Estradiol-induced enhancement of fear extinction in female rats: the role of NMDA receptor activation

Running title: Estradiol, NMDAr activation, and fear extinction

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Abstract

Converging cross-species evidence indicates that fear extinction (the laboratory basis of exposure therapy for anxiety disorders) in females is modulated by endogenous and exogenous estradiol. The mechanisms underlying estradiol's influences on fear extinction are largely undefined. However, one likely candidate is the NMDA-receptor (NMDAr), activation of which is necessary for estradiol-mediated enhancements in structural and functional neural plasticity, as well as extinction consolidation in males. Here, we demonstrate that systemic co-administration of the non-competitive NMDAr antagonist, MK801, blocked the enhancement of fear extinction by systemic estradiol in ovariectomized rats. In intact rats, MK801 during diestrus (rising estradiol) prevented the enhancement in extinction recall in rats that received extinction training during proestrus (peak estradiol). Systemic administration of the partial NMDAr agonist D-cycloserine (DCS) prior to extinction training facilitated extinction in ovariectomized rats, mimicking the effects of estradiol. In intact rats, DCS administered on the afternoon of proestrus and the morning of estrus (declining estradiol) facilitated extinction in rats that received extinction training during metestrus (low estradiol). Finally, DCS also facilitated extinction in ovariectomized rats when administered immediately after extinction training. Combined, these findings suggest that endogenous and exogenous estradiol enhance fear extinction via NMDAr-dependent mechanisms. Moreover, these findings raise the possibility that fear extinction deficits during periods of low endogenous estradiol levels can be reversed by increasing NMDAr activation via DCS administration, either well prior to, or immediately after, extinction training.

Key Words: Fear Extinction; Fear Conditioning; Anxiety Disorders; Estradiol; NMDA; D-Cycloserine

1. Introduction

Anxiety disorders are more prevalent, burdensome, and associated with poorer treatment outcomes, in women relative to men (Li & Graham, 2017; McLean et al., 2011). Despite this, current neurobiological theories of fear extinction, the learned reduction in fear that occurs following repeated exposure to a fear-eliciting conditioned stimulus, are founded on preclinical research that has mostly been conducted in males (Lebron-Milad & Milad, 2012). Studies of extinction and the pharmacological adjuncts that augment it provide important insights about how to improve exposure therapy, the recommended psychological treatment for anxiety disorders that was based on extinction (Graham & Milad, 2011). It is therefore critical that contemporary models of extinction are informed by studies conducted in females.

The sex hormones estradiol and progesterone substantially modulate extinction in females. Extinction is enhanced during phases of the reproductive cycle characterized by high hormonal levels, and impaired during low hormonal phases, an effect observed in rats, non-anxious women, and clinically anxious women (reviewed in Glover et al., 2015; Li & Graham, 2017). Furthermore, estradiol administration, or pharmacological enhancement of estrogen receptors (at the time of extinction training), facilitates extinction in rats and women (Graham & Milad, 2013; Graham & Scott, 2018; Zeidan et al., 2011). Finally, we have recently demonstrated that higher estradiol levels during exposure therapy for spider phobia are associated with improved treatment efficiency and effectiveness, suggesting that the relationship between estradiol and extinction in laboratory studies translates comparably to clinical treatment settings (Graham et al., 2018). Estradiol enhancements in fear extinction are associated with alterations in ventromedial prefrontal cortex (vmPFC), hippocampus, and amygdala activity, key components of the extinction neurocircuitry (Zeidan et al., 2011). Beyond this, the mechanisms underlying hormonal modulation of extinction are poorly

understood. The acute effects of estradiol on extinction are likely driven by rapid facilitation of cell signaling, long-term potentiation (LTP), and epigenetic modifications supporting memory consolidation (Frick, 2015). However, cyclic fluctuations in extinction may also be attributable to longer-term effects of estradiol that are subject to biphasic regulation by progesterone, as highlighted by our recent study in which we subjected ovariectomized (OVX) rats to a hormonal replacement regime mimicking cyclic fluctuations in sex hormones (Graham & Daher, 2016). We demonstrated that two treatments of estradiol alone facilitated extinction, whereas progesterone augmented and then attenuated estradiol's effects in a time-dependent manner. As extinction training took place 48-72 h following estradiol treatment, the augmentation of extinction could not be attributed to rapid effects of estradiol on cell signaling processes. Moreover, in intact rats, pharmacological inactivation of the progesterone receptor during proestrus (peak estradiol and progesterone) maintained extinction at optimal levels 48 h later during metestrus (basal estradiol and progesterone), when extinction is typically impaired. This suggests that, when unopposed by progesterone, cyclic increases in estradiol facilitate long-term enhancements in extinction that are sustained after estradiol levels decline.

