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Sex differences in fear responses: Neural circuits

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Abstract

Women have increased vulnerability to PTSD and anxiety disorders compared to men. Understanding the neurobiological underpinnings of these disorders is critical for identifying risk factors and developing appropriate sex-specific interventions. Despite the clear clinical relevance of an examination of sex differences in fear responses, the vast majority of pre-clinical research on fear learning and memory formation has exclusively used male animals. This review highlights sex differences in context and cued fear conditioning, fear extinction and fear generalization with a focus on the neural circuits underlying these behaviors in rodents. There are mixed reports of behavioral sex differences in context and cued fear conditioning paradigms, which can depend upon the behavioral indices of fear. However, there is greater evidence of differential activation of the hippocampus, amygdalar nuclei and the prefrontal cortical regions in male and female rodents during context and cued fear conditioning. The bed nucleus of the stria terminalis (BNST), a sexually dimorphic structure, is of particular interest as it differentially contributes to fear responses in males and females. In addition, while the influence of the estrous cycle on different phases of fear conditioning is delineated, the clearest modulatory effect of estrogen is on fear extinction processes. Examining the variability in neural responses and behavior in both sexes should increase our understanding of how that variability contributes to the neurobiology of affective disorders.

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1. Introduction

Post-traumatic stress disorder (PTSD) is twice as common in women as in men following a traumatic experience (Breslau, 2009; Kessler et al., 1995). This increased risk among women persists even when controlling for variables such as the cause and severity of trauma (Olf et al., 2007; Kessler et al., 2012). Furthermore, women are up to 60% more likely to suffer from an anxiety disorder such as panic attacks and phobias compared to men (de Jonge et al., 2016; McLean et al., 2011). The reasons for this increased disease burden arise from multiple interactions between neurobiological, physiological and sociocultural factors (Tolin and Foa, 2006). PTSD and anxiety disorders can be characterized by abnormalities in fear learning and extinction (Rothbaum and Davis, 2003; Mahan and Ressler, 2012). Indeed, altered fear inhibition, processing of contextual information, fear extinction and fear generalization are all hallmarks of PTSD (Wessa and Flor, 2007; Blechert et al., 2007;

Milad et al., 2008; Jovanovic et al., 2009, Rougemont-Bücking et al., 2011). However, the majority of preclinical studies elucidating the neural circuitry and molecular mechanisms of fear conditioning have used male rodents (Predergast et al., 2014; Lebron-Milad and Milad, 2012). Delineating sex differences in fear learning and memory processes will ultimately contribute to the development of effective therapeutic interventions for both sexes.

Associative fear learning and its neural substrates have been subjects of intense investigation for the past 40 years (reviewed in Pape and Pare, 2010; Herry and Johansen, 2014). This type of learning is rapid, robust, readily quantified, and its stimulus parameters tightly controlled. While fear conditioning involves coordinated activity across multiple brain regions, this review emphasizes the neural structures and pathways which are differentially recruited by male and female rodents (Fig. 1). The amygdala integrates sensory information about both discrete cues and context with aversive input in both rodents and humans (Delgado et al., 2006). Context fear conditioning and both cued and context fear extinction require interactions between the cortex, hippocampus and amygdala (Yavas et al., 2019). In rodents, these cortical regions include the prelimbic cortex (PL) and infralimbic cortex (IL) of the medial prefrontal cortex. In humans, the analogous cortical regions are the dorsal anterior cingulate cortex and the ventromedial prefrontal cortex, respectively (Phan et al., 2002). Importantly, both men and women with PTSD show altered activity of these regions in imaging studies (Shin et al., 2005; Williams et al., 2006; Frewen et al., 2011). Indeed, PTSD can be characterized by hyperactivation of the amygdala to aversive stimuli and hypoactivation of the ventromedial prefrontal cortex in regulating the amygdala (Milad and Quirk, 2012).

In humans, sex differences result from a combination of biological sex and gender, a term for both self and societal perception of sex (McCarthy, 2016). Gender is influenced by both psychological and cultural factors (Mauvais-Jarvis et al., 2020). In rodents, sex differences are due to biological sex alone, which is driven by sex chromosomes. Indeed, sex differences can be viewed as a proxy for factors such as sex-linked genes, or hormones (Maney, 2016). While examining how sex differences in fear responses are manifested at the neuronal level in animal models has considerable translational significance, the distinction between these responses of the brain and body and the conscious feelings of fear and anxiety cannot be directly explored using animals. While non-human animals can detect threats and respond defensively, we cannot determine whether they also experience subjective conscious feelings of fear (LeDoux and Pine, 2016). With these caveats in mind, this review highlights recent advances in our understanding of sex differences in context and cued fear conditioning, fear extinction, and fear generalization, with an emphasis recent developments in understanding the neural circuitry underlying these behaviors.

2. Context fear conditioning

Context can be broadly defined as the external environment and internal physiological state of an animal (Spear, 1973). Deficits in context processing can lead to inappropriate behavioral responses. PTSD is perhaps the most representative example, as memories of the traumatic event are experienced in neutral or safe environments (Ressler and Mayberg, 2007). In Pavlovian fear conditioning, animals are placed in a conditioning

chamber which serves as an environmental context. When aversive stimuli are delivered (usually electric footshocks), animals form an association between the context and the aversive unconditioned stimulus (US). When they are returned to the conditioning chamber, conditioned responses such as freezing, occur (reviewed in Maren et al., 2013).

In order for this context-US association to occur, animals must first form a representation of the context, and then must associate this encoded context with an aversive stimulus. In this first step, elements of an environment (lighting, sounds, odorants, space) become bound into a unitary contextual representation (Rudy and O'Reilly, 2001; Rudy, 2009). There is evidence that females have slower acquisition of context representations: short time intervals between exposure to the context and the delivery of the footshock selectively impair learning in females (Wiltgen et al., 2001). The idea that males are better able to encode context representations is supported by studies showing that males display stronger context fear conditioning than females (Maren et al., 1994; Pryce et al., 1999; Poulos et al., 2015; Mizuno et al., 2012; Gresack et al., 2009), even at longer (14 day) retention intervals (Colon et al., 2018).

Others, however, report no behavioral sex differences in context fear conditioning (Dachtler et al., 2011; Kosten et al., 2005; Urien et al., 2021), or observe sex differences only under specific experimental conditions (Wiltgen et al., 2001) or in specific strains (Pryce et al., 1999). Furthermore, others find stronger context fear conditioning in females (Moore et al., 2010; Keiser et al., 2017). Reconciliation of these apparently conflicting results takes into account sex differences in the behavioral expression of fear, the estrous cycle of females and differential activation of the neural circuits underlying context fear conditioning in males and females.

One explanation for the reduced levels of freezing in females to an aversive context might involve different behavioral responses to the context. For example, when rats are given the chance to avoid an aversive context, females show more entries into the non-shock arm of a Y-maze (Shanazz et al., 2002). This suggests that females might express fear memory of a context with a more active avoidance response. This sex difference in the behavioral expression of fear memory is also seen during cued fear conditioning (see below). There are also numerous examples of male and female rodents using different learning strategies to solve spatial navigation tasks (Hawley et al., 2012; Grissom et al., 2013).

