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Dopamine D1 Receptor Activation Rescues Extinction Impairments in Low-Estrogen Female Rats and Induces Cortical Layer-Specific Activation Changes in Prefrontal–Amygdala Circuits

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Post-traumatic stress disorder (PTSD) is twice as common in women as in men; it is a major public health problem whose neurobiological basis is unknown. In preclinical studies using fear conditioning and extinction paradigms, women and female animals with low estrogen levels exhibit impaired extinction retrieval, but the mechanisms that underlie these hormone-based discrepancies have not been identified. There is much evidence that estrogen can modulate dopaminergic transmission, and here we tested the hypothesis that dopamine–estrogen interactions drive extinction processes in females. Intact male and female rats were trained on cued fear conditioning, and received an intraperitoneal injection of a D1 agonist or vehicle before extinction learning. As reported previously, females that underwent extinction during low estrogen estrous phases (estrus/metaestrus/diestrus (EMD)) froze more during extinction retrieval than those that had been in the high-estrogen phase (proestrus; PRO). However, D1 stimulation reversed this relationship, impairing extinction retrieval in PRO and enhancing it in EMD. We also combined retrograde tracing and fluorescent immunohistochemistry to measure c-fos expression in infralimbic (IL) projections to the basolateral area of the amygdala (BLA), a neural pathway known to be critical to extinction retrieval. Again we observed diverging, estrous-dependent effects; SKF treatment induced a positive correlation between freezing and IL-BLA circuit activation in EMD animals, and a negative correlation in PRO animals. These results show for the first time that hormone-dependent extinction deficits can be overcome with non-hormone-based interventions, and suggest a circuit-specific mechanism by which these behavioral effects occur.
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INTRODUCTION

Women are twice as likely as men to develop post-traumatic stress disorder (PTSD) (Breslau *et al*, 1999); however, little is known about the neurobiological mechanisms related to this discrepancy. PTSD patients exhibit a disruption in the activity of both the prefrontal cortex (PFC) and amygdala (Hughes and Shin, 2011; Shin *et al*, 2006), which work together to regulate fear expression. In the rat brain, the infralimbic (IL) region of the PFC mediates the memory of extinguished fear through projections to the basolateral amygdala (BLA; Quirk and Mueller, 2008; Sierra-Mercado *et al*, 2011). Although extinction circuitry is well established, most extinction research uses male subjects, and to date, the functioning of this circuitry in the female brain has received far less attention (Lebron-Milad and Milad, 2012).

Recent research has demonstrated that fluctuating estrogen levels can mediate extinction in female rats and humans in a highly time-dependent manner, with hormonal status on the day of extinction learning, but not extinction retrieval, determining successful fear suppression during extinction retrieval (reviewed in Lebron-Milad and Milad, 2012). Specifically, female rats and women who undergo extinction learning when estrogen levels are low exhibit impaired extinction retrieval compared with those who undergo extinction learning when estrogen levels are high (Milad *et al*, 2009, 2010). In addition, low estrogen levels induced by hormonal contraceptive administration also result in poor extinction retrieval measures in both female rats and women (Graham and Milad, 2013). These findings suggest that high estrogen levels during extinction are critical to successful learning and consolidation in females, but the mechanisms by which estrogen modulates extinction processes are not known.

One intriguing possibility is that estrogen increases the efficacy of dopamine (DA) activity in the PFC, thus promoting optimal freezing suppression after extinction.

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Dopaminergic projections from the ventral tegmental area (VTA) to the PFC are more dense in females than in males (Kritzer and Creutz, 2008), which may indicate that the female PFC is relatively more primed to respond to DA activity. In addition, evoked DA release in the PFC fluctuates across the estrous cycle (Thompson and Moss, 1997), suggesting a modulatory role for ovarian hormones in DA signaling. In humans, estrogen–DA interactions in PFC function have been observed in women with variations in the gene for catechol-*O*-methyltransferase (COMT), the enzyme that breaks down synaptic DA (Jacobs and D'Esposito, 2011). In this study, elevated DA levels in women with the *met/met* allele prevented PFC-mediated cognitive deficits associated with low estrogen phases of the menstrual cycle.

