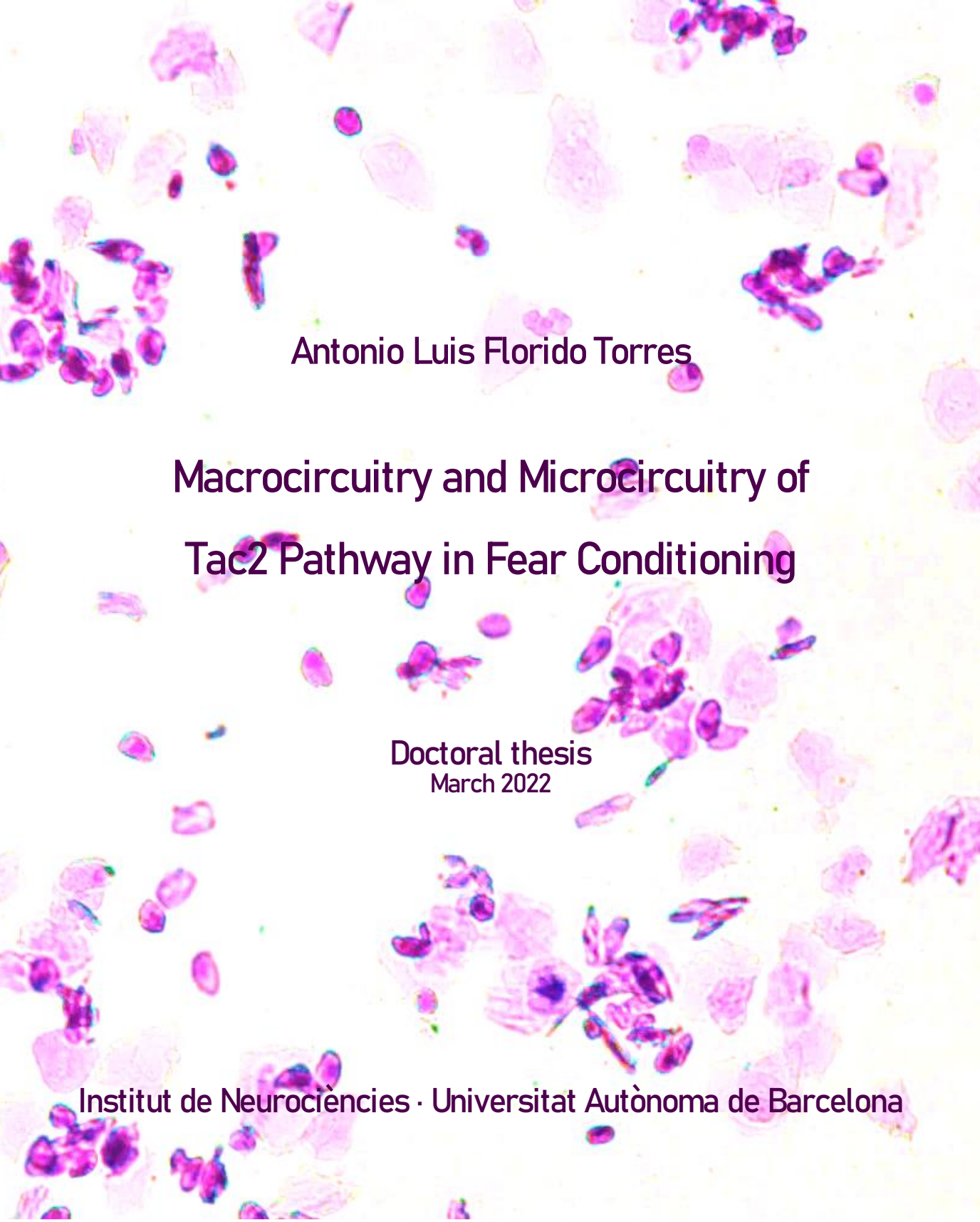
Universitat Autònoma de Barcelona

MACROCIRCUITRY AND MICROCIRCUITRY OF THE TAC2 PATHWAY IN FEAR CONDITIONING

Antonio Luis Florido Torres



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Doctoral thesis
March 2022

Institut de Neurociències · Universitat Autònoma de Barcelona

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INc Institut de Neurociències

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Tutor: Marc Pallarès i Anyó

DOCTORAL THESIS

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To Carlos Florido and Trinidad López

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With love,

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INDEX

ABSTRACT

Fear-based disorders are highly disabling conditions and current treatments are not successful in many cases. Women present almost three times more life prevalence of these disorders than men. Recent research has shown that centromedial amygdala (CeM) tachykinin 2 (Tac2) neurons are crucial for the regulation of fear conditioning (FC) in male mice. Here, we have found that systemic administration of a Neurokinin-3-Receptor (Nk3R) antagonist (osanetant) after cued-fear acquisition altered memory consolidation in a sex-opposite manner. Further, this pharmacological manipulation altered the normal rise in testosterone to FC in male mice. Interestingly, females only exhibited memory consolidation alterations when the drug was given in the proestrus stage of the estrous cycle (high estradiol and progesterone). Concordantly, chemogenetic silencing of CeM-Tac2 neurons after FC mimicked the memory consolidation alterations observed after systemic osanetant administration. Moreover, we used the novel object recognition paradigm to test the effect of a systemic blockade of Nk3R during memory consolidation. Further, we assess the expression of Estrogen Receptor Alpha, Estrogen Receptor Beta and Androgen Receptor and heterodimerization with Nk3R in the medial prefrontal cortex (mPFC) and dorsal hippocampus (DH) of mice. Nk3R systemic antagonism elicited decreased memory consolidation in males while it enhanced it in females during proestrus. Nk3R analysis in the different subregions of the mPFC and the DH showed a higher expression in males than females. Moreover, females presented upregulation of the Androgen receptor in the CA1 and the Estrogen receptor beta in the cingulate cortex, CA1, and Dentate Gyrus. Overall, males presented an upregulation of the Estrogen receptor alpha. We also explored the heterodimerization of GCPR membrane sex hormones receptors with the Nk3R. We found a higher percentage of Nk3R-membrane G-Protein Estrogen Receptors heterodimers in both mPFC and DH in females

suggesting an interaction of estradiol with Nk3R in memory consolidation. However, males presented a higher percentage of Nk3R-membrane G-Protein Androgen Receptors heterodimers in the Cingulate and Prelimbic Cortex pointing at the interaction of testosterone with Nk3R in memory consolidation. These data propose novel ideas on functional interactions between NK3R, sex hormones, Estrogen receptors, and Androgen receptors in memory consolidation which may be relevant for healthy individuals and psychiatric patients.

INTRODUCTION

Fear is an adaptive reaction that an organism presents to the appearance of threats that can put its physical or mental integrity at risk (Davis, 1997; Maren, 2003). It is a fundamental process for the survival of a species since it allows identifying situations of danger and responding accordingly to them. Typically, the appearance of a threat elicits reactions that have been called the fight, flight, or freeze responses (Jones & Monfils, 2016). The fear response is an emotional reaction that involves cognitive, physiological, endocrine, motor, and sensory components oriented towards fighting the threat or escaping from it when this threatening situation appears (J. LeDoux, 2012; J. E. LeDoux, 2014; Watson & Rayner, 1920).

Fear responses can be innate or acquired through classical conditioning (Maren, 2003; Ren & Tao, 2020). To demonstrate the acquisition of fear responses, Watson and Rayner carried out a famous study known as the Little Albert experiment (Beck et al., 2009; Mertens et al., 2020; Meulders, 2020). This research aimed to establish:

1) if a healthy child could acquire a fear response to an unknown animal (a white rat),

2) if this fear response could be transferred to other elements that were similar to the animal,

3) and in case of acquiring the fear response, how long this memory lasted in time.

At the start of the experiment, little Albert showed no fear of the rat. However, after presenting a loud noise that caused the child to cry whenever the child approached the rat, the rat acquired aversive connotations and its mere presentation was able to elicit a fear response in the child. Not only that, but little Albert generalized his fear response

to other objects and animals that presented a resemblance to the white rat (a dog, a coat, or wool). The third phase of the experiment could not be carried out, reportedly because the mother withdrew the child from the experiment.

This experiment shows the pavlovian nature of most of the fears humans present during their lifespan. The study shows that a neutral stimulus (NS), which does not initially produce a fear response, when it is repeatedly paired with a stimulus that innately produces a fear response (unconditioned stimulus, US; unconditioned response, UR), acquires the ability to elicit a fear response (conditioned response, CR) by itself in the absence of the US, thus becoming a conditioned stimulus (CS) (Fanselow & Wassum, 2016; Fendt & Fanselow, 1999; Maren, 2003; Sah, 2017; Sah et al., 2020a; VanElzakker et al., 2014). These acquired fears may be restricted to a specific cue, showing effective discrimination of the US, or appear in the presence of not conditioned cues, thus showing generalization of the CR to other stimuli which may resemble, or not, the original CS (Ahmed & Lovibond, 2015; Asok et al., 2019; Bergstrom, 2020; Dunsmoor & Paz, 2015; Dymond et al., 2015; Grosso et al., 2018; Laufer et al., 2016; Olivera-Pasilio & Dabrowska, 2020; Struyf et al., 2015). Similar to other conditioned behaviors, fear responses can also be extinguished. Fear extinction, the basis of exposure therapy for fear-related disorders, consists of repeated presentations of the CS alone, in absence of the US. Therefore, the CR diminishes along with the different appearances of the CS (Chang et al., 2009; Farrell et al., 2013; Lebrón-Milad et al., 2013; Lonsdorf et al., 2017; Myers & Davis, 2007; Walker et al., 2002).

In the laboratory, fear conditioning (FC) is a valid and widespread tool to assess fear learning, retention, and extinction (Beckers et al., 2013; Blechert et al., 2007; Daviu et al., 2014; Florido et al., 2021; Lonsdorf et al., 2017). Usually, rodents are introduced in a sound-

attenuating chamber with a grid floor that delivers footshocks as the US. Different stimuli may be used as a CS (f.i. a tone or a light), and they are usually presented co-terminating with the footshock. Freezing behavior, which is considered as the lack of any movement but those needed for breathing, is usually considered a valid index of fear in rats and mice (D. C. Blanchard et al., 2001; Eilam, 2005; Fanselow, 1994; Gladwin et al., 2016; Roelofs, 2017; Roelofs et al., 2010). Notwithstanding, other behaviors (for instance, darting) are starting to be studied as possible valid indexes of fear in rodents as a possible source of sex differences in fear responses (Archer, 1975; R. J. Blanchard & Blanchard, 1969; Gruene et al., 2015). Animals present an increase in freezing behavior with the different presentations of the CS+US pairings in the conditioning phase. Afterward, animals are tested for fear expression using a fear extinction paradigm, in which the presentation of the US alone decreases the freezing response along with the session.

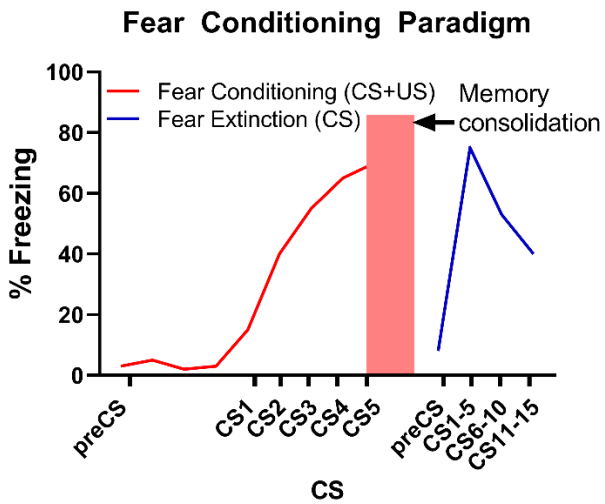

Figure 1. Schematic representation of a fear conditioning task. In red, the acquisition of fear through repeated pairings of the conditioned stimulus with the unconditioned stimulus. In blue, the fear expression/extinction phase in which only the conditioned stimulus is presented repeatedly.

Because of its pavlovian nature, this procedure allows the experimenter to take a deep look into the brain mechanisms of short-term or working memory, memory consolidation, and long-term memory of fear. Further, and importantly, it is also considered a valuable tool to explore the mechanisms underlying fear extinction and the retention of extinction.

1. Neurobiology of fear.

Fear memories are stored and retrieved in the brain by a cluster of different areas with high interconnectivity between them (do Monte et al., 2016; J. E. LeDoux, 2000, 2012). This brain circuitry is highly conserved among mammals, which allows the use of animal models of fear assuring translatability of the results to the human population. Classically, the circuitry responsible for emotional processing in the brain has received the name of the limbic system (Mogenson et al., 1980; Rajmohan & Mohandas, 2007; Rolls, 2015, 2019; Sokolowski & Corbin, 2012). The limbic system is a group of brain areas such as the amygdaloid complex, hippocampus, corpus callosum, septum, olfactory complex, thalamus, prefrontal cortex, nucleus accumbens, and hypothalamus. These areas are linked and support the acquisition, retention, expression, and extinction of learned fears as well as innate fears.

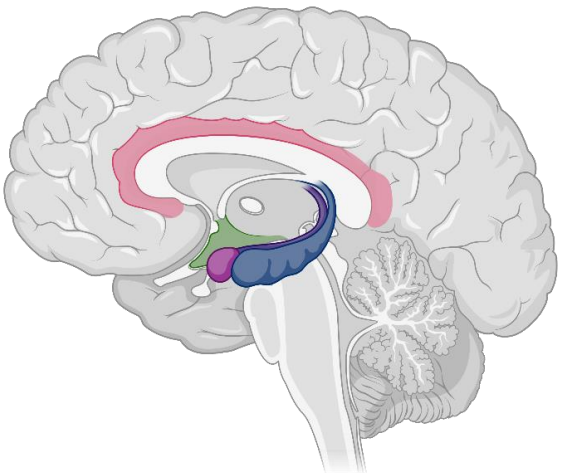

Figure 2. Sagittal plane of the human brain showing brain regions of the limbic system.

1. 1. Fear Conditioning.

The amygdaloid complex is a group of nuclei located in the medial region of the temporal lobe. Anatomically, it is conformed by the Lateral Amygdala (LA), Basal Amygdala (BA), Medial Amygdala (MeA), and Central Amygdala (CeA) (Eleftheriou, 1972; Gallagher & Hollandt, 1994; Humphrey, 1972; Lammers, 1972; Sah et al., 2003). Sensory information from the thalamus and sensory cortices reach the Basolateral Amygdala, which processes threatening cues and drives rapid fear responses through direct projections from the CeA to other brain areas such as the Periaqueductal Grey Matter, Hypothalamus, or the Bed Nucleus of the Stria Terminalis (J. LeDoux, 1992; Ressler, 2010). Among these responses, we may find increased blood pressure and heart rate, bradycardia/tachycardia, panting, respiratory distress, corticosterone/cortisol release, increased startle response or freezing (Eilam, 2005; Gladwin et al., 2016; Hermans et al., 2013; Koba et al., 2016; Natl et al., 2020; Öhman, 2007; Proverbio et al., 2015; Roelofs, 2017).

