Universitat Autònoma de Barcelona

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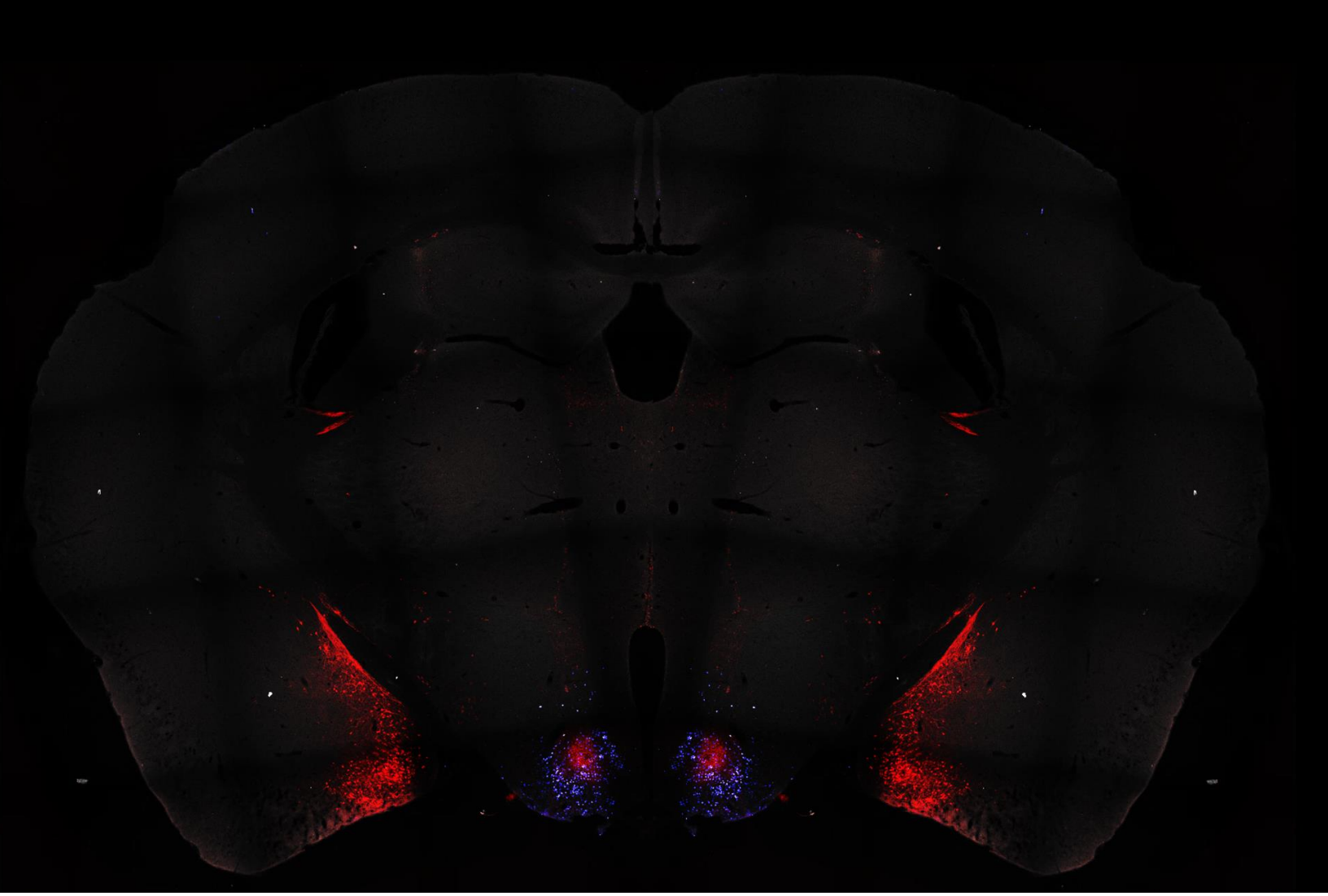
**Eric Raúl Velasco García**



**Doctoral thesis**

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Ph.D. program in Neurosciences. Institute of Neuroscience (Faculty of Medicine).

Universidad Autónoma de Barcelona (UAB). December 2022.

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To Torben and Chelo

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# Index

# List of Abbreviations

| | |
|---|---|
| **ACTH** | Adrenocorticotropic hormone |
| **ANS** | Autonomic nervous system |
| **BLA** | Basolateral amygdala |
| **BNST** | Bed nucleus of the stria terminalis |
| **cAMP** | Cyclic AMP |
| **CeA** | Central amygdala |
| **CNO** | Clozapine N-oxide |
| **CR** | Conditioned response |
| **CREB** | cAMP-response element binding |
| **CRH** | Corticotrophin releasing hormone |
| **CS** | Conditioned stimulus |
| **DMH** | Dorsomedial hypothalamus |
| **DREADDs** | Designer Receptors Exclusively Activated by Designer Drugs |
| **EF** | Early follicular |
| **ER** | Estrogen receptor |
| **ERE** | Estrogen response element |
| **FC** | Fear conditioning |
| **FE** | Fear extinction |
| **FE1** | Fear extinction one |
| **FER** | Fear extinction recall |
| **FPS** | Fear potentiated startle |
| **GABA** | γ-Aminobutyric acid |
| **GnRH** | Gonadotropin-releasing hormone |
| **HPA** | Hypothalamic-pituitary-adrenal axis |
| **IL** | Infralimbic cortex |
| **IMO** | Immobilization stress |
| **KO** | Knock out |
| **LF** | Late follicular |
| **LUT** | Luteal phase |
| **MAPK/ ERK** | mitogen-activated protein kinases, extracellular signal-regulated kinases |
| **MeA** | Medial amygdala |
| **mPFC** | Medial prefrontal cortex |
| **mRNA** | Messenger RNA |
| **NA** | Noradrenaline |
| **NK3R** | Neurokinin 3 receptor |
| **NMDA** | N-methyl-D-aspartate receptor |
| **PAC1R** | Pituitary adenylate cyclase-activating polypeptide type I receptor |
| **PACAP** | Pituitary adenylate cyclase-activating polypeptide |
| **PAG** | Periaqueductal gray |
| **PFC** | Prefrontal cortex |
| **PKA** | Protein kinase A |
| **PI3K/ AKT** | Phosphoinositide 3-kinase/protein kinase B |
| **Prl** | Prelimbic cortex |
| **PTSD** | Posttraumatic stress disorder |
| **PVN** | Paraventricular hypothalamus |
| **SNP** | Single nucleotide polymorphism |
| **Tac2** | Tachykinin 2 |
| **US** | Unconditioned stimulus |
| **VIP** | Vasointestinal activating peptide |
| **VMHdm** | Ventromedial hypothalamus, dorsomedial part |

# Abstract

Stress and fear-based disorders are common and disabling medical conditions that affect women disproportionately but no clear mechanism for this has been defined yet. Faulty signaling through the pituitary adenylate cyclase activating peptide (PACAP), and its receptor (PAC1R), has been associated with posttraumatic stress disorder (PTSD) in women exclusively. Moreover, the alterations in fear processing, specially impairments in fear extinction (FE), are hallmark in these disorders and it is unknown whether the PACAP-PAC1R system is implicated in this phenomenon. Therefore, we hypothesized that PACAP-PAC1R would be causally implicated in the alterations in FE in females. The shifting hormonal states in women are another source possibly increasing their risk for stress and fear-based disorders. Studies have shown that sex and sex hormones can influence FE, but it is unknown yet whether these hormonal variations are associated with a vulnerability for trauma processing. For this, we hypothesized that sex hormones would modulate brain processes under low-stress conditions like FE, but under high stress conditions their role would be negligible. The aims of this thesis were first, to test whether PACAP-PAC1R is implicated in a pathological FE in females and women and second, to clarify the influence of the shifting hormonal states over stress and fear memory processing. To approach this, we performed translational studies in two cohorts of traumatized women and female rodents exposed to immobilization stress (IMO). A fear conditioning-FE paradigm was used, taking fear potentiated startle magnitude and freezing response as outcome measures. For the hormonal aim of the thesis, the posttraumatic symptom intensity was inquired, and a systematic review of the role of sex and sex hormones was performed. With our results, we provide evidence for the validity of immobilization stress as a PTSD-like model in female mice which induces long-lasting impairments in FE. We also confirmed that the PACAP-PAC1R system is implicated in the appearance of this pathological behavioral phenotype by upregulating its activity in the amygdala and hypothalamus. A circuit connecting the medial amygdala (MeA) to the ventromedial hypothalamus dorsomedial part (VMHdm) was identified as crucial for the appearance of this impairments in FE and the upregulation of PACAP-PAC1R function. In women, we confirmed the initial hypothesis by showing that the SNP rs2267735 in the *ADCYAP1R1* is associated with impairments in FE in a cohort of traumatized women.

Regarding the hormonal influence, this thesis provides evidence about the null contribution of shifting hormonal states to trauma processing in animals and humans. Further, it was confirmed that estradiol modulates FE memory consolidation, and that sex and sex hormones can modulate FE by altering the function of the hypothalamus, prefrontal cortex, and hippocampus. This thesis provided a PACAP-PAC1R molecular-circuit mechanism by which females are at increased risk for psychopathology after traumatic stress. Also, we showed that sex hormones are important for FE processing but their role in trauma processing is minimal. These findings confirm and extend the role of the PACAP-PAC1R in stress and fear-based disorders psychopathology for women. Moreover, it identifies the MeA to VMHdm as a crucial point of stress sensitization of fear circuits. Further studies may delve deeper into the consequences of this circuit dysfunction by extending the findings to other behavioral and molecular domains and possibly providing pharmacological targets for the modulation of stress and fear memories in females.

# Introduction

## Anxiety, Stress, and Fear-based disorders

Anxiety, Stress and Fear-related disorders are a cluster of mental conditions with fear and anxiety symptoms as their core manifestation. They include generalized anxiety disorder, panic disorder, specific phobias, posttraumatic stress disorder, agoraphobia, and obsessive-compulsive disorder. Their specific categorization has changed through time depending on the edition and type of medical classification system (American Psychiatric Association, 2022; World Health Organization, 2019). For this work, we will refer to them as stress and fear-related disorders (Flores et al., 2018). Posttraumatic stress disorder (PTSD) is of special interest as it is one of the few disorders in which a single environmental exposure can trigger a mental disorder, thus offering the opportunity to study its underlying neurobiology. PTSD may develop following exposure to a threatening or horrific event, or series of events, and may include reexperiencing, avoidance, hyperactivation, and dissociation symptoms that persist over time and cause significant functional impairment (World Health Organization, 2019).

According to the last report by the World Health Organization, anxiety disorders are the most common type of mental disorder followed by mood disorders, affecting up to one in four depending on the country (GBD 2019 Mental Disorders Collaborators, 2022). These disorders are not only very common but also very persistent, as the 12-month prevalence and the lifetime prevalence numbers are similar (Craske et al., 2017). This persistence, along with an early age of onset, makes them leading causes of disease burden in the world (GBD 2019 Mental Disorders Collaborators, 2022). Moreover, having an anxiety or depressive disorder increases the risk of adverse health outcomes in other domains, e.g., suicide, and cardiovascular disease (Clarke and Currie, 2009; Seligowski et al., 2022). Despite causing considerable costs and disability, no specific treatments for these disorders exist. Current treatments are targeted to slow down disease progression but are neither curative nor preventive. As a result, psychotherapy and antidepressants work for most, but not all patients, with a large proportion of them dropping out of treatment or experiencing relapses (Batelaan et al., 2017; Edlund et al., 2002; Imel et al., 2013). It is imperative to increase the knowledge about the neurophysiopathology underlying stress and fear-based disorders given that their burden has not decreased in the last 30 years despite the existence of evidence-based interventions (GBD 2019 Mental Disorders Collaborators, 2022). Moreover, there are expected increases in their

economic and social impact as a consequence of population stressors like COVID-19, economic crises, and war (Santomauro et al., 2021).

## The role of sex in stress and fear-based disorders

The distribution of mental disorders is not equal among sex. Males tend to have more chronic psychotic and neurodevelopmental disorders, while females are more affected by anxiety and mood disorders (GBD 2019 Mental Disorders Collaborators, 2022). Panic disorder, specific phobias, and PTSD affect women in a 2.0/ 3.0 to 1 ratio compared to males (Bangasser and Valentino, 2014; GBD 2019 Mental Disorders Collaborators, 2022). In addition, women have longer disease courses, greater impairments, and greater comorbidity as part of their clinical presentation (World Health Organization, 2019). The reasons underlying this biased prevalence are unknown but are thought to arise from the interplay between physiological and environmental factors (Bangasser and Valentino, 2014; Tolin and Foa, 2006). Moreover, the lack of successful and specific treatments is obscured by the historical under-representation of females and women in basic and clinical research (Zucker and Beery, 2010). Thus, the inclusion of sex as a biological variable, either by specifically focusing on females or by including both sexes, seems a logical approach for studying the underlying mechanisms behind the sex-biased prevalence of stress and fear-based disorders (Clayton and Collins, 2014).

For this thesis, sex, or sex differences, will be considered as the biological trait determined genetically by the presence of sex chromosomes that promote male or female phenotypes. Gender, defined as the social, environmental, and cultural construct by which a person self-identifies, is not the focus of our studies.

# How are fear and stress processed in the brain?

## Fear neurobiology

Fear is a brain state that facilitates the execution of rapid behaviors or body functions that preserve the individual´s integrity in the presence of a threat (Adolphs, 2013). Innate fear responses are triggered by stimuli like predators, aggressive conspecifics, pain, or dangerous environments, and the defensive mechanisms elicited by them do not require previous experience (Blanchard and Blanchard, 1989). In turn, acquired fear responses are a subset of implicit and basic associative learning processes by which organisms establish causal relationships, an example being classical (Pavlovian) conditioning (Thompson and Kim, 1996). Both, innate and acquired fear, trigger the formation of fear memories to decrease the probability to face the same threat in the future or increase the preparedness to face the challenge. In this work, we will refer to fear as the brain state elicited by threats for which objective measures are described, rather than the subjective feelings arising from threat exposure or anticipation.

## Fear neurobiology: fear learning

During classical fear conditioning (FC) a neutral stimulus is repeatedly paired with a naturally threatening stimulus (unconditioned stimulus, US) so that an association forms and the neutral stimulus becomes a conditioned stimulus (CS) with the capacity to elicit a fearful, conditioned response (CR) (Pavlov, 1927). This associative memory is usually referred to as learned fear and it is a model used to study fear neurobiology (LeDoux, 2014). Three processes are described by which an initial fearful association is stored long-term. 1) Synaptic consolidation, at the neuronal level by the potentiation of co-activated neurons, 2) Systems consolidation, at the macrocircuit level by the recruitment of remote brain structures that stabilize the memory and allow its further long-term recall, 3) Reconsolidation, by which recalled memories are updated and integrated with new environmental information (McKenzie and Eichenbaum, 2011; Winocur and Moscovitch, 2011). The appearance of CRs can be specific to a stimulus or generalized to similar stimuli. Also, contextual cues are integrated into the fear memory enneagram with the capacity to elicit CRs (Maren et al., 2013). In naturalistic scenarios, discrete

cues are hardly disentangled from contextual cues, therefore fear memories are embedded into contexts.

## Fear neurobiology: fear extinction

Fear extinction (FE) is the process by which the CRs decrease over time after repeated presentations of the fear-eliciting CS in the absence of an aversive outcome or US (Myers and Davis, 2007). FE, like FC, is influenced by the context. In a process known as fear renewal, previously context extinguished CRs can re-appear if the organism is re-exposed to the CS in a different context to which FE took place. Also, FE memories weaken with time, and they may suddenly reappear in what is known as the spontaneous recovery of fear. Further, CRs can be fully recovered by the sole presentation of the US after FE has taken place in what is known as fear reinstatement (Myers and Davis, 2007).

The processes by which FE occurs are still under investigation but can be divided into 1) memory-weakening processes, which mostly debilitate previously potentiated synapses and are dominant if the extinction procedures take place shortly after fear learning. 2) the formation of a new inhibitory memory, as both fear and extinction memories coexist and are differentially expressed depending on factors like time, context, and internal states (Myers et al., 2006). In a newer theory, a third not-observable event, a latent cause, is related to both the CS and the US. Organisms rely on statistical inference to elucidate how likely is that a specific latent cause produces the observed stimuli associations (CS, US, context). Then, when the US is omitted and a mismatch is detected, a new inference is made about a new previously unknown latent cause that signals the CS, but not the US, assigning this association to a new cluster. The organism's behavioral response is related to which cluster is judged to be associated with the current presentation of cues. That way, a CS-US mismatch can be updated into the old CS-US cluster (erasure mechanism) or can be assigned to the new cluster (new memory formation) (Dunsmoor et al., 2015).

When studying the stability and strength of fear and extinction memories, the time points of assessment are relevant as the presentation of unreinforced CSs can reflect different time-dependent processes. Unreinforced CSs appearing shortly (minutes) after fear learning evaluate

the short-term memory retrieval and the weakening of previously potentiated synapses. In contrast, the presentation of unreinforced CSs after a consolidation period (usually hours, or days, e.g., 24h) evaluate processes involved in the long-term storage of fear and extinction memories, including fear memory recall and the formation of a new inhibitory extinction memory. If unreinforced CSs were further presented 24h after fear extinction, fear extinction recall, fear extinction retention and the temporal stability of extinction memories would be evaluated (Myers and Davis, 2007).

## Fear measures in animals and humans

In rodents, common readouts of fear are freezing, changes in blood pressure, heart rate, respiratory rate, and ultrasonic vocalizations. In humans, researchers rely on subjective fear ratings, skin conductance response, the contraction of facial musculature in fear-potentiated startle, pupillary size, and heart rate, among others. For this work, freezing, defined as the absence of movements except for respiration, will be used as the outcome measure of fear during a cued-FC paradigm. Freezing will be automatically scored as the percentage of time an animal remains immobile during each CS presentation (Milad et al., 2011). In humans, a fear-potentiated startle differential paradigm will be used in which two CS are shown but only one is paired with the US. This outcome measure assumes that the natural startle reflex to a loud probe, measured as the magnitude of the contraction of the orbicularis muscle, will be enhanced by the presence of a fearful stimulus (Blumenthal et al., 2005). The methods used in this thesis are shown in Figure 1.

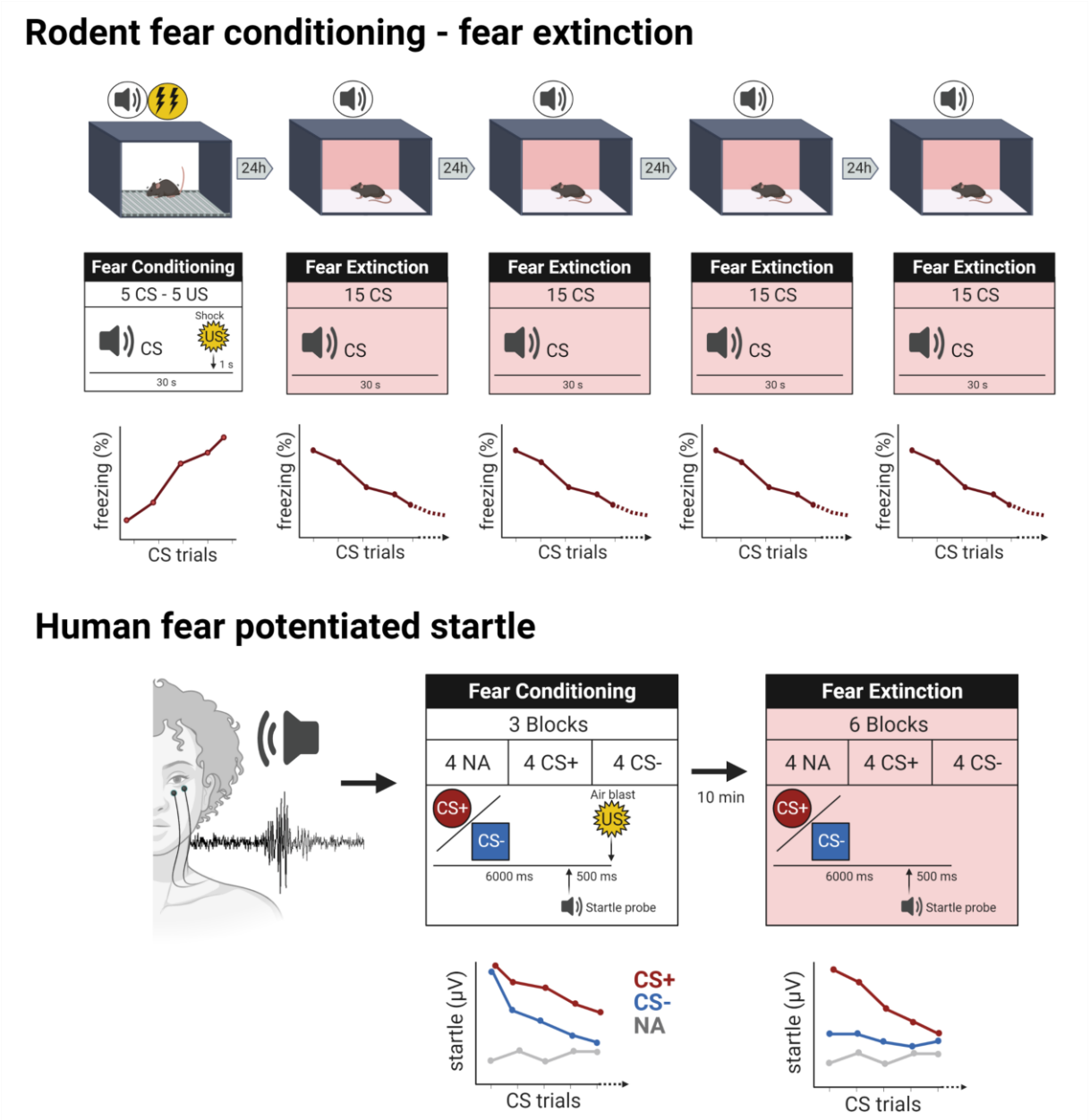


**Figure 1.** At the top, the fear conditioning (FC) and fear extinction (FE) protocol used in this thesis. During FC an electric shock was paired with a tone (CS) 5 times. After 24h, mice were put back in the fear chambers with changes in context, and the tone was presented 15 times for each FE session. The % of time animals were freezing during the CS was used as the measure of fear. At the bottom, humans taking a fear-potentiated startle (FPS) task were presented with two CS, one paired with a US (CS+) and other not (CS-), there were also trials with the startle probe alone (NA). During FC, 12 trials of each CS type were presented. During FE, 24 trials of each CS were presented and the CS+ was never paired with the US. The magnitude of the FPS response (μV) was used as the measure of fear.

## Fear neurobiology: brain circuits

FC and FE are processed in the brain through specific brain structures and interconnecting circuits (Figure 2). Research identified the amygdala, hippocampus, and prefrontal cortex as core regions for FC and FE processing. Fear learning is thought to arise from the potentiation of nociceptive (US) and auditory (CS) thalamic inputs converging in the lateral amygdala, this information is then conveyed into the adjacent basolateral amygdala (BLA) which integrates contextual and predictive information from the hippocampus and prefrontal cortex (PFC). Information flows to the central amygdala (CeA), the major output station, triggering behavioral and autonomic responses to threats through projections to the hypothalamus and periaqueductal gray (Herry and Johansen, 2014). Shortly after the memory is acquired, fear retrieval relies on amygdala microcircuits and the prelimbic cortex (Prl). However, with time fear memories are embedded into cortical systems and the activation of corticothalamic circuits (auditory cortex, Prl, paraventricular thalamus) is needed for their retrieval (Do-Monte et al., 2015). In FE, different neuronal populations in the BLA, periaqueductal gray, and thalamus encode prediction errors. Moreover, top-down inputs from the infralimbic cortex (IL) target inhibitory amygdala circuits to decrease fear expression (Milad and Quirk, 2012). Similarly, inputs from the hippocampus targeting both, the prefrontal cortex and the basolateral amygdala, signal safety and promote fear extinction (Maren et al., 2013).

The structures are considered core nodes for fear processing, but their communication depends on inputs, outputs, and redundant circuits. The periaqueductal gray (PAG) is a central output pathway but also relays incoming nociceptive stimuli (Tovote et al., 2016). The bed nucleus of the stria terminalis (BNST) integrates information about external and internal conditions. Its function is related to the recruitment of the fear circuit upon the anticipation of threats, anxiety, and the persistence of threat responses (Avery et al., 2014). The insula integrates nociceptive, somatosensory stimuli with cognition (Uddin, 2015). Stimuli undergo high-level processing in their respective cortices being either visual, olfactory, auditory, or somatosensory. With time, associative memories are embedded into cortical circuits that can be accessed through cortico-limbic circuits (Kitamura et al., 2017). The hypothalamus regulates threat responses to innate and acquired threats, it is also an integration node for neuroendocrine and metabolic responses to stress and the main output station of cortisol secretion (Silva et al., 2016).

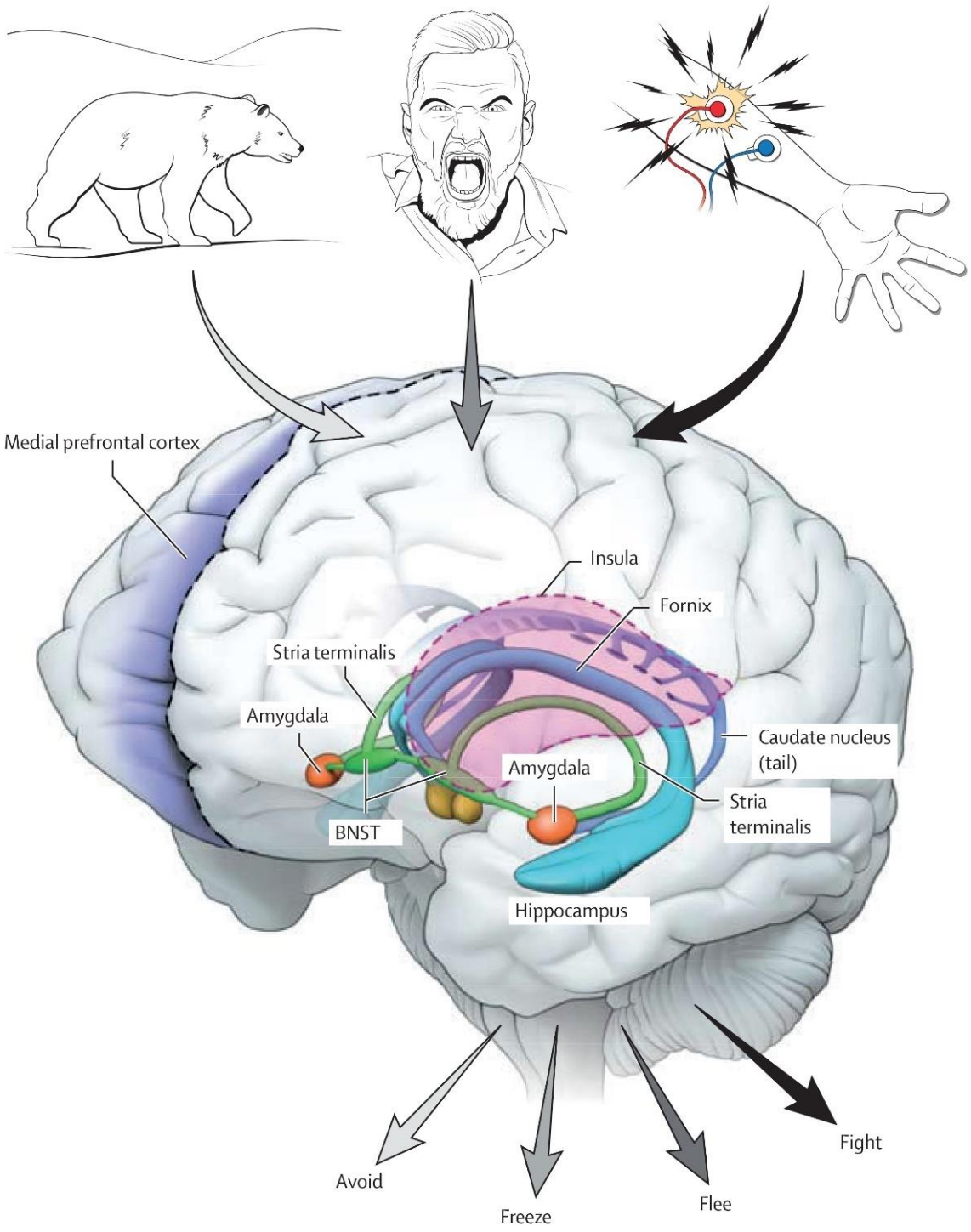


**Figure 2.** In the top, examples of innate fearful stimuli that are processed by the brain´s fear circuit: hippocampus in light blue, amygdala in orange, bed nucleus of the stria terminalis and stria terminalis in green, hypothalamus in yellow, insula in pink, and medial prefrontal cortex in purple. The circuits between these structures and other effector structures (not shown) trigger defensive responses shown at the bottom. **Figure extracted from (Penninx et al., 2021).**

## Stress neurobiology

Stress is defined as an actual or anticipated threat to homeostasis or well-being. The information about internal or external stressors recruits two main systems, the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal axis (HPA). Both systems rely on neuronal circuits that highly overlap with fear and memory circuits, so that the response is adequately tailored to previous experience or anticipated outcomes (Ulrich-Lai and Herman, 2009). The ANS provides an immediate response to stressors through sympathetic and parasympathetic innervation of organs leading to enhancements in alertness, arousal, and the mobilization of resources. ANS responses are short-lived and limited by reflex parasympathetic activation. In turn, the HPA is activated after minutes or hours of facing a stressor and results in the secretion of glucocorticoids that amplify the sympathoadrenal response (Ulrich-Lai and Herman, 2009).

Stressors can be categorized as reactive (physical) or anticipatory (psychological). Reactive stressors represent an actual challenge to homeostasis signaled by sensory systems (e.g., hemorrhage, infection, pain), while anticipatory stressors represent a condition where a challenge to the current well-being is predicted (e.g., aggressive conspecific, predators, aversive environment) (Herman et al., 2003). Both stressor modalities are processed by distinct, yet overlapping, brain circuits. Circuits processing anticipatory anxiety are integrated into reactive circuits at multiple levels, thus the stress response is tuned according to internal and external sensory information and previous experience (Herman et al., 2003).

## Stress neurobiology: brain circuits

Research has shown that reactive stressors rely on structures like the brainstem, bed nucleus of stria terminalis, and hypothalamus. In turn, anticipatory stressors recruit the hippocampus, prefrontal cortex, and amygdala (Ulrich-Lai and Herman, 2009)**.** The ANS is activated by brainstem, medullary and spinal cord nuclei through the sympathetic and parasympathetic arms. In the hypothalamus, the paraventricular nucleus (PVN) is a central node for stress processing. It receives important inputs from the nucleus of the solitary tract and dorsomedial hypothalamus (DMH) to regulate autonomic responses to physical stressors, while the circumventricular organs (median preoptic nucleus, subfornical organ) respond to fluid and volume challenges.

The locus coeruleus and the rostral ventrolateral medulla stimulate sympathetic responses, while the dorsal motor nucleus of the vagus nerve and the nucleus ambiguous mediate parasympathetic responses (Ulrich-Lai and Herman, 2009). The activity of the locus coeruleus involves rapid sympathetic responses that recruit higher-order structures like the hippocampus, amygdala, and prefrontal cortex, that modulate the HPA axis.

Anticipatory stressors rely mostly on limbic structures as they integrate multi-modal sensory incoming information with memory. Moreover, these structures are subject to modulation by arousal and attention systems (locus coeruleus, raphe nuclei). The PFC modulates top-down responses with the Prl preferentially inhibiting and the IL triggering autonomic responses (Radley et al., 2006). The amygdala is considered a hub of stress integration able to switch from a top-down to a bottom-up control of stress responses. Among its nuclei, the CeA regulates responses to systemic stressors, the medial amygdala (MeA) to psychogenic stressors, and the BLA to acute and chronic stressors. The hippocampus, like the Prl, provides negative feedback to the HPA axis for stress response termination but it can also engage with the amygdala to promote a bottom-up control of stress responses (Herman and Mueller, 2006). High activity in the hippocampal-amygdala circuit promotes plasticity and storage of long-term memories (Arnsten, 2009). The modulation of the HPA activity by limbic structures is indirect, as there are few direct connections to the PVN. Connections from Prl to the PVN are relayed through the BNST, but the connections between the amygdala and PVN are still largely unknown but thought to involve the BNST and other hypothalamic nuclei (Dayas et al., 2001; Herman et al., 2003; Zhang et al., 2021). The relaying of signals through the BNST and other hypothalamic nuclei positions these structures as integration nodes between physiological processes and limbic inputs.

When an organism faces a stressor, the SNA activates rapidly and relies on catecholaminergic signaling from the adrenal medulla and sympathetic nerves to mobilize resources and prepare the organism for the challenge (e.g., increased heart rate, mydriasis, increased muscle blood flow) (De Kloet et al., 2005). The PVN activates the HPA response by releasing corticotropin-releasing hormone (CRH), oxytocin, and vasopressin, which stimulate the release of adrenocorticotropic releasing hormone (ACTH) that acts on the adrenal glands to release glucocorticoids to the bloodstream. Glucocorticoids act through mineralocorticoid and

glucocorticoid receptors and have widespread effects on the body and brain. They can modulate gene transcription, neuronal excitability, and neuronal plasticity (Joëls et al., 2012). Glucocorticoids can promote acute increases or decreases in neuronal excitability, but their capacity to modulate gene transcription through nuclear receptors make them able to regulate the thresholds for neuronal depolarization at longer time courses. These delayed effects of glucocorticoids are especially important in limbic structures as they can relate to memory processes, and promote the retention and consolidation of memories (De Kloet et al., 2005). The function of the HPA axis is restored to basal levels by glucocorticoids through a negative feedback system at the hypothalamus, pituitary gland, and limbic structures (De Kloet et al., 2005).

## Stress sensitization

Stress exposure can increase the excitability of the HPA axis and sympathoadrenal systems even after stressors have disappeared in what is termed stress sensitization. This enhanced reactivity has been described for chronic stressors and includes increased activity in the paraventricular thalamus and locus coeruleus along with plastic changes in the amygdala and hippocampus (Herman, 2013). In acute stressors, the nature of the initial stressor plays a major role in the appearance of stress sensitization as it is mostly observed after high-intensity emotional or systemic stressors (Belda et al., 2015). Notably, behavioral sensitization persists longer than the sensitization of the HPA axis and reflects the adaptation of neuronal circuits to stress (Belda et al., 2015). In what could be contrary to stress sensitization, stress habituation is observed after the organism repeatedly faces mild stressors with the magnitude of the stress responses decreasing over time (Herman, 2013).

## Animal models to study stress and fear-based disorders

Exposure to a single stressor is used as an animal model to study the neurobiology of PTSD (Armario et al., 2008). Different stressful procedures can be used to induce stress sensitization, like electric shocks, exposure to predator odor, or restraint (Maren and Holmes, 2016). Among the available models, the immobilization stress (IMO) is the procedure that elicits the highest HPA response in comparison with other single-stress exposure models. IMO is an intense psychological stressor that is conducted by attaching the four limbs of the rodent to an IMO board using duct tape and gently surrounding the immobilized animal with a fastener (Figure 3). Rodents remain immobilized for 2 hours after which they are put back into their home cages and left undisturbed until further behavioral testing (Figure 4). IMO exposure induces a posttraumatic behavioral phenotype characterized by depressive-like, anxiety-like behaviors and impairments in fear extinction (Andero et al., 2013, 2011; Wingo et al., 2018). The mechanisms underlying acute stress-induced sensitization are still unclear, but they are thought to occur in a glucocorticoid-independent manner and to rely on neuropeptide signaling (Belda et al., 2012). The deficits in FE elicited by IMO can manifest as impairments in FE consolidation or reductions in within-session fear. Notably, the effects of a single stressor are dependent on sex as the same chronic restraint stress protocol can induce opposite effects in males in females, for example by impairing FE in males but facilitating it in females (Baran et al., 2009; Olave et al., 2022; Wingo et al., 2018).

The neuronal correlates mediating the impairments in fear extinction after stress are somewhat defined for chronic stress protocols. Chronic stress induces dendritic hypertrophy in BLA neurons and dendritic retraction in the hippocampus and mPFC. Also, the mPFC-BLA connectivity is enhanced and associated with increased plasticity in the BLA neurons and stronger fear memories (Maren and Holmes, 2016). The neuronal correlates for stress sensitization after exposure to the strongest emotional stress protocol, the IMO, are still unknown. Moreover, it is yet unknown whether these adaptations in the stress circuit occur in females, as only males have been used to approach research questions after IMO (Maren and Holmes, 2016).

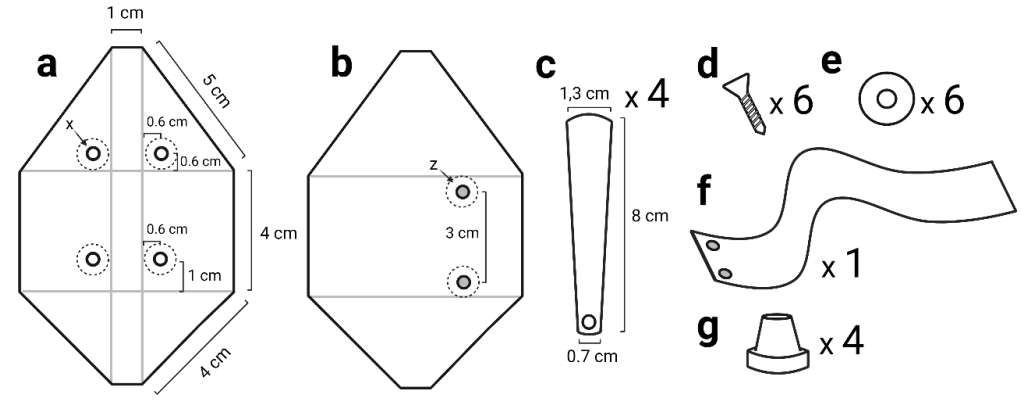


**Figure 3**. Graphical representation of an IMO board. (a) Top side. (b) Bottom side. (c) Metal arms. (d) Flathead screws. (e) Metal washers. (f) Hook-and-loop fastener. (g) Adhesive rubber leg. **Figure extracted from Velasco ER et al., (under revision).**

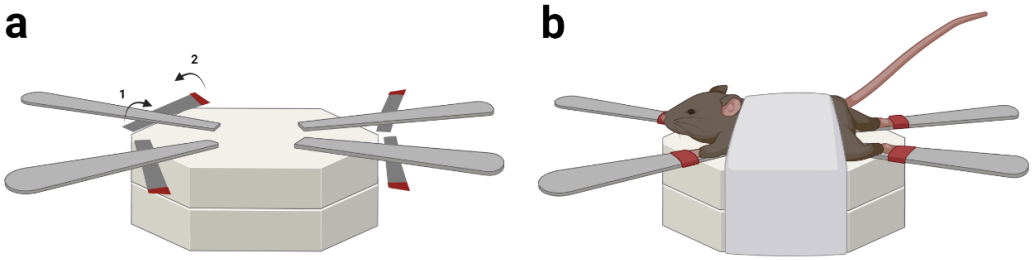


**Figure 4.** Graphical representation of the stress immobilization procedure. (a) Preparation of the immobilization board with the tape strips on each metal arm. (b) Mouse positioned in the stress immobilization board. **Figure extracted from Velasco ER et al., (under revision).**

## Fear dysregulation in stress and fear-based disorders

Exposure to acute traumatic stress (e.g., sexual abuse, or vehicle accidents) can modify brain function permanently. Most individuals exposed to trauma recover but others have long-lasting symptoms (Maren and Holmes, 2016). In vulnerable subjects, repeated exposure to trauma reminders results in hyperactivity of the ANS and HPA-axis, with the consequent hypersecretion of glucocorticoids and the development of metabolic and cardiovascular disorders (Chrousos and Gold, 1992). There are some pathological HPA responses in these individuals that include higher basal cortisol levels, deficits in the suppression of cortisol secretion, and blunted ACTH responses to CRH (De Kloet et al., 2005). Notably, treatment success requires the normalization of HPA responses, and relapses are associated with HPA disturbances (Zobel et al., 2001). In clinical populations, some studies have found altered cortisol levels in PTSD patients but without identifying a clear direction (Yehuda, 2002).

Patients suffering from stress and fear-related disorders have disturbances in fear processing that include a heightened conditioning to danger cues, fear generalization, impaired FE, and decreased inhibitory learning of safety cues (Dunsmoor and Paz, 2015; Garfinkel et al., 2014; Jovanovic and Norrholm, 2011; Lissek et al., 2005). Also, individuals with PTSD can create FE memories but have problems retaining them (Wicking et al., 2016). Some of these deficits in fear processing have been found to have neuronal correlates for PTSD patients consisting of increased amygdala reactivity to threats, smaller hippocampal volumes, and impairments in PFC function (Admon et al., 2013; Logue et al., 2018; McLaughlin et al., 2014; Milad et al., 2009b; Stevens et al., 2013). Thus, researchers have hypothesized that fear alterations, specially FE impairments, may reflect intermediate phenotypes in several stress and fear-based disorders, which can be further quantified and studied to understand the mechanisms underlying complex behavioral clinical entities like PTSD (Meyer-Lindenberg and Weinberger, 2006). Besides, the study of FE is relevant because of its clinical extension in the form of exposure therapy (Vervliet et al., 2013). Exposure therapy is one of the main psychotherapy techniques used to treat anxiety disorders, phobias, and PTSD (Powers et al., 2010).

To study the dysregulations in the fear circuit, the classical FC- FE models are valuable due to their high translational potential. Stress and fear circuits are similar between rodents and humans

and the impairments in FE have been mapped to neuronal signatures that are shared among species (Milad and Quirk, 2012).

## What positions an organism vulnerable to the detrimental effects of stress?

Psychiatric disorders are complex clinical entities where a set of altered behavioral states can be observed or experienced, but the underlying brain mechanisms remain largely unknown. According to the diathesis-stress model, the origin of a mental disorder arises from the interaction of an individual vulnerability with the environment (Figure 5). A diathesis, or vulnerability, can be a specific genetic configuration, psychological trait, or biological feature. Thus, this model can explain the susceptibility of certain individuals to develop a mental disorder and it can additionally determine which individuals will develop it based on surpassing an arbitrary threshold (Lazarus, 2003). Environmental factors leveraging individuals towards disease include the exposure to severe acute trauma, certain types of stressors, adverse childhood experiences, or substance abuse (Garcia-Esteve et al., 2021; Tortella-Feliu et al., 2019).

The question arises of whether some specific biological traits promote a vulnerable phenotype for the development of stress and fear-based disorders. These biological traits or features could be used when designing primary prevention strategies, at screening interventions or emergency care to identify individuals at risk. Some biological features that are known to contribute to a vulnerable phenotype include functional polymorphisms of genes that alter HPA responses to stress, or the function of serotonin and glucocorticoid receptors (Binder et al., 2004; Caspi et al., 2003; Wüst et al., 2004). Notably, being a woman is identified as a biological risk factor for stress and fear-related disorders giving 2 to 3 times the risk for PTSD even after controlling for individual and environmental variables (Tolin and Foa, 2006). In this work, we focused on exploring two biological traits that are specifically related to a greater vulnerability to stress and fear-based disorders in women.

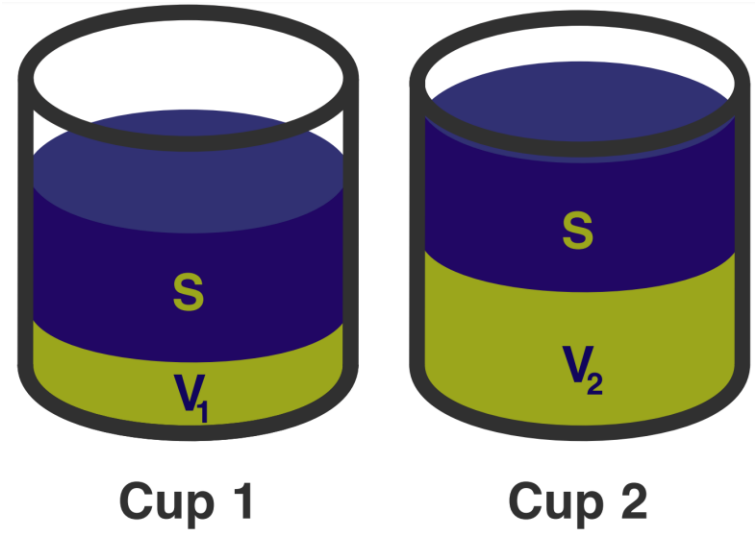


**Figure 5**. Graphical representation stress (S) and individual vulnerability (V) interaction. Under the same stressor, a person with cup 2 (V2) would be more vulnerable than a person with cup 1 (V1) due to its individual vulnerability.

## Female-specific risk factors

Sex differences in brain function at birth and early life are related to an increased vulnerability for males to suffer from neurodevelopmental and conduct disorders. In turn, the increased vulnerability in women to depressive, stress, and fear-related disorders is evident after puberty (GBD 2019 Mental Disorders Collaborators, 2022). This has led to the hypothesis that sex differences in the prevalence of stress and fear-related disorders may be related, at least in part, to sex hormones (Lebron-Milad and Milad, 2012). Sex hormones are related to differences in fear extinction and other types of memory. However, it is difficult to draw a clear picture due to conflicting findings, even in studies from the same laboratory (Lebron-Milad and Milad, 2012). Other potential contributions to the sex differences observed in stress and fear-related disorders are brain signaling systems that undergo specific regulation by sex hormones. An example of this is the pituitary adenylate cyclase-activating peptide (PACAP), and its receptor (PAC1R), which is a neuropeptide system tightly regulated by sex hormones and associated with a specific risk for PTSD in women (Ressler et al., 2011). Despite the relevance of this vulnerability biological trait for women, the underlying mechanisms are just starting to be explored.

## Hormonal systems

After puberty, adult humans and rodents are exposed to changing levels of sex hormones. In males, different hormonal levels of testosterone between and within males are related to their hierarchical position, stress exposure, competition with conspecifics, social cues, and reproduction (Sapolsky, 2005). For females, between-subject differences are observed but largely unexplored, while within-female differences follow their reproductive stages. Shifting hormonal levels are observed during their fertile life, and high or low levels are associated with pregnancy/ lactation and menopause respectively (Sundström-Poromaa and Gingnell, 2014).

In humans, the menstrual cycle lasts 28 days (± 5 days) and is divided into 4 phases. The Early Follicular phase (EF) (~ 0-7 days) has low levels of sex hormones that are followed by rising estradiol levels that peak during the Late Follicular phase (LF) (~ 10-15 days). After ovulation, mid-estradiol and high progesterone levels are detected in the Mid-Luteal phase (ML) (~ 17-25 days) until a sharp drop in hormonal levels occurs in the Late Luteal phase (~ 26-28 days). In rodents, the estrous cycle lasts 4-5 days and is composed of 4 phases. The proestrus is characterized by high peaks of estradiol and progesterone followed by ovulation. In estrus, hormonal levels are low and extend into the initial part of the metestrus. In metestrus, progesterone levels increase and remain high until mid-diestrus and return to low basal levels before the next proestrus phase (Lebron-Milad and Milad, 2012). Despite some similarities, the estrous and menstrual cycles are not equivalent (Figure 6). For research purposes, the proestrus is considered a “high hormone state” and the metestrus/diestrus a “low hormone state” (Lebron-Milad and Milad, 2012).

