quirk & Mueller, 2008). Therefore, acquisition of extinction can be manipulated prior to the first extinction trial, whereas consolidation of extinction can be manipulated immediately following the initial extinction session (Millan et al., 2011). Similarly, retrieval of an extinction memory can be manipulated before subsequent extinction trials, and reconsolidation can be manipulated immediately after a subsequent extinction trial (Millan et al., 2011).

The neural circuitry for extinction learning includes the infralimbic medial prefrontal cortex (IL-mPFC), dorsal hippocampus (DH) and nucleus accumbens (NAc). In particular, IL-mPFC has been shown to have a crucial role in extinction of drug seeking. In the self-administration paradigm, local infusions of inactivating agents in the IL-mPFC such as gamma-Aminobutyric acid (GABA) receptor agonists reinstated the previously extinguished drug seeking response (Peters, LaLumiere, & Kalivas, 2008). IL-mPFC also plays an important role in the

consolidation of the extinction memory. Inactivation of IL-mPFC via GABA receptor agonists following daily extinction sessions impaired extinction retention (LaLumiere, Niehoff, & Kalivas, 2010). Moreover, potentiating AMPA receptors in IL-mPFC after extinction training augmented extinction retention in a model of cocaine seeking (LaLumiere et al., 2010). Taken together, these findings highlight the role of IL-mPFC in regulating the consolidation and expression of extinction of drug seeking.

Effects of 17β-estradiol (E_2) on learning and memory

As suggested earlier, extinction does not eliminate or result in forgetting of the initial conditioned response; rather, extinction involves new learning that the CS no longer predicts the reinforcer (Bouton et al., 2006; Milad & Quirk, 2002; Santini, Muller, & Quirk, 2001). A wealth of scientific literature has shown that E_2 can enhance learning and memory through the activation of signaling cascades and modulation of synapse structure and function, which contributes to the rewiring of neural circuits (Woolley, 2007; Srivastava et al., 2011; Frick, 2015). E_2 also enhances drug-associated memories in rodents and women (Lynch et al., 2001; Larson et al., 2007; Segarra et al., 2014). Therefore, since extinction is a form of learning, E_2 may have the potential to enhance extinction of cocaine seeking.

Estrogens can mediate their effects via two different modes of action: the classical "genomic" mechanism, and the rapid "non-genomic" cell-signaling mechanism. In the genomic mode of action, estrogens bind to intracellular estrogen receptors (ERs), such as ERα and ERβ, which act as nuclear transcription factors. The binding of estrogens to ERs causes estrogen-receptor complexes to bind to estrogen response elements (ERE) in the DNA to influence gene transcription. In the non-genomic cell signaling mode of action, estrogens can bind and rapidly activate ERs (e.g. ERα, ERβ, G-coupled; Srivastava & Evans, 2013) or neurotransmitter receptors

(e.g. metabotropic glutamate receptors or N-methyl-D-aspartate receptors; Lewis, Kerr, Orr, & Frick, 2008; Boulware, Heisler, & Frick, 2013) located at the cell membrane, which then go on to activate cell-signaling pathways that eventually lead to changes in gene expression and epigenetic alterations (Levin, 2005; Vasudevan & Pfaff, 2007; Frick, Kim, Tuscher, & Fortress, 2015). ERs are expressed in many brain regions, including the hypothalamus, entorhinal cortex, perirhinal cortex, hippocampus, amygdala, prefrontal cortex, and cerebellum (Shughrue, Scrimo, & Merchenthaler, 1998; Kuiper, Shughrue, Mechenthaler, & Gustafsson, 1998; Osterlund, Gustafsson, Keller, & Hurd, 2000; Shughrue & Merchenthaler, 2001; Spencer et al., 2008) and both receptors are located in regions that are critical for the neural circuitry of extinction. Activation of these two types of ERs via E_2 may have differential behavioral consequences such as initiating copulatory behaviors, and improving performance in spatial learning and memory tasks (Luine, Richards, Wu, & Beck, 1998; Brann, Dhandapani, Wakade, Mahesh, & Khan, 2007; Clipperton-Allen, Almey, Melichercik, Allen, & Choleris, 2011; Cornil & Charlier, 2010; Almey et al., 2014).

Effects of E_2 on hippocampal memory

The effects of E_2 on cognitive function have been extensively examined in rodent models and a majority of research has focused on how E_2 regulates hippocampal plasticity and memory. However, there are limited studies that examine the role of E_2 in IL-mPFC and extinction. DH is involved in the circuit that mediates extinction of drug seeking (Peters, Kalivas, & Quirk, 2009). Furthermore, DH has been implicated in extinction as temporary inactivation of this region impairs extinction of conditioned fear (Corcoran & Maren, 2001). Therefore, literature on the effects of E_2 in the hippocampus can give us insight into how E_2 might act in the IL-mPFC to facilitate extinction of cocaine seeking. For example, OVX rodents that have a dramatic loss of

circulating E_2 show impaired performance on hippocampal-dependent tasks such as Morris water maze (Gresack & Frick, 2006; Markham, Pych, & Juraska, 2002; Lowry, Pardon, Yates, & Juraska, 2010; Daniel, Roberts, & Dohanich, 1999; Monteiro, de Mattos, Ben, Netto, & Wyse, 2008), radial arm maze (Fader, Johnson, & Dohanich, 1999; Daniel, Fader, Spencer, & Dohanich, 1997; Bimonte & Denenberg, 1999; Heikkinen, Puolivali, Liu, Rissanen, & Tanila, 2002; Gibbs & Johnson, 2008), object placement, and object recognition (Luine, Jacome, & MacLusky, 2003; Wallace, Luine, Arellanos, & Frankfurt, 2006; Hamilton et al., 2011; Fonseca et al., 2013).

Exogenous treatment of E_2 to young OVX rodents has generally been shown to improve spatial memory. For example, acute or chronic systemic administration of E_2 enhances spatial memory in the Morris water maze, radial arm maze, and object placement (O'Neal, Means, Poole, & Hamm, 1996; Daniel et al., 1997; Fader et al., 1999; Luine et al., 1998; Bimonte & Denenberg, 1999; Sandstrom & Williams, 2001; Luine et al., 2003; Li et al., 2004; Gresack & Frick, 2004; Walf, Rhodes, & Frye, 2006; Inagaki, Gautreaux, & Luine, 2010; Tuscher, Fortress, Kim, & Frick, 2015). Exogenous E_2 can also enhance object recognition memory (Luine et al., 2003; Gresack & Frick, 2006; Lewis et al., 2008; Jacome et al., 2010; Frick, Fernandez, & Harburger, 2010; Tuscher et al., 2015). To validate the role of the hippocampus in spatial memory and object memory consolidation, intrahippocampal infusions of E_2 enhance both forms of memory (Packard, Kohlmaier, & Alexander, 1996; Packard & Teather, 1997; Fernandez et al., 2008; Pereira, Bastos, de Souza, Ribeiro, & Pereira, 2014; Zhao, Fan, & Frick, 2010; Fortress, Fan, Orr, Zhao, & Frick, 2013; Boulware et al., 2013). A similar pattern has been observed when ER-specific agonists were administered to rodents. Administration of a ERβ selective agonist, diarylpropionitrile (DPN), but not ERα agonist, propyl pyrazole triol (PPT), mimicked the mnemonic effects of E_2 in the radial arm maze (Liu et al., 2008), Morris water maze (Rhodes &

Frye, 2006), object placement, and object recognition (Jacome et al., 2010; Walf et al., 2006, Walf, Koonce, & Frye, 2008; Frick et al., 2010) in OVX rodents. Conversely, studies have also reported that E_2 either does not improve or, in some cases, inhibits performance on some memory tasks. This variability may be due to dose, duration of treatment, age at the onset of treatment, route of administration, and difficulty of task (Dohanich, 2002; Frick, 2009; Acosta et al., 2009; Holmes, Wide, & Galea, 2002; Gresack & Frick, 2006; Markham & Juraska, 2007; Markowska & Savonenko, 2002; Garza-Meilandt, Cantu, & Claiborne, 2006). Taken together, E_2 has been shown to improve memory in a variety of behavioral tasks dependent on the hippocampus and may act as a key mediator underlying extinction of cocaine seeking in females.

Effects of E_2 on extinction memory

E_2 modulates neural plasticity which can lead to strengthened learning and memory. Furthermore, E_2 can influence neural plasticity within the circuitry underlying extinction of fear conditioning. The fear extinction network is well-characterized and includes the amygdala, hippocampus, and medial prefrontal cortex (mPFC; Quirk, Russo, Barron & Lebron, 2000; Corcoran & Maren, 2001; Santini, Ge, Ren, de Oritz, & Quirk, 2004; Bouton et al., 2006; Likhtik, Popa, Apergis-Schoute; Fidacaro, & Pare, 2008; Myers et al., 2011). Specifically, IL-mPFC is critical for the retrieval and consolidation of extinction of fear memories (Quirk et al., 2000; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011) and extinction of drug seeking (Peters et al., 2008, Otis, Fitzgerald, & Mueller, 2014; Hafenbreidel, Rafa Todd, Twining, Tuscher, & Mueller, 2014). Because many of the same brain regions are recruited for the expression of fear- and drug-related extinction memories (Peters et al., 2009), literature on either type of memory could provide insight into the role of E_2 in extinction of cocaine seeking.

The role of E_2 in extinction of fear conditioning has been examined in humans as well as rodents. E_2 can enhance recall extinction of fear memories in both women and female rodents (Graham & Milad, 2013). This study was conducted in women who used hormonal contraceptives (HC) that contain estrogens and progesterone that inhibit ovulation by decreasing production of E_2 (Rivera, Yacobson, & Grimes, 1999). Importantly, combined HCs induce stable reductions in circulating E_2 compared to naturally cycling women during the follicular (high E_2) phase of the menstrual cycle (Pluchino et al., 2009). Women using HCs exhibited significantly poorer extinction recall compared to naturally cycling women as shown by higher skin conductance responses during fear conditioning and extinction. Moreover, administration of E_2 to naturally cycling women significantly reduced skin conductance responses and enhanced their ability to recall extinction memories (Graham & Milad, 2013). Extinction impairment was also observed in female rats treated with HCs, in which freezing behavior was used as an index to measure fear responses during extinction. HC-treated rats exhibited impaired extinction and continued to show elevated freezing responses in the presence of the CS compared to vehicle-treated rats. However, extinction impairment was reversed in HC-treated rats when serum E_2 levels were restored either by exogenous treatment of E_2 or by terminating HC treatment after fear conditioning (Graham & Milad, 2013). Additionally, systemic injections of ERβ agonist (DPN) but not ERα agonist (PPT) prior to extinction training reduced freezing responses and facilitated extinction recall of fear in female rats (Zeidan et al., 2011). Thus, E_2 is necessary for extinction of fear memories in women and female rodents and this effect may be differentially regulated by ERβ.

E_2 is also an important regulator of synaptic plasticity in females and males, in both rats and humans. Females have similar extinction recall to males when they undergo extinction during high E_2 phase of their menstrual cycle but have impaired extinction recall when they undergo

extinction during the low E_2 phase (Milad, Igoe, Lebron-Milad, & Novales, 2009, Milad et al., 2010). However, E_2 also plays an important role in the male brain. E_2 can be synthesized from circulating testosterone by the enzyme aromatase (Sharpe, 1998; Jones, Boon, Proietto, & Simpson, 2006; Gillies & McArthur, 2010). Inhibiting conversion to estrogens using an aromatase inhibitor significantly impaired extinction recall in male rats (Graham & Milad, 2014). Therefore, the effects of E_2 on extinction memories are not limited to females but include males as well.

The effects of E_2 on extinction of drug seeking has been examined in our labs. Similar to results observed following extinction of fear conditioning, systemic administration of E_2 facilitated extinction in a cocaine CPP paradigm (Twining, Tuscher, Doncheck, Frick, & Mueller, 2013). In this study, OVX rats were conditioned to associate a context with the rewarding effects of cocaine. During conditioning, rats received 2 days of E_2 treatment followed by 2 days of vehicle treatment to model naturally cycling rats. Systemic E_2 injections were either paired or explicitly unpaired with cocaine conditioning sessions. Another group of rats received E_2 daily during conditioning to test whether non-cycling groups exhibited similar acquisition or extinction patterns. Regardless of E_2 treatment during conditioning, all rats expressed a cocaine CPP which suggests that E_2 levels during conditioning did not affect subsequent CPP expression. Furthermore, during extinction, rats either received E_2 or vehicle prior to each extinction trial. Administration of E_2 to OVX rats promoted faster extinction compared to vehicle-treated rats. E_2-treated rats extinguished within a week, whereas vehicle-treated rats continued to perseverate for over 5 weeks. Importantly, extinction impairment in vehicle-treated rats was reversed by administration of E_2. Overall, this study has clear implications that E_2 may have therapeutic potential for women addicted to cocaine.

