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performed only at the time of SPS (Nahvi, Nwokafor et al. 2019). Even though stress can inhibit the activity of the hypothalamic-pituitary-gonadal system (Rivier and Rivest 1991), vascular estrogen levels and local estrogen levels (via increased aromatase activity) increase in female organisms during acute stress (Shors, Pickett et al. 1999, Dickens, Cornil et al. 2011, Oyola and Handa 2017). Enhanced vascular estradiol levels also potentiates stress-enhanced HPA activity (Viau and Meaney 1991, Seale, Wood et al. 2004, Figueiredo, Ulrich-Lai et al. 2007, Heck and Handa 2019). Together these findings raise the possibility that enhanced estrogen levels during SPS activates nuclear estrogen receptors, which facilitates SPS effects on emotional memory in female rats.

In Experiment 2, administration of 17β estradiol had no effect on conditioned freezing in SPS rats during extinction testing trials, which suggests further enhancing estrogen receptor activation prior to SPS does not modulate the impact SPS has on emotional memory regulation and/or expression. However, in Experiment 2, SPS decreased freezing, during extinction training and testing relative to control animals. These effects were opposite in direction to SPS effects observed in Experiment 1. These results could be interpreted to mean that SPS enhances acquisition and maintenance of extinction memory, but such an interpretation is counter to results obtained in Experiment 1 and multiple studies that have consistently observed (in a few cases in female rats) that SPS disrupts the retention of fear extinction (Yamamoto, Morinobu et al. 2008, Knox, George et al. 2012, Knox, Nault et al. 2012, Keller, Schreiber et al. 2015, Knox, Stanfield et al. 2016, Canto-de-Souza, Demetrovich et al. 2021, Ferland-Beckham, Chaby et al. 2021, Mancini, Marchetta et al. 2021, Omura, Fuchikami et al. 2021). Furthermore, even though conditioned freezing in control rats during fear conditioning and early trials of extinction training were equivalent between Experiments 1 and 2, conditioned freezing during extinction testing

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was enhanced in control rats in Experiment 2 relative to Experiment 1. Together these findings raise the possibility that there could have been a group effect in rats in Experiment 2. It is possible that rats in Experiment 2s had heighted arousal/stress prior to commencing any experimental protocols (e.g. housing conditions prior to arriving at the University of Delaware). Heightened arousal/stress can lead to enhanced freezing during extinction testing (Maren and Chang 2006). Furthermore, as arousal and emotion-like intensity increase, defense behavior changes from freezing and enhanced startle to action that involves increased locomotor activity (e.g. avoidance) (Davis and Astrachan 1978, Blanchard and Blanchard 1989, Lang, Davis et al. 2000, Luyten, Vansteenwegen et al. 2011, Fadok, Krabbe et al. 2017). Such a behavioral transition would be associated with decreased freezing. If this were true, then control animals in Experiment 2 (for unidentified reasons) would have higher arousal/emotion-like intensity, which could have disrupted retention (or expression) of extinction in control rats. When Experiment 2-rats were exposed to SPS, this could have caused a shift in behavioral strategy during extinction testing, which manifested as a decrease in freezing. The consistent finding that SPS rats behaved differently to control rats during extinction testing in Experiments 1 and 2 demonstrates that SPS changes emotional memory expression and/or regulation in female rats. However, the discrepancy between the direction of SPS effects on freezing between Experiments 1 and 2 (as well as the difference in freezing in control rats during extinction testing between Experiments 1 and 2) highlight the need to examine multiple defense behaviors in female rats that could more reliably measure emotional memory regulation and SPS effects on emotional memory regulation. It also raises the possibility that under different initial conditions (e.g. Experiment 1) enhancing estrogen receptor activation could further exacerbate SPS effects (unlike the findings in Experiment 2).