Estrogens are the main sex hormone in females. Estradiol is the most potent estrogen secreted from the ovaries but its synthesis is also documented to a lesser extent in the adrenal glands, peripheric tissues, and the brain (Melmed et al., 2019). In males, testosterone is aromatized to estradiol by 5α-reductase. Estrogens bind mostly to estrogen receptors (ERs) that are located in intracellular compartments or the membrane, along with the G protein-coupled estrogen receptor. The binding of estradiol to membrane ERs increases intracellular calcium and activates cyclic AMP/ Protein kinase A (cAMP/ PKA), mitogen-activated protein kinases, extracellular signal-regulated kinases (MAPK/ ERK), and the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways that converge at nuclear targets (Cover et al.,

2014). ERs are differentially distributed in the brains of males and females, with females also experiencing fluctuations in ERs expression due to a differential regulation by the hormonal cycle (Mendoza-Garcés et al., 2011). Progesterone is a steroid hormone produced cyclically by the corpus luteum in females, and independently from the cycle, in the adrenal gland and brain of both sexes. It is tightly regulated by gonadotrophins and serves as a substrate for cortisol and allopregnanolone, a positive allosteric modulator of the GABA-A receptor ($GABA_AR$) (Melmed et al., 2019). Progesterone dynamics are different between humans and rodents mainly due to a distinct regulation of the corpus luteum between species (Accialini et al., 2017). In turn, testosterone is the dominating sex hormone in males with a circadian pattern of secretion and anabolic and androgenic effects in both sexes. It binds to androgen receptors and triggers genomic and non-genomic effects although many of its functions are mediated through its conversion to dihydrotestosterone or estradiol in target tissues (Melmed et al., 2019).

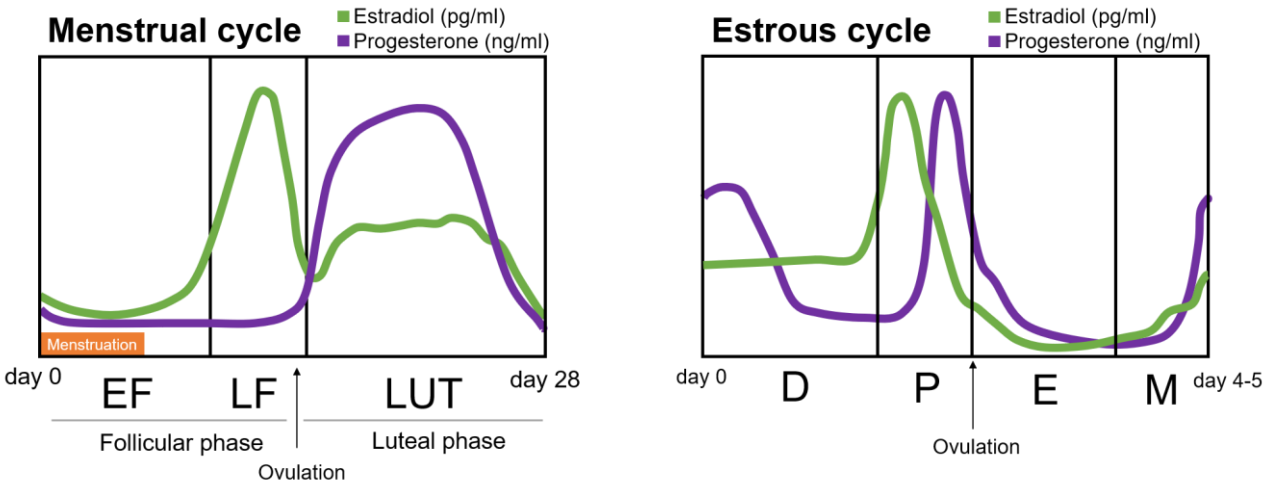


**Figure 6**. Graphical representation of the menstrual (left) and estrous (right) hormonal cycles. Estradiol (green) and Progesterone (violet) relative concentrations are shown. The menstrual cycle is divided into follicular and luteal phases (LUT). The follicular phase is subdivided into early follicular (EF) and late follicular (LF). The estrous cycle is divided into Diestrus (D), Proestrus (P), Estrus (E,) and Metestrus (M). **Figure extracted from (Velasco et al., 2022).**

There are direct and indirect ways to assess hormonal levels. The direct method implicates the analysis of sex steroids in blood with immunoassays or mass spectrometry. However, this approach is invasive and represents a stressful procedure that can interfere with fear and stress readouts. In turn, the indirect methods can be easily conducted before experimental settings and induce low levels of stress (Florido et al., 2021a). For this thesis, hormonal levels in mice were measured only when they were the main outcome of interest and no further behavioral testing was necessary. For the rest of the cases, we used the vaginal cytology method which is an easy and precise way to monitor for estrous cycles in rodents (Caligioni, 2009). In humans, the allocation into menstrual cycle phases was based on the last menstrual period date (Schmalenberger et al., 2021).

## Hormonal modulation of the stress response

There are differences in the neuroendocrine and neurophysiological responses to stress during the distinct phases of the menstrual cycle. For example, higher basal cortisol levels are observed in the follicular phase and greater increases in cortisol to psychogenic stressors are seen in the luteal phase (Collins et al., 1985; Montero-López et al., 2018). Moreover, estradiol stimulates HPA responses, induces milder activations of the stress circuit, and modulates the transcription of glucocorticoid receptors and CRH (Goldstein et al., 2010; Kudielka and Kirschbaum, 2005). Progesterone is secreted from the adrenal glands upon stressors and exerts a negative feedback over the HPA axis (Crowley and Girdler, 2014). The role of testosterone is less clear since its release upon stressors seems to be associated with the valence of the stimulus and an anticipatory challenge effect (Hermans et al., 2007; Sapolsky, 2005; Van Honk et al., 2005).

Notably, animal models show that females are somewhat resilient to the effects of chronic and acute stress showing greater ACTH and total corticosterone responses (Altemus, 2006; McCormick et al., 2002). These findings contrast with the observed vulnerability of females to develop stress and fear-related disorders. Moreover, the role that the menstrual/ estrous cycle has over acute stress processing is not systematically explored in animals and its study is anecdotical in natural scenarios in humans (Bryant et al., 2011; Ferree and Cahill, 2009). One study that explored this research question in secondary analyses showed that the luteal phase was related to increased flashback memories, although no overall severity for PTSD symptoms

was reported (Bryant et al., 2011). Nevertheless, this analysis is biased as the researchers included in the menstrual cycle groups women that were taking oral contraceptives or already in menopause (Bryant et al., 2011). Therefore, although this study gave some clues about the effect of hormones upon trauma, it is not known yet if experiencing an acutely stressful event at a specific phase of the menstrual/ estrous cycle is related to worse outcomes. In this work, we aimed to explore whether there is a window of vulnerability in the menstrual/ estrous cycle during which stress exposure is more detrimental.

## Hormonal modulation of fear: fear extinction

Several reports indicate the existence of sex differences in FE and modulation by sex hormones (Lebron-Milad and Milad, 2012). Studies approaching research questions regarding sex differences in FE have used heterogeneous methods, different outcome measures, and varying task settings that result in non-conclusive findings. For example, studies of cued-FE studying sex differences have reported that females have impairments in FE (Baker-Andresen et al., 2013; Baran et al., 2010, 2009; Fenton et al., 2016, 2014; Greiner et al., 2019); no impairments in FE (Blume et al., 2017; Gruene et al., 2015; Lebron-Milad et al., 2012; Merz et al., 2012; Milad et al., 2009a); or enhancements in FE (Baran et al., 2010; Gilman et al., 2015; Moore et al., 2010). Similarly, contextual-FE studies show that females have better FE (Barker and Galea, 2010; Dalla and Shors, 2009; Daviu et al., 2014; R R Gupta et al., 2001; Stephen Maren et al., 1994); worse FE (Matsuda et al., 2015); or no differences in FE (Blume et al., 2017).

The inclusion of the hormonal status in the analyses improves the reproducibility of findings with most studies showing positive effects for FE consolidation with high estradiol levels (Cover et al., 2014). However, studies haven't been able to determine if the specific vulnerability for FER impairments arises in all women during the low-estrogen phases of the menstrual cycle or if the vulnerability pertains mostly to a subset of women experiencing chronic low-estrogen states or large hormonal shifts. Studies using rodents support the role of specific phases of the estrous cycle, but these findings have not been replicated in humans. Notably, there are large inter-individual differences in the levels of sex hormones during equivalent phases of the menstrual cycle and the comparison of individuals with high vs low

hormonal levels introduces bias to the conclusions (Sundström-Poromaa and Gingnell, 2014) (Figure 7).

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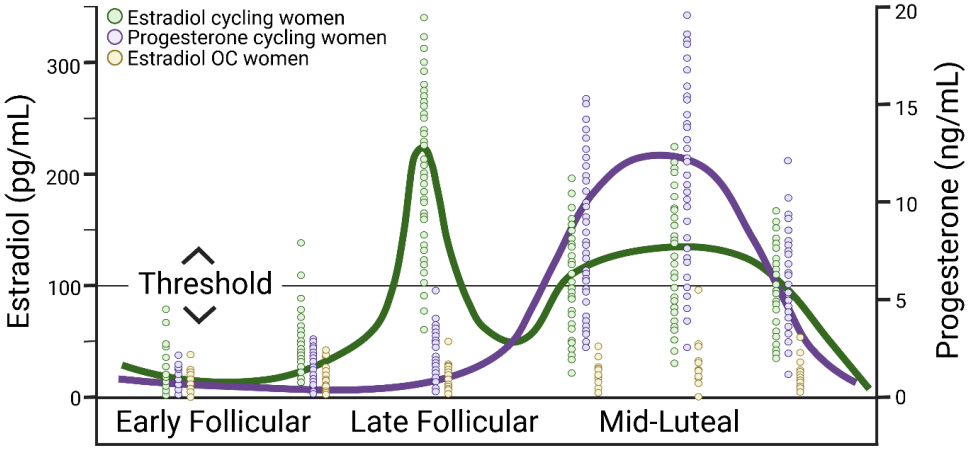


**Figure 7**. Graphical representation of the menstrual cycle and the inter-individual variability in the absolute levels of hormones. Estradiol (green) and progesterone (violet) levels in cycling women 18-42 years. Assumed estradiol levels (yellow) in women taking oral contraception. An arbitrary threshold is commonly used to split samples into high hormone/ low hormone groups. **Figure extracted from Velasco ER et al., (under revision) and adapted from (Sundström-Poromaa and Gingnell, 2014).**

## Neuropeptide systems: PACAP

The PACAP (encoded by *ADCYAP1*)- PAC1R (encoded by *ADCYAP1R1*) is an ancestral neuropeptide system tightly conserved throughout evolution (Vaudry et al., 2009). It shares more than 50% similarity with the vasoactive intestinal polypeptide (VIP) family and belongs to the VIP-secretin-GHRH-glucagon superfamily (Sherwood et al., 2000). PACAP-PAC1R is expressed in the central and peripheric nervous system, cardiovascular, endocrine, and immune systems (Sherwood et al., 2000). In the brain, the greatest expression is observed in the hypothalamus, but it is also detected in several extra-hypothalamic areas where it regulates learning, memory, and the stress response (Shen et al., 2013). The two splice variants of PACAP (PACAP 27, PACAP38) bind to their specific receptor, the PAC1R, with high affinity and with lower affinity to the VPAC1 and VPAC2 receptors (Shen et al., 2013). PACAP family receptors recruit mainly $G_s$ proteins to stimulate adenylate cyclase and increase cyclic-AMP (cAMP).

cAMP activates protein kinase A, exchange proteins activated by Camp, extracellular signal-regulated kinase, enhance N-methyl-D-aspartate (NMDA) receptor activity, and promote gene expression through cAMP-response element binding (CREB) phosphorylation (Lu et al., 2022). $G_{q/11}$ and $G_{i/o}$ proteins are also recruited by PAC1R/ VPAC1/ VPAC2 receptors to a lesser extent which activate phospholipase C, protein kinase C, enhance NMDA receptor function and mobilize intracellular calcium (Lu et al., 2022).

## PACAP regulation of the stress response

PACAP increases the firing rate of neurons, induces the phosphorylation of CREB, and stimulates CRH release in the PVN leading to increases in corticosterone and enhanced behavioral stress responses (Agarwal et al., 2005; Norrholm et al., 2005). Also, PACAP activates the autonomic nervous system enhancing sympathetic activity and suppressing parasympathetic activity (Tanida et al., 2010). In addition, the expression of PACAP mRNA in the ventromedial hypothalamic nucleus (VMH), PVN, and the arcuate nucleus (ARC) is tightly regulated by sex hormones (Apostolakis et al., 2004; Moore et al., 2005). PACAP-PAC1R system's integrity is necessary for appropriate stress responses. Transgenic mice with knocked-out PACAP show attenuations in ACTH secretion, lower steroidogenic production in the adrenal cortex, and lower plasma cortisol levels after restraint stress (Stroth and Eiden, 2010). Under physiological conditions, the role of this system is to maintain the activation of the stress circuit and the secretion of catecholamines in the face of a sustained or chronic stressor, but acute HPA responses are PACAP-independent (Stroth and Eiden, 2010).

The chronic activation of the stress circuit is associated with maladaptive structural and functional changes in the hippocampus, amygdala, PVN, and BNST (Herman, 2013; Radley et al., 2015). Some of these maladaptive processes are regulated by PACAP. Mice lacking PACAP fail to develop anxiety and depression after chronic stressors (Lehmann et al., 2013). In addition, PACAP is the main signal maintaining the release of catecholamines from the adrenal medulla as it has the capacity to stimulate catecholamine biosynthetic pathways (Stroth et al., 2013). Therefore, PACAP acts as the primary regulator of sustained stimulus-induced secretion of catecholamines during prolonged acute (3h) systemic and psychological stressors (Stroth et al.,

2013). Also, PACAP infusions in the BNST promote the appearance of anxiety-like behaviors (Hammack et al., 2009).

PACAP is related to pathological stress responses in women. PACAP38 blood levels are associated with PTSD symptoms in women but not men (Ressler et al., 2011). Moreover, a single nucleotide polymorphism (SNP) (rs2267735) in the PAC1R gene is associated with greater FPS responses, greater amygdala reactivity to threat, and greater PTSD symptoms in traumatized women but not men (Almli et al., 2013; Ressler et al., 2011; Stevens et al., 2014)). Some studies had difficulties replicating this effect, but further studies showed that this was related to the trauma load of the samples. The risk allele "C" is associated with greater PTSD symptom severity but only in females with high trauma load (Almli et al., 2013; Chang et al., 2012; Lind et al., 2017; Uddin et al., 2013). In addition, studies have suggested that the allele risk may act in a dose-dependent manner (Lind et al., 2017). This sex-specific risk is thought to be associated with an estrogen-dependent regulation of PACAP. The *ADCYAP1R1* SNP rs2267735 is located within an estrogen response element (ERE) that regulates gene transcription when bound to ERα. Under physiological conditions, *ADCYAP1R* transcription is enhanced by estradiol but the SNP in the PAC1R1 gene compromises the binding of estradiol to the ERE resulting in lower *ADCYAP1R* mRNA transcripts and greater PTSD symptoms (Hammack and May, 2015; K. Mercer et al., 2016). Hence, high estrogen levels are thought to compensate for the disbalances in *ADCYAP1R* transcription in subjects with the risk genotype, but during low estrogen states, they result in vulnerability to the detrimental effects of stress (K. B. Mercer et al., 2016).

## PACAP modulation of fear: fear extinction

Given the widespread expression of PACAP-PAC1R in cortical and limbic structures, it would be expected to be implicated in fear regulation, but few studies have explored this. One study showed that fear conditioning in mice is associated with increases in *Adcyap1r1* in the amygdala (Ressler et al., 2011). Another one demonstrated that larger increases in *Adcyap1r1* transcripts are observed in mice that were exposed to IMO before fear learning, suggesting that PACAP-PAC1R may be related to stronger fear memories (Andero et al., 2014). No studies to date have

explored whether PACAP-PAC1R modulates FE. Studies in female rodents have identified adaptations in the PACAP-PAC1R system after chronic stress in the BNST and prefrontal cortex but without describing behavioral correlates (Hammack and May, 2015). In humans, a neuroimaging study found that the risk genotype was associated with higher hippocampal activations during contextual fear learning in a small cohort of females (Pohlack et al., 2015). Increasing data shows that the PACAP-PAC1R system is a crucial regulator of the stress response and may be related to psychopathology in women. Still, the regulation of specific stress and fear circuits by PACAP remains largely unexplored. Notably, the hypothalamus, which is the area with the highest expression of PACAP, has not been considered when studying the effects of stress or PACAP adaptations.

## Circuit convergence

As discussed in the previous sections, the processing of stressors and fear memories rely on overlapping circuits. This seems logical since interpreting environmental stimuli according to experience is relevant for controlling physiological responses and behavior. Connections between the IL, CeA, BST, and autonomic centers trigger activations of the SNA, while connections between the Prl, MeA, BLA, hippocampus, BNST and hypothalamic nuclei mediate HPA responses (Ulrich-Lai and Herman, 2009). For fear learning and FE, the connections between the amygdala, Prl, IL, and hippocampus are considered central for plasticity and memory formation. However, the contributions of other brain structures that are part of the extended stress circuit remain largely unexplored. PACAP is well positioned to interface between stress and fear circuits in these structures. For example, the BNST is crucial for anxiety and persistent defensive responses to threats, and exposure to sustained stress alters PACAP function in some of its subnuclei (Hammack and May, 2015). Further, the hypothalamus acts as an integration center for stress processing by regulating physiological and endocrine responses, with some of its subnuclei expressing high levels of PACAP. Sex hormones exert ample effects on the function of the BNST and hypothalamus but also regulate the transcription of PACAP-PAC1R. Therefore, a genetic vulnerability, like the one arising from *ADCYAP1R1* SNP rs226773, may be able to impair PACAP-PAC1R function specifically in these nuclei (Figure8).

Previous works in our laboratory showed how studying the dynamics of neuropeptides shortly after behavioral procedures can inform about relevant functions in specific brain structures (Andero et al., 2014, 2013). Therefore, studying the dynamics of PACAP-PAC1R shortly after IMO or FC-FE may inform us about its implication in these behaviors. Further, by combining a transgenic mouse with a chemogenetic approach researchers have been able to isolate the function of a neuropeptide system in a specific brain structure. In our lab, this approach was used to demonstrate the necessity of the Tachykinin 2 (*Tac2*) pathway in the central amygdala for fear memory consolidation (Andero et al., 2014). The *Tac2* encodes for Neurokinin B and *Tac2r* for its receptor the Neurokinin 3 Receptor (NK3R). They are a neuropeptide system highly expressed in the limbic system and with an important regulation by sex hormones. Our laboratory demonstrated how manipulations of the *Tac2* circuits in the central amygdala can cause a sex-opposite regulation of fear memories that is dependent on the regulation of sex hormones (Florido et al., 2021b). For this work, we will try to identify the function of PACAP-dependent brain circuits using the DREADDs chemogenetic approach. DREADDs stands for Designer Receptors Exclusively Activated by Designer Drugs and consist of a viral vector inserting a modified muscarinic receptor exclusively activated by Clozapine-N-Oxide (CNO) in neurons expressing Cre recombinase. However, since there are no PACAP-Cre mice available, we will use a second viral vector to express Cre recombinase in the neurons of interest.

PTSD is a complex clinical entity with alterations in several types of behaviors. Circuit approaches have already shed light on the principle components underlying some of the behavioral alterations in anxiety (Kim et al., 2013). To understand the pathophysiologic mechanisms deriving organisms into disease, we need to know which brain regions, genes, proteins, and cell types are involved. The FC-FE model has increased our understanding about the role of the amygdala, prefrontal cortex, and hippocampus in threat processing. However, specific studies need to be conducted to dissect the precise components of memory that are affected. Different brain circuits regulate the consolidation and retrieval of memories, sensitization processes, generalization, and renewal, and many of them are associated with increased symptomatology in patients with stress and fear-based disorders (Ressler, 2020). Following trauma exposure, some individuals fail to inhibit trauma-related fear reactions. These impairments in fear inhibition and FE are hallmarks of these disorders and cataloged under a

negative valence domain, specifically in the constructs of acute threat (fear), potential threat (anxiety), and sustained threat (chronic stress) (Ressler, 2020). FE impairments are considered intermediate phenotypes, behavioral traits in the continuum of health and disease, which when escalated promote maladaptive stress responses and leverage individuals toward the manifestation of PTSD symptoms (Ramikie and Ressler, 2016).

The two main aims of this project are: First, to clarify the extent to which structural and molecular factors related to sex influence FE. Second, to bridge the gap between biological features and clinical symptoms by focusing on the mechanisms of disease and studying the influence of PACAP regulation over the function of stress and fear circuits.

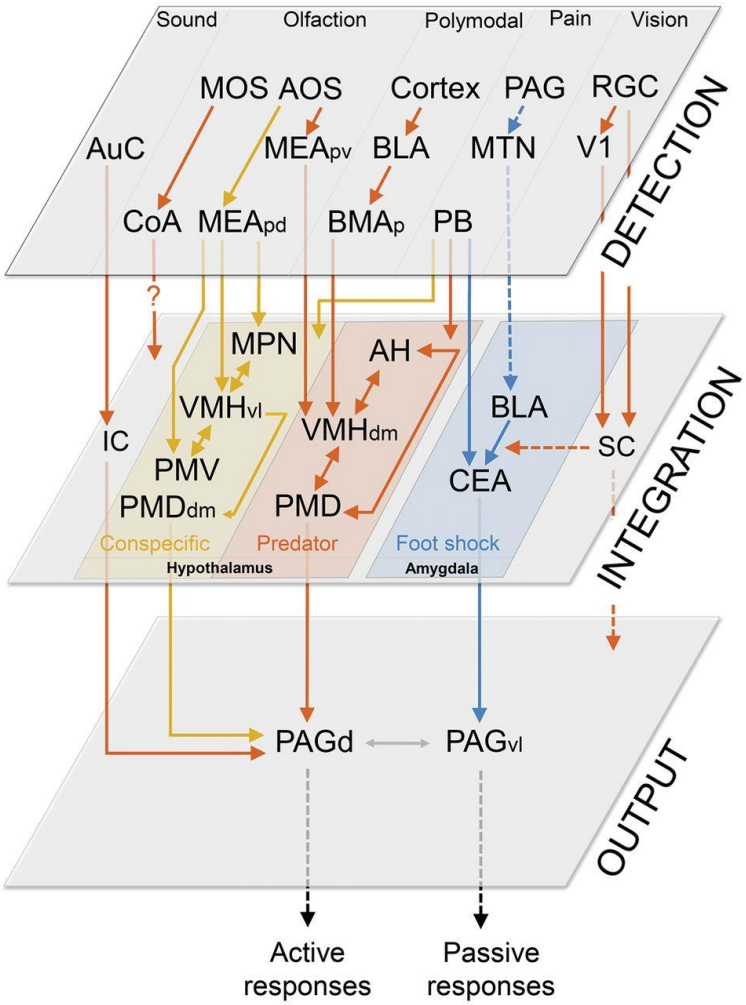


**Figure 8.** Neural circuits involved in processing threats in rodents. Brain structures are shown in levels according to their function: detection (upper plane), integration (middle plane), output (lower plane). Auditory inputs reach the auditory cortex (AuC) that projects to the inferior colliculus (IC) and output at the dorsal periaqueductal gray (PAGd). Visual stimuli are detected by the retinal ganglion cells (RGN) and primary visual cortex (V1) to reach the superior colliculus (SC) that projects to the amygdala and brainstem. Olfactory cues are used to detect predators (orange) and conspecifics (yellow). The main and accessory olfactory system (MOS, AOS) projects to the cortical amygdala (CoA), medial amygdala- anteroventral, and posteroventral (MeAad, MeApd). Polymodal inputs, including pain, arrive through the amygdala- basolateral, central (BLA, CeA), parabrachial nuclei (PB), and midline thalamic nuclei (MTN). The MeA and BLA send projections to a medial hypothalamic defensive circuit for integration: the anterior hypothalamus (AH), medial preoptic nucleus (MPN), ventromedial hypothalamus- ventrolateral and dorsomedial (VMHvl, VMHdm), and premamillary nucleus- dorsal, ventral, and dorsomedial (PMD, PMV, PMDdm). Information outputs in a scalable manner through the PAGd and PAGvl as active or passive responses. **Figure extracted from** (Bianca A Silva et al., 2016)**.**

# Hypotheses and objectives

The hypotheses are oriented towards identifying brain structures where fear and stress signaling converge and which are under neuropeptide regulation (Tac2/ PACAP-PAC1R). We aimed to detect the long-range circuits arising from these brain structures that could underlie the appearance of a posttraumatic behavioral phenotype after IMO. Finally, we decided to review the current evidence to clarify whether high hormonal states can influence FE and to hypothesize about their role in processing severe stressors. Given the successful results obtained in the Tac2 pathway in our laboratory (Florido et al., 2021b), the objectives for this thesis were re-focused to explore exclusively the PACAP-PAC1R system and its circuits.

# General objectives

1. Test whether PACAP is implicated in pathological fear extinction memories after trauma in female mice and women and define the brain structures and circuits related to the appearance of this posttraumatic behavioral phenotype.
2. Clarify the sex differences in fear extinction and define whether changing hormonal states can influence FE and the processing of severe stressors.

# Hypotheses and specific objectives

**1. PACAP and FE**

*Based on the findings in male mice demonstrating stronger fear memories after IMO, IMO exposure in females will be related to impairments in FE measured as greater freezing rates. Given the wide expression of PACAP in limbic structures and its strong implication in the stress response. It is expected that the FE impairments will be related to increases in PACAP-PAC1R function (mRNA regulation, neuronal activity) in structures interfacing between fear and stress circuits like the amygdala, BNST, and hypothalamus. Also, the activity in the circuits interfacing fear and stress will be causally implicated in the appearance of the impairments in FE. The risk genotype in the ADCYAP1R1 SNP rs2267735 in women will be related to impairments in FE as measured with a fear-potentiated startle task.*

- **Objective 1.** To expose male and female mice to IMO to induce stress sensitization and promote stronger fear memories and impairments in FE. Animals will undergo

FC 6 days after IMO, and 24h after will take 4 consecutive FE sessions, freezing levels will be used as the measure of fear.

- **Objective 2.** To explore PACAP-PAC1R regulation after IMO-FC-FE in structures interfacing stress and fear circuits like the amygdala, BNST, or hypothalamus. mRNA levels will be measured by qPCR after the first FE session and PACAP+ neuron activity will be measured using c-Fos and FosB/ ΔFosB as markers of neuronal activity after 4 FE sessions.
- **Objective 3.** To disrupt brain circuits connecting integration structures, like the hypothalamus or BNST, to cortical/ subcortical structures, like the amygdala or hippocampus, to prevent the enhancement of fear memories by IMO. A mixed chemogenetic approach with viral vectors (retrograde Cre-inserting adenovirus and a DREADDs adenovirus) will be used. 1h before IMO, a parenteral dose of CNO will be given to inhibit the circuit and FE will be tested in 4 consecutive sessions.
- **Objective 4.** To test for impairments in FE related to the risk genotype in the *ADCYAP1R1* SNP rs2267735 in a cohort of traumatized women taking a fear-potentiated startle task.

## 2. Hormonal regulation of FE and Stress

*The inconclusive findings about the sex differences in FE arise from the lack of a systematical analysis in the context of the existing structural and functional sex differences in the brain. Based on previous research, we expect to find a role for shifting hormonal states in learning taking place under mildly stressing conditions like FE, but not during intense and acute stressing conditions.*

- **Objective 5.** To perform a systematic review of the role of sex in FE memories to identify the underlying structural and functional correlates.
- **Objective 6.** To test whether there exists a window of vulnerability during the distinct phases of the menstrual/ estrous cycle in a cohort of traumatized women/ IMO females that results in greater posttraumatic symptoms/ greater impairments in FE. Posttraumatic symptoms will be evaluated with the Acute Stress Disorder Interview 3 weeks after suffering sexual abuse, FE impairments will be measured as freezing during FC and 4 FE sessions.

# Results

# Article 1

# PACAP-PAC1R modulates fear extinction via the ventromedial hypothalamus

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# PACAP-PAC1R modulates fear extinction via the ventromedial hypothalamus

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Exposure to traumatic stress can lead to fear dysregulation, which has been associated with posttraumatic stress disorder (PTSD). Previous work showed that a polymorphism in the PACAP-PAC1R (pituitary adenylate cyclase-activating polypeptide) system is associated with PTSD risk in women, and PACAP (*ADCYAP1*)-PAC1R (*ADCYAP1R1*) are highly expressed in the hypothalamus. Here, we show that female mice subjected to acute stress immobilization (IMO) have fear extinction impairments related to *Adcyap1* and *Adcyap1r1* mRNA upregulation in the hypothalamus, PACAP-c-Fos downregulation in the Medial Amygdala (MeA), and PACAP-FosB/ΔFosB upregulation in the Ventromedial Hypothalamus dorsomedial part (VMHdm). DREADD-mediated inhibition of MeA neurons projecting to the VMHdm during IMO rescues both PACAP upregulation in VMHdm and the fear extinction impairment. We also found that women with the risk genotype of *ADCYAP1R1* rs2267735 polymorphism have impaired fear extinction.

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Exposure to traumatic stress is one of the main environmental factors leading to disease[1]. Post-traumatic stress disorder (PTSD) is a debilitating mental disorder that occurs in some individuals exposed to traumatic stress. Moreover, PTSD may involve different symptoms including flashbacks, intrusive thoughts, and fear processing alterations[2,3]. PTSD prevalence ranges from 3.9 to 20%, and sociodemographic risk factors are key for its development[4,5].

Importantly, women have a twofold likelihood of developing PTSD compared to men[6,7]. It is widely demonstrated that sex hormones in women are crucial for the maintenance and modulation of PTSD and its symptomatology[8–10]. However, only two prior studies have addressed the question of whether the specific phase of the menstrual cycle at the moment of trauma matters for the development of PTSD. Trauma experienced during the luteal phase appears to be associated with increased flashbacks but without increasing overall PTSD severity[11]. In contrast, no effect of the menstrual cycle phase during trauma was found in people with PTSD[12]. Nevertheless, both studies have some methodological limitations inherent to retrospective analyses and included women regardless of having irregular menstrual cycles or taking hormonal contraceptives.

The PACAP (encoded by *ADCYAP1*)-PAC1R (encoded by *ADCYAP1R1*) (pituitary adenylate cyclase-activating polypeptide and its type I receptor) neuropeptide system regulates neuroendocrine stress responses[13,14]. The rs2267735 single nucleotide polymorphism (SNP) in *ADCYAP1R1*, located within an estrogen response element, has been suggested as a specific biomarker of PTSD in women but not men[15]. In these women, the CC genotype in the rs2267735 is associated with lower *ADCYAP1R1* expression and higher PTSD symptoms, possibly resulting from differential SNP-dependent estrogen receptor transcriptional regulation of *ADCYAP1R1*[16]. Moreover, rs2267735 regulates amygdala and hippocampus response to threatening stimuli in women with PTSD[17].

An important feature of PTSD is the impaired fear extinction (FE) to both stimuli related to trauma and newly aversive stimuli unrelated to trauma. Along this line, animal models are a valuable tool to uncover molecular mechanisms of FE and PTSD-like symptomatology. For example, we have previously shown that acute stress immobilization (IMO) impairs FE in male mice[18] and shares molecular mechanisms with people with PTSD[19]. With this model, researchers have studied some of the mechanisms of traumatic stress, although it is important to note that such models are most likely insufficient to capture features of complex forms of trauma in humans, such as sexual assault. The inclusion in research studies of people who have experienced such trauma has the potential to help better understand the development of PTSD and inform about relevant variables to consider to support patients. Such studies must be planned in a context of an ethical and sensitive framework to avoid exacerbating the effects of past traumatic experiences.

Studies of PACAP-PAC1R system in female rodents have identified the bed nucleus of the stria terminalis (BNST) in mice[16], and the prefrontal cortex in rats[20], as important structures for fear processing. Notably, the highest levels in the brain of PACAP-PAC1R are in the hypothalamus[21,22], but this brain region has not been associated with PTSD or fear processing.

Male lab animals are typically used in research studies focused on the neurobiological responses to stress. Female mice have often been disregarded with the argument of increased variability, given their cyclical hormonal fluctuations[23]. Despite this argument having been debunked, it is still held as a false construct in some cases[24–26]. Here, we used translational approaches to investigate the association of traumatic stress with hormonal cycle phases. Also, we explored the molecular mechanisms driving FE impairments in female mice subjected to IMO. We observed altered PACAP expression and neuronal activation in the ventromedial hypothalamus (VMHdm) and Medial Amygdala (MeA) that could be rescued by the inhibition of MeA to VMHdm projections during acute stress in female mice. In addition, to incorporate a translational connection to trauma in human participants, we investigated the association of a PAC1R genetic polymorphism with FE in a group of women that experienced trauma.

## Results

**IMO stress in female mice induces deficits in fear extinction regardless of the estrous cycle phase at trauma**. We showed previously that IMO produces impairments in FE in male mice, a deficit that is also observed in people with PTSD[19]. Here, we explored whether IMO exposure induced impairments in FE in naturally cycling female mice by exposing them to IMO or compensatory handling (control group) for 5 min and subjecting them 6 days later to a fear conditioning (FC) task followed by four consecutive FE tests (Fig. 1a). Results showed that female mice exposed to IMO had greater freezing rates during the FE tests compared to the control, non-stressed group ($F(1,61) = 12.528$, $P = 0.001$) (Fig. 1b). Results in male mice were on the same line (Supplementary Fig. 1a, b). The effect of IMO was specific for FE tests in both sexes since no significant interactions with stress were observed during FC.

Changes in bodyweight in rats after IMO are considered a marker of stressor intensity[27,28]. In our experiments, IMO exposure resulted in decreased weight gain in males and females, with female mice gaining overall less weight than males ($\chi^2(3) = 36.484$, $P < 0.001$, control males vs IMO males, $P = 0.020$; control females vs IMO females, $P < 0.001$, control females vs control males, $P = 0.007$, IMO females vs IMO males, $P = 0.006$) (Supplementary Fig. 1c). Repeated-measures analyses showed that males had their weight gain decreased by IMO ($F(1,20) = 16.952$, $P = 0.001$; control t1 vs IMO t1, $P = 0.09$; control t2 vs IMO t2, $P = 0.009$; control t1 vs control t2, $P < 0.001$; IMO t1 vs IMO t2, $P = 0.009$) (Supplementary Fig. 1d), but female mice experienced a slight weight loss ($F(1,37) = 8.937$, $P = 0.005$; control t1 vs IMO t1, $P = 0.065$; control t2 vs IMO t2, $P = 0.690$; control t1 vs control t2, $P = 0.038$; IMO t1 vs IMO t2, $P = 0.045$) (Supplementary Fig. 1e). Moreover, some evidence suggests that stress can increase grooming[29], but we found no differences in the number of grooming episodes in IMO or control female mice during FE1 (Supplementary Fig. 1f).

Female hormonal cycles modulate neurotransmitter and hypothalamic–pituitary–adrenal axis (HPA) function and affect spatial learning, short-term memory tasks, and FE consolidation[30–32]. Still, the interaction of stress with the hormonal cycles is less clear, especially to answer the question of whether the estrous cycle phase at the moment of trauma is associated with a greater vulnerability to develop a more severe post-traumatic behavioral phenotype. We monitored the estrous cycle of a group of naturally cycling female mice with vaginal smear cytology and subjected them to IMO during the proestrus (high estradiol/high progesterone) and metestrus (low hormones) phases of the estrous cycles (Fig. 1c, d). These phases were selected based on previous research[33]. We used the estrous cycle and stress as between-subject variables and FE sessions as within-subject variables. We found a main effect of stress ($F(1,24) = 5.419$, $P = 0.029$) without a main effect of cycle ($F(1,24) < 0.001$, $P = 0.995$), or an interaction cycle * stress ($F(1,24) = 0.245$, $P = 0.625$). Although, our analyses revealed slightly different freezing rates during FC given by an

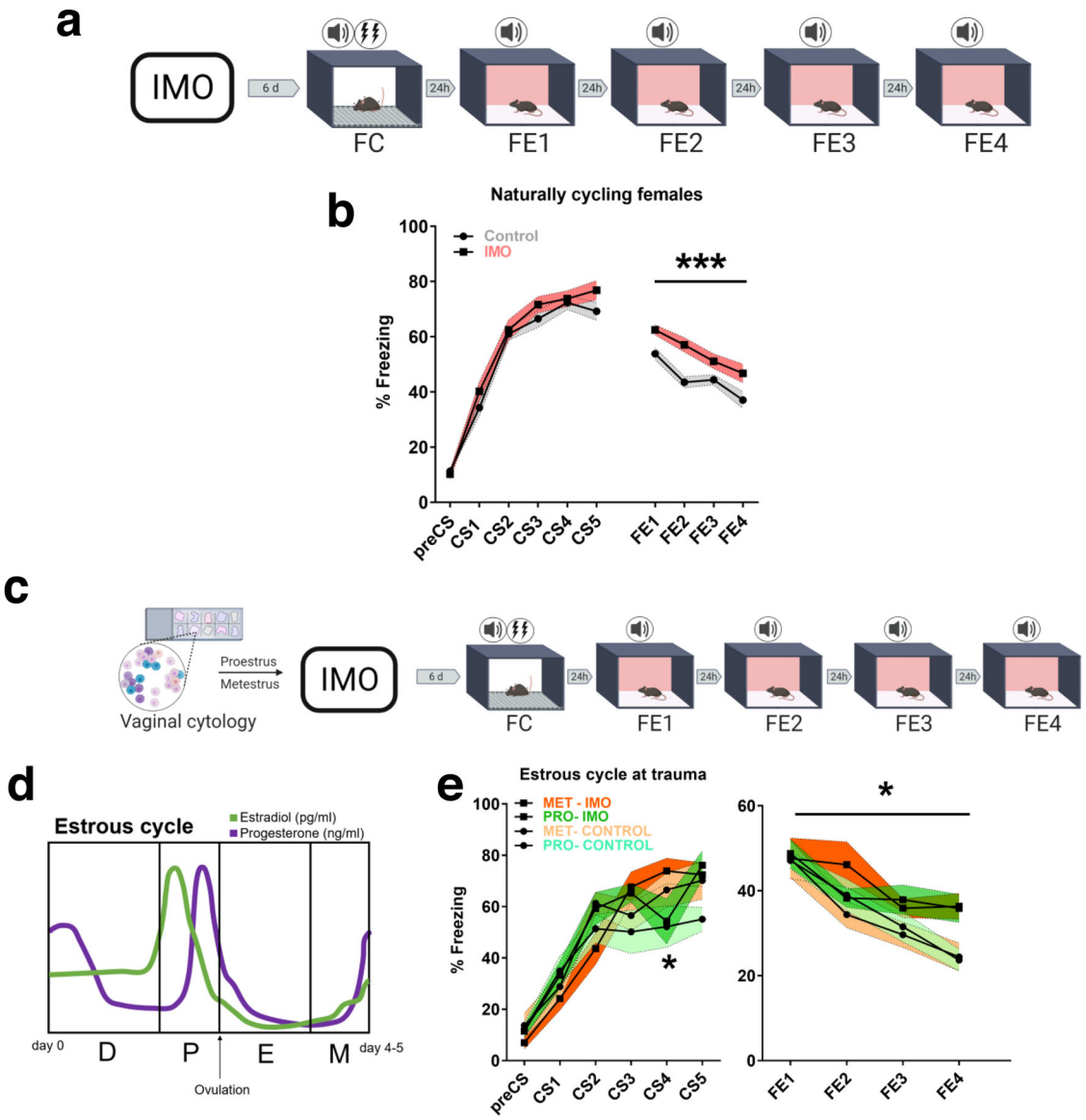



**Fig. 1 Long-lasting alterations of behavior after trauma and its association with the estrous cycle phase. a** Schematic representation of the behavioral protocol. **b** Fear learning and fear extinction in naturally cycling females undergoing IMO or compensatory handling (control: $n = 31$, IMO: $n = 32$) ($P = 0.001$). **c** Methods used to monitor the estrous cycle before the behavioral procedures. **d** Estrous cycle and related hormonal levels in female C57BL/6 mice. **e** Fear learning, and fear extinction in proestrus or metestrus females ($n = 7$ per group) ($P = 0.029$). Data are expressed as mean ± SEM. $^{*}P \leq 0.05$, $^{***}P \leq 0.001$. In **b**, **e**, main effect stress, main effect estrous cycle, main effect FE session or CS, and FE session* stress or CS*stress, estrous cycle*stress, FE session * estrous cycle, FE session * stress * estrous cycle interactions were analyzed using repeated-measures ANOVA, Bonferroni corrections were made for multiple comparisons. Asterisks above a line indicate significant main effect stress in repeated-measures ANOVA. CS conditioned stimulus, D diestrus, E estrus, FC fear conditioning, FE fear extinction session, IMO immobilization stress, M metestrus, Met metestrus, P proestrus, Pro proestrus. Source data are provided as a Source Data file.

interaction of CS * cycle, specifically during CS4 ($F(4,96) = 2.644$, $P = 0.038$; CS4 proestrus vs metestrus $P = 0.042$) and independently from stress ($F(4,96) = 1.197$, $P = 0.317$) (Fig. 1e and Supplementary Fig. 1g). This indicates that the estrous cycle phase at the time of trauma is not associated with differences in the increased freezing levels during FE induced by IMO. In addition, no changes in weight gain were observed in cycle monitored female mice between IMO and control groups ($t(13) = 0.079$, $P = 0.938$) (Supplementary Fig. 1h), neither in repeated measures analyses ($F(1,13) < 0.001$, $P = 0.994$) (Supplementary Fig. 1i).

Sex hormones have cyclical fluctuations that interact with stress hormone responses[31,34]. In rats, there are basal and stress-induced corticosterone concentrations that are higher in proestrus compared to estrus and diestrus[35]. Still, these differences seem to be constrained to an early corticosterone response to brief acute stressors[36]. Based on our findings regarding the estrous cycle, we hypothesized that differences in basal hypothalamic–pituitary-gonadal axis (HPG) hormonal levels would result in similar recovery profiles of HPA hormones. A group of female mice in proestrus or metestrus were subjected to IMO and hormonal regulation was assessed shortly after trauma (60 min) (Supplementary Fig. 2a). Regarding the HPA axis, corticosterone was robustly upregulated in IMO females regardless of the estrous cycle phase ($\chi^2(3) = 116.159$, $P < 0.001$; proestrus basal vs proestrus IMO, $P < 0.001$; metestrus basal vs metestrus IMO, $P < 0.001$; proestrus IMO vs metestrus IMO,

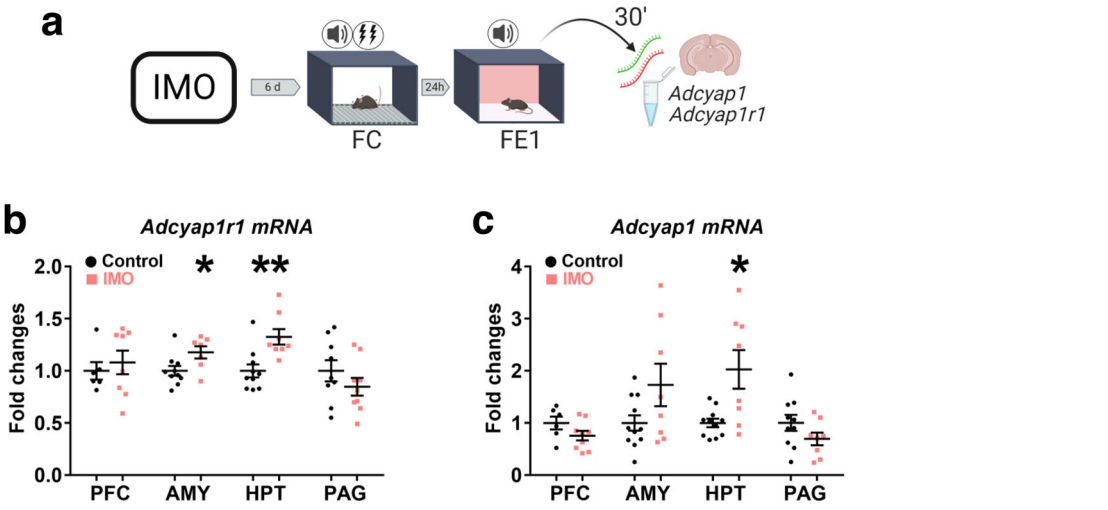



**Fig. 2 *Adcyap1-Adcyap1r1* regulation during early FE (FE1). a** Representation of the methods used to evaluate *Adcyap1, Adcyap1r1* regulation after FE1. **b** *Adcyap1r1* mRNA regulation 30 min after FE1 in prefrontal cortex (PFC) (control: $n = 6$, IMO: $n = 8$), amygdala (AMY) (control: $n = 10$, IMO: $n = 7$) ($P = 0.030$), hypothalamus (HPT) (control: $n = 10$, IMO: $n = 8$) ($P = 0.002$), and periaqueductal gray (PAG) (control: $n = 9$, IMO: $n = 9$). **c** *Adcyap1* in PFC (control: $n = 6$, IMO: $n = 9$), AMY (control: $n = 11$, IMO: $n = 8$), HPT (control: $n = 11$, IMO: $n = 8$) ($P = 0.020$), and PAG (control: $n = 10$, IMO: $n = 8$). Data are means ± SEM. $^*P \leq 0.05$, $^{**}P \leq 0.01$. Two-tailed *t* tests were conducted. IMO immobilization stress, FC fear conditioning, FE fear extinction. Source data are provided as a Source Data file.

$P = 0.886$) (Supplementary Fig. 2b). Additional steroids in the HPA axis, deoxycorticosterone ($\chi^2(3) = 62.322$, $P < 0.001$; proestrus basal vs proestrus IMO, $P < 0.001$; metestrus basal vs metestrus IMO, $P < 0.001$; proestrus IMO vs metestrus IMO, $P = 0.570$) (Supplementary Fig. 2c) and dehydrocorticosterone ($\chi^2(3) = 139.334$, $P < 0.001$; proestrus basal vs proestrus IMO, $P < 0.001$; metestrus basal vs metestrus IMO, $P < 0.001$; proestrus IMO vs metestrus IMO, $P = 1.000$) followed the same direction (Supplementary Fig. 2d).

Regarding the HPG axis, we found basal differences in progesterone levels between proestrus and metestrus ($\chi^2(3) = 65.065$, $P < 0.001$; proestrus basal vs metestrus basal, $P < 0.001$). After stress, the levels of progesterone decreased in the metestrus IMO group compared to metestrus basal ($P < 0.001$) with no changes in progesterone regulation after stress in proestrus ($P = 1.000$) (Supplementary Fig. 2e). There were differences in the basal testosterone levels ($\chi^2(3) = 13.756$, $P = 0.003$; proestrus basal vs metestrus basal, $P = 0.046$) and stress increased testosterone levels in metestrus females (metestrus basal vs metestrus IMO, $P = 0.002$), but not in proestrus females (proestrus basal vs proestrus IMO, $P = 1.000$) (Supplementary Fig. 2f). Furthermore, we found no differences in basal estradiol levels ($\chi^2(3) = 9.410$, $P = 0.024$; proestrus basal vs metestrus basal $P = 0.280$), but IMO decreased estradiol in proestrus females (proestrus basal vs proestrus IMO, $P = 0.029$), but not in metestrus females (metestrus basal vs metestrus IMO, $P = 1.000$) (Supplementary Fig. 2g). These results provide evidence for a robust upregulation of the HPA axis in IMO females in both estrous phases with similar 60 min post-stress hormonal profiles. Moreover, basal differences in HPG hormones showed a differential regulation by IMO.