One candidate pathway by which estradiol could enhance extinction is via the N-methyl-D-aspartate receptor (NMDAr). Estradiol facilitates NMDAr binding, phosphorylation, and transmission in the hippocampus and PFC (Bi et al., 2001; Galvin & Ninan, 2014; Smith and McMahon, 2005; Weiland et al., 1992; Woolley et al., 1997), and pharmacological blockade of NMDAr prevents estradiol's impact on brain functioning (e.g., facilitated LTP; Smith & McMahon, 2006; Smith et al., 2016) and morphology (e.g., augmented dendritic spine density; Woolley & McEwen, 1994; Smith & McMahon, 2005). Extensive evidence demonstrates that extinction in male rats is dependent on NMDAr activation within the mPFC, hippocampus, and amygdala (reviewed in Singewald et al.,

2015); surprisingly, the role of NMDAr in extinction in females has not been systematically examined. However, estradiol's enhancement of other kinds of learning, such as object recognition, is NMDAr-dependent (Lewis et al., 2008; Vedder et al., 2013). Therefore, the present study examined the role of NMDAr in the long-term effects of estradiol on extinction in female rats. Similar to our past work (Graham & Daher, 2016), we took a two-pronged approach in which, to achieve optimal experimental control, initial experiments examined the role of NMDA in extinction in OVX rats treated with an estradiol replacement regime that mimics natural increases in estradiol during the estrous cycle (Woolley & McEwen, 1993). We subsequently validated these outcomes in intact, cycling rats to examine the role of NMDA in extinction during natural increases in estradiol during the estrous cycle. Experiment 1 assessed whether enhanced extinction in estradiol-treated OVX rats was blocked by co-administration of the NMDAr antagonist, MK801, 48 h prior to extinction training. Experiment 2 assessed whether enhanced extinction in intact rats extinguished during proestrus (peak estradiol) was blocked by MK801 administered 24 h prior to extinction training during diestrus (rising estradiol). Experiment 3 assessed whether the partial NMDAr agonist, D-Cycloserine (DCS), mimicked estradiol's effects on extinction in OVX rats when administered 48 h prior to extinction training. Experiment 4 assessed whether extinction was enhanced during metestrus (basal estradiol) by administering DCS on the afternoon of proestrus, and the morning of estrus (declining estradiol), 24 h prior to extinction training. Note that in experiments involving OVX rats (i.e., 1 and 3), extinction training was timed to take place 48 h after hormone and drug treatments because past research has demonstrated that the effects of estradiol on extinction (Graham & Daher, 2016) and brain morphology/functioning (Smith & McMahon, 2005; Woolley & McEwen, 1993) are evident in OVX rats at this time point. Thus, the timing of treatments in OVX rats mimicked the effects of the natural rise in estradiol during proestrus in intact rats. Combined, Experiments

1-4 demonstrated long-term effects of NMDAr modulation on extinction. To determine whether NMDAr modulation has also immediate effects, Experiment 5 assessed whether DCS administered immediately after extinction training enhanced extinction recall in OVX rats.

2. Materials and Methods

2.1 Subjects

Experimentally naïve Sprague Dawley-derived female rats, aged 10-12 weeks, obtained from a commercial supplier (Animal Resources Centre, Perth, Australia), were used. Rats were housed in groups of eight in plastic cages (67x30x22 cm) in a 20-22ºC colony room. They were maintained on a 12 hr light-dark cycle (lights on at 7am) with food and water available ad libitum. In Experiments 1, 3 and 5 rats received bilateral ovariectomy, and in Experiments 2 and 4 vaginal smears were conducted daily (between 8-10am) to determine estrous cycle phase in intact rats; as detailed previously (Graham & Daher, 2016). Procedures were approved by the Animal Care and Ethics Committee at UNSW Australia and followed guidelines outlined in *The Australian Code Of Practice For The Care And Use Of Animals For Scientific Purposes* (8th edition, 2013).