In summary, OVX female rats demonstrate impaired memory on a variety of behavioral tasks, as well as extinction of cocaine seeking, and these deficits can be rescued by E_2 replacement (Frick et al., 2010; Twining et al., 2013; Graham & Milad, 2013; Tuscher et al., 2015). E_2 can exert its actions via ERα and ERβ which are expressed in the mPFC, amygdala, and hippocampus (Shughrue et al., 1998; Osterlund et al., 2000), structures involved in extinction learning and consolidation (Quirk & Mueller, 2008). Binding of E_2 to its receptors can stimulate intracellular signaling cascades, protein synthesis, and growth factor induction (Spencer et al., 2008; Frick, 2012; Frick et al., 2015) and potentially promote extinction through these mechanisms.

Effects of E_2 intrinsic excitability

The physiological mechanisms underlying the memory-enhancing effects of E_2 have been studied via electrophysiological recordings in hippocampal pyramidal cells. E_2 has the ability to enhance intrinsic excitability, which is defined by intrinsic membrane properties that affect how efficiently synaptic inputs are transformed into action potentials (APs; Wong & Moss, 1991; Kumar & Foster, 2002; Carrer, Araque, & Buno, 2003; Wu, Adelman, & Maylie, 2011). The hippocampus is necessary for associative learning and the degree of intrinsic excitability is positively correlated to the ability to learn hippocampus-dependent tasks (Thibault & Landfield, 1996; Moyer, Power, Thompson, & Disterhoft, 2000; Tombaugh, Rowe, & Rose, 2005; Wu et al., 2011). In agreement, an increase in excitability is reported following successful learning (Moyer, Thompson, & Disterhoft, 1996; Thompson, Moyer & Disterhoft, 1996).

Conversely, a lack of E_2 reduces excitability in hippocampal neurons. OVX rats that did not receive any E_2 treatment over a long period of time (~5 months) had reduced intrinsic membrane excitability of hippocampal pyramidal neurons compared to OVX controls that were

deprived of E_2 for 10 days (Wu et al., 2011). Slices from these rats required a larger depolarizing current to initiate firing compared to control OVX rats. Interestingly, bath-application of E_2 increased intrinsic excitability in the control OVX rats but not in the long-term E_2-deficient OVX rats. E_2 enhances excitability by suppressing the afterhyperpolarization (AHP) that follows action potentials. The AHP has multiple components, and is comprised of a fast AHP (fAHP), medium AHP (mAHP), and slow AHP (sAHP). Furthermore, the AHP acts as a negative feedback regulating excitability and spike frequency adaptation (Storm, 1990; Sah & Davies, 2000). E_2 can regulate the slow Ca^{2+}-activated K^+ current (sI_{AHP}) that mediates the sAHP in CA1 pyramidal neurons in hippocampal slices from OVX female rats (Kumar & Foster, 2002; Carrer et al., 2003). The sI_{AHP} in OVX rats was significantly larger compared to control rats, and bath-application of E_2 reduced the sI_{AHP} (Carrer et al., 2003). Overall, E_2 promotes excitability by suppressing sI_{AHP} in hippocampal neurons, which contributes to synaptic plasticity as well as learning and cognitive abilities. However, the role of E_2 in IL-mPFC neurons remains unknown.

Mechanisms underlying the role of E_2 in learning and memory

The molecular targets of E_2 are diverse but mounting evidence from clinical and pre-clinical studies suggest that Brain-derived neurotrophic factor (BDNF) may be a key mediator in regulating the memory-enhancing effects of E_2 (Singh, Meyer, & Simpkins, 1995; Aguirre & Baudry, 2009; Hill, 2012; Wu, Hill, Gogos, & VanDenBuuse, 2013; Fortress, Kim, Poole, Gould, & Frick, 2014). BDNF is an important regulator of neuroplasticity in different brain regions. BDNF is the most abundant neurotrophin in the central nervous system and has two receptors, its high-affinity receptor tropomyosin-related kinase B (TrkB) and the low-affinity receptor $p75^{NTR}$, which is a non-specific receptor for all neurotrophins (Chao, 2003; Huang & Reichardt, 2003; Reichardt, 2006). BDNF and its receptor TrkB play an important role in learning and memory.

Genetic and pharmacological disruptions of BDNF or its TrkB receptors have led to impairments in learning tasks. For example, heterozygous BDNF mutant mice were significantly impaired on spatial learning in a water maze test (Linnarsson, Bjorklund, & Ernfors, 1997). Spatial learning via the radial arm maze is positively correlated with enhanced BDNF mRNA as well as phosphorylated TrkB in the hippocampus (Mizuno, Yamada, Olariu, Nawa & Nabeshima, 2000; Mizuno, Yamada, He, Nakajima, & Nabeshima, 2003). Many of the trophic effects of E_2 and BDNF are similar, or even identical, which have led to the hypothesis that these signaling molecules can act synergistically to influence behavior, synaptic structure, and physiology of neurons.

Interactions between E_2 and BDNF

E_2 and BDNF are signaling molecules that can facilitate extinction of fear and drug seeking. As previously mentioned, exogenous treatment of E_2 enhanced extinction of fear conditioning in both female rats and humans (Zeidan et al., 2011; Graham & Milad, 2013). Facilitated extinction was also reported in a cocaine CPP paradigm when OVX female rats received systemic injections of E_2 (Twining et al., 2013). BDNF has been shown to play a similar role in extinction memory in male rats. For example, a single infusion of BDNF in IL-mPFC reduced conditioned fear (Peters, Dieppa-Perea, Melendez, & Quirk, 2010). Interestingly, infusions of BDNF into prelimbic mPFC, a structure involved in the expression of drug seeking behavior, were sufficient to suppress cocaine seeking behavior in a self-administration model. This type of suppression would normally occur only after extinction (Berglind et al., 2007). Additionally, a study by Otis et al., (2014) directly observed facilitated extinction of a cocaine CPP when TrkB receptors were potentiated by an infusion of BDNF in IL-mPFC of male rats. Extinction was impaired when BDNF activation was blocked by a highly selective TrkB receptor

antagonist, ANA-12 (Otis et al., 2014). Taken together, BDNF signaling in IL-mPFC regulates extinction of drug seeking and conditioned fear (Peters et al., 2010; Otis et al., 2014). However, the interaction of E_2 and BDNF in extinction of cocaine seeking in female rats is unclear.

An underlying mechanism of learning and memory involves dendritic plasticity. In general, E_2 is associated with greater dendritic spine density on pyramidal cells in the hippocampus and mPFC (Woolley & McEwen, 1993; Murphy, Cole, Greenberger, & Segal, 1998; McEwen & Elves, 1999; Christensen, Dewing, & Micevych, 2011; Tuscher, Luine, Frankfurt, & Frick, 2016). Similarly, BDNF/TrkB signaling has been shown to influence dendritic spine and synaptic density in hippocampal and cortical neurons (Gottmann, Mittmann, & Lessmann, 2009; Yoshii & Constantine-Paton, 2010; Luine & Frankfurt, 2013). Previous studies have reported that BDNF may be necessary for E_2 regulation of dendritic spines and ultimately synaptic transmission. E_2-mediated increases in dendritic spine density are attenuated by inhibiting TrkB receptors with K-252a in hippocampal slice cultures (Sato et al., 2007). Moreover, in aromatase knockout mice, there was a large decrease in both BDNF protein as well as dendritic spine density and this was rescued by treatment with estradiol benzoate (Sasahara et al., 2007). Thus, E_2 may regulate BDNF protein levels and spine density in the mPFC and hippocampus, regions crucial for extinction.

Regulation of dendritic morphology leads to the modulation of synaptic plasticity (Bourne & Harris, 2008). Previously, both BDNF and E_2 have been shown to be important modulators of synaptic plasticity by enhancing N-methyl-D-aspartate (NMDA) receptor mediated currents (Woolley, 1998; Foy et al., 1999; Poo, 2001; Lu, 2003; Cohen-Corey, Kidane, Shirkey, & Marshak, 2010; Otis et al., 2014). Furthermore, the effects of E_2 on synaptic transmission have been shown to be mediated by upregulation of BDNF (Scharfman, Mercurio, Goodman, Wilson,

& MacLusky, 2003). BDNF can have neurophysiological effects at different points of the estrous cycle, as BDNF expression is high during proestrus when E_2 levels are elevated compared to other phases of the cycle (Scharfman et al., 2003). In hippocampal slices, repetitive hilar stimuli evoked population spikes, inducing hyperexcitability in the CA3 region during the proestrus phase of the cycle. Stimulation to the CA3 region in slices derived from OVX rats did not evoke any spike responses. Furthermore, hyperexcitability in CA3 during proestrus was reversed by a non-specific Trk receptor antagonist, K-252a (Scharfman et al., 2003). Overall, this investigation suggests that E_2 may enhance synaptic transmission in hippocampal neurons via a BDNF-dependent mechanism.

Mechanisms implicated in synaptic plasticity and learning involve new protein synthesis and activation of cell-signaling cascades (Frick, 2012). Both E_2 and BDNF share common signal transduction pathways and transcription factors crucial for synaptic plasticity. These include signaling through the extracellular regulated protein kinase (ERK; Yamada & Nabeshima, 2003; Toran-Allerand et al., 1999; Boulware et al., 2013), the phosphatidylinositol 3-kinase (PI3-K; Znamensky et al., 2003; Mizuno et al., 2003; Fortress et al., 2013), Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII; Sawai et al., 2002; Blanquet, Mariani, & Derer, 2003) and cAMP-response-element-binding protein (CREB; Ernfors & Bramham, 2003; McEwen, 2001; Boulware et al., 2005). Furthermore, E_2 regulates BDNF protein expression in a variety of brain regions. E_2 replacement in young adult OVX female rats enhances BDNF protein levels in the olfactory bulbs (Jezierski & Sohrabji, 2000, 2001), hippocampus (Allen & McCarson, 2005; Gibbs 1998; Fortress et al., 2014), cortex (Allen & McCarson, 2005; Sohrabji, Miranda, & Toren-Allerand, 1995), amygdala (Liu et al., 2001; Zhou, Zhang, Cohen, & Pandey, 2005), septum (Gibbs 1999, Liu et al., 2001), dorsolateral area of the bed nucleus terminalis and the lateral habenular nucleus (Gibbs,

1999). E_2 has also shown to increase BDNF expression in the entorhinal cortex of OVX, aged female rats (Bimonte-Nelson, Nelson, & Granholm, 2004) as well as in the hippocampus of gonadectomized male mice (Solum & Handa, 2002). A possible explanation for the interaction between E_2 and BDNF may due to the gene encoding BDNF, which contains a sequence similar to the ERE. This sequence is also found in other estrogen-regulated genes (Sohrabji et al., 1995).This suggests that the ER-ligand complex binds to the ERE-like motif on the BDNF gene and protects DNA from DNAase degradation, thereby regulating BDNF expression (Sohrabji et al., 1995).

A substantial amount of literature provides evidence that estrogenic regulation of BDNF is necessary for synaptic plasticity and memory (Srivastava et al., 2011; Luine & Frankfurt, 2013; Fortress et al., 2014). Regulation of BDNF by E_2 has been observed in brain structures such as the hippocampus and PFC, which are critical for memory formation (Luine & Frankfurt, 2013). Furthermore, low levels of BDNF and E_2 can be associated with anxiety-like behaviors (Gouveia et al., 2004), depression, (Russo-Neustadt & Chen, 2005; Marcus et al., 2005) and impaired memory formation (Matsushita et al., 2005; Bian, Zhang, Zhang, & Zhao, 2005). Specifically, both E_2 and BDNF have facilitated extinction of a cocaine CPP, independently (Twining et al., 2013; Otis et al., 2014). Therefore, this suggests that the regulation of BDNF by E_2 may play an important role in extinction of cocaine seeking.

Clinical relevance

Drug-associated memories can be strengthened by environmental stimuli (cues) that promote compulsive drug taking and trigger relapse (Carter & Tiffany, 1999; Shalev, Grimm, & Shaham, 2002; See, 2005). Both clinical and preclinical studies of substance use disorders suggest that females are more vulnerable than males to addiction, including initiation of drug taking,

bingeing, and relapse (Anker & Carroll, 2010). Treatment of addictive disorders can be modeled through extinction and has been shown to be effective in preclinical investigations. Specifically, E_2 facilitates extinction in female rodents (Zeidan et al., 2011; Twining et al., 2013; Graham & Milad, 2013), therefore, it may be more clinically relevant to investigate mechanisms underlying E_2-induced facilitation of extinction. Furthermore, pharmacological activation of BDNF or E_2 signaling would enhance extinction-based therapies for learning and memory related disorders, such as addiction.

AIMS

Aim 1

In the present study, we aimed to investigate the mechanisms mediating E_2-induced facilitation of extinction of cocaine seeking. Data from our lab have shown that E_2 is necessary for extinction of a cocaine-induced CPP and lack of it results in persistent deficits of extinction (Twining et al., 2013). The goal of this study was to target BDNF/TrkB signaling as a potential mechanism for E_2-induced enhancement of extinction as direct infusions of BDNF in the IL-mPFC has shown to facilitate extinction in male rats (Otis et al., 2014). To date, this mechanism has not been examined in female rats. Our hypothesis is that E_2 will facilitate extinction of cocaine seeking and this effect will be impaired by systemic or IL-mPFC infusions of Trk receptor antagonists. Since we aimed to impair acquisition of extinction, pharmacological manipulations were made prior to extinction trials.