During human and rodent estrous cycles, both estrogen and progesterone levels fluctuate (Fig. 2). In rodents, the estrous cycle consists of four approximately 12–24 h long stages: proestrus, estrus, metestrus and diestrus, which can be characterized by fluctuations in levels of the potent biologically active estrogen 17β estradiol (E2). E2 levels are elevated during proestrus and early estrus, they plunge during metestrus, and begin to rise again during diestrus (reviewed in Taxier et al., 2020). In humans, the menstrual cycle has an average twenty-eight day duration with fluctuating levels of estrogen and progesterone. The follicular phase begins on the first day of menstruation and ends at ovulation on Day 14, followed by the luteal phase. E2 peaks in the late follicular phase and rises again during the mid-luteal phase whereas progesterone peaks in the mid-luteal phase. Both E2 and

progesterone levels are lowest in the early follicular and late-luteal phases (Guyton and Hall, 2006).

Numerous studies have examined the neurophysiological effects of E2 on the hippocampus. Exogenous E2 increases dendritic spine density on pyramidal neurons in the CA1 region (Woolley and McEwen, 1993) as well as in the amygdala (de Castilhos et al., 2008) and the prefrontal cortex (Khan et al., 2013). Within the hippocampus, E2 increases glutamate binding to NMDA receptors (Woolley et al., 1997) and enhances long-term potentiation at CA3-CA1 synapses (Foy et al., 1999; Smith and McMahon, 2005). Several excellent reviews describe the effects of E2 on hippocampal-dependent learning and memory, and the mechanisms of E2 signaling (Taxier et al., 2020; Frick, 2015).

The effects of E2 on context fear conditioning seem to depend on dose and treatment duration. Long-term estrogen treatment in ovariectomized mice enhances context fear conditioning and fear-potentiated startle, and increases corticotropin-releasing hormone mRNA expression in the central nucleus of the amygdala (CE; Jasnow et al., 2006; Hiroi and Neumaier, 2006). This effect seems to be dose-dependent, as high chronic levels of E2 (either 1000 nM or 50 µg) increase context fear conditioning and anxiety-like behavior on the elevated plus maze, while low doses (either 200 nM, 0.05 µg, or 5 µg) do not (McDermott et al., 2015; Matsumoto et al., 2018). To test the chronic effects of E2, it is usually administered via drinking water, or via capsule implantation in ovariectomized females. However, when E2 is administered as one injection prior to behavioral testing, the opposite behavioral effects are observed. Indeed, one injection of a high level of E2 (10 µg, s.c.) either 30 min or 4 h before training impairs context fear conditioning (Barha et al., 2010; Gupta et al., 2001), while low levels (0.3 µg, s.c.) of E2 facilitate context fear conditioning as well as spatial working memory (Barha et al., 2010). In naturally cycling females, the proestrus phase, which coincides with high levels of E2, can be associated with lower levels of context fear conditioning (Cushman et al., 2014), including fear responses to a sustained cue (Vantrease et al., 2022). However, others find no effect of estrous cycle on context fear conditioning (Keiser et al., 2017). Further research is needed to clarify how naturally cycling levels of E2 affect the acquisition and consolidation of context fear conditioning.

3. Context fear conditioning: neural circuits

The neural structures and molecular mechanisms underlying context fear conditioning are beyond the scope of this article, but several excellent reviews provide these details (Maren et al., 2013; Chaaya et al., 2018). In brief, the hippocampus is essential for encoding the spatial properties of the context (Holland and Bouton, 1999; Fanselow and Dong, 2010), consistent with its role in spatial representation and navigation. The basolateral amygdala (BLA), composed of the lateral (LA) and basal (BA) nuclei is crucial for integrating context representations with somatosensory information about the US (Anagnostaras et al., 2001). The prelimbic region (PL) of the prefrontal cortex processes aspects of both the spatial and emotional aspects of context fear conditioning (Zelikowsky et al., 2014). The experiments detailing the contribution of these structures to context fear conditioning have

been conducted almost entirely in male rodents (rats and mice) or in both males and females but without explicitly analyzing sex differences, except as described below.

Both male and female rodents recruit the dorsal hippocampus during consolidation of context fear conditioning (Keiser et al., 2017). Both protein kinase A and cAMP response element binding protein (CREB) are required in both sexes for long term fear memories (Abel et al., 1997; Bourtchuladze et al., 1994). However, males show greater phosphorylated extracellular signal-regulated kinase (ERK) and phosphorylated CREB in the dorsal hippocampus during context fear conditioning acquisition and consolidation (Gresack et al., 2009; Kudo et al., 2004).

Indeed, evidence suggests that during the expression of context fear conditioning in rodents, males shift towards hippocampal activation and females shift towards BLA activation (Fig. 3A). The hippocampus and BA compete during context fear conditioning (Biedenkapp and Rudy, 2009). Context fear retrieval increases FOS in both the proximal and distal CA1, and the CA3 and dentate gyrus regions of the hippocampus in male mice more than in females (Colon and Poulos, 2020; Keiser et al., 2017). In contrast, the BLA is activated in response to footshock and context exposure to a greater extent in females than in males (Colon and Poulos, 2020). Moreover, context fear expression increases FOS activity in the BA in females more than males (Keiser et al., 2017). If males rely on hippocampal processes more than females, training paradigms that favor hippocampal processing should produce greater fear conditioning in males. Indeed, when the interval between exposure to the context and the delivery of the footshock is shortened, male mice acquire context fear conditioning more than females (Wiltgen et al., 2001). The balance between activating hippocampal and amygdala circuits during context fear conditioning also has implications for generalization of context fear (see below).

4. Extended amygdala circuits

Differential activation of the bed nucleus of the stria terminalis (BNST) in males and females also affects the expression of context fear conditioning. Indeed, there are volumetric and neurochemical differences in the BNST of males and females (del Abril et al., 1987; Allen and Gorski, 1990; Hines et al., 1992). The BNST plays a role in sex-specific behaviors such as aggression in males (Trainor et al., 2010; Masugi--Tokita et al., 2016) and maternal behavior (Klampfl et al., 2014). Moreover, areas of the BNST that are involved in reproductive behavior have different patterns of connectivity in males and females, which are attributable to different hormonal influences (Polston et al., 2004; Stefanova and Ovtscharoff, 2000). In male rodents, lesions and temporary inactivation of the BNST have established that the dorsal anterior portion is necessary for the expression but not acquisition of context fear (Sullivan et al., 2004; Duvarci et al., 2009; Pelrine et al., 2016). Moreover, both FOS and ARC expression are increased in the anterolateral BNST following context fear expression in males. However, this increase in immediate-early gene activity is not seen in females (Urien et al., 2021; Urien and Bauer, 2022).