2.2 Pharmacological Manipulations

2.2.1 *Estradiol.* In Experiment 1, OVX rats received two s.c. injections of β-Estradiol, spaced 24 h apart (Sigma-Aldrich, Australia; 10 µg in 150 µL sesame oil), or an equivalent volume of sesame oil. We have previously demonstrated that this quantity and dose, which mimic the rise in natural estradiol across the cycle (Woolley & McEwen, 1993) augment extinction in OVX rats (Graham & Daher, 2016).

2.2.2 *MK801.* In Experiments 1 and 2, OVX and intact rats received s.c. injections of MK801 dissolved in saline (Sigma-Alrich, Australia; at a dose of 0.2 mg/ kg of body weight, in a volume of 1mL/ kg of body weight), or an equivalent volume of saline. This dose counteracts the impact of estradiol treatment (identical to that described above) on NMDAr transmission, LTP, and CA1 dendritic spine density in OVX rats (Smith & McMahon, 2005; Woolley & McEwen, 1994).

2.2.3 *D-Cycloserine (DCS).* In Experiments 3, 4, and 5, OVX and intact rats received s.c., injections of DCS dissolved in saline (Sigma-Alrich, Australia; at a dose of 15 mg/ kg of body weight, in a volume of 1mL/ kg of body weight). This dose enhances extinction in male rats (reviewed in Singewald et al., 2015).

2.3 Apparatus

2.3.1 *Conditioning and Extinction Chambers.* Two sets of four Med Associates experimental chambers, designated Context A and B, were used for conditioning, extinction training, and recall. Contexts differed in visual and tactile features, and were identical to those described previously (Graham & Daher, 2016; Milligan-Saville & Graham, 2016).

2.3.2 *Conditioned and Unconditioned Stimuli.* The CS was a white noise (4 dB above background noise) delivered through the sidewall speaker, and the US was a scrambled foot-shock (1 s, 0.6 mA) delivered through the floor.

2.4 Procedure

2.4.1 *Handling and context pre-exposure.* Rats were handled for 4-5 min each day for three consecutive days; after handling on each day rats were individually placed in Context A for 10 min.

2.4.2 *Fear conditioning.* Rats were placed in context A and after a 2 min adaptation period the CS was presented for 10 s and coterminated with the shock US. Rats received 3 CS-US pairings (intertrial interval 85-135 s, with an average of 110 s).

2.4.3 *Hormone/ drug administration.* In all experiments hormone/drug administration occurred after fear conditioning to avoid introducing potentially confounding effects on the acquisition or consolidation of learned fear. In Experiment 1, rats received two injections of estradiol or sesame oil, spaced 24 hr apart, each immediately followed by MK801 or saline. In Experiment 2, rats received a single injection of MK801 or saline on the afternoon of diestrus. In Experiment 3, DCS or saline was administered to OVX rats twice, spaced 24 hr apart. In Experiment 4, DCS or saline was administered to intact rats twice, on the afternoon of proestrus, and the morning on estrus, approximately 16 hr apart. In Experiment 5, DCS was administered to OVX rats once, immediately after extinction training. Note that the differences in the number of MK801 injections in Experiments 1 and 2, and the differences in the timing between MK801 or DCS injections and extinction training in Experiments 1-4, are due to slight differences in the timing of the effects of estradiol on fear extinction between estradiol-treated OVX rats, and intact, cycling rats.

2.4.4 *Extinction training.* Rats were placed in Context B and after a two min adaptation period, received 6 x 2 min CS presentations, with an intertrial interval of 2 min. In Experiments 1 and 3, extinction training occurred 48 hr after the final drug injection. In Experiment 2, extinction training occurred the day after conditioning during metestrus (for the Metestrus Saline group), or the day after the drug injection during proestrus (for the Proestrus MK801 and the Proestrus Saline groups). In Experiment 4, extinction training occurred the day after the final drug injection during metestrus. In Experiment 5, extinction training occurred the day after conditioning.

