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Orexin-1 receptor blockade and sex effects on the acquisition and extinction of active avoidance

Laia Vila Solés

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Ph. D. Thesis

Neuroscience

Orexin-1 receptor blockade and sex effects on the acquisition and extinction of active avoidance

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Impediments to action advances action.
What stands in the way becomes the way.
Marcus Aurelius

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List of abbreviations

1WPA	One-way passive avoidance
2-AA	Two-way active avoidance
A1	Acquisition session 1
A2	Acquisition session 2
A3	Acquisition session 3
A4	Acquisition session 4
A5	Acquisition session 5
AB	Accessory amygdala
ACh	Acetylcholine
AChE	Acetylcholinesterase
Amy	Amygdala
Arc	Arcuate nucleus
BA	Basal amygdala
BF	Basal forebrain
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
BSA	Bovine serum albumin
CeA	Central amygdala
CeM	Central amygdala
CA1	Cornus ammonis 1
CA2	Cornus ammonis 2
CA3	Cornus ammonis 3
CR	Conditioned response
CRF	Corticotropin-releasing factor
CS	Conditioned stimulus
DA	Dopamine
DAB	3, 3′- Diaminobenzidine
DAG	Diacylglycerol
DBB	Diagonal band of Broca
dCA1	Dorsal cornus ammonis 1
dCA2	Dorsal cornus ammonis 2
dCA3	Dorsal cornus ammonis 3
dHPC	Dorsal hippocampus
vHPC	Ventral hippocampus
DMH	Dorsomedial hypothalamus
DMSO	Dimethyl sulfoxide
DORAs	Dual receptor antagonists
DS	Dorsal striatum
EPM	Elevated plus maze
E1	Extinction 1
E1	Estrone
E2	Extinction 2
E2	17β-estradiol
E3	Estriol
EZM	Elevated zero maze
FC	Fear conditioning

FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GIRKs	G-protein-gated inward rectifier potassium channels
Glu	Glutamate
GnRH	Gonadotropin-releasing hormone
HPA	Hipothalamic-pituitary-adrenal
HPC	Hippocampus
IC	Insular cortex
ICSS	Intracranial Self-Stimulation
Icv	Intracerebroventricular
IL	Infralimbic cortex
IP_3	Inositol triphosphate
Itc	Intercalated cells of the amygdala
ITI	Intertrial interval
KO	Knockout
LA	Lateral amygdala
LAd	Dorsal lateral amygdala
LAv	Ventral lateral amygdala
LC	Locus coeruleus
LD	Light/dark test
LDT	Laterodorsal tegmental nucleus
LHA	Lateral hypothalamic area
LH	Luteinizing hormone
LH	Lateral hypothalamus
LhB	Lateral habenular nucleus
LPO	Lateral preoptic area
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MBT	Marble burying test
MCH	Melanin-concentrating hormone
MFB	Medial forebrain bundle
MGm/PIN	Thalamic posterior intralaminar nucleus
MiTg	Midbrain tegmental nucleus
MLDA	Mesolimbic dopaminergic system
mPFC	Medial prefrontal cortex
MS	Medial septum
MWM	Morris water maze
NAcc	Nucleus accumbens
NA^{LC}	Noradrenergic neurons in the locus coeruleus
NMDAR	N-methyl-D-aspartate-receptor
NPY	Neuropeptide Y
NS	Neutral stimuli
NSCC	Non-selective cation channels
OCD	Obsessive-compulsive disorder
OF	Open field test
OI	Optimal intensity
OIS	Optimal intensity search
OV	Ovariectomy
OVX	Ovariectomized

Ox	Orexin
Ox-A	Orexin A
Ox-B	Orexin B
OX1R	Orexin 1 receptor
OX2R	Orexin 2 receptor
PAG	Periaqueductal gray
PBS	Phosphate buffer saline
PCA	Principal components analysis
PFA	Perifornical area
PFC	Prefrontal cortex
PH	Posterior hypothalamus
PKC	Protein kinaseC
PLC	Phospholipase C
PMA	Platform-mediated avoidance
POA	Preoptic area
PPTg	Pedunculopontine tegmental nucleus
PrL	Prelimbic cortex
PTC	Pavlovian threat conditioning
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
ROIs	Regions of interest
RNS	Reward nervous system
RSC	Retrosplenial cortex
RT-3d	Retention three days after the acquisition
RT-10d	Retention ten days after the acquisition
SB	SB334867
SEM	Standard error mean
SLEA	Sub-lenticular extended amygdala
SORAs	Selective orexin receptor antagonists
SR	Spontaneous recovery test
US	Unconditioned stimulus
TBS	Tris-buffered saline
TMN	Tuberomammillary nucleus
vCA1	Ventral cornus ammonis 1
vCA3	Ventral cornus ammonis 3
Veh	Vehicle
VMH	Ventromedial hypothalamus
VP	Ventral pallidum
VTA	Ventral tegmental area

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Summary/Resum

Anxiety-related disorders affect millions of people worldwide, significantly impacting different aspects of their life and compromising their overall wellbeing. Avoidance behavior stands out as one of the core symptoms of these disorders, representing a behavioral response triggered by excessive fear and anxiety, where individuals attempt to "avoid" or repel perceived threads or stimuli. When avoidance levels are not optimal, it becomes maladaptive, leading to the development of mental disorders. Notably, fear- and anxiety-related disorders are more prevalent in women. Animal research using pavlovian and instrumental paradigms proves invaluable in elucidating sexual differences in these emotional learning processes, including and their extinction, and in exploring the underlying physiological and neural mechanisms. Recent studies have highlighted the direct impact of sex hormones on emotional memories, yet their role in the acquisition and extinction of avoidance behavior is yet to be studied.

Orexins are a type of neuropeptide produced by neurons in the lateral hypothalamus which project widely across the brain. Since their discovery, they have been linked to a wide variety of physiological and cognitive functions, such as anxiety and emotional memory. However, their specific role in active avoidance behavior remains unclear, and understanding their modulation could offer valuable insights into enhancing psychological and psychiatric treatments for traumatic memories.

Intracranial self-stimulation (ICSS) in the medial forebrain bundle (MFB) has been shown to facilitate the acquisition and consolidation of different types of memories. However, its effects on the extinction of aversive memories is yet to be studied.

This dissertation aims to study active avoidance behavior, with a particular focus on the involvement of the orexinergic system - specifically, orexin-1 receptors (OX1R)- and sexual differences. Additionally, it seeks to explore a potential facilitating treatment of the extinction behavior.

In **Study I**, we observed that female rats exhibit a faster and stronger acquisition, as well as a slower extinction of avoidance conditioning, compared to male rats. These

findings enabled us to establish the most appropriate conditioning protocols to address the remaining objectives set forth. In **Study II**, we evaluated the effects of OX1R blockade on the acquisition and extinction of 2-AA conditioning in both male and female rats. We observed that OX1R selective blockade impaired acquisition but not extinction, specifically in males. Moreover, patterns of c-Fos expression suggest certain sex-dependent functional differences in extinction circuitry. Finally, we observed that elevated levels of sex hormones during proestrus, may have anxiolytic effects and positively modulate the extinction of two-way active avoidance (2-AA) conditioning in females. To elucidate whether the poor performance exhibited by male rats resulting from OX1R blockade are linked to alterations in fear and anxiety levels, **Study III** examined sex differences and the effects of selective OX1R blockade on non-conditioned fear and anxiety tests. We observed that males exhibit more anxious and fear-related behaviors, which remain unaffected by OX1R blockade. These findings provide insight into the typically lower level of conditioning observed in males, but do not fully explain the detrimental effects of OX1R blockade. In **Study IV**, we evaluated whether the stimulation of the brain's reinforcement system through ICSS could potentially enhance the extinction of 2-AA conditioning in female rats, which indeed proved effective.

This dissertation sheds light on the differential involvement of OX1R in the acquisition and extinction of active avoidance behavior, emphasizing sexual differences in this conditioning. Furthermore, this thesis provides insights into the potential influence of sex hormone levels on anxiety, fear, and active avoidance behavior in female rats. Finally, we propose MFB-ICSS as a potential treatment to facilitate the extinction of avoidance behavior in females.

Els trastorns relacionats amb l'ansietat afecten milions de persones arreu del món, impactant significativament diferents aspectes de la seva vida i comprometent el seu benestar general. El comportament d'evitació és un dels símptomes fonamentals d'aquests trastorns, representant una resposta conductual desencadenada per nivells de por i ansietat excessius, on els individus intenten "evitar" o eludir estímuls o amenaces percebudes. Quan els nivells d'evitació no són òptims, esdevé una conducta desadaptativa, conduint al desenvolupament de trastorns mentals. És remarcable el fet que els trastorns relacionats amb la por i l'ansietat siguin més prevalents en dones. La recerca animal que utilitza paradigmes de condicionament pavlovians i instrumentals demostren ser molt rellevants per a esbrinar les diferències sexuals en aquests processos d'aprenentatge emocional, incloent la seva extinció, i per explorar els mecanismes fisiològics i neurals subjacents. Estudis recents han destacat l'impacte directe de les hormones sexuals en les memòries emocionals, però el seu paper en l'adquisició i extinció de la conducta d'evitació encara està per estudiar.

Les orexines són un tipus de neuropèptid produït per neurones en l'hipotàlem lateral que projecten de manera àmplia per tot el cervell. Des del seu descobriment, s'han vinculat amb una àmplia varietat de funcions fisiològiques i cognitives, com ara l'ansietat i la memòria emocional. No obstant això, el seu paper específic en el comportament d'evitació activa encara no està clar, i comprendre la seva modulació podria oferir idees molt valuoses per millorar els tractaments psicològics i psiquiàtrics de les memòries traumàtiques.

L'autoestimulació intracranial (AEIC) al feix prosencefàlic medial (FPM) del ha demostrat la facilitació de l'adquisició i consolidació de diferents tipus de memòries. No obstant això, els seus efectes en l'extinció de memòries aversives encara no s'han estudiat.

Aquesta tesi doctoral té com a objectiu estudiar el comportament d'evitació activa, amb un enfocament particular en la implicació del sistema orexinèrgic - específicament, els receptors orexin-1 (OX1R) - i el dimorfisme sexual. A més, busca explorar un possible tractament facilitador del comportament d'extinció.

En l'**Estudi I**, vam observar que les rates femelles exhibeixen una adquisició més ràpida i forta, així com una extinció més lenta del condicionament d'evitació, en comparació amb les rates mascles. Aquests resultats ens van permetre establir els protocols de condicionament més apropiats per abordar els objectius restants. En l'**Estudi II**, vam avaluar els efectes del bloqueig selectiu de OX1R en l'adquisició i extinció del condicionament de la tasca d'evitació activa de dos sentits (2-AA) tant en rates mascles com femelles. Vam observar que el bloqueig selectiu de OX1R va afectar l'adquisició però no l'extinció, específicament en els mascles. A més, els patrons d'expressió de c-Fos suggereixen certes diferències funcionals dependents del sexe en el circuit d'extinció. Finalment, vam observar que nivells elevats d'hormones sexuals durant la fase de proestrus poden tenir efectes ansiolítics i modular positivament l'extinció del condicionament de 2-AA en femelles. Per esbrinar si el baix rendiment exhibit per rates mascles com a resultat del bloqueig de OX1R està vinculat a alteracions en els nivells de por i ansietat, l'**Estudi III** va examinar les diferències sexuals i els efectes del bloqueig selectiu de OX1R en proves de por i ansietat no condicionades. Vam observar que els mascles exhibeixen més comportaments ansiosos i relacionats amb la por, que no es veuen afectats pel bloqueig de OX1R. Aquests resultats proporcionen informació sobre el nivell típicament més baix de condicionament observat en els mascles, però no expliquen completament els efectes perjudicials del bloqueig de OX1R. En l'**Estudi IV**, vam avaluar si l'estimulació del sistema neural del reforç a través de l'AEIC podria millorar potencialment l'extinció del condicionament de 2-AA en rates femelles, la qual cosa de fet va ser efectiva.

Aquesta tesi doctoral posa de manifest la implicació diferencial dels OX1R en l'adquisició i extinció de la conducta d'evitació activa, posant èmfasi en les diferències sexuals en aquest condicionament. A més, aquesta tesi proporciona idees sobre la potencial influència dels nivells d'hormones sexuals en l'ansietat, la por i el comportament d'evitació actiu en rates femelles. Finalment, proposem el FPM-AEIC com a possible tractament per facilitar l'extinció del comportament d'evitació en femelles.

GENERAL INTRODUCTION

1. Active avoidance behavior

1.1 Fear and anxiety

Fear and anxiety are phylogenetically adaptive emotions of our daily life, and optimal levels of both in the correct situations or environments are adaptive (Beckers et al., 2023). In response to a life-threatening situation, such as a fire, appropriate levels of fear activate neural mechanisms that allow us to escape from the situation as quickly as possible. However, exposure to fearful or anxious situations can result in the formation of enduring traumatic emotional memories which can be resistant to change. The development of these traumatic memories not only adversely impacts the emotional well-being of people, but also manifests in maladaptive behaviors, affecting their daily lives.

It is important to distinguish fear and anxiety as separate constructs (Bolles, 1975; Perusini & Fanselow, 2015). Despite being related, there are critical differences regarding behavioral, functional, pharmacological and neural levels of study. There is a clinical differentiation between fear-based disorders, like phobias, and anxiety-based disorders, such as panic disorder or social anxiety (Grillon, 2008). Moreover, both fear- and anxiety-related disorders exhibit distinct patterns of physiological reactivity (Lang et al., 2016), as well as different activity in neural circuits (Daniel-Watanabe & Fletcher, 2022; Davis et al., 2010). Fear can be defined as an immediate response to a threatening or noxious stimulus, serving the purpose of guiding the individual away from potential danger. It can be observed in different behaviors, such as fight, flight or freezing responses. On the other hand, anxiety can be defined as a sustained response designed to make the subject move toward the perceived danger in approach-avoidance conflict situations. Moreover, while anxiety is sensitive to anxiolytic drugs, fear is not (Fernandez-Teruel et al., 2019; McNaughton & Corr, 2004). Nonetheless, it is noteworthy to mention that there is a functional overlap between fear and anxiety (McNaughton & Corr, 2004).

Animal models have elucidated the factors and mechanisms that contribute to the development of fear- and anxiety-related disorders. Additionally, the use of the

fear conditioning paradigm enables the independent examination of fear and anxiety. While fear-based studies pair a stimulus with an aversive one, resulting in the development of a conditioned response to the conditioned stimulus, the presentation of the aversive stimulus in anxiety-based studies is unpredictable (Davis et al., 2010). Thus, fear models center around the actual presence of an aversive stimulus, while anxiety models focus on the anticipation of such a stimulus (Daniel-Watanabe & Fletcher, 2022). Consequently, animal research has demonstrated that fear and anxiety engage distinct neural circuits, with the amygdala playing a crucial role in fear, and the bed nucleus of the stria terminalis (BNST) being relevant for anxiety (Davis et al., 2010).

The models of study aforementioned are known as conditioned paradigms. In addition, fear and anxiety can also be assessed in animals using ethological or non-conditioned tests. In non-conditioned paradigms, animals are set in different environments, some of them being more potentially dangerous than others. In both models though, animals exert both active and passive coping strategies, but different defensive responses can be observed when comparing animals' performance in both paradigms (de la Mora et al., 2010).

1.2 Non-conditioned paradigms

The use of non-conditioned paradigms exploits the innate fear response of rodents towards novel or potentially threatening objects or environments. The results of these tests are useful since they inform about how animals tend to react or how they react after a drug administration in terms of fear and anxiety.