Three neurophysiologically distinct cell types have been identified within the anterolateral BNST (Daniel et al., 2017). These can be distinguished based on their spiking and

rectification properties and responses to depolarizing and hyperpolarizing current injection (Hammack et al., 2007; Rodríguez-Sierra et al., 2013). They also differ in their expression of serotonin receptor subtypes, and corticotropin releasing factor (CRF) (Dabrowska et al., 2013; Guo et al., 2009). As CRF acts within the BNST to increase anxiety-like behaviors (Sahuque et al., 2006), CRF + Type III neurons are thought to be “anxiety on” neurons, while Types I and II are “anxiety off” (Dabrowska et al., 2013; Daniel and Rannie, 2016). There is evidence that there are more CRF-containing neurons in the anterolateral BNST in females (Uchida et al., 2019), and that the overall number of neurons in the female anterolateral BNST is greater than the male (Guillamon 1988). CRF + neurons also differ in the timing of their responses to noxious stimuli in males and females (Yu et al., 2021). Electrophysiological characterization of Types I and II BNST neurons reveals no effect of estrous cycle and no difference in firing properties between males and females (Smithers et al., 2018). However, when correlating neuronal activity with behavior, sex differences are observed. For males, time spent in the open arms of the elevated plus maze correlates with lower resting membrane potentials of Type I neurons and higher firing rates of Type II neurons. No correlations between neuronal activity and behavior are seen in females (Smithers et al., 2018). Finally, serotonin differentially affects neuronal excitability and behavior in males and females. Although 5HT-1A receptor protein levels do not significantly differ between males and females, knockdown of these receptors in the BNST increases excitation and fear responses in males only, not in females, without affecting anxiety-like behaviors (Marcinkiewicz et al., 2019).

The BNST receives strong glutamatergic input from the basal nuclei of the amygdala, with the basolateral nucleus projecting to the anterolateral portion and the basomedial nucleus projecting the anteromedial portion (Krettek and Price, 1978; Dong et al., 2001). The anterolateral BNST sends robust projections to the CE, which are mainly GABAergic (Sun and Cassell, 1993; Poulin et al., 2009). Several recent studies suggest that these circuits are differentially activated during context fear expression in males and females. When animals are exposed to a sustained 8 min auditory stimulus followed by an aversive footshock, males exhibit greater fear expression than females when tested 4 days later (Vantrease et al., 2022). Fear responses to sustained cues, or diffuse environmental threats paired with aversive stimuli are dependent on the BNST (Walker et al., 2009; Torrisi et al., 2018). When the specific BLA-BNST pathway is inhibited, using chemogenetic techniques, fear expression to the sustained cue is reduced, but only in males not in females (Vantrease et al., 2022). This indicates that the BLA-BNST pathway is active during sustained fear responses, but only in males.

Regardless of estrous cycle, BLA neurons in females have higher spontaneous firing rates compared to males (Blume et al., 2017, 2019). Moreover, the frequency and amplitude of mEPSCs, as well as the number of spines in both the LA and BA are higher in pyramidal neurons of females compared to males (Blume et al., 2017). When both pyramidal and stellate neurons are examined, others find no sex differences in mEPSCs and spine density, but increased spine length in females (Guily et al., 2022) However, when BLA neurons specifically projecting to the BNST are examined, the opposite pattern is observed. Males have greater spontaneous activity in BNST-projecting BLA cells, and these neurons are more excitable by depolarizing current injections. These physiological properties correspond to

smaller I_{AHPs} in these neurons in males compared to other BLA subpopulations (Vantrease et al., 2022). In contrast, BNST-projecting neurons in females have lower spontaneous activity than other subpopulations of BLA neurons, and the decreased excitability of these neurons corresponds with larger I_{AHPs} (Vantrease et al., 2022). Finally, there are fewer BNST-projecting BLA neurons overall in females compared to males (Vantrease et al., 2022). Together, these findings identify a specific sexually dimorphic pathway within the extended amygdala (Fig. 3A).

The BNST and CE are reciprocally connected (Dong et al., 2001; Sun and Cassell, 1993). They both receive glutamatergic inputs from the BLA and their efferents target the same brain stem structures (Dong et al., 2001; Krettek and Price, 1978; Holstege et al., 1985). Indeed, the BNST has been classified as belonging to the extended amygdala complex (Alheid, 2003; Walker and Davis, 2008). FOS protein expression increases in the male BNST during the expression of context fear (Lemos et al., 2010; Urien and Bauer, 2022, but see Sasaki Russell et al., 2020). However, neither FOS nor ARC expression increases in the female BNST following context fear expression (Urien et al., 2021; Urien and Bauer, 2022). Furthermore, when analysis of ARC expression is restricted to CE-projecting BNST neurons, only males continue to exhibit increased activity following context fear expression (Urien et al., 2021). Activity in the BNST-CE pathway does not increase in female rodents. Taken all together, these data suggest that in males but not females, context fear expression activates both a BLA-BNST and BNST-CE circuit. Interestingly, a study of resting-state fMRI effective connectivity shows greater connectivity in both the BLA-BNST and BNST-CE pathways in men compared to women (Hofman and Straube, 2019). Whether these pathways are differentially activated in men and women during fear conditioning tasks is currently not known.

It should be noted that neurotoxic lesions of the BNST do impair the expression of context fear conditioning in both males and females (Urien et al., 2021). This suggests that a subpopulation of neurons within the BNST of females does contribute to context fear expression, or that neurons outside the specific anterodorsal BNST may be recruited in females. Clearly, the behavioral function of the BLA-BNST and BNST-CE circuit in females requires further study.

In sum, while there are mixed reports of behavioral sex differences in context fear conditioning, there is more evidence of different neural circuit recruitment in male and female rodents. To enhance reproducibility and allow comparisons between experiments, nearly all studies of context fear conditioning use freezing as a behavioral index of fear. However, both the BNST and CE project to multiple downstream targets including brainstem areas involved in behavioral responses, hypothalamic areas involved in autonomic modulation and the parabrachial nucleus (Radley et al., 2009; Dong et al., 2001; Petrovich and Swanson, 1997). As described above, the BNST and CE may be differentially activated in males and females during context fear conditioning. This suggests that a broader characterization of fear expression might reveal differences between males and females. Indeed, as described below, multiple behavioral measures of fear expression are used in cued fear conditioning experiments.

5. Generalization of context fear expression

In humans, many anxiety disorders and PTSD are characterized by an inability to reduce fear responses in non-threatening contexts (Jovanovic and Ressler, 2010). Instead, fear responses are generalized to neutral or safe contexts (Lissek et al., 2010; Lopresto et al., 2016). In animal models, one way this can be represented is by a generalization of fear responses to a neutral context which is different from the conditioning context. As described above, context fear conditioning requires the animal to form a representation of the context and then associate it with an aversive stimulus. When exposure times to these contexts are reduced, or the interval between exposure context and shock delivery is reduced, animals form incomplete representations of each context and generalization occurs (Westbrook et al., 1994). Lengthening the time between training and retrieval by weeks or months also leads to an increase in fear responses to neutral contexts (Wiltgen and Silva, 2007).

Female rodents exhibit greater fear generalization to neutral contexts than males (Keiser et al., 2017; Asok et al., 2019). At long (3 weeks), but not short (1–2 days) time intervals between training and testing, females exhibit greater fear responses in the neutral context when they are tested in the neutral context before the conditioning context (Keiser et al., 2017; Asok et al., 2019). Females rodents are thus predisposed to freeze more in a neutral context when it is presented before the training context, while male rodents do not exhibit this bias. However, pre-exposing female rodents to the training context prior to training reduces fear generalization (Keiser et al., 2017; Asok et al., 2019). These data suggest that generalization in females occurs when the representation of the training context is weak, and that preexposure can allow them to form a more detailed context representation (Fanselow, 1990; Urcelay and Miller, 2014).