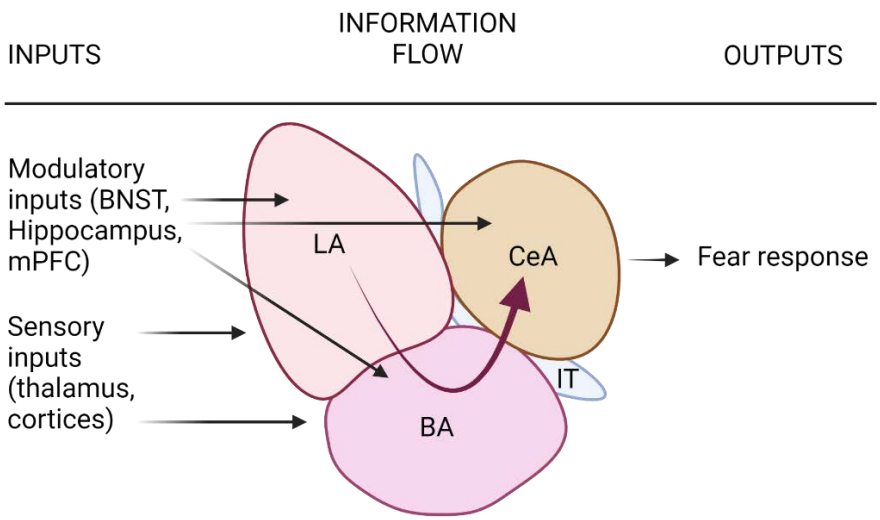

Figure 3. Schematic representation of the main inputs and outputs of information in the Amygdala. LA = Lateral Amygdala, BA = Basal Amygdala, CeA = CeA, BNST = Bed Nucleus of the Stria Terminalis, mPFC = medial Prefrontal Cortex, IT = Interstitial Nuclei Black arrows indicate inputs/outputs. The purple arrow indicate the information Flow in the Amygdala.

The Lateral Amygdala receives direct inputs from sensory cortices and the thalamus. Some of these projections can elicit by themselves innate fear responses, such as painful stimuli. Others, like those that transfer tones or light information do not (Bauer et al., 2001; Maren & Quirk, 2004; Quirk et al., 1997; Sah et al., 2020b). Recent research uncovered the long-term potentiation molecular mechanisms that allow visual or tone information to elicit a fear response after repeated pairings with unconditioned stimuli (Dityatev & Bolshakov, 2005; Lynch, 2004; Maren, 1999; Vazdarjanova & Maren, 2000). Glutamatergic neurons in the Lateral Amygdala are dense in N-Methyl-D-Aspartic Acid (NMDA) receptors. The NMDA receptor is a Calcium channel that presents a Magnesium ion blocking the entrance of calcium inside the cell. When a neutral stimulus appears, such as a tone, it induces the binding of Glutamate to the NMDA receptor. Notwithstanding, Calcium ions cannot penetrate the cell due to the blockade caused by Magnesium. The presentation of an unconditioned stimulus excites the dendrites of these glutamatergic cells, causing the Magnesium ion to move outside the channel. Under that situation, if a neutral stimulus appears in this situation, Glutamate binds to the NMDA receptor and therefore creates a calcium current inside the postsynaptic cell. The entrance of calcium initiates a potentiation of the auditory-amygdalar projection through diverse mechanisms mediated by a Calmodulin. Among these mechanisms, two downstream signaling cascades have been specially studied: the Protein-Kinase A (PKA)

activation (Matsushita et al., 2001; Vitolo et al., 2002) and the Phosphoinositide 3-Kinase (PI3K) activation (Lin et al., 2001). Both downstream cascades converge in the cellular nucleus, enhancing the transcription of genes, such as CREB or b-Catenin, that strengthen the synapse by the formation of new synaptic buttons with the presynaptic neurons, or the transcription and posterior translation to the membrane of α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic Acid (AMPA) receptors. These receptors will later allow the Lateral Amygdala neuron to depolarize in the presence of Glutamate and the absence of an additional potential coming from innate strength connections.

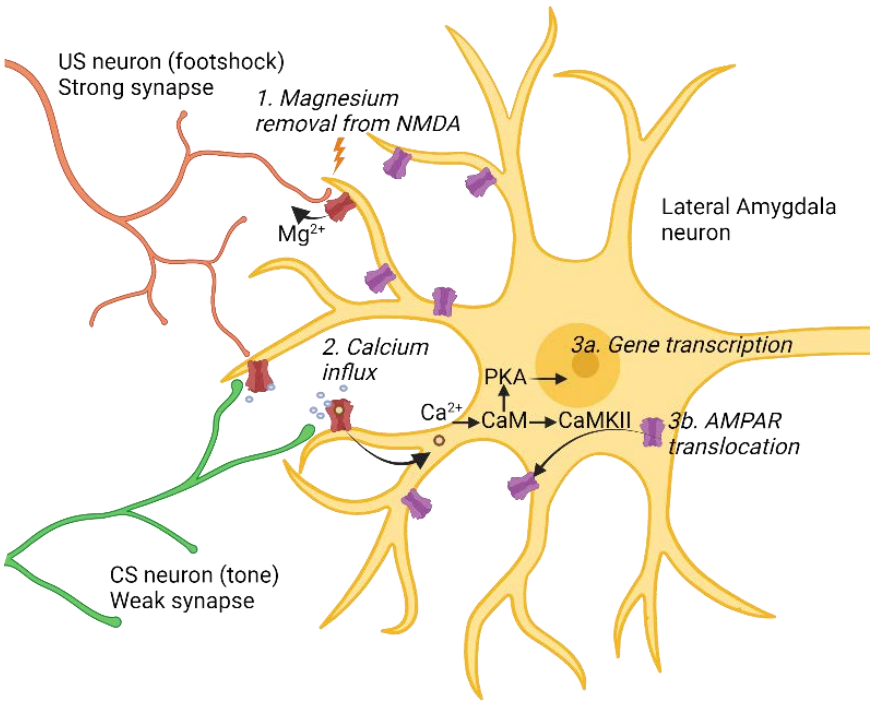

Figure 4. Representation of molecular mechanisms that mediate long-term potentiation in the Lateral Amygdala. US = Unconditioned Stimulus; CS = Conditioned Stimulus; in red NMDA receptors; in purple AMPA receptors; blue dots indicate Glutamate and yellow dots Calcium ions.

Synaptic plasticity in the Lateral Amygdala is required for fear learning, in a process that cognitively receives the name of memory consolidation. The administration of a Mitogen-activated protein (MAP)-kinase inhibitor in the Lateral Amygdala, a protein necessary for synaptic plasticity, before a fear conditioning task, impairs the consolidation of new fear memories as shown by a lower freezing response in comparison with animals that locally received vehicle (Duvarci et al., 2005; Schafe et al., 2000, 2008).

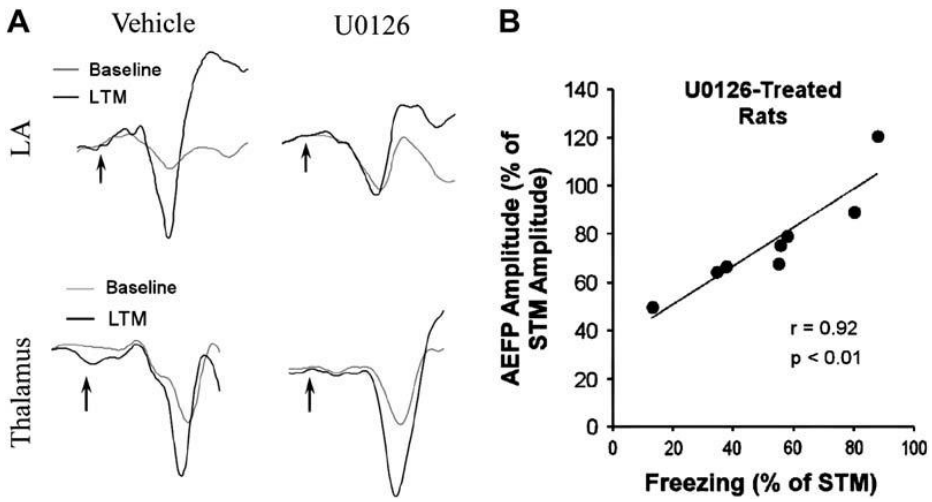

Figure 5. Synaptic plasticity in the Lateral Amygdala (LA) is required for auditory fear conditioning. (A) In this experiment, rats received injections of a MAP kinase inhibitor (U0126) or vehicle into the LA prior to fear conditioning. In vehicle-treated animals (left traces) field potential responses to the auditory CS (arrows) were enhanced during a long-term memory test (LTM) in both auditory thalamus (lower traces) and in the LA (upper traces). However, in animals injected with U0126 into the LA (right traces), neural plasticity was impaired in the LA but not in the auditory thalamus. These results therefore suggest that the MAP kinase inhibitor impaired synaptic plasticity locally in the LA. (B) The impairment in neural plasticity in the LA was correlated with impairments in the consolidation of auditory fear conditioning. Animals that showed greater impairment in neural plasticity displayed less fear responses to the CS during a long-term memory test. This suggests that plasticity in the LA is required for auditory fear learning and memory. Figure extracted from Sigurdsson et al., 2006.

Memory consolidation involves a series of mechanisms that includes synapses potentiation and systems consolidation. Thus, during fear conditioning, synaptic plasticity is not only observed in the Lateral Amygdala, but also in other areas such as the sensory thalamus, the CeA, or the Bed Nucleus of the Stria Terminalis. Altogether, these molecular mechanisms trigger the remodeling of the system cytoarchitecture to permit a rapid fear response in the presence of the newly conditioned stimulus. Systems consolidation of fear memory is strongly regulated by the hippocampal formation, as demonstrated by a lack of systems consolidation after the damage of the dorsal or ventral hippocampus (Oh & Han, 2020; Quillfeldt, 2019; Sutherland et al., 2008; Wiltgen & Tanaka, 2013).

1. 2. Fear Extinction.

As beforementioned, fear responses can also be diminished by the mere presence of the Conditioned Stimulus in the absence o an Unconditioned Stimulus in a process known as fear extinction (Chang et al., 2009; Lonsdorf et al., 2017; Myers & Davis, 2006; Walker et al., 2002). During fear extinction, the freezing response executed by the rodent decreases with the repeated presentation of tones until reaching low values (Velasco et al., 2019). In the beginning, it was thought that this process was mediated by a depotentiation of the previously potentiated synapse. Instead, recent research has manifested that the time-lapse that takes between fear conditioning and extinction is what determines the mechanisms that underlie the decrease of the fear response (Myers et al., 2006). In this sense, short time intervals between fear conditioning and extinction may activate depotentiation mechanisms that decrease the strength of the long-term potentiation in the making. Notwithstanding, most of the fear extinction paradigms

occur after the consolidation of fear memories has occurred, therefore involving different mechanisms.

Fear responses may be reinstated after fear extinction by exposing the animals to the unconditioned stimulus or a stressor (Laurent & Westbrook, 2010; Morris et al., 2005). Further, the mere pass of time after fear extinction may cause the spontaneous recovery of the fear response (Cruz et al., 2014; Quirk, 2002; Schiller et al., 2008). These phenomena suggest that, instead of a depotentiation process, the previously potentiated synapsis is temporarily silenced by a systemic process triggered by fear of extinction. In this line, fear research has highlighted the major role of the medial prefrontal cortex in the acquisition of this inhibition over the fear responses. Fear extinction is considered a new form of learning (Giustino & Maren, 2015; Marek et al., 2018; Milad & Quirk, 2002), in which inhibitory connections from the medial Prefrontal Cortex to the Amygdala are long-term potentiated. Similar to the acquisition of fear memories, the acquisition of extinction memories involves molecular mechanisms aimed at the inhibition of the amygdalar output and the reduction of the fear response (Santini et al., 2004). In this sense, fearful memories compete with medial Prefrontal Cortex inputs in the production of a fear response. This pathway is strongly modulated by the Hippocampus, as shown by a highly dependent context efficacy of fear extinction (Bouton et al., 2008; Maren et al., 2013; Milad et al., 2005).

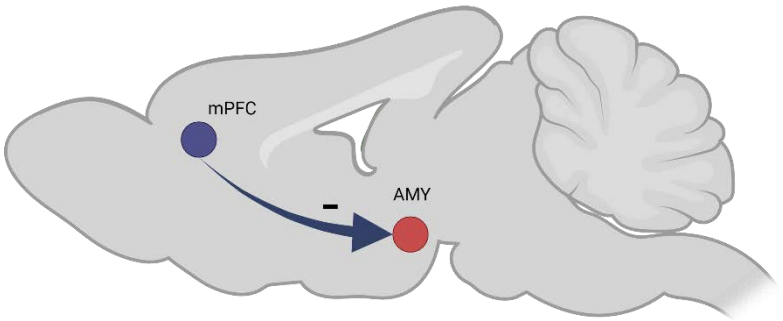

Figure 6. Sagittal plane of the mouse midbrain showing inhibitory projections from the medial Prefrontal Cortex to the Amygdala. These modulatory connections are involved in fear extinction and therefore, potentiated with the acquisition of an extinction memory. The amygdala and medial Prefrontal Cortex compete in the formation of a fear response. mPFC = medial Prefrontal Cortex; AMY = Amygdala.

1. 3. Contextual and auditory fear conditioning.

In animal models, the Conditioned Stimulus usually employed is a tone (auditory fear conditioning) or a specific context (contextual fear conditioning). Both processes converge in the Amygdala, but they differ in the brain areas that encode and transmit environmental information. In the case of auditory fear conditioning, tones are perceived by the auditory system, which involves the thalamus and auditory cortices that project directly to the Amygdala. In the case of contextual fear conditioning, contextual information is encoded by the Hippocampal formation and later sent through direct projections to the Amygdala.

2. Fear-related disorders and sex.

2. 1. Sex-dependent incidence of psychiatric disorders.

Mental disorders do not distribute equally among the world population. The World Health Organization publishes yearly a study comparing Disability-Adjusted Life-Years (DALYs) between men and women in some of the most frequent psychopathologies, across different periods of the lifespan ("Global, Regional, and National Burden of 12 Mental Disorders in 204 Countries and Territories, 1990-2019: A Systematic Analysis for the Global Burden of Disease Study 2019," 2022).

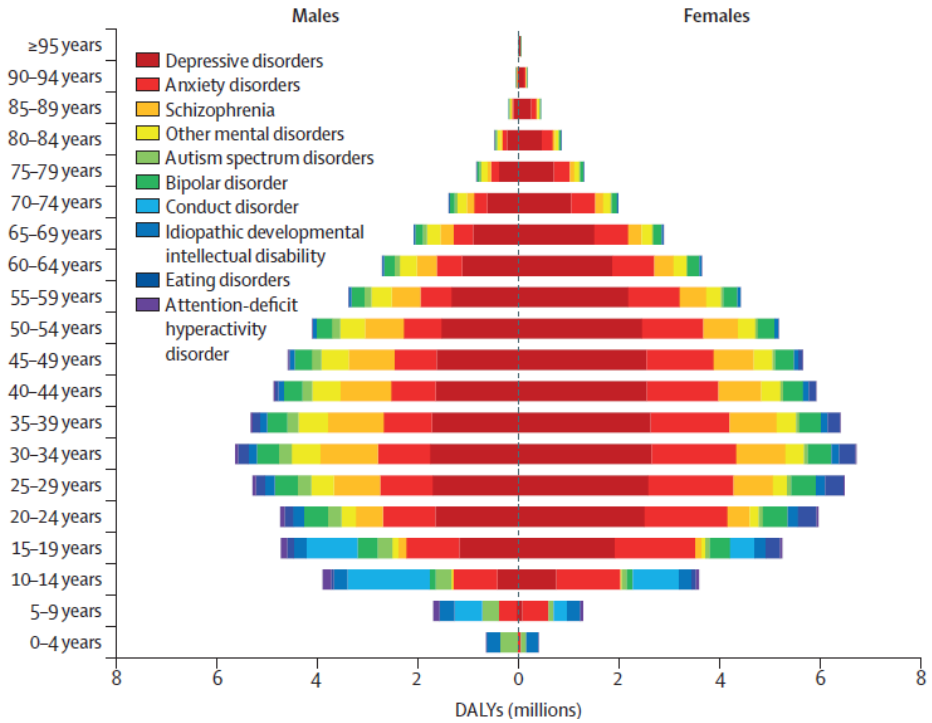

Figure 7. Global DALYs by mental disorder, sex and age, 2019 DALYs = disability-adjusted life-years. Figure extracted from GBD 2019 Mental Disorders Collaborators, 2022

While mental disorders cause more disability to males between 0 and 14 years old, this tendency is reversed in the range from 15 years old until the end of life. As observed, women tend to present more disability caused by depressive or anxiety disorders, while males' disability is more related to developmental intellectual disorders or schizophrenia.