One of the most popular tests in animal psychology used to assess emotional responses is the open field (OF) test, which was created by Calvin Hall in 1934 (Hall, 1934). This test became popular due to the simplicity of the apparatus and procedure, the easiness to assess and interpret behaviors and the sensitivity to genetic, physiological and pharmacological manipulations (Walsh & Cummins, 1976). It consists of placing the animal in a novel open space that is surrounded by walls and allowing it to freely explore the space. The test can be performed in a range of 2 to 20 min, although it is usually performed in short periods of time. Hall

originally proposed that the use of this test could offer an index of timidity of animals, typically rodents, by the assessment of the amount of defecations. Currently, the behavioral variables most commonly recorded and analyzed are: number of crossings of the lines marked on the floor, frequency of rearing, grooming ("protracted washing of the coat") (Prut & Belzung, 2003), defecations and micturitions. The OF test is suggested to be a good tool to assess non-pathological anxiety that arises when facing a stressful or threatening response, since the anxiolytic effects of some drugs are not observed in the test (Prut & Belzung, 2003).

The light/dark (LD), based on the model proposed by Crawley and Goodwin (1980), is also used to assess unconditioned responses. This test takes advantage of rodents' natural aversion to brightly illuminated spaces and their tendency to explore. It relies on the conflict between rodents' desire to explore a new context and their initial neophobic response to unfamiliar environments. The test apparatus consists of a two-compartment cage typically with one-third designated for the dark compartment and two- thirds for the light compartment, separated by a wall with an opening no larger than 7 cm (Bourin & Hascoët, 2003).

Another approach-avoidance conflict test is the elevated zero maze (EZM), which is modified from the classic elevated plus-maze (EPM). In this test, the aversive nature of open arms causes subjects to predominantly stay in the closed arms, making the time spent in the open arms a reliable measure of anxiety. The EZM offers an advantage over the EPM, because it avoids the ambiguity of classifying the central square as either open or closed arm, eliminating potential conflicts for the researcher (Shepherd et al., 1994).

Finally, the marble burying test (MBT) is a classical behavioral test analyzing rodents' innate tendency to bury different objects, which is intended to assess anxiety-related behaviors. This test could also offer potential insight into obsessive-compulsive symptoms (Angoa-Pérez et al., 2013; Santana-Santana et al., 2021).

While these tests effectively measure fear and anxiety level in animals, considering alternative models becomes crucial when the objective shifts from assessing

baseline responses to deciphering how they create, consolidate, retrieve and erase emotional memories.

1.3 Conditioned paradigms

Memory is the cognitive process responsible for encoding, storing, and retrieving the knowledge of our past experiences. Typically, the term "memory" refers to the ability to conscious retrieval of past events, a form known as declarative or explicit memory. This particular memory type is mainly hippocampal-dependent and is characterized by its flexibility. Beyond this conventional understanding of memory, there are other memory systems that are equally important for an individual's survival. As advanced living organisms, it is crucial for us not only to recall past events, but also to remember the emotions associated with those experiences. Neuroscientists refer to this function as emotional memory, which is responsible for acquiring, consolidating and retrieving information regarding the emotional importance of past experiences. This memory system is also referred to as non-declarative or implicit memory, and it is characterized by its inflexibility (LeDoux, 1993) and it is the system where fear and anxiety-related memories are categorized.

Pavlovian fear conditioning (FC) has served as a key translational tool for investigating the psychological and neurobiological mechanisms underlying fear, anxiety and fear- and anxiety-related memories. The acquisition of this task is considerably fast and it results in long-lasting memories (Pavlov, 1927). The FC paradigm involves associating an initially innocuous stimulus or neutral stimulus (conditioned stimulus, CS) with a noxious stimulus (unconditioned stimulus, US), leading to the acquisition of fear of the CS. Thus, a CS, usually an acoustic tone, predicts the occurrence of an aversive and biologically threatening stimulus, usually a footshock (US). After enough pairings, the presence of the CS will evoke a learned fear response with physiological and behavioral responses (LaBar & Cabeza, 2006; LeDoux, 2000; Maren & Quirk, 2004). Two distinct paradigms are employed in FC. The first is contextual FC, where animals are placed in a conditioning chamber with specific environmental features. During this process, noxious stimuli or US are

delivered, leading subjects to form an association between the aversive stimulus and the context, which will then become a CS. When animals are placed once again in the chamber after the training session, freezing behavior is observed as a conditioned response (CR) (LeDoux, 2000). The second paradigm is cued FC, where animals associate an aversive US with a CS, which can be a light, a tone, or a combination of both. When animals are reintroduced to the chamber after the training session, the CS elicits fear responses (Maren et al., 2013).

Once animals have learned the fear response, researchers can study their ability to modify this memory through extinction sessions. These sessions consist of subsequent presentations of the CS without pairing it to the US. Experiencing the CS without any adverse consequence inhibits the conditioned responses. As the extinction of this fear learning is context-dependent, if the animal is placed in a different environment from where the extinction took place and is exposed to the CS, the CR would be elicited (LaBar & Cabeza, 2006).

Classical conditioning paradigms provide a straightforward method which leads to fast results in studying the neural basis of fear and anxiety. However, this paradigm proved inadequate in explaining complicated human responses to fearful events. Consequently, instrumental conditioning, developed by Burrhus Frederic Skinner (Skinner, 1937), emerged as the chosen paradigm to study complex human responses. This paradigm posits that if an US is paired with a CS, the presentation of the CS will elicit an experimenter-designed response in the subject in order to either avoid or seek a stimulus. This type of conditioning recognizes that the acquisition of complex responses or behaviors result from their outcome. Such responses are integral to the core symptomatology of certain anxiety and fear-related disorders in clinical settings, including avoidance behavior (see *1.4 Avoidance behavior*).

1.4 Avoidance behavior

Avoidance behavior refers to the behavioral response performed in order to evade threatening situations or stimuli, serving a critical role in ensuring survival by preventing potential harm. However, non-optimal levels of avoidance can

significantly disrupt overall life and wellbeing. At this point, avoidance loses its adaptive function and becomes maladaptive, contributing to a wide variety of mental disorders, such as fear- and anxiety-related disorders (Krypotos et al., 2015; LeDoux et al., 2017). Excessive avoidance, even in the absence of an actual threat, can result in the bypassing of some thoughts and situations to manage discomfort, which could ultimately impair interpersonal and environmental interactions. Conversely, insufficient or deficient avoidance can lead to the recurrence of aversive thoughts, severely impacting mood and emotional wellbeing.

In psychology, avoidance behavior and anxiety are strongly connected, since both concepts are linked to how people cope with threatening, stressful or fearful situations. Although avoidance behavior provides short-term relief, it can exacerbate anxiety in the long-term by hindering the ability to cope with and learn from fear-inducing situations. Thus, avoidance behavior is a core symptom of anxiety-related disorders, constituting a maladaptive coping mechanism that perpetuates or intensifies anxiety levels.

Anxiety-related disorders are the most prevalent psychiatric disorder, affecting around 30% of the population at some point in their lives and they have a substantial impact on individuals' quality of life by influencing various aspects (Bandelow & Michaelis, 2015). In Europe alone, anxiety-related disorders affected 61.5 million people in 2011, with a higher prevalence in women than in men (Kundakovic & Rocks, 2022; Wittchen et al., 2011). Avoidance is the central feature of a wide spectrum of mental disorders that belong in the category of anxiety and fear-based disorders. Excessive avoidance is the core symptom of several mental disorders, such as anxiety disorders, Obsessive Compulsive Disorder (OCD), Post-Traumatic Stress Disorder (PTSD) (Kashdan et al., 2009), and social phobia (Krypotos et al., 2015). On the other hand, a deficiency in avoidance can contribute to the development of major depression (Ottenbreit et al., 2014) and is indicative of higher suicide rates (American Psychiatric Association, 2013; Dixon et al., 1991; Servatius, 2016).

In order to understand avoidance behavior, elucidating the neural circuits responsible for fear and anxiety is of paramount importance. Research in animal behavior provides a valuable avenue for comprehending the functioning of this coping mechanism and discerning the origins of adaptive or maladaptive avoidance. By precisely manipulating the neurobiological correlates of behavior in a controlled manner – an approach not feasible in human studies – researchers gain insight into the mechanisms at play. The ability to alter specific neural structures and pathways yields fast and reliable results, broadening our understanding of how these structures work. Last, but not least, advancements in animal research not only expand our knowledge but also contribute to the development of new pharmacological or behavioral therapeutic approaches.

In a laboratory setting, behavioral avoidance refers to the conditioned responses that subjects learn to perform in order to reduce or prevent contact with aversive or negative situations. Two distinct types of avoidance procedures exist: passive and active avoidance. In passive avoidance procedures, also known as inhibitory avoidance, a response during the warning signal presentation leads to the introduction of an aversive stimulus. Over time, subjects learn that by inhibiting the response, the noxious event will be completely avoided. In active avoidance procedures, a warning signal is presented followed with a noxious stimulus unless the subjects perform an experimenter-designated response. Consequently, this response terminates the presence of the negative stimulus. Unlike passive avoidance, in this procedure the performance of a response entirely prevents the occurrence of a noxious stimulus (Krypotos et al, 2015; LeDoux et al., 2017).

Active avoidance paradigms used in research vary in types and features. Firstly, these paradigms can be classified based on the presence or absence of warning signals alerting subjects to an imminent threatening stimulus. Signaled active avoidance paradigms incorporate a warning signal that precedes the noxious stimulus, while unsignaled active avoidance or Sigman avoidance paradigms do not.

Additionally, active avoidance tasks differ in the behavioral response or outcome they elicit in animals. A commonly used task is the lever-press signaled

avoidance, where animals must press a lever during the warning signal presentation to avoid a footshock (Beck et al., 2011). Another task is the platform-mediated avoidance (PMA) task, where animals press a lever to obtain food pellets and can avoid a footshock by stepping onto a platform during the warning signal presentation. After the tone offset, they can return to the lever (Bravo-Rivera et al., 2014). Lastly, shuttle avoidance is another relevant task for studying active avoidance. This task requires animals to cross to the other compartment of a chamber when the CS is presented in order to avoid a footshock, and it can be one-way or two-way (explained in detail in *1.5 Two-way active avoidance*) (Modaresi, 1975).

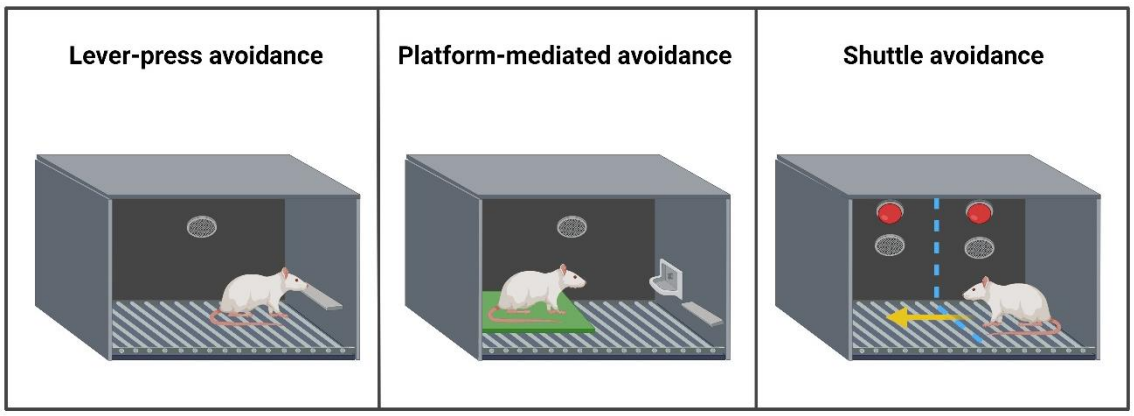


Figure 1. **Representation of different signaled active avoidance tasks**.

In the 1930s, psychologist Orval Hobart Mowrer initiated the study of avoidance behavior by combining classical Pavlovian and instrumental conditioning to assess conditioned avoidance as the main variable indicating aversion in signaled active avoidance paradigms (Mowrer, 1939, 1940). Mowrer merged Pavlov's and Thorndike's law of effect theory and formulated the two-factor theory (Mowrer, 1951), which posited that avoidance responses are reinforced by reducing conditioned fear. The two-factor theory, together with the work of his colleague Neal Miller, was pivotal for the study of fear and anxiety (Miller, 1948; Mowrer, 1951) and shaped the research trajectory for several decades (Masterson & Crawford, 1982; Overmier & Brackbill, 1977; Rescorla & Solomon, 1967; Schoenfeld, 1949). However, theoretical concerns regarding the two-factor theory findings that emerged between the 1940s and 1980s lead to a decline in interest in instrumental

conditioning and active avoidance, especially regarding the nature of the reinforcement and the assumption that the avoidance response is instrumental.

The fundamental hypothesis of the two-factor theory by Mower proposed fear reduction as the reinforcer of avoidance response (Miller, 1941, 1948, 1951; Mowrer, 1947, 1951; Mowrer & Lamoreaux, 1946). It posits that once active avoidance behavior is learned, every trial is an extinction trial, implying that we should expect fear to decrease quickly and extinction to be facilitated. However, this contradicts findings of persistent avoidance behavior and the consequent resistance to extinction (Kamin et al., 1963; Mineka, 1979; Solomon et al., 1953; Solomon & Wynne, 1954). Habit creation was proposed as an explanation, suggesting that learned responses become a habit, and persist without the reinforcing stimulus.

Complicating matters, avoidance behavior did not co-vary with conditioned fear levels and the subjective nature of fear in non-verbal animals, such as rodents, became a challenge. One theoretical solution to the problem of the two-factor theory was a change in the terminology regarding the main construct of the theory from *fear* to *threat*. Similarly, consensus dictated that fear conditioning should be known as Pavlovian threat conditioning (PTC). By changing these terms, the theory now focuses on assessing defensive processes, instead of fear states. This shift in terminology prompted a realization within the field that it must distinguish between the circuits responsible for fear and those underlying defensive responses. The second issue raised questions about the assumed instrumentality of avoidance responses. Fear reduction was hypothesized by Mowrer as the mechanism reinforcing avoidance behavior. When fear reduction mechanisms started to be problematic, the instrumentality of avoidance was questioned. Bolles defended that avoidance behavior was a species-specific flight response to the CS learned through Pavlovian learning instead of a learned behavior driven by fear reduction mechanisms (Bolles, 1970). However, and in favor of the two-factor theory, two arguments emerged to highlight that Pavlovian classical conditioning theory inadequately explains avoidance behavior. Firstly, in avoidance procedures, an experimenter-defined response is required – either to avoid or induce the presentation of a negative stimulus. This is incompatible with the automatic and

innate responses induced by Pavlovian conditioning. Secondly, the learned response in avoidance procedures neutralizes the negative event, whereas classical conditioning involves a stimulus causing a response. These arguments led to the categorization of avoidance procedures as instrumental conditioning procedures (Krypotos et al., 2015).

To address how avoidance behavior is reinforced if fear reduction is not the driving mechanism, it was proposed that different reinforcers act together. The termination of the CS (unpleasant and threatening) serves as a negative reinforcer, while the offset of the CS, once it has been avoided, becomes a safety cue. Thus, both negative reinforcement through threat termination and positive reinforcement via safety signals could equally contribute to avoidance behavior learning.

All the aforementioned theoretical challenges, together with the lack of a well-defined neural circuit underlying active avoidance hindered research in this field. Consequently, Pavlovian conditioning regained prominence (Masterson & Crawford, 1982; McAllister & McAllister, 1991; Overmier & Brackbill, 1977; Rescorla & Solomon, 1967). Nonetheless, as learning theory advanced over the years, the theoretical concerns were addressed and active avoidance still remains a promising experimental procedure being applied in research to investigate the neural mechanisms of fear- and anxiety-related disorders.

A particularly useful paradigm is the signal active avoidance, which can be empirically tested through the two-way active avoidance task.