2.4.5 *Extinction recall.* Rats were placed in Context B, and following a 1-minute adaptation period, received a single 2 min CS presentation. In all experiments, for all groups, extinction recall was tested 24 h after extinction training, with the exception of the Metestrus saline group in Experiment 2, for whom extinction recall occurred 36 h after extinction training. This design of Experiment 2 allows for all groups to be conditioned and tested during the same estrous phase (diestrus), and for the time between conditioning and extinction recall to be equivalent across groups. However, it introduces an unavoidable confound in which the interval between conditioning and extinction training, and the interval between extinction training and extinction recall, are different between the metestrus and proestrus groups. Nonetheless, it should be noted that the critical comparison in Experiment 2 is between the two proestrus groups (for whom the time intervals across the various experimental phases is identical); the metestrus group was included merely to allow comparisons to a group for whom extinction recall is typically poor. Moreover, we have previously shown that the difference in extinction recall between proestrus and metestrus groups is evident irrespective of whether the time intervals across the experimental phases are held constant or allowed to vary (Graham & Daher, 2016; Milligan-Saville & Graham, 2016). An overview of the experimental timeline for each experiment is depicted in the first panel of each figure.

2.5 Scoring and Statistics

2.5.1 *Scoring.* Rats were scored for freezing, defined as the absence of movement except that required for respiration (Fanselow, 1980), during extinction training and recall. Freezing was scored using a time-sampling procedure whereby every three seconds the animal was scored as either “freezing” or “not freezing”. A percentage score was calculated for each animal to determine the proportion of total observations spent freezing.

2.5.2 *Statistical analyses.* In Experiment 1, a univariate Analysis of Variance (ANOVA) with the between subjects factors of hormone treatment (Estradiol or Sesame Oil) and drug condition (MK801 or Saline) was used to analyze pre-CS freezing (during the adaptation periods prior to extinction training and recall) and CS-elicited freezing during extinction recall. A mixed-model ANOVA with the same between subjects factors as above, and the within-subjects factor of extinction trial, was used to analyze CS-elicited freezing during extinction training. In Experiments 2, 3, 4, and 5, one-way ANOVAs were used to examine group differences in pre-CS freezing prior to extinction training and recall, CS-elicited freezing during recall. Mixed-model ANOVAs with the between subjects factor of group, and the within-subjects factor of extinction trial, were used to analyze CS-elicited freezing during extinction training. Main effects and interactions were investigated further with independent-samples t-tests or Student Newman Keuls test where appropriate.

3. Results

3.1 Experiment 1: Does MK801 block estradiol-induced augmentation of fear extinction in OVX rats?

In Experiment 1, MK801 was co-administered with estradiol treatment in OVX rats to determine whether this prevents estradiol-associated increases in extinction recall. Pre-CS freezing levels for all experiments are depicted in Table 1. There were no differences in pre-CS freezing during the adaptation period prior to extinction training (no main effects or interaction, largest $F(1,34) = .9$). CS-elicited freezing during extinction training and extinction recall is depicted in Figure 1. CS-elicited freezing decreased throughout extinction training (significant effect of trial, [$F(5,170)=11.97$, $P<0.0001$, partial $\eta^2 = .26$]; Figure 1B). Averaged across trials, MK801-treated rats exhibited higher CS-elicited freezing than Saline-treated rats, (main effect of MK801 treatment [$F(1,34)=10.35$, $P=0.003$, partial $\eta^2 = .23$]), but

no main effect of hormone treatment or interaction (largest F(1,34) = 2.36). Post-hoc tests indicated that Estradiol-Saline rats exhibited significantly less freezing than Estradiol-MK801 rats on trial 3 (P<0.05), with no other group differences on this trial (P=.17) or other trials (smallest P=.054).

MK801-treated rats exhibited significantly higher pre-CS freezing during the adaptation period prior to extinction recall, indicated by a main effect of MK801 treatment (F(1,34)=8.87, P=0.005, partial η^2 = .21) but no main effect of hormone treatment or interaction (largest F(1,34) = 1.23). Pre-CS freezing was included as a covariate in the subsequent analysis of CS-elicited freezing during extinction recall. This analysis revealed no significant effect of pre-CS freezing (F(1,33)=1.49) or hormone treatment (F(1,33)=3.32), but a significant effect of MK801 treatment (F(1,33)=7.2, P=0.01, partial η^2 = .18) that was qualified by a significant interaction between MK801 and hormone treatment (F(1,33)=4.13, P=0.05, partial η^2 = .11; Figure 1C). This interaction was due to Estradiol-Saline rats exhibiting significantly reduced CS-elicited freezing relative to all other groups (P<.05), whereas all other groups did not differ (P=.41).