Even more interesting, other studies address sex differences in fear or stress-related disorders. In this line, Bangasser and Valentino published a study (Bangasser & Valentino, 2014) comparing the lifetime prevalence of stress-related disorders between men and women.

	Lifetime prevalence		Ratio
	Female (%)	Males (%)	Female:Male
Panic	6.2	3.1	2.0
Generalized anxiety	7.1	4.2	1.7
Any anxiety disorder	36.4	25.4	1.4
PTSD	9.7	3.6	2.7
Major depression	20.2	13.2	1.5
Any affective disorder	24.4	17.5	1.4
Alcohol abuse	7.5	19.6	0.4
Drug abuse	4.8	11.6	0.4
Migraine	18.2	6.5	2.8
Insomnia	12.9	6.2	2.1
Irritable bowel syndrome	14.5	7.7	1.9

Table 1. Sex differences in the prevalence of stress-related disorders. Adapted from Bangasser and Valentino, 2014.

Women present a higher lifetime prevalence of fear-related disorders compared to men. Specifically, Post-traumatic Stress Disorder (PTSD) presents a 2.7 female to male ratio, indicating that females present almost three times the lifetime prevalence of PTSD in men, and Panic disorder is twice as prevalent in women than in men. In contrast, men present a higher life prevalence for other stress-related disorders, such as alcohol or drug abuse (0.4 female to male ratio).

Taking together the data from different sources, it is concluded that women present a much higher life prevalence of fear-related disorders that causes a high disability if we compare these variables to men.

Notwithstanding, basic and clinical research has classically suffered from a bias towards the study of the male subject, either in the human population, animal models, or cellular studies. This bias affects all fields of biomedical research, but it is especially relevant in Neuroscience and Physiology.

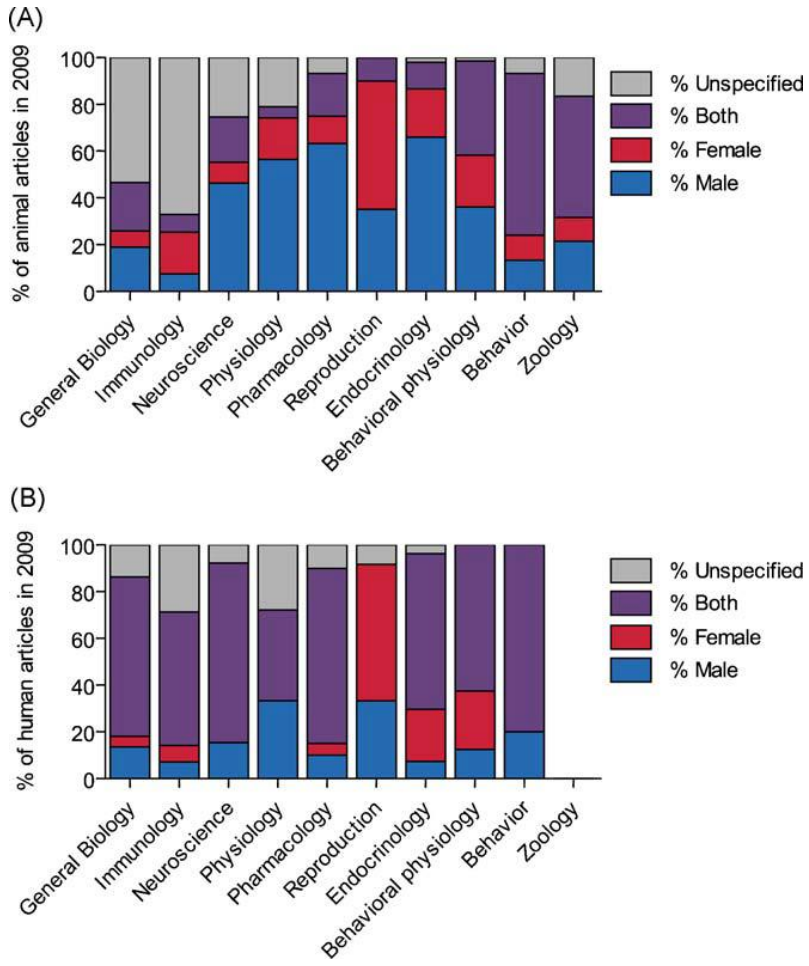

Figure 8. Distribution of studies by sex and field in 2009. (A) Percent of articles describing non-human animal research that used male subjects, female subjects, both male and female subjects, or did not specify the sex of the subjects. (B) Percent of articles describing human research in the same categories. The zoology category was excluded because of insufficient use of human subjects in this field to form an accurate estimate. Extracted from Beery and Zucker, 2011.

After observing this ~ 1:5 female to male bias in Neuroscience research, it is clear that research in females has been neglected in the last decades. This is especially surprising given the fact that the lifetime prevalence of some psychopathologies in females, for instance, fear-related disorders, is at least twice compared to males (Beery & Zucker, 2011).

Typical concerns about the use of females in Neuroscience include conventions or hormonal fluctuations caused by the estrous cycle (Shansky, 2019). This is an important question to address, due to their implications for future research and the efficacy and effectiveness of treatments. Recent research has highlighted that the variability in sex hormones is equal in males and females (Chen et al., 2021; Miguel-Aliaga, 2022; Shansky & Murphy, 2021). While females present hormonal variability in terms of circulating estradiol concentration across 3 to 5 days in rodents (or 27 to 29 days in humans), males also present a great variability in circulating testosterone levels regulated by circadian rhythms or seasons. Thus, the reason for the female-related variability in neural processes because of sex hormones variability is not valid to avoid research in females. Moreover, although conventionalism may have led neuroscientists to usually perform experiments in male subjects, this practice may a caused misgeneralization or results. The lack of reproducibility and generalization of results is important because of what it implies for treating psychopathologies. Poor knowledge of the female brain may have led to treatments against pathologies that do not reach the desired efficacy (Clayton & Collins, 2014; Zucker & Beery, 2010).

In female rodents, the estrous cycle is defined as the period that occurs between two consecutive phases of the sexual receptiveness of the animal. It is characterized by sex hormones fluctuations that modulate the sexual behavior of the mouse (E. Allen, 1922; Hansel & Convey,

1983). Moreover, in the last years, it has been established that this cycle also modulates other aspects of animal physiology such as brain function and, subsequently, behavior and cognitive processes (Cora et al., 2015; Florido et al., 2021; Marcondes et al., 2002). Although females present androgens, the typical sex hormones in male subjects, the concentration of these substances is quite low in comparison to males. Besides, these androgens typically serve as precursors for the synthesis of estrogens, the main sex hormones in females (Hammes & Levin, 2019; Turcu et al., 2014). There have been identified three types of estrogens in females: estrone, estradiol, and estriol. Estrone is synthesized by aromatase from Progesterone and Estriol is aromatized androsterone, while Estradiol -the main sex hormone in females- is synthesized from testosterone by aromatization (McEwen, 2001). Other sex hormones that fluctuate during the estrous cycle include Progesterone, Follicle Stimulating Hormone (FSH), and Luteinizing Hormone (LH) (Velasco et al., 2019). According to the fluctuations of sex hormones across the estrous cycle, researchers have divided this cycle into four stages: proestrus (presenting a high concentration of Estradiol and Progesterone), Estrous (with a strong decay of sex hormones levels), Metestrus (representative for having the lowest concentration of sex hormones) and Diestrus (presenting a relatively higher concentration of Progesterone compared to Estrus and Metestrus but low Estradiol levels) (Maeng et al., 2015; Milad et al., 2009).

The estrous cycle differs from the menstrual cycle in women in different aspects. For instance, the phases of the menstrual cycle are divided into Early Follicular, Late Follicular, Mid Luteal, and Late Luteal. Estradiol peaks during the Late Follicular phase and stabilizes during mid-luteal, while the mid-luteal phase is characterized by a strong peak of Progesterone. Another difference between the rodent and human sex

hormones cycle is menstruation (Reed & Carr, 2018). While rodents do not present bleeding after ovulation, women show a typical bleeding every 28 days indicative of the loss and reabsorption of the oocyte.

The estrous cycle has been shown to have a determinant role in fear extinction in female rodents and women. In rodents, peaks of Estradiol and Progesterone typical of the Proestrus phase of the estrous cycle enhance fear extinction, while this same process is enhanced in women after ovulation during the mid-luteal phase of the menstrual cycle (Velasco et al., 2019).

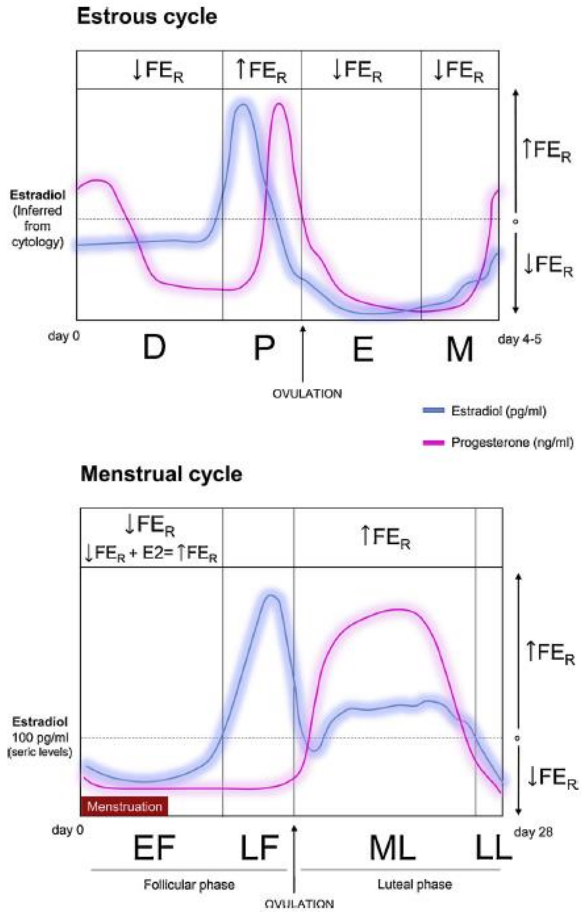

Figure 8. Scaled representation of estradiol and progesterone levels during the distinct phases of the estrous (rodent) and menstrual cycle (human). The result of subjecting females to Fear Extinction (FE) training during each phase is shown at the top as fear extinction recall (FER). The FER of females 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. Extracted from Velasco et al., 2019

2. 2. Aversive memories and fear-related disorders.

Aversive memories are a strong component of a group of disorders known as fear-related disorders (de Quervain et al., 2017; C. S. Kogan et al., 2016). These abnormalities in human behavior have in common aberrant processing of threatening cues, that lead to an uncontrolled overactivation of the fear neurocircuitry. Current research is focused on increasing the effectiveness of treatments or finding new therapeutic targets that may have fewer side effects and increased efficacy (Flores et al., 2018). Nowadays, treatments for fear-related disorders are oriented towards the use of benzodiazepines, that tone down the amygdala and related structures, and antidepressant drugs to promote synaptic plasticity, conjugated with exposure therapy in a cognitive-behavioral framework (Abraham et al., 2012; Acheson et al., 2013; Akirav et al., 2006; Bystritsky et al., 2013; de Quervain et al., 2017; Milad et al., 2014; Singewald et al., 2015). Although these treatments have shown some efficacy, this is limited and patients usually remit in a short time. Further, little research has focused on the prevention of this type of aberrant fear, probably because of the short time window to act on memory formation after an aversive association such as a traumatic event. Notwithstanding, the hours that follow the association of aversive events with neutral cues are an opportunity for medical intervention. An attenuated fear consolidation process would probably result in a decrease in symptoms frequency and severity.

3. Neuropeptidergic modulation of fear memories.

Neuropeptides are cerebral proteins that act as neurotransmitters and neuromodulators (Hökfelt et al., 2018; Nusbaum et al., 2017; Russo, 2017). These proteins regulate multiple brain processes, such as energy homeostasis, analgesia, sleep, sexual behavior, blood pressure or learning, and memory (Beuckmann & Yanagisawa, 2002; Borbély et al., 2013; Clark et al., 1985; Concetti & Burdakov, 2021; Dornan & Malsbury, 1989; Dyzma et al., 2010; Maywood et al., 2021; Ohno & Sakurai, 2008; Rosati et al., 2016; Schüssler et al., 2006; Steiger, 2007; White, 1993; Zaben & Gray, 2013). The neurotransmitter property of neuropeptides is mediated by G-coupled Protein Receptors (GPCRs), which typically modulate intracellular signaling cascades. Therefore, the Neuropeptide-GPCR complex usually acts on several signaling cascades that promote diverse and complex effects on neurons (Marvar et al., 2021).

These peptides have been shown to have a role in fear memory. For instance, Neuropeptide Y (NPY) receptor 1 (Y1R) knockout mice show impaired fear expression compared with wild-type mice; while NPY Y2 Receptor (Y2R) agonism results in increased fear extinction and long-term suppression of fear (Tasan et al., 2016; D. Verma et al., 2012). In the last decades, Vasopressin-Oxytocin interactions, Orexins and Tachykinins have been studied in fear memories due to their expression/interaction with the Amygdala.

3.1. Vasopressin and Oxytocin.

Vasopressin and Oxytocin are neuropeptides that have been shown to be involved in different brain processes, such as aggression (Bosch & Neumann, 2012; Nelson & Trainor, 2007; Oliveira et al., 2021; Pagani

et al., 2011) or anxiety (Landgraf, 2006; Morales-Medina et al., 2016; Neumann & Landgraf, 2012). They are usually studied together since they represent an integrated system where each peptide exerts opposing roles. In the CeA, these peptides have been shown to excite different populations of neurons (Huber et al., 2005) and exert an opposite role in fear memories. CeM presents neurons that excite upon Vasopressin infusion, and these same neurons receive inputs from Oxytocin-expressing neurons in the Centrolateral Amygdala (CeL) that inhibit the CeM neurons (Viviani & Stoop, 2008). Microinfusions of Vasopressin in limbic areas have been shown to increase memory consolidation, whereas infusions of Oxytocin in the same regions decrease consolidation (Kovács et al., 1979). Recent findings highlight that this opposite effect of Vasopressin and Oxytocin are regulated by an opposite regulation of catecholamines in the limbic-midbrain areas and further modulate the activity of the Hipothalamic-Pituitary-Adrenal (HPA) axis (Kovács et al., 1979; Porter et al., 1988; Trembleau et al., 1995).

3.2. Orexins.

The orexinergic system is constituted by two different neuropeptides, Orexin-A and Orexin-B, exclusively synthesized in the Hypothalamus, that bind with different affinity to Orexin-1-Receptor (OX1R) and Orexin-2-Receptor (OX2R). These receptors are G Protein-Coupled Receptors (GPCRs) whose effect is mainly related to the activation of the Phospholipase C (PLC) signaling cascade (Wang et al., 2018).

These neuropeptides have recently been studied in fear-related disorders. Orexinergic projections from the Perifornical Hypothalamus to the CeA have been shown to depolarize OX1R, but not OX2R, triggering fear responses (Dustrude et al., 2018). Further, orexinergic

neurons in the hyopothalamus innervate norepinephrine neurons in the Locus Coeruleus (LC), modulating awareness and vigilance. These neurons in the LC project to the LA, increasing fear conditioning through OX1R. Optogenetic or chemogenetic stimulation of this circuit enhances fear responses, while its inhibition decreases freezing towards fearful cues (Soya et al., 2017).