Taken together, these studies suggest that estrogen influences PFC function at least in part through dopaminergic mechanisms. Although the postsynaptic processes involved in this interaction are not known, the D1 receptor is an attractive candidate. D1 stimulation enhances LTP between PFC layer III and layer V neurons (Goldwater *et al*, 2009; Huang *et al*, 2004), and tightly modulates neuronal activity during PFC-mediated tasks (Arnsten *et al*, 2012; Vijayraghavan *et al*, 2007). IL D1 receptors are reported to be critical to successful extinction in males (Hikind and Maroun, 2008), but their role in females is not known. In the current study, we tested the hypothesis that D1 modulation of extinction is estrous-dependent. To assess the impact of potential estrous–DA interactions on relevant neural circuitry, we also performed fluorescent immunohistochemistry for c-fos in retrogradely labeled, amygdala-projecting IL neurons after extinction retrieval testing.

MATERIALS AND METHODS

Animals

In all, 20 male (250–300 g) and 40 female (175–240 g) Long–Evans rats (8–10 weeks old; Taconic Farms, Hudson, NY) were individually housed upon arrival at Northeastern University. All animals had access to food and water *ad libitum* and were acclimated to the animal facility for 5 days before surgery. Animals were kept on a 12:12 light/dark cycle (lights on at 0700 hours) and all behavior testing was carried out during the light phase of the cycle. Daily vaginal swabbing was performed in females, using the Dorsal Method (Becker *et al*, 2005), to ensure normal estrous cycling in females. Estrous phase designations were made using cytology assessments, as described previously (Shansky *et al*, 2006). All surgical and behavioral procedures were approved and supervised by Northeastern University's Institutional Animal Care and Use Committee.

Retrograde Tracer Injections

All animals underwent stereotaxic surgery in which 0.2 μl Fluorogold (FG; Fluorochrome LLC, Denver, CO) was injected unilaterally into the right BLA, as described in Shansky *et al* (2009). Animals were anesthetized with an intraperitoneal injection of a ketamine (90 mg/kg) and xylazine (4 mg/kg) cocktail. Once deep reflexes were no longer present, animals' heads were shaved and secured into a stereotaxic apparatus (Stoelting, Wood Dale, IL) and animals received a subcutaneous injection of 0.3 ml of 10% buprenorphrine before an incision being made to reveal the skull. Once bregma was clearly visible, a burr hole was drilled above the BLA (-3.0 mm AP, ± 5.0 mm ML; Paxinos and Watson, 2005) and a 1 μl syringe attached to a stereotaxic arm was lowered to 8.0 mm DV. After allowing tissue to settle for 2 min, an infusion pump (Harvard Apparatus, Holliston, MA) delivered FG at 0.02 μm/min. The syringe was left in place for 10 min, and then slowly removed from the brain and the incision was sealed with VetBond surgical glue (3M, St Paul, MN). Animals were kept in cages atop heated recovery pads until righting reflexes returned. At varying intervals throughout surgery, animals received three, 1 ml subcutaneous injections of filtered sterile saline (0.9% NaCl). For 3 days after surgery, animals received subcutaneous injections of 0.3 ml buprenorphine, and were monitored for healthy eating and drinking habits.

Behavioral Equipment

All testing was carried out in a Habitest Modular Rat Test Cage, which was contained inside of an isolation cubicle (Coulbourn Instruments, Whitehall, PA). Each test cage contained a single overhead infrared camera, a house light, stimulus light, and speaker, which were all wall-mounted. Tone presentations were delivered through the wall-mounted speaker in each individual test cage. Test cages were also equipped with a grid shock floor capable of delivering a mild footshock. The conditioned stimulus (CS) was a 5 kHz tone with an intensity of 80 dB, and lasted 30 s. The unconditioned stimulus (US) was a 0.7 mA, 0.5 s footshock that immediately followed the CS during fear conditioning.