Aim 2

In aim 2, we determined the interactions between BDNF and E_2 via electrophysiological recordings in IL-mPFC neurons derived from female OVX rats. We measured intrinsic

excitability which lowers the threshold for synaptic changes and is a neural correlate of learning. A significant amount of attention has been given to synaptic plasticity as a mechanism for learning. However, recent studies have demonstrated that memory storage cannot be exclusively based on a synaptic model and that intrinsic excitability plays a critical role in adaptive behaviors (Frick & Johnston, 2005; Song, Detert, Sehgal, & Moyer, 2012; Zhang & Linden, 2003), such as extinction. E_2 enhances intrinsic physiology in hippocampal neurons (Kumar & Foster, 2002; Carrer et al., 2003) but the effects of E_2 on IL-mPFC neurons remain unknown. Furthermore, there is no known data that BDNF influences intrinsic excitability in the hippocampus or any other regions necessary for extinction. This study aimed to examine whether E_2 enhances intrinsic excitability and if this effect was dependent on BDNF/TrkB signaling. To test this, OVX female rats received systemic injections of E_2 or vehicle to determine if priming with E_2 affected membrane properties as previously shown (Schiess, Joels, & Shinnick-Gallagher, 1988). Rats were euthanized and patch-clamp recordings were conducted in layer 5 of IL-mPFC pyramidal neurons. Throughout recordings, brain slices were incubated with vehicle, E_2 (25 nM), vehicle + K-252a, E_2 + K-252a and excitability was measured. We hypothesized that E_2 would enhance intrinsic excitability of IL-mPFC pyramidal neurons, and that blockade of Trk receptors would attenuate the effects of E_2.

METHODS

Subjects and Surgery

Female Long-Evans rats weighing between 275-300 g were individually housed in clear plastic cages. Rats were maintained on a 14-h light/10-h dark cycle and had unlimited access to water and standard laboratory chow (Teklad, Harlan Laboratories). Rats were weighed and handled daily for approximately 3 days prior to surgery and before the start of experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Milwaukee in accordance with National Institute of Health guidelines.

Surgeries were performed as previously described (Frick et al., 2004; Twining et al., 2013; Otis, Kidane, & Mueller, 2013). Rats were anesthetized with a mixture of ketamine/xylazine (90mg/kg/10.5mg/kg, i.p.) and underwent bilateral OVX using a dorsal approach (Frick et al., 2004). A single, horizontal incision was made along the spine and the ovary was isolated. The tip of the uterus was clamped and ligated and the ovary was removed with a scalpel. The remaining tissue was returned to the abdomen. The same procedure was repeated on the other ovary, and the incision was closed with sterile sutures and wound clips. Next, rats were implanted with a double-barrel guide cannula aimed bilaterally at IL-mPFC (anteriorposterior, +2.8; mediolateral $\pm$ 0.6, and dorsoventral, -4.4 mm relative to bregma). Following surgeries, rats were given an antibiotic (penicillin G procaine, 75,000 units in 0.25 mL) and an analgesic (carprofen, 5.0 mg in 0.1 mL) subcutaneously and then allowed to recover for approximately 7 days before behavioral testing. After completion of behavioral procedures, verification of cannula placements was conducted using a cresyl violet stain.

Drugs

Cocaine HCl (National Institute on Drug Abuse) was dissolved in sterile 0.9% saline at a concentration of 10 mg/mL, and administered systemically at a dose of 10 mg/kg, i.p. To ensure that E_2 levels did not build over time from repeated infusions, a water-soluble form of E_2 dissolved in 2-hydroxypropyl-β-cyclodextrin (HBC) that is metabolized within 24h was used (Pitha & Pitha, 1985). HBC vehicle and HBC-encapsulated E_2 (Sigma Aldrich) were dissolved in sterile 0.9% saline (0.2 mg/mL) and injected i.p. at a dose of 0.2 mg/kg (Gresack & Frick, 2006). ANA-12 (Sigma Aldrich) was dissolved in 1% DMSO in physiological saline (Zhang et al., 2015) and administered i.p. at 0.5 mg/kg, i.p. (Cazorla et al., 2011). K-252a (Sigma Aldrich) was dissolved in vehicle of 50% DMSO in artificial cerebral spinal fluid (aCSF) and infused into the IL-mPFC at 3.5 ng/0.3 µl/side (50 µM; Xin et al., 2014). For electrophysiological recordings, 25 nM of β-estradiol (not HBC encapsulated) was dissolved in 100% DMSO and diluted with aCSF to a final DMSO concentration of 0.0001%. K-252a was dissolved in 100% DMSO and bath-applied at 100 nM and diluted to a final concentration of 0.001% DMSO (Montalbano, Baj, Papadia, Tongiorgi, & Sciancalepore, 2013).

Conditioned place preference

Place preference apparatus

Testing and conditioning were conducted in a 3-chamber apparatus in which 2 larger conditioning chambers (13” x 9” x 11.5”) were separated by a smaller chamber (6” x 7” x 11.5”). The larger conditioning chambers had wire mesh flooring with white walls, whereas the other had gold-grated flooring with a black wall. The center chamber had aluminum sheeting as flooring. All floors were raised 1.5”, with removable trays placed beneath. Removable partitions were

used to isolate the rats within specific chambers during conditioning. During baseline and CPP trials, the doors were removed to allow free access to the entire apparatus. Each of the larger chambers contain two infrared photobeams separated by 3". If the beam furthest from the door was broken, then the rat was determined to be in the larger chamber. If only the beam closest to the center chamber was broken, then the rat was determined to be in the center chamber. During all phases of the experiments the room was kept in semi-darkness.

Conditioning and Testing

A pre-test determined baseline preferences by placing the rats into the center chamber with free access to the entire apparatus for 15 min and recording time in each chamber. Rats spent an equal amount of time in the larger conditioning chambers, but less time in the center chamber. ANOVA revealed an effect of chamber for all rats during baseline test ($F_{(2,204)} = 98.73$, $p < 0.001$), and *post-hoc* analyses confirmed that less time was spent in the center chamber than either of the conditioning chambers ($p < 0.001$). Therefore, an unbiased procedure was used, in which rats were randomly assigned to receive cocaine in one of the two larger chambers, independent of baseline preference scores. After a pre-test, rats were conditioned to associate one chamber, but not another, with cocaine in a counterbalanced fashion over 8 days. Injections were given immediately before placing the rats in their chambers for 20 min conditioning sessions.

Following conditioning, rats went through extinction training in which they were placed into the center chamber and allowed free access to the entire apparatus. In general, when the hypothesis was drug-induced facilitation of extinction, CPP extinction trials lasted for 15 min. Alternatively, when the hypothesis was drug-induced impairment of extinction, CPP extinction trials lasted for 30 min in length.

Experiment one

All rats received daily 0.2mg/kg (Gresack & Frick, 2006) i.p. injections of E_2 throughout conditioning phase. Rats received E_2 treatment one hour before conditioning trials which lasted for 8 days. Conditioning trials consisted of 4 pairings with cocaine and 4 pairings with saline. Following conditioning, rats did not receive any hormone treatment for 2 days before CPP testing (Twining et al., 2013). To determine whether K-252a impairs extinction learning of a cocaine CPP, rats received E_2 injections one hour prior to a CPP trial and were infused with 50 µM of K-252a (Xin et al., 2014) or aCSF vehicle 15 min prior to a CPP trial (30 min). K-252a was infused 15 min prior to a CPP trial as previous studies have shown that infusions of K-252a in the IL-mPFC approximately 15 min prior to testing impaired behavioral performance in other paradigms (Xin et al., 2014). To control for hormonal manipulations, rats received HBC one hour prior to a CPP trial and were also infused with K-252a or aCSF 15 min prior to a CPP trial (30 min). A 30 min time period was chosen for the extinction trials to observe impairment as extinction proceeds faster in controls.

Experiment two

We then examined whether direct inactivation of TrkB receptors impairs extinction by systemically administering the selective TrkB receptor antagonist, ANA-12. One hour prior to each CPP trial (15 min), rats received either systemic injections of 0.2 mg/kg E_2 (Gresack & Frick, 2006) and 0.5 mg/kg ANA-12 (Cazorla et al., 2011; Zhang et al., 2015) or E_2 and vehicle. The dose for ANA-12 was chosen as this dose impaired behavioral performance in other paradigms (Zhang et al., 2015). ANA-12 was systemically administered 1 hour prior to a CPP trial because active concentrations have been detected as early as 30 minutes and up to 6 hours after systemic injections (Cazorla et al., 2011). A 15 min time period was chosen for extinction

trials in this experiment even though the hypothesis was to impair extinction because 30 min extinction trials produced large variability in previous experiments.

Experiment three

As previously mentioned, experimental manipulations of extinction can be made prior to extinction trials, whereas consolidation of extinction can be manipulated immediately following extinction training (Millan et al., 2011). Therefore, to determine whether systemic administration of E_2 is necessary for consolidation of extinction, rats received systemic injections of 0.2 mg/kg E_2 (Gresack & Frick, 2006) or HBC vehicle immediately after daily CPP extinction trials (15 min). Rats were given shortened 15 min extinction trials to limit extinction across trials, as we hypothesized that E_2 would enhance the consolidation of extinction of a cocaine-induced CPP.

Histology

After behavioral testing, rats were euthanized with an overdose of ketamine and perfused with 0.9% saline followed by 10% buffered formalin phosphate. Brains were removed and placed in 30% sucrose/formalin solution. Following brain submersion, 40 µM thick coronal sections were sliced using a microtome from brain regions in which cannula were implanted. Sections were then mounted and stained with cresyl violet. Injector tip locations were confirmed using a rat brain atlas (Paxinos & Watson, 2007).

Data analysis

Drug seeking behavior was analyzed using a three-way ANOVA to compare time within each chamber across trials and between groups (Twining et al., 2013; Otis et al., 2014). When appropriate main interaction effects were detected, Fisher's LSD post hoc tests were used to make pairwise comparisons.

Patch-clamp electrophysiology

Female rats aged 3 months were OVXed and allowed to recover for 4-5 days. Rats received systemic injections of E_2 or HBC for three consecutive days before being euthanized for patch-clamp recordings. They were anesthetized with isofluorane, and their brains were rapidly removed and transferred to ice-cold, oxygenated (95%/O_2/5%CO_2) aCSF containing the following composition (in mM): 124 NaCL, 2.8 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 20 dextrose. Coronal slices were cut 400µM in ice-cold aCSF using a vibrating blade microtome (Leica VT1200). Slices were allowed to recover in warm aCSF (32-35°C) for 30 min followed by incubation in room-temperature aCSF for the remainder of the experiment (until the end of the day). Slices were transferred to a submersion chamber, mounted, and perfused with aCSF (~2 ml/min; 32°C). Pyramidal neurons with visible apical dendrites in layer V of the IL-mPFC were visualized with differential interference contrast using a 60X water-immersion lens on an upright Eclipse FN1 microscope (Nikon Instruments). Whole-cell patch recordings of IL-mPFC pyramidal neurons were obtained using fire polished borosilicate glass pipettes (3-8 MΩ), filled with internal solution containing the following (in mM): 110 K-gluconate, 20 KCL, 10 HEPES, 2 $MgCl_2$, 2 ATP, 0.3 GTP, 10 phosphocreatine; 0.2% biocytin, pH 7.3, and 290 mOsm. Intrinsic excitability were obtained with current clamp using a MultiClamp 700B (Molecular Devices) patch-clamp amplifier connected to a Digidata 1440A digitizer (Molecular Devices). The liquid-liquid junction potential (measured as 13 mV) was compensated for all recordings. All electrophysiological data were analyzed using Clampfit (Molecular Devices).

Experimental Design

After 10 min of stable recordings, neurons were polarized to approximately -60 mV to control for variance in resting membrane potential. A series of 1s current steps were applied (-40

to 500 pA; 10 pA steps) and the number of evoked APs were recorded to measure basic membrane properties such as input resistance and rheobase. To measure input resistance, current pulses were injected and the resulting voltage deflections were measured to create an IV plot. Furthermore, a rheobase was measured which is the minimum amount of current required to elicit a single action potential. Rheobase was analyzed in a subset of pyramidal neurons by applying 1s current steps with 10 pA increments until a single AP is elicited. Intrinsic excitability was measured by applying a 2s depolarizing step every 7.5s and evoked APs were recorded. The level of depolarizing step was adjusted to rheobase and remained constant throughout the experiment (Otis et al., 2013). To measure effects of E_2 on intrinsic excitability, 25 nM of E_2 was bath-applied and baseline current steps were applied every 5 min for approximately 30 min. To ensure that excitability was enhanced by E_2 and not by the current pulses on its own, the same protocols were repeated for the same amount of time in slices that remained in aCSF. To measure effects of K-252a on E_2 induced excitability, slices were bathed in 100 nM K-252a (Montalbano et al., 2013) for approximately 20 min before bath-application of E_2. Following recording, brain slices were fixed in phosphate-buffered formalin overnight.