3.3. The Tac2 pathway in fear memory consolidation.

The Tac2 gene is part of the family of the Tachykinins genes. In mice, there are two different Tachykinin genes, Tac1 and Tac2. The result of the Tac1 gene transcript is a pro-protein that, after synthesis, may produce two different products: substance P and Neurokinin A. The Tac2 is responsible for the synthesis of Neurokinin B (NkB). While substance P and Neurokinin A bind, respectively, to Neurokinin 1 and 2 receptors (Nk1R, Nk2R); NkB binds to the Neurokinin 3 Receptor (Nk3R) (Marvar et al., 2021).

In the Amygdala, Tac2 is expressed in a small portion of the CeA, located in its medial region. This area receives the name of Centromedial Amygdala (CeM). The gene expression profile in the Amygdala is different when compared 30 minutes and 2 hours after fear conditioning. Tac2 transcripts have shown to be one of the main upregulated genes in the CeA when comparing mice that received paired tones with footshocks against those who received unpaired tones and shocks. This data indicates a possible role for Tac2 transcription in the short-term after fear conditioning in mice. Further, the expression of Tac2 is quite restricted to the CeM, which represents the major output of the CeA, and hypothalamus (see Figure 9). Nk3R is also expressed in the CeA, and other brain areas, regulating fear processes. The systemic administration of the potent selective antagonist osanetant

against this receptor from 30 minutes before up to 1 hour after fear conditioning, decreases fear memory consolidation in male mice. These data were also replicated using a site-directed infusion of osanetant and optogenetic silencing of these neurons.

Further, clozapine-N-oxide silencing of CeM-Tac2 neurons using Designer Receptor Exclusively Activated by Designer Drugs (DREADDs), a modified muscarinic receptor that can be inserted in neurons using an Adenoassociate Virus (AAV) (Roth, 2016), reproduced the decrease in fear memory consolidation in male mice (Andero et al., 2014). Notwithstanding, whether these manipulations affect fear memory consolidation in females remains unexplored and needs to be addressed. Moreover, whether Tac2 regulates other types of memory not linked to fear or emotion is also to be elucidated.

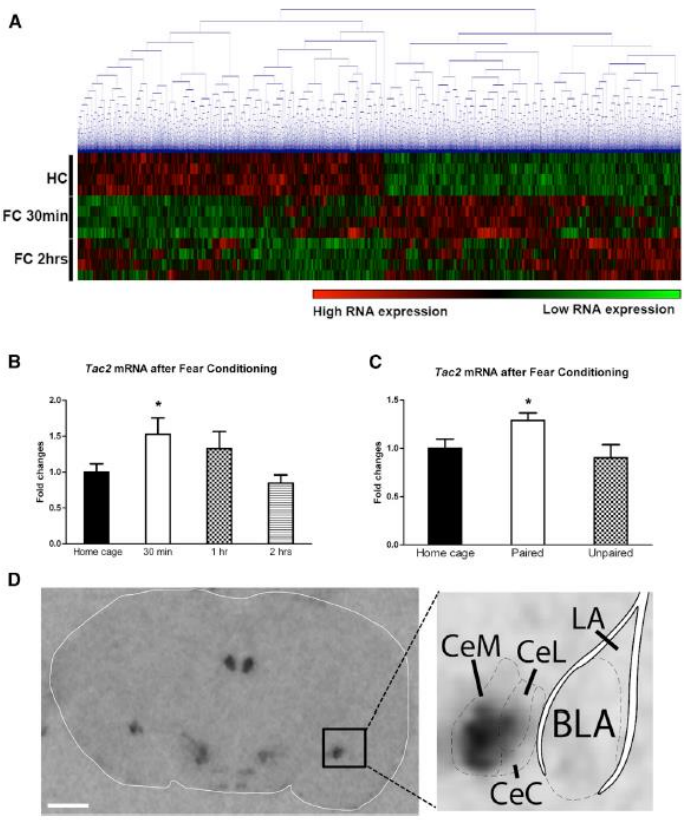

Figure 9. Differential Regulation of Tac2 Gene Expression in the Amygdala during Cued-Fear Conditioning. (A) With average linkage hierarchical clustering of an RNA microarray, there is a differential gene regulation 30 min and 2 hr after auditory fear conditioning (FC) when compared to home cage group (no FC). n = 4 per group. (B) Tac2 mRNA levels are rapidly upregulated in the amygdala during fear consolidation 30 min after fear conditioning. *p % 0.05 versus HC and 2 hr. n = 7–8 per group. (C) Tac2 upregulation occurs when the conditioned stimulus (acoustic tone) and the unconditioned stimulus (electric footshock) are paired but not when they are unpaired. *p % 0.05 versus HC and unpaired. n = 11–15 per group. Mean + SEM is shown. (D) Tac2 expression by radioactive in situ hybridization in the amygdala is restricted to the central amygdala (CeA) with highest expression in the CeM amygdala. Scale bar, 1 mm. Figure extracted from Andero et al., 2014.

Tac2 and its transcript, NkB, have already been studied in the onset of puberty in female mice. It is expressed in Kisspeptin neurons of the hypothalamus and medial amygdala. Tac2 mRNA in the Arcuate Nucleus of the hypothalamus increases before puberty, and an increased expression of Nk3R and NkB are sufficient for the onset of puberty in females (Gill et al., 2012). Considering the previous data, it remains important to elucidate the role of Tac2 in memory formation in both sexes.

HYPOTHESES AND OBJECTIVES

Oxytocin, Vasopressin, Orexins, and Tachykinins have acquired an important role in the modulation of fear memories in recent years. Oxytocin and Vasopressin interactions are widely studied in male and female rodents, and recent studies also include females in the study of Orexins in fear. Notwithstanding, the knowledge of Tachykinins in the modulation of fear memories and related behaviors is restricted to males. To further understand how Tachykinins regulate fear memories, the following hypotheses and objectives are proposed:

1) Osanetant, a potent selective Nk3R antagonist, has previously been shown to decrease fear memory consolidation in male mice when administered after fear conditioning. In this study, we hypothesize that the administration of the same dose of osanetant in females after fear conditioning will reduce fear memory consolidation.

To that end, we will use male and female mice for a fear conditioning task and they will receive osanetant 5 mg/kg 30 minutes after fear conditioning. All mice will be tested for fear expression 24 hours after the administration and freezing will be taken as a measure of fear.

2) Given that chemogenetic silencing of Centromedial Amygdala Tac2 neurons in male mice has shown to decrease fear memory consolidation in male mice, we hypothesize the same effect in female mice.

To address this question, we will use Tac2-Cre mice and we will inoculate an Adeno-associate virus to produce the expression of an inhibitory DREADD (Designer Receptor Activated by Designer Drugs). 8 weeks after the infection, male and female mice will undergo a fear conditioning task and they will receive a single dose of Clozapine-N-Oxide (CNO) 30 minutes after conditioning. Animals will be tested for fear expression 24 hours after the administration of CNO and freezing will be used as a measure of fear.

3) Previous research has shown that, in humans, a chronic administration of an Nk3R antagonist decreases circulating sex hormones concentration. We hypothesize that osanetant will produce a decrease in testosterone in males, and estradiol in females, 30 minutes after its administration (1 hour after fear conditioning). We hypothesize that osanetant will not affect stress steroids concentration, such as corticosterone.

To test this hypothesis. male and female mice will receive fear conditioning and an osanetant injection (5 mg/kg) 30 minutes after conditioning. Mice will be decapitated 30 minutes after the injection of osanetant. Trunk blood and brains will be collected for later analyses of sex steroids through mass spectrometry (testosterone and corticosterone) and ELISA (estradiol).

4) Because osanetant has been shown to modulate fear memory consolidation in male mice, we hypothesize that this modulation could affect other types of not-amygdala-dependent memories such as recognition memory consolidation.

To that end, we will use male and female mice, that will undergo a Novel Object Recognition (NOR) task. Animals will be subjected to the familiarization phase of the NOR and will receive the same dose of osanetant (5 mg/kg) 30 minutes after familiarization. 24 hours after receiving the drug, animals will be tested for novel object recognition by exposing the animals for 15 minutes to the previously familiarized object and a novel one. The relative time exploring the objects will be used as a measure of recognition.

DIRECT AND INDIRECT MEASUREMENTS OF SEX HORMONES IN RODENTS DURING FEAR CONDITIONING

Direct and Indirect Measurements of Sex Hormones in Rodents During Fear Conditioning

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Fear conditioning (FC) is a widely accepted tool for the assessment of learning and memory processes in rodents related to normal and dysregulated acquired fear. The study of sex differences in fear learning and memory is vast and currently increasing. Sex hormones have proven to be crucial for fear memory formation in males and females, and several methods have been developed to assess this hormonal state in rats and mice. Herein, we explain a routine FC and extinction protocol, together with the evaluation of sex hormonal state in male and female rodents. We explain three protocols for the evaluation of this hormonal state directly from blood samples extracted during the procedure or indirectly through histological verification of the estrous cycle for females or behavioral assessment of social hierarchies in males. Although females have typically been considered to present great variability in sex hormones, it is highlighted that sex hormone assessment in males is as variable as in females and equally important for fear memory formation. The readout of these protocols has had a great impact on different fields of fear learning and memory study and appears essential when studying FC. The proven interaction with drugs involved in the modulation of these processes makes sex hormone assessment during FC a valuable tool for the development of effective treatments for fear-related disorders in men and women. © 2021 Wiley Periodicals LLC.

Basic Protocol 1: Fear conditioning and fear extinction
Basic Protocol 2: Blood collection for direct measurement of sex hormone levels in fear conditioning
Basic Protocol 3: Indirect measurement of sex hormones in females during fear conditioning
Basic Protocol 4: Assessment of dominance status in males before a fear conditioning protocol
Support Protocol: Construction of a confrontation tube

Keywords: blood extraction • confrontation tube test • fear conditioning • sex hormones • vaginal smear cytology

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INTRODUCTION

Fear conditioning (FC) is a type of classical conditioning in which a neutral stimulus (e.g., a tone) is paired with an aversive one [an unconditioned stimulus (US), e.g., a mild electric shock] to generate a conditioned response in the presence of the newly conditioned stimulus (CS), even when the US is absent (Maren, 2001). This paradigm may be used to study different phases of the fear learning process: acquisition of fear, fear memory consolidation, fear extinction (FE), FE recall, memory consolidation, reinstatement of the fear response, and renewal or spontaneous recovery of the fear response (Hermans, Craske, Mineka, & Lovibond, 2006; Maren, 2001). FC is widely considered a valid tool to assess learning and memory processes in normal and pathological animal and human models of fear-related behaviors, for example, dysregulation of fear memory in animal models of post-traumatic stress disorder (PTSD) (Andero et al., 2013; Andero, Dias, & Ressler, 2014; Blechert, Michael, Vriends, Margraf, & Wilhelm, 2007).

Sex differences in fear memory have been highlighted at the behavioral and molecular levels of analysis. Indeed, it has been widely described that females express lower rates of extinction memory than males (Velasco, Florido, Milad, & Andero, 2019). Even more, in female rats, the levels of sex hormones inferred by vaginal cytology have been shown to modulate FE memory consolidation after FE training, with higher freezing rates in extinction recall when rats are trained during metestrus (low estradiol levels) in comparison to those trained during proestrus (high estradiol levels) (Milad, Igoe, Lebron-Milad, & Novales, 2009). Further, it has been proven that some drugs are more effective when administered during certain phases of the estrous cycle (Florido et al., 2021; Lebrón-Milad, Tsareva, Ahmed, & Milad, 2013), showing interaction with sex hormones in the modulation of fear memory consolidation. Thus, controlling circulating sex hormone concentration across the FC protocol can be useful for an accurate interpretation of the behavioral outcomes. Assessing differences in sex hormone levels during an FC protocol is relatively easy and may be addressed in different ways. Sex hormones can be directly measured through blood extract analyses or indirectly using behavioral or cytological methods. Notably, the study of sex differences and sex hormone modulation of behavior is not restricted to fear learning and memory; it is becoming increasingly important in other paradigms (Jury, DiBerto, Kash, & Holmes, 2017).

Blood collection is required for a direct and rigorous determination of circulating sex hormone levels. Although there are different methods for blood extraction in rodents, most of them require sedation or terminal procedures or induce stress (see Current Protocols article; Donovan & Brown, 2005; Parasuraman, Raveendran, & Kesavan, 2010). However, tail blood collection in freely moving mice is suggested as a valid method because of the reduced stress experienced by the animal (Aryal, Tae-Hyun, Yoon-Gyoon, & Hyung-Gun, 2011; Fluttert, Dalm, & Oitzl, 2000). Additionally, it is important to note that the direct determination of sex hormones requires analysis once the blood samples are collected. In this context, rodent serum or plasma can be analyzed using different techniques, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), or mass spectrometry. Hence, this approach is laborious and does not enable

researchers to consider the putative differences in sex hormone levels while performing FC studies.

Given the abovementioned limitations of the direct methods, the use of indirect methods could be an appropriate alternative in certain experimental protocols. These indirect methods may facilitate the study of fear because they allow estimation of the hormonal levels during the FC procedures, as well as reducing both the stress induced in the animals and the use of multiple reagents. Indirect methods in females include vaginal smear cytology, vaginal aperture analysis, and vaginal impedance analysis (Ajayi & Akhigbe, 2020). However, due to its low cost and high reliability, vaginal smear cytology is the most appropriate approach to assess the exact moment of the estrous phase in females (Singletary et al., 2005). This specific method allows the experimenter to control the estrous cycle during a specific behavior. Whereas indirect methods in females focus on assessing vaginal states, we have to focus on behavior to indirectly determine the sex hormonal state in males. In fact, studying dominance status between males has arisen as a valid method to indirectly assess basal levels of testosterone, with dominant males presenting higher values for circulating testosterone than submissive males (Bishop & Chevins, 1987; Van Loo, Mol, Koolhaas, Van Zutphen, & Baumans, 2001). There are different behavioral approaches to evaluate dominance status in males (Zhou, Sandi, & Hu, 2018), such as the water competition test or the food competition test. However, these protocols have their limitations, requiring the induction of water or food deprivation in healthy animals and subsequent stress. The confrontation tube test is preferable due to its low cost and simple methodology. Because the tube test allows the experimenter to record voluntary behavior in the absence of deprivation, it allows the quantification of specific dominance-related behaviors. Moreover, animals habituate easily to the task and present minimum stress in response to the experimental situation.

In this article, we will focus on a valid method to collect and store blood samples (Basic Protocol 2) during a basic FC protocol (Basic Protocol 1). Furthermore, we will describe vaginal smear cytology (Basic Protocol 3) and the confrontation tube test (Basic Protocol 4 and Support Protocol) as indirect measures of sex hormones in females and males, respectively, during an FC task (Basic Protocol 1). Of note, there may be other valid methods to perform the experiments explained here.

STRATEGIC PLANNING

The freezing response is a valid tool to quantify fear in rodents (Roelofs, 2017). It is understood as the lack of any movement of the animal except for that required for breathing. Different systems have been developed to evaluate FC and FE in rodents. Some of these systems use highly sensitive weight transducers that, after calibration of the threshold for freezing, give reliable measures of fear. Other systems use video-tracking of the animal for later frame-by-frame analysis to determine the lack of movement or use infrared light beams to detect freezing, among others. It is beyond the scope of this article to describe how to measure freezing during FC; to that end, you can consult other protocols (see Current Protocols articles; Chang et al., 2009; Jones, Agee, & Monfils, 2018; Pickens, Golden, & Nair, 2013).