The effects of estrogen on generalization of context fear are not straightforward. In naturally cycling females, estrous cycle does not affect fear generalization (Keiser et al., 2017). However, when ovariectomized females are administered E2 (15 µg, s.c.) prior to training, they generalize their fear to neutral contexts (Lynch et al., 2014). This effect is mediated by cytosolic estrogen receptors in the dorsal hippocampus (Lynch et al., 2016).

6. Generalization of context fear conditioning: neural circuits

As described above, context fear retrieval may recruit different neural circuits in males and females, with males shifting towards hippocampal activation and females shifting towards BLA activation. Indeed, during aversive memory tasks, hippocampal activity and amygdala activity can suppress each other (Biedenkapp and Rudy, 2009; McDonald and White, 1995; McIntyre et al., 2002). In males but not females, both context fear retrieval and generalization increase FOS expression in both the CA1 and CA3 regions of the hippocampus (Keiser et al., 2017; Colon and Poulos, 2020). Conversely, in females but not males, exposure to both the training context and the neutral context enhance FOS activity in the BA. When animals are pre-exposed to the training context prior to training, context fear conditioning is enhanced in males only, and fear generalization is reduced in females only (Keiser et al., 2017). To date, the contribution of the BNST to context fear generalization in males and females has not been studied. The effects of sex on context fear conditioning

and context generalization are thus complex. If different neural circuits are responsible for context fear retrieval, then the optimal conditions for retrieval may differ in males and females (Lynch et al., 2014; Wiltgen et al., 2001). Alternatively, males and females may use different strategies during context fear conditioning and retrieval (Pellman et al., 2017; Shansky et al., 2018).

7. Cued fear conditioning

In classical Pavlovian cued fear conditioning, animals acquire an association between an aversive US and a conditioned stimulus (CS) such as a tone or light. This association is dependent on contingency, or the ability of the CS to predict US occurrence (Rescorla, 1968). Afterwards, when presented alone, the CS elicits responses characteristic of fear including autonomic (increased heart rate and blood pressure) and behavioral responses (LeDoux, 2000).

Numerous studies have revealed that when freezing to the CS is used as an index of fear, there are no behavioral sex differences (Baran et al., 2009; Clark et al., 2019; Fenton et al., 2014; Greiner et al., 2019; Day and Stevenson, 2020), although some have found reduced (Baran et al., 2010; Pryce et al., 1999) or enhanced (Gresack et al., 2009; Chen et al., 2014) cued fear conditioning in females. Interestingly, sex differences in passive vs. active fear responses have been reported, with females using an active “darting” response to the conditioned stimulus during testing (Gruene et al., 2015; Colom-Lapetina et al., 2019). Females also exhibit more diverse behavioral patterns of responses in other tasks such as the forced swim test (Colom-Lapetina et al., 2017). However, active vs. passive coping strategies in females do not necessarily predict behaviors in other tasks (Colom-Lapetina et al., 2019; Kent et al., 2017). More recently, ultrasonic vocalizations (USVs) have been used as an index of affective state in rodents to model affective disorders (Knutson et al., 2002; Sangiamo et al., 2020; Burgdorf et al., 2020). USVs in the 18–32 kHz range are emitted during fear conditioning (Borta et al., 2006; Litvin et al., 2007), while USVs in the 32–70 kHz range are generally produced during rewarding behaviors (Brudzynski, 2021). Analysis of 22 kHz USVs reveals that females vocalize less frequently than males during cued fear retrieval (Shumake et al., 2014; Willadsen et al., 2021). However, females vocalize more than males at 50 Hz (Tryon et al., 2021). Reliance on one measure of fear responses thus misses the complexity of an animal’s range of behaviors. Further, there may be sex-specific responses to aversive stimuli that result from variability within subsets of populations.

There are mixed reports of the effects of estrous cycle on cued fear conditioning in rodents. Animals conditioned during the proestrus phase, characterized by high levels of E2, show no difference in cued fear acquisition compared to animals conditioned during the metestrus phase, characterized by low E2 levels (Milad et al., 2009). Others, however, find that proestrus is associated with lower levels of cued fear (Blume et al., 2017). Female rodents do show more anxiety-like behaviors during the metestrus and diestrus phases, characterized by low estrogen (Marcondes et al., 2001). In ovariectomized females, however, long-term E2 treatment using capsule implantations enhances cued fear responses (Jasnow et al., 2006, Morgan and Pfaff, 2001), as well as fear-potentiated startle (Hiroi and Neumaier, 2006). As with the effects of estrogen on context fear conditioning discussed above, it is

difficult to reconcile data from naturally cycling females with data from females receiving hormone replacement. Changes in E2 levels are accompanied by variations in progesterone levels. When ovariectomized mice are injected with progesterone alone, they show increased freezing during both cued and context fear retrieval (Frye and Walf, 2008). In healthy women, there is no strong association between menstrual phase and cued fear conditioning (White and Graham, 2016; Milad et al., 2006).

Fluctuating estrogen levels may also impact fear- and anxiety-like behavior through epigenetic mechanisms. Women with PTSD have increased methylation of CpG sites in the *HDAC4* gene which encodes histone deacetylase 4 (Maddox et al., 2018), an HDAC associated with learning and memory (Wang et al., 2011; Kim et al., 2012). Cued fear conditioning in mice increases *Hdac4* mRNA expression in the amygdala during low-estrogen phases of the estrous cycle in mice (Maddox et al., 2018). While sex differences in gene expression, and the genetic factors underlying sex differences in affective disorders are gaining more attention (Oliva et al., 2020; Ponomareva and Ressler, 2021), preclinical studies including both sexes are necessary. Most rodent GWAS and large-scale transcription studies exploring changes in gene expression related to affective disorders have focused only on male animals (Gray et al., 2014; Muhie et al., 2015).

8. Cued fear conditioning: neural circuits

During auditory fear conditioning, sensory information converges in the LA where associative plasticity underlies fear conditioning (Sears et al., 2014; Rogan et al., 1997; Kim and Cho, 2017). The BA receives input from the hippocampus and integrates relevant contextual information (Calandreau et al., 2005). The central amygdala (CE), itself a site of plastic changes during fear learning, is also the main output nucleus of the amygdala (Ehrlich et al., 2009; Ciochi et al., 2010). The prelimbic region (PL) of the prefrontal cortex is active during fear memory recall and its outputs to the BLA are necessary for fear retrieval (Do-Monte et al., 2015).

Neurons in the LA respond to both auditory and aversive stimuli (Romanski et al., 1993), and show significant increases in firing in response to auditory stimuli after fear conditioning (Quirk et al., 1995). Long-term potentiation occurs at input synapses to the LA, and pharmacological blockade of LTP prevents fear conditioning (Rogan and LeDoux, 1995; Bauer et al., 2002). These experiments were conducted entirely in male rats and mice, except as described below.