1.5 Two-way active avoidance conditioning

The two-way active avoidance (2-AA) task is a widely used conflict test for rodents, requiring experimental subjects to shuttle between compartments to avoid an aversive stimulus, usually a footshock (US), which is presented after a signal, usually a light, a tone or both (CS). It is a type of signaled active avoidance task because it uses a warning signal to alert of the impending threatening stimulus. The 2-AA task is performed in a shuttle box, a chamber that is virtually divided into two

compartments (Figure 2), although sometimes a wall with an open door separates them. It is a two-way task because animals must perform the avoidance behavior regardless of the compartment they occupy when the CS appears. During the acquisition of the 2-AA task, subjects may receive the CS and the US in both compartments. However, the US presentation ceases if subjects move to the other compartment (escape response) or it does not appear if subjects shuttle during the CS presentation (avoid). Repeated exposure to these contingencies facilitate the learning of escape and avoidance behaviors. In classical or instrumental conditioning, there are two main forms of conditioning. In a delay conditioning procedure, the US is presented during the final few milliseconds of the CS, and both stimuli end at the same time. In a trace conditioning procedure, the US is presented sometime after the CS. This last type of conditioning is more cognitively complex and it relies on the hippocampus (Bangasser et al., 2006; Dylla et al., 2013; Grover et al., 2022).

The 2-AA task was used to study the basis of fear and anxiety, due to the emergence of the two-factor theory proposed by Mowrer (Mowrer, 1951) (for further details see section: *1.4 Avoidance behavior*). Anxiety is believed to manifest in the initial stages of the learning phase, as subjects attempt to solve the contextual conflict (Fernandez-Teruel et al., 2019). Interestingly, the task not only assesses emotional states but also the coping strategies that animals employ to navigate the situation. Successful task performance consists of three different types of learning occurring sequentially (Diehl et al., 2019; Fernandez-Teruel et al., 2019; LeDoux et al., 2017):

1. **Defensive reactions**: animals learn the association between the tone and the shock and exhibit species-specific defensive behaviors, such as freezing to the tone following the initial pairing. In this phase, PTC mediates these reactions.
2. **Defensive actions**: animals learn that performing a specific response terminates all the stimuli. When animals shuttle randomly during the footshock to the other compartment, they learn that this terminates both the

CS and the footshock. Once avoidance behavior is acquired, it is considered to be instrumental learning.

3. **Defensive habits**: animals develop a tendency to perform avoidance behavior upon the early onset of the warning signal, which becomes a habit. This avoidance behavior in the form of habit is long-lasting and resistant to extinction.

While this task effectively generates instrumental conditioning, it carries certain limitations that should be considered. One proposed limitation revolves around the lack of a secure location throughout the learning phase. Since animals are required to respond in every trial by shuttling to the other compartment to avoid an unpleasant stimulus, occurrence of freezing could potentially interfere with the adaptive response animals are expected to learn. This could be one reason why some animals fail to learn the task. Another limitation lies in the fact that the learned response lacks consequences for animals; in other words, performing the response does not entail any costs. In clinical settings involving humans, avoidance behaviors significantly impair patients' lives. Finally, in this task, animals learn to control the termination of the warning signal and the footshock, a scenario that differs from real-life situations in clinical settings. This high level of control afforded to subjects through predictive environmental cues does not align with the environments encountered by patients suffering from anxiety disorders or PTSD (Diehl et al., 2019).

1.6 Variables assessed in the 2-AA task

The learning outcome of the 2-AA task can be assessed through different variables. Depending on the contingency between the response and the presentation of the stimuli, there are three possible responses the animals can perform: avoidance, escape or non-response, as shown in Figure 2.

The main variable is the number of avoidance responses, indicating how many times subjects crossed to the opposite compartment during the presentation of the CS in time to avoid receiving the US (footshock). This variable is expected to improve

over the course of the trials, with an expected increase of avoidance responses during training. The escape responses elicited while the CS is still active. In this scenario, subjects experience the footshock and move to the other compartment while the CS is ongoing. It is important to distinguish between avoidance and escape responses. In avoidance responses, subjects avoid the upcoming noxious stimuli, whereas in escape responses animals distance themselves from an ongoing aversive event. A third variable assessed in the 2-AA task is the non-responses, representing the number of times that subjects fail to elicit an avoidance response during the CS+US presentation. This behavior should not be confused with an evoked freezing response. If a subject does not perform an avoidance response by moving to the other compartment, it does not necessarily indicate a freezing response. Under normal conditions during the learning phase, animals typically begin by performing escape responses and non-responses. As they gradually learn the contingencies of the task, they are expected to display increasingly more escape responses and eventually transition to avoidance responses, as shown in Figure 2. Another variable measured in this task is the latency of response, which denotes the time elapsed between the appearance of the CS and the initiation of a response. The latency is expected to decrease throughout the learning trials.

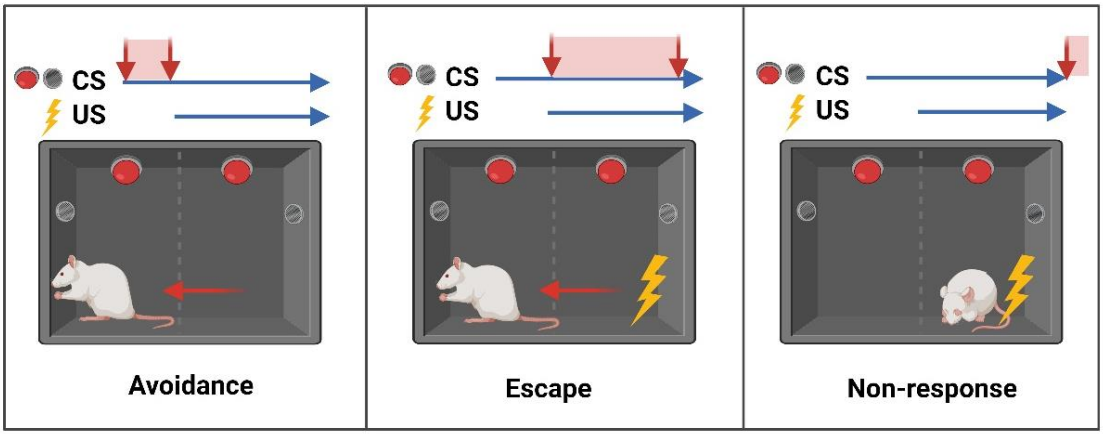


Figure 2. Types of responses that subjects can perform in the 2-AA task. Left. Avoidance response. **Center.** Escape response. **Right.** Non-response. Note that the red segment represents when the subject exhibits a response. If a response takes place before the presentation of the US, it is considered an avoidance response, as the subject successfully avoids the footshock. When animals shuttle during the presentation of the footshock, it is classified as an escape response. If the response occurs after the termination of both stimuli or does not take place at all, it is categorized as a non-response.

Finally, intertrial crossings are also analyzed in order to determine the number of times that subjects cross between the two compartments during the intertrial interval (ITI). The duration of the ITI can range anywhere between 50 s and 70 s. During this interval, locomotor activity is recorded. While intertrial crossings may originally seema measure of unspecific motor activity (Brush et al., 1979; Guenaire & Delacour 1983), they also provide insights into the coping strategies employed by the animals to learn the task (Aguilar et al., 2004; Boix et al., 1988; Gendron & Brush 1996; Wilcock et al., 1981; see review by Fernandez-Teruel et al., 2019).

1.7 Extinction of aversive conditioning

Extinction is a complex phenomenon essential for adapting behavior to new environmental demands. It allows for the modification of learned behaviors previously associated with a fear-eliciting cue, by replacing them with safe ones (Bouton et al., 2021). Exposure therapy, the cornerstone in treating fear-related disorders, relies on exposing individuals to fear-eliciting stimuli in a safe environment. This repeated exposure, devoid of adverse consequences, gradually diminishes fear levels. This therapeutic approach is believed to depend, at least in part, on extinction learning (Maren, 2015).

It should be noted that the term “extinction” can take on different meanings, each carrying distinct implications. Firstly, extinction refers to the experimental procedure used. Secondly, it denotes the resulting effect of this procedure, which is the reduction in fear response. Lastly, it encompasses the cellular processes driving this cognitive process (Myers & Davis 2007). For clarity, from hereon, the term “extinction” will refer specifically to the theoretical process of fear response reduction. In fear extinction procedures, CRs are reduced or suppressed by the presentation of the CS in the absence of the noxious US (Myers and Davis, 2007). However, this response reduction is temporary and context-dependent, and behavioral relapse can occur in different ways (Bouton, 1993; Delamater & Westbrook, 2014; Maren, 2015). Four types of relapse effects can be identified depending on context and stimulus presentation: renewal, spontaneous recovery, reinstatement, and rapid reacquisition.

1. **Renewal effect**: This occurs when a previously extinguished response returns after a change in the context where the conditioning first occurred.
2. **Spontaneous recovery effect**: This happens when the extinguished response resurfaces after enough time has passed following extinction.
3. **Reinstatement effect**: When the original US is reintroduced after extinction, it can trigger the CR again.
4. **Rapid reacquisition effect**: This is observed when the CS is paired with the US again after extinction. The speed of reacquisition varies depending on the similarity between the reacquisition context and the extinction and acquisition contexts. When conditions are similar to those present during extinction, reacquisition is slower; conversely, a fast reacquisition will take place in a context similar to that of the acquisition.

These relapse effects highlight that the original learning is at least partially intact (Bouton et al., 2021). Moreover, these relapse effects strongly suggest that extinction is not the same as forgetting. While forgetting occurs passively over time without CS and fear memories can last for long periods of time (Gale et al., 2004), extinction involves repeated exposure to CS in the absence of US to reduce fear responses. Extinction does not erase fear memories; it instead creates new inhibitory associations between safe cues and fear-related emotions (Bouton, 1993). This cognitive process can be experimentally carried out in both rodents and humans, making it a valuable translational model for treating fear and anxiety disorders (Milad & Quirk, 2012; Myers & Davis, 2007).

Theories of fear learning and extinction theories hinge on the nature of associations between the CS and the US. Various extinction theories propose different mechanisms underlying the nature of the associations established during and after extinction procedures. Among these theories, two main ideas have gained support.

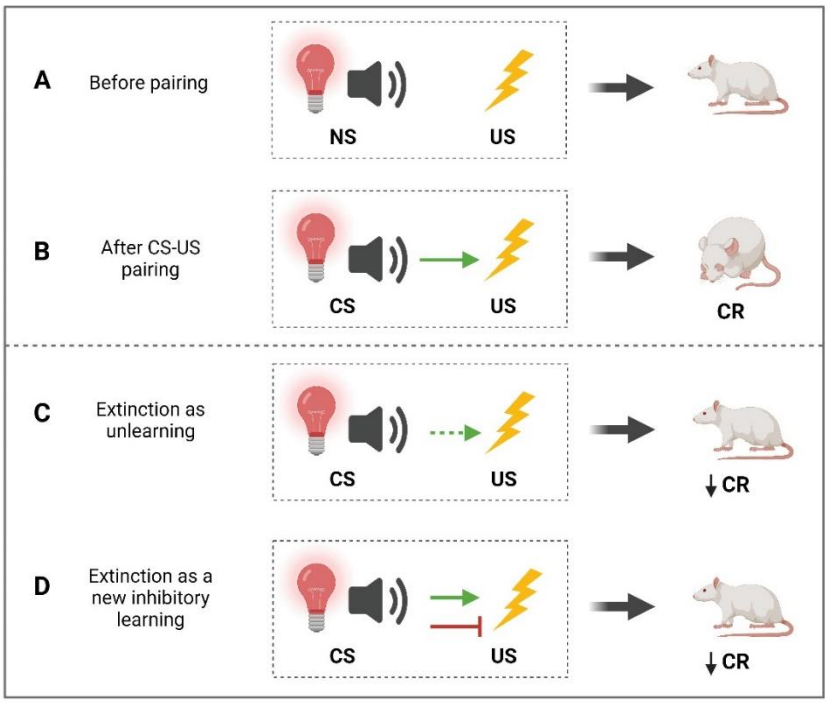


Figure 3. **Acquisition and extinction of fear memories**. **A**. Neutral stimuli do not elicit a conditioned response until they are paired with a noxious stimulus. **B**. The contingent presentation of NS paired with a noxious stimulus, such as a footshock, transforms the NS into a CS; and now the presence of the CS causes a CR. **C**. This model proposes that a decrease in the CR occurs because the previous association is inhibited. **D**. This model proposes that a decrease in the CR is due to the creation of a new inhibitory association. Abbreviations: CR: conditioned response; CS: conditioned stimulus; NS: neutral stimuli; US: unconditioned stimulus.

One prominent model posits the erasure of unlearning of the previously learned CS+US association. According to this view, learning is based on the strength of the association between CS and US, and extinction procedures work to weaken this association (Myers & Davis, 2007). This theory finds support in the work of Rescorla & Wagner (1972) as well as Mackintosh (Delamater & Westbrook, 2014; Mackintosh, 1975; Maren & Davis, 2007). Specifically, it proposes that inhibition or long-term depression of neural circuits underlying the original learning, mediated by N-methyl-D-aspartate-receptor (NMDAR) and certain protein kinases and phosphates, contributes to this psychological phenomenon (Davis, 2011; Myers et al., 2006). However, this mechanism fails to fully account for the recurrence of the CR after extinction training. If erasure of the previous association was the sole mechanism underlying extinction, it could not explain the resurgence of the original association.

A second model proposes that extinction involves the creation of new inhibitory associations, which would strengthen new neural circuits developed during the extinction process. Interestingly, the presence of the aforementioned relapse effects supports the notion that extinction relies on more than unlearning or erasure. While these effects do not rule out the possibility of the first mechanism, they indicate that extinction involves, at least partially, a novel and active learning process (Bouton et al., 2021). There are other models explaining the nature of extinction learning, including non-associative mechanisms that focus on attentional levels (Wagner, 1981). Nevertheless, it is also possible that a unifying framework could accommodate the various models simultaneously.

Successful reduction of fear is predicted by the timing between the conditioning and the extinction procedures. When extinction procedures immediately follow the acquisition phase, fear reduction is minimal, a phenomenon known as immediate extinction deficit (MacPherson et al., 2013; Maren & Chang, 2006; Tapias-Espinosa et al., 2018). Thus, recent exposure to a traumatic experience impedes long-term fear reduction (Maren, 2015). This deficit is associated with reduced activity in the IL cortex (Kim et al., 2010).

1.8 Neural circuitry of the 2-AA task

The evolution of the active avoidance study stemmed from the acknowledgement that active avoidance learning encompasses different sequential learning phases (see *1.5 Two-way active avoidance conditioning*). Therefore, the exploration of the neural basis of this learning task must consider three different neural substrates.

1.8.1 Defensive reactions circuitry

We currently know that the key neuroanatomical area regulating aversive memories is the amygdala (Johansen et al., 2011; LeDoux, 1992; Davis, 1992; Kapp et al, 1992; Repa et al, 2001). It receives information about the CS and the US and sends projections to other brain areas to evoke adaptive behavioral, autonomic, and endocrine responses (LeDoux, 2000). The amygdala can be subdivided into three

main regions with different subdivisions: basolateral (BLA), Central (CeA) and Medial (CeM) amygdala. The BLA amygdala consists of the lateral (LA), basal (BA) and accessory (AB) subregions (LeDoux, 2000). Other subdivisions can be found, like the dorsal lateral amygdala (LAd), ventral lateral amygdala (LAv) or the basal amygdaloid nuclei of the central amygdala. Sensory and auditory afferences regarding the US and the CS converge in the LA nucleus. This information comes from the auditory thalamus, containing the medial subdivision of the medial geniculate nucleus together with the adjacent thalamic posterior intralaminar nucleus (MGm/PIN), and the auditory cortex. Single-unit recording studies have shown that the thalamic pathway is faster at generating plasticity in the amygdala compared to the cortical pathway (Olds et al., 1972; LeDoux, 2000; Maren & Quirk, 2004). Moreover, it seems that the thalamic pathway towards the amygdala is sufficient to elicit defensive/fear responses, since its lesion prevents fear conditioning (LeDoux et al., 1990; Romanski & LeDoux, 1992). A comprehensive illustration of this notion is shown in Figure 4A.