This procedure is typically carried out inside a fear chamber (Basic Protocol 1), approximately 25 × 25 × 25 cm in size, that is equipped with a speaker on the ceiling or the walls that provides tones as well as white noise and with a lightbulb that may dispense visual light cues. To provide footshocks, animals are placed on top of a grid floor equipped with a shocker. This fear chamber is usually placed inside a sound-attenuating chamber that is commonly equipped with a video camera on the front door to record or control the animal's behavior throughout the sessions and a lightbulb to provide general illumination (Fig. 1).

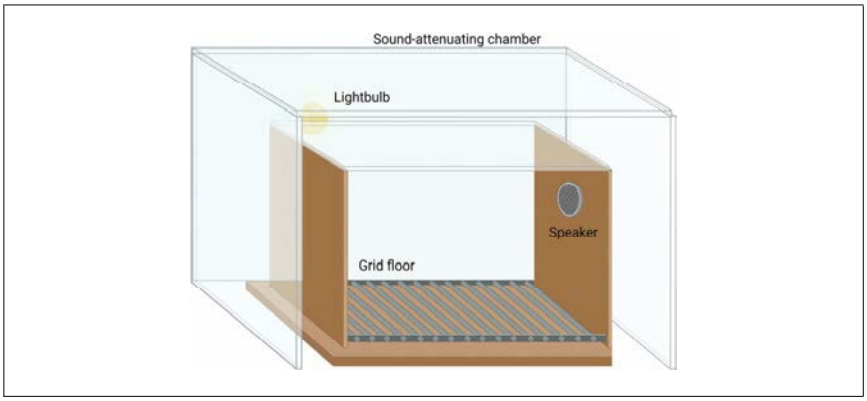

Figure 1 Fear conditioning setup, with fear conditioning chamber used for fear acquisition and extinction. The chamber is equipped with a speaker to deliver tones and a grid floor to deliver footshocks and is located in a sound-attenuating chamber. The latter chamber delivers light through a lightbulb.

NOTE: All protocols involving live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals.

BASIC PROTOCOL 1

FEAR CONDITIONING AND FEAR EXTINCTION

This protocol aims to explain how we perform cued or contextual FC procedures in the laboratory. This protocol is further combined with Basic Protocols 2, 3, and 4 to relate hormonal values to behavioral outcomes during an FC/FE procedure. At the end of day 2, animals will be adequately habituated to the experimental procedure to prevent neophobia from interfering with the behavioral outcome. At the end of day 3, animals will present a conditioned fear response to the tone or context. At the end of day 4, the experimenter will observe, through freezing rates, the memory trace from the day before. Further, animals will acquire a new type of learning, consisting of inhibition of the fear response in absence of the US (FE). At the end of day 5 (and subsequent days if required), the experimenter can evaluate the memory trace generated after FE learning.

Materials

Adult mice or rats (see Critical Parameters and Troubleshooting for specifications for strains and age)
70% (v/v) ethanol
41% (v/v) isopropyl alcohol

Computer (for running software)
StartFear system (Panlab/Harvard Apparatus) or FreezeFrame (ActiMetrics), including software and fear chambers with fan, yellow light, and grid floor (see Fig. 1)
White methacrylate boxes (30 × 15 × 15 cm; with transparent removable top lid with eight holes of 1-cm diameter to allow air renewal; custom made or commercial vendor, e.g., Faberplast)
Shock calibration tool (Panlab/Harvard Apparatus)
Decibel meter (Sound Level Meter, Brüel & Kjaer; for cued FC)
Gray flat floor (to cover grids; custom made or commercial vendor, e.g., Panlab/Harvard Apparatus; for cued FC)
Red-lightbulb lamp (for cued FC)

Day 1: Habituation to the experimental procedure (first session)

1. Make sure all animals (adult mice or rats) in same home-cage are properly identified with either tail marks (lines or numbers) or ear punches.
2. Turn on computer, open software, and turn on fear system equipment to measure freezing (StartFear system or FreezeFrame).
3. Calibrate system according to the manufacturer's instructions and turn on fan installed in the back of the fear chamber.
4. Clean inside of chamber with 70% ethanol.
5. Place one animal inside white methacrylate box and transport it to testing room using a specific route (*route A*).
6. Immediately and gently place animal inside the chamber and let it habituate to testing conditions for 5 min.
7. Using the same white methacrylate box, individually return animal to its home-cage using *route A* in the reverse direction.
8. Repeat steps 4 to 7 until all animals are habituated.

   Use as many boxes and animals as fear chambers installed in the equipment.
9. Clean inside of the chamber with 70% ethanol.

Day 2: Habituation to the experimental procedure (second session)

10. Repeat steps 2 to 9 from day 1.

Day 3: Training/fear acquisition

11. Calibrate fear system following the manufacturer's instructions:
    a. Set shock calibration tool to the desired current intensity (0.3 mA).
    b. For auditory-cued FC, calibrate tone to 75 dB using a decibel meter.
12. Clean inside of the chamber with 70% ethanol and turn on chamber's fan.
13. Bring animal from the vivarium to the experimental room using *route A* in the white methacrylate box.

14a. *For cued FC:* Place animal inside the chamber and start pre-configured acquisition session:
   i. Let animal stay in the chamber for 5 min with the yellow light on and sound off.
   ii. Continue with a 6-KHz tone for 30 s, co-terminating with a 1-s footshock (0.3 mA). Leave an intertrial interval (ITI) of 180 s. Repeat until five trials of CS+US and ITI are completed.

14b. *For contextual FC:* Place animal inside the chamber and start pre-configured acquisition session:
   i. Let animal stay in the chamber for 5 min with the yellow light on and sound off.
   ii. Continue with a footshock (1 s, 0.3 mA) and an ITI of 180 s. Repeat until reaching five trials.

15. Return animal to the vivarium inside the white methacrylate box using *route A* in the reverse direction.
16. Repeat steps 12 to 15 until all animals are conditioned.
17. Clean inside of the chamber with 70% ethanol.

Day 4: Fear extinction

18. Calibrate fear system as explained in step 11.

19a. *For cued FE:*

i. Cover grid floor of the chamber with the gray flat floor and use a red-lightbulb lamp to illuminate interior of the chamber.
ii. Clean chamber with 41% isopropyl alcohol.
iii. Bring animal from the vivarium to the experimental room inside the white methacrylate box using an alternate route (*route B*).
iv. Place animal inside the chamber and start pre-configured extinction session: 5 min with the red light on and fan off, followed by 15 trials of 30-s tones spaced with an ITI of 30 s without tones.
v. Repeat steps 19a, ii, to 19a, iv, until all animals are tested.

19b. *For contextual FE:*

i. Use same context as the one used for day 1.
ii. Clean chamber with 70% ethanol.
iii. Use *route A* to bring animal from the vivarium to the experimental room inside the white methacrylate box.
iv. Place animal inside the chamber and start pre-configured extinction session: record 20 min of animal locomotor activity with the same lighting and flooring as for fear acquisition, without delivering any footshocks.
v. Repeat steps 19b, ii, to 19b, iv, until all animals are tested.

20. Clean all equipment with 70% ethanol at end of the session.

Day 5: Fear extinction recall

21. To test FE recall, repeat steps 18 to 20 from day 4 (FE).

You can test FE recall for just 1 day or for consecutive days depending on the requirements of your design.

BASIC PROTOCOL 2

BLOOD COLLECTION FOR DIRECT MEASUREMENT OF SEX HORMONE LEVELS IN FEAR CONDITIONING

Important considerations

The maximum volume of blood that can be extracted in each collection is critical to avoid hypovolemia (see Critical Parameters and Troubleshooting below).

Habituation to the blood collection procedure

To minimize the stress induced by blood extraction, all animals must be habituated to the procedure. To habituate the animals, perform steps 1 to 12 of the blood collection protocol but skip steps 7 and 9 (do not make the incision or collect blood). It is recommended to alternate the habituation with the blood collection procedure during the entire protocol (i.e., on alternating days).

Repeated sampling

If the protocol requires more than one blood sample collection, follow steps 1 to 15 but perform a new incision in the opposite lateral tail vein at ~2 mm from the last incision.

Materials

Adult mice or rats (see Critical Parameters and Troubleshooting for specifications for strains and age)

Permanent marker or ear punch
250-ml beaker (optional; for mice)

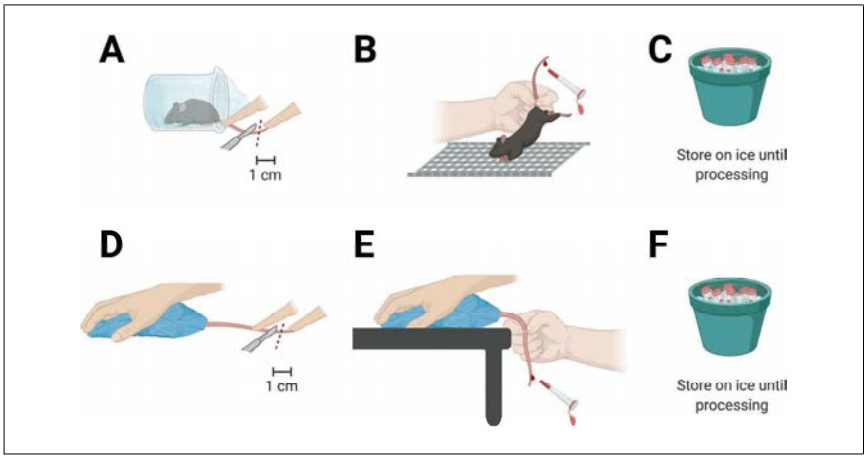

Figure 2 Procedure of blood extraction in rats and mice. For mice: (**A**) Place the animal in a beaker, extend the tail, and make a small incision at 1 cm from the tip of the tail using a scalpel. (**B**) Place the animal on a wire-bar rack to permit grasping and collect blood drops with a capillary tube. If blood flow is inadequate, massage the tail. (**C**) Keep the blood samples on ice for up to 4 hr until further processing. For rats: (**D**) Restrain the animal by using a piece of cloth, extend the tail, and make a small incision at 1 cm from the tip of the tail using a scalpel. (**E**) Place the animal on the edge of the bench and collect blood drops with a capillary tube. If blood flow is inadequate, massage the tail. (**F**) Keep the blood samples on ice for up to 2 to 4 hr until further processing.

Piece of cloth material (~25 × 20 cm; optional; for rats)
Scalpel [scalpel blade holder holding scalpel blade (size 11, Swann-Morton®, cat. no. 0203)]
Wire-bar rack (optional; for mice)
Anticoagulant-treated capillary blood collection tubes (Microvette® CB 300 K2 EDTA, Sarstedt, cat. no. 16.444), pre-labeled
Gauze sponges
Refrigerated centrifuge (Centrifuge 5424 R, Eppendorf), 4°C
0.5-ml Eppendorf tubes, pre-labeled

Blood collection

1. Identify all animals (adult mice or rats) in the same home-cage by marking tail of the animal with lines or numbers using a permanent marker or ear punches.
2. Bring home-cage from the vivarium to the experimental room. Perform blood collection in a different room from the one used for behavioral testing (see Basic Protocol 1). Avoid odors/cleansing products used during the FC paradigm (see Basic Protocol 1).
3. Take an animal from home-cage by gently grasping the tail at the base.
4. Restrict major movements and exploratory behavior while avoiding full restraint:
   a. *For mice:* Fill a 250-ml beaker with paper towels and, holding the tail, place animal's head near the mouth of the beaker. Allow animal to enter the beaker, keeping its tail outside the beaker (Fig. 2A).
   b. *For rats:* Have a second person restrain animal by wrapping and holding it in a piece of cloth material (make a "cylinder" out of the wrapping material and the rat; Fig. 2D).
5. Maintain tension of tail and wait for animal to stop fidgeting.
6. Visualize lateral tail vein in the distal one-third of the tail.

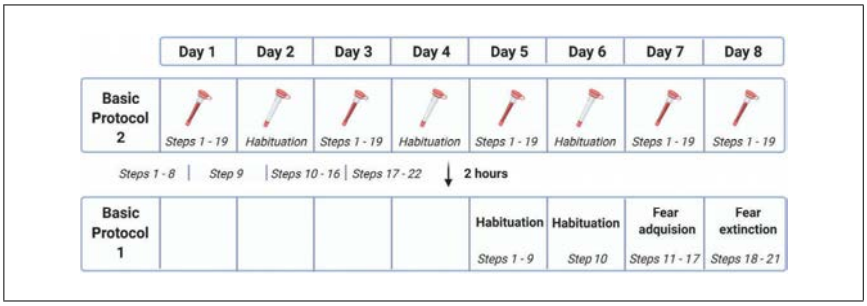

Figure 3 Schematic example of a routine procedure combining direct measures of sex hormones with fear conditioning and extinction.

7. Extend tail with one hand and, with the other hand, made a small incision at an angle perpendicular to tail at 1 cm from the tip of the tail using a scalpel (Fig. 2A and 2D).
8. Place animal in an appropriate position for blood collection:
   a. *For mice:* Remove animal from the beaker and place on a wire-bar rack to permit grasping (Fig. 2B), allowing the animal to roam freely during blood collection.
   b. *For rats:* Keep animal wrapped in the cloth but place it on the edge of the bench so that the tail hangs down (Fig. 2E).
9. Collect blood drops into a capillary tube (separated from the outer tube of a pre-labeled anticoagulant-treated capillary blood collection tube).
10. Optional: Massage tail by passing the thumb and index finger from the base to the tip of the tail if blood flow is inadequate.

    This step should not take longer than 2 to 3 min. Experience is necessary to master this procedure.
11. Once the required volume of blood is collected, apply a gauze sponge with gentle pressure on the bleeding site to ensure hemostasis. If necessary, help hemostasis by applying some ice in the area of the incision.
12. Place animal back into its home-cage when the bleeding has stopped.
13. Close capillary tube by first putting on the small cap (the inferior one) and then closing the lid and place tube in the pre-labeled outer tube.
14. Process all samples as soon as possible after collecting (see steps 16 to 19). If this is not possible, keep samples on ice or at 4°C for up to 4 hr until further processing (Fig. 2C and 2F).
15. Wait ≥2 hr to proceed to a behavioral test (i.e., Basic Protocol 1; see Fig. 3 for an example procedure).

Plasma separation

16. Centrifuge tubes for 15 min at 8000 × *g*, 4°C (Fig. 4A).
17. Carefully remove tubes from the centrifuge.
18. Immediately transfer supernatant into a pre-labeled, clean polypropylene tube (1.5-ml Eppendorf tube; Fig. 4B).

    Following centrifugation, the resulting supernatant is designated as plasma.
19. Assay immediately or freeze plasma samples at −80°C until use (Fig. 4C).

    Avoid repeated freeze/thaw cycles.

    Plasma samples could be employed for sex steroid determination through RIA, mass spectrometry, or ELISA, depending on your laboratory setup and experimental goals.

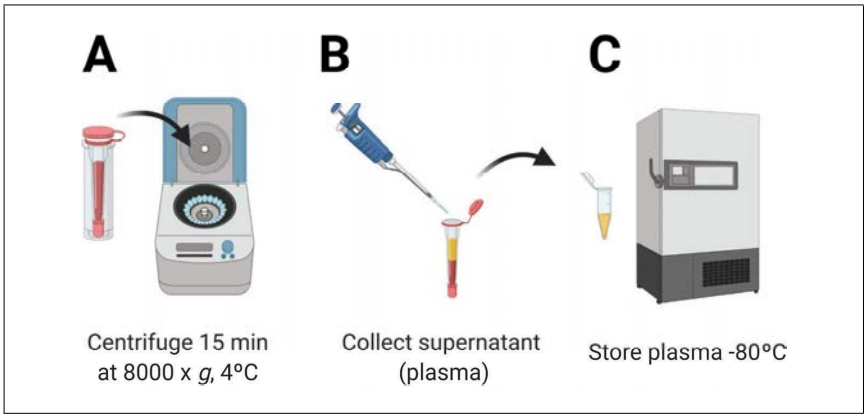

Figure 4 Plasma separation protocol. (**A**) Centrifuge the tubes for 15 min at 8000 × *g*, 4°C. (**B**) Transfer the supernatant plasma into a clean Eppendorf tube. (**C**) Store the plasma samples at −80°C until further processing.