Excitatory input to the LA and synaptic plasticity is stronger in females than in males, (Blume et al., 2017; Chen et al., 2014, Fig. 3B). Similarly, an fMRI study in humans revealed greater changes in activation of the amygdala during fear conditioning in females compared to males (Lebron-Milad et al., 2012). Orchiectomy in males enhances cued fear conditioning and LTP at input synapses to the LA. This enhancement is reduced when supplemental testosterone is administered. In contrast, ovariectomy in females reduces fear conditioning and LTP in the LA which is restored by E2 administration (Chen et al., 2014). The synthesis of E2 can be blocked using drugs that inhibit the P450 enzyme aromatase. In the hippocampus, the inhibition of aromatase impedes LTP (Di Mauro et

al., 2015). Aromatase is also expressed in the amygdala (Wagner and Morrell, 1996). In both the hippocampus and amygdala, pharmacological inhibition of aromatase decreases spine density and LTP in females but not in males (Vierk et al., 2012). This suggests that neuron-derived E2 modulates synaptic plasticity in the amygdala in females only. Together these findings suggest that in females, E2 enhances synaptic plasticity in the LA which could contribute to enhanced cued fear conditioning.

Sex differences in GABAergic interneuron activity within the BLA also have the potential to influence cued fear conditioning. The calcium-binding proteins parvalbumin (PV) and calbindin (CB) are expressed in about 50% of BLA GABAergic neurons (Mascagni et al., 2009; McDonald and Mascagni, 2001). PV + neurons preferentially synapse on the soma of their target cells and provide the main source of inhibition to BLA excitatory neurons (Muller et al., 2006). Roughly 25% of GABAergic neurons containing calretinin (CR); these cells target other interneurons (Mascagni et al., 2009). Finally, a minority of GABAergic interneurons contain somatostatin (SOM; McDonald and Pearson, 1989).

PV + interneuron activity has direct effects on the magnitude of fear learning (Wolff et al., 2014). During presentations of the CS, PV + neurons are excited and enhance auditory responses in BLA neurons through inhibition of SOM + interneurons which disinhibits the principal excitatory neurons (Wolff et al., 2014). During the low E2 phase of diestrus, the number of PV + neurons in the female BLA increases (Blume et al., 2017). This could contribute to the facilitation in cued fear learning and anxiety-like behavior seen during the diestrus phase. The number of SOM + interneurons depends on sex chromosomes, with XX chromosomes increasing SOM expression (Puralewski et al., 2016). As SOM + neurons regulate cued fear responses (Wolff et al., 2014), these neurons may contribute to sex differences in cued fear. Finally, female guinea pigs have a higher density of calbindin-containing interneurons within the BLA (Równiak et al., 2015). Given the enhancing effects of E2 on synaptic plasticity in the LA, and the contribution of GABAergic transmission to increased excitatory activity in the LA, it is surprising that most find no difference in cued fear conditioning in males and females. Perhaps focusing on freezing behavior as a measure of fear misses the variability of fear responses in females. Or compensatory activity in other amygdala regions in males yields equivalent behavioral responses.

The PL is also a site of plasticity during fear learning and is active during fear memory recall (Sotres-Bayon and Quirk, 2010). *In vivo* electrophysiological recordings from the PL reveals that females show sustained activity in this area which parallels increased fear expression (Fenton et al., 2014). Gamma oscillations in the PL are specifically stronger during cued fear recall in females compared males (Fenton et al., 2016). Interestingly, chemogenetic inhibition of PL cortical ensembles does not affect freezing behavior to conditioned cues, but does reduce conditioned suppression of food seeking in females but not males (Giannotti et al., 2019). This suggests that the contribution of PL to sex-dependent fear expression depends on the behavioral task.

Unlike context conditioning, cued fear conditioning is not hippocampal-dependent (Kim and Fanselow, 1992). However, trace conditioning involves a temporal separation of the cue from the US and does depend on hippocampal activity (McEchron et al., 1998). Trace

conditioning also requires persistent neuronal firing in the prefrontal cortex to maintain the representation of the cue across the trace interval and link it with an outcome during memory formation (Gilmartin and Helmstetter, 2010). In males, prefrontal activity during the trace interval requires cholinergic signaling. Trace conditioning can be disrupted by a low dose of a muscarinic antagonist in males, but only at higher doses in females in estrus or metestrus. Females in proestrus are insensitive to the effects of muscarinic receptor antagonism in the prefrontal cortex (Kirry et al., 2019). In contrast, inhibition of PACAP receptors in the prefrontal cortex impairs trace cued conditioning in females (Kirry et al., 2018). Thus, while there appears to be no difference in the magnitude of fear expression in trace conditioning tasks between males and females, this set of studies offers an example of males and females engaging different neuromodulatory systems within the prefrontal cortex during fear expression. Further, the contribution of these modulatory systems to trace fear conditioning are regulated by the estrous cycle in female rodents.

9. Generalization of cued fear conditioning

The inability to inhibit fear is a phenotype of several anxiety disorders and PTSD (Jovanovic et al., 2012; Pitman et al., 2012). Fear inhibition processes include fear extinction, safety learning, and fear-safety discrimination (Milad and Quirk, 2012; Marshall et al., 2014; Day et al., 2016). Fear generalization occurs when cues that resemble the initial CS also elicit fear responses. While some generalization of fear responses provides an adaptive benefit for an animal's survival, overgeneralization to harmless stimuli is seen in anxiety disorders and PTSD (Lissek, 2012). Several recent excellent reviews provide details on the behavioral and neural mechanisms of fear generalization (Dunsmoor and Paz, 2015; Asok et al., 2019).

Most studies of fear generalization use a CS + which is paired with an aversive stimulus and predicts threat, and a CS- which is unpaired with the aversive stimulus and predicts safety. Generalization is defined by equivalent freezing to the CS+ and CS-, whereas discrimination produces greater freezing to the CS + than the CS-. Recent studies of sex differences in cued fear discrimination in rodents have produced mixed results. Some show successful discrimination in both males and females (Clark et al., 2019; Gilman et al., 2015). Others show more generalization in females (Day et al., 2016; Aranda-Fernandez et al., 2016; Greiner et al., 2019), or more generalization in males (Foilb et al., 2017). When animals are trained using CSs that are explicitly unpaired with the US, females show greater fear responses than males to the unpaired CS (Urien and Bauer, 2022). Training protocols can also differentially affect fear generalization. After one day of training, females show greater discrimination, but after two or three days, males show greater discrimination between CS+ and CS- (Day et al., 2016). In contrast, research in humans suggests that females show reduced discrimination between CS+ and CS- compared to males (Lonsdorf et al., 2015; Gamwell et al., 2015). Very little research has examined the effects of estrus or menstrual cycle on fear discrimination and generalization in either rodents or humans. There is some evidence that women in the luteal phase of the menstrual cycle associated with higher levels of estrogen and progesterone, discriminate between the CS+ and CS- better than either men or women in the follicular phase (Merz et al., 2012; Glover et al., 2013). In contrast, one study found no effect of menstrual cycle phase or contraceptive use on fear discrimination in healthy females (Lonsdorf et al., 2015). In rodents, however, estrogen

replacement in gonadectomized male and female impairs conditioned inhibition, a form of safety learning (Toufexis et al., 2007). Conditioned inhibition occurs when both the fear and safety cues are presented simultaneously, resulting in reduced fear responses (Rescorla, 1969). A recent review compares rodent and human clinical sex differences in conditioned inhibition responses (Krueger and Sangha, 2021).