Behavioral Procedures

One week after surgery, animals began a 3-day fear conditioning, extinction, and extinction retrieval paradigm. Fear conditioning consisted of five habituation CS tones, followed by seven CS tones that were each paired with the US. Mean intertrial interval was 4 min (2–6 min range). The following day, animals received an intraperitoneal injection of SKF38393 (10 mg/kg; Sigma-Aldrich, St Louis, MO) or saline vehicle (VEH) 30 min before behavior testing. Extinction learning consisted of 20 tone presentations, with a mean intertrial interval of 4 min (2–6 min range). Extinction learning took place in the same cages with the following contextual changes: opaque plexiglass floor placed over grid floor, different 'stimulus' light instead of 'house' light, house fan turned off, and peppermint soap (Dr Bronner's Magic All-One!) spread beneath the floor in order to introduce a different smell into the cage. The following day, animals underwent extinction retrieval in the same context as extinction learning. Retrieval consisted of three tone presentations, with a mean intertrial interval of 4 min (3.5–4.5 min range).

Behavioral Analysis

Freezing behavior, defined as the cessation of all movement but that required to breathe (Milad *et al*, 2009), was used as

a measure of conditioned fear. Freezing during tone presentations was measured using FreezeFrame software (Coulbourn Instruments) output from an infrared camera. Experimenters blind to treatment groups reviewed freezing behavior manually based on software output.

Sacrifice and Tissue Processing

Animals were anesthetized by CO_2 inhalation 25–35 min after termination of extinction retrieval, and then were immediately transcardially perfused with 100 ml of 1% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS), followed by 500 ml of 4% PFA with 0.125% glutaraldehyde in 0.1 M PBS. Brains were extracted, placed in 10 ml of 4% PFA in 0.1 M PBS and stored overnight at 4 °C. After 24 h, the brains were switched into 10 ml of 0.1% sodium azide in 0.1 M PBS for long-term storage. Fifty micrometer, IL-containing coronal sections were cut on a vibratome and stored in 0.1% sodium azide in PBS.

For immunostaining of c-fos and FG+ neurons in the IL, two to three sections per animal were isolated and washed three times for 10 min in PBS, and then washed in a blocking buffer containing 5% normal donkey serum and 0.5% Triton X-100 in PBS for 1 h. Sections were then incubated with polyclonal rabbit anti-FG 1:20 000 (52-9600; Flourochrome LLC) and polyclonal goat anti-c-fos 1:2000 (sc-52; Santa Cruz Biotechnology, Dallas, TX) for 48 h at 4 °C. Sections were rinsed three times for 10 min in PBS and incubated for 1 h at room temperature in appropriate secondary antibodies: donkey anti-rabbit IgG conjugated to Cy3 (1:200, 711-165-152; Jackson Immuno Research, West Grove, PA) and donkey anti-goat IgG conjugated to Cy2 (1:200; 715-225-151; Jackson ImmunoResearch). Sections were then washed three times for 10 min in PBS, rinsed in DAPI (D9542; Sigma-Aldrich)-containing PBS, mounted, dried, and coverslipped using PermaFluor mounting media (TA-030-FM; Thermo Scientific, Waltham, MA).

Imaging and Quantification

Two-dimensional images of cortical layers III and V (identified using DAPI staining) were captured with a ×20 objective and a zoom of 1.5 on an Olympus FV1000 confocal laser-scanning microscope using Fluoview software (Olympus America, Center Valley, PA). Two images per layer, per hemisphere were collected from each section. Images were imported to ImageJ software, and FG+ neurons were counted manually by an experimenter blind to experimental conditions. Colocalization was determined by computing the percentage of FG+ neurons that were also c-fos+.

Experimental Groups

All animals were randomly assigned to treatment groups (SKF or VEH) before behavioral testing. Females were assigned to estrous groups based on *post hoc* assessment of estrous phase on the day of extinction learning (Milad *et al*, 2009). Animals in proestrus were classified as PRO; those in estrus, metaestrus, or diestrus were classified as EMD (estrus/metaestrus/diestrus). Animals were removed from final analyses if they did not reach the criteria for successful fear conditioning (>40% freezing across first two extinction tones; Sotres-Bayon *et al*, 2007) or if FG injection did not hit the BLA. Final *n*'s were: male VEH (8); male SKF (9); EMD VEH (7); EMD SKF (9); PRO VEH (8); and PRO SKF (8).