Immunohistochemistry

To confirm that patch-clamp recordings were from IL-mPFC neurons, biocytin-filled pyramidal neurons were washed in 0.1 M PBS, followed by 1% NaBH4 in PBS and 10% normal goat serum (NGS). The slices incubated overnight with 3% NGS, 0.2% Triton-X, and PBS. After 24 hours, slices were washed in PBS and incubated for 2h with a green fluorescent antibody (streptavidin, 1:250; Invitrogen).Slices were washed with PBS before being mounted with antifade mounting medium and coverslipped. Slices were visualized using 20X magnification with green fluorescent light, to locate neurons and verify that they were pyramidal.

Electrophysiologial data analysis

Electrophysiological data were analyzed using Clampfit (Molecular Devices). Basic neuron properties were examined: input resistance, resting membrane potential, AP half-width, and AP amplitude. To analyze slow afterhyperpolarization (sAHP), voltage was recorded 1s following current offset which was then subtracted from baseline voltage before current injection (Kaczorowski, Davis, & Moyer, 2012). Basic measures of intrinsic excitability were analyzed before and immediately following drug application. Finally, to analyze AP frequency, the average number of spikes was plotted against time.

RESULTS

Trk receptor blockade in IL-mPFC facilitated extinction of a cocaine CPP

Previous work conducted in our lab has shown that E_2, when administered daily, facilitates extinction of cocaine seeking in female rats (Twining et al., 2013). The mechanism underlying E_2-induced facilitation of extinction, however, is unknown. BDNF has a role in regulating the memory-enhancing effects of E_2 in other paradigms (Singh et al., 1995; Aguirre & Baudry, 2009; Fortress et al., 2014) and facilitates extinction of a cocaine CPP in male rats (Otis et al., 2014). BDNF targets a Trk receptor, and therefore we determined the necessity of Trk receptor activation for E_2-induced facilitation of extinction in OVX female rats. We targeted Trk receptors using a non-selective antagonist, K-252a. We hypothesized that K-252a would prevent the E_2-induced facilitation of extinction. For this experiment, rats were surgically implanted with bilateral guide cannula aimed at IL-mPFC (for all IL-mPFC sites, see Figure 1A). Rats were pretested, conditioned, and subjected to daily CPP trials. Rats were given lengthened extinction trials (30 min) to promote learning, as we hypothesized that K-252a would impair extinction (Otis et al., 2014). One hour prior to each CPP trial, all OVX female rats received 0.2 mg/kg of E_2 (Gresack & Frick, 2006) systemically to facilitate extinction of a cocaine CPP (Twining et al., 2013). Rats were given a single bilateral IL-mPFC infusion of K-252a (n = 8; 3.5 ng/0.3 µl/side; Xin et al., 2014) or aCSF vehicle (n = 8) 15 min prior to a 30 min extinction trial (Figure 1B). A mixed model three-way ANOVA revealed a significant chamber by trial by group interaction ($F_{(10, 140)} = 2.502$, $p < 0.05$), and subsequent two-way ANOVA indicated a chamber by trial interaction for K-252a-treated rats ($F_{(10, 70)} = 5.961$, $p < 0.001$), but not vehicle-treated rats ($F_{(10, 70)} = 0.882$, $p > 0.05$).

Post hoc analysis confirmed that both groups spent significantly more time in the previously cocaine-paired chamber during the first CPP trial ($p < 0.05$), indicating that both groups expressed a CPP. During subsequent extinction trials (i.e., trials 2,3,5, and 6), aCSF-infused rats continued to spend significantly more time in the cocaine-paired chamber than in the saline-paired chamber (*post hoc* $p < 0.05$), while K-252a-treated rats spent an equivalent amount of time in the cocaine-paired and saline-paired chambers during extinction trials 2,3,4, and 6 (*post hoc* $p > 0.05$). Overall, we did not observe the expected E_2-induced facilitation of extinction shown previously by our lab (Twining et al., 2013), and contrary to expectations K-252a facilitated extinction of a cocaine CPP.

We repeated the experiment, and extended the infusion protocol to include daily infusions of K-252a across four days instead of one. Previous work in our lab has demonstrated that four daily systemic injections or infusions into IL-mPFC of NMDA receptor antagonists impaired extinction (Otis et al., 2014; Hafenbreidel et al., 2014). In addition to systemic injections of 0.2 mg/kg E_2 (Gresack & Frick, 2006), rats received 3.5 ng/0.3 µl/side of K-252a (n = 8; Xin et al., 2014) infusions or vehicle aCSF (n = 10) into IL-mPFC (Figure 2A for all IL-mPFC infusion sites) 15 min prior to each of the first four extinction trials (30 min; Figure 2B). Rats did not receive any infusions during subsequent extinction trials (i.e., 5 and 6; Otis et al., 2014). ANOVA revealed no significant chamber by trial by group interaction ($F_{(10, 160)} = 0.761$, $p > 0.05$). However, there was a significant chamber by trial interaction, ($F_{(10, 160)} = 2.768$, $p < 0.05$), and a significant effect of chamber overall ($F_{(2, 32)} = 35.613$, $p < 0.001$). *Post* hoc analysis confirmed that both K-252a-treated and aCSF-treated rats spent more time in the previously cocaine-paired chamber than in the saline-paired chamber during the first trial ($p < 0.05$). However, K-252a-treated rats spent equivalent time in the cocaine-paired and saline-paired chambers (*post hoc* $p >$

0.05) during all subsequent trials (i.e. 2-6). aCSF-treated rats spent significantly more time in the cocaine-paired chambers during trials 2 and 4. Although E_2-treated rats did extinguish across days, inhibition of Trk receptors did not impair this effect and rather appeared to facilitate extinction. Thus, at the dose used in this study, Trk receptors in the IL-mPFC do not mediate E_2-induced facilitation of extinction of cocaine seeking.

Figure 1

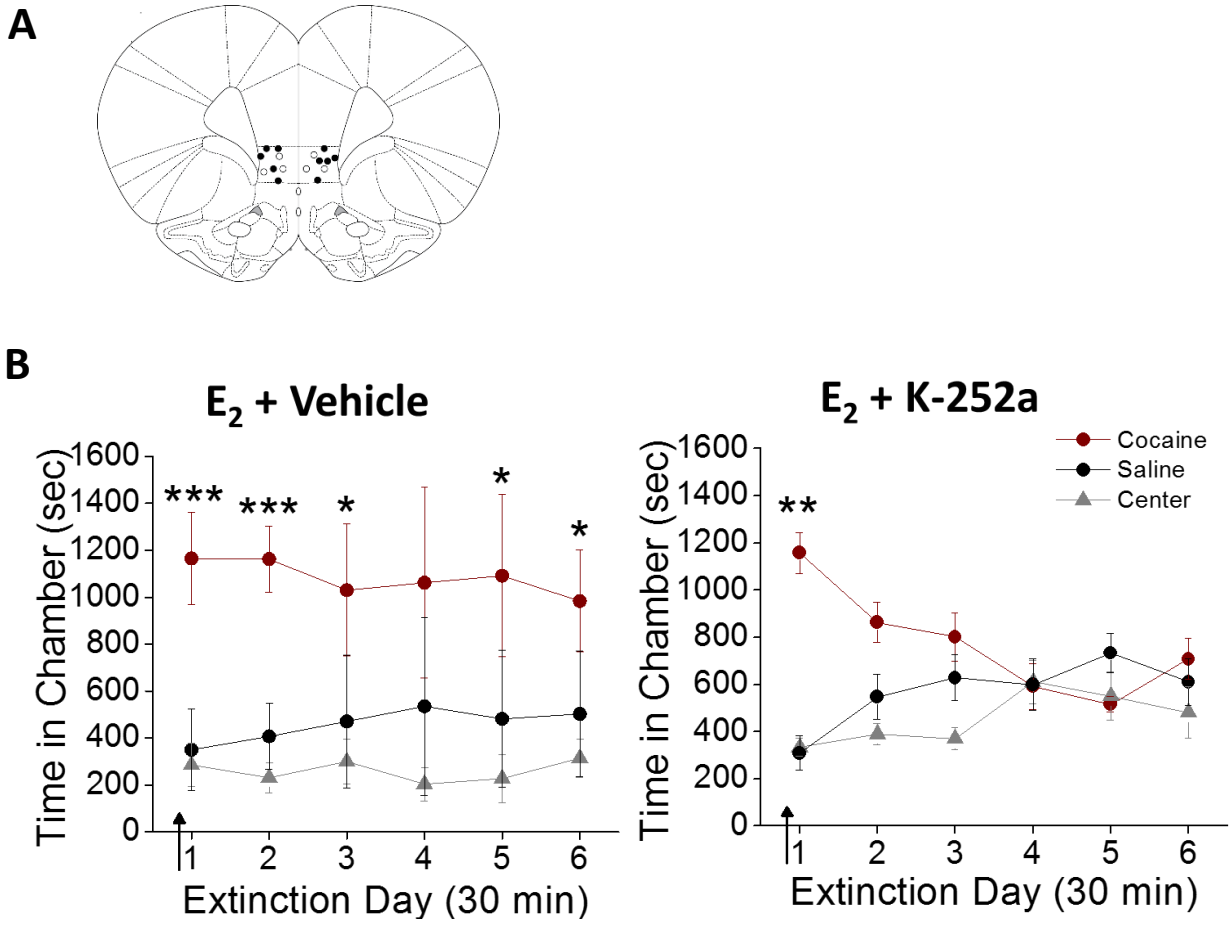

Figure 1. A single IL-mPFC infusion of non-selective Trk receptor antagonist facilitated extinction of a cocaine CPP. (A) Coronal drawings (bregma, +3.72 mm; Paxinos & Watson, 2007; Copyright 2007) showing placements of injector tips for K-252a (n = 8) and vehicle (n = 8). Following conditioning IL-mPFC infusion (arrow) of **(B)** Trk receptor antagonist, K-252a prior to the first CPP trial facilitated extinction. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

Figure 2

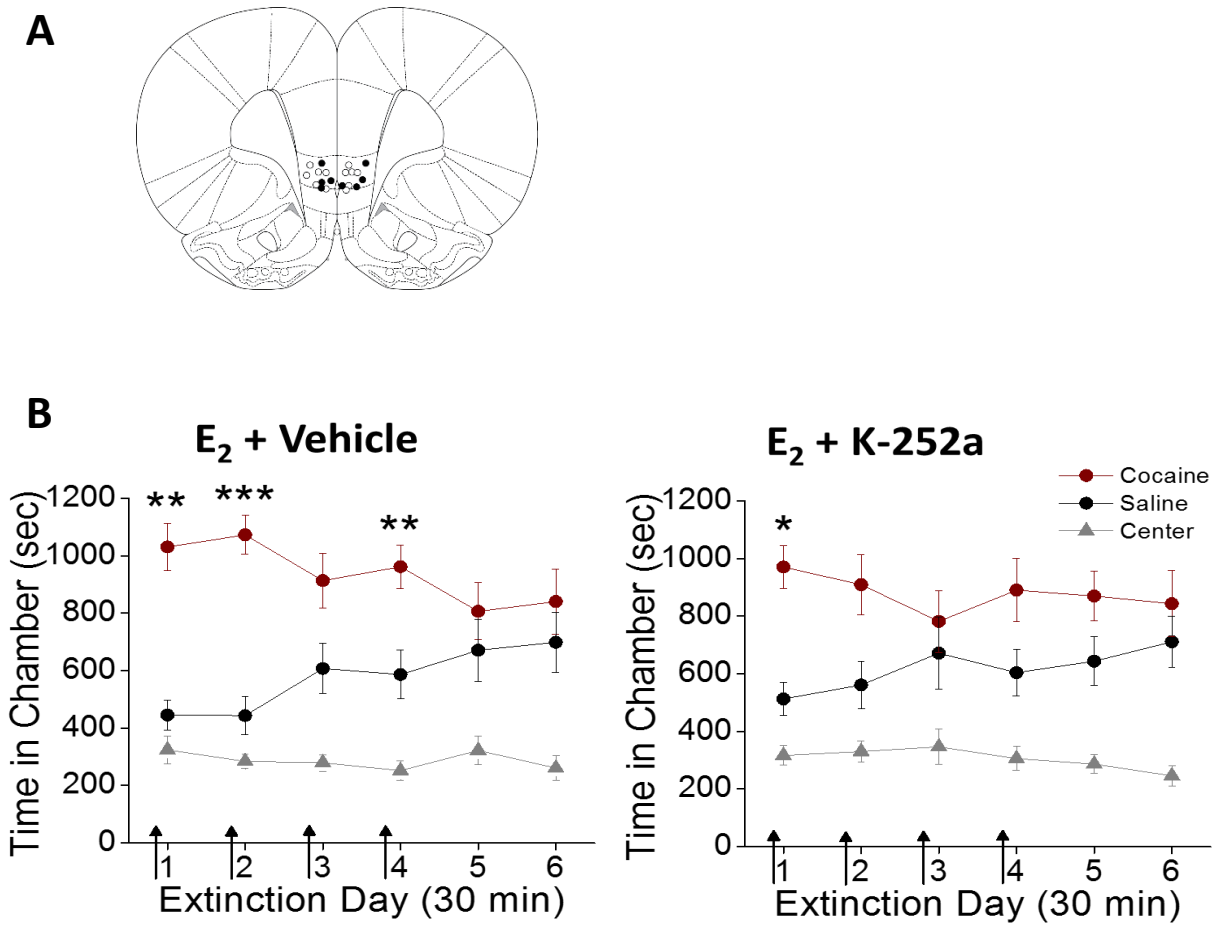

Figure 2. Multiple IL-mPFC infusions of non-selective Trk receptor antagonist facilitated extinction of a cocaine CPP. (A) Coronal drawings (bregma, +3.72 mm; Paxinos & Watson, 2007; Copyright 2007) showing placements of injector tip placements for K-252a (n = 8) and vehicle (n = 10). Following conditioning IL-mPFC infusions (arrows) of **(B)** Trk receptor antagonist, K-252a prior to the first four CPP trials facilitated extinction. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