Once the LA receives sensory information regarding the CS and the US, it creates an association between them (Maren, 2005). The LA is of paramount importance for learning the association between the tone and shock. The information regarding the CS and the US converge in this nucleus (Romanski et al., 1993; Bordi & LeDoux, 1994) and increase its neuronal activity (Rogan & LeDoux, 1995; Maren, 2000). This increase in activity induces long-term potentiation, which eventually activates intracellular processes to promote gene transcription and protein synthesis leading to cytoskeletal changes (Lamprecht & LeDoux, 2004; Rogan et al., 1997). Thus, the activity of the LA is necessary for learning the association between a neutral and an aversive stimulus. Several studies have proven this assertion by disrupting the activity of the LA (LeDoux et al, 1990; Muller et al, 1997).

Afferences from cortical and subcortical areas into the LA, are then projected to other amygdaloid nuclei. Specifically, the LA innervates the CeA both directly and indirectly through the BLA (Pitkänen et al, 1997; Paré & Smith, 1998). Although the CeA could be seen as a passive relay between the amygdala and other brain regions, it is critical in the acquisition of PTC. Hence, its activity is essential for fear memory

recall and fear-driven behavior (Li et al, 2013). The CeA sends amygdalar projections to elicit a wide range of physiological responses in response to the CS. Freezing responses are obtained through CeA projections to the periaqueductal gray (PAG) (LeDoux et al, 1988; Kim et al, 1993), while neuroendocrine and autonomic reactions are generated through CeA projections to the hypothalamic and brainstem regions (Kapp et al, 1979; LaBar & Cabeza, 2006; LeDoux et al, 1988; Maren & Quirk, 2004).

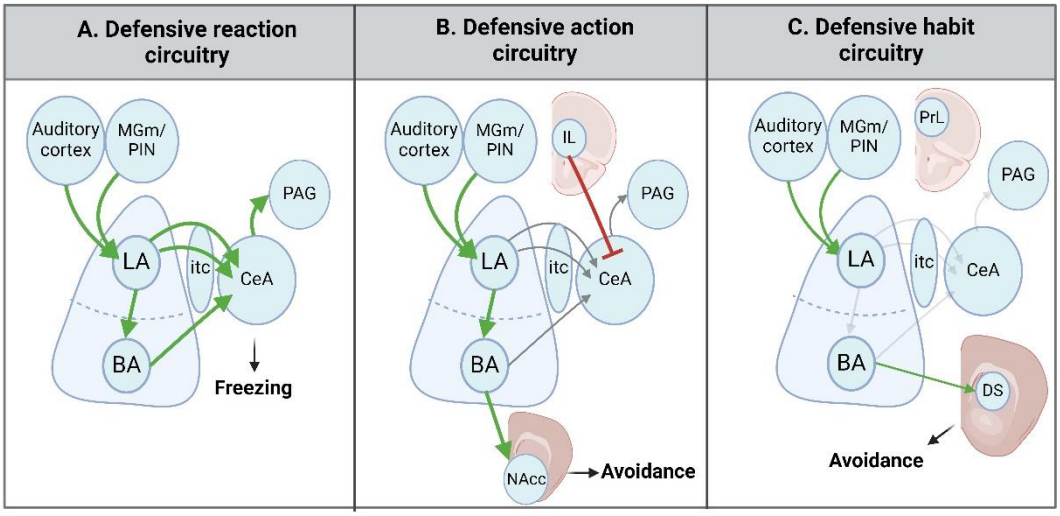


Figure 4. Neural circuits underlying the different learning stages of the two-way active avoidance task. **A.** Defensive reaction circuitry. **B.** Defensive action circuitry. **C.** Defensive habit circuitry. Abbreviations: BA: basal amygdala; CeA: central amygdala; DS: dorsal striatum; IL: infralimbic cortex; itc: intercalated cells of the amygdala; LA: lateral amygdala; MGm/PIN: adjacent thalamic posterior intralaminar nucleus; NAcc: nucleus accumbens; PAG: periaqueductal gray; PrL: prelimbic cortex.

1.8.2 Defensive action circuitry

The repetition of the paired CS and US presentation elicits different types of responses in animals. The first mechanism that animals will use when the shock appears is freezing. However, crossing to the other virtual compartment becomes increasingly probable as animals carry out different behaviors. Once subjects realize that crossing over terminates the presentation of the footshock, they learn that by actively responding during the CS presentation, they can avoid the aversive stimulus.

Once avoidance behavior is acquired, the significance of the CeA seems to diminish. Notably, electrolytic lesions of the CeA facilitate avoidance behavior in poor performers (Choi et al, 2010). Although the amygdala remains a key player in the avoidance response circuitry, the defensive response of avoidance seems to be regulated by different pathways than the defensive reaction of freezing. Specifically, avoidance involves the connections between the LA and BA amygdala (Amorapanth et al., 2000). Subsequently, the BA amygdala sends strong projections to the nucleus accumbens (NAcc) (McDonald, 1991, Wright et al, 1996; Boeke et al 2017). These projections are crucial for associating external sensory cues with biologically important goals (Wright et al, 1996).

There is ongoing debate regarding whether NAcc activity facilitates or hinders active avoidance. Some studies, either using signaled active avoidance, platform-mediated avoidance or lever pressing avoidance, have shown that NAcc inactivation and BA-NAcc connection disruption impairs avoidance (Ramirez et al, 2015; Bravo-Rivera et al, 2014; Fernando et al, 2014). Additionally, dopamine release in NAcc during the warning signal presentation facilitates avoidance, suggesting that dopamine release may serve as a cue for successful avoidance (Wenzel et al, 2015; Oleson et al, 2013). Other studies, however, show that NAcc neurotoxic lesions actually facilitate the acquisition and retention of a 2-AA task (Lichtenberg, 2014; Lorens et al, 1970). These studies suggest that a decrease in dopamine could decrease anxiety levels, reducing freezing behavior and consequently, facilitating avoidance. Regardless, NAcc dopamine release and connections with the amygdaloid region seem critical for avoidance behavior. Further research is necessary to elucidate the functional implications of this region for such complex behavior.

Another brain region that seems critical for the transition from freezing to avoidance behavior is the infralimbic cortex (IL). The IL cortex inhibits the CeA, which leads to the inhibition of the freezing behavior, typical defensive behavior in rats (Moscarello & LeDoux, 2013). This inhibition is crucial for subjects to shift from the defensive reaction pathway (LA-CeA-PAG) towards the defensive action pathway (LA-BA-NAcc) and actively avoid the US. Interestingly, IL activation

correlates with higher levels of avoidance, and c-Fos levels in the IL can distinguish good and poor performance in an unsignaled active avoidance task (Martínez et al., 2013).

While the BNST was traditionally associated with conditioned reactions to threatening conditions, recent studies indicate its role in active avoidance behavior (Guerra et al., 2023). Notably, this effect is sex-dependent, with BNST activation being necessary for male rats, but not for female rats, in performing avoidance behavior. Additionally, the ventral hippocampus mediates the context-dependence of avoidance behavior in the shuttle box (Oleksiak et al., 2021). It is suggested that the ventral hippocampus contributes to diminishing avoidance responses outside the context in which they were originally learned. A comprehensive illustration of these circuits is shown in Figure 4B.

1.8.3 Defensive habit circuitry

Once avoidance behavior is firmly established, other brain regions participate in transforming this learned experimenter-designed behavior into a habit. This marks a transition from an outcome-dependent defensive action, characteristic of the learning phase, to an outcome-independent defensive habit, observed in the latter stages of training when avoidance behavior becomes a habit.

A notable difference in brain area activation related to habit circuitry is the diminished dependence on the amygdala. While the amygdala is necessary for the performance of defensive reactions (LA-CeA) and for defensive actions (LA-BA), different studies using Sidman and discriminative active avoidance show that once a habit is acquired, the activity of the amygdala is no longer needed (Lázaro-Muñoz et al., 2010; Poremba & Gabriel, 1999). A proposed hypothesis suggests an intrastriatal shift between the ventral striatum and the dorsal striatum (DS). During the active avoidance learning phase, the NAcc is essential for stimulus-response association. However, as learning progresses, NAcc activation levels decrease, and the activity is believed to transfer to the DS, facilitated by midbrain DA (dopamine) neurons (Everitt & Robbins 2005, 2016). Electrophysiological data from subjects

performing a T-maze and lesion studies with a lever pressing paradigm indicate distinct involvement of different DS regions based on the learning stage. Thus, the dorsomedial striatum is involved during the acquisition and retention of the task (Yin et al, 2005), while the dorsolateral striatum is involved in habitual performance (Balleine & O'Doherty, 2010; Thorn et al, 2010; Yin et al, 2004). Future studies focusing on the habit learning of avoidance behavior are required to confirm that these brain areas activation patterns are consistent with active avoidance becoming a habit.

The prelimbic cortex (PrL) emerges as another critical region for habit learning. In a study using a platform-mediated avoidance task, PrL activity levels correlated with avoidance behavior resistant to extinction (Bravo-Rivera et al, 2015). An illustration depicting these circuits is shown in Figure 4C.

1.8.4 Extinction of avoidance behavior circuitry

As explained in previous sections, active avoidance behavior requires both pavlovian and instrumental learning. Logically, the neural circuits underlying the extinction of active avoidance behavior recruits neural circuits overlapping both types of learnings. Figure 5 depicts circuits between prefrontal cortex, nucleus accumbens, basolateral amygdala and ventral hippocampus involved in the learning of this behavior.

The medial PFC is a pivotal area for the extinction of avoidance behavior through its connections to the BLA and ventral striatum, as shown in retrograde labeling studies (Martínez-Rivera et al., 2019). Moreover, chemical inactivations of the IL cortex impair the extinction of avoidance in a PMA task during the first extinction session and the subsequent extinction sessions (Bravo-Rivera et al., 2014). It seems that the IL inhibition of the behavioral response leads to optimal performance in extinction learning. Supporting this idea, optimal spontaneous recovery performance levels are negatively correlated with c-Fos IL activity in a 2-AA task (Tapias-Espinosa et al, 2018). Similarly, extinction training increases the

activity of the IL in a lever press paradigm (Jiao et al., 2015). This IL influence on extinction learning depends on its downstream pathways, such as the circuit IL-BLA and IL-NAcc (Martínez-Rivera et al., 2019). Moreover, the PL-NAc pathway also seems to be essential for the extinction of avoidance behavior (Bravo-Rivera et al., 2015).

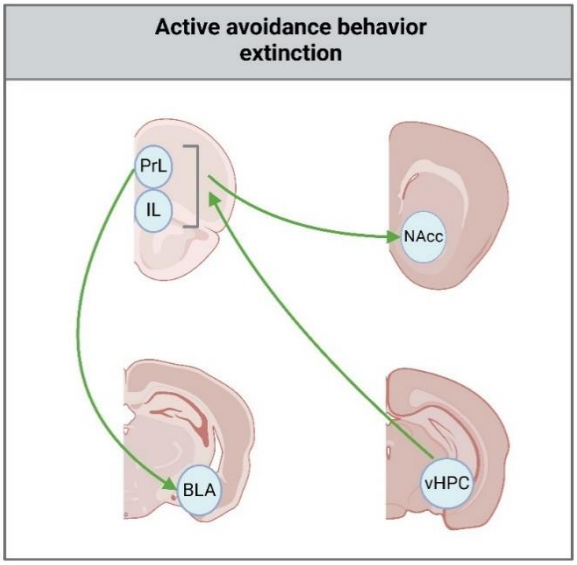


Figure 5. **Neural circuits underlying the active avoidance behavior extinction**. Abbreviations: BLA: basolateral amygdala; IL: infralimbic cortex; NAcc: nucleus accumbens; PrL: prelimbic cortex; vHPC: ventral hippocampus.

Additionally, successful avoidance extinction learning involves interactions between the vHPC to the PL and the IL cortices. BDNF seems to be a key molecule regulating these pathways, since extinction learning increased its expression and a knockdown BDNF model in the vHPC impaired the extinction learning (Rosas-Vidal et al., 2018).

2. Orexins

2.1 Introduction

Orexins are a type of excitatory neuropeptides synthesized by neurons in the lateral and dorso-posterior hypothalamus. Orexins play a crucial role in regulating various physiological and cognitive functions, such as food intake, sleep-wake cycle, energy homeostasis, addiction, reward, and memory functions.

The discovery of orexins and their receptors took place in 1998 by two separate research groups. De Lecea et al. (1998) identified the mRNA sequence encoding the *prepro*-hypocretin protein precursor, which gives rise to two neuropeptides: Hypocretin A and Hypocretin B. De Lecea et al. (1998) named these neuropeptides "hypocretins" due to their secretion in the hypothalamus and their shared amino acids with the gut hormone secretin. Meanwhile, Sakurai et al., (1998) identified the two orphan G-protein-coupled-receptors to which these two neuropeptides bind: orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R). Sakurai et al., (1998) named these neuropeptides "orexins", in reference to the term *orexis* from the Greek *appetite*, since orexins were primarily associated with food intake at the time. In the same year, Peyron et al., (1998) mapped orexin projections, revealing dense and complex projections throughout the brain, suggesting their involvement in a wide variety of physiological functions.

Interestingly, prior to these discoveries Paut-Pagano et al., (1993) had observed neurons in the hypothalamus, while studying central prolactin neurons involved in sleep regulation, which were later confirmed to be orexin neurons by Risold et al., (1999). They corroborated that the peptide sequence for prolactin neurons described by Paut-Pagano et al., (1993) and the *prepro*-hypocretin peptide precursor were the same.

Simplifying the nomenclatures, and according to Gotter et al., (2012), the gene and mRNA sequence follows the term "hypocretin" (HCRT in humans and Hcrt in

rodents), while the precursor peptide, the two neuropeptides (Ox-A, Ox-B), and the two types of receptors (OX1R, and OX2R) are named after "orexin".

The HCRT gene in humans is located on chromosome 17q21 (Peyron & Kilduff, 2017), while Hcrt gene in rodents is on chromosome 11 (De Lecea et al., 1998). However, despite the difference in chromosome location, both HCRT and Hcrt genes share the same order and orientation (Miyoshi et al., 2001), and both mouse and human genes consist of 2 exons.