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Group differences in freezing were analyzed using two-way ANOVAs corrected for multiple comparisons, with factors of sex/estrous phase or drug condition and trial number for fear conditioning and extinction. For extinction retrieval, freezing across the three tone presentations was averaged for each animal, and group means were then analyzed using two-way ANOVAs with factors of sex/estrous phase (males, PRO, EMD) and drug condition (VEH *vs* SKF). Sidak's multiple comparison *post hoc* tests were conducted when interactions were observed. Percent FG-labeled neurons also positive for c-fos were averaged for each animal, and then group means were calculated. Between-group differences were assessed by two-way ANOVAs. Pearson's correlations were performed to measure the relationship between layer V activation and freezing during extinction retrieval. For all analyses, significance was set at $p<0.05$.

RESULTS

Behavior

Freezing behavior during each tone presentation in fear conditioning, extinction learning, and extinction retrieval is shown in Figure 1. For all comparisons, female animals were grouped by estrous phase during extinction learning, based on findings by Milad *et al* (2009).

In fear conditioning analyses of freezing to tone, an expected significant effect of trial number was observed ($F_{11,286}=75.9$, $p<0.0001$), but no significant main group effect was observed ($F_{3,26}=2.1$, $p=0.13$), and no trial × group interaction was observed ($F_{33,286}=0.86$, $p=0.69$), indicating comparably successful learning in all groups. Similarly, a significant main effect of trial was observed for extinction learning ($F_{3,78}=107.1$, $p<0.0001$), but no main effect of group ($F_{3,26}=1.1$, $p=0.37$), and no trial × group interaction ($F_{9,78}=1.1$, $p=0.37$) was observed. However, a significant group × treatment interaction was observed in extinction retrieval analyses ($F_{2,42}=25.95$, $p<0.0001$). *Post hoc* analyses revealed that PRO-VEH females froze less than EMD-VEH females ($p=0.006$), indicating enhanced extinction retrieval, and replicating findings in Milad *et al* (2009). In VEH groups, neither group of females differed from males. In males, SKF administration had no effect on freezing during extinction retrieval ($p=0.99$), but in females, SKF administration affected extinction retrieval freezing in an estrous-dependent manner. SKF administration during extinction learning resulted in greater freezing during extinction retrieval for PRO females ($p=0.002$), and less freezing in EMD females ($p<0.0001$).