***Adcyap1- Adcyap1r1* upregulation is related to fear extinction deficits in a female PTSD-like mouse model**. High PACAP levels and *ADCYAP1R1* genetic variants are related to greater post-traumatic symptoms and fear responses in women[15]. Further, *Adcyap1r1* mRNA is upregulated 2 h after FC in male mice[15,37], but few studies have studied its regulation in female mice[38]. We exposed a group of naturally cycling female mice to IMO or compensatory handling for 5 min, 6 days later they took a FC task followed by one session of FE (FE1). After FE1 (30 min), brains were removed to study *Adcyap1-Adcyap1r1* mRNA regulation (Fig. 2a). We found greater freezing levels in female mice exposed to IMO ($F(1,19) = 4.741$, $P = 0.042$) (Supplementary Fig. 3a, b) and *Adcyap1r1* transcripts were upregulated in the amygdala ($t(15) = -2.396$, $P = 0.030$) and hypothalamus ($t(16) = -3.586$, $P = 0.002$) of IMO female mice (Fig. 2b). Similarly, *Adcyap1* mRNA was upregulated in the hypothalamus of IMO female mice ($t(9.217) = -2.795$, $P = 0.020$) (Fig. 2c). Moreover, mean freezing levels during FE1 were not correlated with *Adcyap1* or *Adcyap1r1* mRNA regulation in the amygdala (*Adcyap1r1*, $r = -0.213$, $P = 0.411$; *Adcyap1*, $r = -0.075$, $P = 0.759$) or hypothalamus (*Adcyap1r1*, $r = -0.237$, $P = 0.343$; *Adcyap1*, $r = -0.240$, $P = 0.322$) (Supplementary Fig. 3c). These results demonstrate that IMO exposure increased the regulation of *Adcyap1r1* transcripts in the hypothalamus–amygdala and *Adcyap1* in the hypothalamus of females after a FE test.

**Activity profiles of PACAP + neurons in the amygdala and hypothalamus are related to FE deficits observed after IMO**. Then, we subjected a group of naturally cycling female mice to the same behavioral task and collected tissue 90 min after FE4 to perform an immunofluorescence study (Fig. 3a). We used c-Fos+ and FosB/ΔFosB+ as markers of recent and repeated neuronal activity[39,40], and colocalized them with PACAP + neurons. We first validated a PACAP antibody with a conditional KO approach and then quantified signal colocalization in areas implicated in IMO or FE according to previous research[41,42] (Supplementary Fig. 4a–c). Results showed that shortly after the last FE session, IMO females had fewer PACAP-c-Fos+ neurons in the basolateral amygdala (BLA) ($t(5.339) = 3.159$, $P = 0.023$) and in the MeA ($t(8) = 3.797$, $P = 0.005$) compared to controls (Fig. 3b). In turn, we observed decreases in PACAP-FosB/ΔFosB+ in the ventrolateral periaqueductal gray (PAGvl) ($t(10) = 2.397$, $P = 0.037$) and a marked increase in the VMHdm ($t(8) = -4.982$, $P = 0.001$) (Fig. 3c). PACAP neurons with double colocalization, PACAP-c-Fos+ & FosB/ΔFosB+, mirrored the previous results with lower colocalization observed in the BLA ($t(8) = 3.078$, $P = 0.015$) and MeA ($t(8) = 2.446$, $P = 0.040$), and greater colocalization in the VMHdm ($t(8) = -2.639$, $P = 0.030$)

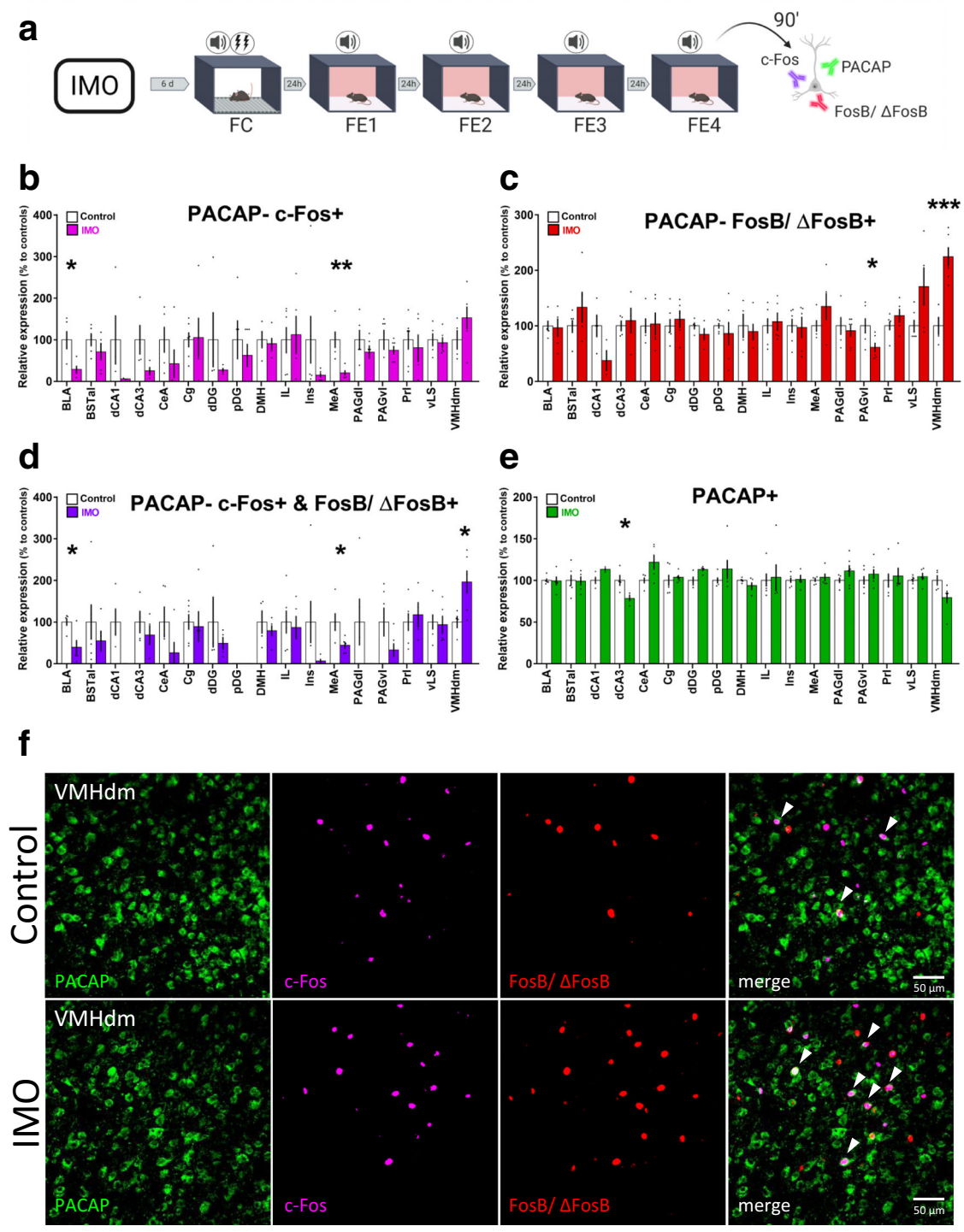



**Fig. 3 Immobilization stress induces changes in PACAP+ neuronal activation after a fear extinction task. a** Schematic representation of the behavioral and immunohistochemical methods. **b** Quantification of cellular colocalization of PACAP-c-Fos+ (BLA and MeA $n = 5$ per group) (BLA: $P = 0.023$, MeA: $P = 0.005$). **c** Quantification of cellular colocalization of PACAP-FosB/ΔFosB+ (PAGvl $n = 6$ per group, VMHdm control: $n = 4$, IMO: $n = 6$) (PAGvl: $P = 0.037$, VMHdm: $P = 0.001$). **d** Quantification of double colocalization of PACAP-c-Fos+ and FosB/ΔFosB+ (BLA and MeA $n = 5$ per group; VMH control: $n = 4$, IMO: $n = 6$) (BLA: $P = 0.015$, MeA: $P = 0.040$, VMHdm: $P = 0.030$). **e** Quantification of PACAP+ cells (dCA3 $n = 4$ per group) (dCA3: $P = 0.028$). **f** Representative confocal images of PACAP, c-Fos, FosB/ΔFosB colocalization in the VMHdm. White arrowheads signal triple colocalization. Scale bar 50 µm. Results are presented as relative expression compared to controls (in %) and extracted from cells per $mm^2$ normalized to DAPI. Data are presented as means ± SEM. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Two-tailed *t* tests or Mann–Whitney's *U* tests were used. BLA basolateral amygdala, BSTal bed nucleus of the stria terminalis anterolateral part, dCA1 dorsal CA1, dCA3 dorsal CA3, CeA central amygdala, Cg cingulate cortex, dDG dorsal dentate gyrus, pDg posterior dentate gyrus, DMH dorsomedial hypothalamus, FC fear conditioning, FE fear extinction, IL infralimbic cortex, IMO immobilization stress, Ins insular cortex, MeA medial amygdala, PAGdl dorsolateral periaqueductal gray, PAGvl ventrolateral periaqueductal gray, Prl prelimbic cortex, vLS ventral lateral septum, VMHdm ventromedial hypothalamus dorsomedial nucleus. Source data are provided as a Source Data file.

(Fig. 3d). See Fig. 3f for a representative image. We also detected a reduction of PACAP+ signal in the dorsal CA3 of IMO females ($t(6) = 2.893$, $P = 0.028$) (Fig. 3e). Mean freezing levels during FE were negatively correlated with PACAP-c-Fos+ neurons in MeA ($r = -0.716$, $P = 0.020$) and positively correlated with PACAP-FosB/ΔFosB+ neurons in VMHdm ($r = 0.715$, $P = 0.020$) (Supplementary Fig. 4d–g). These results show that increased freezing levels during four FE sessions in IMO female mice are associated with decreased activation of PACAP+ neurons in MeA and repeated activation of PACAP+ neurons in the VMHdm.

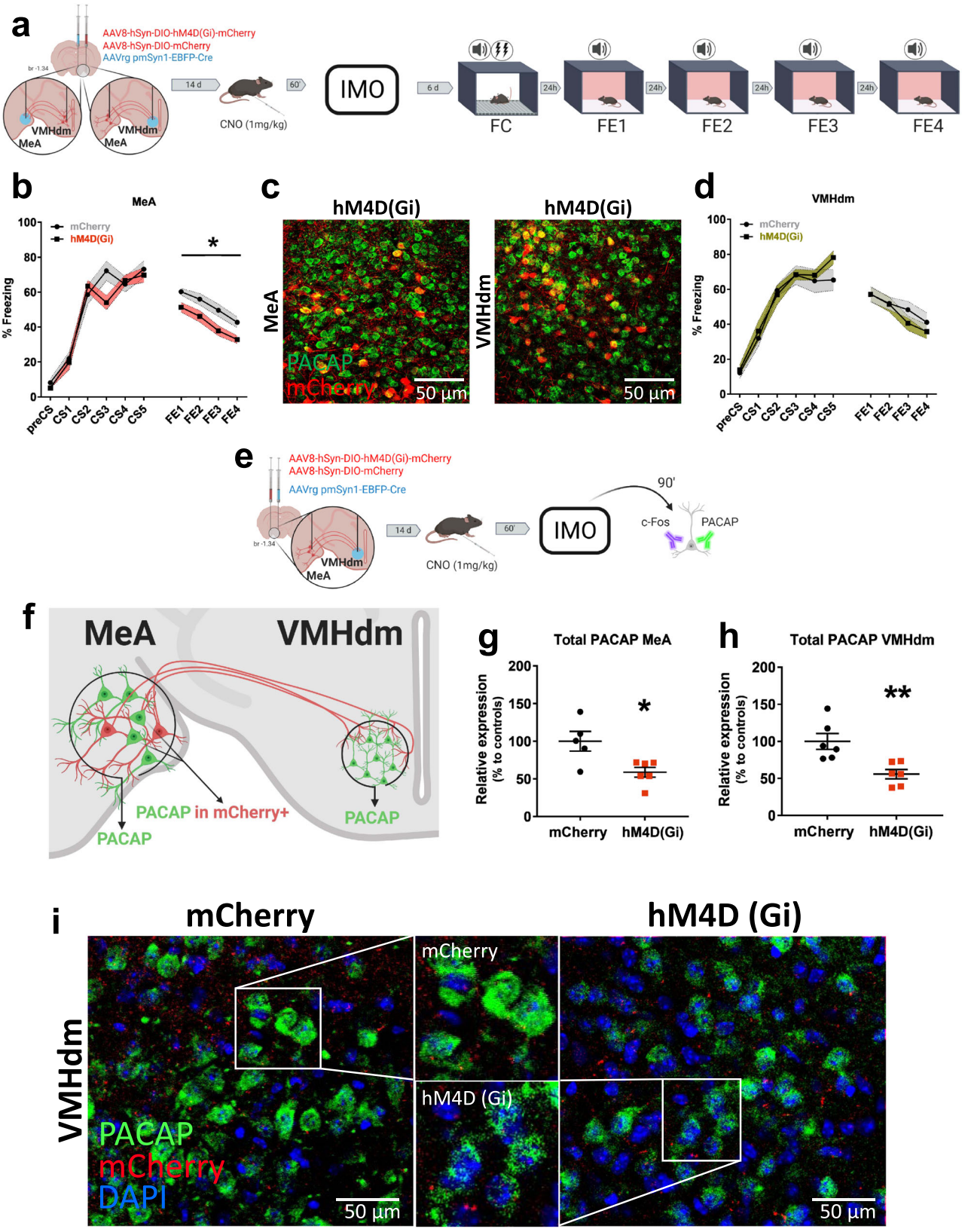
**Inhibition of the MeA to VMHdm projections rescues fear extinction deficits and PACAP upregulation after IMO**. Connections between the amygdala and the hypothalamus are involved in stress processing and fear learning. The MeA processes sensory cues and it is strongly activated by emotional stressors[43,44]. The VMHdm receives extensive projections from the MeA, and its repeated activity is related to persistent defensive behaviors in response to heterogeneous threats[45]. A MeA-VMH circuit was previously implicated in aggression and conspecific/predator threat responses, but not in emotional stressors[46,47]. Therefore, since it is known that IMO exposure strongly elicits an upregulation of c-Fos in the MeA[41,43], we hypothesized that trauma would increase MeA activity and enhance signaling to the VMHdm to trigger increases in PACAP function, which would further promote VMHdm activation. However, since MeA-VMH share reciprocal connections and the amygdala is a possible inhibitory feedback mechanism for VMH function[48–50], we tested for circuit directionality in separate groups of naturally cycling female mice. We used a non-PACAP-selective combined chemogenetic retrograde AAV approach to inhibit the activity of this circuit by injecting AAV-hM4Di-DREADD (AAV8-hSyn-DIO-hM4D(Gi)-mCherry) or AAV-DIO-mCherry (AAV8-hSyn-DIO-mCherry) and AAV5rg-pmSyn-EBFP-Cre, so that hM4D(Gi) receptors were expressed in VMHdm-projecting MeA neurons or MeA-projecting VMHdm neurons (Fig. 4a, c). mCherry and enhanced blue fluorescent protein (EBFP) reporters were used to verify injection sites (Supplementary Fig. 5). To trigger circuit inhibition, we administered Clozapine N-oxide (CNO, 1 mg/kg I.P.) 1 h before the exposure to IMO; 6 days later mice were exposed to a FC task followed by four consecutive FE tests (Fig. 4a). Our results showed that the deficits in FE induced by IMO were rescued by the inhibition of MeA to VMHdm projections during IMO ($F(1,16) = 5.250$, $P = 0.036$) (Fig. 4b), but not by the inhibition of VMHdm to MeA projections ($F(1,12) = 0.196$, $P = 0.666$) (Fig. 4d).

We used the same approach to study the short-term dynamics of PACAP after trauma in mice (90 min after IMO) (Fig. 4e, f). The overall PACAP levels were significantly lower in the MeA ($t(9) = -2.99$, $P = 0.015$) and VMHdm ($t(10) = -3.544$, $P = 0.005$) of animals that had MeA to VMHdm projections inhibited during IMO (Fig. 4g–i). However, PACAP levels were not significantly lower in mCherry+ neurons in the MeA ($t(9) = -2.203$, $P = 0.055$) (Supplementary Fig. 6a, b). Furthermore, inhibition of MeA to VMHdm projections resulted in a greater number of overall c-Fos+ ($U = 1.500$, $P = 0.009$) and PACAP-c-Fos+ neurons ($U = 1.000$, $P = 0.009$) in the MeA but not in mCherry+ MeA neurons ($U = 15.500$, $P = 0.548$) (Supplementary Fig. 6c, d, f). The chemogenetic inhibition did not change c-Fos+ ($U = 17.500$, $P = 0.937$) or PACAP-c-Fos+ ($t(9) = -1.608$, $P = 0.142$) expression in the VMHdm (Supplementary Fig. 6e, g).

We further explored the effects of MeA to VMHdm circuit inhibition and found differences in c-Fos regulation in brain areas acting as afferent/efferent inputs to this circuit or as structures receiving collateral projections. The chemogenetic inhibition resulted in lower activations of PACAP+ neurons in the anterior hypothalamus ($t(10) = -2.614$, $P = 0.026$) and bed nucleus of the stria terminalis medial anterior part ($t(10) = -2.287$, $P = 0.045$). Also, lower overall c-Fos counts were observed in the anterior hypothalamus ($t(10) = -2.547$, $P = 0.029$), bed nucleus of the stria terminalis medial anterior part ($t(10) = -2.526$, $P = 0.030$), Prelimbic Cortex ($t(10) = -2.783$, $P = 0.019$) (Supplementary Fig. 7a–c). Despite our approach was not specific to PACAP+ neurons, we observed that around 80% of mCherry+ neurons had PACAP+ immunolabeling (Fig. 5a, b). Moreover, there was prominent VGLUT2 expression in the VMHdm where PACAP+ neurons were located (Fig. 5c). Of note, we did not find significant differences in PACAP expression in the VMHdm between basal and 90 min post-IMO females ($t(9) = 0.642$, $P = 0.537$) or males ($t(5.153) = -1.114$, $P = 0.315$). No effects for sex or stress on PACAP expression were found in the VMHdm (stress * sex, $F(1,18) = 1.665$, $P = 0.213$; sex, $F(1,18) = 0.013$, $P = 0.909$; stress, $F(1,18) = 0.130$, $P = 0.722$) or MeA (stress * sex, $F(1,19) < 0.001$, $P = 0.996$; sex, $F(1,19) = 0.477$, $P = 0.512$; stress, $F(1,19) = 2.548$, $P = 0.127$) (Supplementary Fig. 7d–f). In sum, these experiments showed that PACAP dynamics in the VMHdm after trauma in mice are influenced by the activity of neurons projecting from the MeA. Their inhibition rescues the post-traumatic FE-deficient phenotype and it is associated with lower PACAP levels in MeA and VMHdm, enhanced activity of the MeA, and decreased activity in structures related to the threat detection system.

**No association between the menstrual cycle phase and post-traumatic symptoms after a traumatic experience in women**. We studied a cohort of women attending the Emergency Department at the Hospital Clínic de Barcelona (Supplementary Table 1 for demographics) shortly after suffering sexual abuse (<96 h) and documented their menstrual cycle phase at the time of trauma (early follicular, late follicular, luteal) (Fig. 6a). We assessed post-traumatic symptoms 3 weeks after trauma with the Acute Stress Disorder Interview, a clinical tool previously validated for post-traumatic symptom assessment[51]. Our results showed that all women had similar post-traumatic symptom scores regardless of their menstrual cycle phase at trauma ($F(2,111) = 0.088$, $P = 0.915$) (Fig. 6b). The lifespan distribution of PTSD prevalence suggests that age can moderate post-traumatic symptoms[52]. The mean age in our sample was 30 ± 10.4 years and it was not correlated with post-traumatic symptom scores ($r = 0.001$, $P = 0.987$) (Supplementary Fig. 8a). Furthermore, we observed that some women were not conscious during trauma which could have affected their ability to process the event. In our study, there was no significant difference in post-traumatic symptom severity between women that were conscious during trauma compared to women that were not-conscious women ($t(152) = 1.949$, $P = 0.053$) (Supplementary Fig. 8b). We repeated the analysis controlling for consciousness status and the effect of the menstrual cycle phase and found no significant effect ($F(2,96) = 0.226$, $P = 0.798$).

In our sample, 17.6% of women had a history of previous sexual abuse in adulthood (PSAA) and 20.5% history of previous aggression in adulthood (PAA) (Supplementary Table 2). However, for these women their mean post-traumatic symptom scores did not differ compared with women without a history of PSAA ($U = 1790.500$, $P = 0.635$) or PAA ($U = 2350.000$, $P = 0.655$). We analyzed separately to take into account childhood trauma history and found similar symptom scores in women with a history of sexual (CSA; $U = 2473.000$, $P = 0.366$) or physical abuse (CPA; $U = 2586.000$, $P = 0.144$), but women who had experienced emotional abuse had greater symptom scores (CEA;




$U = 2515.000$, $P = 0.016$). To know if these variables were interacting with the menstrual cycle phase, we performed additional independent analysis introducing them as between-subject factors. Mean post-traumatic symptom scores were similar regardless of menstrual cycle phase and history of PSAA ($F(2,91) = 0.449$, $P = 0.639$), PAA ($F(2,101) = 0.248$, $P = 0.781$), CEA ($F(2,85) = 1.672$, $P = 0.194$) or CPA ($F(2,87) = 0.211$, $P = 0.811$). A cycle * CSA interaction was observed given by different scores in women in EF ($F(2,85) = 4.481$, $P = 0.014$; EF no CSA vs EF CSA, $P = 0.033$).

**Fig. 4 Effect of the chemogenetic inhibition of the medial amygdala-ventromedial hypothalamus circuit during immobilization stress. a** Schematic representation of the methods and timeline of experiments. **b** Fear learning and fear extinction in mice with a temporal inhibition of MeA to VMHdm projections (hM4D(Gi)) during IMO vs controls (mCherry) (mCherry: $n = 10$, hM4D(Gi): $n = 8$) ($P = 0.036$). **c**, left panel: representative confocal images showing PACAP+ (green) neurons and mCherry+ (red) cell bodies in the MeA in animals receiving hM4D(Gi) in MeA; right panel: cell bodies in the VMHdm in animals receiving hM4D(Gi) in VMHdm. Scale bar 50 µm. **d** Fear learning, and fear extinction in mice with a temporal inhibition of VMHdm to MeA projections (hM4D(Gi)) during IMO vs controls (mCherry) (mCherry: $n = 6$, hM4D(Gi): $n = 8$). **e**, **f** Methods used to assess PACAP expression shortly after IMO in animals with inhibited MeA to VMHdm circuitry (hM4D(Gi) vs controls (mCherry)). **g**, **h** Effect of a temporal inhibition of MeA to VMHdm projections on PACAP expression shortly after IMO in the MeA (mCherry: $n = 5$, hM4D(Gi): $n = 6$) ($P = 0.015$) and VMHdm (mCherry: $n = 6$, hM4D(Gi): $n = 6$) ($P = 0.005$). Results are presented as relative expression compared to controls (in %) and extracted from PACAP levels normalized to DAPI ($n = 5$–6 per group). **i** Representative confocal images displaying differences in the expression of PACAP in the VMHdm of animals receiving a temporal inhibition of MeA to VMHdm projections during IMO (hM4D(Gi)) left panel vs controls (mCherry) right panel. Respective magnifications are shown in the middle part upper panel (mCherry) and lower panel (hM4D(Gi)). Scale bar: 50 µm. Data are means ± SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. In **b**, **d**, main effect treatment, main effect FE session, and FE session * treatment interaction were analyzed using a repeated-measures ANOVA. Asterisks above a line indicate significant main effect treatment in repeated-measures ANOVA. In **g**, **h**, two-tailed *t* tests were used. CNO clozapine N-oxide, FC fear conditioning, FE fear extinction, IMO immobilization stress, MeA medial amygdala, VMHdm ventromedial hypothalamus dorsomedial nucleus. Source data are provided as a Source Data file.

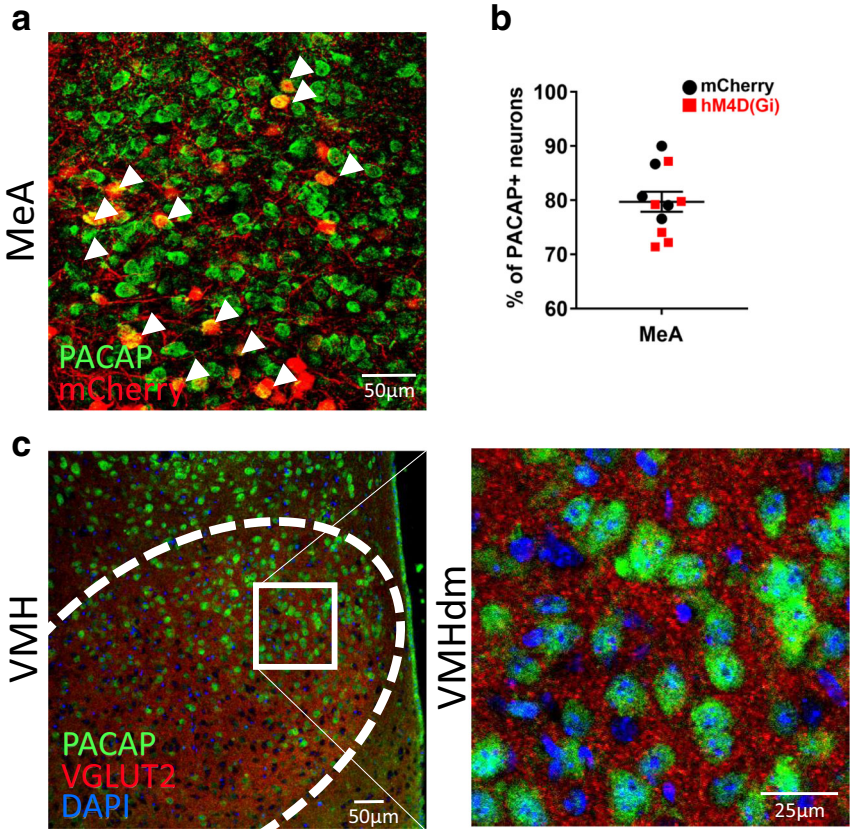



**Fig. 5 Sites of delivery of viral vectors in MeA and VMHdm. a** Representative image showing the colocalization of mCherry+ neurons with PACAP+ immunolabeling in the MeA. White arrowheads signal colocalization. **b** Quantification of the proportion of mCherry+ cells with PACAP+ immunolabeling of animals receiving AAV5rg-pmSyn-EBFP-Cre in the VMHdm and either AAV-hM4Di-DREADD or AAV-DIO-mCherry in the MeA (mCherry: $n = 5$, hM4D(Gi): $n = 6$). **c** Representative image showing a prominent VGLUT2 expression in the VMHdm where PACAP+ neurons are enriched. Data are expressed as mean ± SEM. Scale bars **a**, 50 µm; **c** left panel scale bar 50 µm, right panel 25 µm. MeA medial amygdala, VMHdm ventromedial hypothalamus dorsomedial nucleus. Source data are provided as a Source Data file.

Then, we analyzed for post-traumatic sub-symptoms (dissociation, re-experiencing, avoidance, hyperactivation) and observed that the menstrual cycle phase at trauma was not a factor related to the severity of post-traumatic sub-symptoms as measured in the cohort (Supplementary Fig. 8c). However, women that were conscious during the sexual assault had greater severity of re-experiencing symptoms ($U = 1708.000$, $P = 0.001$) (Supplementary Fig. 8d). In addition, participants that were diagnosed with PTSD in medical follow-ups (median 321 days, range 46–1339) (Supplementary Table 3) had greater post-traumatic symptom severity in the assessment at the 3rd week post trauma ($t(41.871) = 2.738$, $P = 0.009$) (Fig. 6c). In these women, the menstrual cycle phase was not associated with PTSD diagnosis, 4 of 20 women in the early follicular phase were

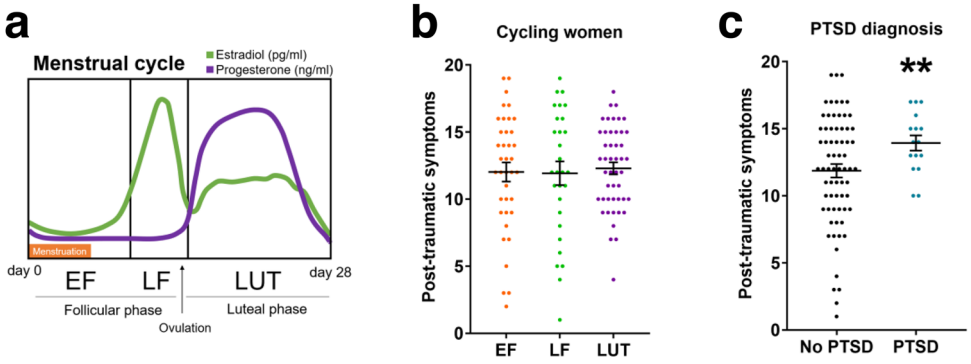



**Fig. 6 Post-traumatic symptoms and menstrual cycle phases. a** Menstrual cycle and related hormonal levels in women. **b** Post-traumatic symptom scores of women at 3 weeks post trauma analyzed by menstrual cycle phase at trauma (EF: $n = 38$, LF: $n = 29$, LUT: $n = 47$) **c**, or PTSD diagnosis within the first year of follow-up (PTSD: $n = 16$, No PTSD: $n = 69$) ($P = 0.009$). Data are expressed as mean ± SEM. $^{**}P \leq 0.01$. In **b**, the main effect menstrual cycle was analyzed in a one-way ANOVA. In **c**, post-traumatic symptoms were analyzed using a two-tailed *t* test. EF early follicular, LF late follicular, LUT luteal phase, PTSD post-traumatic stress disorder. Source data are provided as a Source Data file.

diagnosed with PTSD, 1 of 15 in late follicular phase, and 5 of 32 in luteal phase ($P = 0.548$, two-sided Fisher's exact test). These findings suggest that the menstrual cycle phase at the moment of trauma is not a factor associated with the severity of post-traumatic symptoms.

***ADCYAP1R1* genetic variants are related to fear extinction deficits in traumatized women**. PACAP-PAC1R is known to be regulated after stress exposure and prior work suggested that traumatized women carrying a risk genotype in the *ADCYAP1R1* rs2267735 SNP have greater post-traumatic symptoms[15,53]. More specifically, the homozygous "CC" allele has been linked to increased total PTSD symptom severity[15], elevated hyperarousal[15], and greater emotional numbing[54]. Further, a recent meta-analysis revealed that the "C" allele of rs2267735 conferred a significant risk for PTSD in a combined dataset comprised of both sexes; the risk that only remained in a female subset when each sex was examined separately[55]. Taken together, the presence of the "CC" genotype together with low estradiol levels, decreased *ADCYAP1R1* expression, and exposure to traumatic stress can produce a phenotype, including both higher PTSD symptoms and elevated conditioned fear responses[56]. For this reason, the "CC" allele has been identified as a "risk allele" within this pathway in women as compared to "G" carriers who have been repeatedly classified as "low-risk"[55,57]. Our prior studies of extinction in this population have indicated that lower estrogen levels were associated with higher startle responses during the early phase of extinction, which was in turn associated with symptoms of PTSD[58–60]. However, we had not examined PAC1R genotype effects on FE.

Therefore, we studied fear-potentiated startle during extinction in another cohort of women from the Grady Trauma Project. Care was taken to ensure that participants' trauma was not exacerbated by their participation, as detailed in the Methods section (see Supplementary Table 4 for demographics) (Fig. 7a). Women above 40 years old have a cumulative incidence of irregular cycles starting from 10% at 40 to 80% at 50 (see ref. [61]). Hence, we divided women into two age groups, ≤40 years ($n = 71$) and over 41 years ($n = 49$) and focused our analyses on the younger age group. Of note, in the experiments for Fig. 6b, c, we removed women with irregular cycles from the analyses. In an analysis of covariance (ANCOVA), we analyzed extinction as a repeated measures variable (two levels: early, late), and age and genotype as between-group variables. As in our prior studies where we found interactions with childhood trauma[62,63], we categorized women as having experienced 0–1 types of childhood abuse or 2+ types of childhood abuse from the Childhood Trauma Questionnaire. We also entered the top two GWAS principle components, total lifetime trauma, and baseline startle as covariates in the analyses. At the end of fear conditioning, startle responses were greater to CS+ compared to CS-, ($F(1,111) = 9.082$, $P = 0.003$), but no interaction with age, childhood trauma, or genotype ($P = 0.937$), showing that FC was successful but did not differ between groups. There was a significant reduction of startle from early to late extinction ($F(1,108) = 10.640$, $P = 0.001$), as well as a significant three-way interaction of age * genotype * childhood trauma on startle ($F(1,108) = 4.131$, $P = 0.045$) even when adjusting for covariates (Supplementary Fig. 9a–c). Follow-up analyses with the same control variables found that in women ≤40 years, genotype and childhood trauma had a two-way interaction on startle during early extinction, with the CC genotype associated with a higher startle in those with two or more childhood trauma exposures ($F(1,64) = 4.951$, $P = 0.030$) (Fig. 7b). Notably, we did not see the same associations in the older age category ($F(1,64) = 1.514$, $P = 0.225$) (Supplementary Fig. 9c). These results suggest that women that had experienced a high level of childhood trauma load, and with a risk genotype in the *ADCYAP1R1* rs2267735 SNP have impairments in FE.

## Discussion

In this study, we found that female mice subjected to an acute traumatic stressor developed long-lasting alterations of FE that were unrelated to the phase of the estrous cycle at the moment of trauma. In these mice, IMO exposure upregulated *Adcyap1-Adcyap1r1* transcripts in the amygdala and hypothalamus and changed PACAP+ neuronal activation in MeA and VMHdm. We found that PACAP in the VMHdm is relevant for stress responses and that the inhibition of MeA to VMHdm circuit during trauma alters PACAP short-term dynamics and rescues the IMO-induced FE impairments. In addition, in a cohort of women who experienced sexual assault we observed no association for the menstrual cycle phase at trauma with post-traumatic symptom scores at 3 weeks post trauma. Further, women with a history of multiple traumas and a risk genotype in the *ADCYAP1R1* rs2267735 SNP had impairments in FE.

Our data showed that IMO is an acute and intense emotional stressor that produces deficits in FE that are coupled with physiological adaptations to trauma as evidenced by a decreased weight gain in female mice. Female mice exposed to IMO during proestrus or metestrus had similar increased freezing levels during FE and similar post-stress corticosterone levels. However, mice in proestrus and metestrus presented differences in the

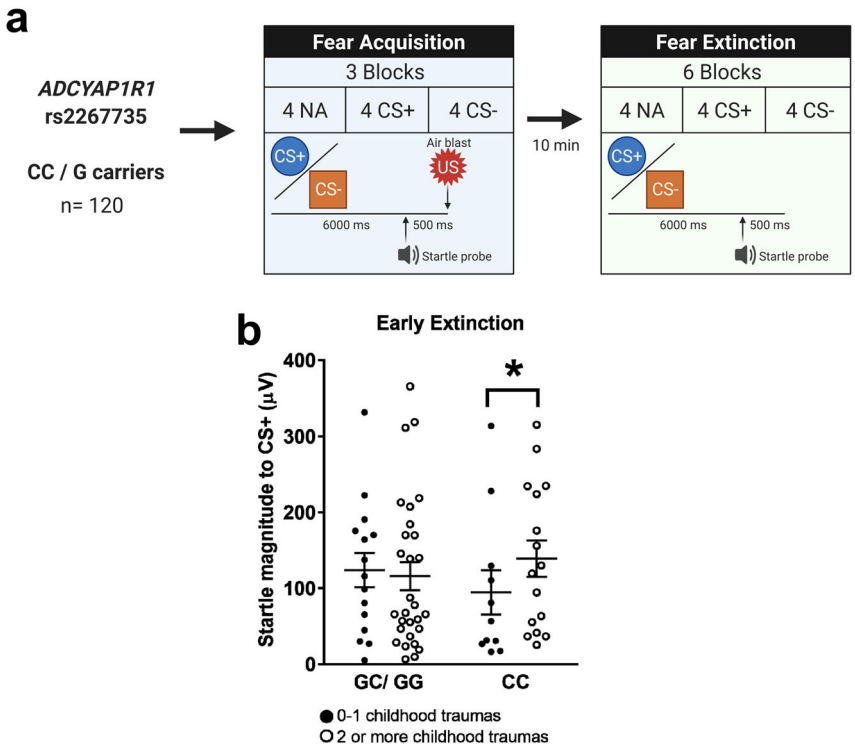



**Fig. 7 *ADCYAP1R1* rs2267735 genotype effects during early FE in highly traumatized individuals. a** Methods used for the assessment of the influence of rs2267735 genotype over FE ($n = 71, 49$). **b** Fear-potentiated startle magnitude of cycling women (≤40 years old) with CC or GC/GG genotypes during early FE and its relation to childhood trauma exposure ($n = 71$) ($P = 0.030$). Analyses for >40 years old ($n = 49$) are shown in Supplementary Fig. 9c. Data are means ± SEM. $^*P \leq 0.05$. Fear-potentiated startle data in **b** were analyzed with an ANCOVA with extinction as repeated measures variable, age, genotype, and childhood trauma as between-subjects variables. Covariates included GWAS principal components, total lifetime trauma, baseline startle. None of the CS+ presentations was paired with an US during the FE phase. CS+ reinforced conditioned stimulus, CS- non-reinforced conditioned stimulus, NA noise probe alone, US unconditioned stimulus. Source data are provided as a Source Data file.

regulation of progesterone, testosterone, and estradiol after stress. These differences have been previously characterized for progesterone and they have shown to vary depending on the type of stressor[64]. To note, the effects of IMO over FE and weight gain in females with cycle monitorization were attenuated compared to females without cycle monitorization. This attenuation may be related to their exposure to repeated manipulations for cytology assessment and the habituation of their stress response, although the exact mechanisms remain to be discovered[65–67].

The menstrual cycle is known to influence performance in emotional tasks. For example, menstrual phases with high estradiol levels are related to better FE consolidation, less subjective distress, and attenuations in the activity of cortical and subcortical arousal regions[30,68,69]. However, research focused specifically on the effects of traumatic injury found that trauma experienced in distinct phases of the menstrual cycle was associated to similar PTSD symptom scores, but women experiencing trauma in the mid-luteal phase reported having more flashbacks[11]. Our findings are in line with this study in the sense that the menstrual cycle phase at trauma was not a factor associated with overall greater post-traumatic symptomatology. However, we did not find greater re-experiencing symptoms associated with any menstrual cycle phase after performing a more detailed segmentation into menstrual cycle phases and controlling for irregular cycles and oral contraceptive use.

Hence, the menstrual cycle phase and hormonal levels may not be factors influencing the encoding of traumatic memories, rather the recollection of aversive memories or the performance during mildly stressful tasks. Research studies that have found an association for the luteal phase with greater flashback memories perform the encoding and retrieval of memories during the same luteal phase or during the early follicular phase (low hormones)[11,70,71]. Indeed, this memory retrieval effect was also found in women with PTSD that were evaluated during the early follicular phase and who showed greater avoidance, fear-related and overall symptom severity compared to women evaluated during the mid-luteal phase[10]. Altogether our results suggest that the hormonal cycle phase at trauma is not associated with the further development of a more severe post-traumatic phenotype. Since our extinction paradigm in both mice and women is unrelated to the trauma, our conclusions about the lack of effects of the cycle during trauma do not necessarily predict whether extinction-based therapies for the memories acquired during the trauma itself would be different based on the cycle at trauma.

Previous research showed that PACAP-PAC1R function is altered in female clinical populations but its mechanistic contribution to disease remains elusive[15,16]. PACAP is implicated in central and sympathoadrenal responses to emotional stressors[72] and *Adcyap1r1* transcripts increase after FC in both male and female mice[15,16]. Here, we found that female mice exposed to IMO upregulated *Adcyap1-Adcyap1r1* mRNA in the amygdala and hypothalamus after a FE session when compared to animals receiving compensatory handling. Thus, exposure to traumatic stress can prime PACAP-PAC1R for overactivation under a FE task. In addition, in our cohort of highly traumatized women the CC genotype in *ADCYAP1R1* rs2267735, which has been associated with vulnerability[15,17], produced impairments in the early phases of FE. Notably, this risk genotype is associated with lower *ADCYAP1R1* expression and decreased function of estradiol as an adaptive stress response[16]. The alterations in FE were clear in

women ≤40 years old but only trend level in older women, an effect that may be related to lower estradiol levels with increasing age, but also to other age-related variables.

Concerning the neural mechanisms of action, we observed that IMO induced impairments in FE, deficits in MeA function, and increased the activation of PACAP+ neurons in the VMHdm. Brain circuits between the amygdala–hypothalamus are involved in stress and threat processing. In mice, the MeA is strongly activated by IMO and modulates behavioral and hypothalamic neuroendocrine responses[43,73], while the stimulation of VMH can induce fear responses like immobility or avoidance[74]. Together, the MeA-VMH are part of a medial hypothalamic defensive system that is implicated in the generation of innate and conditioned defensive responses to conspecific/predator cues[75–77]. Since the MeA is a major source of input to the medial hypothalamic defensive circuit that is strongly activated by IMO, we hypothesized that it could be relaying signals to the VMHdm to increase PACAP function that further facilitated VMHdm activation. In our results, a single traumatic exposure in female mice induced functional changes in the hypothalamus, through the MeA to VMHdm projections, that were related to deficits in FE but not fear learning. These changes in FE may be related to the functional restructuration of basal behavioral circuits that facilitate the organism's survival or coping mechanisms.

We also found a neuronal signature of trauma after FE sessions, the lower levels of PACAP-c-Fos+ neurons in the MeA after FE4 are related to a previously unknown process of neuronal adaptation after a traumatic stressor. The reasons for this decrease in PACAP-c-Fos+ expression weeks after IMO exposure, once the post-traumatic phenotype is established, could be simply reflecting the neuronal signature of trauma. However, PACAP-c-Fos+ expression in the MeA is likely necessary for normal FE to occur. Future studies focused on the effects of trauma over FE should be able to clarify this. The VMH displays high interconnectivity with hypothalamic and diencephalic structures playing a central role in homeostasis by regulating mating, feeding, aggression, and metabolism[75,78]. Previous research showed that excitatory activity in the VMHdm increases defensive responses through "one-to-many" wiring configurations[74,79]. Moreover, a strong activation in the VMH leads to persistent activity and persistent defensive behaviors that rely on neurotransmitter release and recurrent excitatory networks[45]. Notably, a study that used a model of foot shocks as a stressor in males found that MeA-VMH synapses were potentiated after the shocks and related to stress-induced increases in aggression[80]. Our findings are in line with these studies and suggest that the VMHdm in female mice may have undergone synaptic potentiation after IMO and promoted an aversive internal state that facilitated coping with threatening stimuli but to the detriment of FE.

We speculate that the neuronal ensembles representing sensory and neuroendocrine aspects of the acute stress response were altered by the chemogenetic inhibition of MeA to VMHdm neurons. The increase in c-Fos+ neurons in the MeA after chemogenetic inhibition may be secondary to these neurons being embedded in intra-amygdala inhibitory circuits or feedback loops within brain-wide stress-response circuits. Both, the amygdala ensembles, and intra-amygdala inhibitory circuits are crucial for fear learning and fear extinction[81–83]. In our results, we observed that the manipulation of the MeA to VMHdm circuit influenced brain-wide neuronal activity in a larger threat detection system. This suggests that the information processed by the MeA and relayed to the VMHdm or other structures is crucial for processing sensory and neuroendocrine aspects of trauma. In addition, the specific inhibition of the MeA to VMHdm circuit resulted in short-term changes in PACAP dynamics after trauma. Higher PACAP levels in the MeA and VMHdm of control animals shortly after IMO were related to the appearance of a FE-deficient post-traumatic behavioral phenotype. Also, there is evidence demonstrating that PACAP signaling can increase glutamatergic function through NMDA-dependent mechanisms in the VMH[84,85]. These findings suggest a glutamatergic role in the VMHdm after exposure to traumatic stress that is relevant in our model. Future studies may delineate if trauma-induced changes in VMH function are dependent on this mechanism.

Classical FE studies have shown that amygdala–hippocampal–prefrontal circuits are the main drivers of FE, but stress exposure can promote structural and functional changes[42,86]. These FE circuits are embedded into a larger defensive circuitry which ultimately executes appropriate behaviors in the face of a threat. The VMH is capable of promoting aversive internal states and is well-positioned to mediate the integration of stress and fear-related behaviors through its direct connections with the BLA, BNST, PAG, or the paraventricular thalamus[74,87–89]. Interestingly, some evidence shows a role for the VMH in humans for the induction of panic attacks[90]. Given that most of these results come from studies that have used male rodents, they should be carefully considered when translating findings to female rodents or humans. In our paradigm, we were mainly engaging the VMHdm and the defensive parts of the amygdala which are reported to work similarly in males and females[74,91]. However, some subnuclei in the MeA and VMH are sex-dimorphic, influenced by sex hormones, and regulate reproductive and defensive responses to conspecifics[77,92]. Future studies should consider the type of stressor used when studying sex differences. In humans, traumatic stress secondary to interpersonal violence or sexual abuse has a large pathogenic potential and is one of the most common sources of trauma in the population[93–95]. In sum, our results shed light on the role of PACAP-PAC1R in VMH for acute stress processing in female mice and show that the MeA to VMHdm circuit regulates PACAP short-term dynamics after an intense acute stressor and the appearance of a FE-deficient post-traumatic behavioral phenotype.

These findings come along with limitations. In the mouse study we only selected the two most representative and classically studied phases of the estrous cycle: proestrus (high hormones), metestrus (low hormones), it remains to be determined if these findings extend to each phase of the estrous cycle. The chemogenetic manipulation we used was successful for rescuing trauma-induced impairments in FE and PACAP regulation, although it was not specific for PACAP neurons.

In our study of women who had experienced trauma, we attempted to determine the menstrual phase at time of trauma carefully as possible, using a method based on the last menstrual period date and reported cycle regularity. However, for this calculation, we had to exclude a proportion of young women with irregular cycles or women in and near menopause. A further limitation is that we had a high proportion of missing information regarding the history of previous trauma exposure which could have affected the analyses on the moderation of menstrual cycle*trauma history. Unlike our results on post-traumatic symptom severity, our analyses on PTSD diagnosis and menstrual cycle phase rely on a small sample size and should be interpreted accordingly. In humans, experiencing violence and aggression have a high potential for leading to mental health conditions such as PTSD. Future studies focused on testing specifically for memories directly related to the trauma may inform if the menstrual cycle stage should be taken into account for people receiving extinction-based therapies. Importantly, these studies must ensure that the participants are not retraumatized by their participation.

In sum, we demonstrate that the menstrual cycle stage at the moment of traumatic stress is not associated with the severity in post-traumatic symptoms. Furthermore, in a mouse model, stage of estrous cycle at time of modeling trauma does not affect PTSD-like behavior. We also found that the PACAP-PAC1R system is important for fear extinction in highly traumatized women and a PTSD-like model in female mice. Additionally, our study discovered that high levels of PACAP in the VMHdm lead to vulnerability to the negative consequences of stress whereas low levels of PACAP may result in resilience. Growing evidence points at the PACAP-PAC1R system as crucial for stress processing, with its dysfunction being associated with worse outcomes in women. Further studies may potentially find therapeutic interventions involving the PACAP-PAC1R system. However, one of the problems, from a pharmacology point of view, is the widespread expression of the PACAP-PAC1R system. We have previously proposed that targeting specific neuronal populations that colocalize with PACAP in emotional areas of the brain could offer advances to the current understanding of fear and stress processing[37].