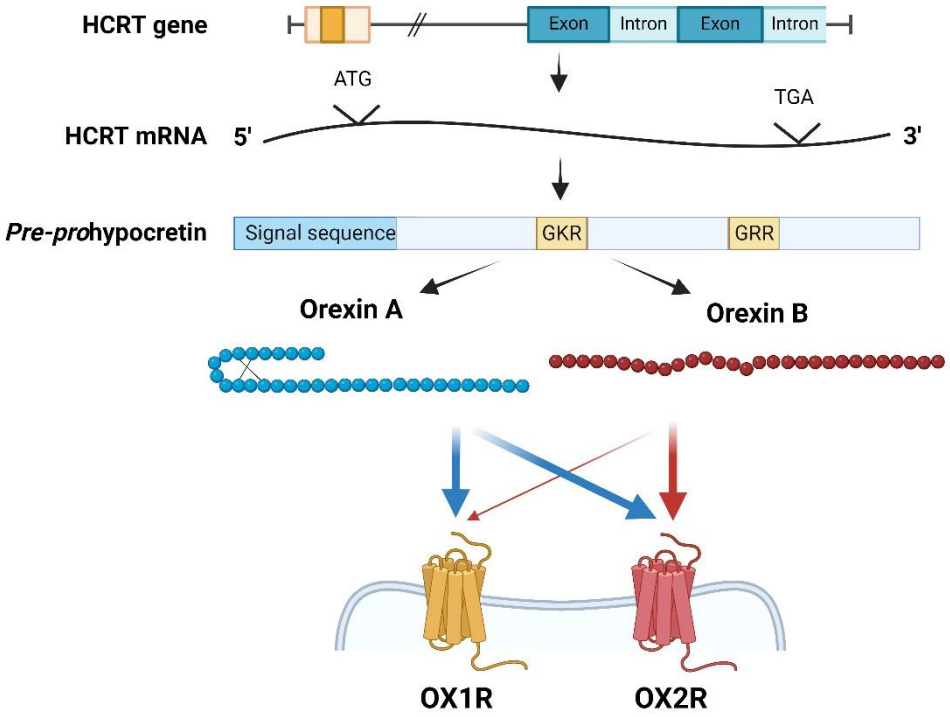


Figure 6. Orexin neuropeptides synthesis and receptor binding affinities. The HCRT gene encodes HCRT mRNA. The translation of the HCRT mRNA produces the *prepro*-hypocretin precursor protein, and its cleaving generates two forms of neuropeptides: orexin A and orexin B, which bind with different affinities to the G-protein-coupled receptors OX1R and OX2R. Binding affinities are shown by arrow width. Abbreviations: HCRT: hypocretin; OX1R: orexin-1 receptor; OX2R: orexin-2 receptor.

The HCRT gene encodes the HCRT mRNA sequence, which is translated into the 130-amino-acid *prepro*-hypocretin precursor peptide. When the precursor peptide is cleaved, it produces a 33 amino acid peptide, called Ox-A, which is intramolecularly stabilized by two disulfide bridges, and a 28 amino linear acid peptide called Ox-B (Gatfield et al., 2010; Gotter et al., 2012; Khoo & Brown, 2014; Wang et al., 2017). Ox-A and Ox-B are endogenous ligands to OX1R and OX2R, which

are G-protein-coupled receptors. OX1R has one-order-of-magnitude greater affinity for Ox-A than Ox-B. However, OX2R has similar affinities for both neuropeptides (Cohen et al., 2020; Sakurai, 2006), as shown in Figure 6.

2.2 Orexin receptors cellular signaling

OX1R and OX2R signal transduction pathways are complex and promiscuous. Both receptors are G-protein-coupled receptors which can activate various pathways leading to distinct neuronal activity patterns. They are capable of coupling with different types of Gα proteins, including G_q, G_s and G_i (Kukkonen and Leonard, 2014), each with its own functional implications for neuronal activation. For example, G_q proteins are excitatory, leading to depolarization, while G_i proteins are inhibitory, causing hyperpolarization. In addition, the net result of these signaling cascades will vary depending on the agonist concentrations.

However, orexins and their receptors primarily exert excitatory effects on neurons by increasing intracellular Ca^{2+} (Ammoun et al., 2003; Khoo & Brown, 2014; Sakurai et al., 1998; Zhu et al., 2003), although inhibitory activity can also be observed in specific cases (Sakurai, 2006). OX1R and OX2R couple to $G\alpha_q$ proteins and activate phospholipase C (PLC), increasing the production of second messengers like diacylglycerol (DAG) and inositol triphosphate (IP_3) via membrane phospholipids, resulting in the activation of protein kinase C (PKC). PKC then phosphorylates calcium channels to promote Ca^{2+} influx (Kohlmeier et al., 2004). Additionally, orexin receptors can activate non-selective cation channels (NSCC) through Gq proteins, facilitating calcium and sodium influx, thus promoting membrane depolarization. (Kukkonen & Leonard, 2013).

Although not entirely confirmed, it is plausible that OX1R and OX2R also increase calcium levels through the classical inositol triphosphate (IP_3) pathway involving calcium release from the endoplasmic reticulum. Taken all together, these pathways result in a net increase of intracellular calcium levels, which facilitate membrane depolarization and generation of action potentials, resulting in an excitatory neuronal response (Gatfield et al., 2010; Kukkonen & Leonard, 2014).

Furthermore, orexin receptors signaling can activate adenylyl cyclase to produce cAMP, which further activates protein kinase A and cAMP-regulated channels (Patel et al., 2001; Holmqvist et al., 2005). OX1R activation can also inhibit adenylyl cyclase via Gi coupling (Holmqvist et al., 2005; Urbanska et al., 2012).

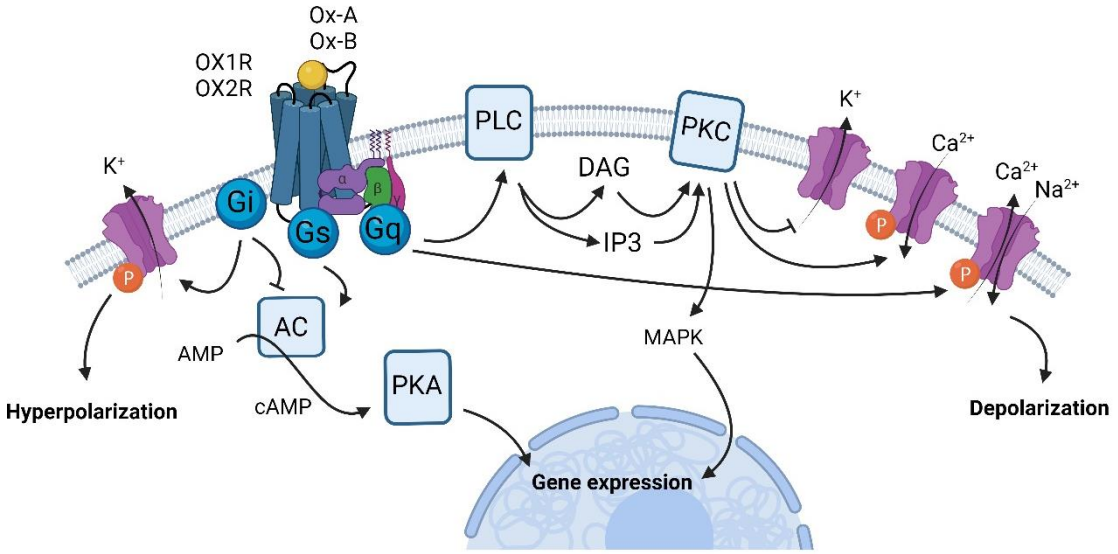


Figure 7. **Orexin receptors signaling cascades**. Orexin neurons' signaling produces excitatory effects and gene expression changes, although they can also produce inhibitory effects in some cases. Abbreviations: AC: adenylyl cyclase; DAG: diacylglycerol; Ox-A: orexin A; Ox-B: orexin B; OX1R: orexin-1 receptor; OX2R: orexin-2 receptor; PKA: protein kinase A; PKC: phospholipase C; PLC: phospholipase C; MAPK: members of the mitogen-activated protein kinase cascade.

Moreover, OX1R/OX2R can activate pathways that promote gene expression. For instance, PKC phosphorylates extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) pathway (Ammoun et al., 2006, Gorojankina et al., 2007). The MAPK/ERK pathway is of biological relevance due to its involvement in several cellular processes such as synaptic plasticity and cell survival (Thomas & Huganir, 2004).

As previously mentioned, orexin neurons can also have inhibitory effects under certain circumstances. This inhibition may occur through the activation of G-protein-gated inward rectifier potassium channels (GIRKs) by $G\alpha_{i/o}$ proteins. These channels create a K^+ outward current, which hyperpolarizes the cell membrane and decreases cell excitability, resulting in an inhibitory neuronal response. Such

inhibitory effects of orexins have been observed in the POMC-expressing neurons in the hypothalamic arcuate nucleus (Sakurai, 2006; Kano et al., 2019).

2.3 Orexin circuits

Orexin (Ox) neurons are restricted to the lateral hypothalamic area (LHA), perifornical area (PFA), dorsomedial hypothalamus (DMH) and the posterior hypothalamus, as shown in Figure 7. Despite their localized distribution, orexin neurons project widely throughout the brain.

The distribution of orexin receptors has been studied in rats by examining levels of OX1R and OX2R mRNA (Marcus et al., 2001; Trivedi et al., 1998). These studies have shown that the anatomical distribution of orexin receptors closely mirrors that of orexin fibers. While there is an overlap in the regions where both receptors are expressed, certain areas exhibit differential expression patterns, indicating distinct physiological roles for each receptor. For example, the PFC, the hippocampus and the LC predominantly express OX1R (Marcus et al., 2001), whereas the NAcc and the tuberomammillary nucleus predominantly express OX2R (Marcus et al., 2001; Sakurai, 2006; Trivedi et al., 1998).

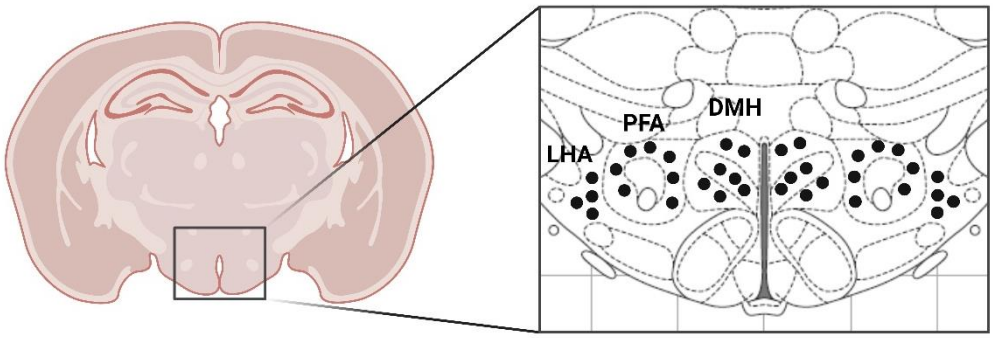


Figure 8. **Location of orexin neurons throughout the hypothalamus**. **Left**. coronal section around Bregma anterior-posterior -3.12. **Right**. Orexin neurons (black dots) distributed throughout the hypothalamus. Abbreviations: DMH: dorsomedial hypothalamus; LHA: lateral hypothalamic area; PFA: perifornical area. Image adapted from Paxinos & Watson (2007).

The densest inputs to Ox neurons originate in the hypothalamic region, specifically in the lateral hypothalamic area (LHA), the posterior hypothalamus (PH), the DMH, the ventromedial hypothalamus (VMH), the paraventricular nucleus (PVN), the arcuate nucleus (Arc) and the the tuberomammillary nucleus (TMN)

(Canteras et al., 1994; Li & de Lecea, 2020; Sakurai et al., 2005; Yoshida et al., 2006). These orexinergic local circuits create a net positive-feedback mechanism, involving Ox neurons and other neuron types. The hypothalamic region comprises 11 non-neuronal cell types, 15 GABAergic neuron types, and 15 glutamatergic neuron types, including Ox (Mickelsen et al., 2019). This positive-feedback mechanism can be mediated through both indirect and direct orexin local circuits. The indirect pathway consists of Ox neurons' activation of local glutamatergic neurons, which in turn excite orexin neurons (Li et al., 2002). In the direct pathway orexin neurons activate other Ox neurons via the activation of OX2R (Yamanaka et al., 2010). Overall, the physiological effects of this positive-feedback mechanism in the hypothalamic area contributes to the increase of arousal and attention levels (Li & de Lecea, 2020).

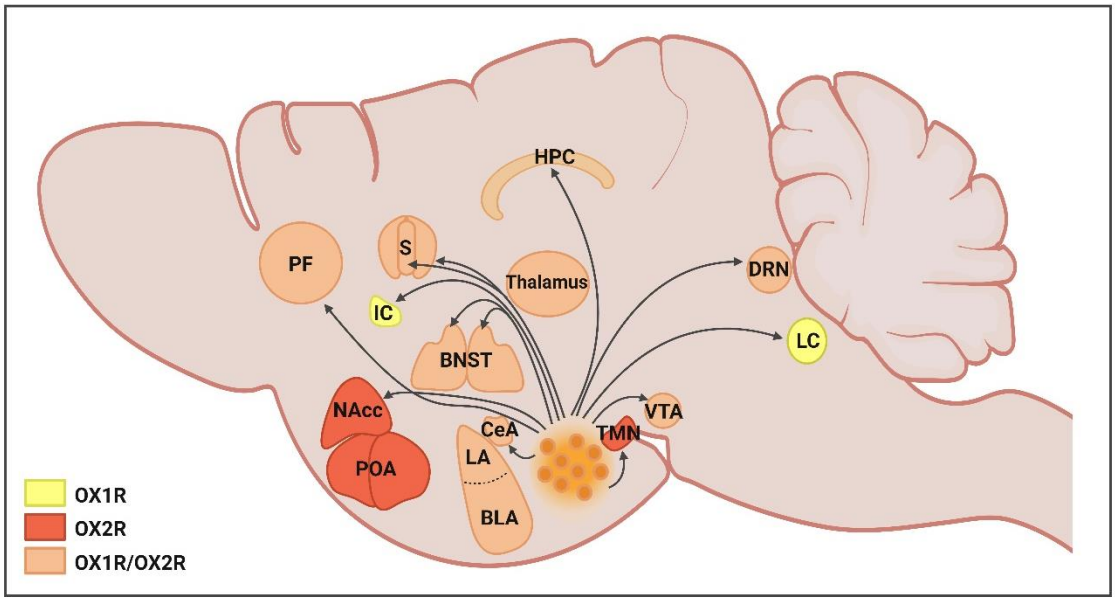


Figure 9. **Orexin neurons' projections throughout the brain**. Mouse brain sagittal section with gray arrows showing neuronal projections. Orexin neurons are depicted with orange dots located in the hypothalamic area. Abbreviations: Arc: arcuate nucleus; BF: basal forebrain; BLA: basolateral amygdala; BNST: bed nucleus of the stria terminalis; CeA: central amygdala; DRN: dorsal raphe nucleus; IC: insular cortex; LA: lateral amygdala; LC: locus coeruleus; LS: lateral septum; NAcc: nucleus accumbens; POA: preoptic area; PVN: paraventricular nucleus; S: septum; TMN: tuberomammillary nucleus; VP: ventral pallidum; VTA: ventral tegmental area.

Orexin neurons also receive inhibitory inputs from four local sources. Leptin, an adipose-derived hormone that signals energy reserves states relative to fat levels, modulates Ox' activity. During negative energy balance states caused by fasting

conditions, low leptin levels lead to Ox neuron activation, increasing arousal and food seeking behaviors (Diano et al., 2003; Louis et al., 2010; Leinninger et al., 2011; Goforth et al., 2014). Additionally, melanin-concentraning hormone (MCH) neurons, intermingled within Ox neurons in the LHA, modulate Ox neuron activity (Konadhode et al., 2015). GABAergic neurons in the LH also inhibit Ox neurons, with some active during sleep states, contributing to decreased arousal and promoting sleep (Hassani et al., 2010). Finally, neurons releasing neuropeptide Y (NPY) located in the LHA could also inhibit Ox neurons (Mickelsen et al., 2019), regulating physiological functions, such as food intake, energy homeostasis, circadian rhythm and cognition. Although NPY neurons were initially thought to excite Ox neurons, they seem to inhibit Ox neurons both presynaptically and postsynaptically (Reichmann & Holzer, 2016).

Different tracing techniques have been employed to determine the monosynaptic projections towards Ox neurons, addressing the challenge posed by their dispersed location. The techniques include orexin/GFP:TTC transgenic mouse lines (Sakurai et al., 2005), and retrograde tracing using cholera toxin B subunit, as well as anterograde tracing using biotinylated dextrans (Yoshida et al., 2006). However, these methods have limitations and may exhibit low sensitivity levels for certain regions. Below is a summary of the validated structures that innervate orexin neurons and those projected by Ox neurons.