Trk receptor blockade in IL-mPFC did not impair extinction of a cocaine CPP in the absence of E_2

Trk receptor blockade did not impair extinction when rats were administered E_2 prior to each CPP trial but facilitated extinction of a cocaine CPP. To control for hormonal manipulations, we investigated whether Trk receptor antagonist infusions into IL-mPFC would block extinction in the absence of E_2. Following conditioning, all OVX female rats received HBC vehicle one hour prior to a CPP trial (Twining et al., 2013). Rats were given a single bilateral IL-mPFC infusion of K-252a (n = 6; 3.5 ng/0.3 µl/side; Xin et al., 2014) or aCSF vehicle (n = 6) 15 min prior to a 30 min extinction trial (Figure 3). A mixed model three-way ANOVA revealed a significant effect of chamber overall ($F_{(2, 20)} = 26.06$, $p < 0.001$) and a significant chamber by trial interaction ($F_{(2, 80)} = 3.206$, $p < 0.01$). However, there was no significant group by chamber by trial interaction ($F_{(8, 80)} = 1.840$, $p > 0.05$). *Post hoc* analysis confirmed that both vehicle-treated and K-252a-treated rats showed a significant preference for the cocaine paired chamber on trial 1 (*post hoc* $p < 0.05$). However, both groups did not show a significant preference on all subsequent trials (*post hoc* $p > 0.05$). Inconsistent with previous findings (Twining et al., 2013), HBC-treated rats did show evidence of extinction, although extinction training was not extended for the 16-32 day procedure used previously in which CPP expression was initially poor but stabilized across days. In addition, blocking Trk receptors in IL-mPFC at the selected dose did not impair extinction of a cocaine CPP.

Figure 3

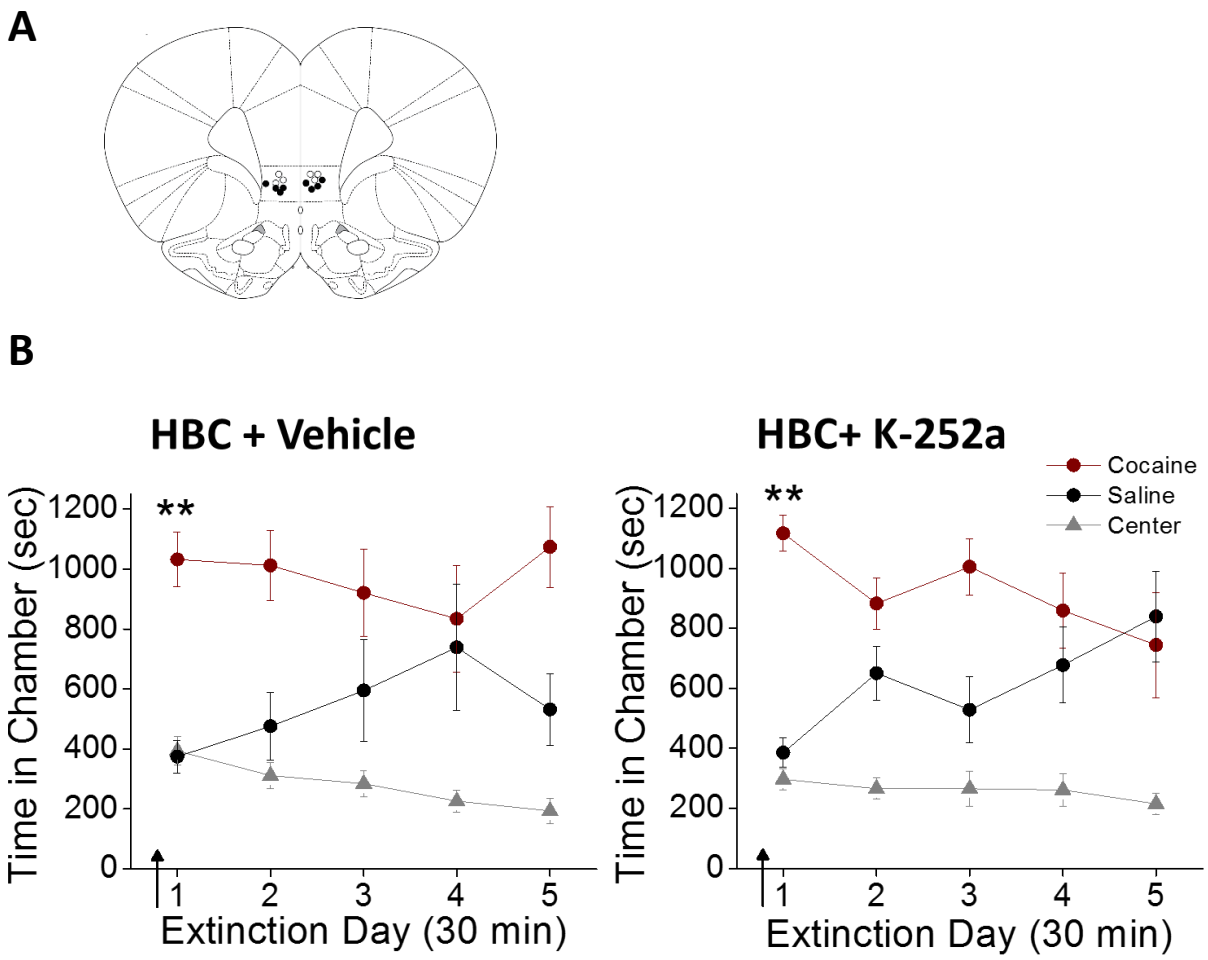

Figure 3. A single IL-mPFC infusion of non-selective Trk receptor antagonist did not disrupt extinction of a cocaine CPP in the absence of E_2 (A) Coronal drawings (bregma, +3.72 mm; Paxinos & Watson, 2007; Copyright 2007) showing placements of injector tips for K-252a (n = 6) and vehicle (n = 6). Following conditioning IL-mPFC infusion (arrow) of **(B)** Trk receptor antagonist, K-252a prior to the first CPP trial had no effect on extinction.
** $p < 0.01$

E_2-induced facilitation of a cocaine CPP was disrupted by systemic injections of TrkB receptor antagonist

The previous experiments indicate that an infusion of the non-selective receptor antagonist, K-252a, in IL-mPFC did not impair extinction of a cocaine CPP. This suggested that Trk receptors in the IL-mPFC may not play a role in mediating the effects of E_2 in the enhancement of extinction. Thus, we examined whether TrkB receptors mediated extinction by using systemic injections of an antagonist. K-252a does not cross the blood-brain barrier, so we used a selective TrkB receptor antagonist, ANA-12, that is known to cross the blood-brain barrier effectively (Cazorla et al., 2011; Zhang et al., 2015). Second, in the experiments that we conducted so far (Figure 1, Figure 2, Figure 3), we reported a large amount of variability in the vehicle-treated rats with 30 min extinction trials and this variability was not observed previously in male rats (Otis et al., 2014). Furthermore, E_2-induced enhancement of extinction in female rats was reported with 15 min extinction trials (Twining et al., 2013). Therefore, for this experiment, 15 min extinction trials were used, which is consistent with previous data collected from OVX female rats (Twining et al., 2013). All rats received 0.2 mg/kg of E_2 (Gresack & Frick, 2006) one hour prior to each CPP trial. In addition to E_2 injections, either rats received a systemic injection of 0.5 mg/kg of ANA-12 (n = 9; Cazorla et al., 2011; Zhang et al., 2015) or vehicle (n = 10) one hour before a CPP trial (Figure 4). This dose has been reported to impair performance in a number of behavioral paradigms (Zhang et al., 2015). Furthermore, rats received systemic injections of ANA-12 one hour prior to a CPP trial as active concentrations of ANA-12 have been detected as early as 30 minutes and up to 6 hours after an injection (Cazorla et al., 2011). ANOVA revealed no significant trial by chamber by group interaction ($F_{(10, 170)} = 0.1203$, $p > 0.05$). However, there was a significant effect of trial by chamber ($F_{(10,170)} = 3.926$, $p < 0.001$) and

an overall effect of chamber ($F_{(2, 34)} = 38.457$, $p < 0.001$). *Post* hoc analysis confirmed that both ANA-12-treated and vehicle-treated rats spent more time in the previously cocaine-paired chamber than in the saline-paired chamber during the first trial ($p < 0.001$). However, vehicle-treated rats did not show a significant preference for the cocaine-paired chamber during subsequent trials (*post hoc* $p > 0.05$). ANA-12-treated rats showed a significant preference for the cocaine-paired chamber during trials 2, 3, and 5 (*post hoc* $p < 0.05$). Previous experiments in this particular study did not report an E_2-induced facilitation of extinction consistently. However, in this experiment, E_2-facilitated extinction appears to be impaired by TrkB receptor antagonist, ANA-12.

Figure 4

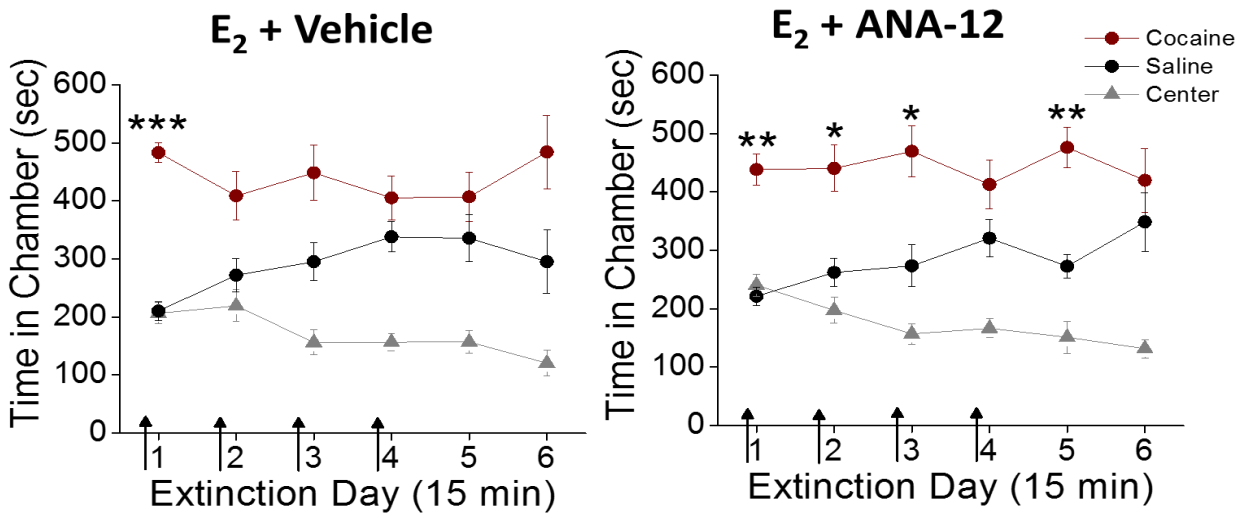

Figure 4. Injections of selective TrkB receptor antagonist disrupted E_2-induced facilitation of extinction of a cocaine CPP. Systemic injections (arrows) of ANA-12 (n = 9) or vehicle (n = 10) before the first 4 CPP trials impaired E_2-induced facilitation of extinction. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

E_2 potentiates IL-mPFC pyramidal neuron excitability

Intrinsic excitability of hippocampal pyramidal neurons is potentiated by E_2 by suppressing sAHP following action potentials (Kumar & Foster, 2002; Carrer et al., 2003; Wu et al., 2011). E_2 can enhance intrinsic excitability, which is an underlying cellular mechanism of successful learning (Moyer et al., 1996; Thompson et al., 1996) and may offer a potential mechanism for extinction. To date, whether E_2 enhances intrinsic excitability in the IL-mPFC pyramidal neurons remains unknown. To test this, female OVX rats received systemic injections of E_2 for three days before they were euthanized for recordings to ensure the effects of excitability with E_2 priming. Previously, E_2 priming has been shown to affect membrane properties and excitability in neurons (Schiess et al., 1988). Furthermore, we used patch-clamp electrophysiology to record APs evoked by brief current pulses in layer 5 IL-mPFC pyramidal neurons (Figure 5A). Bath-application of E_2 (25 nM) increased the number of evoked APs in slices extracted from rats that had previously received systemic injections of E_2 (Figure 5C). To ensure that excitability is drug-dependent and not due to the current injections, cells in the control group (n = 8) received the same number of current injections over time as the experimental group (n = 9; Figure 5C). ANOVA revealed a significant effect of treatment ($F_{(2,105)} = 8.139$, $p < 0.001$), an effect of time ($F_{(5, 105)} = 2.511$, $p < 0.05$) but no treatment by time interaction ($F_{(5, 105)} = 1.527$, $p > 0.05$). Furthermore, E_2 caused membrane depolarization and decreased rheobase, which are also indicative of increased excitability and this was not observed in control slices treated with aCSF (Otis et al., 2013; Table 1). E_2 did not reduce sAHP which suggests that E_2 does not increase excitability by reducing sAHP in IL-mPFC pyramidal neurons.