10. Cued fear generalization: neural circuits

As described above, individual LA neurons encode the association between the CS and the aversive US and enhance their responsiveness to the CS after fear conditioning (Quirk et al., 1995). Neuronal activity in the LA is also necessary for the generalization of fear (Ghosh and Chattarji, 2015). Further, as animals shift their behavior from cue discrimination to generalization, a larger proportion of LA neurons fail to distinguish between the CS+ and CS- (Ghosh and Chattarji, 2015). When FOS is used as an index of neuronal activity, there is no correlation between FOS expression in the BLA and discriminative ability, and no sex differences (Foilb et al., 2021). Although the BNST is not necessary for cued fear conditioning, lesions of the BNST reduce fear generalization (Duvarci et al., 2009). In both males and females, fear discrimination is associated with increased FOS expression in the BNST, but this increase is greater in males than in females (Foilb et al., 2021). Regions of the CE also regulate fear generalization (Ciocchi et al., 2010). Changes in inhibitory input from the lateral portion of the CE to neurons in the medial portion can affect fear responses to both the CS+ and CS- (Ciocchi et al., 2010). However, these studies were conducted only in male rodents. When fear discrimination is correlated with FOS expression in the CE, no sex differences are observed (Foilb et al., 2021). Thus to date, there are no clear behavioral sex differences in cued fear discrimination in rodents and very few studies of the differential contribution of neural structures to fear discrimination in males and females.

11. Cued fear extinction

When animals are presented with a CS that was previously paired with a US, they learn that the CS no longer predicts the US. This phenomenon, cued fear extinction, is a new type of learning that inhibits the original fear memory (Bouton et al., 2006; Myers and Davis, 2002). Since it forms the theoretical basis for exposure therapy, the psychological and neurobiological mechanisms of fear extinction have been the subject of intense research over the past decades (Singewald and Holmes, 2019; Tovote et al., 2015). In PTSD and anxiety disorders, fear extinction processes are disrupted (Sevenster et al., 2018; Zuj and Norrholm, 2019).

Cued fear extinction can be divided into two phases: within-session extinction when the CS is repeatedly presented, and between-session extinction when recall of the extinction memory is tested, usually 24 h after the extinction procedure. Females show reduced within-session extinction when compared to males, manifested as increased freezing behavior (Baker-Andresen et al., 2013; Baran et al., 2009; Clark et al., 2019). This deficit persists to between-session recall, as females exhibit more resistance to extinction (Fenton et al., 2014; Clark et al., 2019). Moreover, females show more spontaneous recovery of fear over time (Fenton et al., 2014; Matsuda et al., 2015).

Cued fear extinction in female rodents is clearly influenced by estrous cycle. During metestrus and diestrus when E2 levels are low, female rodents show reduced fear extinction recall compared to proestrus when E2 and progesterone levels are high (Milad et al., 2009; Chang et al., 2009; Gruene et al., 2015; Blume et al., 2017). Ovariectomy and hormonal contraceptives which reduce circulating E2, also reduce fear extinction in rats (Parrish et al., 2019). To establish a causal relationship between E2 levels and extinction learning, females in metestrus given E2 (15 µg/kg, s.c.) show enhanced extinction recall, whereas blocking estrogen signaling in females in proestrus reduces extinction recall (Maeng et al., 2017; Milad et al., 2009). Finally, E2 treatment enhances fear extinction in a dose-dependent manner (Graham and Scott, 2018).

A similar pattern of results is seen in human studies. Women with high E2 levels exhibit greater cued extinction recall than women with low E2 levels, as measured by E2 serum levels (Zeidan et al., 2011; Graham and Milad, 2013; Hwang et al., 2015). Low levels of estrogen are associated with impaired extinction in women with PTSD compared to non-traumatized controls (Glover et al., 2012). In addition, women with PTSD report more severe anxiety and re-experiencing symptoms during low-estrogen phases of their menstrual cycle (Bryant et al., 2011; Glover et al., 2012; Maeng and Milad, 2015). Lower estrogen levels are also associated with impaired fear inhibition in both traumatized and healthy women (Glover et al., 2013). Further, E2 levels modulate overall arousal levels in women with PTSD, with low E2 levels increasing this impairment while higher E2 levels might be protective (Sartin-Tarm et al., 2020). To establish a causal relationship between E2 levels and extinction recall, administration of E2 in naturally cycling early-follicular phase women prior to extinction learning enhances recall of extinction (Graham and Milad, 2013). Thus, E2 levels have a clear modulatory effect on fear extinction.

12. Cued fear extinction: neural circuits

The BLA is a critical site of plasticity for the acquisition and storage of cued extinction memory (Quirk and Mueller, 2008; Pape and Pare, 2010). This plasticity includes NMDA- and MAPK/ERK-dependent plasticity of principal neurons, as well as an increase in perisomatic inhibition of principal neurons (Sotres-Bayon et al., 2007; Herry et al., 2006; Trouche et al., 2013). Subpopulations of neurons within the BA targeting the infralimbic subdivision (IL) of the prefrontal cortex are recruited and exhibit plasticity during fear extinction (Senn et al., 2014). The IL itself is necessary for the retrieval of extinction of auditory fear conditioning memories (Quirk et al., 2000; Sierra-Mercado et al., 2011). Groups of intercalated neurons located between the BLA and CE receive input from the prefrontal cortex and contribute to the expression of fear extinction (Likhtik et al., 2008).

Using iontophoretic application of GABA or glutamate, the inhibition/excitation ratio of LA and BA neurons across the estrous cycle can be ascertained (Blume et al., 2017). During proestrus when estrogen levels are high, there is a shift towards stronger inhibition in the LA. This parallels faster extinction learning compared to the diestrus phase when there is less inhibition in the LA (Blume et al., 2017, Fig. 3C). Further, E2 treatment has been shown to reduce EPSP amplitude in BLA neurons *in vitro* (Womble et al., 2002). When E2 (15 µg/kg, s.c.) is injected prior to extinction learning in low-estrogen metestrus rats, it enhances

extinction and reduces c-FOS expression in the amygdala (Zeidan et al., 2011). Together these data suggest that the effects of the estrous cycle on fear extinction are at least partly mediated by reduced excitation and enhanced inhibition of BLA neurons by E2.

Fear extinction recall depends on the IL and interactions between the IL and the amygdala (Senn et al., 2014; Sierra-Mercado et al., 2011). E2 treatment induces greater activity in the IL during extinction memory recall, as indexed by FOS expression (Zeidan et al., 2011; Maeng et al., 2017, Fig. 3C). E2 itself potentiates intrinsic excitability of IL neurons (Yousuf et al., 2019). Similarly, in humans, women with high E2 levels exhibit greater activation of the ventromedial prefrontal cortex and amygdala compared to low E2 women (Zeidan et al., 2011).