3.2 Experiment 2: Does MK801 during diestrus block augmentation of fear extinction in intact rats during proestrus?

In Experiment 2, MK801 was administered during the natural rise in estradiol during diestrus in intact cycling rats to determine whether this prevents the proestrus-associated enhancements in extinction recall. There were no differences in pre-CS freezing during the adaptation period prior to extinction training (F(2,35)=1.5). CS-elicited freezing during extinction training and extinction recall is depicted in Figure 2. CS-elicited freezing decreased throughout extinction training, (significant effect of trial [F(5,175)=13.33, P<0.0001, partial η^2 = .28]; Figure 2B). There was no group by trial interaction

(F(10,175)=1.88), however there was a main effect of group (F(2,35)=3.34, P=0.03, partial η^2 = .18), due to Proestrus-MK801 rats exhibiting significantly greater CS-elicited freezing relative to Proestrus Saline rats (P<0.05); CS-elicited freezing in Metestrus-Saline rats did not significantly differ from either group (smallest P = .13).

There were no differences in pre-CS freezing during the adaptation period prior to extinction recall (F(2,35)=1.01), but there was a significant group difference in CS-elicited freezing during extinction recall (F(2,35)=5.95, P=0.01; Figure 2C). Proestrus-Saline rats exhibited significantly lower CS-elicited freezing than Proestrus-MK801 and Metestrus-Saline rats (P<0.05); the latter two groups did not differ (P=.23).

3.3 Experiment 3: Can DCS mimic the effects of estradiol in OVX rats?

In Experiment 3 DCS administration was timed to take the place of estradiol treatment in OVX rats, to determine whether mimicking the treatment regime used in Experiment 1, with DCS in place of estradiol, could enhance extinction in OVX rats. There were no differences in pre-CS freezing during the adaptation period prior to extinction training (F(1,22)=1.68). CS-elicited freezing during extinction training and extinction recall is depicted in Figure 3. CS-elicited freezing decreased throughout extinction training (significant effect of trial [F(5,110)=8.99, P<0.0001, partial η^2 = .29]; Figure 3B). There was no group by trial interaction (F(5,110)=1.73), and no main effect of group (F(1,22)=3.32), however as inspection of the means indicated that groups did appear to differ substantially in their freezing levels during extinction training, exploratory post-hoc ANOVAs were conducted on each extinction trial. This analysis indicated that DCS-treated rats exhibited significantly reduced freezing on trials 2 (F(1,22)=5.85, P=0.02), 3 (F(1,22)=6.31, P=0.02), and 4 (F(1,22)=7.91, P=0.01), but not trials 1, 5, or 6 (largest F(1,22) = 1.09).

There were no differences in pre-CS freezing during the adaptation period prior to extinction recall ($F(1,22)=.15$), but during extinction recall DCS-treated rats exhibited significantly lower CS-elicited freezing than saline-treated rats ($F(1,22)=9.03$, $P=0.01$; Figure 3C).

3.4 Experiment 4: Does DCS during declining estradiol enhance extinction in intact rats during metestrus?

In Experiment 4, DCS administration was timed to take place during the natural fall in estradiol levels during the estrous cycle in intact rats to determine whether DCS during this hormonal period could prevent the metestrus-associated impairments in extinction recall. DCS-treated rats exhibited significantly higher pre-CS freezing during the adaptation period prior to extinction training ($F(1,23)=6.32$, $P=.02$). CS-elicited freezing during extinction training and extinction recall is depicted in Figure 4. CS-elicited freezing decreased throughout extinction training (significant effect of trial [$F(5,115)=9.3$, $P<0.0001$, partial $\eta^2 = .29$]; Figure 4B). There was no group by trial interaction ($F(5,115)=1.76$), and no main effect of group ($F(1,23)=3.11$), however as inspection of the means indicated that groups did appear to differ substantially in their freezing levels during extinction training, exploratory post-hoc ANOVAs were conducted on each extinction trial. This analysis indicated that DCS-treated rats exhibited significantly reduced freezing on trials 3 ($F(1,23)=4.31$, $P=0.05$), and 4 ($F(1,23)=6.14$, $P=0.02$), but not on trials 1, 2, 5, or 6 (largest $F(1,23) = 1.96$).

There were no differences in pre-CS freezing during the adaptation period prior to extinction recall ($F(1,23)=.72$), but during extinction recall DCS-treated rats exhibited significantly lower CS-elicited freezing than saline-treated rats ($F(1,23)=5.91$, $P=0.02$; Figure 4C).

3.5 Experiment 5: Does DCS improve extinction recall when administered immediately after extinction training in OVX rats?