Figure 5

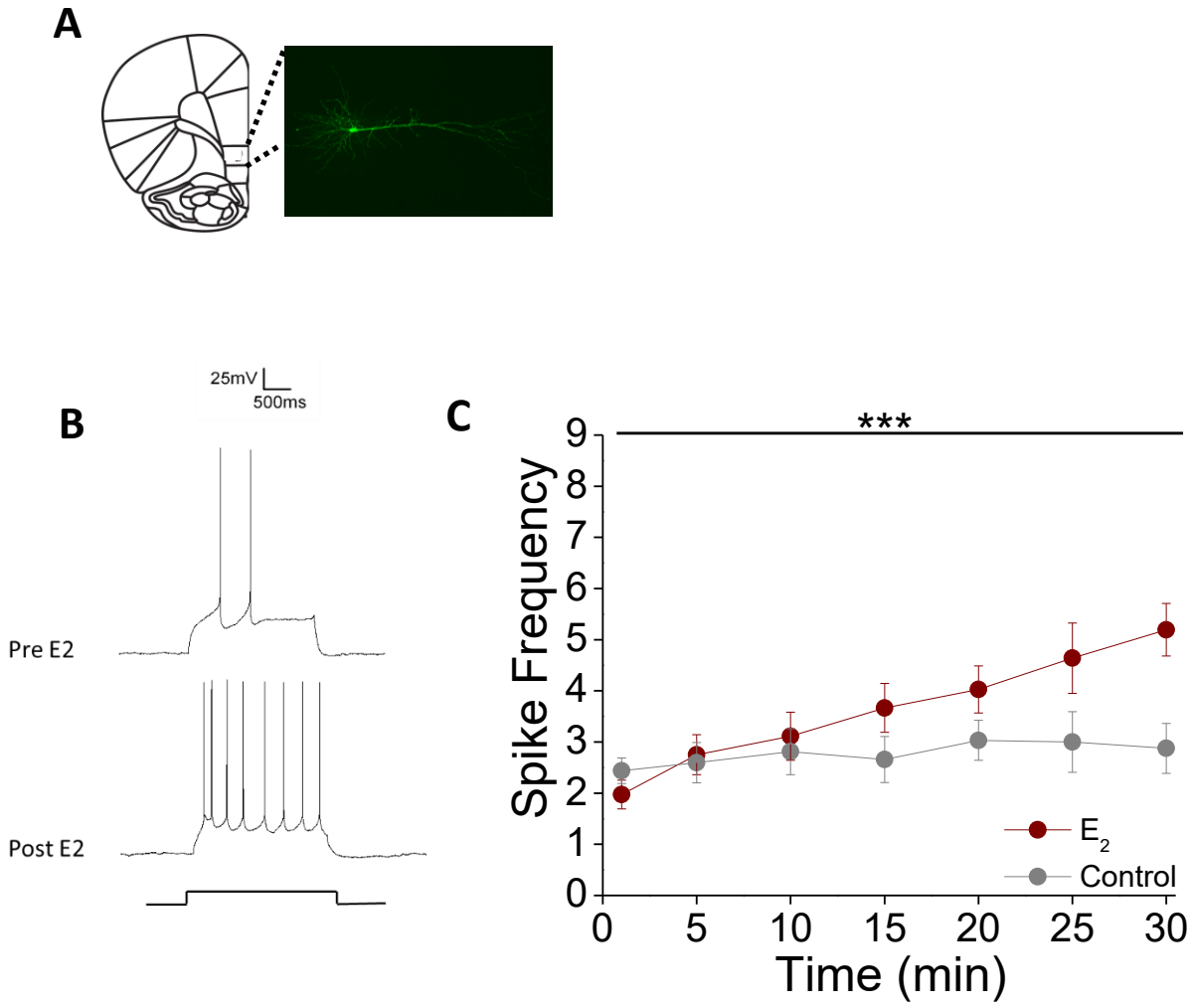

Figure 5. Bath-application of E_2 potentiates IL-mPFC pyramidal neuron excitability in slices from OVX rats that were primed with E_2. (A) Photomicrograph of a biocytin-filled pyramidal neuron. **(B)** Individual traces of current-evoked APs from an IL-mPFC pyramidal neuron before and after E_2 application. **(C)** E_2 increases AP frequency. *** $p < 0.001$. Error bars indicate SEM.

Table 1. Effects of E_2 on intrinsic excitability of IL-mPFC pyramidal neurons in slices from OVX rats primed with E_2

Drug	Time	R_N (MΩ)	Vm (mV)	Rheo (pA)	sAHP (mV)	AP_{hw} (ms)	AP_{amp} (mV)
E_2	Pre	277 ± 35	-60 ± 0.6	33 ± 6	0.15 ± 0.3	1.25 ± 0.1	38.8 ± 2.3
	Post	318± 44*	-59 ± 0.0*	27 ± 6*	-0.01 ± 0.2	1.20 ± 0.1*	33.8 ± 2.6
aCSF	Pre	347 ± 30	-59 ± 1	24 ± 3	-0.8 ± 0.3	1.50 ± 0.1	39 ± 1.3
	Post	403 ± 44**	-59 ± 0.8	23 ± 4	-0.08 ± 0.2	1.50 ± 0.1	33 ± 1.4*

E_2, 17β-estradiol; R_N, input resistance; Vm, resting potential; Rheo, rheobase; sAHP, slow afterhyperpolarization; AP_{hw}, Action Potential half-width; AP_{amp}, Action Potential amplitude. *$p < 0.05$, **$p < 0.001$ as compared to pre-E_2 application.

To control for hormonal manipulations prior to recording, we systemically injected OVX female rats with HBC vehicle for three days before they were euthanized for patch-clamp recordings. The brain slices extracted from these rats were incubated in 25nM E_2 or aCSF and the number of evoked APs were recorded from IL-mPFC (Figure 6A). To ensure that excitability is drug-dependent and not due to the current injections, cells in the control group (n = 9) received the same number of current injections over time as the experimental group (n = 9; Figure 6C). Bath-application of E_2 (25 nM) increased the number of evoked APs in slices extracted from rats that previously received HBC injections (Figure 6C). ANOVA revealed an effect of treatment ($F_{(2,112)} = 14.976$, $p < 0.001$), but no effect of time ($F_{(5,112)} = 1.916$, $p > 0.05$) or treatment by time interaction ($F_{(5,112)} = 0.263$, $p > 0.05$). E_2 did not cause membrane depolarization or reduce sAHP in IL-mPFC neurons (Table 2). Thus, bath-application of E_2 enhances excitability in IL-mPFC pyramidal neurons without E_2 priming.

Figure 6

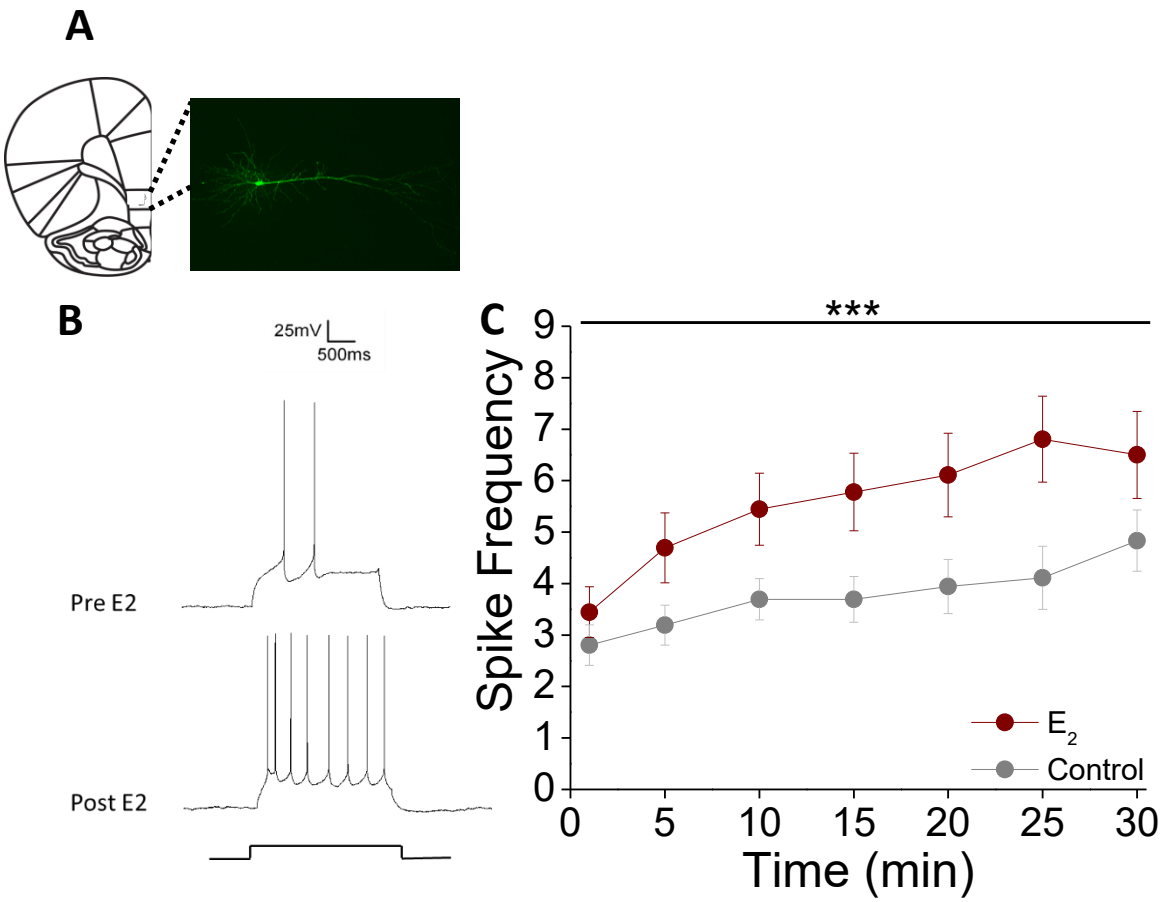

Figure 6. Bath-application of E_2 potentiates IL-mPFC pyramidal neuron excitability in slices from OVX rats that were not primed with E_2. (A) Photomicrograph of a biocytin-filled pyramidal neuron. **(B)** Individual traces of current-evoked APs from an IL-mPFC pyramidal neuron before and after E_2 application. **(C)** E_2 increases AP frequency. *** $p < 0.001$. Error bars indicate SEM.

Table 2. Effects of E_2 on intrinsic excitability of IL-mPFC pyramidal neurons in slices from OVX rats that were not primed with E_2

Drug	Time	R_N (MΩ)	Vm (mV)	Rheo (pA)	sAHP (mV)	AP_{hw} (ms)	AP_{amp} (mV)
E_2	Pre	273 ± 38	-59 ± 0.5	29 ± 4	-0.03 ± 0.1	1.0 ± 0.0	38.9 ± 2.9
	Post	306± 44*	-58 ± 0.5	19 ± 4**	-0.1 ± 0.2	0.95 ± 0.0**	33.9 ± 3.0*
aCSF	Pre	309 ± 52	-57 ± 0.5	36 ± 7	-0.4 ± 0.2	1.1 ± 0.0	34.6 ± 2.7
	Post	310 ± 21	-57 ± 0.8	28 ± 6**	-0.1 ± 0.2	1.07 ± 0.0**	30.6 ± 3.1*

E_2, 17β-estradiol; R_N, input resistance; Vm, resting potential; Rheo, rheobase; sAHP, slow afterhyperpolarization; AP_{hw}, Action Potential half-width; AP_{amp}, Action Potential amplitude. *$p < 0.05$, **$p < 0.001$ as compared to pre-E_2 application.

Trk receptor blockade prevented E_2-induced potentiation of IL-mPFC pyramidal neuron excitability

Intrinsic excitability of cortical pyramidal neurons is potentiated by Trk receptor activation (Marongiu, Imbrosci, & Mittmann, 2013) and may offer a potential a mechanism by which Trk receptors mediate extinction. Whether Trk receptor blockade prevents E_2-induced enhancement of intrinsic excitability in IL-mPFC pyramidal neurons is unknown. Bath-application of E_2 enhances excitability in IL-mPFC neurons with or without prior systemic E_2 administration (Figure 5, Figure 6). To examine the role of Trk receptors in E_2-induced excitability of IL-mPFC neurons (Figure 7A), OVX female rats received systemic HBC vehicle injections before they were euthanized for patch-clamp recordings. Slices were incubated for 20 min in 100 nM K-252a (Montalbano et al., 2013) and E_2 (25 nM) was bath-applied. The effect of E_2 was blocked by bath-application of K-252a (Figure 7B), indicating that Trk receptor blockade prevents E_2-induced potentiation of IL-mPFC neuron excitability. Comparing neurons treated with E_2 (n = 9), E_2 + K-252a (n = 7), and control (n = 9) ANOVA revealed an effect of treatment ($F_{(4,147)} = 4.932$, $p < 0.001$), but no effect of time ($F_{(5,147)} = 1.750$, $p > 0.05$, or treatment by time interaction ($F_{(10,112)} = 1.061$, $p > 0.05$). E_2 reduces rheobase, AP half-width, and AP amplitude and this change is not present in the presence of K-252a (Table 3). Thus, E_2 potentiates excitability in IL-mPFC neurons via a Trk receptor-dependent mechanism.