The densest extra-hypothalamic projections are found in some brainstem nuclei, including the locus coeruleus (LC), the raphe nuclei, the reticular formation, the preoptic area, and the cholinergic neurons of the basal forebrain, involved in the regulation of sleep/wake cycles and anxiety-related responses (Henny & Jones, 2006; Li & De Lecea, 2020; Peyron et al., 1998; Sakurai et al., 2005; Yoshida et al., 2006).

Orexin neurons also strongly project to mesocorticolimbic areas, indicating their involvement in reward and addiction, including the ventral tegmental area (VTA), the septal nuclei, the BNST (Giardino et al., 2018) and the amygdaloid nuclei. Ox neurons also project to other reward-related structures, such as the shell of the

NAcc and the prefrontal cortex (PFC) (Baldo et al., 2003; Giardino et al., 2018; Peyron et al., 1998).

Moreover, the lateral and medial septum also send dense projections to orexin neurons, innervating the medial and dorsal orexin fields (Sartor & Aston-Jones, 2012). The thalamus, particularly the medial portion of the lateral habenular nucleus (LhB), along with the midbrain tegmental nucleus (MiTg), the retrochiasmatic area, the laterodorsal tegmental nucleus (LDT), and the PAG also send dense projection to Ox neurons (Cohen et al., 2020).

Finally, orexin neurons project to cortical regions involved in cognitive function such as the CA1, CA3, DG and the entorhinal region of the hippocampus (HPC) (Elahdadi Salmani et al., 2022), as well as the insular cortex (IC) (Li & de Lecea, 2020; Nambu et al., 1999; Peyron, et al., 1998).

2.4 Orexin receptor ligands

Orexin receptors are widely distributed throughout the CNS and their functions have been associated with the neuromodulation of different physiological functions, including sleep and sleep disorders. This underscores the interest of pharmaceutical companies in finding therapeutic approaches that promote sleep (LeBold et al., 2013).

2.4.1 Orexin receptor antagonists

Research on pharmacological treatments for sleep has explored different types of dual orexin receptor antagonists (DORAs). Some candidates, such as Almorexant, Suvorexant, SB-649868 and MK-6096, have been investigated, but certain issues have been encountered. Almorexant caused tolerability issues (Hoever et al., 2010) and SB-649868 was withheld due to toxicology concerns. In contrast, Suvorexant and MK-6069 showed promise in improving sleep and insomnia (LeBold et al., 2013). However, only Suvorexant has been approved for clinical use in humans

(Saitoh & Sakurai, 2023). Given the need to address psychiatric disorders involving OX1R and sleep disorders involving OX2R, pharmaceutical companies have shifted their focus to developing selective orexin receptor antagonists (SORAs).

The first OX1R selective antagonist was SB-334867 (Porter et al., 2001; Smart et al., 2001), which showed good selectivity for the OX1R, rapid absorption and brain penetration after intraperitoneal administration (Porter et al., 2001). It exhibits a higher affinity for OX1R than for OX2R. However, it may also have off-target affinities to other receptors, including adenosine A2A or serotonin 5-HT_{2C}.

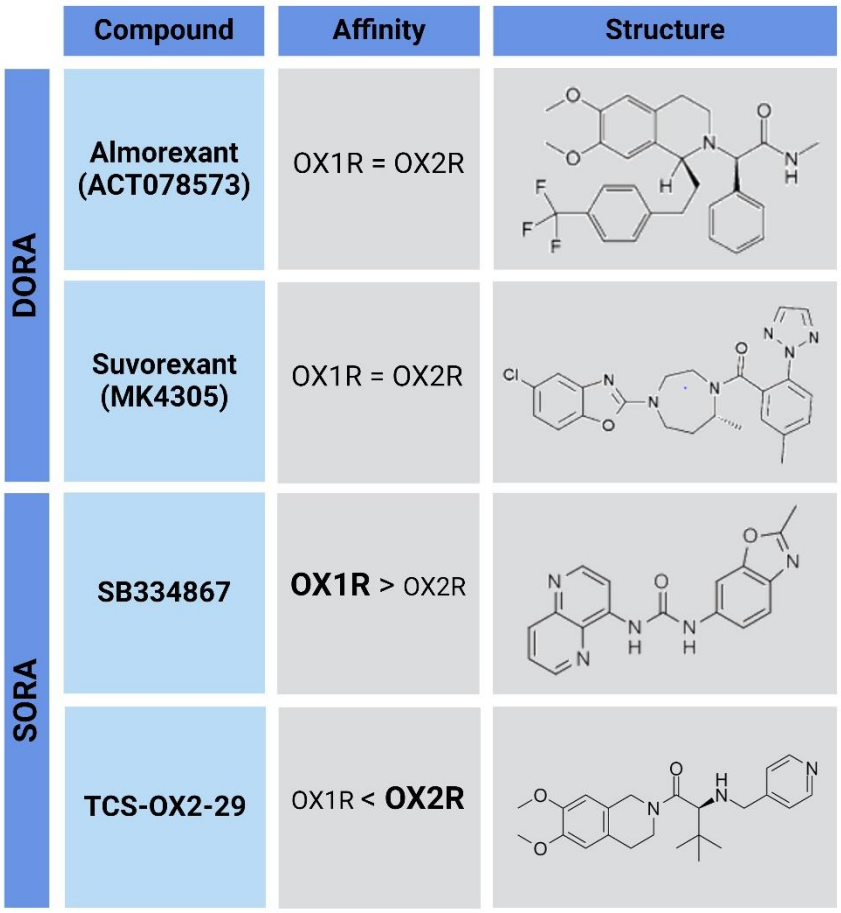


Figure 10. **Representative list of orexin receptor antagonists**. Note that the receptor affinity is included in general terms in the second column. (Data from LeBold et al., 2013; Mieda & Sakurai, 2013).

The first OX2R selective antagonist was TCS-OX2-29, developed by Banyu Pharmaceuticals (Hirose et al., 2003). It presents a significantly higher affinity for OX2R than for OX1R. Its advantageous characteristics include water solubility and

increased selectivity (Boss et al., 2009). Other OX2R selective antagonists include JNJ-10397049 (Faedo et al., 2012) and EMPA (Malherbe et al., 2009).

2.4.2 Orexin receptor agonists

The development of orexin receptor agonists holds significant promise for both understanding receptors function and providing therapeutic options for disorders such as hypersomnia. Despite not receiving much attention until recent years, advancements in research have led to the discovery and design of three main types of agonists: peptidic, non-peptidic and dual agonists.

The first OX1R-selective peptide discovered was orexin A(2−33), which lacks the two disulfide bonds of Ox-A, but exhibits the same activity as the native neuropeptide. The first OX2R-selective peptides to be identified were [Ala11,D-Leu14]orexin B and [Ala11,D-Leu15]orexin B (Asahi et al., 2003). The shortests segments to show activity against both receptors or exclusively OX2R were orexin B(8−28) and orexin B(10−28) (Lang et al., 2004).

Efforts have been made to design small-molecule OX2R-selective agonists. The first non-peptidic small molecule OX2R agonist was Yan7874 (Yanagisawa, 2010). However, its pharmacological characterization found off-target cytotoxic activity (Turku et al., 2017). Currently, YNT-185 and TAK-925 are potential candidates for optimal OX2R-selective agonists, showing promise in improving hypersomnia (Fujimoto et al., 2022; Nagahara et al., 2015).

Finally, small-molecule OX1R/OX2R dual and OX1R-selective agonists have also been developed. Among them, RTOXA-43 has been identified as a dual agonist (Zhang et al., 2021) and YNT-3708 stands out as the most potent OX1R agonist discovered to date (Iio et al., 2023; Saitoh & Sakurai, 2023).

2.5 Orexin functions

Given the widespread distribution of Ox neurons throughout the CNS, orexin functions are multiple and diverse. They modulate autonomic (Subramanian & Ravichandran, 2021), physiological and cognitive functions, including regulation of arousal and sleep/wake functions, feeding behavior and energy homeostasis, reward-seeking behavior, stress response and learning and memory, among others (Sakurai et al., 1998; Sakurai, 2014; Sakurai & Mieda, 2011). Below is a comprehensive overview of the most significant functions associated with orexins.

2.5.1 Feeding behavior and metabolism

The first function attributed to orexins was the regulation of feeding behavior, since administration of orexin A stimulates food intake (Sakurai et al., 1998; Haynes et al., 2000) and icv administration of OX1R antagonist, anti-orexin antibody or genetic ablation of orexin neurons decreases food consumption (Haynes et al., 2000; Yamada et al., 2000; Hara et al., 2001).

Orexin neurons are bilaterally distributed within the LHA (Bernardis & Bellinger, 1996; Sakurai, 1999). Glucose, leptin and ghrelin are important homeostatic signals for modulating feeding behavior and metabolism. High levels of glucose and leptin decrease the activity of Ox neurons (Burdakov et al., 2005; Yamanaka et al., 2003), and low levels of glucose or ghrelin activate Ox neurons (Briski & Sylvester, 2001; Otlivanchik et al., 2015; Toshinai et al., 2003). Orexins, in turn, activate neurons expressing NPY in the arcuate nucleus, another crucial hypothalamic nucleus involved in food intake regulation (Dube et al., 2000; Jain et al., 2000; Yamanaka et al., 2000). This suggests that Ox neurons not only influence feeding behavior and arousal but also serve as integrators, sensing nutritional factors and modulating arousal, awareness, and wakefulness, to regulate energy expenditure and adapt behavior for food-seeking purposes. Thus, orexins may play a critical role in individual survival by promoting active states when food-seeking behavior is necessary (Kotz, 2006; Sakurai, 2006; Willie et al., 2001).

Regarding orexin receptors functions, it is suggested that OX1R is primarily implicated in the food-seeking function, while OX2R regulates wakefulness and energy expenditure to enable food-seeking behavior (Sakurai, 2014; Haynes et al., 2000).

2.5.2 Sleep and arousal

Orexins are considered to be arousal-promoting peptides due to their involvement in the sleep-wake cycle by promoting wakefulness (Sakurai, 2007; Saper et al., 2005). They achieve this by activating brain regions involved in the sleep/wake cycle, such as the dorsal raphe (Brown et al., 2001), tuberomammillary nucleus (Bayer et al., 2001), and LC (Hagan et al., 1999; Horvath et al., 1999). Notably, orexin neurons are inhibited during sleep by GABAergic neurons from the ventrolateral preoptic area (Sakurai et al., 2005; Tsujino & Sakurai, 2013).

Deficits in orexin function lead to narcolepsy, a neurological disorder characterized by excessive daytime sleepiness, cataplexy, harsh transitions between wakefulness and REM sleep, and disruptions in maintaining wakefulness (Chemelli et al., 1999). Patients with narcolepsy exhibit a significant reduction, ranging from 80 up to 100%, in the number of Ox neurons (Peyron et al., 2000; Thannickal et al., 2000) and orexin-A levels in cerebrospinal fluid (Nishino et al., 2000) in comparison to healthy individuals. Moreover, mice lacking the orexin gene with null mutations in OX2R, OX1R/OX2R or a deletion of the *prepro*-orexin gene display a similar phenotype to humans with narcolepsy (Chemelli et al., 1999; Hara et al., 2001). Conversely, orexin antagonists, including SORAs and DORAs, have been explored as potential treatments for insomnia (Subramanian & Ravichandran, 2021).

Initially, it was believed that OX2R was the primary mediator of the sleep/wake cycle since it is abundantly expressed in the histaminergic TMN, and OX1R-knockout mice do not show robust impairments on sleep and awake states (Willie et al., 2003). However, studies on double-receptor knockout mice revealed a more severe phenotype in comparison to OX2R-knockout mice (Willie et al., 2001). Thus, although OX2R appears critical for maintaining wakefulness, both receptor

pathways are necessary for proper sleep and wakefulness regulation (Sakurai, 2007; Tsujino & Sakurai et al., 2013).

2.5.3 Reward

Orexins play an important role in modulating the brain's reward system due to their anatomical connections with the dopaminergic mesolimbic pathway. Early studies demonstrated dense projections from orexin neurons in the LH to reward-related regions, including the VTA and the NAcc (Peyron et al., 1998; Wise, 2004). This system is responsible for processing the rewarding effects associated with innate elements, such as food and sex. Activation of orexin receptors within theVTA via Ox-A or Ox-B infusions increase DA release in the NAcc and PFC (Vittoz et al., 2008). Additionally, studies have shown that Ox-A icv infusions cause a dose-dependent increase of intracranial-self stimulation thresholds (Boutrel et al., 2005), suggesting a role in reward processing.

Orexin neurons also influence addictive behavior and drug-seeking through various mechanisms. They depolarize VTA neurons and interact with neurotransmitters, such as glutamate through NMDAR (Borgland et al., 2006; España et al., 2011), potentially enhancing drug-seeking behavior. Additionally, icv Ox-A infusions have been linked to a dose-dependent reinstatement of cocaine seeking behavior, with SB334867 blocking this effect (Boutrel et al., 2005). Similarly, Harris et al., (2005) showed that Ox neurons' activation increased the preference for drug- and food-related cues and it reinstated a previously extinguished drug-seeking behavior.

Furthermore, Ox neurons play a role in stress-related reinstatement of drug seeking (Boutrel et al., 2005), possibly through activation of the hipothalamic-pituitary-adrenal (HPA) axis (Bonnavion et al., 2015). Moreover, corticotropin-releasing factor (CRF), a peptide hormone involved in stress response, depolarizes orexin neurons. It has been proposed that this effect contributes to the maintenance of the stress response (Winsky-Sommerer et al, 2004). It has also been reported that CRF is involved in the consolidation of aversive memories (Isogawa et al, 2013).

Thus, CRF could mediate orexin neurons' activity through CRF-expressing BNST neurons (Giardino et al., 2018). The activity of orexin neurons would potentiate arousal effects, and this could contribute to the consolidation of emotional memories (Flores et al., 2014).

When considering the role of orexin receptors in reward and drug-seeking behavior, it is important to note that the VTA exhibits similar expression for both OX1R and OX2R (Sakurai et al., 1998; Trivedi et al., 1998). Studies using SB334867 have concluded that the OxA-OX1R pathway is critical in drug relapse, drug-seeking behavior and the duration of abstinence periods (James et al., 2017). Specifically, OX1R activation seems to be more relevant for drug-seeking behavior tested on morphine and cocaine (Harris et al., 2005), nicotine (Hollander et al., 2008; Pasumarthi et al., 2006; Plaza-Zabala et al., 2012a) and alcohol (Lawrence et al., 2006). On the other hand, OX2R seems to play a critical role specifically in alcohol seeking behavior, suggesting potential therapeutic targets for the treatment of alcohol use disorders (Baimel et al., 2015). DORAs have shown promise in reducing alcohol-induced anxiety and sleep disruptions (Campbell et al., 2020).

In conclusion, orexins do not directly influence the positive or hedonic effects of drugs. Instead, orexin signaling regulates behavioral responses in situations where there is a high motivation to obtain drugs (James et al., 2017), underscoring their importance in addiction and substance-seeking behaviors.

2.5.4 Fear and anxiety

Orexin neurons are also involved in emotional processes and emotional memory. They become activated in response to emotionally salient stimuli, regardless of whether they carry positive or negative valence (Blouin et al., 2013). This activation is believed to integrate and coordinate arousal levels, autonomic and stress responses to appropriately respond to the emotional situation (Blouin et al., 2013; Sakurai, 2007, 2014).