The CE can be subdivided into lateral (CeL) and medial (CeM) portions which mediate fear responses. Neurons within the CeL can be classified as “fear on” or “fear off” neurons, which are reciprocally connected, differ in their neurochemical profiles, show plastic changes during fear learning and extinction, and gate fear expression and generalization (Ciocchi et al., 2010; Haubensak et al., 2010). The CeM is regulated by the CeL and functions as the main output of the CE to regulate behavioral fear responses (Duvarci and Pare, 2014). E2-mediated enhancement of fear extinction learning is associated with increased FOS expression in the CeL (Maeng et al., 2017). Twenty-four hours later, when fear extinction recall is assessed, animals receiving E2 injections (15 µg/kg, s.c.) show reduced FOS expression in the CeM and greater extinction recall (less freezing). Moreover, examining the ratio between FOS activation in the CE and IL reveals that E2 modifies interactions between the two regions. Extinction learning produces stronger CE modulation while extinction recall produces stronger IL modulation (Maeng et al., 2017). The IL also sends excitatory projections to the BLA which mediate fear extinction (Likhtik et al., 2005; Herry et al., 2008). When the morphology of neurons within the IL projecting to the BLA are examined, males with faster rates of extinction show increased dendritic arborization of these neurons and lower spine density (Gruene et al., 2015). Despite similar behavioral responses during extinction, females do not exhibit these structural changes. Interactions between the prefrontal cortices and amygdala subnuclei thus exhibit some differences depending on sex and E2 levels. As extinction-based therapies are used to treat many types of anxiety disorders and PTSD, parsing out these interactions has the potential to inform treatments.

13. Conclusion

Sex differences in fear responses are nuanced and complex. The neural structures discussed above contribute to multiple processes, and the behavioral responses are interdependent. For example, fear extinction will depend not only on extinction processes themselves, but on the strength of the original fear memory. In general, studies of fear learning in females have proceeded from the fiction that males are the default sex and that females must be compared to males using the same behavioral tasks and analyzing the same behavioral responses and neural circuitry. Nevertheless, it is possible to draw a few conclusions from the experiments described above.

First, it is clear that a broader range of behaviors, and possibly other measures such as autonomic arousal, can be used to more completely assess behavioral differences between male and female rodents. Indeed, in human fear conditioning studies, skin conductance response (SCR) is often used as a measure of fear, rather than freezing. These differences in readouts of fear can make it difficult to compare rodent and human fear conditioning studies. Recent experiments in which animals are given the opportunity to avoid an aversive stimulus (Shanazz et al., 2002), or in which active responses and USVs are analyzed in addition to freezing responses (Gruene et al., 2015; Tryon et al., 2021) are examples of this type of experiment.

Second, it is difficult to draw conclusions about the effects of estrus and menstrual cycles on context and cued fear conditioning. One notable exception is cued fear extinction in which female rodents and women at high E2 phases of their cycles show greater fear extinction recall compared to low E2 phases. The estrus cycle of rodents and the menstrual cycle of women differ in two important ways. First, the proestrus phase in rodents is characterized by both high E2 and high progesterone levels. In the menstrual cycle, E2 and progesterone levels peak on different days. Second, the duration of each phase in the estrus cycle is 12–24 h. Thus, when female rodents are conditioned on one day and tested 24 or 48 h later, they will be tested in a different estrous phase. Indeed, there is recent evidence that rats conditioned in the diestrus phase and tested in proestrus exhibit lower levels of context fear than animals conditioned and tested in the same estrus phase (Blair et al., 2022). Possible state-dependent effects of estrous cycle phase on fear processes are less of a concern in human studies where the follicular and luteal phases of the menstrual cycle last several days.

Finally, a more nuanced assessment of sex differences will ask what information is encoded by neural circuits and how males and females might use different strategies in approaching behavioral tasks (Tronson, 2018; Shansky, 2018). These types of studies have the potential to uncover variability in neural responses and behavior in both sexes, and increase our understanding of how that variability contributes to the neurobiological underpinnings of affective disorders.

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Data availability

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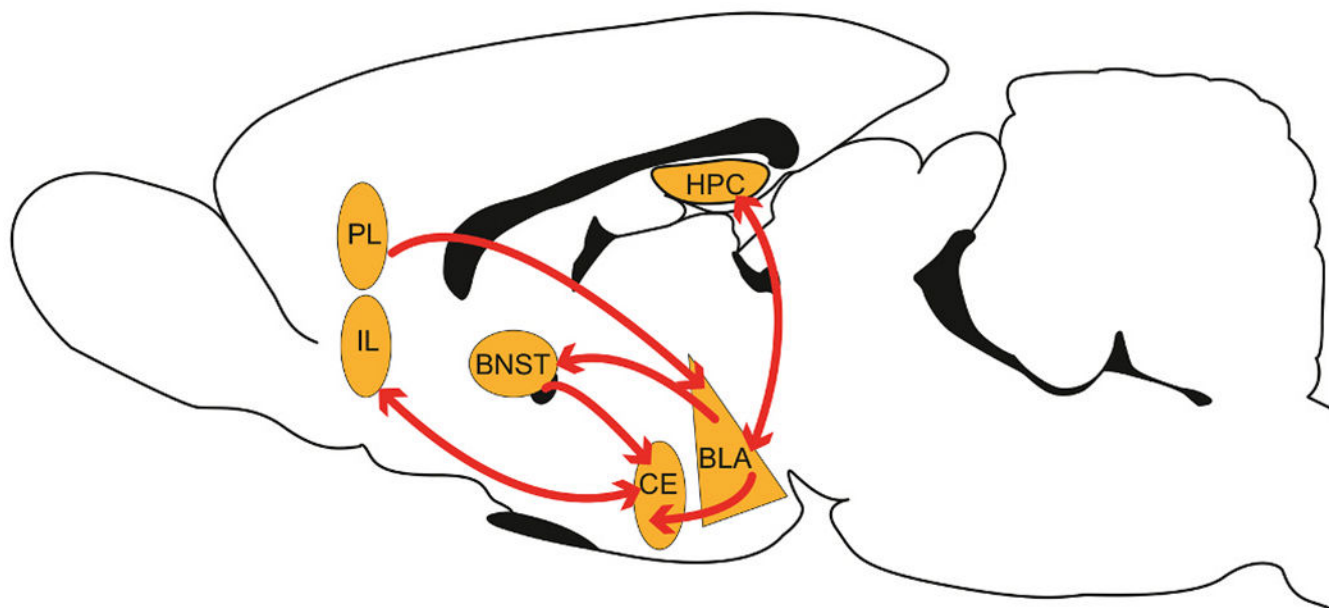


Fig. 1. Schematic representation of neural structures and pathways underlying fear responses with differential activity in males and females. The structures discussed in the text which contribute to differences in fear behaviors include the basolateral nucleus of the amygdala (BLA), the central nucleus of the amygdala (CE), the bed nucleus of the stria terminalis (BNST), the hippocampus (HPC), and the prelimbic (PL) and infralimbic (IL) prefrontal cortices. Arrows denote pathways which are differentially activated in males and females.