## Methods

### Mice

*Ethics and biosecurity protocols for mice experiments*. Ethics protocols were approved for the experiments in mice ref. CEEAH 3603 and biosecurity protocols 345-16 and 407-17 by the Committee of Ethics of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya. Institutional Animal Care and Use Committee (IACUC) protocol for the mice was McLean-based, and 2017N000228. Experiments were carried out following the European Communities Council Directive (2010-63-UE) and Spanish legislation (RD 53/2013).

*Animals*. Behavioral experiments were performed on adult (8–12-week-old) wild-type C57BL/6J male and female mice (Charles River, Spain). Antibody-validation experiments from Supplementary Fig. 4a, b were performed on 6-month-old $PACAP^{flox/flox}$ females. Mice undergoing surgery were housed in pairs, the rest in groups of four and kept under standard conditions of temperature (22 ± 1 °C) and humidity (~40%) in a 12 h light/12 h dark schedule (lights on at 8:00 h), with ad libitum food and water intake[96]. Behavioral procedures and pharmacological manipulations began early in the light phase of the cycle. Male and female mice were housed separately in the same room.

*Immobilization stress in mice*. IMO was performed as previously described[18]. Briefly, IMO was conducted in a room separate from the housing and behavioral paradigms. Each animal was immobilized by gently restraining its four limbs in a prone position to metal arms attached to a wooden board for 2 h (Panlab-Harvard apparatus, Spain). All animals in the same cage received the same treatment—either IMO or compensatory handling. Handling lasted ~5 min per mouse and consisted of letting the animal walk on top of their home cage and in the hands of the experimenter wearing latex gloves. After stress exposure or compensatory handling, animals were returned to their home cage where they remained undisturbed until fear training. FC testing started 6 days after IMO/ handling. Of note, 2 days before IMO/handling exposure mice were habituated to the context of the FC chambers for 5 min.

*Cued-fear conditioning and fear extinction in mice*. FC and FE procedures were carried out with a computerized StartFear system (Panlab-Harvard, Spain) as previously described[26]. Tones and shocks were delivered and controlled using Freezing v1.3.04 software (Panlab-Harvard apparatus, Spain). The fear chamber consisted of a black methacrylate box with a transparent front door (25 × 25 × 25 cm) inside a sound-attenuating chamber (67 × 53 × 55 cm). The same boxes were used for FC and FE. Animals were habituated to the fear chambers for 5 min/day for two consecutive days before IMO. During the cue-dependent FC, mice were placed in the fear chambers for 5 min and then received five trials of a tone (30 s, 6 kHz, 75 dB), as the conditioned stimulus (CS), that co-terminated with a foot-shock (1 s, 0.3 mA), as the unconditioned stimulus (US). The intertrial interval (ITI) was 3 min, and 3 additional min followed the last trial. The FE tests were performed 4 times in consecutive days (FE1, FE2, FE3, FE4) starting 24 h after FC. For FE, mice were placed in the fear chambers for 5 min and then exposed to 15 trials of the 30 s CS tone alone (cued fear) with a 30 s of ITI interval. An additional 30 s interval followed the last trial of FE. Freezing behavior, a rodent´s natural response to fear defined as the absence of movement except for respiration, was scored by a high sensitivity weight transducer system located at the bottom of the experimental chambers which records and analyses the signal generated by the movement of the animal. Freezing was scored when mice remained immobile for more than 1 s and episodes were averaged in 30 s slots using Freezing v1.3.04 software (Panlab-Harvard apparatus, Spain). Different contexts were used for FC and FE tests. Habituation and FC context consisted of a yellow light source (~10 lux), a grid floor of 25 bars (3 mm Ø and 10 mm between bars), the background noise of 60 dB produced by a ventilation fan, and a solution of ethanol 70% as odor. FE context consisted of a red-light source (~10 lux), a gray plexiglass floor covering the bars, no background noise, and CR36 (bronopol 0.26%, benzalkonium chloride 0.08%, and isopropyl alcohol 41%) (José Collado, Spain) as odor. The chambers were carefully cleaned with soapy water before and after each mouse. Also, different routes were used to transport them from their home cages to the testing room on FC and FE days.

*Vaginal smear cytologies in mice*. The estrous cycle was monitored in a cohort of naturally cycling female mice subjected to IMO or compensatory handling. Vaginal cytologies were adapted from known protocols[97], and performed at 9:00–11:00 h. Subsequent behavioral procedures or sample collection took place after 2 h. Vaginal smear cytologies were obtained for 8–12 days before testing to ensure estrous cycle regularity. A vaginal lavage was performed with 10 µl of standard NaCl 0.9% solution using five flushes at the entrance of the vaginal aperture. The vaginal lavage was then placed on an adhesion slide, dried, stained with 0.1% Cresyl violet Acetate (Sigma-Aldrich, Spain), washed twice in distilled water, and read in brightfield microscopy at ×10 or ×20. The estrous cycle phase was determined by the proportions of cells in the cytology (cornified, nucleated, leukocytes) as described in ref. [67]. Proestrus is characterized by a predominance of nucleated cells with few leukocytes or cornified cells; estrous consists mostly of cornified cells; metestrus has a predominance of cornified cells with leukocytes and diestrus is characterized by a predominance of leukocytes. Estrous phase monitoring was carried out from 3 to 9 consecutive days (until the desired phase was identified). Only animals in proestrus or metestrus were selected.

*Reverse transcription and quantitative PCR*. A separate cohort of female mice was sacrificed 30 min after fear expression (FE1). Brains were immediately fresh frozen on isopentane cooled with dry ice and stored at −80 °C. Bilateral tissue punches from the prefrontal cortex, amygdala, hypothalamus, and periaqueductal gray were performed based on the Mouse Brain Atlas by Paxinos[98] and individually stored. Total RNA was isolated and purified from the tissue with Maxwell RSC simplyRNA Tissue Kit #AS1390 (Promega Biotech Ibérica, Spain)[99]. Gene expression changes were detected by relative quantitative reverse transcription PCR FAST 7500 (Applied Biosystems, USA). cDNA was obtained by reverse transcription using the High-Capacity cDNA Reverse Transcription Kit #4368814 (Thermo Fisher, Spain) according to the manufacturer's instructions. TaqMan assays (Applied Biosystems, USA) were used to quantify the expression of *Adcyap1* Mm00437433_m1 (#4331182) and *Adcyap1r1* Mm01326453_m1 (#4331182) normalized to mouse *Gapdh* Mm99999915_g1 (#4352932E, glyceraldehyde-3-phosphate dehydrogenase). Statistics are computed with ddCT, graphics are represented as fold changes obtained with the $2^{-\Delta\Delta Ct}$ method[37].

*Immunohistochemistry*. General procedure: A group of female mice was transcardially perfused with 4% paraformaldehyde (PFA) (Casa Álvarez, Spain) 90 min after the last behavioral procedure. Brains were post-fixed in PFA for 24 h (4 °C) after which they were rinsed (3 ×15 min) with Sorenson's sodium phosphate buffer (PB) 0.1 M consisting of 10.9 g/l sodium phosphate dibasic (Sigma-Aldrich, Spain), 3.2 g/l sodium phosphate monobasic (Sigma-Aldrich, Spain). Further, they were transferred into 30% Sucrose (Sigma-Aldrich, Spain) in PB for 48–72 h at 4 °C. After this, brains were frozen in Isopentane (Sigma-Aldrich, Spain) cooled with dry ice, and stored at −80 °C until sectioning. In all, 30-µm coronal sections were cut on a cryostat and stored at −20 °C in an anti-freeze solution (30% ethylene glycol (Sigma-Aldrich, Spain), 20% glycerol (Sigma-Aldrich, Spain) in 0.1 M phosphate buffer)). For immunohistochemistry, free-floating slices were rinsed thoroughly in KPBS and then incubated for 60 min at room temperature in blocking buffer (5% Donkey Serum and 0.4% Triton-X (Sigma-Aldrich, Spain) in potassium phosphate-buffered saline (KPBS)). Primary antibodies were diluted in 0.4% Triton-X in KPBS and incubated in agitation at 4 °C overnight. The next day, after three rinses in KPBS, slices were incubated in agitation with secondary antibodies in 0.4% Triton-X in KPBS at room temperature for 2 h. After incubation, slides were rinsed in KPBS and DAPI 1:10,000 (Sigma-Aldrich, Spain) to stain cell nuclei. Immediately after, they were mounted on glass slides and coated with Fluoromount-G Mounting Medium (Thermo Fisher, Spain). The following primary antibodies were used: anti-c-Fos (1:500, ab6167) (Abcam, UK), anti-FosB [83B1138] (1:2000, ab11959) (Abcam, UK), anti-PACAP-38 (1:1000, T-4469) (Peninsula Laboratories, USA), anti-PAC1R (1:100, ab54980) (Abcam, UK) and mouse monoclonal anti-VGLUT2 [8G9.2] (1:300, ab79157) (Abcam, UK). As secondary antibodies: AlexaFluor-647 donkey anti-sheep (1:500, A-21448) (Fisher Scientific, Spain), Rhodamine-Red donkey anti-mouse (1:500, 715-295-151) (Jackson Antibodies, UK), and AlexaFluor-488 donkey anti-rabbit (1:1000, 711-545-152) (Jackson Antibodies, UK). For PACAP antibody-validation free-floating slices between bregma −1.1 and −1.8 mm were incubated with primary antibody anti-PACAP-38 (1:5000, T-4469) (Peninsula lab, USA) and secondary antibody Alexa fluor 647 donkey anti-rabbit (1:1000, A-31573) (Invitrogen, USA).

Image acquisition: The following structures were analyzed according to the Mouse Brain Atlas (Paxinos and Franklin, 2001): basolateral amygdala (BLA), bed

nucleus of the stria terminalis anterolateral (BSTal), dorsal CA1 hippocampal region (dCA1), dorsal CA3 hippocampal region (dCA3), central amygdala (CeA), cingulate cortex (Cg), dorsal dentate gyrus hippocampal region (dDG), ventral dentate gyrus hippocampal region (pDG), dorsomedial hypothalamus (DMH), infralimbic cortex (IL), insular cortex (Ins), medial amygdala (MeA), dorsolateral periaqueductal gray (PAGdl), ventrolateral periaqueductal gray (PAGvl), prelimbic cortex (Prl), ventral lateral septum (vLS), ventromedial hypothalamus dorsomedial nucleus (VMHdm). Immunofluorescence images were captured using a Zeiss LSM 700 Confocal microscope and Zen2010 software (Zeiss, Spain) under a dry 20× magnification lens. Five to six images per structure per animal were acquired using four different laser lines (405, 488, 555, and 639 nm). Two-dimensional overview pictures were obtained for the colocalization experiment (8 bit, 1024 × 1024 pixels). For PACAP quantification experiments three-dimensional *Z*-stacks were acquired in 1.5 µm thin optical planes along six planes (9 µm). Two-dimensional overview pictures were obtained by *z* projection (16 bit, 1024 × 1024 pixels) and further analyzed blind to the experimental group in Image J software (Fiji v.1.53c, Rasband W, NIH, USA). For the mosaics, *Z*-stacks (1.51 µm/interval) were acquired using a Leica SP5 confocal microscope (Leica, Spain) with a dry ×20 magnification lens at ×0.5 zoom and processed using the software Leica Application Suite Advance Fluorescence (LAS AF) Version 2.7.3.9727 (Leica Microsystems, Germany). A two-dimensional overview picture was obtained from the maximum intensity projection made in Image J software. For PACAP antibody validation, images were taken using the Echo Revolve fluorescent microscope (Echo, USA), merged using Adobe photoshop, and processed with Image J.

Quantification: For analyses, the average background signal for each picture and channel was manually measured and subtracted, a Gaussian filter (sigma 1) was used to improve signal detection, thresholds were used to segment the images and to create binary masks, masks were visually inspected and corrected. For colocalization experiments, a DAPI mask was overlaid on each channel's mask with the LungJ plugin and colocalized cells were automatically counted using the analyze particles plugin. Automatic and manual counts were compared in a subset of pictures obtaining a high correlation ($r > 0.95$). Labeled neurons were calculated as the average number of cells per $mm^2$. Colocalization counts were normalized to DAPI for each picture and averaged for each mouse. Colocalization results are shown in relation to controls. For PACAP signal quantification, masks for each channel were overlaid to measure the integrated density (intDen = area × mean intensity). Protein levels were normalized to DAPI for each picture and averaged for each mouse.

*Surgeries and viral vector microinjection*. Ovariectomy surgery: Ovariectomy surgeries were performed in a subset of females to use their serum to make the standard curve preparation for the ELISA estradiol kit. Mice were anesthetized with 4% isoflurane for induction, and 2–3% for maintenance, in oxygen at a constant rate of 1.5 l/min. After skin shave and disinfection with EtOH 95% and iodine povidone 10%, ovariectomies were performed making a bilateral incision on the back of the animal, 1 cm lateral to the midline and right over the back limbs line. Adipose tissue was extracted, and the ovary was localized and isolated making a knot with sterile absorbable suture thread (Centauro, Spain) around the oviduct. The ovary was extirpated and the adipose tissue, containing the rest of the oviduct, was returned to the abdominal cavity. The muscle was sewed with sterile absorbable thread and the skin was sewed using sterile silk suture (Centauro, Spain). Mice remained undisturbed for 8 weeks until trunk blood was collected to avoid any effect of previous estradiol.

Stereotaxic surgery: Mice were anesthetized with 4% isoflurane for induction, and 2–3% for maintenance, in oxygen at a constant rate of 1.5 l/min. Animals were placed in the stereotaxic frame (Harvard-Panlab, Spain) and aligned in the antero-posterior (AP) and lateromedial (LM) planes. The coordinates for AAV injection in relation to bregma according to Mouse Brain Atlas by Paxinos and Franklin[98] were (MeA) AP −1.3 mm, ML ±2.0 mm, DV −5.25 mm; (VMHdm) AP −1.3 mm, ML ±0.25 mm, DV −5.25 mm. Animals received bilateral intra-MeA or intra-VMHdm injections of 0.3 µl of virus/side at a constant rate of 0.1 µl/min with a microinfusion pump (Harvard Apparatus, USA). For antibody-validation studies, unilateral infusions of 0.3 µl of virus/side into the (VMHdm) AP −1.3 mm, ML 0.3 mm, DV −5.25 mm and (MeA) AP −1.3 mm, ML 2.0 mm, DV −5.25 mm. The matched contralateral region within the same subject was used as the control condition. After infusions, the needle was left in place for an additional period of 10 min to allow the fluid to diffuse and to prevent reflux, then it was slowly withdrawn during 10 additional min. The skin was closed using a 3-0 Polyamide suture.

Viral vectors: The viral vectors used were AAV-hM4Di-DREADD (AAV8-hSyn-DIO-hM4D(Gi)-mCherry, 1.21E + 13 gc/ml), AAV-control-DREADD (AAV8-hSyn-DIO-mCherry, 1.19E + 13 gc/ml) from Viral Vector Production Unit of Universitat Autònoma de Barcelona and AAV-retrograde-Cre-EBFP (AAVrg pmSyn1-EBFP-Cre; $6 \times 10^{12}$ vg/ml) from Addgene (viral prep # 51507-AAVrg). To inhibit MeA neurons projecting to the VMHdm, mice received bilateral injections of 0.3 µl of AAV-retrograde-Cre-EBFP into the VMHdm and bilateral injections of 0.3 µl of AAV-hM4Di-DREADD or AAV-control-DREADD in the MeA. To inhibit VMHdm neurons projecting to MeA, mice were injected bilaterally with 0.3 µl of AAV-retrograde-Cre-EBFP into the MeA and 0.3 µl of AAV-hM4Di-DREADD or AAV-control-DREADD in the VMHdm. For antibody validation, animals received unilateral 0.3 µl of AAV9-CMV-eGFP-Cre (105545-AAV9) (Addgene, USA).

Injection verification: To detect AAV8-hSyn-DIO-mCherry or AAV8-hSyn-DIO-mCherry serial coronal sections containing the MeA and VMHdm were visualized directly under a Zeiss LSM 700 confocal microscope and images from representative slices were obtained with a dry ×20 objective at 0.5 zoom. Injection sites were histologically verified by overlapping to standard stereotaxic plates[98], direct visualization of needle trajectory and detection of EBFP or mCherry expression. Only animals with at least an ipsilateral pair of injections circumscribed to both the MeA and the VMHdm were included in the study.

*Drugs*. For the activation of the inhibitory designer receptors exclusively activated by designer drugs, animals received an intraperitoneal injection of clozapine N-oxide (CNO) (Tocris, UK) in 0.5% DMSO at 1 mg/kg[37].

*Steroid determination*. For steroid determination trunk blood was collected after decapitation and allowed to clot at 4 °C. Then it was centrifuged (8000×*g*, 15 min, 4 °C) to collect serum and stored at −80 °C until analyzed. Serum levels of testosterone, progesterone, dehydrocorticosterone, and deoxycorticosterone were determined based on previously reported papers[100,101]. Briefly, 20 µl of serum were mixed with 20 µl of labeled internal standard solution. After proteins precipitation with 100 µl of acetonitrile, samples were centrifuged (3000 × *g*, 5 min) and the supernatant was transferred to a clean tube. The mixture was vortexed and transferred to a clean tube. Serum samples underwent a liquid-liquid extraction by the addition of 1 ml of NaCl (saturated solution) and 4 mL of ethyl acetate. Extracts were centrifuged (3000 × *g*, 5 min) and the organic layer was transferred into a clean tube and dried under a nitrogen stream. Dried extracts were reconstituted with 100 µl of methanol and 10 µl were injected into the LC-MS/MS system consisting of an Acquity UPLC system coupled to a triple quadrupole (TQS Micro) mass spectrometer. Steroid detection was performed by selected reaction monitoring (SRM) including two transitions for each analyte. The most specific one was selected for the quantification. Quantification was performed by an external calibration approach using the TargetLynx module of the MassLynx software (Thermo Fisher, Spain). Estradiol was measured with the ELISA kit ES180S-100 (Calbiotech, USA). For standard curve preparation, serum from ovariectomized mice was used adding known concentrations of estradiol (Sigma-Aldrich, Spain): 0, 3, 10, 30, 100, and 300 pg/ml, using denaturalized EtOH (Casa Álvarez, Spain) as a vector for estradiol. Kit instructions were followed as stated, samples were loaded in duplicates and absorbance was read at 450 nm with the microplate reader Varioskan Lux (Thermo Fisher, Spain) controlled with Skanit for microplates v6.0 software (Thermo Fisher, Spain). The average of duplicates was used as estradiol determinations for each sample.

## Humans

*Participants and ethics statement*. For Fig. 6, Supplementary Fig. 8, and Supplementary Tables 1–3, participants ($n = 293$) were recruited from the Emergency Department in the Hospital Clínic de Barcelona, Spain, as part of a specialized protocol that provides first-aid care in sexually abused women. All women attending the ED in the Hospital Clínic de Barcelona were offered medical, psychiatric, and psychosocial counseling regardless of their participation in the study. The first contact occurred in the ED with a quick, multidisciplinary, and coordinated assessment that ensured to avoid revictimization. Throughout this process, the women were accompanied, received medical treatment, and were offered the opportunity to report the offense to the police. At discharge, in-person follow-ups were carried out monthly with telephonic assessments between them. The follow-up aimed to detect and treat any possible effects of trauma, promote treatment adherence, and ensure the person's return to normal functioning. Inclusion criteria for the study were willingness to participate, being older than 18 years, and being a victim of a recent sexual assault. Exclusion criteria were language barriers, being a tourist, having mental disabilities or active psychosis, and women ceasing to participate in the study before post-traumatic symptom assessment at the 3rd week post trauma. For the final cohort, we additionally excluded women with missing last menstrual period dates, women in menopause, and women with irregular menstrual cycles for a final cohort of 170 participants. Participants did not receive payment as the study was part of their treatment plan. All women signed the informed consent approved by the Ethics Committee of Clinical Research in the Hospital Clínic de Barcelona.

For Fig. 7, Supplementary Fig. 9 and Supplementary Table 4, participants were part of the Grady Trauma Project, which is a group of investigators studying civilian trauma based at Grady Memorial Hospital and Emory University School of Medicine in Atlanta, Georgia. The project focuses on PTSD and the clinical and physiological implications of trauma exposure. Participants ($n = 55$ CC genotype, $n = 65$ G allele carriers) were recruited from the General Medical Clínic, Primary Care, Diabetes, Sickle Cell and OB/GYN, and Main Pharmacy Waiting Rooms at Grady Healthcare System, a publicly funded, not-for-profit healthcare system that serves the low-income and homeless population in downtown Atlanta, a city of ~4 million people. Participants of different races and ethnic groups were included, both males and females. Participants were approached in the waiting room for screening by trained study staff and the study procedures were explained to potential participants[60]. Inclusion criteria were willingness to participate and the

ability to understand the informed consent form. Exclusion criteria included: participants with active symptoms of mania, schizophrenia, or other psychoses; participants with current prominent suicidal ideation, intoxicated participants. Participants with special medical conditions that can contribute significantly to psychiatric symptoms, including hypo- or hyperthyroidism, systemic lupus erythematosus, cirrhosis, and dementia. For the startle testing, participants with a positive urine drug test were excluded. All participants were screened for hearing impairments with an audiometer (Grason-Stadler, Model GS1710), and the ones not able to detect tones of 30 dB(A)SPL at frequencies ranging from 250 to 4000 Hz were not included in the study. We balanced three primary concerns in making our decisions about how much and when to pay participants: (1) the primary demand on participants in this study is time and we wanted to compensate participants adequately for this time; (2) the amount of money is not intended to be coercive; (3) we wanted to include participation from subjects across a full range of socioeconomic status. Participants were informed that the research is voluntary; that they were free to stop their participation at any time; participation did not impact their other services in any way; and that they would be paid for their participation. The consent form described the study procedures, including saliva collection for DNA, interviews about trauma history and symptoms, and the acoustic startle procedure. The potential risks and discomforts were also described and reviewed with participants before they signed the informed consent form. We anticipated that some women could experience distress after the FC task. As established in the research protocol of the Grady Trauma Project, all women were able to openly discuss any possible distress with the interviewers, encouraged to address it with their clinicians, and were given phone numbers for further contact. The interviewers were able to identify women in need of psychiatric treatment and made direct referrals to the Grady Healthcare System and other local treatment providers. Women with previously undiagnosed mental disorders, not resulting from their participation in the study, received medical treatment following the Grady Hospital mission regardless of insurance status or ability to pay. Patients were paid $60 in the FPS experiment. All participants provided written consent approved by the Emory University Institutional Review Board and Grady Research Oversight Committee.

*Assessments in clinical populations*. For Fig. 6, Supplementary Fig. 8, and Supplementary Tables 1–3, women received an initial assessment in the emergency department where clinical and demographic variables were collected. Post-traumatic symptoms were evaluated at around 3 weeks post trauma using the Acute Stress Disorder Interview (ASDI), which is a structured clinical interview with 19 items that assess dissociative, re-experiencing, avoidance, and hyperarousal symptoms[51]. The ASDI has good internal consistency ($r = 0.90$), and excellent sensitivity (91%) and specificity (93%) compared to independent clinical diagnosis[51]. Women received periodic clinical follow-ups for up to a year after sexual assault by a trained mental healthcare professional that monitored their disease course and detected the appearance of any mental disorder. In Supplementary Table 1, PTSD diagnoses were obtained in a retrospective analysis of clinical data, many women did not attend medical follow-ups before 1 year and therefore were accounted as unknown for PTSD diagnosis. Reasons to abandon the study included personal decision, migration, symptom attenuation or inability to reach them by phone.

For Fig. 7, Supplementary Fig. 9 and Supplementary Table 4, PTSD symptoms were assessed using the Modified PTSD Symptom Scale (PSS), which is a psychometrically valid self-report scale that assesses post-traumatic symptom severity during the last two weeks with 17 items[102]. The Childhood trauma questionnaire (CTQ) is a self-report tool used to assess three types of childhood abuse: sexual, physical, and emotional. For this study, the brief version of the CTQ was used with high reliability for this population[62]. Previous studies have shown its validity, internal consistency, and stability over time[103].

*Procedures in clinical populations*. Menstrual cycle phase: For the allocation of women into menstrual cycle phases at the moment of trauma, we used the last menstrual period date, which was collected in the first visit to the ED. We used the mean age in our sample (30 ± 10.4 years) to calculate the duration of each menstrual cycle phase according to data from studies with large samples[104]. The segmentation into menstrual cycle phases was based on the hormonal profiles of interest (e.g., estradiol (E2) and progesterone (P4) levels). We discarded the cases with missing last menstrual period dates, irregular menstrual cycles, cycles lengths >35 days, or women using hormonal contraception. Women that were sexually abused between the initial or 9th day of the menstrual cycle (low levels of E2 and P4) were allocated into the early follicular phase; between the 10th and 17th day (high E2, low P4) in the late follicular phase; between the 18th and 27th day (high E2, high P4) in the mid-luteal phase and between 28th and 30th day (a decline of both, E2 and P4) in the late-luteal phase. For analyses, mid-luteal and late-luteal were collapsed into the luteal phase.

Grady Trauma Project: Research participants were approached in the waiting rooms of a public hospital's primary care clinics while either waiting for their medical appointments or while waiting with others who were scheduled for medical appointments. After the subjects provided written informed consent, they participated in a verbal interview and donated saliva for genetic analyses[105]. DNA was extracted from saliva in Oragene collection vials (DNA Genotek Inc, Canada) using the DNAdvance kit (Beckman Coulter Genomics, USA). Genotyping was performed using the Omni-Quad 1 M Bead Chip. Quality control was performed by using the Psychiatric Genomics Consortium PTSD Workgroup guidelines[106]. Briefly, SNPweights software17 was used to assign ancestry. PLINK[107] was used to perform quality control analyses such as SNPs that had a call rate <95% were removed; individuals with missingness with >2%, heterozygosity >0.2, and failing sex checks were removed; variants with significant deviation from Hardy–Weinberg proportions ($P < 1 \times 10^{-6}$ in controls and $P < 1 \times 10^{-10}$ in PTSD cases) were also excluded. Principal components for ancestry were calculated according to the PGC guidelines in each separate ancestry group[106]. We extracted the *ADCYAP1R1* variant, rs2267735, from the genome-wide data. rs2267735's HWE was $P = 0.4$ and $P = 0.8$ in EA and AA, respectively.

Fear-potentiated startle protocol: The protocol consisted of a fear acquisition and a FE phase. During fear acquisition, participants were initially presented to the CSs without any reinforcement to habituate them. Immediately after, they completed FC with the presentation of 36 trials divided into three blocks (12 trials per block) with four trials of each type (CS+, reinforced conditioned stimulus; CS-, non-reinforced conditioned stimulus; NA, a 40 ms, 108 dB noise probe alone). CSs consisted of colored shapes shown on a computer screen for 6 s. As in previous studies[59], a 250 ms, 140 p.s.i. air blast directed to the larynx was used as the US. Intertrial intervals were randomized between 9 and 22 s. After the fear acquisition phase, participants were allowed to rest for 10 min before starting fear extinction phase. The Fear Extinction consisted of 72 trials divided into six blocks (12 trials per block) with four trials of each type (a non-reinforced CS+, CS−, and NA). None of the CS+ presentations were paired with an air blast US during the Fear Extinction phase.

Data acquisition and data analysis: Data were acquired with the electromyography (EMG) module of BIOPAC MP150 for Windows (Biopac Systems, Inc., USA). Data were then filtered, rectified, and smoothed using MindWare software suite (MindWare Technologies, Ltd., USA) and exported for statistical analyses. EMG signal was sampled at a frequency of 1 kHz and filtered with a low-frequency cutoff at 28 Hz and a high-frequency cutoff at 500 Hz. The measure of the acoustic startle response was captured as the maximum amplitude of the eyeblink muscle contraction appearing between 20 and 200 ms after the presentation of the startle probe. All amplitude values were used without coding low responses as zero. For data analyses, fear-potentiated startle was calculated using a Difference Score (startle magnitude to NA – startle magnitude to CS+/CS−) in each conditioning block. Fear Extinction was divided into early (first 4 CS+ trials) and late (last 4 CS+ trials). In an analysis of covariance (ANCOVA), fear extinction was analyzed as a repeated measures variable (two levels: early, late), and age and genotype as between-groups variables.

*Data acquisition and analyses*. Supplementary Data 1: Statistical analyses were performed with IBM SPSS 25.0. Repeated-measures ANOVA, one or two-way ANOVAs or Student's *t* test (two-tailed) were used. Analysis of covariance (ANCOVA) was used for controlling for the effect of covariates over the dependent variable. Data were visualized, normality and equality of variance assumptions were calculated. For related sample analyses, CS trials in FC or mean FE per session were used as within-subject factors and treatment as between-subject factor. Greenhouse-Geisser correction was used when necessary. In all cases, if a statistically significant interaction was found, two-tailed pairwise comparisons were calculated. Bonferroni post hoc analyses were performed if necessary. Mann–Whitney's *U* tests for independent samples or Kruskal Wallis' H test were used for samples not meeting criteria for parametric analyses. Wald's $\chi^2$ with pairwise comparisons was used in Generalized Linear Model for multifactorial analyses that did not meet normality or homoscedasticity. Additional individual comparisons were performed when appropriate. Pearson correlation coefficients were used for quantitative variables. The significance of association between categorical variables with <5 observations per group was calculated with the Fisher's exact test. Data are presented as mean ± or +SEM and statistical significance was set at $P < 0.05$. Detection of outliers was performed using Grubb's test and removed from analyses when necessary. A summary of the statistical analyses is shown in Extended Data. Procedural and schematics were created with BioRender.com. All graphs presented in the figures were designed using GraphPad Prism version 7.0 for Windows (GraphPad Software, USA).

**Reporting summary**. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability

No large-scale datasets were generated in this study. Source data are deposited in the Digital Repository of Documents from the Universitat Autónoma de Barcelona under accession code https://doi.org/10.5565/ddd.uab.cat/259560. Source data are provided with this paper.

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## Author contributions

This study was planned and conceptualized by R.A., E.R.V., and Á.F. Á.F. and E.R.V. made the behavioral experiments and vaginal cytologies. E.S. and Á.F. made the qPCR. Á.F. and E.R.V. made and analyzed IF. A.F. made estradiol ELISA and helped with ovariectomies. E.R.V. made stereotaxic surgeries and DREADD experiments. A.G. and O.P. contributed to the mass spectrometry data. K.J.R., A.L., T.J., and S.N. contributed the FPS data. K.J.R., E.L.N., P.D., and R.R. contributed the antibody validation. A.T., A.R., L.L.G.E., and E.R.V. collected and analyzed clinical data. The paper was written by R.A. and E.R.V. and commented on and discussed by all authors.

## Competing interests

The authors declare no competing interests.

## Additional information

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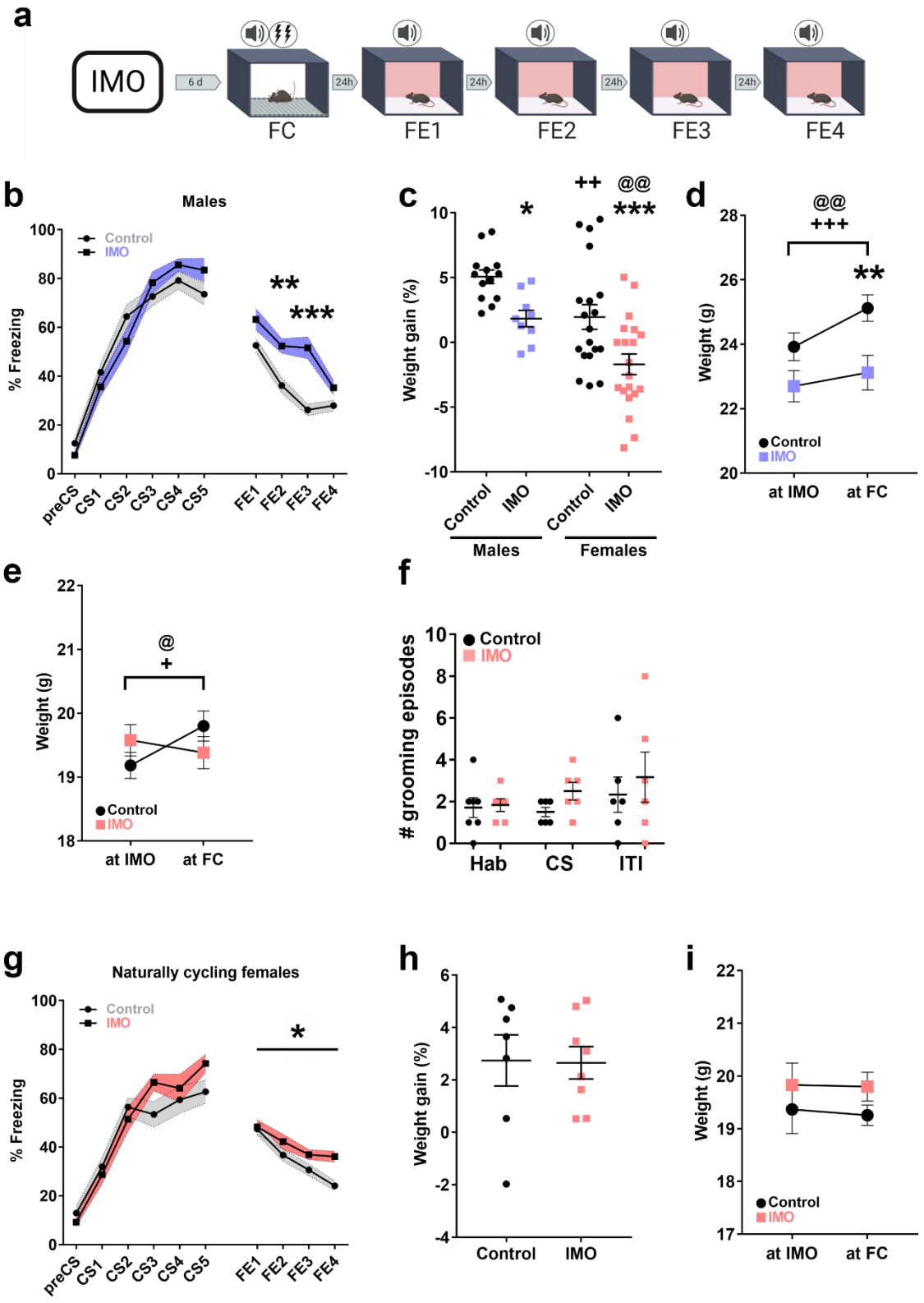
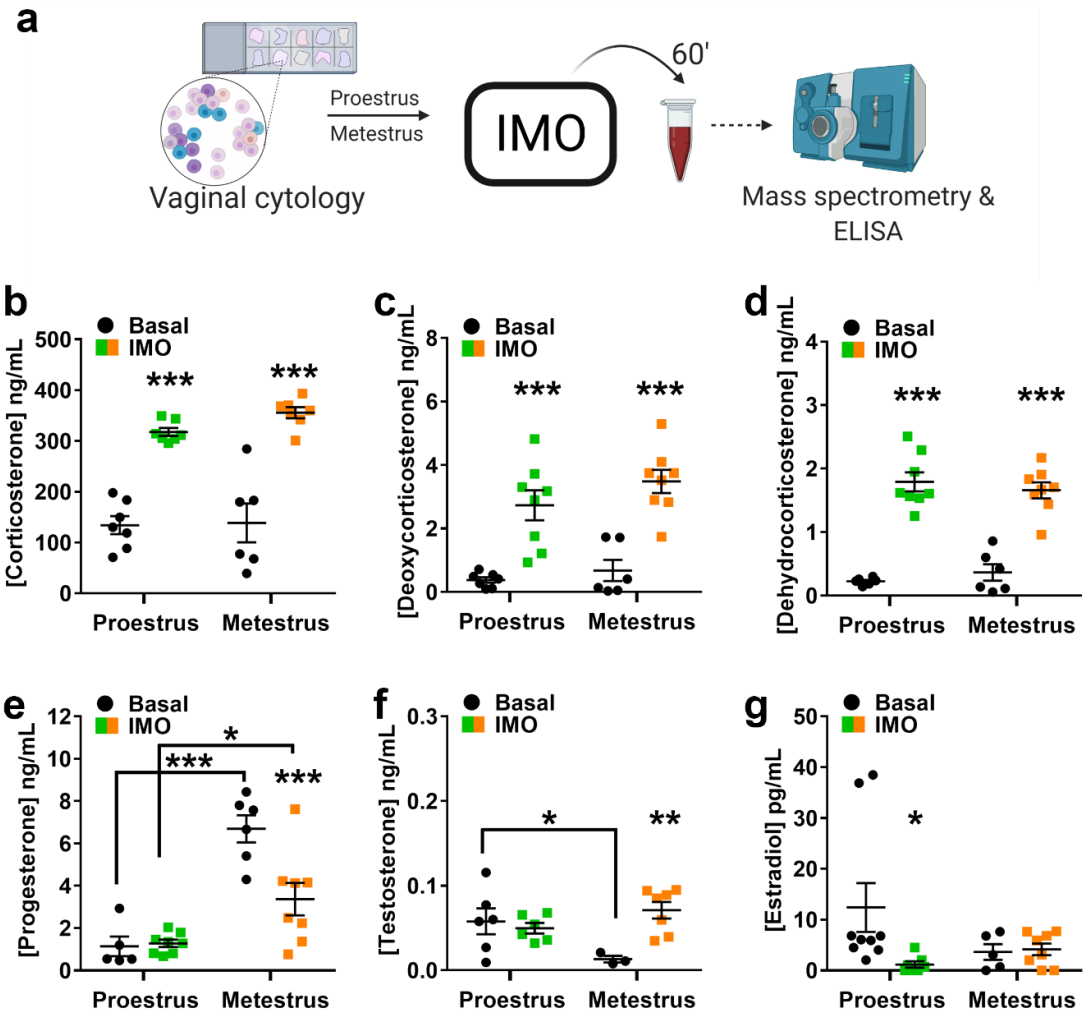
**Supplementary Information for the article:**

PACAP-PAC1R modulates fear extinction via the ventromedial hypothalamus. Velasco ER, Florido A, Flores Á, Senabre E, Gomez-Gomez A, Torres A, Roca A, Norrholm S, Newman EL, Das P, Ross RA, Lori A, Pozo OJ, Ressler KJ, Garcia-Esteve LL, Jovanovic T, Andero R.

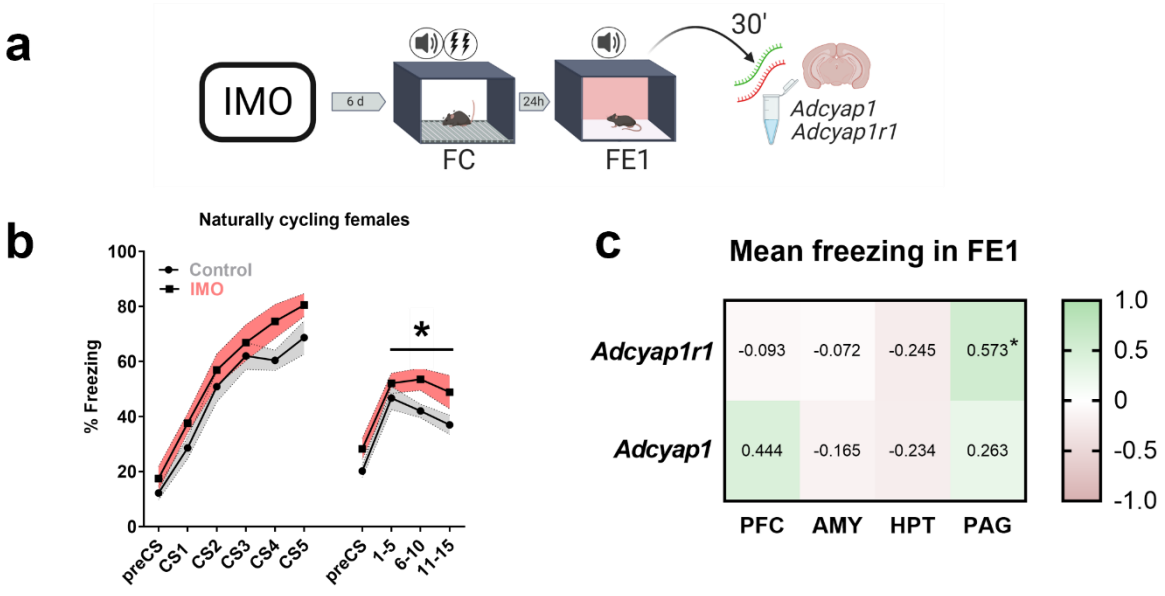
## Supplementary Figures



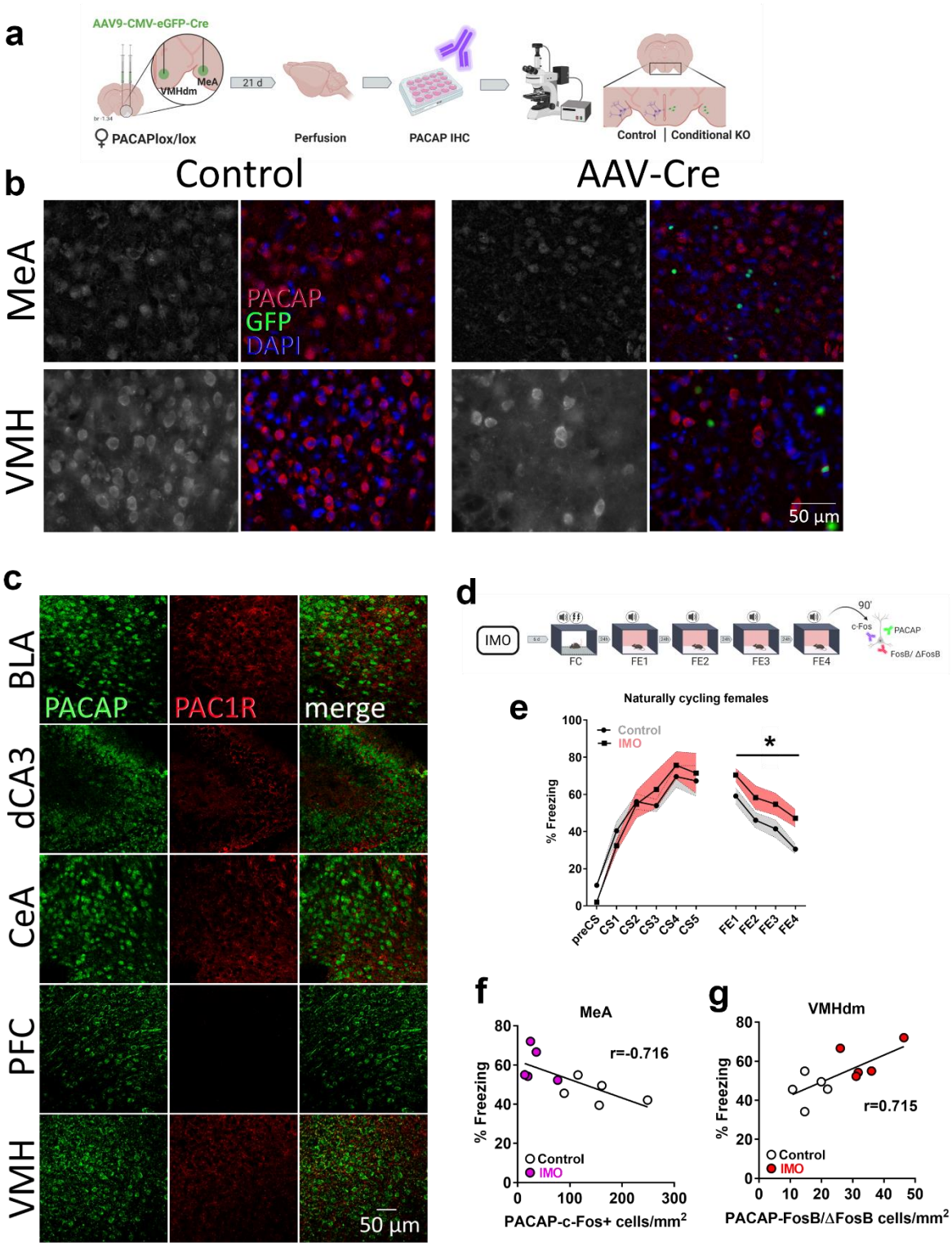
**Supplementary Figure 1: IMO induces behavioral and physiological changes in males and female mice. a,** Representation of the methods used to assess fear learning and fear extinction after IMO. **b,** Fear learning and fear extinction in males exposed to IMO vs compensatory handling (control: n=13, IMO=9) (FE session*stress: p=0.003). **c,** Comparison of the % weight gain of males and females (males control: n=13, IMO=9; females control: n=19, IMO: n=20) (sex*stress p<0.001). **d,** Impact of IMO on males' weight between IMO t1 and FC t2 (control: n=13, IMO=9) (time*stress p=0.001). **e,** Impact of IMO on females' weight between IMO t1 and FC t2 (control: n=19, IMO: n=20) (time*stress p=0.005). **f,** Impact of IMO on number of grooming episodes in females at habituation (Hab), tone presentations (CS), or inter-trial intervals (ITI) during FE1 session (n=6 per group). **g,** Fear learning and fear extinction in females with estrous cycle monitorization exposed to IMO vs compensatory handling, results are shown clustered by treatment (n=14 per group) (p=0.023). **h,** Impact of IMO on % weight gain in females with cycle monitorization shown clustered by treatment (control: n=7, IMO: n=8). **i,** Impact of IMO on cycle monitored females' weight between IMO t1 and FC t2 (control: n=7, IMO: n=8). Data are expressed as mean ± SEM. *p≤0.05, ** p≤0.01, *** p≤0.001. * IMO vs Control, @ IMO vs IMO, + Control vs Control. Asterisks above a line indicate significant main effect stress in repeated-measures ANOVA. In **b, d, e, g, i,** repeated measures ANOVA was used main effect stress, main effect FE session or CS or time, and FE session*stress or CS*stress or time*stress interactions were used. In **c,** a GzLM was conducted. In **f, h,** two-tailed t-tests were used. CS: conditioned stimulus, FC: fear conditioning, FE: fear extinction session, Hab: habituation, IMO: immobilization stress, ITI: intertrial interval.




**Supplementary Figure 2: Regulation of HPA and HPG hormones shortly after IMO in female mice during proestrus or metestrus. a,** Methods used for cycle monitorization, stress procedure, and hormonal analyses. HPA hormonal regulation of **b,** Corticosterone (proestrus: n=7 per group; metestrus basal: n=6, IMO: n=7) (stress*estrus: $p<0.001$), **c,** Deoxycorticosterone (proestrus basal: n=7, IMO: n=8; metestrus basal: n=6, IMO: n=8) (stress*estrus: $p<0.001$), and **d,** Dehydrocorticosterone (proestrus basal: n=6, IMO: n=8; metestrus basal: n=6, IMO: n=8) (stress*estrus: $p<0.001$) 60 min after IMO in proestrus and metestrus females (n=5-8 per group). HPG hormonal regulation of **e,** Progesterone (proestrus basal: n=5, IMO: n=8; metestrus basal: n=6, IMO: n=8) (stress*estrus: $p<0.001$), **f,** Testosterone (proestrus n=6 per group; metestrus basal: n=3, IMO: n=7) (stress*estrus: $p=0.004$), and **g,** Estradiol (proestrus basal: n=9, IMO: n=7; metestrus basal: n=5, IMO: n=8) (stress*estrus: $p=0.024$), 60 min after IMO in proestrus and metestrus females. Data are expressed as mean ± SEM.*$p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$ asterisks above a line for specific comparisons. Data was analyzed with Generalized Linear Model (Wald's $\chi^2$) with pairwise comparisons between groups and Bonferroni corrections. HPA: Hypothalamic-pituitary-adrenal axis, HPG: Hypothalamic-pituitary-gonadal axis, IMO: immobilization stress.