In individuals with narcolepsy, en shown to present impairments on emotional processing is often impaired due to altered amygdala activation in response to

emotional images (de Zambotti et al., 2014; Ponz et al., 2010). Moreover, orexin neurons' activation is associated with anxiety and panic-like behavior in humans and rats (Johnson et al., 2010a). Supporting this notion, the dual orexin antagonist almorexant has been shown to reduce fear-potentiated startle responses in rats (Steiner et al., 2012).

In the following sections, we will delve into the role of orexins on non-conditioned fear and anxiety, followed by their involvement in conditioned fear and emotional memory.

2.5.4.1 Orexin's effects on non-conditioned fear and anxiety

The specific role of orexins on non-conditioned fear and anxiety is controversial due to inconsistent findings in the scientific literature. Researchers employ non-conditioned tasks that involve ethologically relevant stimuli to rodents, such as fear of open or bright spaces, to assess anxiety and fear levels (see section *1.2 Non-conditioned paradigms*).

On one hand, some studies report anxiolytic effects of the orexinergic system. Pan et al., (2020) showed that infusions of Ox-A into the CeA reduced anxiety levels in rodents tested in the open field test and elevated plus maze, without affecting locomotion. Similarly, Khalil & Fendt (2017) reported increased anxiety in orexin-deficient mice across various tests, including the OF, LD and the carnivore odor-avoidance test, without affecting locomotion. Moreover, Han et al., (2021) demonstrated that icv administration of Ox-A results in decreased anxiety levels in the OF and EPM in rats subjected to a single prolonged stress model. These findings imply that the activation of the Ox-A/OX1R pathway might have anxiolytic-like effects and influences avoidance behaviors associated with anxiety.

On the other hand, other studies have suggested a potential anxiogenic role of orexins. Ox-A infusions into the lateral ventricle or the PVN have been shown to increase anxiety in both mice and rats in the OF and the EPM (Li et al., 2010; Suzuki et al., 2005). Similarly, Ox-A infusion into the CeA of Syrian golden hamsters

increases anxiety-like behaviors in the EPM and LD (Avolio et al., 2011). Furthermore, Ox-A infusions into the BNST, but not the septum, increases anxiety levels in the EPM. In the same study, patch clamp techniques revealed that Ox-A depolarized BNST neurons (Lungwitz et al., 2012). These studies point to potential brain targets of orexin neurons' projections, which could be underlying orexin-mediated modulation of anxiety-like behavior.

Moreover, studies using dual receptor antagonists have shown they promote decreased anxiety in a social interaction test in rats previously subjected to contextual fear conditioning (Dong et al., 2015). Similarly, SB334867 infusions reduce anxiety-like behaviors in a seizure rat model tested on the EPM (Kordijaz et al., 2017). In another study, SB334867 decreases anxiety in a cat odor avoidance model, where subjects with OX1R blockade approached the cat odor significantly more in both the first and the second trials (Staples & Cornish, 2014). Lastly, SB334867 has been shown to reduce hypercapnic gas-induced anxiety responses (Johnson et al., 2012b).

Table 1. **Effects of orexins on non-conditioned fear and anxiety tests**.

Study	Task	Area	Treatment / species	Results
Anxiolytic				
Arendt et al., 2014	OF EPM	BLA	Viral shRNA knockdown of OX2R Male mice	↑ anxiety None
Abbas et al., 2015	EPM L/D OF	-	OX1R knockout male mice	↓ locomotor activity ↓ locomotor activity None
Khalil & Fendt, 2017	OF LD Carnivore odor-avoidance test	-	Orexin-deficient female and male mice	↑ anxiety ↑ anxiety ↑ anxiety
Pan et al., 2020	OF EPM	Bilateral CeA	Ox-A / male Wistar rats	↓ anxiety

Han et al., 2021	OF EPM	Icv	Orexin-A /male Wistar rat / SPS model	↓ anxiety ↓ anxiety
Anxiogenic				
Suzuki et al., 2005	EPM OF rat EPM	Unilateral icv	Ox-A / Male mice Ox-A / Male Wistar rats	↑ anxiety ↑ anxiety tendency to anxiogenic
Li et al., 2010	EPM	PVN	Ox-A infusion Sprague-Dawley rats	↑ anxiety
Avolio et al., 2011	EPM LD	CeA	Ox-A Male syrian golden hamster	↑ anxiety
Johnson et al., 2012b	CO2-induced anxiety	Systemic	SB334867 Male Sprague-Dawley rats	↓ anxiety
Lungwitz et al., 2012	EPM	BNST Septum	Ox-A 30 min prior Ox-A 30 min prior Male Sprague-Dawley rats	↑ anxiety = anxiety
Staples and Cornish, 2014	Cat odor avoidance model	Systemic	SB334867 Male Wistar rats	↓ anxiety
Dong et al., 2015	Social interaction test	Unilateral PVT	DORA Male Sprague-Dawley rats	↓ anxiety
Kordijaz et al., 2017	EPM	Left icv	SB334867 Male Wistar rats PTZ kindled rats	↓ anxiety

Table 1. **Effects of orexins on non-conditioned fear and anxiety tests**. Abbreviations: BLA: basolateral amygdala; CeA: central amygdala; DORA: dual orexin receptor antagonist; EPM: elevated plus maze; Icv: intracerebroventricular; L/D: light/dark transition test; OF: open field test; OxA: orexin A; OX1R: orexin 1 receptor; OX2R: orexin 2 receptor; PVN: paraventricular nucleus; PVT: paraventricular nucleus of the thalamus. ↑ increases; ↓ decreases.

Other studies have found different results compared to previous reports. For instance, Aitta-Aho et al. (2016) showed that DREADD-mediated orexin neurons' activation does not affect anxiety levels in the OF, and OX1R blockade does not affect

behavior in the EPM (Staples & Cornish, 2014). Similarly, oral administration of almorexant does not modify the behavior in the EPM (Steiner et al., 2012).

It is crucial to consider the effect of orexins on locomotion. Orexin neurons are an important mediator of arousal levels, as previously described (see *2.5.2 Sleep and arousal*). Thus, their blockade could affect locomotion, potentially dissipate their effects on anxiety and fear responses. While most studies manipulating the orexin system did not observe any effects on locomotor activity, some studies have reported locomotor activity alterations in OX1R KO mice, without affecting anxiety (Abbas et al., 2015), and when blocking OX1R in the first trial of the cat odor avoidance model (Staples & Cornish, 2014).

These conflicting results regarding locomotion could be attributed to two main factors. First, OX1R modulation at lower doses could reduce anxiety, but induce sedative effects at higher doses. Some authors have suggested that orexins mediate the sedative effects of diazepam (Panhelainen & Korpi, 2012), possibly through the recruitment of different neuronal populations depending on the dose used. However, studies using higher doses (30 mg/kg) have not observed locomotor activity alterations (Johnson et al., 2010b). Therefore, dosage alone may not fully explain orexin effects on anxiety and locomotion. Second, the nature of the situation in which animals are exposed and the familiarity of the stressor could be a determining factor for orexin effects on anxiety and fear. Depending on the characteristics of the test employed and the frequency of testing, different brain regions may be activated, and OX1R blockade may yield disparate effects (Staples & Cornish, 2014).

Overall, the results regarding the effects of orexins on non-conditioned fear and anxiety remain inconclusive. The location of action of the drugs used in these experiments appears to be a critical factor that could explain these inconsistent findings. Therefore, further research is needed in this field in order to better understand the role of these neuropeptides in anxiety and fear modulation.

2.5.5 Learning and memory

As discussed previously, OX1R is broadly expressed in memory-related brain areas, including various regions of the hippocampal formation (cornus ammonis 1: CA1; cornus ammonis 2: CA2; dentate gyrus: DG), the retrosplenial cortex (RSC) and the medial prefrontal cortex (mPFC) (Marcus et al., 2001; Trivedi et al., 1998). Orexin-A administration typically enhances implicit (see section *2.5.5.1 Orexins on conditioned fear*) and explicit memory processes.

The selective blockade of OX1R has been proven to affect performance in hippocampus-dependent tasks. For instance, SB334867 impairs the consolidation of both spatial and visual discrimination configurations of the Morris water maze (MWM), affecting both acquisition and the retention (García-Brito et al., 2020). Similarly, other studies have reported impairments in the acquisition, consolidation (Akbari et al., 2006, 2007) and retrieval (Akbari et al., 2006) of the spatial configuration of the MWM with SB334867 administration, while non-spatial visual discrimination configuration remained unaffected.

Interestingly, the effects of OX1R selective blockade appear to be training-dependent, with higher training rates mitigating detrimental effects of OX1R blockade (García-Brito et al., 2018). Additionally, orexin-A icv administration has been found to counteract the detrimental effects of a seizure rat model on MWM learning and memory and to promote neurogenesis in the DG via OX1R-mediated $ERK_{1/2}$ activation (Zhao et al., 2014). Conversely, the DORA Almorexant has been shown to facilitate the consolidation of the MWM when administered orally (Dietrich & Jenck, 2010) and to improve MWM performance when administered intraperitoneally in an Alzheimer's disease mice model (Zhou & Li, 2023). These discrepancies could be due to differences in administration protocols.

Although orexins have been shown to facilitate other types of explicit memory tasks, no effects of i.p administration of SB334867 were observed using a novel object recognition test, which involved declarative memory and emotional

processing, and where OX1R-KO mice did not exhibit any deficits on recognition memory (Flores et al., 2014).

Orexins also appear to play a role in working memory, as orexin KO mice display impairments in a delayed non-matching to place T-maze (Dang et al., 2018). Furthermore, DREADD-mediated orexin neurons' activation improves the short-term mice's performance in a delayed spontaneous alternation T-maze, a form of spatial short-term working memory test (Aitta-aho et al., 2016). Conversely, almorexant administration improves the performance on the Y-shaped maze test (Zhou & Li, 2023).

Molecular analyses of orexin effects on learning and memory-related areas have also shed light on the implication of the orexinergic system in these processes. Wayner et al. (2004) showed that orexin-A infusions into the perforant path-granule cells synapse in the DG enhanced synaptic plasticity by increasing long-term potentiation (LTP), which could be blocked by SB334867. Contradicting results reported by Aou et al., 2003, in which orexin-A infusion into Schaffer collateral/commissural-CA1 synapses impairs spatial learning, may be attributed to differences in dosage and site of application. Additionally, orexin blockade may facilitate memory via its sleep-promoting properties (Dietrich & Jenck, 2010), which is in line with the improvement in the consolidation of declarative memories through sleep observed in healthy human subjects (Gais et al., 2007).

Despite some conflicting reports, it is evident that orexins play a significant role in learning and memory processes. Further research is needed to elucidate their specific role and underlying mechanisms in each task.

Finally, orexins also appear to play an important role in emotional implicit memories, as indicated by several studies exploring their involvement in such processes. Understanding these mechanisms could offer potential therapeutic avenues for treating psychiatric disorders.

2.5.5.1 Orexin's effects on conditioned fear

Salient emotional stimuli activate the limbic system, which activates orexin neurons (Blouin et al., 2013). Thus, specific stressful conditions, such as conditioned fear, involve the activation of orexin neurons (Furlong et al., 2009). Noradrenergic neurons in the locus coeruleus (NA^{LC} neurons) play an important role in the formation of emotional memories, as their activity increases after fear conditioning in rats (Ishida et al., 2002). The projection of NA^{LC} neurons to the LA might be important for the consolidation of fear memory, given that footshock stimulation increases NA release in the LA (Galvez et al., 1996). The highest OX1R expression levels are found in NA^{LC} neurons (Marcus et al., 2001; Sakurai & Mieda, 2011) (see *2.3 Orexin circuits*) and orexin neurons strongly innervate the LC. Thus, the orexin-LC-LA pathway could play a critical role in the formation of emotional memory. Specifically, OX1R is involved in the expression of fear, consolidation of fear memories and maintenance of behavioral fear (Sakurai, 2014; Soya & Sakurai, 2020).

Several studies have investigated the effects of orexins on aversive classical conditioning, particularly in the early stages of a signal active avoidance task. They have revealed that orexins can affect the expression and consolidation of fear memories as well as the maintenance of behavioral fear. Specifically, the Ox-A/OX1R pathway is critical for the consolidation of fear memories. In addition, the two neuropeptides appear to have different levels of involvement in the consolidation of fear memories.

Soya et al. (2017) studied the implications of orexin-NA^{LC} neurons in fear expression and maintenance. They found that intraperitoneal injection of SB334867, as well as various manipulations of NA^{LC} neurons, including genetic ablation, chemogenetic inhibition and optogenetic inhibition, reduced cue-induced freezing response in the late phase of the test. These findings indicate orexin's importance in both fear expression and fear maintenance over time, for which it seems essential.

Additionally, Olsen et al. (2021) demonstrated that Ox-A infusions in the CeA increased activity in a fear conditioned context, thus reducing fear-related behavior like freezing.

Sears et al., (2013) highlighted the necessity of the Ox-A/OX1R pathway activation for the acquisition of threat memories. Their findings showed that the administration of SB334867 before the training session impaired the acquisition and consolidation of FC memory, mediated by the circuit between orexin neurons in the PFH to the LC and to the LA. Similarly, Soya et al., (2013) showed that $OX1R^{-/-}$ mice exhibit impaired freezing responses in both cued and contextual FC paradigms and a decreased response of NA^{LC} neurons. Interestingly, the restoration of OX1R levels in NA^{LC} rescued the freezing behavior, but only in the cued task. Thus, OX1R are critical for the expression and formation of fear memory.

Flores et al., (2014) showed that both orexin A and orexin B could have differential roles in the consolidation of fear memories. Ox-A administration affects both cued and contextual FC, whereas Ox-B only impairs contextual FC. Similar results regarding OX1R and OX2R implication in the acquisition of fear memories were obtained by Soya et al., (2013). As previously mentioned, the OX1R-/- mice impariment was evident in both cued and contextual tasks, whereas the $OX2R^{-/-}$ mice only displayed learning impairments in the contextual FC. These disparate results could be explained by the overlapping yet differential patterns of receptor expression in the brain, with OX2R being highly expressed in the hippocampus (a crucial brain region to decode contextual information) (Johnson et al, 2012a), and OX1R being expressed in both the hippocampus and the amygdala, the latter region being critical for both tasks (Maren et al., 2013). Moreover, Flores et al., (2014) showed that orexin neurons' signaling is necessary for the early encoding of aversive memories, since blocking their receptors did not affect consolidation when administered 4 h after the acquisition session.

However, other studies have failed to report effects of the orexinergic system in FC tasks when using orexin-deficient mice in contextual and cued FC (Khalil & Fendt, 2017).

Table 2. Effects of orexins on classical fear conditioning tasks.

Study	Task	Area	Drug/Treatment	Results
Steiner et al., 2012	Fear-potentiated startle testing	Oral administration	Almorexant F344 male rats	↓ fear-startle response
Sears et al., 2013	FC	Right icv LA Bilateral LC OPG: PFH	pre-training SB334867 TCSOX2229 Male Sprague Dawley rats	↓ consolidation = consolidation
Soya et al., 2013	Contextual FC Cued FC	-	$OX1R^{-/-}$ male mice $OX2R^{-/-}$ male mice	↓acquisition and consolidation in cued/contextual FC ↓acquisition and consolidation in contextual FC
Flores et al., 2014	Contextual FC Cued FC	Systemic (5ml/kg) Systemic (5ml/kg)	post-conditioning SB334867 TCSOX229 OX1R KO Male mice	↓ consolidation of contextual and cued ↓consolidation of contextual
Khalil & Fendt., 2017	Contextual FC Cued FC	-	Orexin-deficient male mice	= learning, consolidation in contextual/cued
Soya et al., 2017	Cued FC	Systemic NA^{LC} neurons NA^{LC} neurons NA^{LC} neurons	Pre-retrevial SB334867 OX1R-/- Chemogenetic inhibition Optogenetic inhibition Male mice	↓ freezing (late phase) ↓ freezing (late phase) ↓ freezing (late phase) ↓ freezing (late phase)

Table 2. Effects of orexins on classical fear conditioning. Abbreviations: FC: fear conditioning; icv: intracerebroventricular; LA: lateral amygdala; LC: locus coeruleus; PFH: perifornical hypothalamus; KO: knockout. ↓ impairs, = no effects.