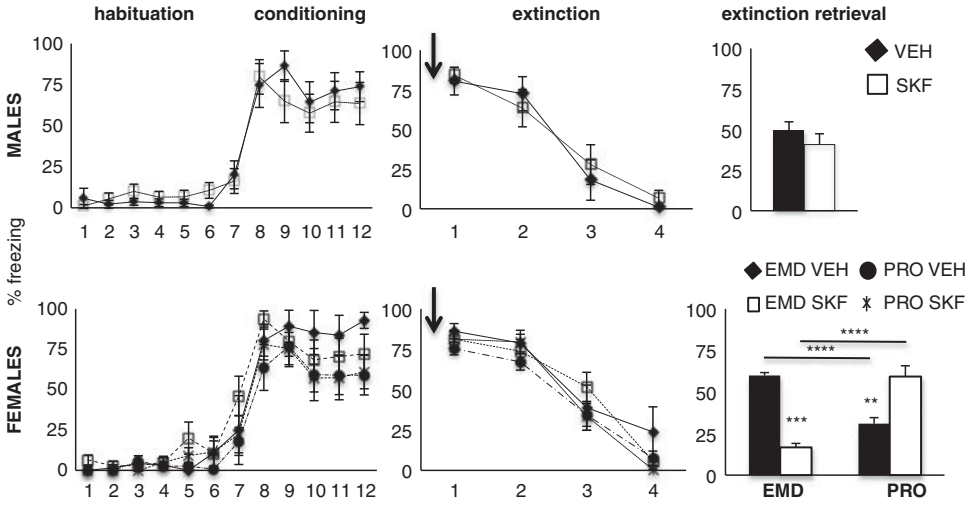

Figure 1 Effects of pre-extinction D1 agonist administration on extinction retrieval in males and females. All data points represent mean ± SEM. No SKF (SKF38393) effects were observed in freezing in males (top panel) across fear conditioning, extinction, or extinction retrieval. In females (bottom panel), vehicle-treated proestrus (PRO) animals exhibited greater extinction retrieval (less freezing) compared with EMD (estrus/metaestrus/diestrus). SKF had no effect on freezing during extinction learning, but induced impaired extinction retrieval in PRO animals, and enhanced extinction retrieval in EMD animals. Arrow denotes injection time point. $^{**}P = 0.006$ compared with PRO-SKF, $^{***}p = 0.002$ compared with EMD-VEH, and $^{****}p < 0.001$ compared with same treatment/opposite estrogen state.

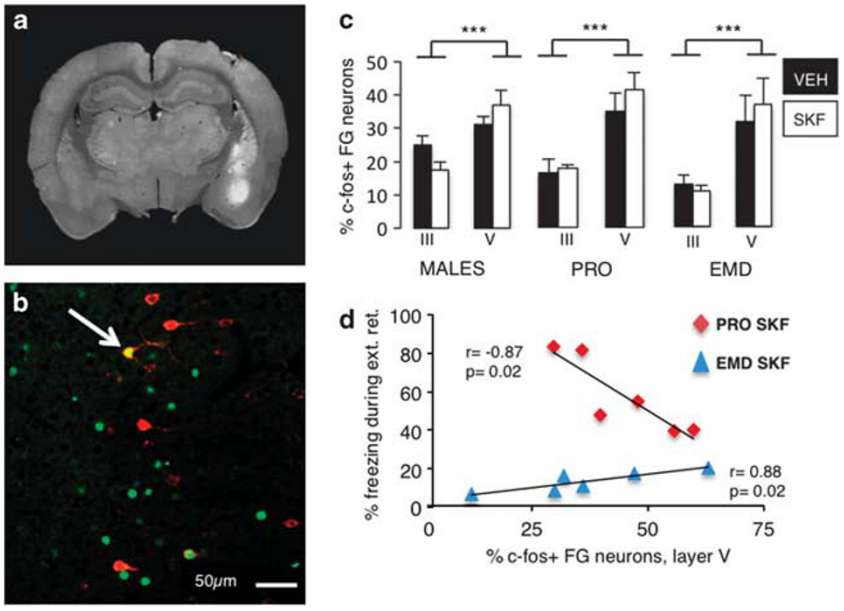

Figure 2 Neuronal activation in basolateral amygdala (BLA)-projecting infralimbic (IL) neurons. (a) Representative fluorescence image showing location and spread of intra-BLA Fluorogold injection. (b) Confocal micrograph demonstrating immunostaining for Fluorogold (red) and c-fos (green) in IL layer V. Retrogradely labeled, c-fos + neurons are characterized by green nuclei inside red cell bodies, example shown with white arrow. (c) Greater activation was observed in layer V compared with layer III for all groups. (d) Freezing behavior during extinction retrieval was negatively correlated with c-fos expression in FG + neurons in PRO-SKF (proestrus- SKF38393) animals, but positively correlated with c-fos expression in FG + neurons in EMD-SKF (estrus/metaestrus/diestrus-SKF) animals. Data points for (c) represent mean ± SEM. $^{***}P < 0.001$ compared with layer III.

c-Fos Activation in IL-BLA Circuitry

Representative FG injection site and tracer spread in the BLA is shown in Figure 2a, and fluorescent immunohistochemistry for FG and c-fos expression in IL layers III and V following extinction retrieval is shown in Figure 2b. No main experimental group effects within were observed for either layer III or layer V ($p > 0.05$ for all comparisons), but a significant main effect of layer was observed for all groups, with layer V expressing a greater percentage of c-fos +, FG-labeled neurons than layer III ($p < 0.001$ for all groups). To probe the relationship between IL-BLA circuit activity and the diverging behavioral responses observed in drug-treated PRO and EMD animals, we ran a regression analysis on freezing during extinction retrieval and layer V activation. We observed a significant negative correlation in PRO animals (Pearson's $r = -0.87$, $p = 0.02$), but a significant positive correlation in EMD animals (Pearson's $r = 0.88$, $p = 0.02$). No other significant correlations were observed.