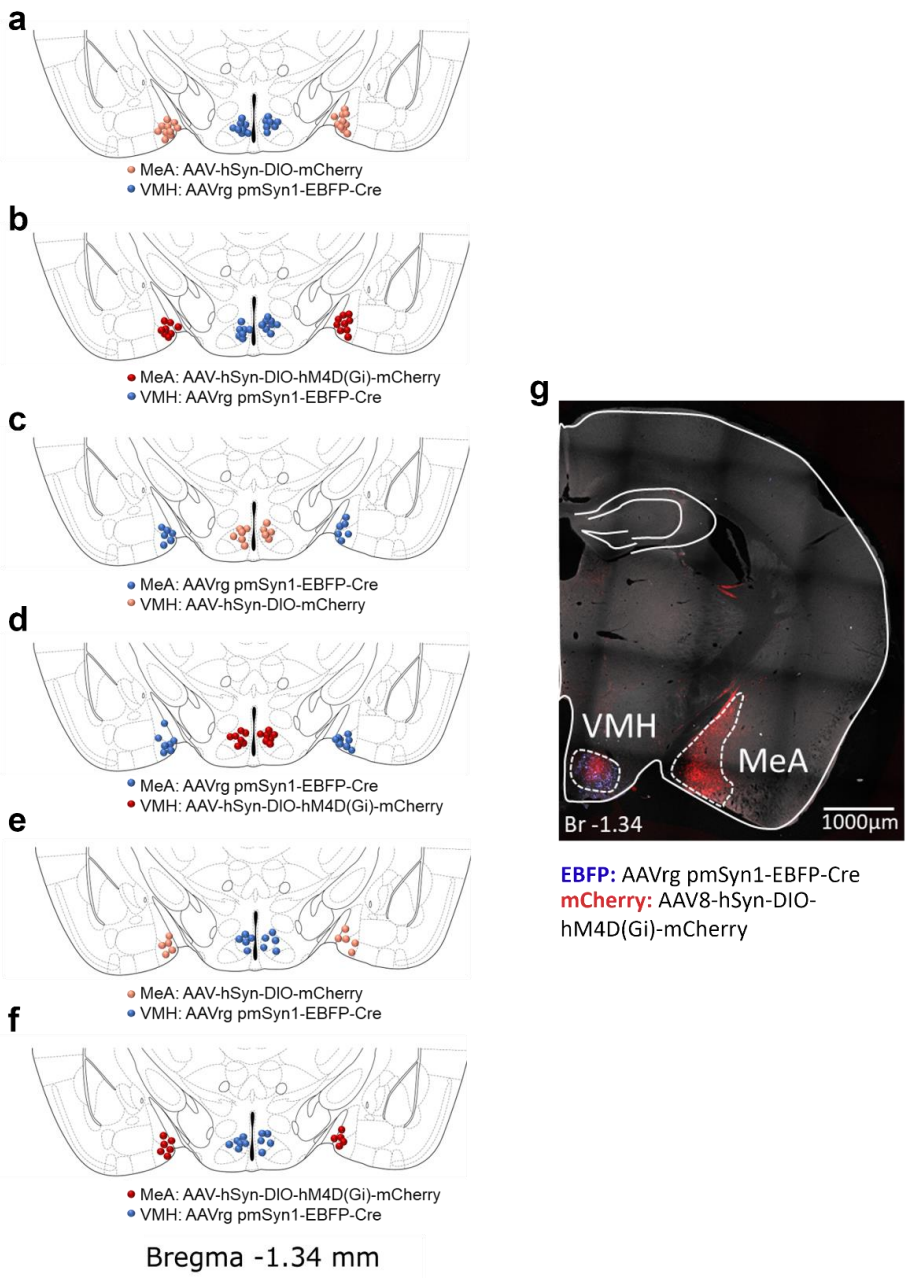



**Supplementary Figure 3: Early FE in traumatized female mice and its association with *Adcyap1-Adcyap1r1* regulation. a,** Representation of the methods used for the behavioral evaluation. **b,** Fear learning and early fear extinction in females exposed to IMO vs compensatory handling (control: n=12, IMO: n=9) (p=0.042). **c,** Correlation of mean freezing scores during FE1 with *Adcyap1* or *Adcyap1r1* mRNA levels. AMY- amygdala, HPT- hypothalamus, PAG- periaqueductal gray, PFC- prefrontal cortex. R values are shown, * signals significant results, magnitude of the correlation is depicted by a color heatmap. In **b,** main effect stress, main effect FE session or CS, and FE session*stress or CS*stress interactions were analyzed using repeated-measures ANOVA. Data are expressed as mean ± SEM. *p≤0.05. Asterisks above a line indicate main effect stress in repeated-measures ANOVA. In **c,** the Pearson correlation coefficient was used. CS: conditioned stimulus, FC: fear conditioning, FE: fear extinction, IMO: immobilization stress.

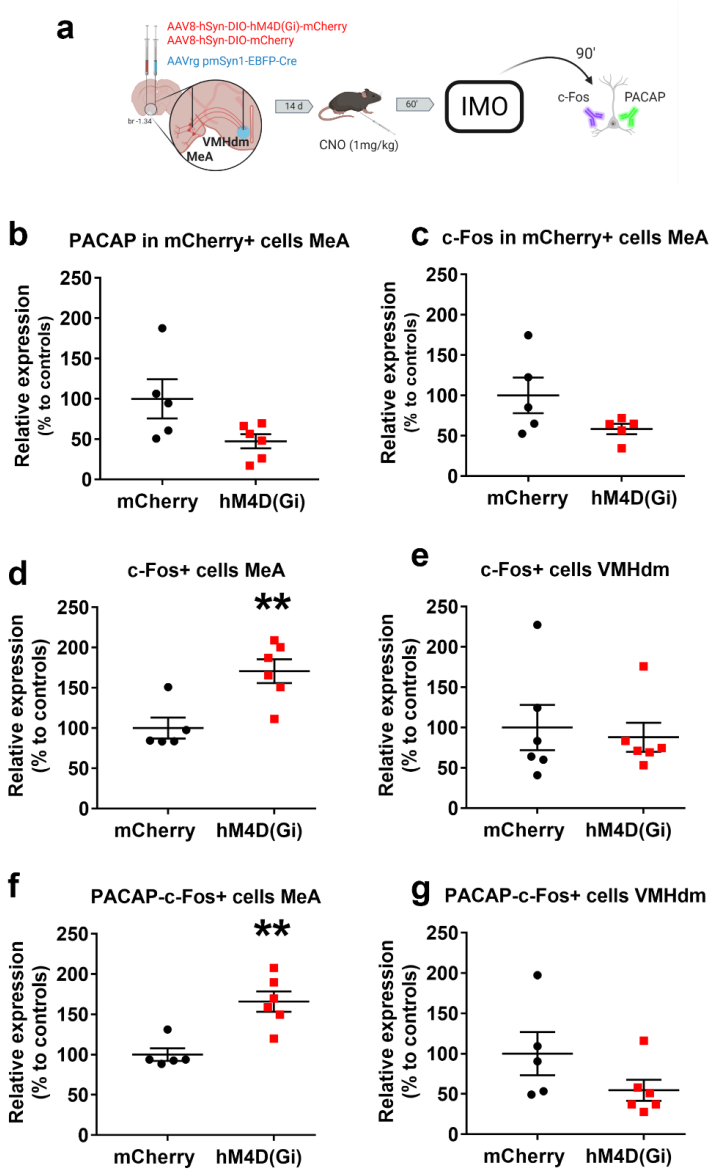



**Supplementary Figure 4: PACAP antibody validation and relation of PACAP neuronal activity to freezing levels in mice. a,** PACAP$^{lox/lox}$ mice were injected unilaterally with AAV9-CMV-eGFP cre into the MeA and VMHdm to conditionally delete *Adcyap1* on one side. IHC slices were prepared with T-4469 anti-PACAP antibody. The contralateral uninfected side served

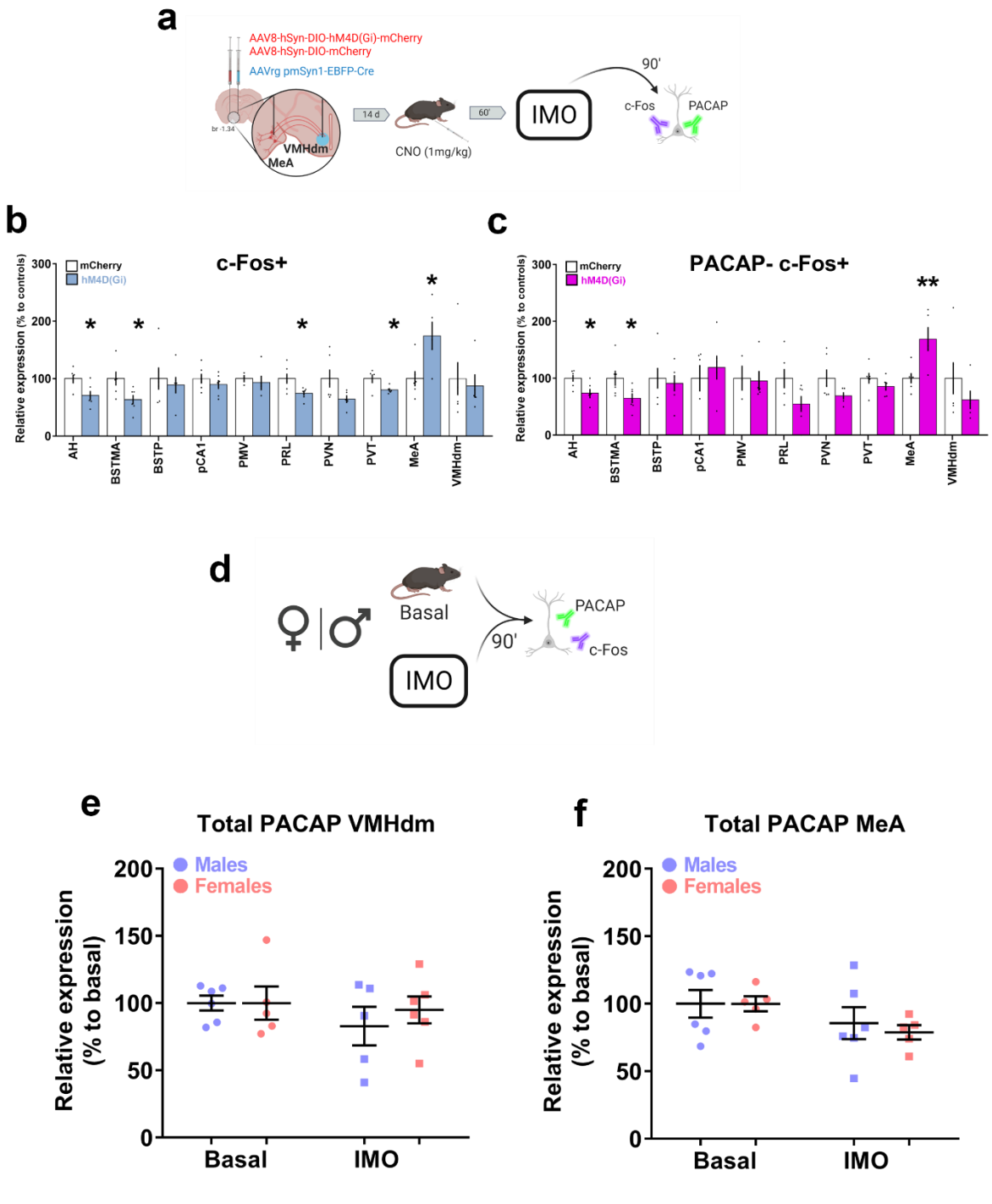
as the control for PACAP expression. **b,** Intact PACAP immunolabels observed in MeA and VMHdm of non-injected side (Control) and fewer PACAP immunolabeled cells are observed in the MeA and VMHdm of the injected conditional KO side (AAV-Cre). **c,** Immunofluorescence study showing PACAP and PAC1R expression in areas of interest. BLA- basolateral amygdala, dCA3- dorsal CA3, CeA- central amygdala, PFC- prefrontal cortex, VMH- ventromedial hypothalamus. Scale bar 50μm. **d,** Schematic representation of the behavioral and immunohistochemical methods. **e,** Fear learning and fear extinction in females exposed to IMO vs compensatory handling (n=6 per group) ($p=0.024$). **f,** Correlation of % freezing in all FE sessions with PACAP-c-Fos+ cells/$mm^2$ in the MeA (n=5 per group). **g,** Correlation of % freezing in all FE sessions with PACAP-FosB/ΔFosB+ cells/$mm^2$ in the VMHdm (control: n=4, IMO: n=6). Data are expressed as mean ± SEM. *$p \leq 0.05$. In **e,** main effect stress, main effect FE session or CS, and FE session*stress or CS*stress interactions were analyzed using repeated-measures ANOVA. Asterisks above a line indicate significant main effect stress in repeated-measures ANOVA. In **f, g,** Pearson correlation coefficients were used. BLA: basolateral amygdala, CMV: cytomegalovirus, dCA3: dorsal CA3, CeA: central amygdala, GFP: green fluorescent protein, IHC: immunohistochemistry study, IMO: immobilization stress, MEA: medial amygdala, PFC: prefrontal cortex, VMH: ventromedial hypothalamus.




**Supplementary Figure 5: Injection verification sites. a-f,** Injections sites were verified for each animal under 20x objective magnification by direct visualization of needle trajectory and detection of EBFP or mCherry. Dots indicate the lowest point of the injector tip. Only animals with a prominent expression of both markers that were circumscribed to the area of interest and with at least an ipsilateral pair of accurate injections were included. Atlas images were adapted from Franklin & Paxinos (2007). **g,** Representative image of the viral vector reporter expression in the injection sites**.** EBFP or mCherry are visualized as blue or red respectively. Scale bar 1000 µm. AAV: adeno-associated virus, AAVrg: retrograde adeno-associated virus, Br: bregma, EBFP: Enhanced blue fluorescent protein, MeA: medial amygdala, VMH: ventromedial hypothalamus.

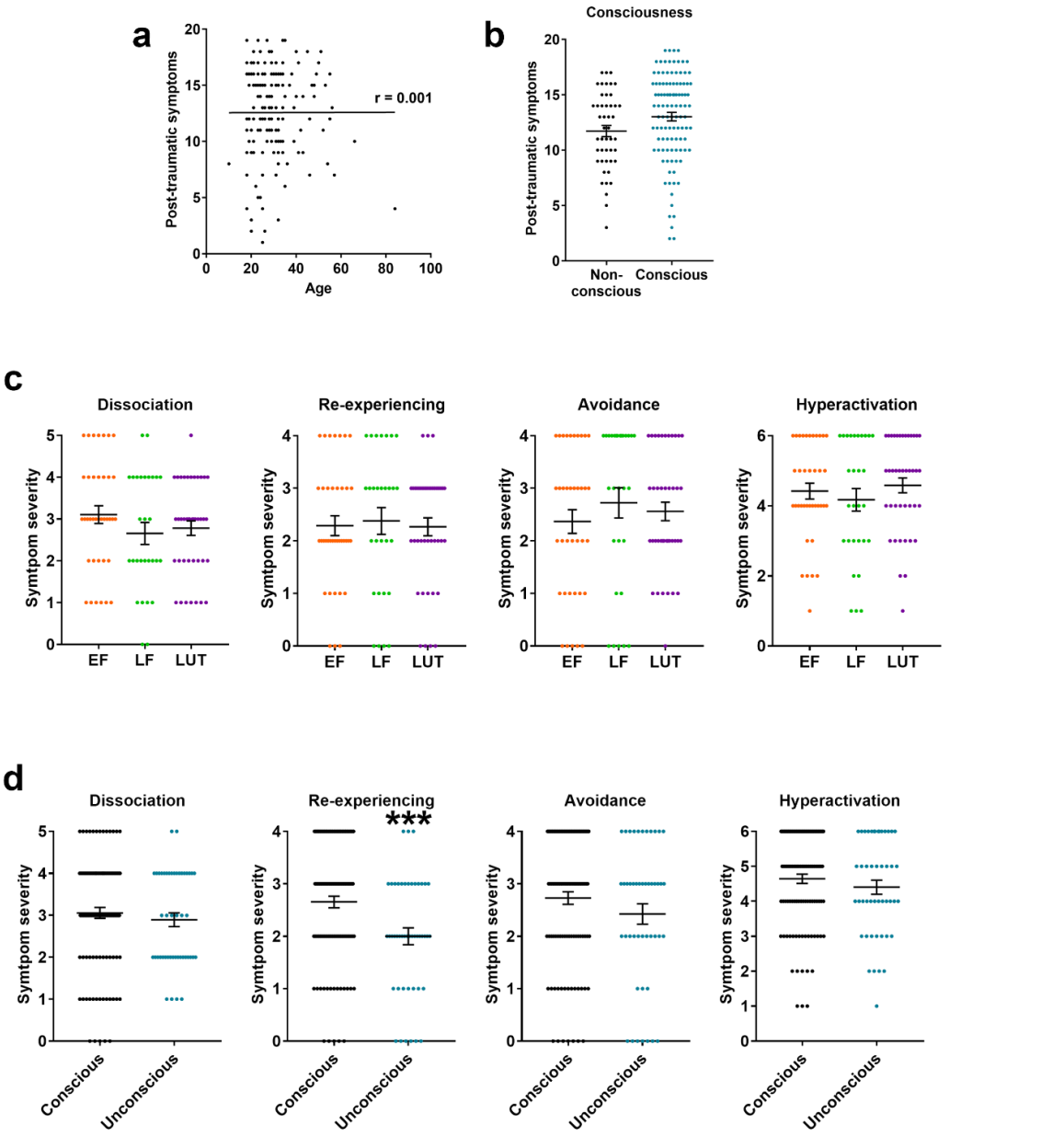



**Supplementary Figure 6: Chemogenetic inhibition of the MeA to VMHdm circuit effects over PACAP levels and c-Fos expression shortly after IMO. a,** Methods used to assess PACAP and c-Fos expression shortly after IMO (90 min) in animals with inhibited MeA to VMHdm circuitry (hM4D(Gi)) vs controls (mCherry). **b,** PACAP expression in mCherry+ cells in MeA (mCherry: n=5, hM4D(Gi): n=6). **c,** c-Fos-mCherry colocalization in MeA (mCherry: n=5, hM4D(Gi): n=6) (n=5 per group). **d, e,** Chemogenetic inhibition effect over c-Fos expression in the MeA (mCherry: n=5, hM4D(Gi): n=6) and VMHdm (n=6 per group) (MeA: p=0.009). **f, g,** Chemogenetic inhibition effect over PACAP-c-Fos+ expression in MeA(mCherry: n=5, hM4D(Gi): n=6) and VMHdm (mCherry: n=5, hM4D(Gi): n=6) (MeA: p=0.009). Results are presented as relative expression to controls (mCherry) (n=5-6 per group). Data are expressed as mean ± SEM. **p≤0.01. Two-tailed t-tests or Mann Whitney U tests were used. CNO: clozapine N-oxide, MeA: medial amygdala, VMHdm: ventromedial hypothalamus dorsomedial nucleus.

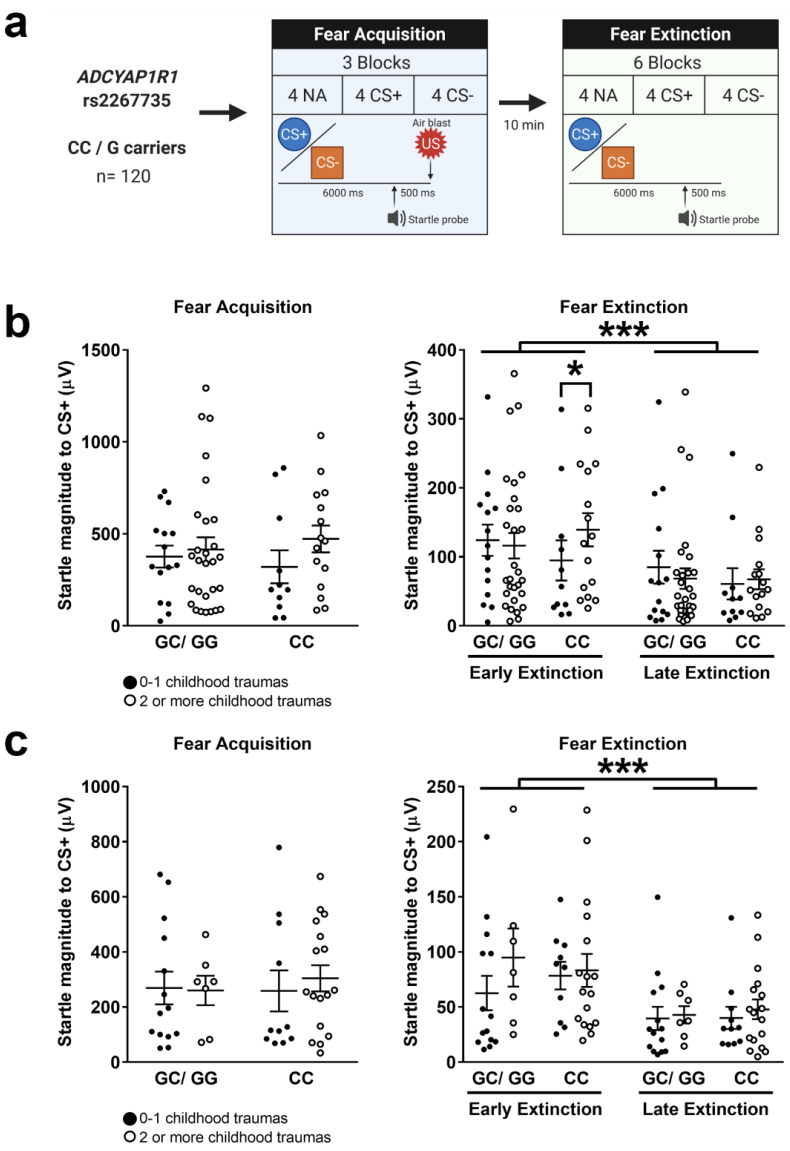



**Supplementary Figure 7: Effect of the chemogenetic inhibition of MeA to VMHdm projections on other brain areas and sex differences in PACAP at basal levels and after IMO exposure. a,** Methods used to assess PACAP and c-Fos expression shortly after IMO (90 min) in animals with inhibited MeA to VMHdm circuitry (hM4D(Gi)) vs controls (mCherry). **b,** Quantification of c-Fos+ neurons (AH, BSTMA, PRL n=6 per group; MeA and PVT mCherry: n=6, hM4D(Gi): n=5) (AH: p=0.020, BSTMA: p=0.027, PRL: p=0.028, PVT: p=0.035,MeA:

p=0.021). **c,** Quantification of colocalization of PACAP-c-Fos+ (AH, BSTMA n=6 per group; MeA mCherry: n=6, hM4D(Gi): n=5) (AH: p=0.020, BSTMA: p=0.045, MeA: p=0.009). **d,** Methods used to assess PACAP levels in basal vs IMO males and females . **e,** Relative expression of PACAP between males and females in the VMHdm (males basal: n=6, IMO: n=5; females basal: n=5, IMO: n=6) and **f,** MeA (males basal: n=6, IMO: n=6; females basal: n=6, IMO: n=5). Results are presented as relative expression to controls, in **b, c** control is animals injected with mCherry; in **e, f,** controls are basal animals (n=5-6 per group). Data are expressed as mean ± SEM. *$p<0.05$, **$p\leq0.01$. For **b,** and **c,** two-tailed t-tests or Mann Whitney U tests were used. For **e,** and **f,** two-Way ANOVA was used. AH: Anterior Hypothalamus, BSTMA: Bed Nucleus of the Stria Terminalis Medial Anterior part, BSTP: Bed Nucleus of the Stria Terminalis Posterior part, CNO: clozapine N-oxide, IMO: immobilization stress, pCA1: Caudal CA1, PMV: Premamilary Nucleus Ventral part, PRL: Prelimbic Cortex, PVN: Paraventricular Nucleus of the Hypothalamus, PVT: Paraventricular Thalamus, MeA: Medial Amygdala, VMHdm: Ventromedial Hypothalamus dorsomedial nucleus.




**Supplementary Figure 8: Relation of posttraumatic symptom and sub-symptom severity with age, consciousness status and menstrual cycle phase. a,** Correlation of age with posttraumatic symptom scores at 3 weeks post-trauma (n=170). **b,** Consciousness status and posttraumatic symptom severity at 3 weeks post-trauma (conscious: n=107, non-conscious: n=47). **c,** Sub-symptom scores (dissociation, re-experiencing, avoidance, hyperactivation) in women allocated in the distinct menstrual cycle phases at trauma (EF: n=38, LF: n=29, LUT: n=47). **d,** Sub-symptom scores in women that were conscious or non-conscious at trauma (conscious: n=107, unconscious: n=47) (Re-experiencing: p=0.001). Data are expressed as mean ± SEM. ***p≤0.001. In **a,** a Pearson correlation coefficient was used. In **b,** a two-tailed t-test was used. In **c,** Kruskal Wallis' H was used. In **d,** Mann-Whitney U Tests were used. EF: Early follicular, LF: Late follicular, LUT: luteal phase.




**Supplementary Figure 9: *ADCYAP1R1* rs2267735 genotype during early FE in traumatized individuals. a,** Methods used to assess of the relation of rs2267735 genotype with FE (n=71,49). **b, c,** Fear potentiated startle magnitude to CS+ in **b,** cycling women (≤40 years old) (n=71) (Early vs late extinction: p<0.001, genotype*childhood trauma: p=0.030) or **c,** women (>40 years old) (n=49) (Early vs late extinction: p<0.001) with CC or GC/GG genotypes during Fear Acquisition, Early FE and Late FE. Data are expressed as mean ± SEM. *p<0.05, ***p≤0.001. Asterisks above a line indicate significant differences between Early and Late FE as assessed with Mann Whitney U tests. None of the CS+ presentations were paired with an US during the FE phase. CS+: reinforced conditioned stimulus, CS-: non-reinforced conditioned stimulus, NA: noise probe alone, US: unconditioned stimulus.

## Supplementary Tables

### Supplementary Table 1. Study participant demographics from Hospital Clínic cohort

| | |
|---|---|
| Age (years), mean ± SD | 30.2 ± 10.4 |
| Pre-existing psychiatric disorders, n (%) | 38 (22.4) |
| Anxiety or depressive disorders | 25 (14.7) |
| Reproductive stage, n (%) | |
| Menopause | 16 (9.4) |
| Reproductive years | 154 (90.6) |
| Hormonal contraceptive use, n (%) | 25 (16.4) |
| Menstrual cycle, n (%) | |
| Irregular cycles | 13 (10.2) |
| Regular cycles (phases) | 114 (89.7) |
| Early follicular | 38 (33.3) |
| Late follicular | 29 (25.4) |
| Luteal | 47 (41.3) |
| **Trauma- related** | |
| Consciousness status, n (%) | |
| Conscious | 107 (62.9) |
| Non-conscious | 47 (27.6) |
| Unknown | 16 (9.5) |
| Meets ASD diagnosis at 3 weeks, n (%) | |
| Yes | 107 (62.9) |
| No | 63 (37.0) |
| PTSD diagnosis 1-year follow-up, n (%) | |
| PTSD | 16 (9.4) |
| No PTSD | 72 (42.3) |
| Unknown | 69 (40.6) |
| Comorbidities diagnosis 1-year follow-up, n (%) | |
| Anxiety disorder | 12 (7.1) |
| Depressive disorder | 19 (11.2) |
| Other | 2 (1.2) |
| No comorbidities | 70 (41.1) |
| Unknow | 67 (39.4) |
| ASDI total score, mean ± SD | 12.64 ± 4.09 |
| Dissociation symptoms (0-5) | 2.98 ± 1.31 |
| Re-experiencing symptoms (0-4) | 2.41 ± 1.16 |
| Avoidance symptoms (0-4) | 2.64 ± 1.27 |
| Hyperarousal symptoms (0-6) | 4.55 ± 1.42 |

Data are presented as mean ± SD or number of subjects and percentage, n (%). ASD: acute stress disorder, ASDI: Acute Stress Disorder Interview, PTSD: posttraumatic stress disorder.

**Supplementary Table 2. Proportion of women with history of trauma exposure from Hospital Clínic cohort**

| Type | Yes, n (%) | No, n (%) | Missing data, n (%) |
|---|---|---|---|
| CSA – Childhood sexual abuse | 56 (32.9) | 81 (47.6) | 33 (19.4) |
| CEA – Childhood emotional abuse | 44 (25.8) | 91 (53.5) | 35 (20.5) |
| CPA – Childhood physical abuse | 55 (32.3) | 82 (48.2) | 33 (19.4) |
| PSAA – Previous SA in adulthood | 30 (17.6) | 113 (66.4) | 27 (15.8) |
| PAA – Previous aggression in adulthood (non-SA) | 35 (20.5) | 128 (75.2) | 7 (4.11) |

Data are presented as number of subjects and percentage, n (%). SA: sexual abuse.

**Supplementary Table 3. Follow-up (in days) of sexually abused women from Hospital Clínic cohort**

| Follow-up (days) | Mean ± SD | Median (IQR) |
|---|---|---|
| First contact | 10.5 ± 7.6 | 9 (12) |
| First follow-up after ASDI | 45.2 ± 11.1 | 43 (16) |
| Days after aggression to PTSD diagnosis | 168.2 ± 217.3 | 75 (174) |
| Last follow-up in all women | 221.7 ± 289.4 | 98 (237) |
| Last follow-up in women with PTSD | 548.0 ± 415.0 | 491 (749) |

Data are presented as mean ± SD or median (range). ASDI: Acute Stress Disorder Interview, IQR- interquartile range, PTSD: posttraumatic stress disorder.

**Supplementary Table 4. Study participant demographics from Grady Trauma Project cohort**

| | Mean ± SD |
|---|---|
| Age, mean (range) | 38.59 (18-62) |
| Race, n (%) | |
| African American | 117 (97.5) |
| Other | 3 (2.5) |
| CTQ, mean ± SD | 46.11 ± 19.83 |
| TEI | 15.49 ± 12.82 |
| PSS Total | 15.49 ± 12.82 |
| PSS Re-experiencing, mean ± SD | 3.72 ± 3.65 |
| PSS Avoidance | 6.41 ± 6.04 |
| PSS Hyperarousal | 5.38 ± 4.50 |
| Genotype G carrier, n (%) | 65 (54.2) |

Data are presented as mean ± SD or number of subjects and percentage (n (%)). CTQ: childhood trauma questionnaire, PSS: PTSD symptom scale, TEI: traumatic events inventory.

# Article 2

# Sex differences in fear extinction

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# Sex differences in fear extinction

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ABSTRACT

Despite the exponential increase in fear research during the last years, few studies have included female subjects in their design. The need to include females arises from the knowledge gap of mechanistic processes underlying the behavioral and neural differences observed in fear extinction. Moreover, the exact contribution of sex and hormones in relation to learning and behavior is still largely unknown. Insights from this field could be beneficial as fear-related disorders are twice as prevalent in women compared to men. Here, we review an up-to-date summary of animal and human studies in adulthood that report sex differences in fear extinction from a structural and functional approach. Furthermore, we describe how these factors could contribute to the observed sex differences in fear extinction during normal and pathological conditions.

## 1. Introduction

### 1.1. Fear learning: Fear conditioning and fear extinction processes

Fear is a neurological process aimed at executing rapid behaviors to preserve ones individual integrity in the presence of a threat (LeDoux, 2014). All fear responses can be categorized as innate or acquired, and those acquired are usually added to the behavioral repertoire of the organism through classical (or Pavlovian) conditioning (Davis, 1994). Classical conditioning is the procedure by which, after a series of repeated matches of a safe stimulus, known as neutral stimulus, with a naturally threatening one, the unconditioned stimulus (US), the neutral stimulus becomes a conditioned stimulus (CS) with the ability to elicit a fearful response, conditioned response (CR) (Pavlov, 1927). The CR can be specific to the tone, showing an effective discrimination, or might occur in presence of different tones, demonstrating generalization of the CR (Dunsmoor et al., 2009; Dunsmoor et al., 2011; Huckleberry et al., 2016). Other stimuli can be used as a CS rather than a tone, as in the case of contextual fear conditioning (FC). Fear memory consolidation is the process by which recent fearful associations are stabilized through the storage in a long-term reservoir (Dudai et al., 2015). Typically, this process is carried out in three temporally segregated stages with some overlap between them (Dudai et al., 2015): first, during synaptic consolidation, synaptic buttons strengthen in response to $Ca2+$ dependent pathways activation after CS presentations (Long Term Potentiation, LTP) (Lynch, 2004; McKenzie and Eichenbaum, 2011). Concurrently, in system consolidation other structures (such as the extended amygdala or the medial prefrontal cortex (mPFC)) are recruited permitting the long term recall of fear memories (Winocur and Moscovitch, 2011). Lastly, fear memories undergo reconsolidation during the recall of the CR and are integrated with new environmental information, permitting the creation of an updated fear memory representation (Schiller et al., 2010).

Fear extinction (FE) refers to the process in which the CRs decline by the successive presentation of a fear-eliciting CS in the absence of an aversive US (Myers and Davis, 2007). This process is also time segregated, and involves the weakening (Long Term Depression, LTD) of previous potentiated synapses, and LTP of other inhibitory pathways (Myers et al., 2006). According to classical LTP studies (Miserendino et al., 1990), FE is mediated by N-methyl-D-aspartate–receptor (NMDA-R) and the signaling action of protein kinases and phosphatases (Michael Davis, 2011; Myers et al., 2006). Furthermore, the extinguished CR might reappear in a different context from that in which FE took place, showing renewal of the CR. Also, the CR might show spontaneous recovery if the animal is presented the CS some time after FE acquisition took place. Moreover, the CR can also be recovered by merely presenting the US some time after extinction, in a process called reinstatement. These three processes (spontaneous recovery, reinstatement and renewal of the CR) highlight that the undergoing processes of FE are aimed towards the acquisition of a new inhibitory learning

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0149-7634/ 

rather than the weakening of a previously potentiated one.

### 1.2. Fear related disorders

In this review we will refer to fear-based disorders as the group of psychiatric conditions that share pathological fear processing and prominent anxiety symptoms as core features in their development, or maintenance (Flores et al., 2018). We will specifically focus on post-traumatic stress disorder (PTSD), phobic disorders and panic disorder. Patients suffering from these conditions show alterations in fear learning that include: a greater conditioning to danger cues, impairments in FE and impairments in inhibitory conditioning to safety signals (Garfinkel et al., 2014; Jovanovic et al., 2012; Jovanovic and Norrholm, 2011; Lissek et al., 2005; Milad et al., 2009a,b; Rougemont-Bücking et al., 2011). In addition, the main psychological treatment available for these patients includes FE procedures (i.e., exposure therapy), thus providing a model with good face validity for exposure therapy (Scheveneels et al., 2016; Vervliet et al., 2013).

Anxiety and fear-based disorders are increasingly recognized as conditions producing a great disease burden and economic impact, also being projected as one of the leading causes of disability and healthcare costs for the next decade (Kessler et al., 2012; Whiteford et al., 2013; Wittchen et al., 2011). Notably, these disorders affect men and women disproportionately. Women are double the risk for panic attacks, specific and social phobias compared to men, and PTSD can reach three times the prevalence in women (de Jonge et al., 2016; Gradus et al., 2017; Steel et al., 2014; Wardenaar et al., 2017). Women are also more likely to have a long disease course, and to present comorbidities (Kessler et al., 2015; McLean et al., 2011; Pigott, 2003). Although the reasons for this biased burden remain largely unknown, they are thought to arise from multiple interactions between physiological, neurobiological, environmental and socio-cultural factors (Kessler et al., 2017; McCutcheon et al., 2010; Olff, 2017; Tolin and Foa, 2006). Due to the high economical and social burdens of fear-related disorders, there is a need to increase research in this field, especially in translational studies, since current treatments work for most, but not all patients. Besides, a considerable proportion of patients drops out of treatment or experiences relapses of symptoms, even after medicial completion (Batelaan et al., 2017; Edlund et al., 2002; Hofmann and Smits, 2008; Imel et al., 2013; Koen and Stein, 2011; Loerinc et al., 2015; Roshanaei-Moghaddam et al., 2011). Research aimed at identifying novel molecular targets and markers of circuit dysfunction will lead to improved interventions, and better quality of life for the people suffering from these disorders.

### 1.3. Sex differences and similarities: a change in the framework

In most mammals, including humans, sex is the biological trait determined genetically by the presence of XX or XY chromosomes. Soon after the XX or XY genotype is set, genes like the *Sry* or *Xist* promote sexo-dimorphic processes that influence brain structure, cellular function, gene expression and a wide range of behaviors (Arnold, 2017, 2009; Davies and Wilkinson, 2006; Du et al., 2004; McCarthy and Arnold, 2011; Sanchis-Segura and Becker, 2016). Apart from these influences, sex hormones shape the organism in three different ways: first, by defining wiring patterns and brain structures during neurodevelopment, also regarded as “organizational effects” (Wallen, 2005). Later, by altering intrinsic functions in the brain depending on their cyclic or sustained presence; like the modulation of hippocampal spines by fluctuating estradiol (E2) levels (Woolley, 1998). The last source of influence arises from the interaction of these sexually determined traits with the environment, making men and women shape their behavior according to social norms, other individuals or their personal adequacy (Berenbaum and Beltz, 2016; Springer et al., 2012). Still, most brain areas are not strictly sexually dimorphic, rather they appear as a continuum of characteristics, or what some authors have described as a mosaic, with several degrees of variability attributable to sex (Joel and McCarthy, 2017). Notably, it must be accounted that many of these differences manifest in the framework of compensation, meaning that organisms of opposite sex use different neurobiological substrates to solve the same problem and converge on the same behavior (De Vries, 2004; Wang et al., 1994). For this reason, once a sex difference is found, it must be contextualized depending on the setting where it is detected (Joel and McCarthy, 2017).

The study of sex differences (or similarities) is not fully considered in the field of neuroscience. In the last years, researchers have produced 5.5 studies in males per 1 in females (Zucker and Beery, 2010); pointing out the evident and growing need to change our approach to science by including female subjects at all levels of research (Clayton and Collins, 2014; Prendergast et al., 2014). Undoubtedly, scientists will need to adapt their approach to research questions (Fields, 2014), but the benefits will overcome the costs by providing improvements in the generalizability of results, increasing the control over data variability and prompting the possibility to develop personalized or even sex-based interventions. Lastly, human research often interchangeably uses the term sex or gender as one variable. Gender is now defined as the social, environmental, cultural and behavioral factors, or choices that influence a person’s self-identity and health (Clayton and Tannenbaum, 2016). From all the reviewed studies here, if any, none performed further confirmation of sex by genotype, or specific analyses of gender preference. For these reasons, we will focus only on studies reporting sex differences.

### 1.4. The study of sex differences in fear extinction

Mixed results are reported when comparing males and females upon cued-FE tasks, with some studies finding impairments for females and others not (Baker-Andresen et al., 2013; Baran et al., 2010, 2009; Fenton et al., 2014; Gruene et al., 2015a, 2015b; Maeng and Milad, 2015; Milad et al., 2009a,b; Voulo and Parsons, 2017). A more consistent picture emerges when the influence of hormones or the estrous (animal)/ menstrual (human) cycle is considered: females undergoing FE training during proestrus (high E2/ high progesterone (P4)) have similar FE recall than males. In contrast, females undergoing FE during metestrus (low E2/ low P4) have impairments in FE recall compared to proestrus females or males (Gruene et al., 2015a; Lebrón-Milad et al., 2013; Milad et al., 2009a,b; Colin D Rey et al., 2014). Fear learning studies that focused on contextual fear conditioning (FC) usually detect that females have lower freezing levels during FE training, and greater extinction rates when compared to males (Maren et al., 1994; Gupta et al., 2001; Dalla and Shors, 2009; Barker and Galea, 2010; Daviu et al., 2014; Bangasser and Wicks, 2017 but see Matsuda et al., 2015; Baran et al., 2009). Although these studies do not control for the estrous cycle, rather they administered E2 or ovariectomized females, finding improvements and impairments in FE respectively. In human studies, men and women acquire fear and extinction similarly but sex differences are observed for FE recall. Women undergoing FE training during the mid-phase of their menstrual cycles, with low E2 levels, or women taking hormonal contraceptives (HC) have less FE recall. In contrast, women with high E2 levels demonstrate better FE recall (Graham and Milad, 2013; Hwang et al., 2015; Lebron-Milad and Milad, 2012; Milad et al., 2010, 2006; Zeidan et al., 2011). In sum, these studies point at important influences of sex hormones over fear memories that could be directly influencing FE consolidation (Lebron-Milad and Milad, 2012). Also, the spontaneous recovery of the CR is more likely to happen in female individuals (Fenton et al., 2016; Matsuda et al., 2015).

### 1.5. Limitations in the study of sex differences in FE

Before reviewing the pertinent studies, it is worth noting some of the limitations found when studying FC and FE. It is possible that the observed sex differences in FE may arise from inherent differences in

fear acquisition, fear memory consolidation or fear expression (Dachtler et al., 2011; Dalla and Shors, 2009; Keiser et al., 2017). This is the case of studies finding significant sex differences in contextual fear acquisition (Blume et al., 2017; Chang et al., 2009). In fact, it is known that males and females possess differently weighted molecular mechanisms for fear memory formation including synaptic kinases, transcription factors and activated genes (Dachtler et al., 2011; Keiser and Tronson, 2016; Mizuno and Giese, 2010; Tronson, 2018). However, just some of these mechanisms are known for FE or FE recall. Also, the molecular signatures of FE in each sex may reflect the engagement of specific cognitive and behavioral strategies used to approach and learn from threats (Mizuno and Giese, 2010; Shansky, 2018; Silva et al., 2013; Tronson, 2018). Researchers have pointed out, that females are more likely to engage in active responses, like darting in rats or the tend-and-befriend response in humans (Gruene et al., 2015a, 2015b; Olff, 2017; Taylor et al., 2000). Therefore, studies in rats relying just on freezing behavior, may be not capturing the full behavioral response of females. Notably, there are no studies to date suggesting that female mice present darting behavior. Additional factors like the dynamics of sex hormones or social interactions leave ample room for methodological differences that could influence results. Examples of this include the techniques and timing used to monitor the estrous cycle, or the effects of social interaction between males (Kikusui, 2013; Maeng et al., 2015; Prendergast et al., 2014). Furthermore, we must account for all the inherent limitations of FC, which include the inability to assess the organism's subjective response, the nature and type of conditioned responses, or the considerable methodological discrepancies between animal and human studies. As an example, most human studies perform FE immediately after FE training, while in animal studies, FE is assessed after a time lapse usually longer than 6 h, being known that both processes recruit specific molecular signatures (Myers and Davis, 2007). Some of these elements are covered in depth elsewhere (Sevenster et al., 2014; Cook et al., 2014; Top et al., 2016) but call for the adaptation of FC and FE methodologies in human and animal studies in order to improve the translationability of results (Flores et al., 2018). Below, we will review FE studies that report sex differences and unless stated, they do not detect significant differences in fear acquisition. Nevertheless, we must encourage readers to make careful considerations given the aforementioned limitations.

## 2. Sex differences in brain systems and molecular pathways involved in fear extinction

### 2.1. Brain structures and neuronal circuits

The structures and circuits implicated in FC and FE act as a dynamic network of connections, with some areas being essential for fear acquisition or fear expression, while others work as relay stations or parallel processing points (Anglada-Figueroa and Quirk, 2005; Nader et al., 2001). Therefore, the correct interaction within this circuitry, and the integrity of its components, are major determinants of the behavioral output. Moreover, circuits involved in fear acquisition, fear expression, FE and anxiety overlap, although using different neuronal substrates. For example, microcircuits in the central amygdala (CeA) or the projections from the basolateral amygdala (BLA) to the mPFC promote fear learning but are also involved in FE and can induce anxiogenic or anxiolytic states (Tovote et al., 2015). The neuroanatomical and functional correlates help us identify key nodes in this distributed network where FE memories are encoded and stored; we review them here considering studies performed in both sexes (Figs. 1 and 2).

#### 2.1.1. Amygdaloid complex

The amygdala is a key hub for fear processing that is mainly composed of a cortical-like structure, the BLA, and a striatum-like structure, the CeA. During FC, auditory (CS) and nociceptive (US) inputs converge in the lateral amygdala (LA) triggering plastic changes (Herry and Johansen, 2014; McGaugh, 2004). The basal amygdala (BA) has prominent connections with the hippocampus and cortical structures, being able to integrate relevant contextual information and internal states (Calandreau et al., 2005; Gründemann et al., 2018; Phillips and LeDoux, 1992). Information flows from these nuclei to the CeA, which is the essential output station that triggers behavioral and homeostatic responses. Besides this, the CeA is also involved in plasticity, nociception and the hierarchical organization of defensive behaviors (Balleine and Killcross, 2006; Cardinal et al., 2002; Ehrlich et al., 2009; Fadok et al., 2017; Isosaka et al., 2015; Li et al., 2013). During FE, different neuronal populations in the BLA signal the CS-US contingency, and partially encode prediction errors (Herry and Johansen, 2014). Moreover, BA projections to the ventral hippocampus, or the prelimbic cortex (PrL) promote fear expression, while BA projections to the infralimbic cortex (IL) promote fear inhibition (Herry et al., 2008; Knapska et al., 2012; Senn et al., 2014). FE requires plastic changes in the amygdala, that can further reduce fear expression by increasing the

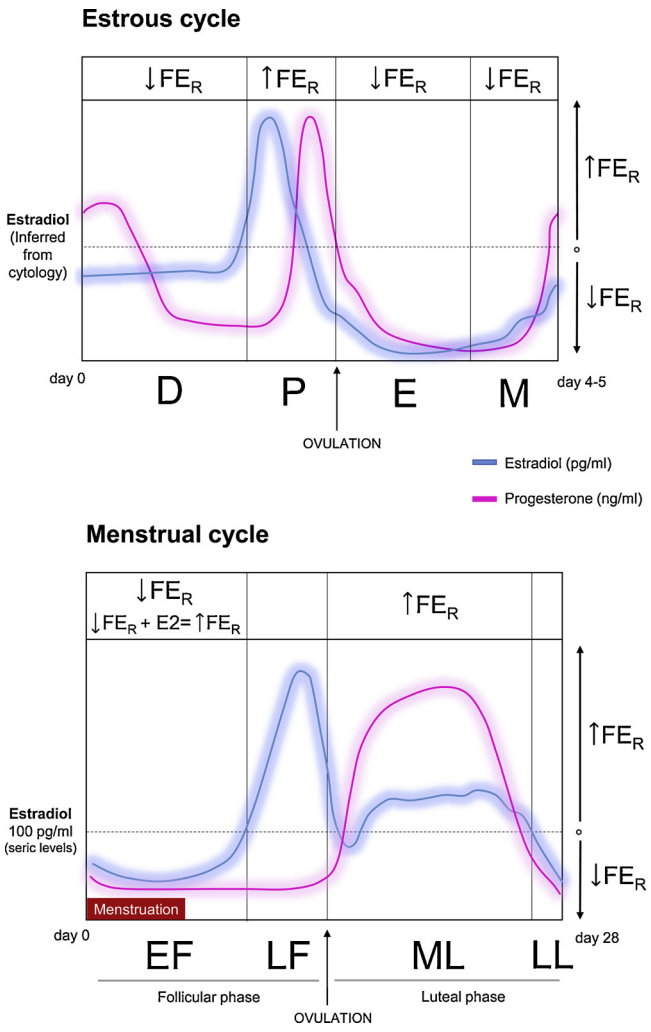


**Fig. 1.** Scaled representation of estradiol and progesterone levels during the distinct phases of the estrous (rodent) and menstrual cycle (human). The result of subjecting females to Fear Extinction (FE) training during each phase is shown at the top as fear extinction recall ($FE_R$). The $FE_R$ of females undergoing FE training under high or low estrogen states appears on the right. * denotes additional within-session effects of the cycle during FE training. D: diestrus, E: estrus, E2: estradiol, EF: early follicular phase, LF: late follicular phase, LL: late luteal phase, M: metestrus, ML: mid luteal phase, P: proestrus. Information obtained from: 1. Milad et al., 2009a,b, 2. Gruene et al., 2015a, 2015b, 3. Rey et al., 2014, 4. Milad et al., 2010, 5. Zeidan et al., 2011, 6 Pineles et al., 2016, 7. Graham and Milad, 2013.