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It should be noted that estrogen has separate effects on emotional learning and memory. Low levels of estrogens, estrogen receptor antagonism, and low levels of estrogen receptor activation, are associated with either deficits in extinction memory (or inhibition of conditioned fear responding) (Milad, Igoe et al. 2009, Milad, Zeidan et al. 2010, Ressler, Mercer et al. 2011, Glover, Jovanovic et al. 2012, Lebron-Milad, Graham et al. 2012, Graham and Milad 2013, Glover, Jovanovic et al. 2015). Also, the results of a clinical study suggests that administration of synthetic estrogen shortly after trauma reduces the incidence of PTSD (Ferree, Wheeler et al. 2012). While the results of this study suggest that activation of nuclear estrogen receptors during SPS facilitates SPS effects in female rats, further research is needed to better understand how activation of estrogen receptors contribute to traumatic stress effects on emotional memory in males vs. females.

Through what neural substrates may enhanced nuclear estrogen receptor activation during SPS lead to potential SPS deficits in emotional memory? SPS lowers glutamate levels in the medial prefrontal cortex (mPFC) (Knox, Perrine et al. 2010, Piggott, Bosse et al. 2019), lowers mPFC neural activity during extinction training (Knox, Stanfield et al. 2016), and disrupts functional connectivity between the mPFC and BLA during extinction training (Knox, Stanfield et al. 2018). Furthermore, SPS-induced deficits in fear memory extinction may be driven by SPS-induced changes in neural function in the mPFC (Canto-de-Souza, Demetrovich et al. 2021, Omura, Fuchikami et al. 2021). Given the critical role of the mPFC in inhibiting fear memory (Quirk, Garcia et al. 2006, Orsini and Maren 2012, Maren and Holmes 2016), activation of nuclear estrogen receptors may act within the mPFC to facilitate SPS effects on emotional memory. The basolateral amygdala (BLA) is critical for expression and inhibition of fear memory (Maren 2001, Cho, Deisseroth et al. 2013, Maren and Holmes 2016, Zhang, Kim et al.

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2020) and is sensitive to SPS. During fear conditioning SPS inhibits internalization of glucocorticoid receptors and enhances PI3K-Akt signaling within the amygdala (Moulton, Chamness et al. 2018, Knox, Della Valle et al. 2021). Inhibiting PI3K-Akt signaling in the BLA during fear conditioning abolishes extinction retention deficits induced by SPS without having effects in control rats (Knox, Della Valle et al. 2021). Thus, activation of nuclear estrogen receptors within the BLA could also facilitate SPS effects on emotional memory. It should be noted that a single administration of 17β-estradiol followed by a time delay of seven days lowered conditioned freezing in control rats during extinction training, which suggests that administration of this ovarian hormone may have a distinct time-delayed effect on emotional reactivity in non-stressed rats, which could be driven by activation of nuclear estrogen receptors (or activation of nuclear estrogen receptors and GPERs).

In this study darting (which can be defined as a sudden increase in locomotor activity that is equivalent to or greater than .2ms/s (Gruene, Flick et al. 2015, Colom-Lapetina, Li et al. 2019, Greiner, Muller et al. 2019, Mitchell, Trettel et al. 2022)) was only observed with footshock onset. Previous studies have observed increased darting in response to an aversive CS (Gruene, Flick et al. 2015), but some studies have not observed darting in response to an aversive CS (Odynocki and Poulos 2019). This suggests that to elicit darting responses with an auditory fear CS, different parameters are needed (e.g. higher frequency tone, different size arena, different fear conditioning protocol). It should also be noted that SPS had effects on darting and freezing during fear conditioning in Experiment 1 and these effects were sensitive to estrogen receptor manipulation. These findings raise the possibility that SPS may have broader effects on fear behavior and these effects are also facilitated, in part, by activation of estrogen receptors during SPS. However, SPS had no effects on freezing early in extinction training (when fear memory

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retrieval occurs) and no SPS effects on freezing or darting were observed in Experiment 2. Together, this makes it difficult to interpret effects observed during fear conditioning in Experiment 1.