Figure 7

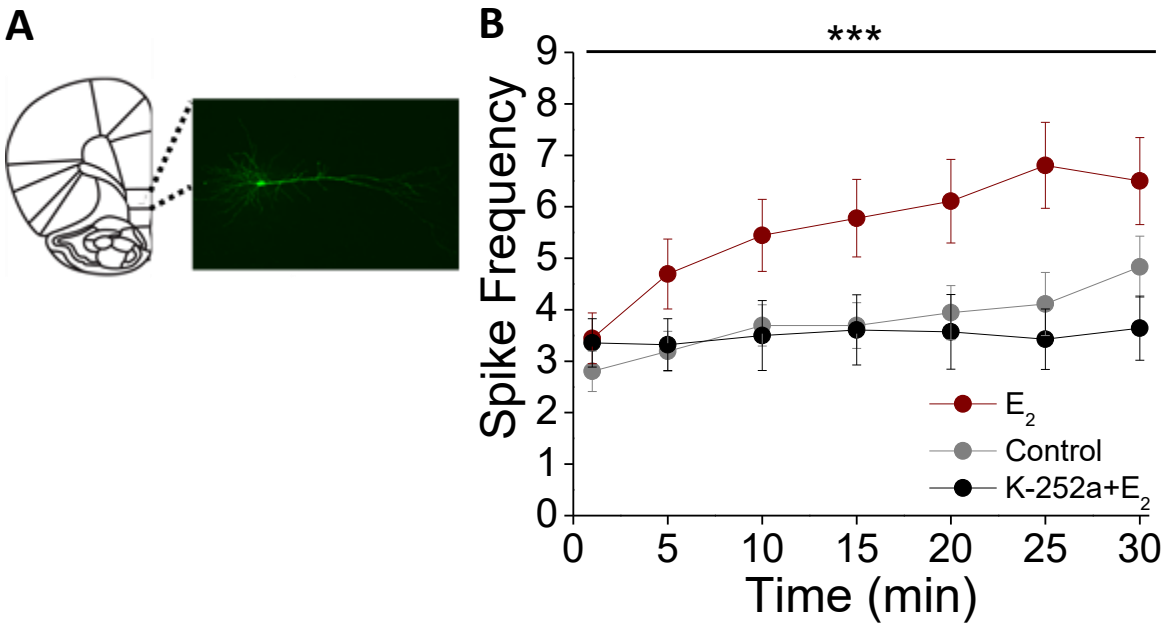

Figure 7. Blockade of Trk receptors prevent E_2-induced potentiation of IL-mPFC neuronal intrinsic excitability (A) Photomicrograph of a biocytin-filled pyramidal neuron. **(B)** Trk receptor blockade prevents E_2-induced increases in AP. *** $p < 0.001$. Error bars indicate SEM.

Table 3. Effects of E_2 and K-252a on intrinsic excitability of IL-mPFC pyramidal neurons

Drug	Time	R_N (MΩ)	Vm (mV)	Rheo (pA)	AP_{hw} (ms)	AP_{amp} (mV)
E_2	Pre	273 ± 38	-59 ± 0.5	29 ± 4	1.0 ± 0.0	38.9 ± 2.9
	Post	306± 44*	-58 ± 0.5	19 ± 4**	0.95 ± 0.0**	33.9 ± 3.0*
E_2+K-252a	Pre	266 ± 39	-59 ± 0.1	40 ± 4	1.5 ± 0.1	32.9 ± 4.0
	Post	322 ± 38*	-60 ± 0.4	37 ± 6	1.4 ± 0.05	29.1 ± 4.1

E_2, 17β-estradiol; R_N, input resistance; Vm, resting potential; Rheo, rheobase; sAHP, slow afterhyperpolarization; AP_{hw}, Action Potential half-width; AP_{amp}, Action Potential amplitude. *$p < 0.05$, **$p < 0.001$ as compared to pre-E_2 application.

E_2 does not facilitate consolidation of extinction of a cocaine CPP

Previous work demonstrated that E_2 is necessary for the extinction of a CPP (Twining et al., 2013), but did not dissociate the role of E_2 in acquisition versus consolidation of extinction. To dissociate these processes, we focused on the consolidation period immediately after an extinction trial. OVX female rats received systemic injections of either 0.2 mg/kg E_2 (n = 6; Gresack & Frick, 2006) or HBC (n = 7) vehicle immediately after each extinction session (Figure 8). A mixed model three-way ANOVA revealed a significant effect of chamber overall ($F_{(2, 22)}$ = 10.4, $p < 0.01$). However, there was no significant group by chamber by trial interaction ($F_{(8, 88)}$ = 1.152, $p > 0.05$) or trial by chamber effect ($F_{(8, 88)}$ = 1.623, $p > 0.05$). *Post hoc* analysis confirmed that the both E_2-treated and vehicle-treated rats showed a significant preference for the cocaine-paired chamber on trials 1 and 2 (*post hoc* $p < 0.05$). Vehicle-treated rats also showed a significant preference on trial 4 (*post hoc* $p < 0.05$). Thus, post-injections of E_2 did not enhance consolidation of extinction of a cocaine CPP.

Figure 8

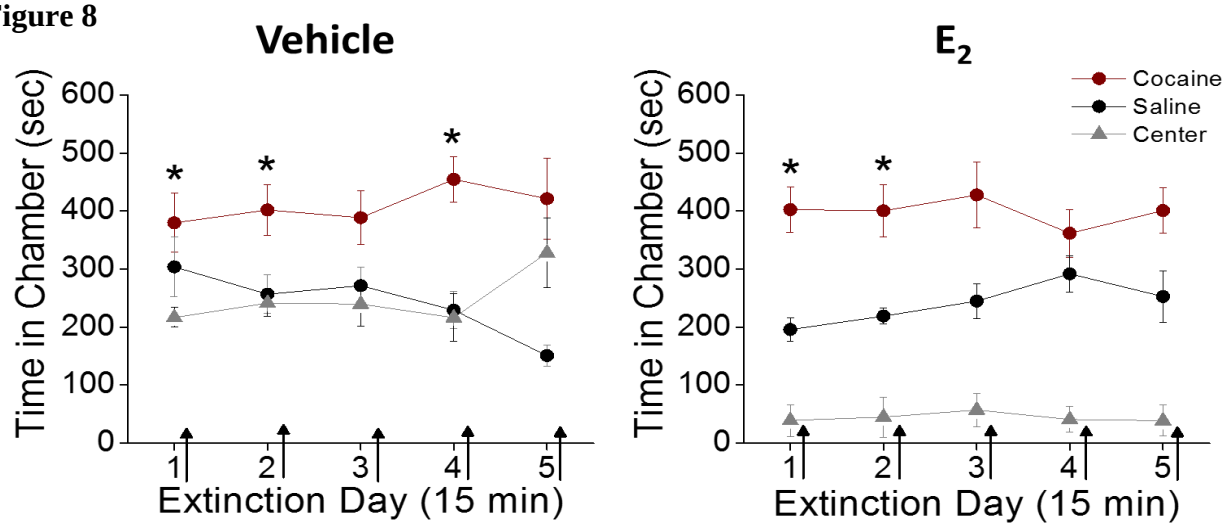

Figure 8. Post-injections of E_2 did not facilitate consolidation of extinction of a cocaine CPP. Systemic injections (arrows) of E_2 (n = 6) or vehicle (n = 7) immediately after a CPP trial did not facilitate consolidation of extinction. * $p < 0.05$.

DISCUSSION

The aim of these experiments was to determine the neurotrophic mechanism underlying E_2-induced facilitation of extinction of a cocaine-induced CPP. Based on previous data from the lab (Twining et al., 2013) we expected that E_2, when administered daily, would mediate extinction of cocaine seeking in female rats. Moreover, we hypothesized that E_2-induced facilitation is likely mediated via BDNF/TrkB signaling. However, systemic injections of E_2 prior to each CPP trial did not consistently facilitate extinction (Figure 1). Inconsistent with previous findings (Twining et al., 2013), extinction was evident in the absence of E_2 (Figure 3). Contrary to expectations, direct infusions of the non-selective Trk receptor antagonist, K-252a in IL-mPFC prior to 30 min extinction trials did not impair but facilitated extinction (Figure 1, Figure 2). These results suggest that Trk receptors in IL-mPFC may not play a direct role in mediating the effects of E_2-induced facilitation of extinction. To examine whether TrkB receptors mediated extinction, a highly selective TrkB receptor antagonist, ANA-12 was systemically administered to female rats in conjunction with E_2 (Figure 4). To reduce the variability observed with 30 min extinction trials, 15 min shortened trials were used (Twining et al., 2013). Female rats that received E_2 and vehicle appeared to show facilitated extinction and this effect was not consistently reported in this study. In contrast, rats that were administered E_2 and ANA-12 had impaired extinction of cocaine seeking (Figure 4). The effects of E_2 on the consolidation of extinction of a cocaine CPP were also examined. Post-extinction injections of E_2 did not enhance the consolidation of extinction of a CPP (Figure 8).

Next, we hypothesized that intrinsic excitability would be potentiated by E_2 in IL-mPFC pyramidal neurons and blockade of Trk receptors would attenuate the effects of E_2. OVX female rats received systemic injections of E_2 or HBC vehicle injections prior to being euthanized for

recordings to investigate whether E_2 priming affects membrane properties (Schiess et al., 1988). Bath-application of E_2 enhanced intrinsic excitability in IL-mPFC neurons, independent of E_2 priming (Figure 5, Figure 6). However, this potentiation was significantly attenuated in the presence of the Trk receptor antagonist, K-252a (Figure 7). Overall, we were unable to consistently replicate the original findings of Twining et al., (2013) and show that E_2 is necessary for the acquisition or consolidation of extinction of cocaine seeking. This study suggests that E_2 may be a modulator and not necessarily a mediator of extinction and future studies are required to elucidate the underlying BDNF/TrkB mechanism. However, we were able to support the hypothesis that E_2 enhances intrinsic excitability via BDNF/TrkB signaling in IL-mPFC neurons. This provides evidence that E_2 and TrkB signaling may be modulating neuronal excitability, thereby, facilitating future synaptic plasticity and learning.

Role of Trk receptors in extinction of cocaine seeking

The results of this study were unable to consistently replicate the findings that E_2 is necessary for the acquisition of extinction of a cocaine CPP (Twining et al., 2013). For example, in Figure 1, rats that received IL-mPFC infusions of vehicle and systemic injections of E_2 prior to a 30 min CPP trial showed a significant preference for the cocaine-paired chamber till extinction day 6. A similar trend was also observed in Figure 2 as rats showed a preference for the cocaine-paired chamber till extinction day 4. This data may suggest that E_2 injections before an extinction trial may be necessary to retrieve the original drug memory. This effect has also been observed in a previous study which demonstrated that following conditioning, OVX rats showed significantly low preferences to the cocaine-paired chamber compared to cycling females (Russo et al., 2003). The retrieval deficit was rescued when OVX females were treated with E_2 (Russo et al., 2003).

Thus, these findings and our results suggest that E_2 may play a role in retrieval of the original drug memory rather than enhancing acquisition of extinction.

E_2-facilitated extinction of a cocaine CPP was observed in Figure 4 when rats were exposed to short 15 min extinction trials, instead of 30 min extinction trials. However, when OVX rats were administered E_2 immediately after a 15 min extinction trial, E_2 did not significantly enhance consolidation of extinction compared to rats that received HBC vehicle (Figure 8). Findings from Twining et al., (2013) showed that 15 min extinction trials were sufficient to consistently facilitate extinction induced by E_2. It is possible that the variable effects of E_2 may be due to small sample sizes of 6-8 rats per group. In summary, the present study shows that 30 min extinction trials appear to enhance retrieval of the original drug memory and 15 min extinction trials provide variable results regarding the role of E_2 in extinction. Thus, these experiments need to be repeated with 15 min extinction trials and larger sample sizes.

Inconsistent with previous findings (Twining et al., 2013), extinction was observed in the absence of E_2 (Figure 3). Data from Twining et al., (2013) demonstrated that E_2 is necessary for extinction of a cocaine CPP as E_2-treated rats extinguished within a week, whereas vehicle-treated rats continued to express a CPP for over 5 weeks. In the present study, rats that received IL-mPFC infusions of vehicle and systemic injections of HBC vehicle expressed a CPP on extinction day 1 but did not show preference for the cocaine-paired chamber during subsequent trials (Figure 3). Issues with replication may be due, in part, to the shorter extinction protocols used in the present experiments. The extinction trial protocol used in the previous study (Twining et al., 2013) lasted between 16-32 days in which CPP expression was initially poor but stabilized across days. Thus, future studies require optimizing a reliable short protocol to examine CPP in females to limit variability across studies.