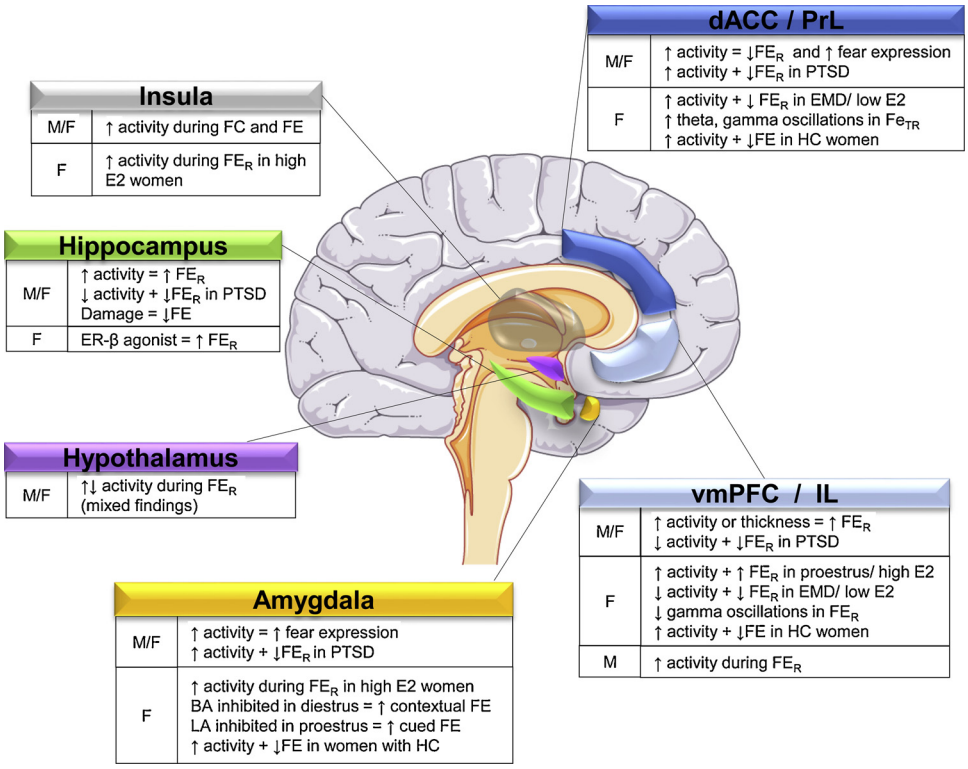


**Fig. 2.** Schematic representation of the brain structures where sex differences in fear extinction are reported. The main findings of animal and human research appear enlisted under each structure. BA: basal amygdala, dACC: dorsal anterior cingulate cortex, E2: estradiol, EMD: estrus, metestrus, diestrus phases of estrous cycle, ER-β: estrogen receptor beta, F: females, FC: fear conditioning, FE: fear extinction: $FE_R$: fear extinction recall, $FE_{TR}$: fear extinction training, HC: hormonal contraceptives, IL: infralimbic cortex, LA: lateral amygdala, M: male, M/ F: male and female, PrL: prelimbic cortex, PTSD: posttraumatic stress disorder, vmPFC: ventromedial prefrontal cortex.

perisomatic inhibition of fear neurons and by potentiating inhibitory synapses from intercalated cells under IL influence (Amano et al., 2010; Davis et al., 2017; Herry et al., 2008, 2006; Sotres-Bayon et al., 2007; Trouche et al., 2013). Thus, the interactions within these circuits modulate fear expression and enable FE encoding and consolidation (Adhikari et al., 2015; Bukalo et al., 2015; Herry et al., 2010). Recent evidence points out additional functions of the amygdala, specifically the CeA, in valence assignment, feeding behavior, and reward-related actions that could render this structure, as an integrator of internal states promoting the engagement into different behaviors (Beyeler et al., 2018; Fadok et al., 2018; Gründemann et al., 2018; Herry and Johansen, 2014; Kim et al., 2017; Paré and Quirk, 2017; Xu et al., 2016). In humans, neuroimaging studies show that the amygdala has restricted activity during FC, and inconsistently activated during FE training (Alvarez et al., 2008; Fullana et al., 2016; Knight et al., 2004; LaBar et al., 1998; Milad et al., 2007b; Phelps et al., 2004). During FE recall, most studies do not report activity in the amygdala (but see Zeidan et al., 2011). However, studies that have observed amygdala activations usually report it to be correlated with activity in the dorsal anterior cingulate cortex (dACC) and greater fear expression (Linnman et al., 2012c).

Anatomical comparisons reveal that males have larger and denser medial amygdalas, whereas females demonstrate more GABAergic neurons and fluctuations in the density of its dendritic spines across the estrous cycle. Notably these differences are not present in the BLA or CeA (Cooke and Woolley, 2005; Morris et al., 2008; Rasia-Filho et al., 2004; Stefanova and Ovtscharoff, 2000). At the functional level, females present a more inhibited LA during proestrus and a more inhibited BA during diestrus that correlate with faster cued-FE and contextual-FE respectively (Blume et al., 2017). Studies that have focused on humans report mixed findings when comparing the amygdala of males and females (Goldstein et al., 2001; Marwha et al., 2017; Ruigrok et al., 2014). However, sex differences are evident when assessing functional activations and resting state functional connectivity (rsFC). The rsFC refers to the spatiotemporal patterns of coupled brain activity that integrate a variety of intrinsic networks involved in cognitive function, memory or salient stimuli detection (Damoiseaux et al., 2006). Regarding fear behavior, there are increases in amygdala-dACC/ dorsomedial prefrontal cortex (dmPFC) rsFC after fear learning, that are positively correlated with behavioral and autonomic measures of fear (Schultz et al., 2012). Likewise, drug-induced decrements in the amygdala-hippocampal rsFC after FE learning relate to greater hippocampal activation and thus enhancements in FE recall (Rabinak et al., 2018). Therefore, it is hypothesized that rsFC changes observed after fear procedures may reflect ongoing memory consolidation, also relating to several behavioral impairments observed in patients with fear-related disorders such as PTSD (Zhu et al., 2017). At rest, men have higher amygdala- ventromedial prefrontal cortex (vmPFC) rsFC and women with low E2 show increased rsFC with the dACC (Engman et al., 2016). When presented with threatening cues, women show greater amygdala reactivity during low E2 phases of the menstrual cycle and this activity is not correlated with arousal measures or cortical activity (Goldstein et al., 2005). A finding that may be explained by the attenuating effects of E2 over the activation of subcortical structures of the arousal system (Goldstein et al., 2010). Most FC-FE studies that include both sexes report no sex differences for the CRs during FE training (Knight et al., 2004; Gottfried and Dolan, 2004; but see Fullana et al., 2018), but some differences are observed for the activity in the amygdala during fear acquisition (Hwang et al., 2015; Lebron-Milad and Milad, 2012). Only one study has found greater activations in the left amygdala and vmPFC during FE recall in women with high E2 compared to women with low E2 (Zeidan et al., 2011).

In the clinical field, PTSD and phobic patients have structural and functional alterations in the amygdala, commonly presenting hyperactivity that is coupled with hippocampal and vmPFC hypoactivity (Engel et al., 2009; Etkin and Wager, 2007a; Ipser et al., 2013; McLaughlin et al., 2014; Michael et al., 2007; Sripada et al., 2012a; Stevens et al., 2013). Likewise, their FE recall impairments correlate with hyperactivations in the amygdala (Milad et al., 2009a,b). In summary, the amygdala is a relevant structure for processing and eliciting conditioned responses regardless of sex. Its basal function seems to be influenced by hormonal levels, with some studies showing

decreased reactivity during high E2 states and hyperactivity during low E2 states; although, this effect may be restricted to specific subnuclei. The CeA, along with the extended amygdala, are under heavy neuromodulatory control and they may be able to integrate multiple inputs to set internal states that facilitate appropriate and scalable behaviors (Fadok et al., 2018; Herry and Johansen, 2014; Paré and Quirk, 2017)

#### 2.1.2. mPFC

The mPFC is a region implicated in fear learning that integrates sensory and contextual information to elicit flexible behavioral adaptations (Giustino and Maren, 2015). Two subdivisions of the mPFC receive the most attention when studying FE in rodents, the IL and the PrL, and they are thought to work as functional homologues of the human vmPFC and dACC respectively (Milad and Quirk, 2012). The IL cortex is involved in the formation of FE memories, activated during FE recall and its IL-BLA projections are necessary for extinction-related plasticity, but its function can be spared during FE recall (Adhikari et al., 2015; Bloodgood et al., 2018; Bukalo et al., 2015; Do-Monte et al., 2015; Herry et al., 2010; Kalisch et al., 2006; Lissek et al., 2013). In comparison, the PrL is implicated in the acquisition and expression of conditioned fear responses by integrating inputs from the BLA, hippocampus and thalamus into cortical networks (Courtin et al., 2014; Do-Monte et al., 2015; Sotres-Bayon et al., 2012). Further detail is discussed in Sotres-Bayon and Quirk, 2010; Giustino and Maren, 2015. In humans, the dorsal parts of the ACC and mPFC (dACC, dmPFC) are relevant for attention, negative emotional responses and the expression and evaluation of fear (Etkin et al., 2011; Milad et al., 2007a). While the ventral parts of the ACC and mPFC (sgACC, pgACC, rACC, vmPFC) exert an inhibitory control over subcortical structures and promote the consolidation of emotional memories, including FE memories (Milad et al., 2007b; Pace-Schott et al., 2015). FE training activates both, the dorsal and ventral parts, from which the vmPFC shows gradual increases (Delgado et al., 2008; Etkin et al., 2011; Fullana et al., 2018). In contrast, during FE recall, prefrontal activations are observed mostly in the ventral ACC and vmPFC when comparing the CR to a CS that underwent FE training against the CR to an unextinguished CS (Fullana et al., 2018; Lebron-Milad et al., 2012; Milad et al., 2007b, 2005).

The mPFC portrays intrinsic and extinction-related sex differences: structurally, the pyramidal neurons in the PrL of female rodents have smaller and less complex apical dendritic arbors (Koss et al., 2014). Also, pre-training electrolytic damage to the IL impairs FE acquisition and its maintenance in females, but in males it only impairs FE recall. Interestingly, this lesion also makes females acquire fear faster (Baran et al., 2010). Successful FE recall induces IL activations in males and females, but females with FE recall impairments (trained during low E2 phases) show persistent PrL activity and hypoactivation of the IL (Gruene et al., 2014; Knapska and Maren, 2009). By measuring prefrontal activity with local field potentials, researchers showed that females have greater freezing levels that correlate with persistent theta (4–12 Hz) and gamma (30–120 Hz) activity in the PrL during FE training. In addition, they fail to produce gamma activations in the IL during FE recall compared to males (Fenton et al., 2016, 2014). Animal studies have revealed that synchronized theta rhythms in the amygdala, mPFC and hippocampus are observed after FC and during fear expression. Moreover, gamma oscillations are involved in cognitive and attentional functions mediated by the prefrontal cortex (PFC) (Karalis et al., 2016; Likhtik et al., 2014; Seidenbecher et al., 2003). These oscillations are important for encoding information, long-range network synchronization, cognitive function, allowing the formation of neuronal ensembles in the short recurring time windows that facilitate synaptic interactions (Herry and Johansen, 2014; Pelletier and Paré, 2004). Thus, the observed sex differences in prefrontal synchronization may contribute to the distinct behavioral responses of males and females during FE.

In humans, anatomical and functional differences result in greater amygdala-vmPFC rsFC in men and greater amygdala-dACC rsFC in women with low E2 levels (Engman et al., 2016; Goldstein et al., 2005, 2001; Ruigrok et al., 2014). Prefrontal activity seems to be more prominent in women during FE training (dACC and mPFC) and men show higher vmPFC activity during FE recall (Lebron-Milad et al., 2012). However, if hormonal levels are considered, women with high E2 have greater activations in prefrontal structures (rACC, MCC) during FE training and FE recall compared to men and women with low E2 (Hwang et al., 2015; Zeidan et al., 2011). These studies do not report significant differences in fear acquisition but find diverging prefrontal activations among sexes. Notably, women taking hormonal contraceptives (HC) have impairments in FE that relate to greater activations in the ACC, vmPFC, amygdala and thalamus compared to men or women in the luteal phase (high E2/ high P4) (Merz et al., 2012).

In the clinics, PTSD patients exhibit basal and functional alterations in prefrontal function; with additional impairments in FE recall that relate to hypoactivations in the vmFPC-hippocampus and hyperactivations in the dACC (Bluhm et al., 2009; Etkin and Wager, 2007a, 2007b; Milad et al., 2009a,b; Rougemont-Bücking et al., 2011; Shvil et al., 2014). A tractographic study performed in traumatized women reported a positive correlation between FE and the integrity of the cingulum, the main white tract connecting the cingulate and the entorhinal cortex, so that better hippocampal-ACC connectivity predicted lower fear responses during FE (Fani et al., 2015). In sum, the reviewed studies suggest that mPFC function, specifically IL signaling, is important to trigger FE memory formation in both sexes but females are more likely to show persistent PrL activity and lower IL activity resulting in lower FE recall. The observed differences in theta and gamma oscillations may relate to a differential coupling between mPFC-hippocampus-amygdala that render females unable to switch between fear and safety states (Courtin et al., 2014; Lesting et al., 2013; Likhtik et al., 2014; Pelletier and Paré, 2004; Stujenske et al., 2014). However, these studies did not account for hormonal status and it is still unknown if low E2 states influence amygdala-dACC connectivity like suggested by human studies (Engman et al., 2016; Goldstein et al., 2005). For example, high E2 or estrogen receptor beta (ER-β) activation can influence excitatory transmission and synaptic plasticity in the IL through glutamatergic mechanisms (Galvin and Ninan, 2014). Still, it remains to be explored if E2 or P4 levels can influence prefrontal interneurons and thus promote a differential engagement of cortical networks that eventually impacts FE memory encoding or its consolidation (Burgos-Robles et al., 2009, 2007; Courtin et al., 2014).

#### 2.1.3. Hippocampus

FE memory is time and context-dependent, and the hippocampus relays information regarding the location and time where FE learning took place. Its role is crucial for memory processes, and the spatial and non-spatial representation of environmental stimuli (Maren et al., 2013). It enables organisms to encode internal or external contexts to generate optimal predictions and adjustments in their behavior. Moreover, fear reinstatement, renewal and spontaneous recovery implicate time and space-dependent contextual changes that trigger the reappearance of fear behavior (Ji and Maren, 2007). The dorsal hippocampus is involved in the acquisition, contextual encoding and context-dependent retrieval of FE memories (Corcoran et al., 2005; Lissek et al., 2013; Maren et al., 2013; Sierra-Mercado et al., 2011). In comparison, the circuits arising from the ventral hippocampus contribute to fear renewal and promote fear relapse (Knapska et al., 2012; Marek et al., 2018). Human neuroimaging studies that evaluated hippocampal activation during FE find mixed results, probably because of the different degrees of contextual involvement. Deactivations during FE training and activations during FE recall are observed, mostly when FE recall takes place in safe contexts (Fullana et al., 2018; Hatch et al., 2013; Kalisch, 2006; Knight et al., 2004; Milad et al., 2007b).

Sex differences in hippocampal function are found in a variety of tasks (Koss and Frick, 2017). In fear paradigms, males show greater freezing to context which has been related to NMDA-R mediated

mechanisms (Maren et al., 1994). An effect that is likely related to the estrogen actions on hippocampal function. Estrogen-dependent positive modulation of hippocampal spines may result in females having increased spines during high E2 states of their estrous cycle, and ovariectomized rats without estrogen replacement having decreased hippocampal spines (Gupta et al., 2001; Li et al., 2004; Woolley, 1998). In addition, hippocampal NMDA-R activated downstream signaling could be important for these sex differences in FE, because phosphorylation of extracellular signal-regulated kinase (ERK) 2 appears to be less sensitive in female mice (Matsuda et al., 2015). A rodent study focused in contextual fear conditioning found that an ER-β agonist dosed in the hippocampus of females enhanced FE recall, thus providing a mechanism for E2 actions in this structure (Chang et al., 2009). Likewise, a human study that considered E2 levels found a positive correlation between E2 levels and FE recall. Also, better FE recall was related to greater activations in the hippocampus, vmPFC, dACC and amygdala (Zeidan et al., 2011).

Regarding clinical populations, PTSD patients have prominent structural and functional hippocampal abnormalities. Men with PTSD show lower amygdala-hippocampus rsFC and women with panic disorder have decreases in hippocampal metabolism (Bisaga et al., 1998; Etkin and Wager, 2007b; Garfinkel and Liberzon, 2009; SHIN et al., 2006; Sripada et al., 2012; Trzesniak et al., 2010; van Rooij et al., 2015). These hippocampal dysfunctions coupled with an overactive amygdala and dACC result in a failure to inhibit fear responses when safety cues or safe contexts are presented (Garfinkel et al., 2014; Jovanovic et al., 2012; Rougemont-Bücking et al., 2011). In conclusion, the encoding of temporal and contextual stimuli is vastly influenced by hormonal states. Females undergo constant shifts in hippocampal function, so that the formation of FE memories using safe contexts as a trigger may be hindered in restricted periods or upon damage. It remains to be determined if hormone-dependent shifts in hippocampal function can influence circuits relevant for FE (Åhs et al., 2015; Knapska et al., 2012; Marek et al., 2018) or the hippocampal inputs to structures that integrate internal states like the CeA, PrL or BNST (Rozeske et al., 2018; Xu et al., 2016; Zelikowsky et al., 2014).

#### 2.1.4. Periaqueductal gray (PAG)

The PAG, also called “central gray”, is a region in the midbrain that coordinates functions like anxiety, fear learning, pain modulation and the onset of rapid defensive responses (Bandler and Shipley, 1994; Rabellino et al., 2016; Tovote et al., 2016). It is recognized as the central output pathway of threat processing, and involved in complex functions, such as the encoding of prediction errors and the relay of expectancy information to higher-order structures (Arico et al., 2017; McNally et al., 2011; Ozawa et al., 2017; Watson et al., 2016). Sex differences in the PAG are documented for sexual behavior, anxiolysis and antinociception (Linnman et al., 2012; Loyd and Murphy, 2009; Schwartz-Giblin and McCarthy, 1995). Moreover, E2 can enhance GABAergic transmission and induce μ-opioid receptor (MOR) internalization, while drops in P4 can alter $GABA_A$ receptor subunit composition, decreasing its inhibitory output (Griffiths and Lovick, 2005; Lovick, 2012; Loyd et al., 2008; Schwartz-Giblin and McCarthy, 1995). In humans, PAG activity is observed in relation to the anticipation of pain, imminent threat confrontation and during FE training (Fullana et al., 2018; Linnman et al., 2012b; Qi et al., 2018). Sex differences exist for PAG’s basal rsFC, with less activation observed in women with high E2 upon fearful stimuli presentation (Goldstein et al., 2010; Kong et al., 2010). Moreover, in FC-FE tasks it is specifically activated to cues that signal danger or anticipate pain (CS+) (Lindner et al., 2015). In the clinics, PTSD patients have shown to have a greater recruitment of the PAG at rest and during threatening or non-threatening situations compared with controls (Harricharan et al., 2016; Rabellino et al., 2016; Steuwe et al., 2014). Overall, the PAG is involved in pain modulation and the execution of behavioral responses, some of which are the main outcomes measured in FE. Sex differences in the PAG are largely understudied, and it is still unknown if hormonal regulation of opioid and GABAergic transmission can influence FE or nociceptive encoding (Ozawa et al., 2017). Moreover, it must be explored if hormonal cycling results in shifting functional states in the PAG that impact how inputs are integrated and behaviors are selected (Fadok et al., 2018; Li et al., 2013).

#### 2.1.5. Hypothalamic nuclei

The hypothalamus regulates autonomic, endocrine and behavioral responses to learned and innate threats (Keifer et al., 2015; LeDoux et al., 1988; Myers et al., 2014; Silva et al., 2016, 2013). It portrays extensive anatomical and functional sex differences related to parenting and sexual behaviors (Bailey and Silver, 2014; Cheung et al., 2015; Forger et al., 2004; Rhodes and Rubin, 1999; Simerly, 2002; Yang et al., 2013). However, the hypothalamus’s involvement in FE is largely unexplored. The expression of estrogen receptors in several hypothalamic nuclei fluctuate throughout the estrous cycle possibly altering its intrinsic activity (Acevedo-Rodriguez et al., 2015; Brown et al., 1992; Frank et al., 2014). When fearful stimuli are presented, the ventromedial hypothalamus (VMH), lateral hypothalamus, and the left amygdala are more activated in men, than women. But when the menstrual cycle phase is considered, women in the late follicular/ mid cycle phase (high E2/ low P4) have attenuations in the stress response circuitry, including the paraventricular hypothalamus, VMH, PAG, dACC and CeA compared to women in the early follicular phase (low E2/ low P4) (Goldstein et al., 2010, 2005). Furthermore, VMH activity may relate to some behavioral alterations seen in patients with fear-based disorders, since electrical stimulation of the VMH elicits panic attacks in humans and some animal models (Kunwar et al., 2015; Wang et al., 2015; Wilent et al., 2010). In line with this, knocking down the vesicular glutamate transporter 2 in the VMH results in decreased fear expression in males only (Cheung et al., 2015). Just few studies have addressed the hypothalamus during FE learning reporting mixed results for its activation (Lebron Milad 2012, Hwang et al., 2015). One study found greater activity in the left-hypothalamus in women, whereas greater activity in the right-hypothalamus was found in men during FE learning, but no further differences were detected during FE recall (Lebron-Milad et al., 2012). In contrast, another study reported no sex differences in hypothalamic activation during FE training or recall, but found that the hypothalamus was highly active along with the threat detection system (amygdala, insular cortex, medial cingulate cortex) in women undergoing FC during high E2 states compared to men or women taking HC (Hwang et al., 2015). Future studies examining the role of the hypothalamus will add to the understanding of the sex differences in fear and FE learning. For example, the VMH is regulated by hormones, but also integrates sensory (medial amygdala, basomedial amygdala) and nociceptive inputs (parabrachial nucleus) to influence relevant structures (dorsal PAG, BNST) that elicit and maintain CRs (Bester et al., 1997; Kunwar et al., 2015; Yang et al., 2013).

#### 2.1.6. Bed nucleus of the stria terminalis (BNST)

The BNST, part of the extended amygdala, acts as an integration node between external sensory information and internal homeostatic or autonomic states (Avery et al., 2014). It can activate the hypothalamus when facing stress, and its multiple subnuclei are strongly regulated by neuropeptides and sex hormones (Glangetas and Georges, 2016; Jennings et al., 2013; Kash et al., 2015; Marcinkiewcz et al., 2016; Tillman et al., 2018). Its function has been related to a sustained state of apprehension or fear, usually defined as anxiety, but it is also implicated in threat processing and responds to phasic and sustained stimuli (Alvarez et al., 2011; Fox et al., 2015; Gungor and Pare, 2016; Lebow and Chen, 2016; Shackman and Fox, 2016a; Torrisi et al., 2018). In fear learning tasks, the BNST contributes to the acquisition, expression, reinstatement and some forms of contextual fear (Fullana et al., 2018; Goode and Maren, 2017; Hammack et al., 2015), but its role in FE is largely unexplored (Ranjan et al., 2017). There is a big gap in

research regarding the influence of the sex differences in the BNST upon fear learning (Allen and Gorski, 1990; Avery et al., 2014). This structure undergoes a sexually dimorphic masculinization early in life and its function is also influenced by hormones in a state-dependent manner (Bangasser and Shors, 2008; Chung et al., 2002; de Vries and Forger, 2015; Kelly et al., 2013; Morishita et al., 2017; Pol et al., 2006; Zhou et al., 1995). Little is known regarding BNST's role in phasic threat response or fear inhibition processes, but its involvement is possibly under reported, at least in human studies (Fox et al., 2015; Shackman and Fox, 2016b). Few studies have observed greater BNST activations in phobic or PTSD female patients (Brinkmann et al., 2017; Münsterkötter et al., 2015; Straube et al., 2007). Furthermore, its role in FE may be of importance due to its capacity to integrate internal states with contextual stimuli, future research will delineate the specific influences of hormones upon its function and how they may relate to FE (Chung et al., 2002; Cooke and Simerly, 2005; Oler et al., 2017).

#### 2.1.7. Insula

The insula is located beneath the lateral sulcus, having an important function detecting salient stimuli and integrating somatosensory, motor and autonomic information with cognitive functions (Craig, 2009; Menon and Uddin, 2010; Namkung et al., 2017; Uddin, 2015). Therefore, it is not surprising that FC studies find it consistently activated during fear acquisition, upon the anticipation of pain or during the delivery of different types of USs (Benson et al., 2014, 2012; Fullana et al., 2016; Gramsch et al., 2014; Sehlmeyer et al., 2009). FE training activates the insular cortex, especially when it takes place in the same context where FC took place (Fullana et al., 2018; Gramsch et al., 2014; Sehlmeyer et al., 2009). Likewise, activations during FE recall are mostly seen when comparing CS + vs CS- (Fullana et al., 2018). Basal differences in its structure and function are described for men and women (Kann et al., 2016; Ruigrok et al., 2014). Importantly, men receiving electric shocks have greater activations in the insula-hippocampus, whereas women taking HC seem to have this activation dampened (Hwang et al., 2015). During FE recall, women have higher insular activity compared to men, but the difference seems to be driven by women with high E2 levels (Hwang et al., 2015; Lebron-Milad and Milad, 2012). Notably, insular dysfunctions are related to several psychiatric disorders, including anxiety and fear-based disorders (Etkin and Wager, 2007a, 2007b; Goodkind et al., 2015; Shin and Liberzon, 2010; Stein et al., 2007). For example, PTSD patients show insular hyperactivity at rest, and during exposure to traumatic and non-traumatic stimuli (Bruce et al., 2013; Fonzo et al., 2010; Simmons et al., 2008; Sripada et al., 2012; Stevens et al., 2013). Only one FE study reported increased insular blood flow during FE training in women with PTSD compared to women without (Bremner et al., 2005). Altogether, the insula plays a crucial role in salient stimuli detection regardless of sex; with some evidence indicating that its function may be modulated by endogenous and exogenous hormones (Hwang et al., 2015; Lebron-Milad and Milad, 2012). Insights into insular dysfunction mechanisms will be of uttermost value to several psychiatric disorders (Goodkind et al., 2015; Menon, 2011).

#### 2.1.8. Summary

In summary, we can conclude that the amygdala, mPFC and hippocampus are implicated in the sex differences observed in FE, but more research is needed to examine the potential role of the BNST, hypothalamus, PAG and insula. The amygdala is consistently shown to react to threats regardless of sex, although it seems that some of its subnuclei may be overactive in females during low E2 phases. Moreover, its role as an integrator of internal states is relevant for FE, allowing females to switch and engage into different response patterns. Regarding the mPFC, IL function is relevant for FE memory formation-consolidation, and females demonstrate persistent PrL and lower IL activations during FE compared to males, which also correlate with greater freezing levels. According to the reviewed studies, mPFC function may follow the menstrual/ estrous cycle shifts rendering it hypoactive in low E2 phases. However, it is not clear if these effects are related to circuits displaying sexual dimorphism, distinct neuromodulation, or changes in connectivity with other structures (e.g., amygdala, hippocampus). Furthermore, the hippocampus is an important structure for the contextual embedding of FE memories receiving a large hormonal influence. E2 positively regulates its dendritic spines and can enhance FE through ER-β activation. The integrity of the hippocampus and its connectivity with the mPFC may be pivotal components of FE memory formation, especially in women and patients with fear-based disorders. Regarding the PAG, it seems that fluctuations in E2 and P4 can promote changes in GABAergic and opioid signaling that further impact its intrinsic activation and inhibitory output. Nevertheless, these effects are largely understudied. Concerning the other reviewed structures, the insula signals and detects salient stimuli regardless of sex but some evidence suggests that women may activate it differently depending on their hormonal levels. Lastly, the BNST and hypothalamus are understudied structures that receive a strong hormonal modulation, which could be relevant for the integration of internal states that impact the appearance and magnitude of the CRs during FE.

### 2.2. Sex differences in molecular mechanisms of fear extinction

FE memory signals the safety of a previously conditioned stimulus in a specific context and this process is highly regulated by several neurotransmitters and intercellular signals at precise time points (Ehrlich et al., 2009). Depending on the temporal characteristics of FE training, immediate or delayed, and the molecular signals presented prior or after training, different mechanisms can be recruited (Maren and Chang, 2006; Myers et al., 2006). The adequate coordination of neurochemical signals allows organisms to learn and ensure that future threats are adequately faced. Nevertheless, dysregulations under certain genetic and environmental conditions can give rise to pathological behavioral responses. Here we will review studies exploring some of these systems in FE, as well as their interaction with sex and hormones.

#### 2.2.1. Glutamate and GABA

Glutamate is an excitatory neurotransmitter that belongs to the family of aminoacidic neurotransmitters. It is synthesized from glutamine in a wide variety of neurons (Meldrum, 2000). γ-Aminobutyric Acid (GABA), an aminoacidic neurotransmitter with inhibitory actions, is produced from the degradation of glutamate by the enzyme Glutamic-Acid Decarboxylase (GAD), which is presented in two isoforms: GAD65 and GAD67 (Meldrum, 2000; Petroff, 2002). The excitatory effects of glutamate are typically produced through ionotropic NMDA-R, α-amino-3-hydroxy-5-methyl-4-isoxazole Propionic Acid receptor (AMPA/ Kainate receptor) or metabotropic receptors (mGluR 1–8) (Sanacora et al., 2008); whereas GABA hyperpolarizes neurons acting on $GABA_A$, $GABA_B$ or $GABA_C$ receptors (Enz, 2001).

Sex differences have been identified within the glutamatergic system in both rodents and humans. First, concentrations of glutamate and GABA are sexually dimorphic in discrete brain nuclei important for FC, such as the nucleus accumbens or the VMH (Frankfurt et al., 1984). Further, concentrations of these neurotransmitters also differ across the estrous cycle in healthy adult female rats. Glutamate presents higher concentrations within the nucleus accumbens in males, but females possess higher levels in the diagonal bands of Broca and the VMH. On the other hand, GABA is more concentrated in the lateral hypothalamus, the habenular nuclei and the VMH of male rats. Notably, these differences arise during the metestrus stage of the estrous cycle, but not in proestrus. Also, there is an increased GABAergic function in response to E2 and the regulation of female sexual behavior by the VMH (Frankfurt et al., 1984). Furthermore, these nuclei are involved in different traits of fear processing, such as predator fear memory by the VMH (Silva et al., 2016) or freezing behavior during the exposure to a CS by the dorsal habenula (Agetsuma et al., 2010). Although the

specific contribution to FE of each of the previous structures is still to be elucidated, they are known to be necessary for normal threat processing. Disruption of GABAergic and glutamatergic neurotransmission, especially during low sex-hormone states in females, might contribute to the prevalent phenotype in fear pathology.

Glutamate is of special interest to FE research due to its implication in LTP. During LTP, glutamate binds to AMPA-R producing a tetanic pulse necessary for the activation of NMDA-R. Further, glutamate binds to NMDA-R allowing $Ca^{2+}$ influx only after magnesium leaves the cation channel in response to a tetanic stimulation. During the early 90's, it was demonstrated that NMDA-R antagonism within the amygdala, but not other areas, blocked the acquisition of the CR in a dose-dependent manner (Miserendino et al., 1990). Upon consideration of the extinction of the CR as a LTP of remote inhibitory synapses, new studies were carried out to examine the involvement of NMDA-R in FE. Surprisingly, pretraining administration of NMDA-R antagonists blocks the acquisition of extinction, while AMPA-R antagonist infusions before FE training have no effect (Zimmerman and Maren, 2010). The AMPA-R is essential for NMDA-R activation and subsequent LTP that leads to memory formation. Interestingly, GluA1, one of the most common AMPA-R subunits, is essential for FC in male mice, but not in females (Dachtler et al., 2011), although it is more expressed in females' hippocampus compared to males (Katsouli et al., 2014). In regards to NMDA-R, female rodents usually perform poorer in NMDA-R dependent tasks, presumably because of a lower activation of NMDA-R during LTP when compared to males (Maren et al., 1994). Notwithstanding, aging produces a downregulation of Glu2N NMDA-R subunit, causing slight LTP decline in males; while this effect is not reported in females (Monfort and Felipo, 2007).

In contrast, the GABAergic system oppositely regulates fear memory formation. While glutamate is involved in depolarization of postsynaptic neuron and associative learning (Riedel et al., 2003), GABA hyperpolarizes postsynaptic membranes (Kalueff and Nutt, 1996). $GABA_A$-R has been widely studied due to its involvement in fear memory within the amygdala, hippocampus and PFC (Davis and Myers, 2002; Makkar et al., 2010). From all GABA receptors, $GABA_A$-R is the main target of a wide variety of available drugs, with their potential anxiolytic effects well described (Holmes and Chen, 2015). In the IL cortex, the pharmacological enhancement of $GABA_A$-R transmission before FE training increases FE acquisition and consolidation in the long term. Also, pre-training infusions of a $GABA_A$-R agonist in the BLA, as well as post-training infusion in the IL cortex, facilitate within-session FE, but produce no effects in successive recalls of that FE memory (Akirav et al., 2006). Particularly, α4-$GABA_A$-R and α5-$GABA_A$-R subunits of the $GABA_A$-R are the most reported sex-dependent mediators of fear memories within the GABAergic system. In males, α4-$GABA_A$-R knockout (KO) present increased fear to context in delay, but not trace auditory FC. In contrast, females lacking α4-$GABA_A$-R subunit express increased fear to context in trace auditory FC, but not delayed (Moore et al., 2010). Additionally, hippocampal deletion of α5-$GABA_A$-R subunit disrupts auditory FC in male and female mice; but producing lower fear expression in males with trace FC, while females display similar fear expression levels in trace and no trace conditions (Yee et al., 2004). Unfortunately, we are not aware of more studies exploring the involvement of these subunits in FE.

### 2.2.2. Cholinergic and monoaminergic systems

*2.2.2.1. Noradrenaline.* Noradrenergic neurons in the locus coeruleus (LC) portray heterogeneous responses during fear and extinction learning that can strongly influence FE processes through its wide projections to the amygdala and mPFC (Quirk and Mueller, 2008; Uematsu et al., 2017). NA acts upon β-adrenergic receptors to increase neuronal excitability and upregulate protein kinase A (PKA) which are crucial processes for neuronal plasticity and FE memory formation (Berlau and McGaugh, 2006; Mueller et al., 2008). Moreover, NA promotes the retrieval of contextual fear memories and rodents with genetic NA depletion, or injected with propranolol (non-selective β-receptor antagonist), have deficits in fear memory retrieval (Murchison et al., 2004; Ouyang and Thomas, 2005). In addition, NA signaling is implicated in the "immediate extinction deficit", an impairment in extinction learning observed when FE is performed immediately after fear acquisition (Giustino et al., 2017). The pharmacological blockade of NA signaling confirms its necessity for normal FE acquisition and FE consolidation (Mueller et al., 2008; Rodriguez-Romaguera et al., 2009). Nevertheless, enhancing NA signaling less consistently improves FE and instead promotes anxiety (Lonsdorf et al., 2014; Morris and Bouton, 2007; Tuerk et al., 2018).

Regardless of the multiple effects of NA on fear acquisition and FE, we are not aware of any study addressing specific sex differences. This is unexpected because it is known that testosterone regulates monoaminergic neonatal development in a sex dependent manner (Stewart and Rajabi, 1994) and that anatomical and functional sex differences exist in the LC (Bangasser et al., 2016, 2011; Mulvey et al., 2018; Valentino et al., 2012). Notably, females are more sensitive to the arousal-enhancing effects of corticotropin-releasing factor (CRF) due to a decreased ability to desensitize CRF1 receptors and differential receptor coupling and trafficking (Bangasser et al., 2018, 2010; Curtis et al., 2006; Valentino et al., 1991). In addition, females present decreases in μ-opioid receptor (MOR) function in the LC compared to males, that can render it overactive specially in stressful situations (Curtis et al., 2012; Guajardo et al., 2017). Besides this, cyclic surges of E2 increase NA synthesis, decrease its degradation but also promote adrenergic receptor internalization; pointing at a plausible mechanism by which females maintain arousal levels at the expense of a decreased ability to influence downstream signaling (Bangasser et al., 2016). The increases in NA tone and the greater LC function in females may confer a heightened susceptibility to develop NA dysregulations and hyperarousal symptoms after increased CRF exposure (Bangasser et al., 2018). Studies in humans performing adrenergic manipulations have found sex-specific effects for emotional processing, amygdala activation and patients' response to treatments (Cahill and van Stegeren, 2003; Kornstein et al., 2000; Lonergan et al., 2013; Poundja et al., 2012; Schwabe et al., 2013) but some others have not (Rothbaum et al., 2008; Steenen et al., 2016). With these data we can assume that FE-related increases in NA may synergize with higher NA levels during high E2 phases, together inducing a stronger recruitment of structures relevant for FE encoding or its consolidation. It is also possible that retrieved fear memories undergo a weaker reconsolidation due to decreased NA influence over intracellular processes, altogether resulting in stronger FE memory formation (Isiegas et al., 2006; Johansen et al., 2011). It remains to be explored if there is a differential recruitment of LC neurons in males and females that could impact its influence over target structures like the mPFC or amygdala.

*2.2.2.2. Dopamine.* DA is involved in arousal, motor control, stress response and several learning theories implicate it in the formation of fear and extinction memories (Abraham et al., 2014; Menezes et al., 2015; Mueller et al., 2010; Rodriguez-Romaguera et al., 2012; Shi et al., 2017). D1 and D2 receptors in the mPFC are involved in FE memory consolidation, and D1 receptor in the BLA is important for within-session FE (Hikind and Maroun, 2008; Mueller et al., 2010). Neuronal activity in the ventral tegmental area is necessary for normal FE learning, likewise promoting mitogen-activated protein kinase (MAPK) phosphorylation in the IL and LA (Brischoux et al., 2009; Gore et al., 2014; Luo et al., 2018). Pharmacological manipulations show that L-dopa and D1 agonists generally enhance FE and FE recall whereas D1 antagonists impair them, and D2 manipulations produce mixed results (Abraham et al., 2016; Haaker et al., 2015; Hikind and Maroun, 2008; Mueller et al., 2010; Zbukvic et al., 2017). Sex differences in DA system are described for baseline or drug-induced DA release, receptor dynamics, DA levels, catechol-O-metyltransferase (COMT) activity and mesocortical projections (Harrison and Tunbridge,

2008; Kritzer and Creutz, 2008; Munro et al., 2006; Paolo, 1994; Riccardi et al., 2011). Additionally, the COMT gene polymorphism (Val$^{158}$Met), enhances DA levels and cortical function alongside interactions with E2. Women with the met/met genotype show improvements in working memory and dorsolateral prefrontal cortex function during the early phase of their cycle (low E2 levels), while val/val women have impairments. This relationship changes in phases near ovulation (high E2 levels), so that met/met women now show impairments, and val/val women have improvements (Jacobs and D'Esposito, 2011). In line with this data, a study reports that D1 agonism in females during low E2 phases reverts their usual FE recall impairment, while females trained during high E2 phases have their FE recall impaired by the drug (Colin D. Rey et al., 2014). Together pointing out that DA signaling follows E2 dynamics and influences PFC function, including FE, as an "inverted U-shape"; exerting a positive influence during low E2 phases and impairing an "optimal" signaling in high E2 phases (Jacobs and D'Esposito, 2011; Colin D. Rey et al., 2014). The basis of this effect is unknown but can relate to the observed differences in mesocortical projections or to a lower DA function that protects females from prefrontal overactivation during high reactivity states, like in low E2 phases. Finally, it must be accounted that DA may also act upon the striatum and influence the cortico-subcortical network connectivity relevant for FE (Correia et al., 2016; Luo et al., 2018; Myers and Davis, 2007).

*2.2.2.3. Serotonin.* Serotonin (5-HT) is produced in the raphe nuclei of the brainstem and implicated in several fear memory processes (Bauer, 2015; Gaspar et al., 2003). Selective serotonin reuptake inhibitors (SSRIs) are one of the most prescribed drugs in psychiatric practice and the first-line pharmacological treatment for mood, anxiety and fear-based disorders (Ravindran and Stein, 2010). When dosed acutely, they inhibit the serotonin transporter (SERT) and lead to a net increase in 5-HT, enhancing anxiety symptoms and increasing fear expression (Marcinkiewcz et al., 2016). On the contrary, chronic doses are needed to obtain clinically significant effects and anxiolysis (Invernizzi et al., 1996; Krishnan and Nestler, 2008). The effects of SSRIs upon FE vary depending on the type of drug, treatment duration and timing of administration. Chronic fluoxetine or escitalopram facilitate FE (Arce et al., 2008; Bui et al., 2013; Deschaux et al., 2013, 2011; Karpova et al., 2011), but chronic citalopram impairs fear acquisition and FE through NR2B NMDA-R subunit downregulation in the BLA (Burghardt et al., 2013). Sex differences in this system include 5-HT receptor distribution, SERT binding potential and the regulation of 5-HT synthesis by E2 through ER-β receptors (Donner and Handa, 2009; Jovanovic et al., 2008; Rubinow et al., 1998; Suzuki et al., 2013). Moreover, studies in animal models report mixed findings for E2-SSRIs interactions. For example, E2 can negatively impact the efficacy of fluvoxamine, but provides benefits for women in the perimenopause (Benmansour et al., 2012; Damoiseaux et al., 2014). A fear learning study found that acute doses of fluoxetine increased fear responses in both sexes during FE training and FE recall. But 14 days of chronic fluoxetine enhanced FE learning and FE recall in females only during low E2 phases (Lebrón-Milad et al., 2013). This effect is similar to the one obtained with D1 agonism and highlights the possibility that increases in monoaminergic signaling may enhance mPFC function during low E2 phases, thus facilitating FE formation. However, this assumption may be overgeneralized, as each monoamine is implicated in discrete processes of FE learning and their interactions with other systems should be considered (Jolas and Aghajanian, 1997; Nestler et al., 1990; West et al., 2009). It will be important to delineate the magnitude of monoaminergic FE enhancement during low E2 phases because they may act as adjuvants to exposure therapy under restricted conditions but may also promote greater fear retrieval.

*2.2.2.4. Acetylcholine (ACh).* ACh binds to muscarinic and nicotinic receptors to regulate several physiologic functions in the central nervous system that include arousal, attention and cognition. Cholinergic transmission is also implicated in neuronal activity synchronization, thereby improving the "signal-to-noise" ratio in the amygdala and facilitating memory encoding in the PFC (Hasselmo, 2006; Unal et al., 2015). Within the FE network, cholinergic neurons act as a relay of sensory pathways and regulate FC and FE by altering synaptic plasticity, firing patterns and neuronal excitability (Knox, 2016). Increases in muscarinic signaling are related to improvements in FE learning and FE recall, while decreased muscarinic signaling usually impairs FE processes (Jiang et al., 2016; Knox and Keller, 2016; Santini et al., 2012; Wilson and Fadel, 2017; Zelikowsky et al., 2013). The role of cholinergic neurotransmission through nicotinic receptors in FE is less clear, since the effects are highly dependent on the length of administration and the hippocampal involvement during a task (Elias et al., 2010; Kutlu and Gould, 2015, 2014).

A contextual fear learning study that explored sex differences reports that muscarinic blockade in males impairs fear memory recall, while females seem unaffected. Nevertheless, animals were exposed to the context only for 5 min and it would be desirable to explore if this male-specific impairment in fear retrieval extends into FE learning (Rashid et al., 2017). Sex differences are observed for nicotinic receptor dynamics, with males (and men) upregulating nicotinic receptors after chronic nicotine exposure, but remaining unaltered in females (Koylu et al., 1997). Moreover in women, P4 levels are associated with lower $\beta_2$-nicotinic receptor expression in cortical and cerebellar areas (Cosgrove et al., 2012). A FC study demonstrated that nicotine exposure affects males and females differently. Males showed impairments in FE when acute low or high doses of nicotine were used, whereas females only were affected by high doses. In contrast, chronic nicotine exposure increased the spontaneous recovery of fear in females only (Oliver et al., 2018; Tumolo et al., 2018). To summarize, although it has been demonstrated that muscarinic and nicotinic receptor activity can modulate fear learning and FE, it is not well defined how they specficially influence FE in each sex. Some studies point out that cholinergic signaling can act upon cortical and BLA interneurons to promote fear learning through disinhibition, but the contribution of this mechanism to FE is largely unexplored (Gozzi et al., 2010; Letzkus et al., 2015). Moreover, tobacco smoking is highly prevalent in patients with psychiatric disorders (Cook et al., 2014; Lawrence et al., 2009) and some of the reviewed studies suggest that chronic nicotine exposure may produce a resistance to FE in men, whereas women are more vulnerable to the spontaneous recovery of fear.

### *2.2.3. Neuropeptides and neurotrophins*

*2.2.3.1. Cannabinoids.* Endocannabinoid (eCB) signaling is crucial for FC and FE. Research has shown that the manipulation of eCBs can alter the acquisition and expression of contextual, but not cued fear memories (Chhatwal and Ressler, 2007; Marsicano et al., 2002). Studies in animals and humans support the notion that agonizing eCB signaling facilitates FE learning (Chhatwal et al., 2005; Lutz, 2007; Das et al., 2013; Dincheva et al., 2015 but see Bowers and Ressler, 2015; Soria-Gómez et al., 2015). Proposed mechanisms for this positive effect include the modulation of synapses in an activity-dependent manner and the stimulation of plasticity at inhibitory synapses (Hill et al., 2010; Trouche et al., 2013; Vogel et al., 2016). In contrast, deletions or blockade of CB1 receptor produces severe impairments in FE due to the blockade of kinase and phosphatase activity (Cannich et al., 2004; Hill et al., 2010; Marsicano et al., 2002; Papini et al., 2015). Also, human studies show that dronabinol (synthetic THC) decreases amygdala reactivity during FE training, whereas increasing hippocampal and vmPFC activity during FE recall (Das et al., 2013; Rabinak et al., 2014, 2013). Notably, the positive effects of eCBs over FE are only demonstrated with acute administrations, as chronic dosing impairs between and within-session FE and threat-safety discrimination (Lin et al., 2008; Papini et al., 2017).