INDIRECT MEASUREMENT OF SEX HORMONES IN FEMALES DURING FEAR CONDITIONING

BASIC PROTOCOL 3

Vaginal cytology is the most reliable and cost-effective procedure to examine the estrous cycle stage in female mice. Endogenous hormonal levels can be inferred indirectly by analyzing the topology of the vaginal cells. The rat and mouse estrous cycle lasts 4 to 5 days, and it is divided into four phases with different hormonal levels: proestrus, estrus, metestrus, and diestrus (Fig. 5A and 5B). In rats, metestrus and diestrus are referred to as diestrus 1 and diestrus 2, respectively.

Some estrous cycle phases are short, so cytology must be performed at the same time each day. To minimize the stress response to manipulation, provide two consecutive days of habituation to cytology procedures before starting testing days. Additionally, leave the animals undisturbed after cytology to allow for any increase in stress hormones to return to baseline levels.

Materials

Cresyl violet acetate stain (Sigma-Aldrich)
Sodium chloride (NaCl)
Adult female mice or rats (see Critical Parameters and Troubleshooting for specifications for strains and age)
Distilled water
DPX mounting medium

Microscopy slides and coverslips (Superfrost™, Fisher Scientific)
Pencil or permanent marker
Piece of cloth material (~25 × 20 cm; optional; for rats)
Slide warmer (optional)
Brightfield microscope with 10× objective (Eclipse 80i)

Preparation

1. Prepare fresh 1% (v/v) cresyl violet acetate stain solution and allow to settle. Prepare fresh 0.9% (w/v) NaCl stock solution.
2. Prepare a different room from the one used for behavioral testing (see Basic Protocol 1), preferably the animals' housing room, for cytology.

   This will avoid additional place conditioning with the experimental room, which may interfere with your behavioral results.

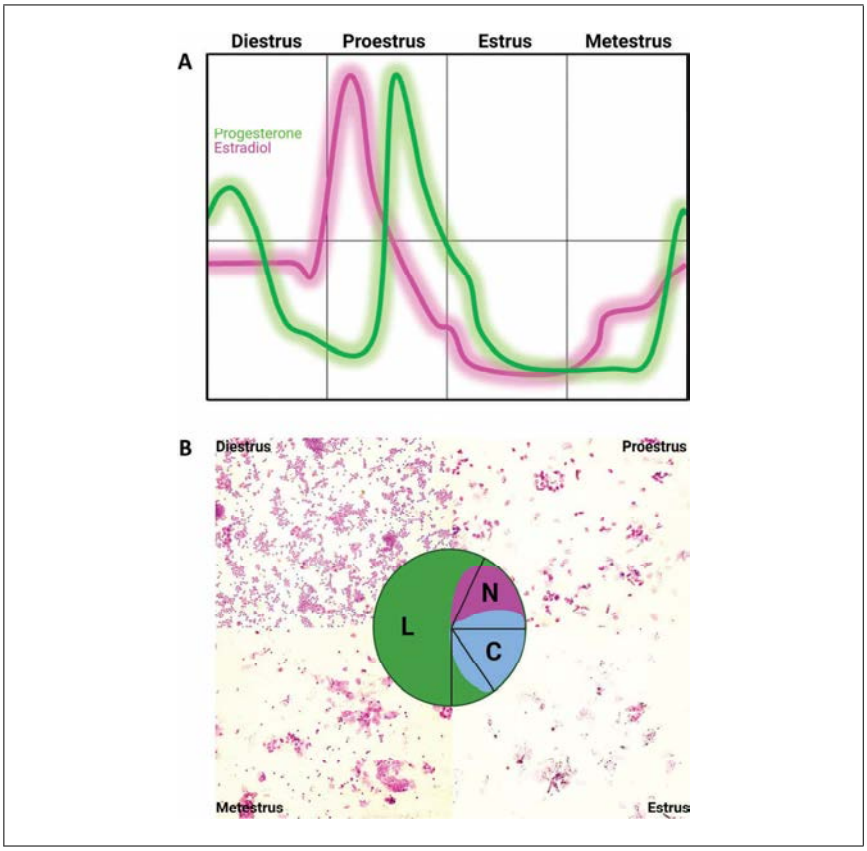

Figure 5 Rodent estrous cycle and characteristics of vaginal secretions. (**A**) Representation of the hormonal levels of estradiol and progesterone over the four stages of the estrous cycle in rodents. (**B**) Characteristics of the cells obtained by vaginal cytology in each phase of the estrous cycle: in proestrus, predominantly nucleated epithelial cells (N); in estrus, cornified epithelial cells (C); in metestrus, three types of cells [nucleated and cornified epithelial cells and leukocytes (L)]; and in diestrus, mainly leukocytes.

3. Make sure all animals (adult female mice or rats) are correctly identified with either ear punches or tail marks.
4. Pre-label microscopy slides using a pencil or a permanent marker.

   Up to 10 vaginal lavage samples can fit on a standard microscope slide (Fig. 6A).
5. Plan to perform vaginal cytology (steps 6 to 19) daily for each animal at the same time (e.g., between 9 AM and 11 AM for consistency in circadian fluctuations of estrous cycle stages) and $\geq$ 2 hr before testing procedures to avoid a possible effect of stress as a consequence of the manipulation (see Basic Protocol 1).

Restraint

6. Correctly identify an animal and proceed to restrain it:
   a. *For mice:* Lift mouse gently by the tail with the dominant hand and place it on a plain surface or any surface that the mouse can grasp with its forelimbs. Place non-dominant hand on the trunk, exerting a little pressure to prevent the mouse from turning. Advance first and second fingers rostrally and grasp skin at the nape (the scruff). With the scruff firmly grasped, turn mouse to the supine position. Place and secure its tail between the fourth and fifth fingers.

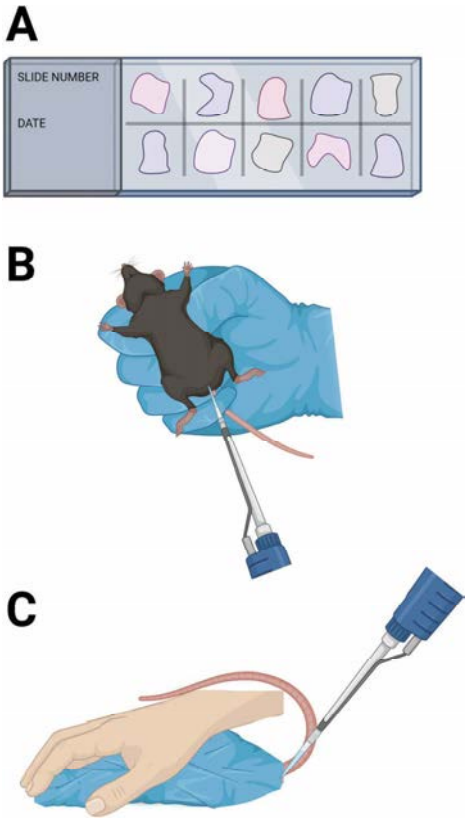

Figure 6 Procedure of vaginal cytology. (**A**) Labeled slide, including 10 vaginal lavage samples appropriately distributed. (**B**) Mouse restraint and insertion of the pipet tip at the entrance of the vaginal opening for vaginal smear cytology. (**C**) Rat restraint and insertion of the pipet tip in the vagina to collect smear cells for vaginal cytology.

The mouse must be correctly restrained so that the animal's movements and hindlimbs do not interfere with the procedure.

b. *For rats:* Have a second person restrain animal by wrapping and holding it in a piece of cloth material (make a "cylinder" out of the wrapping material and the rat).

Restraint of the rat must be achieved with a second person restraining the animal while the other experimenter manipulates the pipet and collects the vaginal smear sample. Experienced researchers may prefer to extract the vaginal smear sample on their own using the dominant hand to restrain the rat and the other hand to collect the sample.

Vaginal lavage

7. Visually inspect vaginal opening. If secretions or urine are observed, gently clean it using a paper towel.
8. Using the dominant hand, fill a pipet with 10 µl of 0.9% NaCl (see step 1) and insert tip into the opening:
   a. *For mice:* With the mouse restrained, slightly tilt the head down and place pipet tip at the entrance of the vaginal opening, inserting it no more than 2 to 3 mm (Fig. 6B).

b. *For rats:* With the rat restrained and the body against a lab table, lift tail and place pipet tip at the entrance of the vaginal opening, inserting it no more than 2 to 3 mm (Fig. 6C).

9. Flush vagina five times with the same solution to collect enough cells, making sure to collect the solution after the final flush.
10. Spread solution on the designated area on the appropriate slide (see step 4). Avoid dispensing solution as drops and be careful not to mix samples from different animals.
11. Return animal to its home-cage, turning it over and releasing the restraint.
12. Repeat steps 6 to 11, using a clean pipet tip for each animal.

Slide processing

13. Dry slides from step 10 at room temperature for 1 hr or with a slide warmer (37°C, 20 min) before staining.

    Slides can be either stored at room temperature in a clean box for 5 to 10 days before staining or stained immediately.

14. Soak slides in 1% cresyl violet acetate stain solution (see step 1) for 5 min.
15. Drain excess staining solution and soak slide in distilled water for 1 min. Repeat this step using clean distilled water.
16. Allow slides to air-dry before proceeding with step 17.

Cytology reading and registering

17. Use a brightfield microscope with a 10× objective to analyze slides.

    If more detail is needed, use a 20× or 40× objective.

18. Infer sex hormone values from the estrous cycle stage presented on the cytology (Fig. 5A).

    To allocate the animal to the corresponding phase of the estrous cycle, observe the proportion of the three main cell types (see Fig. 5B for representative results):

    Proestrus: Predominance of nucleated cells, with few leukocytes or cornified cells.

    Estrus: Mostly cornified cells, with few nucleated cells.

    Metestrus: Predominantly cornified cells and leukocytes, although some nucleated cells can be observed.

    Diestrus: Predominance of leukocytes.

19. After visualizing the slides under the microscope, preserve them long term with DPX mounting medium, cover with coverslips, dry, and store at room temperature.

    For further detail or alternative procedures, see Cora, Kooistra, & Travlos (2015) and Maeng, Cover, Landau, Milad, & Lebron-Milad (2015).

    Follow this protocol for 3 to 4 consecutive cycles to ensure that the animals are correctly cycling and then combine it with Basic Protocol 1 by performing vaginal smear cytology 2 hr before starting behavioral testing.

BASIC PROTOCOL 4 ASSESSMENT OF DOMINANCE STATUS IN MALES BEFORE A FEAR CONDITIONING PROTOCOL

The confrontation tube test is a widely used method to assess social dominance/hierarchy in rodents. This approach offers several advantages over other methodologies for measuring social dominance. Firstly, it does not require complex or expensive equipment (just a methacrylate tube; see Support Protocol), complicated experimental settings, or

laborious animal behavior quantification. Moreover, its results are highly robust. However, it also has some limitations, including the influence that different factors can exert on the results (e.g., age of the animals, strain type, basal stress levels).

Materials

Adult male mice or rats (see commentary below in "Critical Parameters" and "Troubleshooting" for specifications on strains and age)
70% (v/v) ethanol

Confrontation tube, including black barriers (see Support Protocol)
Video camera
30-cm plastic stick (optional)
Stopwatch

Before starting

1. Pair-house adult male mice or rats for 5 weeks before procedure (steps 3 to 15) to establish stable dominance/submission status.
2. Prepare environmental setup by lowering the room light intensity to 10 lux and placing confrontation tube on a stable surface, with the ends of the tube facing similar contexts (such as walls). Place video camera on top of the confrontation tube and focus camera on the tube to record all sessions.

Day 1: Habituation (first session)

3. Clean inside of the confrontation tube with 70% ethanol.
4. Habituate one animal from home-cage to the tube by allowing it three consecutive crossings from one end to the other. If needed, gently help animal fully cross the tube with a 30-cm plastic stick.
5. Return animal to its home-cage and clean tube with 70% ethanol.
6. Habituate another animal from home-cage to the tube as mentioned in step 4.
7. Repeat steps 4 to 6 until all animals are habituated.

Day 2: Habituation (second session)

8. Repeat steps 3 to 7 under the same environmental conditions as on day 1.

Day 3: Confrontation session

9. Repeat steps 3 to 7 under the same environmental conditions as on day 1.
10. Clean inside of tube with 70% ethanol and place black barriers through the slits (Fig. 7A).
11. Allow each animal from a couple to enter tube from opposite ends (Fig. 7B).
12. Once the animals reach the barriers, remove them and start timing 2 min with a stopwatch. Allow animals to confront each other inside the tube for 2 min, or until one of the two is expulsed from the tube (Fig. 7C and 7D). If 2 min pass, expulse animals from the tube using a 30-cm plastic stick and return them to their home-cage.
13. Clean tube with 70% ethanol and repeat steps 11 to 12 until every pair has been tested eight times. Switch tube entrance for each animal in each pair between trials.
14. Calculate proportion of won trials per animal for every session and cage. Designate animal from each couple that presents the highest rate of won sessions as the dominant male.
15. One day after completing the protocol, proceed to Basic Protocol 1 to assess FC parameters depending on hormonal (e.g., testosterone) values inferred from social hierarchies.

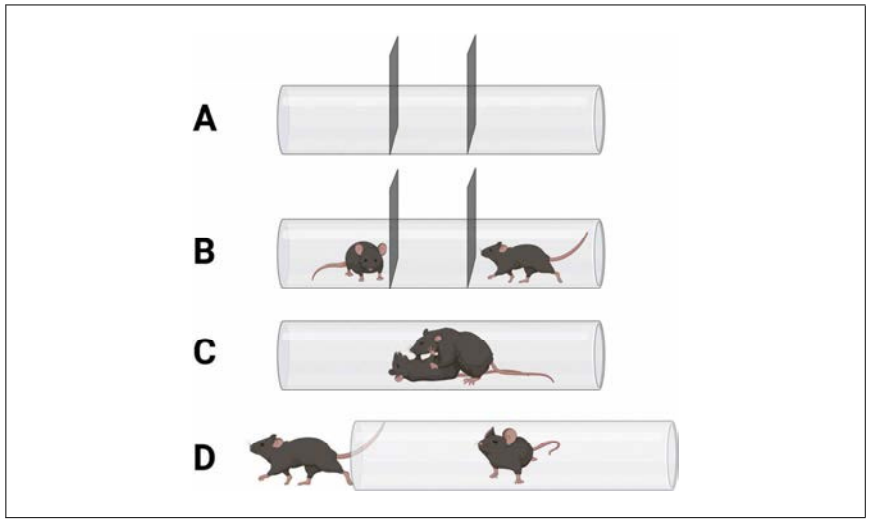

Figure 7 Confrontation tube test protocol. (**A**) Setting up the confrontation tube test. Introduction of black plastic sheets inside previously cut slits aligned with each end of the tube. (**B**) Introduction of the subjects into opposite ends of the tube. (**C**) Interaction of the animals in the middle of the tube, with the more dominant animal showing greater aggression. (**D**) The more dominant animal forces its opponent out of the tube.

SUPPORT PROTOCOL

CONSTRUCTION OF A CONFRONTATION TUBE

The confrontation tube test (Basic Protocol 4) is a valid option to assess social hierarchy in rodents, and it allows the experimenter to infer circulating testosterone concentrations based on the dominance status of the animal. The following protocol generates a confrontation tube in <2 hr using cheap materials that can easily be found. The apparatus resulting from this Support Protocol may be used in Basic Protocol 4 as an alternative to a manufactured version of the confrontation tube.