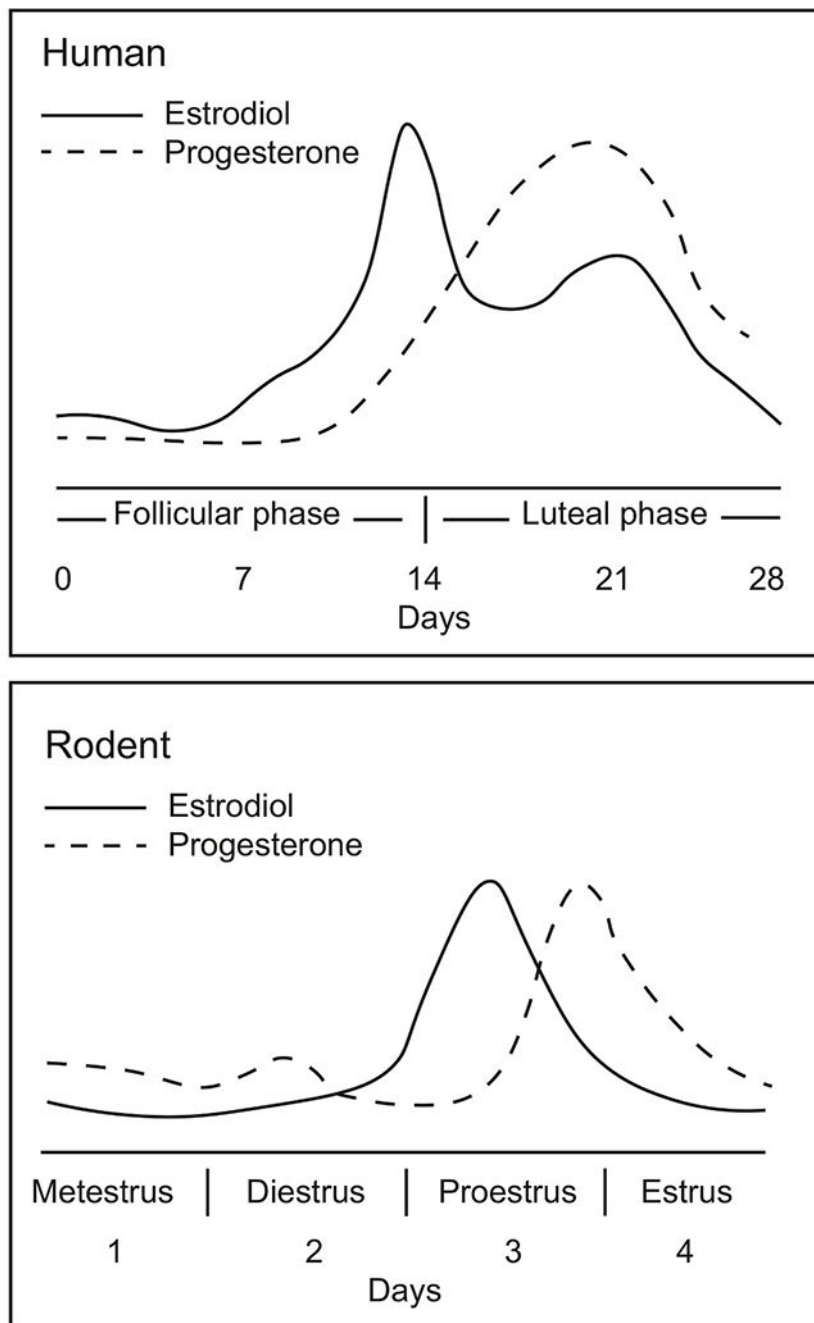


Fig. 2. Estradiol (E2) and progesterone levels vary across the human menstrual cycle (top) and rodent estrous cycle (bottom). The complete cycle in humans is 28 days, while the cycle in rodents is 4 days. In humans, E2 and progesterone levels peak on different days, while in the rodent both hormones peak in the proestrus phase.

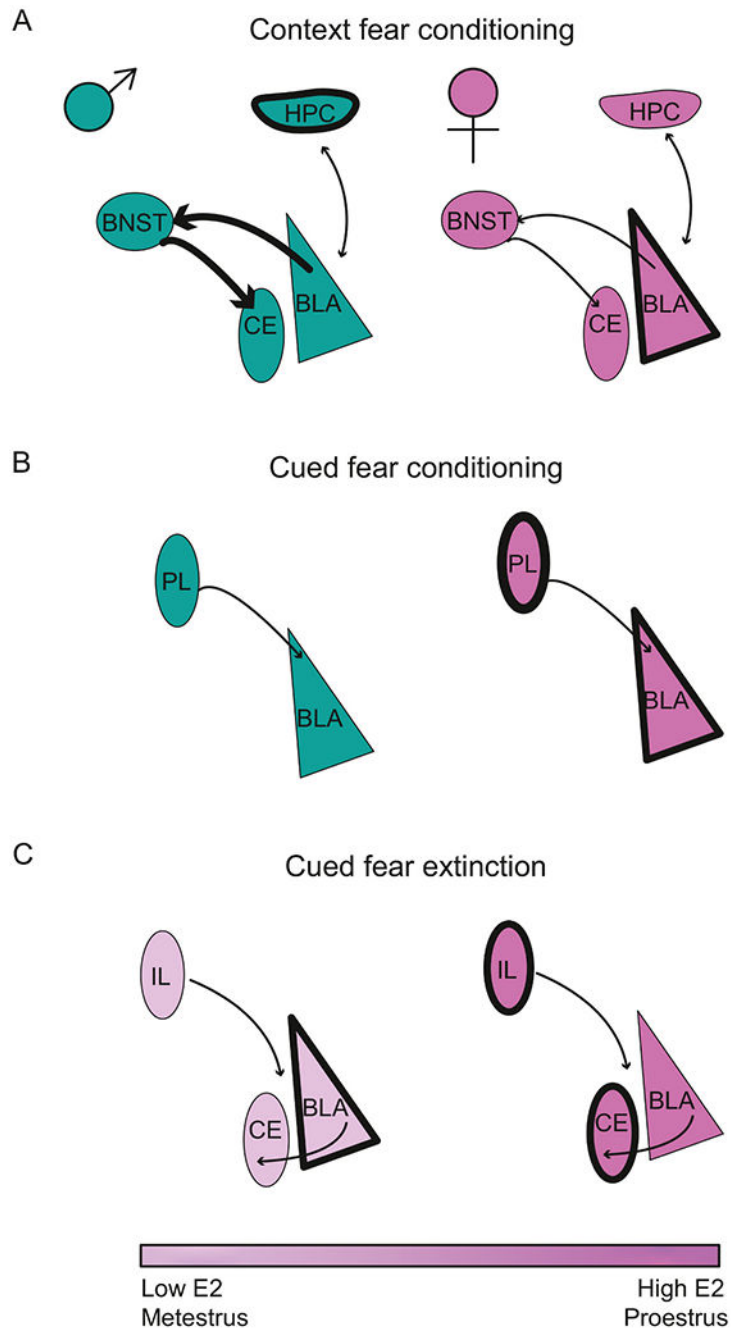


Fig. 3. Schematic representation of neural circuits underlying conditioned fear responses in rodents. Line thickness denotes greater activity within a structure or within a pathway. A) Context fear conditioning is associated with greater HPC activation and increased activity in the BLA-BNST and BNST-CE pathways. In females, context fear conditioning is associated with greater BLA activity. B) Cued fear conditioning is associated with PL and BLA activity in females. C) Modulation of cued fear extinction in female rodents by estrous phase and E2. Metestrus, or low levels of E2, is associated with increased activity in the BLA. Proestrus, or

high levels of E2, is associated with increased IL and CE activity during cued fear extinction recall.

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