There are several region-specific sex differences in eCB levels and

CB1 receptor expression. Compared to naturally cycling females, males and ovariectomized females have higher density of CB1 receptors in the hippocampus, greater CB1 receptor binding in the hypothalamus and lower CB1 binding in the amygdala. Interestingly, the increased hippocampal CB1 expression in ovariectomized females is negatively regulated by the administration of E2 (Bradshaw et al., 2006; Reich et al., 2009; Riebe et al., 2010). Further, cycling females are reported to have fluctuations of eCB levels throughout the estrous cycle in several brain regions (Bradshaw et al., 2006). And various studies accounted cycling females as being more sensitive to the effects of eCBs over nociception, motor movements and neurogenesis (Craft et al., 2013; Krebs-Kraft et al., 2010). Functionally, high E2 levels can potentiate CA1 excitatory transmission in a sex-dependent manner by increasing eCB signaling through the activation of ER-α and promoting a retrograde suppression of GABAergic inhibition (Huang and Woolley, 2012). Lastly, the administration of an eCB antagonist in males can induce differences in the activity of the hypothalamic-pituitary-adrenal (HPA) axis, producing a greater and longer ACTH-dependent corticosteorne diurnal peak (Atkinson et al., 2010). Despite these findings, little research has specifically addressed for sex differences during FE. One study investigated the effects of CB1 agonism and antagonism in females, showing that FE was enhanced with eCB agonists and impaired with antagonists, concluding that eCB effects on FE are not sex-dependent (Simone et al., 2015). Finally, increases in eCB signaling can reverse the stress-dependent alterations in FE in both sexes, but producing different effects in the hippocampus (Zer-Aviv and Akirav, 2016). In sum, few studies have addressed eCB-hormonal interactions upon FE, probably fueled by the positive results obtained with cannabinoid signal enhancements (Gunduz-Cinar et al., 2013). Studies exploring the pharmacokinetics and sex-divergent effects of chronic usage would be useful if considering cannabinoids as adjuvants to exposure therapy.

*2.2.3.2. Opioids*. Opioid peptides are classically involved in pain regulation; and for this reason, used as first line drugs to treat physical trauma. However, they are also consumed as drugs of abuse because of their addictive properties. Several areas of the fear circuitry expressing opiate receptors are also involved in the processing of aversive, cognitive and physiological aspects of pain (Sandkühler and Lee, 2013). For example, opioids act on the intercalated cells of the amygdala and on the PAG to promote FE, hence regulating the encoding of prediction errors and the inhibition of aversive stimuli processing (Ozawa et al., 2017; Roy et al., 2014). Notably, dynorphin and μ opioid receptor (MOR) signaling are implicated in the formation of FE memories in rodents and humans (Bilkei-Gorzo et al., 2012; Likhtik et al., 2008; McNally et al., 2005; Parsons et al., 2010). Gonadal hormones, specifically E2, can interact with the opioid system promoting their release, inducing receptor internalization and altering the rates of receptor homo-heterodimerization (Loyd et al., 2008; Loyd and Murphy, 2009). MOR expression is higher in males compared to cycling females in the ventrolateral PAG, with the lowest expression found during the proestrus phase (Loyd et al., 2008). Moreover, some studies point out that sex and hormones are factors that can influence how painful stimuli are perceived or processed (Chartoff and Mavrikaki, 2015; Craft, 2008; Eckersell et al., 1998; Kelly et al., 2003; Liu et al., 2011; Torres-Reveron et al., 2009).

A study that administered intra-LC doses of a MOR agonist found that females had decreased sensitivity to MOR-mediated inhibition of LC neuronal activity, along with an overall decreased expression of MOR. Also, researchers measured behavioral outcomes using an operant set shifting task, showing that females made more preservative errors, whereas males made more the total errors and premature responses (Guajardo, Synder et al., 2017). This study highlights an important sex dimorphism in opioid function in the LC of females. Opioids are known to counteract the effects of stress-induced LC activation, and to promote the recovery of LC activity to pre-stress levels (Valentino and Van Bockstaele, 2015). A decreased ability to diminish LC hyperactivity after facing stressful events would leave females prone to develop hyperarousal states. A study that focused on the effects of opioid administration on fear learning showed that dosing subcutaneous morphine after fear acquisition resulted in increased fear responses during FE only in females that had low E2 levels. This effect was absent in males, proestrus females or when dosed prior to FE training, demonstrating that acute morphine shortly after trauma can enhance fear responses in a subset of females. However, no further differences were observed during FE recall in any group (Perez-Torres et al., 2015). In sum, the decreased sensitivity of MOR in the LC of females, may hinder their capacity to downregulate LC hyperactivity after facing stressful events. Also, the fluctuation of E2 levels during the estrous cycle can impact the expression of MOR in the PAG; an essential structure that encodes expectancy errors and processes painful stimuli during fear learning tasks. Studies exploring the intracellular mechanisms underlying sex differences in FE will be valuable, opioid receptor activation can influence cAMP expression, and it is known that increased cAMP can delay FE memory formation (Myers and Davis, 2007). Additionally, morphine is commonly dosed after acute trauma and it may promote adverse behavioral outcomes in a subset of women.

*2.2.3.3. Corticotropin-releasing factor (CRF)*. CRF is a peptide hormone involved in the activation of the HPA axis, also regulating neuroendocrine, behavioral and emotional adaptations to stressors (Sherin and Nemeroff, 2011). Localized CRF increases in the BLA during FE training, impair further FE recall but without affecting FE acquisition. On the contrary, CRF decrements improve FE recall (Abiri et al., 2014; Hollis et al., 2016). Fear learning processes are tightly regulated by this peptide, CRF can induce hyperexcitability of principal neurons in the BLA and decrease eCB signaling (Gray et al., 2015; Rainnie et al., 2004). Further, specific impairments of NMDA or $GABA_A$-R function in CRF neurons increase fear expression and impair FE respectively (Gafford et al., 2012; Gilman et al., 2015). Interestingly, CRF is related to the "immediate extinction deficit", pointing out actions over NA transmission, but also a possible convergence of their intracellular signaling cascades (Hollis et al., 2016; Isogawa et al., 2013; Roozendaal et al., 2008). The transcription of CRF is modulated by E2. Higher basal CRF is found in the PVN of females during the proestrus phase, demonstrating also greater upregulation after physical (foot shock) or emotional stressors (Bingaman et al., 1994; Iwasaki-Sekino et al., 2009). Moreover, CRF1 and CRF2 receptors undergo sexually dimorphic changes after puberty, and differences in CRF1 dynamics in the LC are related to an enhanced sensitivity to CRF in females. Specifically, females have a greater coupling of CRF1 receptor with the GTP-binding protein, Gs in unstressed conditions. Also, the association of CRF1 receptor with β-arrestin2, a molecule promoting receptor internalization, occurs in the LC of males only, compromising CRF1 receptor internalization in females (Bangasser and Shors, 2010; Bangasser and Wicks, 2017; Weathington and Cooke, 2012). When administered centrally, CRF induces similar activations in males and females, except for the LC and lateral PAG which are activated only in females (Wiersielis et al., 2016). Nevertheless, another study that evaluated neuronal activity after central CRF administration found negative correlations for E2 levels and c-fos activation in the extended amygdala (Salvatore et al., 2018). In addition, KO of NMDA-R subunit NR1 (*Grin1*) in CRF neurons increased CRs during FE session only in males (Gilman et al., 2015). In sum, CRF enhancements of neuronal excitability seem to be detrimental for FE probably by encouraging an internal state of increased alertness and mobilization of resources (Binder and Nemeroff, 2010). Also, its signaling produces greater activations in the LC of females that are related to a different modulation of CRF1 which may posit females prone to develop arousal dysregulations under high or constant CRF secretion (Bangasser et al., 2018; Bangasser and Wicks, 2017; Curtis et al., 2006). Moreover, it remains to be explored if

CRF projections from structures like the CeA or BNST can influence FE learning or its consolidation in a sex-dependent manner (Ehrlich et al., 2009; McCall et al., 2015; Sanford et al., 2017). The stress-induced sex differences in FE are reviewed somewhere else (Maren and Holmes, 2016; Merz et al., 2018; Merz and Wolf, 2017; ter Horst et al., 2012; Wolf et al., 2015).

*2.2.3.4. Brain-derived neurotrophic factor (BDNF).* BNDF is a neurotrophin that influences neuronal function and survival, also playing roles in neurodevelopment, stress response and memory (Andero and Ressler, 2012). It promotes neuronal excitability (Minichiello, 2009), fear acquisition (Andero et al., 2011) and it is important for FE consolidation (Chhatwal et al., 2006; Choi et al., 2010; Heldt et al., 2007; Peters et al., 2010). Remarkably, intra-hippocampal BDNF produces cue-dependent FE even in the absence of training (Peters et al., 2010). Studies have revealed that BDNF acts as a signaling mediator of estrogen in the brain (Carrer et al., 2003; Scharfman and MacLusky, 2006). High E2 levels upregulate BDNF mRNA and protein levels in the hippocampus, which also fluctuate across the estrous cycle (Gibbs, 1998). The VAL66Met polymorphism in the pro-region of BDNF decreases its secretion, produces deficits in FE, and lower amygdala habituation to emotional stimuli (Gasic et al., 2009; Hariri et al., 2003; Lonsdorf et al., 2015; Soliman et al., 2010). Furthermore, male and female mice with the $BDNF^{Met/Met}$ genotype have impairments in hippocampal function, with females showing additional alterations in the normal fluctuation of plasticity molecules in the hippocampus (Spencer et al., 2010).

Sex differences exist for BDNF function; females with a resistance to FE have lower basal BDNF mRNA levels in the IL and greater methylation at exon IV (Baker-Andresen et al., 2013). In comparison, males subjected to FE have increased BDNF exon I and exon IV mRNA in the mPFC (Bredy et al., 2007). A study that performed a conditional KO of TrkB receptor in parvalbumin interneurons found impairments in FE consolidation for males compared to littermate controls or females (Lucas et al., 2014). Moreover, the authors emphasized on the importance of this differential TrkB-dependent effect, because SSRIs are known to upregulate BDNF in the BLA and promote greater plasticity in parvalbumin interneurons (Karpova et al., 2011). Although the specific mechanism is not known yet, it may follow secondary impairments of NMDA-R function due to the bidirectional glutamatergic-BDNF interactions (Andero and Ressler, 2012; Minichiello, 2009). It remains to be tested if females are endowed with a compensatory mechanism to consolidate FE even in the absence of TrkB signaling. The association of BDNF with psychiatric disorders and its interaction with inter-individual factors like genotype or hormonal status place this neurotrophin at a central point for further studies, especially the ones addressing mental disorders with a sex-biased prevalence (Andero et al., 2014).

*2.2.3.5. Oxytocin- vasopressin.* Oxytocin (OXT) and vasopressin (AVP) are molecules that act as neuropeptides and neurohormones exerting central and peripheric effects. They regulate stress, social behavior and can shape defensive responses, especially to unpredictable threats (Dębiec, 2005; Grillon et al., 2013; Leppanen et al., 2018; Meyer-Lindenberg et al., 2011; Neumann, 2008). Moreover, central OXT promotes weaker fear memory formation, but can also impair FE if dosed prior to FE training (Toth et al., 2012). Nevertheless, the effects of OXT over FE are influenced by factors like the strength of fear memories, the timing of OXT doses and the targeted structures, sometimes producing opposite effects (Huber et al., 2005; Knobloch et al., 2012; Lahoud and Maroun, 2013; Viviani et al., 2011; Zoicas et al., 2014) (Campbell-Smith et al., 2015). Interestingly, in the centro lateral amygdala, OXT can activate a subpopulation of neurons that feedforward inhibit the centro medial amygdala, thereby reducing passive fear responses (freezing, fear potentiated startle) and promoting active fear responses (Terburg et al., 2018; Viviani et al., 2011). In humans, intranasal OXT enhances FE recall but producing transient increases in the CRs of men during FE training (Acheson et al., 2013; Eckstein et al., 2015).

OXT and AVP systems portray structure and species-specific sex differences, with the AVP system usually being more prominent in males and the OXT system in females (de Vries, 2008; De Vries and Panzica, 2006; Dumais and Veenema, 2016; Lee et al., 2009; MacDonald, 2013). Also, both systems are regulated by sex hormones in an organizational and state-dependent manner, but the positive influence of E2 upon OXT is the most notorious (de Vries and Södersten, 2009; Gimpl et al., 2002; Grazzini et al., 1998; Meyer-Lindenberg et al., 2011; Olff et al., 2013; Sippel et al., 2017). Intranasal OXT produces sex-dependent activations in the amygdala and changes in its rsFC (Bethlehem et al., 2017; Domes et al., 2010, 2007; Ebner et al., 2016; Eckstein et al., 2017; Kovács and Kéri, 2015; Lischke et al., 2012; Petrovic et al., 2008; Sripada et al., 2013). In addition, OXT can modulate PFC activity in a sex dependent manner, possibly through actions of interneurons (Li et al., 2016; Luo et al., 2017; Nakajima et al., 2014). It is notable that the effects of OXT are influenced by inter-individual factors such as lifetime experiences and genotype (Bartz et al., 2011; Bradley et al., 2013; Heim et al., 2009; Meinlschmidt and Heim, 2007; Sippel et al., 2017). In the clinics, intranasal OXT exerts positive effects in PTSD patients by activating different neuronal substrates in men and women, also showing beneficial effects for a subset of people after trauma (Koch et al., 2016a, 2016b; Sack et al., 2017; van Zuiden et al., 2017). Unfortunately, these benefits do not seem to generalize to other anxiety disorders (Acheson et al., 2015). Overall, it seems that OXT is a neuropeptide that can influence fear retrieval, acute CRs to threats and FE memory consolidation. Some evidence indicates that OXTR expression is different in males and females in structures like the VMH, but not in the CeA (Uhl-Bronner et al., 2005). However, its functional role in FE, especially in females, remains to be elucidated. When exploring the effects of OXT over FE, researchers must account for sex, hormonal status, genotype and lifetime experiences in order to define the specific conditions under which OXT can positively regulate FE memories (Meyer-Lindenberg et al., 2011)

### 2.2.4. Regulation of fear extinction by gonadal hormones

Fear processes, especially FE, have shown to be strongly regulated by circulating sex hormones. Despite the higher life prevalence of stress and fear related disorders in women, the specific influence of sex hormones on FE remains poorly understood (Bangasser and Valentino, 2014). E2 has proved to enhance FE, either when administered systemically in ovariectomized rats or in the putatively high E2 stages across the estrous cycle (Graham and Daher, 2016; Milad et al., 2009a,b). Further, inhibition of E2 synthesis during FE has shown to reduce auditory FE (Graham and Milad, 2014). In contrast, P4, another hormone that also peaks with E2 during the proestrus phase, is hypothesized to exert opposite functions on FE compared to E2, but mixed results are commonly reported. Allopregnanolone, a P4 metabolite, acts as a positive allosteric modulator of $GABA_A$-R with the capacity to alter its subunit composition. In addition, studies have described a concentration-dependent biphasic effect over $GABA_A$-R that can lead to an allopregnanolone tolerance at high concentrations (Andréen et al., 2009; Pinna et al., 2000; Turkmen et al., 2011). In naturally cycling rats, systemic administration of a P4 receptor antagonist prevents the impairment in FE recall observed in females undergoing FE training during metestrus (Graham and Daher, 2016). Interestingly, if allopregnanolone is artificially infused in the BNST before both, fear acquisition and FE training, it no longer enhances FE. This outcome reflects that the BNST may integrate the temporal profile of internal hormonal states with other ongoing processes (Acca et al., 2017). Altogether, these findings suggest that in naturally cycling females, E2 may exert facilitating effects over FE, while P4 exerts the contrary, probably by involving genetic and epigenetic mechanisms regulating the synthesis of proteins necessary for FE memory consolidation (Tables

**Table 1**
Selected studies that compare sex differences during fear extinction training and fear extinction recall in cued fear conditioning paradigms. Some methodological details that could influence behavioral responses are depicted. * = additional sex differences during fear acquisition or basal outcome measures.

| STUDY | METHODOLOGY | | | | | | | | | RESULTS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Author** | **Species** | **Groups** | **Test sex diff** | **Manipulation** | **via** | **Timing** | **Behavioral paradigm** | **FE recall test** | **Outcome measures** | **FE training** | **FE recall** | **Functional/ anatomical correlates** |
| Merz et al., 2012 | *H* | M, F(Lut), F (HC) | Y | basal | – | – | Diff-cued, imm FE | – | SCR, BOLD | F = M | | F(HC) > M, F(Lut) in AMY, dACC and vmPFC |
| Blume et al., 2017 | *R* | M, F(d), F(p) in FC and $FE_{TR}$ | Y | basal | – | – | S-cued, 4d FE | – | freezing | M = F, F(p) > F(d) | | F(p) with greater LA inhibition |
| Knapska and Maren, 2009 | *R* | just males | N | basal | – | – | S-cued, 24 h FE | 24 h | freezing, c-fos | | M enhanced in FE context, impaired in diff context | Increased c-fos in IL, ITC, DG for FE recall. Increased in BLA, PrL, CeM for fear renewal. CA1 & CA3 c-fos in both |
| Gruene et al., 2014 | *R* | just females | N | basal* | – | – | S-cued, 24 h FE | 24 h | freezing, c-fos | | F enhanced in diff context | Increased c-fos in IL for FE recall |
| Lindner et al., 2015 | *H* | just females | N | basal | – | – | Diff-cued, imm FE | – | FPS, BOLD | | | PAG, vmPFC activation to CS in FE training. Activation to startle probe in CS + during FE training for insula, ACC, thalamus &PAG |
| Baran et al., 2009 | *R* | M, F | N | basal | – | – | S-cued, 1 h and 24 h FE | 24 h | freezing | F fail to extinguish | F fail to extinguish | |
| Voulo and Parsons, 2017 | *R* | M, F | Y | basal | – | – | S-cued, 24 h FE | 24 h | freezing FPS | F = M | M > F only in FPS | |
| Nagaya et al., 2015 | *R* | M, F | Y | Allo / Allo inhibitor / Allo antagonist | Intra-BNST | PRE-FE | S-cued, 24 h FE | 24 h | freezing | F = M | | |
| Antov & Stockhorst 2014 | *H* | M, F(Ef), F (Lf) | Y | basal | – | – | Diff-cued, imm FE | 24 h | SCR | F > M* | F = M | |
| Zeidan et al., 2011 | *R* | just females, F(m) in $FE_{TR}$ | N | ER-α/ ER-β agonist / E2 | SC | PRE-FE/ E2 POST-FE | S-cued, 24 h FE | 24 h | freezing, c-fos | | Enhanced in F(m) | E2 increased c-fos in IL-vmPFC and decreased in amygdala |
| | *H* | just females, F(HE2), F (LE2) | N | basal | – | – | Diff-cued, imm FE | 24 h | SCR, BOLD | | F(HE2) > F(LE2) | F(HE2) greater activation in amygdala HPC, dACC and vmPFC for FE recall, & greater vmPFC for FE training |
| Milad, Zeidan et al., 2010 | *H* | M, F(HE2), F (LE2) | Y | basal | – | – | Diff-cued, imm FE | 24 h | SCR, EXT ret index | F(HE2) =F(LE2). M = F | F(HE2) > F (LE2). M, F(HE2) > F(LE2) | |
| Milad et al., 2009a,b | *R* | M, F(p), F(m) in $FE_{TR}$ | Y | E2/ P/ E2 antagonist/ P antagonist | SC | PRE-FE | S-cued, 24 h FE | 24 h | freezing | F = M. F(m)+E2&PG > F(m), F(m)+E, F(m)+P | F = M. F(p), M > F(m). Enhanced in F(m)+E2 | |
| Milad, Goldstein et al., 2006 | *H* | M, F(Ef), F (Lf) | Y | basal | – | – | Diff-cued, imm FE | 24 h | SCR, EXT ret index | F = M | F(Ef), M > F(Lf) | |
| Hwang et al., 2015 | *H* | M, F(HC), F (HE2), F(LE2) | Y | basal | – | – | Diff-cued, imm FE | 24 h | BOLD | | | F(HE2) > F(LE2) in insula, F(HE2) > M in rACC and insula in FE training. F(HE2) > M in insula and MCC in FE recall |
| Graham and Milad, 2013 | *H* | just FEM F (HC), F(Ef) in $FE_{TR}$ | N | E2 | PO | PRE-FE | Diff-cued, imm FE/ 24 h FE | 24 h | SCR | | Impaired in F (HC). Rescued in F(Ef)+E2 | |
| | *M* | just females, F | N | ER-α/ ER-β agonist /Progestin | SC | PRE-FE | S-cued, 24 h FE | 24 h | freezing | | Impaired in F + P. Rescued with ER agonists | |

(*continued on next page*)

**Table 1** (*continued*)

| STUDY | METHODOLOGY | | | | | | | | | RESULTS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Author** | **Species** | **Groups** | **Test sex diff** | **Manipulation** | **via** | **Timing** | **Behavioral paradigm** | **FE recall test** | **Outcome measures** | **FE training** | **FE recall** | **Functional/ anatomical correlates** |
| Graham and Daher, 2016 | *R* | just FEM. F (OVX), F(d) in FC & $FE_R$ | N | P / E2 / P antagonist | SC | PRE-FE | S-cued, vary FE | 24 h | freezing | | Enhanced in F(m) + P antagonist | |
| Graham and Milad, 2014 | *R* | just males | N | E2 / aromatase inhibitor | SC | PRE-FE, POST-FE | S-cued, 24 h FE | 24 h | freezing | | Impaired by aromatase inhibitor. Rescued by E2 | |
| Maeng & Cover 2017 | *R* | just females, F(m) in $FE_{TR}$ | N | E2 | SC | PRE-FE | S-cued, 24 h FE | 24 h | freezing, c-fos | | Enhanced in F(m) + E2 | E2 increase CEl c-fos and decrease LA c-fos in FE training. Decrease CeM c-fos in FE recall |
| Baran et al., 2010 | *R* | M, F | Y | Bilateral electrolytic lesion IL-PrL | Intra-mPFC | PRE-FC | S-cued, 1 h or 24 h FE | 24 h | freezing | M > F | F > M | |
| Fenton et al., 2014 | *R* | M, F | Y | basal | – | – | S-cued, 24 h FE | 13d | Local field potentials | M > F | M > F | F have persistent theta activation in the PrL during FE training and FE recall |
| Fenton et al., 2016 | *R* | M, F | Y | basal | – | – | S-cued, 24 h FE | 13d | Local field potentials | M > F | M > F | F persistent gamma in PrL in FE training and failure to increase IL gamma in FE recall |
| Lebrón-Milad et al., 2013 | *R* | M, F(p,e), F (m,d) in $FE_{TR}$ | Y | Fluoxetine acute or chronic | IP/ PO | PRE-FE | S-cued, 24 h or 14d FE | 24 h | freezing | Acute impaired in M, F. Chronic enhanced in F(m,d) | Chronic enhanced in F(m,d) | |
| Lebron-Milad et al., 2012 | *H* | M, F | Y | basal | – | – | Diff-cued, imm FE | 24 h | SCR, BOLD | F = M | F = M | FE tr: F > M dACC, mPFC, l-hypothalamus; M > F r-hypothalamus. FE recall: M > F rACC; F > M insula |
| Gruene et al., 2015a, 2015b | *R* | M, F(p), F (e,m,d) in $FE_{TR}$ | Y | basal | – | – | S-cued, 24 h FE | 24 h | freezing | F = M. F(p) > F(e,m,d) | F = M. F(p) > F (e,m,d) | High freezing M have neuroanatomical alterations |
| Mueller et al., 2014 | *H* | M, F | N | basal | – | – | Diff-cued, imm FE | 24 h | EEG | | | dACC theta activity in FE training, vmPFC changes in gamma activity in FE recall |
| Moore et al., 2010 | *M* | M, F | Y | GABAa-α4 KO | – | – | s-cued: trace/ delayed, 24 h FE | – | freezing | F > M with delayed FC. M > F with trace FC | | |
| Wiltgen et al., 2005 | *M* | M, F | Y | GABAa-δ subunit KO | – | – | s-cued: trace/ delayed, 24 h FE | – | freezing | M > F in delayed and trace FC | | |
| Morris and bouton, 2007 | *R* | F | N | Yohimbine | IP | PRE-FE | S-cued, 24 h FE | 24 h | freezing | Enhanced | Enhanced | |
| Mueller et al., 2009 | *R* | M | N | Yohimbine | IP | PRE-FE | S-cued, 24 h FE | 24 h | freezing | Enhanced | | |
| Cain et al., 2004 | *M* | M | N | Yohimbine / Propranolol | SC | PRE-FE | S-cued, 24 h FE spaced/ massed | 24 h | freezing | Yohimbine enhances. Propranolol impairs | Yohimbine enhances | |
| Rey et al., 2014 | *R* | M, F(p), F (e,m,d) in $FE_{TR}$ | Y | D1 agonist | IP | PRE-FE | S-cued, 24 h FE | 24 h | freezing, c-fos | F = M | Impaired in F(p), enhanced in F (e,m,d) | c-fos in IL-BLA projecting neurons was unchanged after FE but correlated with freezing in F(e,m,d) |
| Simone et al., 2015 | *R* | F, F(OVX) | N | CB1 agonist / antagonist | IP | PRE-FE | S-cued, 24 h FE | – | freezing | Agonist low dose enhance, high dose impairs | | |
| Simone et al., 2015 | *R* | M | N | CB1 agonist / antagonist | IP | PRE-FE | S-cued, 24 h FE | – | freezing | Agonist enhance | | |
| Perez-Torres et al., 2015 | *R* | M, F(p), F(m) in FC | Y | Morphine | SC | imm POST- FC | S-cued,24 h FE | – | freezing | M, F(p) + morphine > F(m) + morphine | | morphine increased MOR in amygdala of F(p) & M but not F(m) |

(*continued on next page*)

**Table 1** (*continued*)

| STUDY | METHODOLOGY | | | | | | | | RESULTS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Author** | **Species** | **Groups** | **Test sex diff** | **Manipulation** | **via** | **Timing** | **Behavioral paradigm** | **FE recall test** | **Outcome measures** | **FE training** | **FE recall** | **Functional/ anatomical correlates** |
| Gilman et al., 2015 | *M* | M, F | Y | Grin1 KO in CRF neurons | – | – | Diff-cued, 24 h FE | – | freezing | F > M | | |
| Lucas et al., 2014 | *M* | M, F | Y | TrkB in PV cells KO | – | – | S-cued, 24 h FE | 24 h/ 7d | freezing | | Impaired in M | |
| Baker-Andresen et al., 2013 | *M* | M, F | Y | BDNF agonist | IP | PRE-FE | S-cued: + retrieval, 24 h FE | 24 h | freezing | M > F. Rescued in F by BDNF agonist | Impaired in F | F with lower BDNF mRNA and greater exon IV methylation |

ACTH- adrenocorticotropic hormone, Allo- allopregnanolone, BA- basal amygdala, BDNF- brain derived neurotrophic factor, BLA- basolateral amygdala, BNST- bed nucleus of the stria terminalis, BOLD- blood oxygen level dependent, CB1- cannabinoid receptor type 1, CEl- centro lateral amygdala, CeM- central amygdala, CFC- contextual fear conditioning, d- days, D1- dopamine receptor D1, dACC- dorsal anterior cingulate cortex, DG- dentate gyrus, dHPC- dorsal hippocampus, diff- different, Diff-cued- differential-cued fear conditioning, E2- estradiol, EEG- electroencephalogram, ER-α - estrogen receptor alpha, ER-β- estrogen receptor beta, EXT ret index- extinction retention index, F- female, F(d)- female diestrus, F(e)- female estrus, F(Ef)- female early follicular phase, F(HC)- Female hormonal contraceptives, F(HE2)- female high estradiol, F(LE2)- female low estradiol, F(Lf)- female late follicular phase, F(Lut)- Female luteal phase, F(m)- female metestrus, F(OVX)- female ovariectomized, F(p)- female proestrus, FC - fear conditioning, FE- fear extinction, $FE_R$- fear extinction recall, $FE_{TR}$- fear extinction training, FPS- fear potentiated startle, h- hours, *H*- human, HPC- hippocampus, IL- infralimbic cortex, imm- immediate, IP- intraperitoneal administration, ITC- intercalated cells of the amygdala, LA- lateral amygdala, l-hypothalamus- left hypothalamus, LTP- long term potentiation, M- male, *M*- mouse, M(TESTX)- male orchiectomized, MCC- mid cingulate cortex, MOR- mu opioid receptor, N- no, NMDAr- NMDA receptor, P- progesterone, PAG- periaqueductal gray, PO- oral administration, PRE-FC- previous to fear conditioning, PRE- $FE_R$ - previous to fear extinction recall, PRE- $FE_{TR}$ - previous to fear extinction training, PrL- prelimbic cortex, PV- parvalbumin, *R* – rat, rACC- rostral anterior cingulate cortex, r-hypothalamus- right hypothalamus, SC- subcutaneous administration, SCR- skin conductance response, S-cued- single-cued fear conditioning, SR- spontaneous recovery, TrkB- tropomyosin receptor kinase B, vmPFC- ventromedial prefrontal cortex, Y- yes.

**Table 2**
Selected studies that compare sex differences during fear extinction training and fear extinction recall for contextual fear conditioning paradigms. Some methodological details that could influence behavioral responses are depicted. * = additional sex differences during fear acquisition or basal outcome measures.

| STUDY | METHODOLOGY | | | | | | | | | RESULTS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Author** | **Species** | **Groups** | **Test sex diff** | **Manipulation** | **via** | **Timing** | **Behavioral paradigm** | **FE recall test** | **Outcome measures** | **FE training** | **FE recall** | **Functional/ anatomical correlates** |
| Maren et al., 1994 | *R* | M, F | Y | basal | – | – | CFC,24 h FE | 24 h | freezing | F > M | | M > F HPC LTP & NMDAr activation |
| Gupta et al., 2001 | *R* | M, F, F(OVX) | Y | E2 | SC | PRE-FC | CFC, 24 h FE | 24 h | freezing & FE rates | F(OVX + E2) > F (OVX) | F > F(OVX), M | F(OVX) had greater LTP in DG of HPC, reversed in F(OVX) + E2 |
| Barker and Galea, 2010 | *R* | M(TESTX), F(OVX) | Y | E2 | SC | 15 d PRE-FC | CFC, 24 h FE | 24 h | freezing | F(OVX) > M (TESTX) | F(OVX + E2) > F(OVX), M(TESTX + /-E2) | |
| Daviu et al., 2014 | *R* | M, F | Y | basal | – | – | CFC, 9d FE | 24 h | freezing | F > M | F > M | F > M ACTH & corticosterone levels |
| Chang et al., 2009 | *R* | M, F(p), F(e), F(d) in FC | Y | basal | – | – | CFC, 24 h FE | 24 h | freezing & FE rates | F(p) > M | F(e), F(p) > M | |
| | | F(OVX + E2), F(OVX + P) | N | basal | – | – | CFC, 24 h FE | 24 h | freezing & FE rates | | F(OVX + E2) > F(OVX + P) | |
| | | F(OVX) | N | ER-α/ ER-β agonist | IP | PRE-FC | CFC, 24 h FE | 24 h | freezing & FE rates | | F(OVX + ER-β) > F(OVX + ER-α) | |
| | | F(OVX) | N | ER-β agonist / E2 | Intra-HPC | PRE-FE recall | CFC, 24 h FE | 24 h | freezing & FE rates | | Enhanced for F(OVX + E2/ER-β) | |
| Blume et al., 2017 | *R* | M, F(d), F(p) in FC and $FE_{TR}$ | Y | basal | – | – | CFC, 4d FE | – | freezing | M = F, F(d) > F(p) | | F(d) with greater BA inhibition |
| Matsuda et al., 2015 | *M* | M, F, F(OVX) | Y | basal | – | – | CFC, 24 h | 28d | freezing | F > F(OVX) | M > F | ERK phosphorylation in dHPC appeared sooner in males |
| Matsuda et al., 2015 | *M* | M, F | Y | Basal, 1-week isolation | – | PRE-FC | CFC, 24 h FE | 35d | freezing | (Fear expression) F grouped > F isolated | | |
| Dachtler et al., 2011 | *M* | M, F | Y | GLUR1 KO | – | – | CFC, 24 h FE | – | freezing | Enhanced in M* | | |
| Oliver et al., 2018 | *M* | M, F | Y | Nicotine acute & chronic | IP/ SC | PRE-FE / PRE-FC | CFC, 24 h FE | 24 h | freezing | | Acute impaired F & M. Chronic impaired in M | |
| Tumolo et al., 2018 | *M* | M, F | Y | Nicotine chronic | SC | POST-FE | CFC, 24 h FE | 24 h | freezing | | SR: M > F. Nicotine decreases SR in M and enhances in F | |

ACTH- adrenocorticotropic hormone, Allo- allopregnanolone, BA- basal amygdala, BDNF- brain derived neurotrophic factor, BLA- basolateral amygdala, BNST- bed nucleus of the stria terminalis, BOLD- blood oxygen level dependent, CB1- cannabinoid receptor type 1, CEl- centro lateral amygdala, CeM- central amygdala, CFC- contextual fear conditioning, d- days, D1- dopamine receptor D1, dACC- dorsal anterior cingulate cortex, DG- dentate gyrus, dHPC- dorsal hippocampus, diff- different, Diff-cued- differential-cued fear conditioning, E2- estradiol, EEG- electroencephalogram, ER-α - estrogen receptor alpha, ER-β- estrogen receptor beta, EXT ret index- extinction retention index, F- female, F(d)- female diestrus, F(e)- female estrus, F(Ef)- female early follicular phase, F(HC)- Female hormonal contraceptives, F(HE2)- female high estradiol, F(LE2)- female low estradiol, F(Lf)- female late follicular phase, F(Lut)- Female luteal phase, F(m)- female metestrus, F(OVX)- female ovariectomized, F(p)- female proestrus, FC - fear conditioning, FE- fear extinction, $FE_R$- fear extinction recall, $FE_{TR}$- fear extinction training, FPS- fear potentiated startle, h- hours, *H*- human, HPC- hippocampus, IL- infralimbic cortex, imm- immediate, IP- intraperitoneal administration, ITC- intercalated cells of the amygdala, LA- lateral amygdala, l-hypothalamus- left hypothalamus, LTP- long term potentiation, M- male, *M*- mouse, M(TESTX)- male orchiectomized, MCC- mid cingulate cortex, MOR- mu opioid receptor, N- no, NMDAr- NMDA receptor, P- progesterone, PAG- periaqueductal gray, PO- oral administration, PRE-FC- previous to fear conditioning, PRE- $FE_R$ - previous to fear extinction recall, PRE- $FE_{TR}$ - previous to fear extinction training, PrL- prelimbic cortex, PV- parvalbumin, *R* – rat, rACC- rostral anterior cingulate cortex, r-hypothalamus- right hypothalamus, SC- subcutaneous administration, SCR- skin conductance response, S-cued- single-cued fear conditioning, SR- spontaneous recovery, TrkB- tropomyosin receptor kinase B, vmPFC- ventromedial prefrontal cortex, Y- yes.

1 and 2).

Low circulating E2 has shown to be a vulnerability factor for the development of PTSD (Lebron-Milad and Milad, 2012). Moreover, the chronic suppression of E2 synthesis by monophasic hormonal contraceptives in women or by the administration of progestin in rats, results in a low-extinction phenotype, which can be easily reverted by terminating treatments or by systemically administering an E2 receptor agonist (Graham and Milad, 2013). Additionally, women with low salivary E2 present higher skin conductance response during FE training in comparison to women with high E2 (Wegerer et al., 2014). Serum E2 concentrations can predict exposure therapy efficacy in women with spider phobia (Graham et al., 2018). Moreover, women with fear-based disorders taking HC display reductions in treatment efficacy and increased post-treatment symptoms (Li and Graham, 2016). Little research has been conducted regarding E2 role in other fear-based disorders such as panic disorder. Women at high risk for panic attacks have shown precipitation of panic disorder after taking HC (Deci et al., 1992). In contrast, estrogen replacement therapy is reported to be effective at reducing panic symptoms (Chung et al., 1995). Likewise, in men, pentagastrin-induced panic symptoms are reduced after a 3-day pretreatment with ethinyl E2 (an estrogen receptor agonist) (McManus et al., 2001).

The role of testosterone, the primary sex hormone in males which lacks fluctuating properties, in regard to FE remains controversial. On the one hand, some studies report testosterone does not play a role in male rodents in FC, FE or FE recall (Anagnostaras et al., 1998; McDermott et al., 2012). On the other hand, several studies report a strong modulation of FE acquisition and retention by male and female gonadal hormones. Further, blocking aromatase enzyme with fadrozole during FE training impairs FE recall 24 h later in males (Graham and Milad, 2014). Also, dosing males with a GnRH agonist that increases the synthesis of testosterone enhances FE memory consolidation (Maeng et al., 2017). One hypothesis for this effect is that testosterone acts by its conversion to E2, producing over FE all the facilitating effects that were previously described (Graham and Milad, 2014). This could explain why FE appears more stable in males, while it presents disruptions in females during low E2 stages. Although it's controversial, the current literature on E2 in males is hypothesized to be as important as in females for the consolidation of the FE memory. Testosterone levels seem unaltered in male patients with PTSD, but when analyzing a subset of PTSD patients without any comorbidity, higher testosterone levels are observed in comparison to controls and males with comorbid PTSD (Karlović et al., 2012). In line with this study, abnormalities in testosterone concentration have also been found in American survivors of the Iranian Hostage Crisis, presenting higher salivary testosterone than healthy controls (Rahe et al., 1990). In contrast, some other studies report lower testosterone in cerebrospinal fluid of combat veterans with current PTSD (Mulchahey et al., 2001). The existence of a SNP within the gene encoding for the 5-α-reductase (SRD5A2), an enzyme that degrades testosterone into dihydrotestosterone, correlates with more serious PTSD symptoms in men, but not women (Gillespie et al., 2013).

#### 2.2.5. Summary

There are several molecular mechanisms implicated in the sex differences observed in FE, some of which are directly influenced by the dynamics of gonadal hormones. E2 and testosterone emerge as crucial elements in FE memory formation with the capacity to positively regulate its consolidation. As reviewed here, women with high E2 levels, or rodents undergoing FE training during the proestrus phase, have better FE memory recall compared to women with low E2 levels or rodents trained during other estrous phases. Studies also show that glutamatergic transmission is crucial for fear learning and FE acquisition, although GluA1 seems to be essential for fear acquisition in males. Furthermore, several sex dimorphisms are reported for $GABA_A$-R subunits that may impact fear acquisition and FE learning, having additional interactions with P4 metabolites. However, studies exploring the glutamatergic and GABAergic mechanisms of FE in both sexes are scarce. Neurotransmission in the LC is tightly modulated by sex and hormones. Differences in CRF1 receptor dynamics and MOR sensitivity leave females vulnerable to the effects of sustained CRF signaling, and prone to NA overactivation under stressful situations. Further, the effects of CRF over target structures may interact with hormonal levels, because females with high E2 have shown less activation of the extended amygdala in response to CRF. Neurotransmission by eCBs and BDNF generally improves FE in both sexes, from which the latter is positively regulated by E2 and may be related to the enhanced FE recall in females undergoing FE training during high E2 phases. In the case of DA and 5-HT transmission, studies have shown differential effects over FE that depend on the type of drug and the targeted receptor. However, females benefit from increases in DA and 5-HT transmission only during stages with low E2 levels. The other reviewed neurotransmitters produce different effects over FE in each sex, but not always following the same direction. It seems that their actions are subject to factors like the dose, timing of administration and specific effects over target structures.

## 3. Future directions

Our knowledge about FE and the implicated neural circuits will be greatly improved with the arrival of revolutionary technologies. The combination of tools that determine specific neuronal profiles, with others that track neuronal dynamics *in vivo*, will enable researchers to precisely manipulate neuronal populations driving FE behavior. Additional factors like age, social interactions and the environment have not received much focus despite their powerful impact on health and behavior. Future studies must target these variables and provide evidence for additional within-sex effects that have not been accounted in detail yet, potentially improving our understanding of when and how sex differences arise in FE. Examples include, female's reproductive status (Milligan-Saville and Graham, 2016), the role of social interactions in males (Horii et al., 2017), and the changes in fear learning across the lifespan (Kim and Richardson, 2010; Remmes et al., 2016). In humans, accounting for gender as a research variable is gaining recognition in psychiatric and behavioral research (The Lancet Psychiatry, 2016). Gender and gender conformity may be considered as an additional context (i.e. socioeconomic status, marital status) in which a person develops with the capacity to influence perceptions, choices, health and behavior (Short et al., 2013). Its role upon FE is fairly unrecognized, but its inclusion as a 2-step approach questionnaire where participants are asked for their sex assigned at birth and their current gender identity may provide insights about its contributions to fear learning (Clayton and Tannenbaum, 2016).

Research frontiers will expand by the translation of basic research into the clinics. FE mimics exposure therapy procedures and it can be regarded as an useful model to test novel approaches to treat fear-based disorders despite its intrinsic limitations (Milad et al., 2014). Drugs that increase BNDF or cannabinoid signaling could be beneficial interventions for exposure therapy regardless of sex. In the case of hormonal interventions, males have shown to improve their FE with the use of a GnRH agonist (Maeng et al., 2017), while females may benefit from exogenous E2 or an ER-B agonist during their naturally low E2 stages (Maeng and Milad, 2015). However, a tight monitoring of the menstrual cycle/ hormonal levels is necessary since adverse outcomes are possible if E2 dosing is not timely constrained (Cover et al., 2014). Drugs increasing monoaminergic function in the mPFC during low E2 stages in females may render similar benefits as E2 (Inagaki et al., 2010).

Other aspects that may be relevant for women undergoing exposure therapy include the acknowledgement of SSRIs intake and a high nicotine consumption. Furthermore, hormonal contraception seems to be related with lower levels of FE, altered rsFC and changes in HPA axis reactivity (Engman et al., 2018; Graham and Milad, 2013; Hertel et al.,

2017; Petersen et al., 2014). Thus, it seems urgent to explore the specific outcomes of exposure therapy in women using hormonal contraception. Lastly, the tailored timing of exposure therapy sessions during putatively high E2 phases may result in better clinical outcomes. Nevertheless, we lack studies that track fear learning in a within-subjects design throughout the menstrual cycle. It is still necessary to define the exact time windows in which E2 benefits FE as it is not known if the benefits can be obtained during the peri-ovulatory phase, the mid luteal phase or following drastic hormonal shifts? (Maeng and Milad, 2015). Future studies performing a systematic control of the menstrual/estrous cycle and the hormonal status will inform about the specific factors to account for when performing fear research in women and females.

## 4. Conclusions

Here we have discussed all the studies on sex differences in FE that we are aware of. Despite an exponential growth in the number of papers focused on FE during the last 20 years, few studies using animal models have included both sexes in their design. Moreover, studies in humans and FE scarcely test for sex differences or systematically control for hormonal status. During the last years, this research bias has started to change, and now more studies are focused on sex differences in FE in both animals and humans. The main reason fueling this change implies the consensus about the need to focus on the female brain. For example, it is evident that including sex as a variable in FE is giving us a better understanding of the mechanistic processes underlying it, either by delineating the influence of sex hormones, or by revealing different brain connectivity patterns, among others. In addition, actual medical interventions begin to focus on personalized treatments. Thus, the understanding of how sex and hormonal status alter FE will be beneficial for designing specific treatments for men and women when appropriate.

The need to account for sex and the hormonal status when performing fear research is highlighted by studies that demonstrated women and female rodents seem to be generally hyper-responsive to threats during low E2 hormonal phases, also presenting impairments in FE. The results from this review can be summarized into 5 points: 1) Sex hormones modulate FE and its consolidation but the exact underlying molecular mechanisms remain largely unknown. Seemingly, putative high E2 levels and P4 shifts exert positive and negative effects on FE respectively. 2) Hormonal fluctuations may determine different functional states in females, as some neurotransmitter/ neuropeptides follow these hormonal shifts, potentially influencing neuronal circuits relevant for FE. Examples include the changes in hippocampal spine density, differences in PAG's inhibitory output, and the persistence of CeA/BNST-mediated behaviors. 3) The prominent sex differences in LC function, render it overactive in females under certain conditions. Moreover, greater NA signaling can impact fear retrieval, FE encoding, and FE consolidation. 4) PFC function seems to be regulated differently by monoamines throughout the menstrual/estrous cycle, so that increases in monoaminergic transmission during low E2 phases generally exert a positive influence over FE and the opposite occurs in phases with high E2. An observation that warrants further research since most drugs used to treat fear-based disorders target these systems. 5) There are apparent sex differences in the molecular mechanisms of FE consolidation related to glutamate, males with impaired glutamatergic function are unable to consolidate FE while females seem unaffected. The possibility of an alternative compensatory mechanism for FE consolidation in females should be explored.

Besides the small amount of research focused on females in FE, we must also account for the additional limitations in this review: There are considerable gaps in the mechanisms and circuits implicated in the retrieval of memories, specially FE memories. This is a crucial factor since memories become embedded into distributed networks with the passage of time and much of the reviewed studies are focused on the retrieval of FE in the short term. For example, it seems that FE memories are weakly stored into long-lasting engrams and the role of the striatum, thalamus and dorsolateral prefrontal cortex remains to be explored. Added to this, molecular signatures for FE and FE recall are scarce, and some of the conflicting findings may be explained by mechanisms applying only to a subset of neurons e.g. interneurons vs pyramidal neurons. Thus, the polymodal profiling of the studied neurons along with technical improvements in single-cell research will increase our understanding about their role in the micro and macrocircuits that regulate fear learning. Coupled with these theoretical frontiers, the ample methodological differences make it difficult to directly compare studies. Immediate and delayed FE are known to recruit specific molecular machinery, making them not completely interchangeable. Also, most studies submit subjects to non-naturalistic tasks or scenarios and rely solely on freezing response to measure fear learning. Thus, improvements in fear research will be achieved by the measurement of multiple CRs, the standardization and inclusion of subject's hormonal status and the increased use of pathological fear learning animal models. Overall, the analysis of the sex differences in FE can give important insights about possible circuit and molecular dysregulations underlying the pathophysiology of fear-based disorders.

## Declarations of interest

None.

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# Discussion

One of the main aims of this thesis was to frame the role of PACAP-PAC1R in pathological fear extinction. Also, we pursued to delineate the extent to which sex and sex hormones are implicated in the processing of FE and traumatic memories.

We report that IMO exposure induces in impairments in FE in male and female mice. In females, IMO-FC-FE upregulated PACAP-PAC1R mRNA transcripts in the amygdala and hypothalamus after one FE session. Further, the impairments in FE observed after 4 sessions were associated with lower activations in amygdala PACAP+ neurons and a persistent activity of PACAP+ neurons in the VMHdm. A brain circuit connecting the MeA and VMHdm, which is part of a hypothalamic defensive circuit, was identified as necessary for the upregulation of PACAP in the amygdala and hypothalamus and the appearance of the impairments in FE. The inhibition of this circuit during IMO prevented the detrimental consequences of trauma. In that way, this thesis delineated a circuit-mechanism by which PACAP is implicated in the transformation of an acutely stressful experience into long-lasting changes in behavior. In addition, it was concluded that hormonal levels, especially high estrogen states, are related to improvements in FE memory consolidation. We also confirmed our hypothesis about the null effect of high-or-low hormonal states over the appearance of more severe posttraumatic behavioral phenotypes in female mice and women.