Materials

Methacrylate tube (30 cm long with inner diameter of 3.5 cm for mice; 48 cm long with inner diameter of 5 to 5.5 cm for rats; custom cut by supplier; Servicio Estación)
Permanent marker
Saw with carbide-tipped blade (e.g., Black+Decker BDEJS600C)
Black plastic sheet (0.05 cm thick; e.g., black plastic folder)
Methacrylate plate (30 × 10 × 0.2 cm for mice; 48 × 15 × 0.2 cm for rats; custom cut by supplier; Servicio Estación)
Instant glue (e.g., Loctite Perfect Pen)

1. Draw lines on a methacrylate tube with a permanent marker:
   a. *For mice:* Draw two lines at 13 cm from each end of tube, perpendicular to the length of the tube and from the midpoint of one side to the midpoint of the other.
   b. *For rats:* Draw two lines at 20 cm from each end of tube, perpendicular to the length of the tube and from the midpoint of one side to the midpoint of the other.
2. Use a saw with a carbide-tipped blade to cut through the lines to create two aligned slits at the length specified from each end of the tube (see step 1).

   This cut should be diameter wide so that the sheet in step 3 fits fully across the diameter.
3. Cut two identical barriers to fit into the slits using a black plastic sheet (Fig. 7A).

4. Glue tube to the middle line of a methacrylate plate using instant glue in order to create a platform so that the tube can be firmly placed on top of a flat surface.

COMMENTARY

Background Information

Determination of sex hormones (Basic Protocols 3 and 4 and following Basic Protocol 2) has proven to be an additional tool for the interpretation of behavioral outcomes in fear tasks (Basic Protocol 1) in both males and females. Circulating sex hormone levels can be easily inferred based on behavior in males (Basic Protocol 4) or vaginal smear cytology in females (Basic Protocol 3), providing rapid and low-cost methods that allow the experimenter to manipulate learning and memory processes in certain hormonal states. Further, these approaches may be combined with direct measures (see Basic Protocol 2 for blood collection) that eventually provide a full picture of the role of sex hormones in fear learning and memory formation.

Circulating sex hormones have been shown to modulate extinction memory, a special type of fear memory that is formed when the previously conditioned stimulus is presented repeatedly without an US (Myers & Davis, 2007). In this context, the role of sex hormones is poorly understood (Bangasser & Valentino, 2014), and females' circulating sex hormones have been historically misconstrued as a research problem due to their variability, even though variability in testosterone in males may occur at even higher rates (Shansky, 2019). Progesterone and estradiol are hypothesized to exert opposite effects in the formation of fear memories (Graham & Daher, 2016), although mixed results are commonly reported in the effects of progesterone on FE memory. Estradiol has proven to facilitate FE when given systemically to ovariectomized rats or during those phases of the estrous cycle that present high estradiol, such as proestrus (Milad et al., 2009). Besides, the inhibition of estradiol synthesis leads to reduced FE learning (Graham & Milad, 2014), and the administration of oral contraceptives reduces FE in a phenotype that can be easily reversed by terminating the treatment or using estradiol agonists (Graham & Milad, 2013). All these data support the role of estradiol in the facilitation of extinction memory. In contrast, progesterone antagonists administered during metestrus have been shown to revert the decreased extinction caused by the lack of sex hormones (Graham & Daher, 2016).

Regarding the age of the animal, sex hormones have already been shown to play a relevant role in fear memory acquisition and retention during adolescence. Adolescent female mice in metestrus, diestrus, or proestrus have shown impaired FE and retention, with suppression of these effects by gonadectomy. On the contrary, males present impaired FE after orchidectomy (Perry et al., 2020). These results contrast with the previous results found in adult rodents, showing an impairment effect of estradiol on FE when animals are in the adolescent stage of their lifespan. Allopregnanolone, a compound that results from progesterone metabolism, has been shown to reduce auditory and contextual FC when infused into the basolateral amygdala, whereas it is only effective for contextual FC when infused into the bed nucleus of the stria terminalis (Acca, Mathew, Jin, Maren, & Nagaya, 2017). Of note, the effects of certain drugs, such as the effect of a neurokinin 3 receptor antagonist on fear memory consolidation or fluoxetine on FE, have proven to be enhanced by the presence of high levels of circulating sex hormones during proestrus (Florido et al., 2021; Lebrón-Milad et al., 2013).

In regard to testosterone, the reported results regarding its role in FC, FE, and memory consolidation are controversial. Some reports state that circulating testosterone does not contribute to these processes (Anagnostaras et al., 1998; McDermott, Liu, & Schrader, 2012). Meanwhile, other studies have shown a relevant role for testosterone in the consolidation of FE memory. Administration of fadrozole, an antagonist of the aromatase enzyme that converts testosterone into estradiol, has shown to decrease extinction memory consolidation in male rats (Graham & Milad, 2014). In contrast, administration of a gonadotropin-releasing hormone agonist in males, which enhances testosterone release by the testicles, facilitates FE memory consolidation (Maeng et al., 2017). The most accepted hypothesis for these results is that testosterone exerts its facilitating effects on memory consolidation through its conversion to estradiol, therefore acting on memory consolidation in a similar manner to putative circulating estradiol in females. Although more research is needed to decipher the specific mechanism by which

estradiol modulates extinction memory, these data point to an explanation for the high variations in FE recall in females in comparison to males.

Critical Parameters and Troubleshooting

Age and strain of rodents

Age and strain are two crucial parameters to take into consideration when selecting rodents for the experimental protocols explained above.

In this context, first, it is important to take into account the ontogeny of the limbic system to obtain optimal learning curves in Basic Protocol 1. For instance, it has been demonstrated that mice under 10 days of age (postnatal day 10, or P10) present impaired FC to odors (Sullivan, Landers, Yeaman, & Wilson, 2000). In Long Evans rats, it has been demonstrated that rats on P18 present conditioned fear to a cue (such as a tone) but do not acquire contextual FC until P23 (Rudy, 1993). Additionally, the decline in memory related to age is also a crucial factor when selecting the age of animals to test. In mice, 20-month-old C57BL/6J show impaired retention at 48 hr post-training in cued FC, and 24-hr retention of contextual FC is already impaired at 11 months old (Liu et al., 2003). In rats, 12-month-old Sprague-Dawley do not present age-related memory deficits in FC tasks (Cuppini et al., 2006), and 22-month-old Wistar rats present increased contextual FC (Doyère, Gisquet-Verrier, De Marsanich, & Ammassari-Teule, 2000).

Second, to correctly assess the sex hormone state through the methods described above, it is important to consider the onset of puberty, understood as those changes in gonads and sexual hormones that accompany the transition to adulthood. In mice, sexual maturity appears around P30 in females and P33 in males, whereas in rats, females present their first estrous cycle around P40, and males' sexual maturation occurs around P40 (Schroeder, Notaras, Du, & Hill, 2018). Importantly, reproductive senescence in rats and mice typically takes place between 9 and 12 months of age (Koebele & Bimonte-Nelson, 2016). The estropause in females is characterized by persistent estrus in vaginal smear cytology, with moderate to high levels of estradiol and moderate progesterone related to the lack of feedback from the ovaries to the hypothalamus (Koebele & Bimonte-Nelson, 2016). Male rodents typically present a decline in testosterone serum levels, as known as andropause, between 12 and 14 months old (Cheng et al., 2009).

Interestingly for hormonal assays (Basic Protocols 3 and 4 and following Basic Protocol 2), differences in sex hormone concentration have been determined depending on the strain. For example, diverse values for estradiol in serum have been reported regarding the mouse strain chosen. This might be an important consideration depending on the serum volume requirements of your chosen technique to detect sex steroids. In proestrus, C57BL/6J female mice typically yield values between 5 and 25 ρg/ml, with the most recent work with highly sensitive techniques reporting concentrations around 8 ρg/ml (Hokenson et al., 2021; Nilsson et al., 2015). In contrast, CD-1 or ICR mice have shown concentrations in serum around 55 to 120 ρg/ml (Lee, Kim, Chung, Kim, & Yang, 2013; Walmer, Wrona, Hughes, & Nelson, 1992). These differences might be of interest when selecting a detection method because some methods may have high detection limits (e.g., 11.2 ρg/ml for RIA; Nelson, Felicio, Osterburg, & Finch, 1981) that might be incompatible with the amount of serum extracted in female mice.

To conclude this section, our recommendation in the use of rodents, independent of the species and strain, is to select young and healthy adults that may present normal conditioning and extinction and adequate levels of sexual steroids in serum to avoid issues with concentration during determination.

Blood volume and stress during blood extraction

As explained above, blood extracts from mice or rats may be obtained using different methodologies (see Current Protocols article; Donovan & Brown, 2005; Parasuraman et al., 2010). Whereas other methods present the advantage of more abundant collection, tail-nick extracts are restricted to a small volume of blood per unit time. The total circulating blood volume of a rodent is estimated to be ~8% of its body weight (OACU, n.d.). Thus, large-volume blood extracts may cause hypovolemic shock to the animal, so ethical as well as experimental parameters should be considered. To avoid hypovolemia, blood can be extracted by tail-nick at different rates: 1% of total blood every 24 hr (for 25-g mice, extract 14 to 18 µl/24 hr; for 250-g rats, extract 138 to 175 µl/24 hr), 7.5% over 7 days, or 10% over 2 to 4 weeks, for example. The volume of blood required depends on the type of assays planned

to be performed to evaluate the levels of sexual hormone expression. For instance, Basic Protocol 2 is designed to obtain 20 μl plasma from each blood sample in order to assess the hormone levels by using mass spectrometry assays. Other methods, such as ELISA or RIA, require a bigger amount of plasma for analyses. However, if a higher volume of blood is required for further analysis, up to 0.5 ml trunk blood could be collected by decapitating the animal.

Because this particular methodology does not require anesthesia or terminal interventions, it is especially relevant when different sex hormone measurements over time are required for a particular procedure. The tail vein can be used for repeated or serial blood collection without anesthesia. Moreover, it has been proven to be a minimally invasive method of blood sampling, showing low corticosterone increases in response to the extraction, which allows multiple samples to be taken from the same animal with as little stress as possible. This approach appears ideal in comparison to those extraction methods that require the animal's full restraint during sampling, allowing the animal to be less stressed during extraction due to free mobility in mice or some degree of mobility in rats. Another aspect to consider is that blood sample quality decreases with prolonged bleeding times. Therefore, it is important to not extend the collection of blood to >3 min.

Unestrous females and vaginal occlusion

Inter-individual variability is present in the estrous cycle of females and also in vaginal and vulvar morphology. In the first case, some females may present alterations in their estrous cycle, with prolonged states of estrus and diestrus in response to genetic or environmental factors (Maeng et al., 2015). In some cases, such as pseudopregnancy, females may present a continuous state of diestrus in vaginal cytology readings (Basic Protocol 3), showing alterations in sex hormone fluctuations. These cases are easily identified during the 2-week monitoring process before procedures, and these females must be excluded from experiments due to abnormal cycling.

In our experience, a small percentage of outbred animals from commercial vendors present vaginal occlusion. This phenomenon is characterized by a congenic closure of the vulva, causing vaginal smear accumulations in the lower part of the abdomen that create a liquid bag that very much resembles a scrotum, with the urethra pointing upward and looking like a penis. These animals should be carefully inspected to avoid incorrect sexing. Further, they are not suitable for indirect measures of sex hormones, and their use must be avoided for experimental purposes due to abnormalities in the estrous cycle caused by possible infections of the genitalia.

Difficulties reading vaginal smear cytology

Under the microscope (Basic Protocol 3), some samples may be difficult to read. This might be caused by dirt accumulated over the slides, detritus in the sampling, or incorrect pipet use. To avoid issues during readings, use clean slides and fresh 0.9% NaCl solution, as well as a new pipet tip for each animal. Clean to remove secretions and urine in the vaginal opening before performing the lavage. Furthermore, spread the vaginal lavage fluid generously on the slide to prevent cell accumulation on the same plane and avoid using an excess of oil-based markers on the slides. For correct staining, allow for the 1% cresyl violet solution to settle before staining or filter the solution before every use. Always use clean distilled water to wash the slides after staining.

Some samples may be lost during the staining process. To avoid that, use slides that allow cell adhesion and let the slides completely dry before staining. Do not shake or make rough movements during staining and washing. If cellularity is low, repeat sampling and staining the next day. Avoid re-sampling the same day.

For a troubleshooting guide for reading vaginal smear cytology, please see Table 1.

Stress during the confrontation tube test

Indirect behavioral measures of sex hormones may be stressful for the animal if it is not correctly habituated or if it is exposed to stressful environmental conditions during testing. When performing the confrontation tube test (Basic Protocol 4), some animals may stay inside the tube for >2 min per trial, with little to no movement through the tube, indicating an effect of stress.

Make sure all animals take their time to walk forward through the tube during the habituation sessions. This procedure might take ~10 min per animal per day. Otherwise, animals may not be sufficiently trained to walk forward in the tube, and this might generate stress during the test sessions. During the first couple of sessions, animals may be again stressed due to the presence of their companion in the other part of the tube. This

Table 1 Troubleshooting for Common Problems in Reading Vaginal Smear Cytology

Issue	Possible cause	Solution
Difficulties in reading the sample	Dirt accumulated over the slide, detritus in the sample, incorrect pipet use	Use clean slides Make fresh 0.9% NaCl solution each day Clean to remove secretions and urine from the vaginal opening before performing the lavage Use a new pipet tip for each animal
	Cell accumulation on the same plane	Spread the vaginal lavage fluid generously on the slide and avoid forming drops
Incorrect staining	Problems with the 1% cresyl violet solution	Allow for the 1% cresyl violet solution to settle before staining or filter the solution before every use Always use clean distilled water to wash the slides after staining
Lost samples during the staining process	Cells not adhered to the slides	Use slides that allow appropriate cell adhesion and let the slides completely dry before staining Do not shake or make rough movements during staining or washing
Low cellularity	Misplaced pipet tip during sample collection	Repeat the next day and avoid re-sampling the same day

effect should disappear due to the habituation of the animals to the task throughout the sessions. If the stress effect persists, recalibrate the lighting and make sure no strange noises are present during the habituation and test sessions. Additionally, place the tube test on a large platform so animals can get outside the tube easily with no risk of harming themselves. Moreover, urine or feces from previously tested mice may interfere with the task. Make sure to clean the tube with 70% ethanol after every trial and completely dry it with a paper towel.

Understanding Results

For direct hormonal measures (following Basic Protocol 2), make sure that assumptions for the general linear model (GLM) are met. Otherwise, use non-parametric tests or switch to the generalized linear model (GzLM) for more than two-factor analyses. Basal testosterone in males may present high variability, with increased serum testosterone in dominant males in comparison to submissive ones, although these differences may not always reach statistical significance in small sample sizes ($n < 14$) due to inter-individual variability in testosterone pulses. Additionally, inter-individual variation of estradiol in females that present similar vaginal smear cytology (Basic Protocol 3) is frequent. Thus, within-subject experimental designs are recommended for this type of procedure, especially with low sample sizes.

Indirect hormonal measures (Basic Protocols 3 and 4) follow a qualitative analysis approach. In females (Basic Protocol 3), make annotations of the estrous stage that they present every day to ensure correct cycling and optimal manipulation according to the different stages of the estrous cycle. In males (Basic Protocol 4), quantify the proportion of won trials per session and determine the winner male from each pair in each session. After that, quantify the proportion of won sessions per animal to obtain a measure of the dominant and submissive animals in each pair. Consider that a high number of sessions (at least six) is necessary to correctly assess hierarchies. Animals may encounter difficulties in completely learning the task during the first two sessions, so you will need a high enough number of sessions to achieve representative behavior.