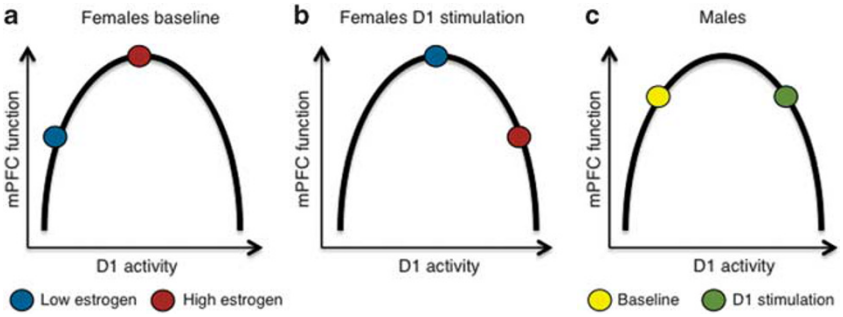

Figure 3 Model of estrogen–dopamine interactions on the inverted-U curve. (a) On the basis of our findings, we hypothesize that low estrogen individuals exhibit impaired medial prefrontal cortex (mPFC) function partly because of suboptimal D1 signaling. (b) When D1 receptors are stimulated, low estrogen individuals improve, whereas high estrogen animals fall to the far end of the inverted-U, and exhibit impairment. (c) Lack of a behavioral effect of SKF in males could be accounted for by a shift from just suboptimal to just supraoptimal D1 signaling.

DISCUSSION

The present study resulted in several novel findings. Most notably, SKF administration affected extinction retrieval in an estrous-dependent manner in female animals, producing impairments in animals with high estrogen levels, and rescuing deficits in low-estrogen animals. To our knowledge, this is the first demonstration of estrous–DA interactions in fear extinction processes, suggesting that fluctuating levels of estrogen may determine the efficacy of dopaminergic actions in the prefrontal cortex. In addition, we found that SKF treatment induced layer-specific, estrus-dependent correlations between IL-BLA circuit activation and freezing during extinction retrieval.

Our behavioral findings add to a small but growing body of literature on the role of estrogen in extinction in both animals and humans. Here, we replicate previous reports that animals in low-estrogen estrous phases during extinction learning subsequently exhibit high levels of freezing, indicating impaired extinction retrieval (Milad *et al*, 2009). Zeidan *et al* (2011) found that these impairments can be rescued with either postextinction injections of estradiol, or pre-extinction injections of the estrogen receptor β-agonist, diarylonilpropronitrile. The same group also showed that women with naturally low estrogen levels also exhibit impaired extinction retrieval, as well as reduced activation of the ventromedial PFC (vmPFC). Finally, pre-extinction estradiol administration in women also enhanced extinction retrieval (Graham and Milad, 2013). Taken together, these studies provide compelling evidence that a low-estrogen state during extinction confers a disadvantage for the maintenance of fear suppression.

In the present study, we demonstrated that these impairments can be prevented by administration of a D1 agonist, suggesting that low estrogen-related deficits can be rescued without direct activation of estrogen signaling mechanisms. It is unlikely that enhanced extinction retrieval in EMD-SKF animals is due to a drug-induced increase in estrogen, as SKF38393 has no effect on estradiol synthesis (Mayerhofer *et al*, 1999). Our findings are consistent with studies of women with different genotypes for COMT, an enzyme that metabolizes synaptic DA. In carriers of the *met/met* allele, decreased COMT activity (and thus higher DA levels) is correlated with better performance on PFC-mediated cognitive tasks (Egan *et al*, 2001). Jacobs and D'Esposito (2011) found that while *val/val* women with low estrogen levels exhibited impaired PFC function compared with those with high estrogen, performance in low-estrogen *met/met* women was comparatively enhanced. As in our work, these findings suggest that increased DA signaling can prevent prefrontal impairments that are due to low estrogen levels. Our data indicate that this may be due to actions at the D1 receptor, which has been extensively studied in the context of PFC-mediated tasks (Arnsten, 2007), and which has been shown in male rats to be necessary for successful extinction (Hikind and Maroun, 2008).