## IMO is a strong emotional stressor in female mice

Our results showed that IMO is a strong and intense emotional stressor capable of inducing FE impairments in females. Previous research showed that acute intense stressors could result in differential effects based on sex (Baran et al., 2009; Olave et al., 2022). While exposure to naturalistic stressors resulted in sex-divergent effects, exposure to intense psychological stressors like IMO induced impairments in line with our findings (Gagliano et al., 2014; Pooley et al., 2018). One strength of our behavioral paradigm that its ability to induce impairments in FE in mice mirror the ones in humans with PTSD, both showing an adequate acquisition of FE memories but with difficulties to maintain them through time (Wicking et al., 2016). In addition, alterations in bodyweight are related to the intensity of the stressor and we found that IMO was equally intense in both sexes as both showed a decreased weight gain between IMO and FC days (Gagliano et al., 2014; Márquez et al., 2002). The impairments in FE in females after IMO

exposure were evidenced from the first FE session but no differences were detected during FC, pointing at a possible sensitization of fear circuits by stress. With these results, we expand previous findings showing that IMO can alter fear expression in male mice and demonstrate that this model can be used to study long-lasting alterations of FE in female and male mice (Andero et al., 2014, 2013, 2011). Notably, we observed attenuations in the effect of IMO over FE and body weight in females that were monitored for their estrous cycles. This effect is possibly related to the repeated manipulations to which these mice were exposed because of the repeated vaginal cytologies, with the consequent habituation of their stress responses (Martí and Armario, 1998; Ueno et al., 2020).

## *Adcyap1-Adcyap1r1* are upregulated during FE in females

We found that PACAP-PAC1R gene transcripts are upregulated after a fear extinction session in the amygdala and hypothalamus. Previous research in males demonstrated that FC was related to an upregulation of *Adcyap1r1* and *Tac2* transcripts in the amygdala and that IMO could further increase transcript levels (Andero et al., 2014; Ressler et al., 2011). In females, it was known that proestrus and exogenous estradiol can increase *Adcyap1r1* in the BNST which was also observed after FC in ovariectomized female mice (K. Mercer et al., 2016; Moore et al., 2005; Ressler et al., 2011). Besides these two studies, PACAP-PAC1R regulation in female mice remained largely unexplored. Our results attempted to fill this gap by providing a biological correlate in naturally cycling females for the *Adcyap1-Adcyap1r1* regulation and the altered FE observed after traumatic exposure.

Based on the literature, we were expecting to find upregulations in PACAP-PAC1R function in brain structures processing fear and stress. The brain areas to study were the PFC, amygdala, PAG and hypothalamus. Initially, we included the hippocampus as an area of interest but due to methodological difficulties it was not further explored. Also, we didn't include the BNST in the mRNA levels assay because of its small size and the methodological difficulties to obtain a precise sample. In the results we found increases in *Adcyap1r1* in the amygdala during fear expression, but not in the PFC, which replicated the findings from previous research in males (Andero et al., 2014; K. B. Mercer et al., 2016; Ressler et al., 2011). Notably, the increases of *Adcyap1* and *Adcyap1r1* observed in the hypothalamus suggested that this neuropeptide system

may be playing an important role in the appearance of the long-lasting consequences of stress. In the amygdala, we observed an increase of *Adcyap1* but without reaching statistical significance. This lack of significance was related to an increased variability in *Adcyap1* transcripts that may reflect a differential regulation at amygdala subnuclei. Alternatively, this may be associated to a previously unknown hormonal-dependent regulation of *Adcyap1* in the amygdala. We also tested for associations between the *Adcyap1-Adcyap1r1* transcript levels with freezing during FE1. We found moderate significant correlations in the PAG for freezing with *Adcyap1r1*, and near significant with *Adcyap1* (p=0.052) that were independent of the treatment group. This result is somewhat expected as neuronal activity in the PAG is related to increased freezing behavior and increases in mRNA transcripts of this neuropeptide system are related to enhanced neuronal activity (Tovote et al., 2016).

## *PACAP*+ neuronal activity is altered in females with IMO and correlated to freezing levels

We observed that PACAP+ neurons are hypoactive in the amygdala and hyperactive in the VMHdm of IMO females and that their activity is correlated to freezing levels. With the mRNA results, we demonstrated that IMO primes PACAP-PAC1R for overactivation during FE. However, human studies demonstrate the implication of this neuropeptide system in the appearance of chronic and sustained posttraumatic symptoms (Ressler et al., 2011). To explore PACAP-PAC1R's implication in the longer term, we analyzed PACAP+ neuronal activity after 4 FE sessions. We used an immunofluorescence study and colocalized PACAP neurons with neuronal activity markers c-Fos (recent activity) and FosB/ ΔFosB (repeated activity) (Nestler, 2015). Based on our hypothesis, we selected brain structures based on their implication in the processing of stress and FE but also included areas that could be acting as integration nodes (Maren and Holmes, 2016; Milad and Quirk, 2012; Bianca A Silva et al., 2016). We found that PACAP neurons were widely distributed in the limbic system and hypothalamic structures but with variable degrees of expression. For example, most of the neurons in the VMHdm were PACAP+, while only a minority were PACAP+ in the BLA. The immunolabeling observed for PACAP was confirmed by contrasting with the results of previous studies (Lein et al., 2007;

Vaudry et al., 2009). We performed a validation of the PACAP antibody by injecting an adenovirus in the hypothalamus and amygdala which induced a conditional KO.

Overall, we observed that IMO exposure resulted in lower recent activity (PACAP-c-Fos+ neurons) in the hippocampus-amygdala complex after the 4$^{th}$ FE session. Although, this difference only reached statistical significance in the BLA and MeA. It's known that activity in the amygdala and the hippocampus is necessary for the creation of new associative cue and context-dependent extinction memories (Maren and Holmes, 2016). Moreover, the activity in the PFC- IL, which is thought to trigger FE learning, was unaltered between IMO and control groups. Overall, suggesting that IMO females have an adequate engagement of the IL that triggers the formation of FE memories, but they experience a lower engagement of structures necessary for their stabilization, storage and expression (Maren and Holmes, 2016). Also, the lower activations of PACAP+ neurons in the amygdala during FE may reflect a signature of trauma. Although, they could also be related to an adaptive mechanism of stress sensitization by which traumatic stressor tunes down the function of both structures to facilitate the appearance of passive threat responses.

IMO exposure also resulted in greater activity of the VMHdm during FE compared to control females (PACAP-FosB/ ΔFosB+). This increased activity likely reflects persistent and repetitive activations of the VMHdm throughout FC and the 4 FE sessions. The VMHdm is embedded within a hypothalamic defensive circuit for which its behavioral correlates have been delineated for responses to predator and conspecific threats (Silva et al., 2016). Other studies have found that increased activity in the VMHdm is related to enhanced and persistent defensive responses and an aversive internal state (Kennedy et al., 2020; Wang et al., 2015). These persistent responses to threat have been hypothesized to arise from a mixture of recurrent hypothalamic excitatory circuits and upregulation of neuropeptide function (Kennedy et al., 2020). Therefore, the persistent activity we found in PACAP+ neurons of the VMHdm is likely related to persistent freezing responses to threat (cue) and which may be explained by the combination of intra-hypothalamic recurrent circuits and the dynamics of PACAP regulation after IMO. We also detected lower PACAP-FosB/ ΔFosB+ in the PAGvl which was somewhat unexpected as greater activity in this structure is related to greater freezing levels, this result

may be explained by the existence of different neuronal subtypes controlling escalating defensive responses (Tovote et al., 2015).

We colocalized the two activity markers with PACAP (PACAP-c-Fos+ & FosB/ ΔFosB+) finding similar results: lower activity in the BLA and MeA, and greater activity in the VMHdm. In addition, we performed correlations for freezing levels during the whole FE sessions and found a strong negative correlation for freezing levels with PACAP-c-Fos+ cells/ mm2 in the MeA (r=-0.716) and a strong positive correlation with PACAP- FosB/ ΔFosB+ cells/ mm2 in the VMHdm (r=0.715). Altogether, these results pointed us that the interplay between the MeA and the VMHdm was possibly related to the greater freezing levels observed during FE. Also, we confirmed our hypotheses about the implication of the PACAP-PAC1R system in the regulation of neuronal activity in brain structures related to fear and stress processing.

## The inhibition of a MeA to VMHdm circuit rescues the impairments in FE and PACAP upregulation

Previous research indicates there is a strong connectivity from the MeA to the VMHdm and some neuronal projections from the VMHdm to the MeA (Canteras et al., 1994; Pardo-Bellver et al., 2012). Further, the MeA is strongly activated by emotional stressors, especially IMO, and it is implicated in the appearance of threat responses (Dayas et al., 1999; Bianca A Silva et al., 2016; Úbeda-Contreras et al., 2018). For these reasons, we hypothesized that the activation of the MeA during IMO could be relaying strong signals to the VMHdm which could trigger a persistent aversive internal state (Kennedy et al., 2020). Given the reciprocal connections, we decided to approach this circuit bidirectionally using a highly specific chemogenetic approach. This approach combines a retrograde-Cre viral vector with an adenovirus that inserts a DREADDs inhibitory receptor. Further, DREADDs inhibitory receptors are activated by the injection of CNO. This approach allowed us to manipulate (inhibit) specific neurons projecting to the structure where the retrograde-Cre virus was inserted. Our hypothesis sustained that high activity in the MeA drove sustained increases in hypothalamic function. To test this, we inhibited this circuit during the exposure to IMO and then subjected the mice to the FC-FE

protocol. This approach was chosen due to its high specificity, its capacity to establish a causal mechanism, and its previous experience in our laboratory.

Our results showed that the inhibition of MeA to VMHdm projections rescued the impairments in FE elicited by IMO exposure. In this case, our controls were animals undergoing surgery as well but receiving a DREADDs control virus that does not insert the artificial inhibitory receptor but a reporter. Notably, the inhibition of VMHdm neurons projecting to the MeA did not result in any significant change in freezing levels. With this result, we proved the necessity of this circuit for the appearance of a posttraumatic behavioral phenotype in mice after IMO and confirmed our initial hypothesis about MeA hyperactivity. Other researchers that used inescapable foot shocks as a stress model, found that the synapses from MeA to VMH projecting neurons underwent synaptic potentiation, and that this potentiation further facilitated the appearance of learned aggressive behaviors (Nordman et al., 2020). Our results are in line with this study as we found that a defensive behavior (freezing) was potentiated by the increases in the activity of the MeA to VMHdm circuit. Nevertheless, we were not able to study molecular markers of synaptic plasticity.

This finding is novel in the sense that the hypothalamus is long known to be an integration node for stress processing, but its relation to fear learning and fear extinction was previously unknown. Moreover, this circuit is essential for the sensitization of the stress response in the face of IMO which may be related to the integrative role of the MeA, processing olfactory inputs and acting as a positive regulator of the HPA axis (Cádiz-Moretti et al., 2016; Ma and Morilak, 2005). Studies have shown that the MeA is involved in the expression of fear-potentiated startle and neuroendocrine responses to discrete cues, but not freezing (Nader et al., 2001; Walker et al., 2005; Yoshida et al., 2014). Nevertheless, MeA inputs to the VMHdm are essential for processing predator and conspecific cues and can trigger freezing responses (Li et al., 2004; Pérez-Gómez et al., 2015; Bianca A Silva et al., 2016). The modulation of fear by the MeA is carried in conjunction with the BLA. The BLA has a role in the consolidation of memories and the MeA in the retrieval of fear and its contextual modulation (Takahashi et al., 2007). Our results contrast with previous research on cued-FC where the role of the MeA was not found to be necessary for freezing (Nader et al., 2001). However, in these paradigms, the auditory cue

was isolated, and the fear circuits were not previously sensitized by exposure to an acute intense stressor. Therefore, our findings suggest that the MeA may be recruited after stress to modulate fear responses. The potentiation of the fear circuitry's function by MeA may be secondary to its function as modulator of the HPA axis response, its interconnectivity with the BLA, or to an enhanced processing of contextual cues.

With our chemogenetic approach, we performed a non-PACAP selective manipulation of MeA to VMHdm circuits. Thus, we wanted to test whether the mechanism underlying the increased freezing responses during FE could be related to an increase in VMH activity facilitated by the neuropeptide PACAP (Kennedy et al., 2020). Given the association of increased PACAP levels and enhanced neuronal activity (Vaudry et al., 2009), we hypothesized that the persistent activity in the VMHdm may be associated with increases in PACAP. For this, we inhibited MeA to VMHdm circuit during IMO and quantified PACAP regulation shortly after IMO (90 min). Our results showed that animals that had the circuit inhibited had lower levels of PACAP in both the MeA and the VMHdm, suggesting that the upregulation of PACAP by IMO was dependent on the activity of the neurons in this circuit. Moreover, we found that the lower levels of PACAP were related to an overactivation of the MeA as measured by PACAP-c-Fos+ neurons, but not in VMH-projecting MeA neurons. This finding was somewhat unexpected but may be explained by the disruption of intra-amygdala inhibitory circuits by DREADDs manipulation, which could have resulted in the overactivation of other non-VMH-projecting MeA neurons (Ehrlich et al., 2009; Whittle et al., 2021). The neuroendocrine correlates of this inhibition remain to be explored.

We also detected that MeA to VMHdm circuit inhibition was able to influence neuronal activity in a brain-wide fear and stress circuitry. Lower neuronal activity in PACAP+ and non-PACAP+ neurons were detected in the anterior hypothalamus and medial anterior BNST, which are implicated in persistent anxiety states and the regulation of HPA responses respectively (Anthony et al., 2014; Duvarci et al., 2009). Our study was focused on females, and we did not find literature reporting sex differences in PACAP regulation after stress. Therefore, we measured PACAP levels in MeA and VMHdm under basal conditions and shortly after IMO (90') in males and females, but without finding significant differences. Thus, this suggested that

PACAP undergoes similar upregulation after IMO regardless of sex. In addition, we performed an immunohistochemistry study labeling glutamatergic neurons and found them to be highly enriched in the VMH. It is known that PACAP can increase glutamatergic function, but the role of this neurotransmitter system in the adaptations of MeA to the VMHdm circuit shall be studied in the future (Cho et al., 2012; Resch et al., 2014).

In sum, our results point to the crucial role that the MeA to VMH circuit plays in the appearance and maintenance of a posttraumatic behavioral phenotype characterized by impairments in FE. The capacity of this circuit to modulate fear responses may be related to its role in integrating environmental, homeostatic, and internal states. The persistent activity in the VMH is related to the maintenance of an aversive internal state that facilitates the appearance of defensive threat responses. Notably, some human research has found that both of these structures are essential for the processing of threats and the VMH has been implicated in the appearance of panic attacks (Alvarez et al., 2008; Wilent et al., 2010). PACAP plays a key role in these structures and facilitates the appearance of behavioral adaptations in the face of intense stressors. The sensitization of this circuit seems to rely on enhanced PACAP signaling but its interaction with other neurotransmitter systems remains to be determined by future studies.

## The risk genotype in the ADCYAP1R1 is related to impairments in FE in women

Given the aforementioned results in female mice and the wide support for PACAP-PAC1R dysfunction playing a role in PTSD in women, we hypothesized that the risk genotype in the *ADCYAP1R1* SNP rs2267735 would be related to impairments in FE. To explore this, we genotyped a cohort of traumatized women that had undergone a FC immediate-FE task that quantified fear-potentiated startle as a measure of fear. This cohort of women was part of a larger pool of subjects receiving medical care for general or psychiatric-related illnesses. Other studies in this cohort had already demonstrated that women with high blood levels of PACAP had greater posttraumatic symptoms and enhanced dark startle reactivity (Ressler et al., 2011). Also, previous reports showed that the risk genotype could predict better the posttraumatic symptoms if the history of trauma exposure was considered in the analyses (Almli et al., 2013).

For this reason, we decided to further divide women into low-trauma and high-trauma exposure using the scores of the Childhood Trauma Questionnaire.

Our initial analyses showed a trend for higher startle magnitudes during early FE in women with the risk genotype. We performed separate analyses by age (cutoff 40 years old) because previous reports showed that the vulnerability conferred by the risk genotype is related to estradiol dynamics (K. B. Mercer et al., 2016). The results we observed confirmed the hypothesis showing greater fear measures (fear-potentiated startle magnitudes) in the early phase of FE but only for women with a history of multiple trauma exposure and in the younger age group. The increase of fear-potentiated startle responses in the older age group did not reach statistical significance but remained trend level. These results are in line with the reports suggesting an interaction of estradiol with the risk genotype in *ADCYAP1R1*, but we could not discard that other unknown age-related variable may be influencing this effect as well. This risk genotype has been associated with increased hyperarousal symptoms (Ressler et al., 2011). According to the dimensional categorization of the behavioral alterations in PTSD patients, the impairments in FE we found could be cataloged under the negative valence domain which is also related to symptoms like avoidance, psychologically distressing reactions to cues, and hypervigilance (Fenster et al., 2018).

## Trauma experienced at different hormonal states results in a similar posttraumatic phenotype in women and female mice

We hypothesized that sex hormones would be able to modulate brain function under mildly stressful conditions, as shown in prior studies of FE (Lebron-Milad and Milad, 2012). However, traumatic stressors would induce such an intense activation and recruitment of stress and fear circuits, that phasic modulations by sex hormones would be obscured by ceiling effects, as an insufficient response under these conditions may compromise survival.

We were able to address this question using a translational approach. In female mice, we used the IMO-FC-FE model taking freezing response as the outcome measure. In women, we inquired about the intensity of posttraumatic symptoms at 3 weeks post-trauma. Our results in female mice showed that experiencing IMO during proestrus (high hormones) or metestrus (low

hormones) resulted in similar impairments in FE as evidenced by overall greater freezing levels in the IMO group. We found a slight interaction of cycle with IMO during FC that did not change the interpretation of results and likely arose from the high variability within subjects. We also observed that the increases in freezing response and decreases in weight gain were attenuated compared to females without cycle monitorization. We assume that this attenuation of the effect of stress is secondary to the habituation of the stress response due to repeated manipulations during vaginal cytologies (Martí and Armario, 1998; Ueno et al., 2020).

Another possible source of vulnerability could arise from differential regulation of the HPA and HPG axis after trauma. We knew from previous reports that basal and stress-induced increases in corticosterone concentrations are higher during the proestrus phase (Figueiredo et al., 2002; J. P. ter Horst et al., 2012). However, higher basal cortisol levels are observed during the follicular phase in humans, and the greatest increases in anticipatory stress occur in the luteal phase (Collins et al., 1985; Montero-López et al., 2018). These results evidence a dissociation between animal and human literature, to remind us of that estrous and menstrual cycle phases are similar but not equivalent. This also suggests that high progesterone levels are associated with greater corticosterone release under stress.

Thus, we assumed that IMO during proestrus (high estradiol, high progesterone) could be related to greater increases in corticosterone. Ideally, we should have performed a time course of corticosterone dynamics and measured it at several time points. However, given the negative influence of the hormonal cycle upon behavior and the economic and ethical limitations that this would imply, we only measured it in one-time point (60') after IMO. To our advantage was the state-of-the-art technology that allowed us to get the most accurate measurements and to include some corticosterone metabolites to map the possible enzymatic pathways affected. The results we obtained were not able to reject the null hypothesis. We found a slight non-significant difference in corticosterone levels but that favored greater corticosterone levels after IMO during metestrus and not proestrus. No differences were observed for corticosterone metabolites. This result contrasted with previous findings in animal research where greater basal and stress-induced corticosterone levels in proestrus were reported (J P ter Horst et al., 2012). This discrepancy could have resulted from the comparison of findings in different species, we

work with mice while most studies use rats, but is also possible that we failed to capture the differences because we took a single time point measurement.

When analyzing the regulation of sex hormones after IMO, we found that basal progesterone levels were higher during metestrus and underwent a sharp downregulation shortly after IMO. This result was also at odds with previous fear and stress research, which pointed to greater basal progesterone levels during proestrus (Lebron-Milad and Milad, 2012). In addition, our results showed greater basal testosterone a trend for greater estradiol levels in proestrus, which is in line with the literature about the rodent's estrous cycle (Nilsson et al., 2015; Pfaus et al., 2015). The discrepancies found in our mice and fear literature could be explained by two factors. First, the studies in the field of fear assume that high progesterone occurs only during proestrus when it actually has a second peak during metestrus (Lebron-Milad and Milad, 2012; Nilsson et al., 2015; Pfaus et al., 2015)**.** It is possible that this finding was overlooked in previous research, but it could also have been simplified to fit a priori-hypotheses. Rodents have a differential regulation of the corpus luteum, hence different progesterone dynamics than humans, and this biological difference is usually not considered in the studies of rodent fear memory (Lebron-Milad and Milad, 2012)**.** Second, early proestrus has high estradiol and late proestrus has high progesterone, and this raises the possibility that our sample of mice was composed mainly of females in the early proestrus phase.

In sum, HPA axis regulation seems to be similar at 60 min post IMO regardless of the estrous cycle phase. However, sex hormone levels undergo a differential regulation after IMO that is likely related to the changing basal levels during the estrous cycle. Studies in rodents in our lab showed a neuropeptide-dependent differential regulation of estradiol and testosterone levels was related to stronger and weaker fear memories respectively (Florido et al., 2021b). However, further research is needed to clarify whether the differential sex hormone regulation observed after IMO is related to differences in other types of behavior appearing after trauma.

To extend these findings in humans, we evaluated the intensity of posttraumatic symptoms in women 3 weeks after exposure to sexual abuse. Women were allocated based on the last menstrual period date in one of the three phases of the menstrual cycle: early follicular (low hormones), late follicular (high estradiol), luteal (high estradiol, high progesterone). The late luteal phase is of special interest because of the increased risk of psychopathology, but we were

unable to include this group because of its short span and the low number of subjects (Handy et al., 2022)**.**

By analyzing the effect of the menstrual cycle phase over posttraumatic symptoms we found convergent results with animal data. The experience of sexual abuse during a specific phase of the menstrual cycle was not associated with worse posttraumatic symptoms at 3 weeks. Moreover, we confirmed that women that further developed PTSD had greater posttraumatic symptoms at the 3-week assessment. Previous research was unable to establish whether the menstrual cycle phase at trauma was relevant for the development of a more severe phenotype. One study that explored this relationship allocated women into follicular and luteal phases, but they were unable to group specifically women with low hormonal levels. Further, they introduced bias in their analyses by including women in menopause and with oral contraceptives into the experimental groups (Bryant et al., 2011).In this study, they reported that women suffering trauma (motor vehicle accidents) during the luteal phase had more flashbacks in further evaluations but without increasing overall PTSD severity. Our findings are in line with this last result, in the sense that we did not find overall greater posttraumatic symptoms related to any of the menstrual cycle phases. However, greater flashbacks during the luteal phase were not present in our cohort. When controlling for other peritraumatic variables, we found that being conscious during sexual abuse was related to greater re-experiencing symptoms. In our cohort, 18% of women that completed follow-ups were diagnosed with PTSD. However, this number must be taken with caution since 25% of women were lost during follow-ups and this result may be confounded by attrition. Also, we tested for the association of the menstrual cycle phase with PTSD diagnosis and found non-significant associations. Nevertheless, this finding has to be taken with caution because dividing the small number of PTSD women into cycle phases resulted in a low number of subjects per group and a loss of statistical power.

Altogether our results in animals and humans are in line with our initial hypothesis about the null contributions of the hormonal cycle phase during trauma for the development of a more severe posttraumatic behavioral phenotype. Our measured outcomes included freezing levels during FE and the intensity of posttraumatic symptoms at 3 weeks, future studies may extend our findings and explore the effect of the menstrual cycle phase in other behavioral domains altered by trauma.

## The sex differences in FE have structural and functional correlates

The study of the sex differences in FE becomes relevant given the pathophysiologic and therapeutic role that FE carries for stress and fear-based disorders. There are contradictory findings in the literature about the role of sex in FE. Despite a widespread consensus in the field about the positive role of estradiol for FE, this premise has been unable to be applied to the clinics for the benefit of patients (Lebron-Milad and Milad, 2012). The sex differences in FE may arise under very specific methodological and individual conditions. This systematic review aimed to clarify the discrepancies in the field by analyzing the sex differences in FE in the context of brain structure and function. Our initial hypothesis sustained that sex hormone were able to influence FE by differentially modulating brain activity and neurotransmitter function. Results from other works in the field have provided some idea about where is that these sex differences arise, also providing some functional correlates for them (Lebron-Milad and Milad, 2012; Milad and Quirk, 2012). Still, there is much to add to these findings as FE is a complex behavior arising from the interplay of several brain circuits and facilitated by aversive internal states.

Sex hormones can shape brain structure and function by determining sexo-dimorphic circuits in the immature brain and by regulating brain function in the mature brain (Wallen, 2005). For this review, the focus was on the study of the activation effects of sex hormones over mature brain structures rather than the structural changes facilitated by them during development. Considering the literature of sex differences in FE, the results of females taking cued-FC paradigms point in all directions, while the contextual-FC paradigm shows that females have overall less fear than males. Also, estrous cycle phases with high estradiol and doses of exogenous estradiol were found to be beneficial for FE and FE recall in cued-FC paradigms but are largely unexplored in contextual-FC paradigms. In humans, men and women learn and extinguish fear similarly, but the differences arise during FE recall. Nevertheless, the overall picture is obscured by contradictory findings between absolute hormonal levels and menstrual cycle phases. The positive effects of estradiol are only seen in studies using sample split approaches which create two groups, high estradiol vs low estradiol, based on arbitrary thresholds.

Our analysis showed that the sex differences in FE are associated with changes in brain function and brain reactivity. In the classical model of FE, the amygdala is a central structure processing sensory and aversive contingency. FE learning relies on top-down control of amygdala reactivity, for which the IL to BLA circuits is thought to be essential. Additionally, the hippocampus activity is tuned to contextual cues leveraging the expression of fear or extinction memories (Herry and Johansen, 2014; Milad and Quirk, 2012). Previous work had noted the strengthening of IL to BLA synapses by estradiol as the potential source for improved FE recall (Lebron-Milad and Milad, 2012). However, there are changes in neuronal signaling at a whole brain level rather than a single circuit. Low estradiol promotes greater activations of the stress and fear circuit which includes the Prl, amygdala, and hypothalamus, which are structures essential for fear expression (Goldstein et al., 2010). These effects are mirrored in FE studies showing that greater activity in these structures is associated with greater fear measures (Hwang et al., 2015; Lebron-Milad and Milad, 2012; Zeidan et al., 2011). Nevertheless, these findings are characterized by their methodological limitations and contrasting findings which show for example high activations in both fear-promoting and fear-inhibiting prefrontal structures during FE (Hwang et al., 2015; Zeidan et al., 2011). Animal studies have suggested that an estrogen-dependent modulation of hippocampal spines in females may be related to the sex differences in FE (Gupta et al., 2001). Therefore, fear memories may be predominantly expressed during low estrogen states by the interaction of low signaling in the hippocampus coupled with an enhanced reactivity of the stress circuit.

Shifting hormonal levels can promote different functional states in females that are followed by cyclical changes in neurotransmitter function. The PFC is an important node undergoing shifting modulation by monoamines throughout the menstrual/ estrous cycle phases. This is exemplified by studies showing that increases in dopaminergic signaling enhance PFC function during low estrogen phases, but impair it during high estrogen phases (Jacobs and D'Esposito, 2011; Rey et al., 2014). Similarly, increases in serotoninergic function are associated with improvements in FE but only during low estrogen phases (Lebrón-Milad et al., 2013). This effect in the shape of an inverted U has been described previously for the effects of stress on several neurobiological endpoints (Sapolsky, 2015). According to some researchers, the sex differences in FE may be also associated with pre-existing wiring and functional patterns between males and females that are further modulated by changing hormonal levels (Frankfurt

et al., 1984). For example, differences in glutamatergic function make males rely completely upon this neurotransmitter for FE memory consolidation while females with arrests in glutamatergic function are still able to consolidate FE (S Maren et al., 1994; Monfort and Felipo, 2007). Another example is the sexo-dimorphic regulation of the locus coeruleus in the face of stress, the main noradrenergic nucleus. Noradrenaline signaling is essential for the upregulation of signaling cascades that enhance neuronal function and promote memory consolidation (Rodriguez-Romaguera et al., 2009). Under arousing conditions, CRF signaling in the locus coeruleus of females induces sustained noradrenergic signaling due to a decreased ability to desensitize CRF1 receptors (Bangasser and Wicks, 2017). Moreover, cyclic estradiol levels can induce changes in noradrenaline synthesis and the internalization of adrenergic receptors (Bangasser and Wicks, 2017). Therefore, this neurotransmitter system seems crucial for the appearance of sex differences in the behavioral and endocrine responses to arousing conditions.

After analyzing the literature about the influences of the estrous/ menstrual cycle and hormonal levels over FE we observed that proestrus seems to be associated with lower freezing rates during cued-FER in rodents, but the effects of the menstrual cycle in humans are not conclusive. This divergence arises from the inherent limitations in these studies including the use of different methodologies, a lack of systematic segmentation into menstrual cycle phases, the use of different control groups to detect experimental effects (EF vs ML; EF vs LF), and the use of heterogeneous measures and indexes to assess FE memory. In addition, menstrual cycle effects are not reported or tested systematically possibly because the analyses are secondary to the main hypothesis and rely on low sample sizes. Notably, the effects of the menstrual cycle phase over FE seem subtle and influenced by actual or prior stress exposure (Antov and Stockhorst, 2014; Blume et al., 2017; Goldstein et al., 2010, 2005; Hwang et al., 2015; Zeidan et al., 2011)

Estradiol emerged as a strong modulator of FE memories with important effects on their consolidation, but also promoting their encoding and retrieval (Graham and Daher, 2016; Graham and Milad, 2013; Milad et al., 2009a; Zeidan et al., 2011). Individuals with high estrogen states show greater inhibitory control that is related to enhancements in prefrontal and hippocampal function along with facilitated long term potentiation. In turn, low estrogen states are detrimental to FE consolidation affecting healthy and traumatized populations (Glover et al., 2013; Graham and Milad, 2013; Milad et al., 2010; Wegerer et al., 2014; Zeidan et al.,

2011). Estrogen exerts its actions through intracellular signaling and gene transcription, but fast-paced responses are also triggered by membrane receptors. The interaction of estradiol with other signaling systems may account for the greater likelihood of women experiencing adverse and long-lasting consequences after stress. Progesterone levels seem to influence FE by enhancing the positive effects of estradiol on FE under constrained circumstances (Graham and Daher, 2016; Milad et al., 2009a). Still, much research remains ahead to determine the exact contribution of progesterone to FE. Progesterone effects are related to a specific time-course of events and the actions by its metabolite allopregnanolone can modulate circuits in opposite directions. Testosterone seems to exert a positive influence over FE. It can decrease conditioned responses in males and females but also improve FE memory consolidation, especially in the early phases (Graham and Milad, 2013; Maeng et al., 2017). The mechanisms of its effects include direct actions through androgen receptors and its aromatization to estradiol (Melmed et al., 2019). However, some studies suggest that these effects could arise from its interaction with glucocorticoids and the modulation of the stress response system (Josephs et al., 2017; Pace-Schott et al., 2015). The chronic intake of oral contraceptives can cause structural and functional changes that can lead to impairments in FER (Graham and Milad, 2013; Petersen and Cahill, 2015; Wen et al., 2021; White and Graham, 2016). Notably, these impairments can be rescued by restoring estrogen signaling before FE (Graham and Milad, 2013). Furthermore, the effects of OC over FE are not exclusive to fear processing but are included in the context of an overall increased emotional reactivity and impaired emotional processing (Figure 9).

## Limitations

We must acknowledge that the main experimental findings from this thesis come from a well-controlled laboratory model of fear learning under non-naturalistic conditions. The animal model of PTSD we used has high face validity, but its construct validity remains low. With our findings, we improved its construct validity by providing evidence about PACAP implication in FE impairments in both humans and female mice after IMO. One inconvenience of this model is that the posttraumatic behavioral phenotype was measured using central tendency statistics in all subjects, while in humans only a minority of individuals exposed to trauma and expressing enduring symptoms of PTSD are measured (Breslau et al., 1998; Hendriksen et al., 2014).

Future researchers may explore our findings according to individual responses to threats in mice (Sillivan et al., 2017).

In our model, we only tested for alterations in FE after IMO. However, IMO exposure affects other domains of behavior as demonstrated by other works in our laboratory (Wingo et al., 2018). We didn't explore measures of hyperarousal or sympathetic activity, which could have revealed associations with the neuronal activity in the MeA (Fenster et al., 2018). In addition, for the PACAP-PAC1R study we focused specifically on females because of the unknown physio-pathological mechanisms of their stress maladaptation. Future studies shall explore whether our findings about the role of MeA to VMHdm circuit in FE apply to males as well. The chemogenetic manipulation we performed was successful in rescuing the impairments in FE and PACAP upregulation, but it was not specific to PACAP neurons. We described how around 80% of the infected cells in the MeA also expressed PACAP immunolabeling. For the study of the brain regulation of PACAP, we focused on structures known to be relevant for fear and stress processing, although we did not perform a full brain-wide scan. Relevant areas that remained to explore include the anterior parts of the hypothalamus, several subnuclei in the BNST, premamillary nuclei, and the septo-hippocampal system.

For testing whether there was a window of vulnerability for trauma during the menstrual cycle we selected only the two most representative phases of the estrous cycle. Also, we used the last menstrual period date to allocate women into the menstrual cycle phase and this method may be contaminated by a recall bias. Further, we had to exclude a proportion of women reporting irregular cycles and near-in-menopause, making it possible that our results are suited specifically to young-normally cycling women only. Finally, the results obtained with sex hormone measures after IMO are puzzling and probably explained by the blood sampling taking place early in the proestrus phase. Still, given that we only explored hormonal regulation at one-time point we were not able to prove or discard this possibility.

## Areas of future research

Future studies still have much to add to our understanding of the sex-biased prevalence of stress and fear-based disorders. With this work, we attempted to elucidate one mechanism implicated

in stress and fear-based disorders by decomposing a complex phenomenon into its principal components and neuronal correlates. Future studies that use this experimental approach will contribute to the identification of the affected pathways across diagnostic boundaries by which mental disorders arise (Cuthbert, 2014). Still, much more precision can be achieved by the combination of tools that allow for the manipulation of specific neuronal profiles with others that track neuronal dynamics in vivo. The identification of the affected brain circuits and their behavioral correlates will likely provide pharmacological targets for the development of novel treatments and help to understand how maladaptive processes arise. Also, the inclusion of sex and hormonal cycles in research will delineate the conditions under which positive or negative effects on behavior are seen. For example, basic research suggests that drugs that increase BDNF or cannabinoid signaling may be beneficial for FE in both sexes, but males may benefit from a GnRH agonist while females will do it by an ER-β agonism during naturally low estrogen phases (Cover et al., 2014; Maeng et al., 2017; Maeng and Milad, 2015).

The PACAP-PAC1R system is a highly expressed neuropeptide with functions in multiple domains. Most of these effects can be included under the umbrella of increased neuronal activity, neuronal survival, and gene transcription (Vaudry et al., 2009). The tracing of the phylogenetic evolution of PACAP has shown its importance for basic functions in vertebrates and invertebrates (Vaudry et al., 2009). Future studies focused on stress will likely benefit by including measures of PACAP function, as research has demonstrated its important role in the maintenance of HPA function under chronic or persistent stressful conditions. Moreover, more studies are needed to delineate in-vivo its specific interactions with estradiol, so that we can delimitate the mechanisms by which PACAP dysfunction results in a vulnerability for females. In the future, we should be able to predict which individuals are at increased risk for stress and fear-based disorder based on their genetic and environmental information. PACAP genotype, environmental features of trauma, and impairments in FE have emerged as possible biomarkers of stress vulnerability. We provided evidence about a novel brain structure, the VMHdm, which was not previously included in the classical FE circuit. Its increased activity promotes an aversive internal state that hampers normal FE. Future studies exploring the role of other hypothalamic areas, or the BNST, will likely shed light on previously unknown processes contributing to the expression of fear memories that are resistant to extinction.

Regarding FE specifically, cross-sectional studies haven't been able to distinguish if the specific vulnerability for impairments in FE recall arises in all women during low-estrogen phases of the menstrual cycle or if the vulnerability pertains to a subset of women experiencing chronic low-estrogen states or large hormonal shifts. Evidence from rodent studies supports the role of the specific phases of the estrous cycle but these findings have not been replicated in humans. Notably, there are large inter-individual differences in the levels of sex hormones during equivalent phases of the menstrual cycle (Sundström-Poromaa and Gingnell, 2014). Longitudinal studies are needed to answer whether women experiencing large hormonal shifts during the menstrual cycle are at risk for impaired FE. The inclusion of subgroup trajectories into FE analyses may help to answer these questions, improve the translation of findings and enrich our understanding of individual features not evidenced in group analyses (Duits et al., 2021; Galatzer-Levy et al., 2017; Pöhlchen et al., 2020). There are reports of specific trajectory behavioral phenotypes emerging during FC and FE that are more likely associated with specific sex (Gruene et al., 2015; Leen et al., 2021). These trajectory phenotypes found during FE could be compared to the ones observed in traumatized populations, which have shown that sex, along with other individual or environmental factors, are crucial factors in leveraging individuals towards resilience or a chronic disease course (Galatzer-Levy et al., 2018, 2013; Orcutt et al., 2004).

Also, large hormonal shifts in sensible periods of women's life may represent windows of vulnerability or protection (Maeng and Milad, 2015; Rehbein et al., 2021). The female-biased prevalence of stress and fear-based disorders debuts around adolescence, which is an important developmental period with hormonal shifts and a generally impaired FE (Baker et al., 2014; Patton et al., 1996). Notably, more studies are needed to determine the time and mechanisms by which sex differences and the gonadal modulation of FE memory appear, given the contradictory findings of estradiol effects over FE on adolescents and adults (Perry et al., 2020). The influence of estradiol over FE can change during a lifetime, as the positive relationship between estradiol and FE is only observed in nulliparous females but not in women or rodents with reproductive experience (Milligan-Saville and Graham, 2016; Tang and Graham, 2020). Thus, pregnancy can induce permanent brain changes that mitigate the effects of estrogen over FE, but the mechanisms of this effect remain unknown.

The inter-species differences in hormonal cycles call for caution when designing translational studies, especially regarding differences in progesterone dynamics. Studying estradiol and progesterone together, instead of isolating effects to estradiol, will force researchers to consider the temporal sequence of events triggered by hormonal exposure since rapid hormonal shifts could be carrying vulnerability or protection windows, rather than absolute high or low hormonal levels. Still, much research is needed on females since many of the studies to date are focused on male rodents and the findings do not necessarily extend to females or humans.

Finally, there are plenty of opportunities to improve the quality of the evidence from FE studies on sex differences. The inclusion of absolute and relative hormonal measures, or the computation of progesterone/estradiol ratios, can minimize the bias introduced by sample-split approaches (Seligowski et al., 2020). Future studies with larger sample sizes and enough statistical power will allow us to delineate the specific effects of each phase of the menstrual cycle over FE. In consequence, studies approaching these questions with inadequate sample sizes and an uneven distribution of individuals at different menstrual cycle phases among experimental groups will difficult our capacity to compare findings between studies.

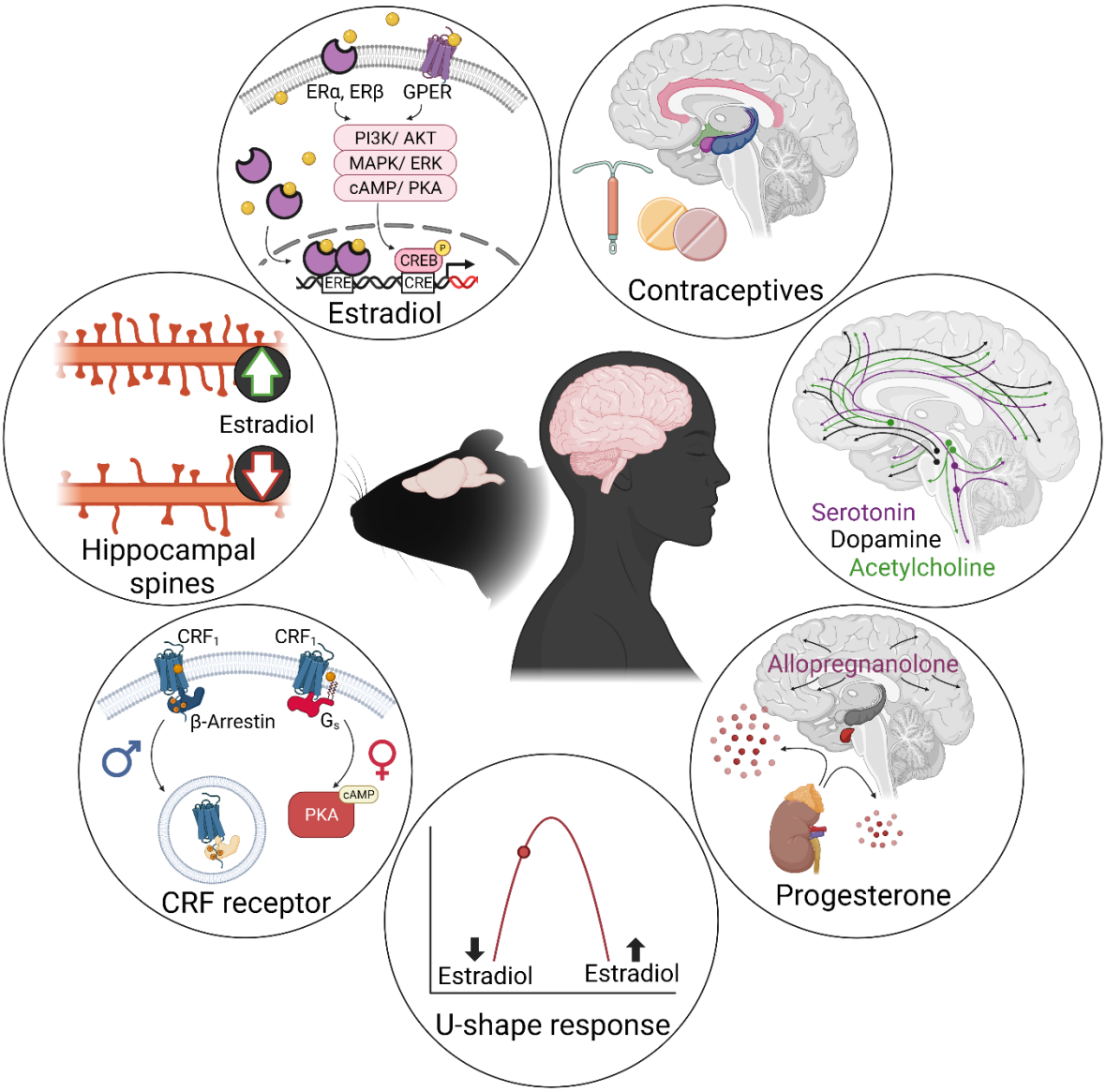


**Fig. 9.** Summary depicting some mechanisms implicated in the sex differences and sex hormone regulation of FE. cAMP: cyclic AMP, cAMP/ PKA: cyclic AMP/ protein kinase A pathway, CREB: cyclic AMP response element-binding protein, CRF1: corticotropin-releasing hormone receptor 1, ERE: estrogen response element, ERα: estrogen receptor α, ERβ: estrogen receptor β, GPER: G protein-coupled estrogen receptor 1, Gs: Gs alpha subunit of heterotrimeric G protein receptor, MAPK/ERK: mitogen-activated protein kinases, extracellular signal-regulated kinases pathway, PI3K/ AKT: phosphoinositide 3-kinase/ protein kinase B pathway, PKA: protein kinase A. **Figure extracted from Velasco ER., et al., (under revision).**

# Conclusions

This work aimed to determine the contribution of the PACAP-PAC1R and hormonal systems over the regulation of stress and fear extinction memories in females. The impairments in fear processing, including FE, are a hallmark of stress and fear-based disorders. However, their mechanisms are just being discovered. Based on the results obtained in this thesis, we can conclude:

1. The PACAP-PAC1R system is implicated in FE impairments in female rodents and women.
2. In female mice, IMO is a strong and intense emotional stressor that induces impairments in FE that are similar to the ones observed in males.
3. The stress-sensitization effect of IMO is specific for FE and not FC, and it is attenuated if females are repeatedly manipulated before stress exposure.
4. The alterations observed during the first FE session are associated with an upregulation of *Adcyap1r1* and *Adcyap1* in the amygdala and hypothalamus.
5. IMO exposure induces alterations in FE throughout 4 sessions that are associated with lower recent activations of PACAP+ neurons in the MeA (PACAP-c-Fos+) and greater repeated activations of PACAP+ neurons in the VMHdm (PACAP- FosB/ ΔFosB+) compared to controls.
6. The projections from the MeA to the VMHdm, which are part of a medial hypothalamic defensive circuit, are necessary for the appearance of impairments in FE and PACAP upregulation after IMO.
7. The inhibition of MeA to VMHdm circuit rescues FE impairments and induces changes in neuronal activity in a brain-wide stress-response circuit that includes the anterior hypothalamus, medial anterior BNST, and Prelimbic cortex.
8. There are no basal or IMO-induced sex differences in PACAP regulation
9. The risk genotype in *ADCYAP1R1* SNP rs2267735 is associated with impairments in early FE in a cohort of polytraumatized and relatively young women
10. Trauma experienced at distinct phases of the estrous/ menstrual cycle is not associated with differences in FE or posttraumatic symptoms.
11. IMO exposure during proestrus or metestrus results in similar regulation of HPA axis hormones 60 minutes after stress exposure, but differences in estradiol, testosterone, and progesterone regulation were detected.

12. Estradiol exerts a positive influence over FE consolidation but the inconsistent methodologies and the failure to account for the time-constrained dynamics of sex hormones have generated contradictory findings in studies using absolute hormonal levels vs estrous/ menstrual cycle phases.
13. The sex differences in FE are associated with a differential regulation of locus coeruleus function, monoaminergic regulation of PFC, glutamatergic mechanisms of FE consolidation, and hippocampal spine formation.

In sum, this thesis used a translational approach to provide information about the biological mechanisms by which females are at increased risk for stress and fear-related disorders. We identified brain structures and circuits where stress and fear memories converge and trigger long-lasting changes in brain function and behavior after trauma. Also, our work sums up the evidence about the deleterious effects of the risk genotype in *ACYAP1R1* over fear and stress in women. The conclusions of our work highlight the importance of continuing the study of the PACAP-PAC1R and supports its use as a biomarker of a maladaptive stress response. Much research remains to be conducted to reconcile the contradictory findings on FE, but promising results suggest that estradiol may be beneficial for vulnerable subjects during FE. Future studies may answer if the improvements of FE by estradiol can be translated into the clinics by for example carrying exposure therapy sessions under high estrogenic states to potentiate its effectiveness.

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# Other scientific activity

**Scientific and review articles**

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**Technical Reports**

Florido, A., Perez-Caballero, L., **Velasco, E. R.,** Molina, P., Marin-Blasco, I., & Andero, R. (2021). Direct and Indirect Measurements of Sex Hormones in Rodents During Fear Conditioning. Current protocols, 1(5), e102. https://doi.org/10.1002/cpz1.102

**Book chapters (pending for publication)**

**Velasco ER,** Florido A, Marin I, Molina P, Perez-Caballero L, Andero R. Stress immobilization inducing fear extinction deficits in male and female mice. Neuromethods (Ed. Springer Nature).

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**Congresses**

PACAP-PAC1R system in females: contributions to fear learning and adaptations to Stressors. **Velasco ER,** Florido A, Flores A, Senabre E, Torres A, Roca A, García-Esteve L, Andero R. FENS Virtual Forum Neuroscience 2020. Glasgow, Escocia. 07.2020.

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**Outreach Activities**

2021, 2022 Barcelona International Youth Science Challenge, Fundació Catalunya la Pedrera. The neurobiology of trauma: effects on behavior and brain function.

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