Time Considerations

When using direct measures of sex hormones (following Basic Protocol 2), consider that animals must stop bleeding and return to basal conditions before behavioral manipulations (Basic Protocol 1). Usually 2 hr after the manipulations is enough time to recover basal corticosterone levels and avoid any stress effect during FC or FE. The time required to complete this protocol will depend on the sample size of animals that you want to test. Consider that, for a home-cage of four animals, blood extracts are usually collected within 5 min of touching the home-cage.

In natural cycling females, as specified in Basic Protocol 3, vaginal smear lavages must be performed 1 hr after the onset of light exposure to avoid an effect of the light changes. All vaginal samples must be obtained around the same time day after day to avoid circadian fluctuation of sex hormones in females (Williamson et al., 2019) and get comparable readings. During monitoring of the estrous cycle, during the 2 weeks before the experiments, samples must be obtained at the beforementioned time but may be stored for several days for later analysis. The time necessary for sampling a home-cage of four animals is <3 min. This timing should be considered when planning the experiment and selecting an appropriate sample size of animals so as not to exceed a reasonable time for sample collection (Basic Protocol 3).

For dominance/submission determination in pairs of male mice (Basic Protocol 4), each pair must be housed together for ≥5 weeks before testing. This will result in a stable social hierarchy between them. After the completion of Basic Protocol 4, dominance and submission status will remain unaltered unless environmental changes are made in the vivarium, so there is no need to keep performing additional indirect measures of sex hormone levels. It takes 8 days to fully complete Basic Protocol 4. The time needed for testing each pair of mice depends on the simple size chosen. Note that each trial should not be >2 min. The completion of the Support Protocol should take <2 hr.

It is noteworthy that Basic Protocol 2, for direct determination of sex hormones in circulating blood, may be combined with indirect methods (Basic Protocols 3 and 4). Because indirect measures are usually qualitative and hard to computerize for reliable statistical analysis, quantitative measures of sex hormones might be beneficial for the experimenter to establish correlations between the two measures or perform quantitative statistical tests.

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Conflict of Interest statement

The authors declare no conflict of interest.

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Data sharing not applicable – no new data generated

Literature Cited

Acca, G. M., Mathew, A. S., Jin, J., Maren, S., & Nagaya, N. (2017). Allopregnanolone induces state-dependent fear via the bed nucleus of the stria terminalis. *Hormones and Behavior*, *89*, 137–144. doi: 10.1016/j.yhbeh.2017.01.002.

Ajayi, A. F., & Akhigbe, R. E. (2020). Staging of the estrous cycle and induction of estrus in experimental rodents: An update. *Fertility Research and Practice*, *6*, 5. doi: 10.1186/s40738-020-00074-3.

Anagnostaras, S. G., Maren, S., Decola, J. P., Lane, N. I., Gale, G. D., Schlinger, B. A., & Fanselow, M. S. (1998). Testicular hormones do not regulate sexually dimorphic Pavlovian fear conditioning or perforant-path long-term potentiation in adult male rats. *Behavioural Brain Research*, *92*, 1–9. doi: 10.1016/S0166-4328(97)00115-0.

Andero, R., Brothers, S. P., Jovanovic, T., Chen, Y. T., Salah-Uddin, H., Cameron, M., … Ressler, K. J. (2013). Amygdala-dependent fear is regulated by Oprl1 in mice and humans with PTSD. *Science Translational Medicine*, *5*, 188ra73–188ra73. doi: 10.1126/scitranslmed.3005656.

Andero, R., Dias, B. G., & Ressler, K. J. (2014). A role for Tac2, NkB, and Nk3 receptor in normal and dysregulated fear memory consolidation. *Neuron*, *83*, 444–454. doi: 10.1016/j.neuron.2014.05.028.

Office of Animal Care and Use (OACU) (n.d.). *Animal Research Advisory Committee Guidelines*. National Institutes of Health Office of Intramural Research. Retrieved from https://oacu.oir.nih.gov/animal-research-advisory-committee-guidelines.

Aryal, B., Tae-Hyun, K., Yoon-Gyoon, K., & Hyung-Gun, K. (2011). A comparative study

of the pharmacokinetics of traditional and automated dosing/blood sampling systems using gabapentin. *Indian Journal of Pharmacology*, *43*, 262–269. doi: 10.4103/0253-7613.81512.

Bangasser, D. A., & Valentino, R. J. (2014). Sex differences in stress-related psychiatric disorders: Neurobiological perspectives. *Frontiers in Neuroendocrinology*, *35*, 303–319. doi: 10.1016/j.yfrne.2014.03.008.

Bishop, M. J., & Chevins, P. F. D. (1987). Urine odours and marking patterns in territorial laboratory mice (Mus musculus). *Behavioural Processes*, *15*, 233–248. doi: 10.1016/0376-6357(87)90009-X.

Blechert, J., Michael, T., Vriends, N., Margraf, J., & Wilhelm, F. H. (2007). Fear conditioning in posttraumatic stress disorder: Evidence for delayed extinction of autonomic, experiential, and behavioural responses. *Behaviour Research and Therapy*, *45*, 2019–2033. doi: 10.1016/j.brat.2007.02.012.

Chang, C., Knapska, E., Orsini, C. A., Rabinak, C. A., Zimmerman, J. M., & Maren, S. (2009). Fear extinction in rodents. *Current Protocols in Neuroscience*, *47*, 8.23.1–8.23.17. doi: 10.1002/0471142301.ns0823s47.

Cheng, J., Hu, W., Toung, T. J., Zhang, Z., Parker, S. M., Roselli, C. E., & Hurn, P. D. (2009). Age-dependent effects of testosterone in experimental stroke. *Journal of Cerebral Blood Flow and Metabolism*, *29*, 486–494. doi: 10.1038/jcbfm.2008.138.

Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal cytology of the laboratory rat and mouse: Review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicologic Pathology*, *43*, 776–793. doi: 10.1177/0192623315570339.

Cuppini, R., Bucherelli, C., Ambrogini, P., Ciuffoli, S., Orsini, L., Ferri, P., & Baldi, E. (2006). Age-related naturally occurring depression of hippocampal neurogenesis does not affect trace fear conditioning. *Hippocampus*, *16*, 141–148. doi: 10.1002/hipo.20140.

Donovan, J., & Brown, P. (2005). Blood collection. *Current Protocols in Neuroscience*, *33*, A.4G.1–A.4G.9. doi: 10.1002/0471142301.nsa04gs33.

Doyère, V., Gisquet-Verrier, P., de Marsanich, B., & Ammassari-Teule, M. (2000). Age-related modifications of contextual information processing in rats: Role of emotional reactivity, arousal and testing procedure. *Behavioural Brain Research*, *114*, 153–165. doi: 10.1016/S0166-4328(00)00223-0.

Florido, A., Velasco, E. R., Soto-Faguás, C. M. et al. (2021). Sex differences in fear memory consolidation via Tac2 signaling in mice. *Nature Communications*, *12*, 2496. doi: 10.1038/s41467-021-22911-9.

Fluttert, M., Dalm, S., & Oitzl, M. S. (2000). A refined method for sequential blood sampling by tail incision in rats. *Laboratory Animals*, *34*, 372–378. doi: 10.1258/002367700780387714.

Graham, B. M., & Daher, M. (2016). Estradiol and progesterone have opposing roles in the regulation of fear extinction in female rats. *Neuropsychopharmacology*, *41*, 774–780. doi: 10.1038/npp.2015.202.

Graham, B. M., & Milad, M. R. (2013). Blockade of estrogen by hormonal contraceptives impairs fear extinction in female rats and women. *Biological Psychiatry*, *73*, 371–378. doi: 10.1016/j.biopsych.2012.09.018.

Graham, B. M., & Milad, M. R. (2014). Inhibition of estradiol synthesis impairs fear extinction in male rats. *Learning and Memory*, *21*, 347–350. doi: 10.1101/lm.034926.114.

Hermans, D., Craske, M. G., Mineka, S., & Lovibond, P. F. (2006). Extinction in human fear conditioning. *Biological Psychiatry*, *60*, 361–368. doi: 10.1016/j.biopsych.2005.10.006.

Hokenson, R. E., Short, A. K., Chen, Y., Pham, A. L., Adams, E. T., Bolton, J. L., … Baram, T. Z. (2021). Unexpected role of physiological estrogen in acute stress-induced memory deficits. *The Journal of Neuroscience*, *41*, 648–662. doi: 10.1523/JNEUROSCI.2146-20.2020.

Jones, C. E., Agee, L., & Monfils, M.-H. (2018). Fear conditioning by proxy: Social transmission of fear between interacting conspecifics. *Current Protocols in Neuroscience*, *83*, e43. doi: 10.1002/cpns.43.

Jury, N. J., DiBerto, J. F., Kash, T. L., & Holmes, A. (2017). Sex differences in the behavioral sequelae of chronic ethanol exposure. *Alcohol*, *58*, 53–60. doi: 10.1016/j.alcohol.2016.07.007.

Koebele, S. V., & Bimonte-Nelson, H. A. (2016). Modeling menopause: The utility of rodents in translational behavioral endocrinology research. *Maturitas*, *87*, 5–17. doi: 10.1016/j.maturitas.2016.01.015.

Lebrón-Milad, K., Tsareva, A., Ahmed, N., & Milad, M. R. (2013). Sex differences and estrous cycle in female rats interact with the effects of fluoxetine treatment on fear extinction. *Behavioural Brain Research*, *253*, 217–222. doi: 10.1016/j.bbr.2013.07.024.

Lee, H., Kim, H., Chung, Y., Kim, J., & Yang, H. (2013). Thymocyte differentiation is regulated by a change in estradiol levels during the estrous cycle in mouse. *Development & Reproduction*, *17*, 441–449. doi: 10.12717/DR.2013.17.4.441.

Liu, R., Liu, I. Y., Bi, X., Thompson, R. F., Doctrow, S. R., Malfroy, B., & Baudry, M. (2003). Reversal of age-related learning deficits and brain oxidative stress in mice with superoxide dismutase/catalase mimetics. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 8526–8531. doi: 10.1073/pnas.1332809100.

Maeng, L. Y., Cover, K. K., Landau, A. J., Milad, M. R., & Lebron-Milad, K. (2015). Protocol for studying extinction of conditioned fear in naturally cycling female rats. *Journal of Visualized Experiments*, 52202. doi: 10.3791/52202.

Maeng, L. Y., Taha, M. B., Cover, K. K., Glynn, S. S., Murillo, M., Lebron-Milad, K., &

Milad, M. R. (2017). Acute gonadotropin-releasing hormone agonist treatment enhances extinction memory in male rats. *Psychoneuroendocrinology*, *82*, 164–172. doi: 10.1016/j.psyneuen.2017.05.015.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. *Annual Review of Neuroscience*, *24*, 897–931. doi: 10.1146/annurev.neuro.24.1.897.

McDermott, C. M., Liu, D., & Schrader, L. A. (2012). Role of gonadal hormones in anxiety and fear memory formation and inhibition in male mice. *Physiology and Behavior*, *105*, 1168–1174. doi: 10.1016/j.physbeh.2011.12.016

Milad, M. R., Igoe, S. A., Lebron-Milad, K., & Novales, J. E. (2009). Estrous cycle phase and gonadal hormones influence conditioned fear extinction. *Neuroscience*, *164*, 887–895. doi: 10.1016/j.neuroscience.2009.09.011.

Myers, K. M., & Davis, M. (2007). Mechanisms of fear extinction. *Molecular Psychiatry*, *12*, 120–150. doi: 10.1038/sj.mp.4001939.

Nelson, J. F., Felicio, L. S., Osterburg, H. H., & Finch, C. E. (1981). Altered profiles of estradiol and progesterone associated with prolonged estrous cycles and persistent vaginal cornification in aging C57BL/6J mice. *Biology of Reproduction*, *24*, 784–794. doi: 10.1095/biolreprod24.4.784.

Nilsson, M. E., Vandenput, L., Tivesten, Å., Norlén, A. K., Lagerquist, M. K., Windahl, S. H., … Ohlsson, C. (2015). Measurement of a comprehensive sex steroid profile in rodent serum by high-sensitive gas chromatography-tandem mass spectrometry. *Endocrinology*, *156*, 2492–2502. doi: 10.1210/en.2014-1890.

Parasuraman, S., Raveendran, R., & Kesavan, R. (2010). Blood sample collection in small laboratory animals. *Journal of Pharmacology and Pharmacotherapeutics*, *1*, 87–93. doi: 10.4103/0976-500X.72350.

Perry, C. J., Ganella, D. E., Nguyen, L. D., Du, X., Drummond, K. D., Whittle, S., … Kim, J. H. (2020). Assessment of conditioned fear extinction in male and female adolescent rats. *Psychoneuroendocrinology*, *116*, 104670. doi: 10.1016/j.psyneuen.2020.104670.

Pickens, C. L., Golden, S. A., & Nair, S. G. (2013). Incubation of fear. *Current Protocols in Neuroscience*, *64*, 6.27.1–6.27.11. doi: 10.1002/0471142301.ns0627s64.

Roelofs, K. (2017). Freeze for action: Neurobiological mechanisms in animal and human freezing. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*, 20160206. doi: 10.1098/rstb.2016.0206.

Rudy, J. W. (1993). Contextual conditioning and auditory cue conditioning dissociate during development. *Behavioral Neuroscience*, *107*, 887–891. doi: 10.1037/0735-7044.107.5.887.

Schroeder, A., Notaras, M., Du, X., & Hill, R. A. (2018). On the developmental timing of stress: Delineating sex-specific effects of stress across development on adult behavior. *Brain Sciences*, *8*, 121. doi: 10.3390/brainsci8070121.

Shansky, R. M. (2019). Are hormones a "female problem" for animal research? Outdated gender stereotypes are influencing experimental design in laboratory animals. *Science*, *364*, 825–826. doi: 10.1126/science.aaw7570.

Singletary, S. J., Kirsch, A. J., Watson, J., Karim, B. O., Huso, D. L., Hurn, P. D., & Murphy, S. J. (2005). Lack of correlation of vaginal impedance measurements with hormone levels in the rat. *Contemporary Topics in Laboratory Animal Science*, *44*, 37–42.

Sullivan, R. M., Landers, M., Yeaman, B., & Wilson, D. A. (2000). Neurophysiology: Good memories of bad events in infancy. *Nature*, *407*, 38–39. doi: 10.1038/35024156.

van Loo, P. L. P., Mol, J. A., Koolhaas, J. M., van Zutphen, B. F. M., & Baumans, V. (2001). Modulation of aggression in male mice: Influence of group size and cage size. *Physiology and Behavior*, *72*, 675–683. doi: 10.1016/S0031-9384(01)00425-5.

Velasco, E. R., Florido, A., Milad, M. R., & Andero, R. (2019). Sex differences in fear extinction. *Neuroscience and Biobehavioral Reviews*, *103*, 81–108. doi: 10.1016/j.neubiorev.2019.05.020.

Walmer, D. K., Wrona, M. A., Hughes, C. L., & Nelson, K. G. (1992). Lactoferrin expression in the mouse reproductive traci during the natural estrous cycle: Correlation with circulating estradiol and progesterone. *Endocrinology*, *131*, 1458–1466. doi: 10.1210/endo.131.3.1505477.

Williamson, C. M., Lee, W., DeCasien, A. R., Lanham, A., Romeo, R. D., & Curley, J. P. (2019). Social hierarchy position in female mice is associated with plasma corticosterone levels and hypothalamic gene expression. *Scientific Reports*, *9*, 7324. doi: 10.1038/s41598-019-43747-w.

Zhou, T., Sandi, C., & Hu, H. (2018). Advances in understanding neural mechanisms of social dominance. *Current Opinion in Neurobiology*, *49*, 99–107. doi: 10.1016/j.conb.2018.01.006.

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