The results of the study did not support our hypothesis that direct Trk receptor blockade in IL-mPFC impairs E_2-facilitated extinction of cocaine seeking. IL-mPFC infusions of a non-selective Trk receptor antagonist, K-252a did not impair but facilitated extinction of a cocaine CPP (Figure 2, Figure 3). A possible explanation for these results may be that K-252a did not affect the new extinction memory, but instead affected the original drug-memory by disrupting reconsolidation. Reconsolidation involves re-stabilizing a memory after the memory has been retrieved and made labile again. For example, following reactivation of an alcohol self-administration memory, administration of NMDA receptor antagonist MK-801 resulted in a strong trend for reconsolidation disruption when tested the following day (Wouda et al., 2010). Similar effects were observed in studies in our lab that aimed to disrupt extinction of a drug-memory in a self-administration model via NMDA receptor antagonists. Infusions of NMDA receptor antagonists appeared to facilitate extinction rather than disrupting it. However, IL-mPFC infusions of NMDA receptor antagonists immediately after short reactivation sessions confirmed that reconsolidation of the original drug memory had been disrupted instead of extinction memory (Hafenbreidel et al., *unpublished)*. Thus, even though Trk receptor blockade has previously been shown to disrupt extinction (Xin et al., 2014), these studies indicate that Trk receptors may also be implicated in reconsolidation of the drug-memory.

The present study was partially able to support the hypothesis that TrkB receptor blockade impairs E_2-induced facilitation of extinction. Rats that were administered systemic injections of TrkB receptor antagonist, ANA-12 and E_2 continued to show a preference for the cocaine-paired chamber during short 15 min extinction trials, demonstrating impaired extinction (Figure 4). Rats that received vehicle and E_2 treatment only expressed a CPP on extinction day 1 and continued to show similar preferences for the cocaine- and saline-paired chambers, indicating facilitated

extinction. The results of this particular experiment is in agreement with our hypothesis that TrkB receptor blockade impairs E_2-induced facilitation of extinction. However, it is important to note that we did not observe clear E_2-facilitated extinction in other experiments in this study. To reduce variability in our results, it is necessary to repeat the experiment with larger sample sizes and 15 min extinction trials. Furthermore, to ensure that TrkB receptor blockade impairs extinction, future work should also control for hormonal manipulations and include groups that do not receive E_2 treatment during extinction.

Another possible explanation for the discrepancy in our behavioral data may be stress, which can alter BDNF protein or mRNA expression (Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003; Franklin & Perrot-Sinal, 2006); therefore, E_2 withdrawal or administration may have variable effects on behavior if the rodent has not been handled carefully. Furthermore, prior behavioral handling has also been shown to modify the effects of E_2, and thus, influence changes in spine synapses. For example, OVX rats treated with estradiol benzoate, which also had a 1-day Morris water maze testing did not have increased CA1 spine synapse densities. However, increases in spine synapse densities were observed in behaviorally naïve OVX rats that received hormone treatment (Frick et al., 2004). Thus, stress or prior experiences such as conditioning may have altered the effects of E_2 or BDNF, which may add to the discrepancies in our behavioral data.

A limitation of the present study was that we were unable to test different doses of K-252a or ANA-12 that may impair extinction. Since we did not see differences between groups that were treated with Trk receptor antagonists (Figure 3), we can speculate that the selected doses may not be sufficient to impair memory. The dose for K-252a was chosen from a previous study (Xin et al., 2014), which was infused in IL-mPFC to impair extinction of a conditioned taste aversion

paradigm in male rats. Furthermore, the dose for ANA-12 has previously been used to reduce self-administration of cocaine in male rats (Vassoler, White, Schmidt, Sadri-Vakili, & Pierce, 2013). Thus, due to sex differences or experimental parameters these doses may not apply to impair extinction of a cocaine CPP in female rats. For future studies, it may be important to test different doses of K-252a and ANA-12 on extinction without E_2 administration. Furthermore, the lowest dose of K-252a and ANA-12 that impairs extinction should be chosen to ensure that Trk receptor antagonists do not impair extinction memory on its own but through an E_2-mediated mechanism (Floresco & Jentsch, 2011).

In summary, due to variability in our results, we are unable to conclude that E_2 mediates extinction of cocaine seeking as previously demonstrated by Twining et al., (2013). Issues with replication may be due, in part, to the shorter extinction protocols as we did not run the 16-32 extinction trial protocol used previously. Low sample sizes may also contribute to variability in our data. However, the study was able to demonstrate an interaction between E_2 and BDNF in a CPP paradigm, and this interaction has not been observed in other behavioral paradigms. Thus, the present study provides insight into the E_2-BDNF interaction and its role in learning and memory.

E_2 potentiates intrinsic excitability in IL-mPFC pyramidal neurons via a BDNF-dependent mechanism

Our findings support the hypothesis that E_2 enhances intrinsic excitability in IL-mPFC neurons via a BDNF-dependent mechanism. These findings in IL-mPFC are novel and the first to highlight the E_2 potentiated mechanism underlying extinction of drug seeking or fear conditioning. This study aimed to measure intrinsic excitability which lowers the threshold for synaptic changes and is a neural correlate of learning. A significant amount of attention has been

given to synaptic plasticity as a mechanism for learning. However, evidence from recent studies show that learning and patterns of neuronal activity produce diverse and global changes by regulating membrane properties. Furthermore, changes in excitability are often expressed as changes in resting and voltage-dependent channels and ion pumps (Daoudal & Debanne, 2003; Mozzachiodi & Byrne, 2010; Sehgal, Song, Ehlers, & Moyer, 2013). Thus, enhanced intrinsic excitability has been shown to be correlated with enhanced learning. For example, increased intrinsic excitability has been observed following eyeblink conditioning and trace fear conditioning, (Kaczorowski & Disterhoft, 2009; McKay, Matthews, Oliveira, & Disterhoft, 2009; Song et al., 2012), which are widely used paradigms to study basic mechanisms of learning and memory. Specifically, intrinsic plasticity has been shown to be important for extinction. *In vitro* studies have demonstrated that fear conditioning depressed IL-mPFC excitability, which was demonstrated by a decreased number of APs and increased sAHP. Moreover, extinction reversed the conditioning-induced effects and increased number of APs (Santini, Quirk, & Porter, 2008). Thus, non-synaptic or intrinsic plasticity plays a crucial role in shaping adaptive behaviors such as extinction.

Our results reveal that E_2 enhances intrinsic excitability in IL-mPFC parallel earlier findings from hippocampal pyramidal neurons (Kumar & Foster, 2002; Carrer et al., 2003; Wu et al., 2011), which implicate the crucial role of E_2 in learning and memory. In addition to increasing spontaneous firing in hippocampal neurons, bath-application of E_2 can enhance baseline synaptic responses by increasing the amplitude of excitatory postsynaptic potential (EPSPs) in CA3-CA1 synapses in slices from male and female rodents (Teyler, Vardaris, Lewis, & Rawitch, 1980; Wong & Moss, 1992; Foy et al., 1999; Fugger, Kumar, Lubahn, Korach, & Foster, 2001). E_2 can also initiate a rapid and reversible increase in kainate-induced currents in CA1 hippocampal

neurons (Wong & Moss, 1991, 1992). Enhancement of kainate-induced currents by E_2 was blocked by a blocker of protein kinase A (PKA) suggesting that E_2 may activate intracellular second messenger systems to modulate neuronal function (Gu & Moss, 1996). E_2 has been shown to potentiate long-term potentiation (LTP) in CA3-CA1 synapses in slices from female rats (Bi, Broutman, Foy, Thompson, & Baudry, 2000; Foy, 2001; Woolley, 2007). LTP is the persistent strengthening of synapses and the underlying mechanism of synaptic plasticity, learning, and memory (Malinow, Schulman, & Tsien, 1989). Thus, the short and long-term effects of extinction may be mediated by E_2-enhanced intrinsic excitability and synaptic potentiation.

Similar to the effects of E_2 on synaptic plasticity, perfusion of BDNF induces LTP in the hippocampus (Ying et al., 2002; Ji et al., 2010). The role of Trk receptors in LTP have also been shown in mice carrying a mutated TrkB receptor ($TrkB^{2478A/2478A}$), which cannot be phosphorylated. Brain slices from $TrkB^{2478A/2478A}$ mice show reductions in late LTP in CA3-CA1 synapses (Lai et al., 2012). However, the role of BDNF or Trk receptors in intrinsic excitability remains unclear. For example, BDNF has shown to reduce excitability in visual cortical cultures (Desai, Rutherford, & Turrigiano, 1999), but TrkB receptor activation enhanced excitability in slices or cortical neurons (Marongiu et al., 2013). TrkB receptor activation also enhanced NMDA-receptor mediated currents in IL-mPFC neurons from male rats (Otis et al., 2014). Our results advance the findings by demonstrating the role of Trk receptors in non-synaptic plasticity in IL-mPFC, a region crucial for extinction.

In summary, our data reveal that E_2 enhances non-synaptic plasticity in IL-mPFC pyramidal neurons. Previous work has been predominantly focused on E_2-enhanced excitability and synaptic activity in the hippocampus (Wong & Moss, 1991; Kumar & Foster, 2002; Carrer et al., 2003). Thus, our findings that E_2-induced potentiation of intrinsic excitability in IL-mPFC

complements previous work in the hippocampus. Since both the hippocampus and IL-mPFC are engaged in extinction, these findings suggest that E_2 plays a critical role in modulating extinction. Finally, our results suggest that E_2-enhanced excitability in IL-mPFC neurons is mediated by BDNF/TrkB receptor activation and this interaction may likely regulate extinction across paradigms.

Future directions

Despite the currently conflicting data regarding the role of E_2 in extinction of a cocaine CPP, previous research shows that E_2 enhances performance on a wide range of cognitive tasks as well as extinction in other paradigms (Luine et al., 2003; Milad et al., 2009; Tuscher et al., 2015). E_2 replacement or elevated E_2 levels are associated with improved spatial reference memory in a water maze task (Gresack & Frick, 2006), object recognition (Lewis et al., 2008; Jacome et al., 2010), and extinction of conditioned fear in female rats and humans (Zeidan et al., 2011; Graham & Milad, 2013). Conversely, low levels of E_2 are associated with extinction failure in females with post-traumatic stress disorder (Glover et al., 2012), Furthermore, low levels of E_2 in both rats and humans leads to extinction impairment of conditioned fear (Graham & Milad, 2013). Thus, the effects of E_2-induced enhancement of extinction that vary in a CPP paradigm, may be more robust in other paradigms such as fear conditioning, or cocaine self-administration

In addition to investigating the role of BDNF as an underlying mechanism for E_2-induced facilitation of extinction, it is important to target another neurotrophic factor, basic fibroblast growth factor (bFGF). Studies conducted in our lab have shown that cocaine-related learning increases bFGF protein expression in IL-mPFC. Furthermore, blocking bFGF in IL-mPFC reverses this enhanced expression and facilitates extinction of cocaine seeking (Hafenbreidel, Twining, Rafa Todd, & Mueller, 2015). bFGF has also shown to reduce intrinsic excitability in

hippocampal pyramidal neurons by inhibiting voltage-gated Na+ (Hilborn, Vaillancourt, Rane, 1998) and K+ currents (Cuppini, Ambrogini, Lattanzi, Ciuffoli, & Cuppini, 2009). Reduced excitability is associated with decreased synaptic plasticity (Gelinas & Nguyen, 2005), thus, increased bFGF expression would potentially reduce extinction-related synaptic plasticity. Previously, E_2 has been shown to interact with bFGF as loss of circulating E_2 resulted in high bFGF expression (Flores, Salmaso, Cain, Rodaros, & Stewart, 1999). E_2 replacement reduced bFGF expression (Flores et al., 1999), which suggests that E_2 may enhance extinction by reversing the maladaptive overexpression of E_2 observed with cocaine-related learning. E_2 may also have the potential to reduce intrinsic excitability and promote extinction-related plasticity. Thus, bFGF may be a promising target to elucidate the mnemonic and neural mechanisms, underlying E_2-induced enhancement of extinction.

CONCLUSION

Persistent drug seeking is maintained by drug-associated memories, through which cues can elicit craving and relapse. Impairing drug-associated memories may dampen the motivation to seek drugs and limit relapse susceptibility. Specifically, women are more vulnerable than males to key phases of addiction, which include initiation, bingeing, and relapse (Anker & Carroll, 2010). Rehabilitation programs that use exposure therapy, in which addicts are exposed to a drug-associated cue in the absence of drug reinforcement have had limited success without any pharmacological adjuncts (Conklin & Tiffany, 2002). Thus, pharmacological enhancement of E_2 or BDNF/TrkB signaling may prove to be clinically relevant for treatment of disorders involving maladaptive memories and behavioral inflexibility such as addiction or posttraumatic stress disorder.

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