Conclusion

The results of this study demonstrate that SPS effects on emotional memory can be obtained in female rats and this effect is likely driven, in part, by activation of nuclear estrogen receptors at the time of SPS. However, differential SPS effects on freezing during extinction testing (increases vs. decreases) were observed in Experiments 1 and 2 as well as enhanced freezing during extinction testing in control rats in Experiment 2 relative to Experiment 1. These observations raise the possibility that changes in parameters of how fear conditioning and extinction training are conducted are needed to better understand emotional memory regulation in female rats as well as characterize what effects SPS is having on emotional memory regulation in female rats. One study has reported that increasing the post-stress incubation period after SPS renders female rats sensitive to SPS effects (Mancini, Marchetta et al. 2021). Other parameters to consider are conducting behavioral experiments in the active cycle of rodents (i.e. in the dark) and/or examining the effects of SPS on emotional memory in behavioral paradigms that allow for the measurement of additional defense behaviors (e.g. avoidance).

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Author contributing statements

M.B. – Conducted experiments, did pubmed searches on the manuscript and helped write the manuscript

D.K. – Designed experiment, helped edit and write the manuscript, and analyzed data

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Figure Captions

Fig 1 Experimental design used in this study.

Fig 2 Effects of ICI182,780 administration prior to SPS on fear and extinction memory measured using freezing and max. velocity. A) In SPS/vehicle rats (n = 9) enhanced freezing, early in fear conditioning, was observed though both SPS/vehicle and control/vehicle animals (n = 11) acquired fear memory in an equivalent manner. Conditioned freezing during extinction training was equivalent between SPS/vehicle and control/vehicle rats, but enhanced in SPS/vehicle rats during extinction testing. B) Administration of ICI182,780 (.005mg/kg: n = 9, .05mg/kg: n = 8) had no effect on freezing in control rats. C) Administration of ICI182,780 (.005mg/kg: n = 10, .05mg/kg: n = 10) prior to SPS lowered conditioned freezing during fear conditioning, but had no effect on freezing during extinction training. Administration of .05mg/kg of ICI182,780 lowered conditioned freezing during extinction testing in SPS rats. D) Darting (defined as increases in max. velocity over baseline) was only observed during fear conditioning. Max. velocity during extinction training and testing was attenuated in SPS/vehicle rats. E) ICI182,780 administration had no effects on max. velocity in control rats, but F) enhanced max. velocity in SPS rats during fear conditioning and extinction training. B – baseline, FC – fear conditioning, ExtTr – extinction training, ExtTest – extinction testing. Black * represents $p < .05$ for stress comparisons, while red * represents $p < .05$ for drug comparisons.

Fig 3 Effects of G-1 administration prior to SPS on fear and extinction memory. A) Administration of .001mg/kg of G-1 (n =12) had no effect on freezing or B) max. velocity when compared to vehicle-treated SPS animals (n =13).

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Fig 4 Effects of 17β estradiol administration prior to SPS on fear and extinction memory measured using freezing and max. velocity. A) In SPS/vehicle animals (n = 10) freezing was decreased during extinction training and testing relative to control/vehicle animals (n = 14). B) Administration of 17β estradiol in control rats (.018mg/kg: n = 9, .18mg/kg: n = 10) decreased conditioned freezing during extinction training and the baseline period of extinction testing. C) Administration of 17β estradiol (.018mg/kg: n = 10, .18mg/kg: n = 10) prior to SPS enhanced conditioned freezing during extinction training. D) Darting (defined as increases in max. velocity over baseline) was only observed during fear conditioning. SPS increased max. velocity during extinction training and testing. E) 17β estradiol administration enhanced max. velocity in control rats during extinction training and testing, but F) had no effect on max. velocity in SPS rats. Black * represents $p < .05$ for stress comparisons, while red * represents $p < .05$ for drug comparisons.

Fig 5 Effects of estrogen hormone receptor manipulation on changes in max. velocity at CS-tone (T) and footshock (F) onset during fear conditioning in SPS and control rats. In all fear conditioning experiments darting was observed during the onset of F, but not at the onset of T. A) Darting during F3 onset was lower in SPS/vehicle rats relative to control/vehicle rats. Administration of ICI182,780 had no effects on max. velocity in B) control rats, but enhanced darting in C) SPS rats. D) Administration of G-1 had no effects on darting during fear conditioning in SPS rats. E) In Experiment 2 darting was equivalent between SPS/vehicle and control/vehicle rats. Administration of 17β estradiol had no effects on darting in F) control or G) SPS animals.