In Experiment 5, DCS was administered immediately after extinction training to determine whether, as has been observed in males, DCS has an acute enhancing effect on extinction recall in females. There were no differences in pre-CS freezing during the adaptation period prior to extinction training ($F(1,19)<.0001$). CS-elicited freezing during extinction training and extinction recall is depicted in Figure 5. CS-elicited freezing decreased throughout extinction training (significant effect of trial [$F(5,95)=5.08$, $P<0.0001$, partial $\eta^2 = .21$]; Figure 5B). There was no group by trial interaction ($F(5,95)=.74$), and no main effect of group ($F(1,19)=.33$).

There were no differences in pre-CS freezing during the adaptation period prior to extinction recall ($F(1,19)=.43$), but during extinction recall DCS-treated rats exhibited significantly lower CS-elicited freezing than saline-treated rats ($F(1,19)=5.57$, $P=0.03$; Figure 5C).

4. Discussion

The present experiments provide novel evidence that estradiol-induced augmentation in extinction may be NMDAr-dependent. In particular, co-administration of MK801 with estradiol prevented enhanced extinction recall in OVX rats (Experiment 1), and MK801 treatment during diestrus (coincident with rising estradiol levels) prevented the cyclic enhancement in extinction recall in intact rats extinguished during proestrus (Experiment 2). Superficially, these findings mirror those obtained in male rats, in whom extinction is impaired when MK801 is administered shortly prior to (e.g., 40 min) or after extinction training, suggesting that NMDAr activation is necessary for the acquisition and consolidation of extinction memories in males (reviewed in Singewald et al., 2015). However, in the

present study MK801 was administered 48 h (Experiment 1) or 24 h (Experiment 2) prior to extinction training, and so it is unlikely that acute effects of MK801 on NMDAr activation during extinction account for the results. This is supported by a past report that MK801-treated OVX rats exhibited comparable LTP magnitude to control rats by 12 h post-treatment, suggesting that MK801 has cleared from the system at this time point (Smith & McMahon, 2005). This is also supported by the absence of hyperlocomotion in MK801-treated rats during extinction training in Experiments 1 and 2, unlike that which occurs when MK801 is in the system, resulting in almost negligible freezing (Chan & McNally, 2009; Langton & Richardson, 2009). Moreover, in Experiment 1, estradiol-treated rats that received MK801 exhibited worse extinction recall relative to estradiol-treated rats that received saline, whereas extinction recall was comparable amongst oil-treated rats irrespective of whether they received MK801 (although, in the latter case, ceiling effects cannot be ruled out). Thus, we suggest that the effects observed in Experiments 1 and 2 were probably primarily driven by MK801 counteracting estradiol's facilitation of extinction, rather than MK801 impairing extinction per se.

The suggestion that estradiol-induced facilitation of extinction may be NMDAr-dependent fits within a wider literature demonstrating that estradiol facilitates NMDAr-dependent hippocampal synaptic plasticity across a strikingly similar timeframe to that which we have observed here and in the past (Graham & Daher, 2016). For example, in OVX rats, the same estradiol treatment increases CA1 dendritic spine density, evident by 24 h and up to 72 h, but not 120 h, post-treatment (Smith & McMahon, 2005; Woolley & McEwen, 1993). This effect is associated with increased sensitivity of CA1 pyramidal cells to NMDAr-mediated synaptic input (Woolley et al., 1997). Similarly, estradiol facilitates the magnitude of LTP at Schaffer Collateral-CA1 synapses, an effect that is mediated by an estradiol-induced increase in the NMDAr/AMPAr ratio, in the absence of changes in NMDAr