D1 activity drives prefrontal function in an inverted-U manner (Vijayraghavan *et al*, 2007; Arnsten, 2011), but the influence of estrogen on this relationship has not been explored. On the basis of our past (Shansky *et al*, 2004) and current findings, we propose a model in which animals with high estrogen levels exhibit enhanced extinction retrieval because of better D1 signaling, whereas animals with low estrogen are relatively impaired because of suboptimal D1 signaling (Figure 3a). Such differences may be the result of estrous-dependent fluctuations in baseline DA levels, which are highest during PRO (Xiao and Becker, 1994). According to this model, D1 stimulation reverses the estrous cycle effect by shifting both groups to the right on the inverted-U curve; now, low estrogen animals are in optimal D1 signaling ranges, whereas D1 receptors in high estrogen animals are overstimulated, leading to behavioral impairment (Figure 3b). Our present data support this idea, and we look forward to testing this hypothesis on a biochemical level in future studies.

Although we grouped our animals according to estrous phases that are characterized by high *vs* low estrogen levels, a role for progesterone in our findings cannot be ruled out. Progesterone levels fluctuate across the estrous cycle, undergoing a sharp peak shortly after that of estrogen, at the approximate beginning of the night of PRO (Staley and Scharfman, 2005). When administered either alone or with estradiol, progesterone given before extinction learning facilitates extinction retrieval in intact animals (Milad *et al*, 2009), and suppresses freezing in ovariectomized rats (Llaneza and Frye, 2009). In addition, there is evidence that progesterone can interact with dopaminergic systems (Frye and Sora, 2010; Yu and Liao, 2000), but how these interactions might influence extinction learning and

retrieval has not been tested directly. In the current study, we speculate that progesterone has a negligible role in the observed experimental outcomes, as peak progesterone levels occurred outside the traditional time window (either immediately before or 0–4 h after extinction learning), during which effective extinction manipulations can be made (Burgos-Robles *et al*, 2007).

Given the established role for IL D1 receptors in extinction in males (Hikind and Maroun, 2008), our finding that SKF38393 affected extinction retrieval in females but not males was somewhat surprising. This may be due to sex differences in the pharmacokinetics of D1 signaling (Festa *et al*, 2004, 2006), a possibility that could be addressed using a range of doses and time points between drug administration and extinction learning. Alternately, it may be the case that no change in extinction was detected in males because SKF administration moved their PFC function from slightly prepeak to slightly postpeak. Such a shift along the inverted-U would result in no observable change in freezing behavior, despite increases in D1 signaling comparable in magnitude to those in females (Figure 3c). Finally, any observed sex differences could be due to variability in the amount of handling males and females experienced or due to daily vaginal swabbing in females to monitor the estrous cycle. Although males are handled for 3 days postoperatively, and daily during behavioral testing, our females experienced slightly more handling during the few days between postoperative care and the start of fear conditioning as a necessary result of estrous cycle monitoring. As handling experience can affect behavior in a number of different paradigms (Hoffman *et al*, 2010; Hui *et al*, 2006), it is possible that the slight discrepancy in handling of males *vs* females could have affected the outcome in our study. However, sex differences in handling effects are most commonly reported in animals exposed to handling during prepubertal periods (Kosten *et al*, 2007; Raineki *et al*, 2009). Moreover, the most robust effects observed in the current study were those between females with high and low estrogen and not between males and females.