expression or phosphorylation (Snyder et al., 2011), and is evident 24 h, 48 h, but not 72 h, post-treatment (Smith & McMahon, 2005; 2006). These effects also occur in response to rising endogenous estradiol during proestrus (Woolley & McEwen, 1993; Bi et al., 2001), and are completely blocked by NMDAr antagonism, using the similar doses to that employed here (Smith & McMahon, 2005; Woolley & McEwen, 1994; Shors et al., 2004). Combined, this demonstrates that estradiol-induced augmentation of fear extinction and hippocampal plasticity are tightly coupled and potentially dependent on a common neurochemical mechanism (i.e., NMDAr activation). Whether estradiol's effects on extinction are mediated by the NMDAr-dependent facilitation of hippocampal plasticity discussed above should be directly interrogated in future experiments that combine behavioral, pharmacological, electrophysiological, and morphological analyses. The notion of a causal link is certainly plausible, given that hippocampal NMDAr activation is necessary to fear extinction (Fiorenza et al., 2012). However, estradiol also modulates plasticity and NMDAr transmission within other components of the fear extinction circuitry, including the vmPFC and amygdala (Inagaki et al., 2012; Galvin & Ninan, 2014; Kahn et al., 2013; Zeidan et al., 2011), and future studies should investigate the neural loci of estradiol's long-term effects on extinction by systematically targeting this circuitry. Indeed, the present study is limited in that only systemic effects were examined. This design is in line with the aim of understanding the role of ovarian hormone fluctuations in fear extinction (which are, initially, peripheral), but it does preclude the ability to draw conclusions regarding the central mechanisms of estradiol's influence on extinction.

Consistent with the suggestion that NMDAr activation may mediate estradiol- and cyclic- associated enhancements in extinction, we also found that mere activation of NMDAr via DCS administration mimicked the effects of estradiol treatment on fear extinction in OVX rats (Experiment 3) and prevented extinction impairments in intact rats extinguished

during metestrus (Experiment 4). Such results are consistent with a vast body of work demonstrating that DCS enhances extinction in rats, and exposure therapy in clinical populations (reviewed in Singewald et al., 2015). However, while past work has clearly demonstrated DCS must be administered during the memory acquisition/consolidation window to be effective, in the present experiments, DCS enhanced extinction when administered 48 h (Experiment 3) or 24 h (Experiment 4) prior to extinction training – well prior to memory acquisition. How might DCS have exerted such long-term effects on extinction? In the case of intact rats, DCS may have maintained NMDAr transmission at proestrus-like levels. This concept is somewhat consistent with the sample-and hold hypothesis, wherein estradiol-induced increases in synaptic contacts are maintained by subsequent NMDAr activity (Srivastava et al., 2008). However, this explanation does not account for the results in OVX rats, in whom NMDAr activation alone was sufficient to cause a long-term facilitation in extinction in the absence of estradiol priming. To our knowledge, the long-term physiological effects of acute DCS administration have never been examined, and therefore the mechanisms underlying these findings require further investigation.

Irrespective of the exact mechanism, the present findings have several potential implications for the use of DCS in the treatment of anxiety disorders in women. When carefully paired with exposure sessions, DCS has modest clinical efficacy in augmenting treatment outcomes across a wide range of anxiety and trauma disorders (Mataix-Cols et al., 2017). A critical component of exposure therapy involves engaging in additional exposures, outside of the therapeutic setting, that may be either planned or opportunistic (Craske et al., 2014). As these additional exposures are not combined with DCS, they represent lost opportunities for stronger facilitation of treatment outcomes. Our findings raise the intriguing possibility that coordinating DCS administration with particular menstrual cycle phases in women (coincident with declining estradiol levels), rather than with exposure sessions per se,

could create a longer timeframe during which exposures could be enhanced, potentially maximizing the effectiveness of DCS as a treatment adjunct. Of course, this speculative use for DCS does have a potential disadvantage with respect to the finding that, depending on the extent of fear reduction within a given exposure session, DCS augments the outcomes of "successful" exposures, and enhances the reconsolidation of fearful memories from "unsuccessful" exposures (Hofmann, 2014). If used in the proposed way, DCS would theoretically facilitate both processes, potentially leading to worse outcomes if there were a high ratio of unsuccessful to successful exposures. This concern may be somewhat mitigated by the observation that DCS-treated rats in Experiments 3 and 4 exhibited reduced freezing during within-session extinction, suggesting that, used in this way, DCS might also facilitate the rate of fear reduction during extinction. Nonetheless, we also examined the impact of post-extinction training DCS in OVX rats (Experiment 5), and found a similar facilitation in extinction recall to what has been previously documented in male rats (Singewald et al., 2015). This outcome suggests that when strategically administered in conjunction with exposure sessions (in line with its current proposed use), DCS could ameliorate deficits in anxiety treatment outcomes that women appear to experience during periods of low endogenous estradiol (Graham et al., 2018). Future experiments examining the longer-term effects of post-extinction DCS on relapse (e.g., spontaneous recovery) in intact rats and women are required to examine this possibility further.