The results of our c-fos experiment were somewhat surprising. Previous studies that measured neural activation through immediate-early gene expression (Zeidan *et al*, 2011), electrophysiological recordings (Milad and Quirk, 2002), or functional imaging (Phelps *et al*, 2004) indicate a relatively straightforward relationship between activity in IL (in rodents) or vmPFC (in humans) and successful extinction retrieval (but see Chang *et al*, 2010). In these studies, an increase in IL or vmPFC activity correlated with a decrease in fear expression, suggesting that when activated, IL/vmPFC neurons are more effective at suppressing amygdala activity. Here, despite observing robust group differences in freezing during extinction retrieval, we did not find corresponding differences in IL activation.

One notable difference between these studies and ours is that we measured c-fos exclusively in neurons that project from the IL to the BLA, whereas most previous work has measured general activation in the region of interest, without focusing on specific circuits. Our aim was to identify and isolate the primary pathway thought to drive suppression of fear during extinction retrieval (Quirk and Beer, 2006), a neuronal subpopulation that accounts for $<10\%$ of all IL neurons (Gabbott *et al*, 2005). To account for variation in FG uptake, we report the percentage of FG-labeled neurons that were positive for c-fos (as opposed to total cell counts), as an indication of the degree to which the IL-BLA pathway was activated during extinction retrieval. However, we also observed c-fos expression in IL neurons that were not labeled with FG (Figure 2b), suggesting that extinction retrieval may involve not only direct IL-BLA projections but also intra-IL cortical networks. The role of distinct populations of IL neurons in extinction retrieval requires further study.

Although we did not observe any overall group effects, we did observe a greater degree of c-fos expression in layer V IL-BLA neurons compared with layer III, suggesting that IL layer V projections to the BLA are more engaged during extinction retrieval. This finding is consistent with the known connectivity distinctions between IL layers (Gabbott *et al*, 2005). Moreover, dopaminergic projections from the VTA to the PFC synapse predominantly in layer V (Hoover and Vertes, 2007), providing evidence for a potential dopaminergic role in extinction retrieval, even in the absence of pharmacological manipulation. We found that while IL-BLA circuit activity did not correlate with freezing in VEH-treated animals, SKF treatment resulted in diverging correlations between layer V activation and freezing behavior in PRO vs EMD animals. This intriguing effect suggests that the opposing behavioral responses observed in these two groups (impairment in PRO and enhancement in EMD) may be due to a switch in the relationship between IL-BLA circuit activity and fear suppression. D1 agonists have been shown to have a quieting effect on neurons in the PFC, which can help tune intra-PFC networks and enhance signal-to-noise ratios (Vijayraghavan *et al*, 2007). Thus, the correlation between layer V activation and freezing in EMD SKF animals may represent a focusing of IL input onto appropriate 'extinction' neurons in the BLA (Herry *et al*, 2008). In PRO SKF, the inverse relationship could indicate a disruption of this effect, perhaps from competing or interfering estrogen signaling in addition to D1 actions. Indeed, it is likely that in PRO animals (particularly the VEH-treated group) behavior is attributable in part to direct actions at estrogen receptors, as administration of ER agonists before or after extinction learning can enhance extinction retrieval (Graham and Milad, 2013; Zeidan *et al*, 2011). Further elucidation of both estrogen and DA modulation of IL-BLA circuit function will be an exciting and likely informative avenue of future research.

Our findings may be relevant to PTSD in women. Although the neurobiological reasons for women's increased susceptibility to PTSD are unknown, there is compelling evidence that low estrogen levels can impede recovery (Lebron-Milad *et al*, 2012). Female PTSD patients with naturally low estrogen levels show impaired extinction retrieval (Glover *et al*, 2012), and thus the discovery of agents that can enhance the maintenance of fear suppression after extinction (or exposure therapy) in women with low estrogen levels will be an important step in developing improved treatments. In the present study, systemic pre-extinction administration of a DA D1 agonist reversed extinction retrieval impairments in female rats with naturally low estrogen levels, an effect that correlated with cortical layer-specific activation of prefrontal–amygdala circuits. Although much work will be necessary to translate

our findings from animals to humans, further research into estrogen–DA interactions in the PFC will hopefully lead to a more nuanced understanding of the mechanisms that modulate fear suppression in females.

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