To conclude, this study provides novel insights to the potential mechanisms by which estradiol modulates fear extinction in females. The finding that estradiol's enhancement of extinction may be NMDAr-dependent, and, extrapolating from past research, occurs in parallel with estradiol's enhancement of NMDAr-dependent plasticity, offers a useful platform for future investigation of potential neural loci and downstream mechanisms of hormonal modulation of extinction. As such, this study contributes to the ongoing

development of a more robust, sex-specific neurobiological model of extinction. Moreover, the findings indicate potential means of optimizing treatment outcomes for women with anxiety disorders using both established and original applications of DCS.

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Figure Captions

Figure 1. (A) Experimental timeline for Experiment 1 (OVX rats). (B) Mean (±SEM) CS-elicited freezing for groups Estradiol-Saline (n = 9), Estradiol-MK801 (n = 10), Oil-Saline (n = 10), and Oil-MK801 (n = 9) during extinction training in Experiment 1. * = Estradiol-Saline < Estradiol-MK801 ($p < 0.05$). (C) Mean (±SEM) CS-elicited freezing during extinction recall in Experiment 1. * = Estradiol-Saline < all other groups ($p < 0.05$).

Figure 2. (A) Experimental timeline for Experiment 2 (intact rats). (B) Mean (±SEM) CS-elicited freezing for groups Proestrus-Saline (n = 12), Proestrus-MK801 (n = 12), and

Metestrus-Saline (n = 14) during extinction training in Experiment 2. * = Proestrus-Saline < Proestrus-MK801 ($p < 0.05$). (C) Mean (±SEM) CS-elicited freezing during extinction recall in Experiment 2. * = Proestrus-Saline < other groups ($p < 0.05$).

Figure 3. (A) Experimental timeline for Experiment 3 (OVX rats). (B) Mean (±SEM) CS-elicited freezing for groups Saline (n = 12) and DCS (n = 12) during extinction training in Experiment 3. * = DCS < Saline ($p < 0.05$). (C) Mean (±SEM) CS-elicited freezing during extinction recall in Experiment 3. * = DCS < Saline ($p < 0.05$).

Figure 4. (A) Experimental timeline for Experiment 4 (intact rats). (B) Mean (±SEM) CS-elicited freezing for groups Saline (n = 12) and DCS (n = 13) during extinction training in Experiment 4. * = DCS < Saline ($p < 0.05$). (C) Mean (±SEM) CS-elicited freezing during extinction recall in Experiment 4. * = DCS < Saline ($p < 0.05$).

Figure 5. (A) Experimental timeline for Experiment 5 (OVX rats). (B) Mean (±SEM) CS-elicited freezing for groups Saline (n = 10) and DCS (n = 11) during extinction training in Experiment 5. (C) Mean (±SEM) CS-elicited freezing during extinction recall in Experiment 5. * = DCS < Saline ($p < 0.05$).

Table 1. Pre-CS freezing during the adaptation period in Experiments 1, 2, 3, 4, and 5.

Experiment and Phase	**Group M (SEM)**			
Experiment 1	**Estradiol Saline**	**Estradiol MK801**	**Oil Saline**	**Oil MK801**
Pre-Extinction training	12.33 (7)	14 (3.56)	9.4 (5.01)	7.33 (4.26)
Pre-Extinction Recall	2.22 (.88)	30.5 (10.76)*	9.5 (4.04)	22.22 (6.78)*
Experiment 2	**Proestrus Saline**	**Proestrus MK801**	**Metestrus Saline**	
Pre-Extinction training	12 (4.88)	26.5 (7.03)	18.57 (5.29)	
Pre-Extinction Recall	5.42 (2.78)	12.92 (6.83)	5 (2.67)	
Experiment 3	**Saline**	**DCS**		
Pre-Extinction training	19.4 (8.6)	7.7 (2.6)		
Pre-Extinction Recall	26.7 (10.6)	10.7 (11.6)		
Experiment 4	**Metestrus Saline**	**Metestrus DCS**		
Pre-Extinction training	3 (2.7)	13.8 (3.5)*		
Pre-Extinction Recall	4.6 (3.8)	9.4 (4.3)		
Experiment 5	**Saline**	**DCS**		
Pre-Extinction training	9.1 (3.75)	9.09 (3.36)		
Pre-Extinction Recall	13.3 (7.7)	7.55 (4.51)		

*Significantly different to other groups ($P < 0.05$).