Orexin neurons seem to be implicated in the extinction of fear memories as well. In Flores et al., (2014) study, OX1R blockade facilitated the extinction in both contextual and cued FC. Interestingly, this specific blockade was able to rescue the extinction in an inbred mouse strain, 129S1/SvImJ, which is characterized by impaired extinction processes. On the other hand, no effects on extinction were observed when OX2R was blocked (Flores et al., 2014). Another study showed that the specific blockade of OX1R using SB334867 reduced freezing behavior during extinction and increased the activity of two main pro-extinction areas, which are the basolateral amygdala and the infralimbic cortex (Flores et al., 2017). Conversely, an overactivation of the orexin system, by icv Ox-A infusions, negatively affected fear extinction (Ten-Blanco et al., 2022). Furthermore, in a study performing Pavlovian FC in humans, plasma Ox-A levels negatively correlated with fear responses during extinction test only for those who underwent extinction 24 h after acquisition, but not for those who performed it two weeks later. These results suggest the involvement of the Ox-A/OX1R pathway in the consolidation of recent fear memory extinction (Shi et al., 2018).

The results obtained from classical conditioning paradigms outline the importance of orexins in this type of learning. It is therefore logical to think that these neuropeptides could also be involved in instrumental learning. As explained before (see *1.1 Fear and anxiety*), avoidance behavior can be used in instrumental paradigms to study the basis of fear and anxiety-related disorders.

Two main types of tasks are commonly used to assess avoidance behavior in animals: passive and active avoidance tasks (see *1.4 Avoidance behavior*). However, literature assessing the effects of orexins on active avoidance paradigms is limited. Therefore, we will first discuss the effects of orexins on passive avoidance and then on active avoidance paradigms. It is important to note that although both tasks share common features, they differ in cognitive and neuronal functioning.

Several studies have explored the role of orexins in passive avoidance tasks. For instance, Ox-A infusions have been found to enhance learning, consolidation and retrieval of a one way passive avoidance task in rats (Telegdy & Adamik, 2002), as well as memory recall in a T-maze footshock avoidance task and in a step-down passive avoidance task in mice (Jaeger et al., 2002). Additionally, blocking OX1R in the DG and dCA1 impairs the retention, consolidation and retrieval of a passive avoidance task in Wistar rats (Akbari et al., 2008). Another study corroborated the relevance of the Ox-A/OX1R pathway by observing the detrimental effects of SB334867 on the consolidation and retrieval of the passive avoidance task, showing thatOX2R could be involved in the retrieval but not the consolidation of the task. Moreover, infusions of Ox-B facilitate the learning, consolidation and reversal stages of a step-through passive avoidance task in a dose-dependent manner (Ardeshiri et al., 2017). Interestingly, while OX2R blockade alone did not affect learning, it inhibited the facilitative effects of Ox-B (Palotai et al., 2014). However, other studies using DORAs have failed to observe an effect on the passive avoidance task (Dietrich & Jenck, 2010).

Regarding active avoidance, Jaeger et al. (2002) demonstrated that Ox-A icv infusions improves the retention in a T-maze footshock avoidance paradigm (Jaeger et al., 2002), but this effect was only observed when Ox-A was administered immediately after the acquisition of the task. Only one study has researched the effects of orexins on a 2-AA task. In the study by Mavanji et al. (2017), orexin deficient mice displayed an impaired memory 24 hours after 20 trials of training. Interestingly, intra-hippocampal infusions of Ox-A rescued memory impairment and maintained this effect over time when administered for a week.

Table 3. Effects of orexins activation or blockade on passive and active avoidance paradigms.

Study	Task	Area	Treatment	Results
Passive avoidance				
Telegdy and Adamik (2002)	1WPA	Unilateral icv Male Wistar rats	Ox-A pre-learning post-learning	↑ learning, consolidation and retrieval

			pre-retrieval	
Jaeger et al. (2002)	T-maze footshock avoidance One trial step-down	Right icv CD-1 male mice	Ox-A post-learning	↑ memory ↑ memory
Akbari et al. (2008)	Passive avoidance learning	Bilateral DG bilateral dCA1 Male Wistar rats	pre-training SB334867 post-training SB334867 pre-training SB334867	↓ retention ↓ consolidation ↓ retrieval
Dietrich & Jenck (2010)	Passive avoidance task	Oral Male Long-Evans rats	Almorexant	＝memory
Palotai et al., (2014)	Step-through passive avoidance	Right icv Male Wistar rats	Ox-B EMPA	↑learning, consolidation, retrieval ↓Ox-B-induced facilitating effects
Ardeshiri et al. (2017)	Passive avoidance task	Bilateral BLA Male Wistar rats	SB334867 TCS-OX2-29	↓ consolidation/ retrieval at all doses ↓ retrieval at higher doses = consolidation
Active avoidance				
Mavanji et al. (2017)	2-AA task (trace)	- Bilateral dCA1 Female O/A3 mice	Orexin deficient mice intra-HPC Ox-A	↓ memory ↑ memory
Jaeger et al. (2002)	T-maze footshock avoidance	unilateral icv CD-1 male mice	Ox-A post-learning	↑ memory

Table 3. **Effects of orexins on passive and avoidance tasks**. Abbreviations: 1WPA: One-way passive avoidance; 2-AA: Two-way active avoidance; DG: dentate gyrus; dCA1: dorsal cornus ammonis 1; BLA: basolateral amygdala; Ox-A: orexin A. ↑ improves, ↓ impairs, = no effects.

The impairment of the 2-AA task in orexin-deficient rodent models may be attributed to several underlying mechanisms. One such mechanism is the impairment of synaptic plasticity. Studies have demonstrated that orexin-deficient mice exhibit lower levels of phosphorylated CREB, paired-pulse facilitation and LTP (Yang et al., 2013). These reduced parameters show that low levels of orexins impair synaptic plasticity mechanisms crucial for learning. Another potential underlying mechanism involves the loss of signaling from neurotransmitters co-expressed in orexin neurons, such as dynorphin (Chou et al., 2001), pentraxin (Reti et al., 2002) and glutamate (Rosin et al., 2003; Mavanji et al., 2017). However, the fact that Ox-A infusions alone reverse impairments in the 2-AA task suggests that the lack of orexin signaling itself is the main cause of the cognitive impairment.

3. Intracranial self-stimulation

3.1 Introduction

Intracranial self-stimulation (ICSS) is a procedure in which subjects self-administer electrical current in brain regions of the reward nervous system (RNS), such as the medial forebrain bundle (MFB) in the lateral hypothalamus (LH), by performing operant behaviors, such as pressing a lever. It was discovered and described by James Olds and Peter Milner in 1954 (Olds & Milner, 1954). Their research revealed that stimulating some brain regions elicit higher response rates, and induce pain or discomfort in others, leading animals to avoid pressing the lever. They identified septal and hypothalamic areas as particularly responsive areas, where stimulation results in higher response rates.

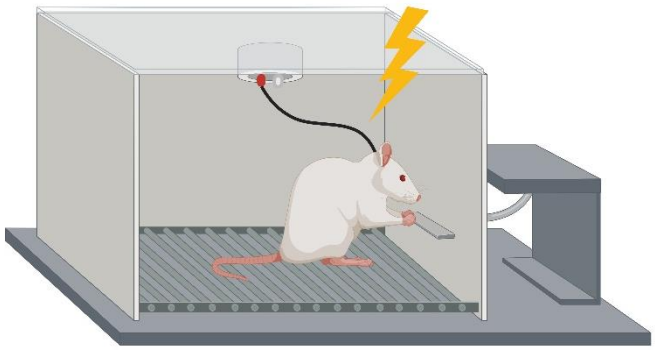


Figure 11. Illustration depicting a rat self-administering ICSS. Illustration depicting a Skinner box with a rat pressing a lever to obtain electrical stimulation in deep brain regions.

3.2 Neural basis of ICSS

Several brain regions, including the septal area, the corpus callosum, the caudate nucleus, cingulate cortex, hippocampus, medial lemniscus, mamillothalamic tract, medial geniculate and tegmentum, have proven initially effective in eliciting ICSS behavior (Olds & Milner, 1954). However, the response rate depends on the stimulated area. Notably, the MFB in the LH is where ICSS behavior is attained more effortlessly, exhibits greater persistence, and elicits higher response rates (Milner in 1991; Olds & Olds, 1963).

The MFB consists of ascending and descending axons arranged along a rostrocaudal axis. It traverses through the LH and extends from the olfactory bulb and medial prefrontal cortex to various brainstem structures (Fig.12). Additionally, there are short interconnecting axons projecting to and from lateral and adjacent hypothalamic regions, contributing to the intricate nature of the pathway. While the descending projections activate regions implicated in the reinforcing aspects of ICSS (Bozarth et al., 1980; Wise, 1996), the ascending fibers also play a role in the general activation of the brain (Takagi et al., 1980).

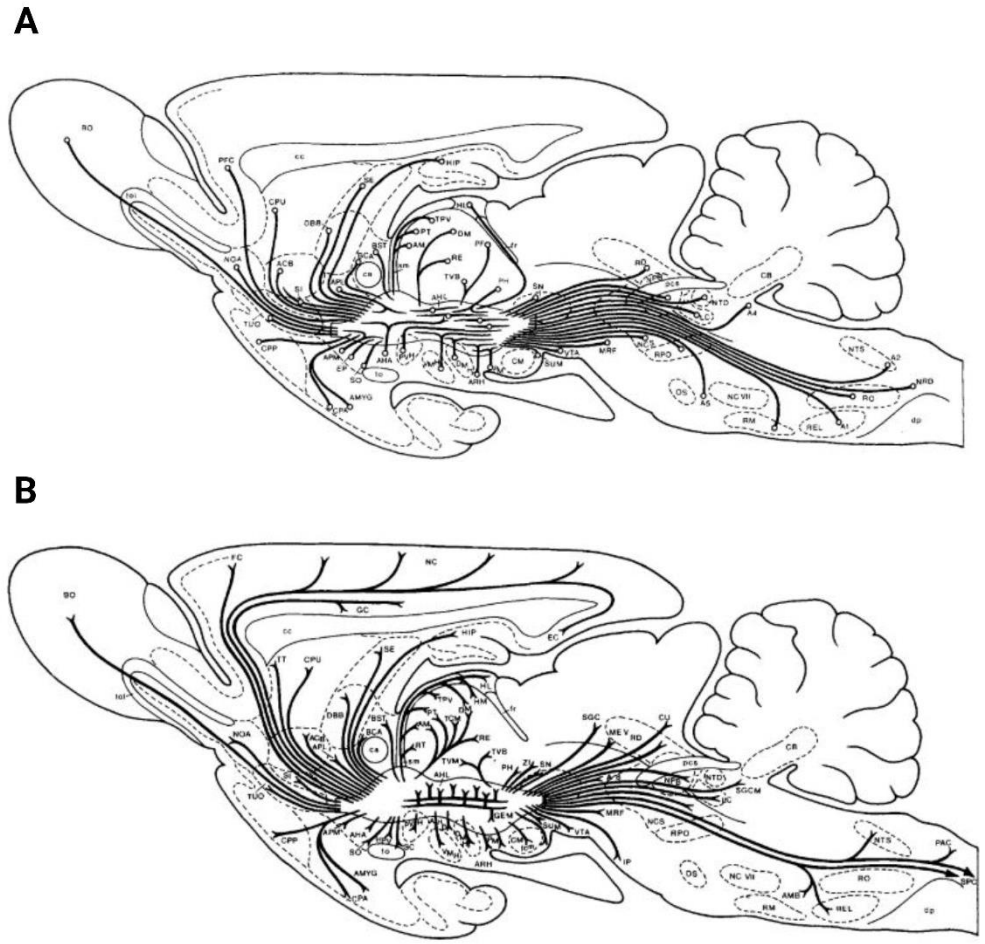


Figure 12. **Diagram representing the medial forebrain bundle circuitry**. **A**. Input of the MFB. **B**. Output of the MFB. Adapted from Nieuwenhuys et al., 1982.

One of the crucial elements of the MFB is the mesolimbic dopaminergic system (MLDA), which is composed of circuits that mediate physiological and cognitive processing of reward (Lewis et al., 2021). It is activated by the myelinated descending fibers from the LH to the VTA (Bielajew & Shizgal, 1986) and it

encompasses ascending dopaminergic fibers from the VTA to the NAcc in the Basal Forebrain (BF), commonly known as the "reward pathway" (You et al., 2001).

The functions of the MLDA system are sustained by the coordinated action of different neurotransmitters. Dopamine plays a pivotal role in the control of reward signals (Lewis et al., 2021). Early indications of dopaminergic involvement in brain stimulation reward were gleaned from investigation into the catecholamine system (German & Bowden, 1974), neuroleptic systems (Edmons & Gallistel 1974; Fouriezos et al., 1978; Franklin, 1978) as well as mapping studies using electrodes and fluorescence histochemistry (Corbett & Wise, 1980). These studies affirm that the rate of ICSS was directly proportional to the density of DA neurons surrounding the electrode tip. ICSS in the MFB has also been shown to be directly associated with an increase in DA levels in the NAcc (Blaha & Phillips, 1990; Hernandez et al., 2012; Klanker et al., 2017; Owesson-White et al., 2008; Phillips et al., 1992; Rada et al., 1998; Taber et al., 1995). Furthermore, supporting the link between ICSS and dopamine, studies have shown that blocking the DA transporter enhances ICSS behavior (Hernandez et al., 2012). On the contrary, lesions in DA neurons (Fibiger et al., 1987) or blocking DA receptors (Fouriezos & Wise, 1976; Lippa et al., 1973) impairs ICSS behavior. However, there are reports suggesting that animals can sustain ICSS behavior even when dopamine depletion occurs within the reward system (Cooper et al., 1974; Phillips & Fibiger, 1978). Thus, the dopaminergic function cannot be the only one supporting ICSS behavior.

Another contributor to the rewarding effects of ICSS in the MFB is acetylcholine (ACh), since its activation simulates dopaminergic neurons in the VTA (Gratton & Wise, 1985; Rada et al., 2000; Yeomans et al., 1985). Additionally, blocking muscarinic receptors (Yeomans et al., 1993) or potentiating nicotinic receptors' activity (Clarke & Kumar, 1984) has been shown to disrupt the rewarding effects of MFB stimulation.

Furthermore, glutamate (Glu) can activate the VTA by establishing connections with adjacent neurons (Dobi et al., 2010; You et al., 2001). Notably, the activation of Glu neurons in both the VTA (Wang et al., 2015) and the pedunculopontine

tegmental nucleus (PPTg) (Yoo et al., 2017) has been linked to reinforcing effects. Additionally, the stimulation of the dorsal raphe nucleus contributes to the reinforcement of ICSS through its Glu projections to the VTA and NAcc (Hernandez et al., 2016; Luo et al., 2015; Qi et al., 2014).

Importantly, the orexinergic system is also involved in reward processing (See *2.5.3 Reward*). Orexin neurons originating from the LH innervate and stimulate DA and non-DA neurons in the VTA (Borgland et al., 2006; Korotkova et al., 2003; Nambu et al., 1999; Peyron et al., 1998). Additionally, orexin-A infusion in the VTA has been shown to enhance LH stimulation (Muschamp et al., 2014), although both intracerebroventricular (icv) or intra-VTA orexin-A administrations increase the ICSS threshold (Boutrel et al., 2005; Hata et al., 2011).

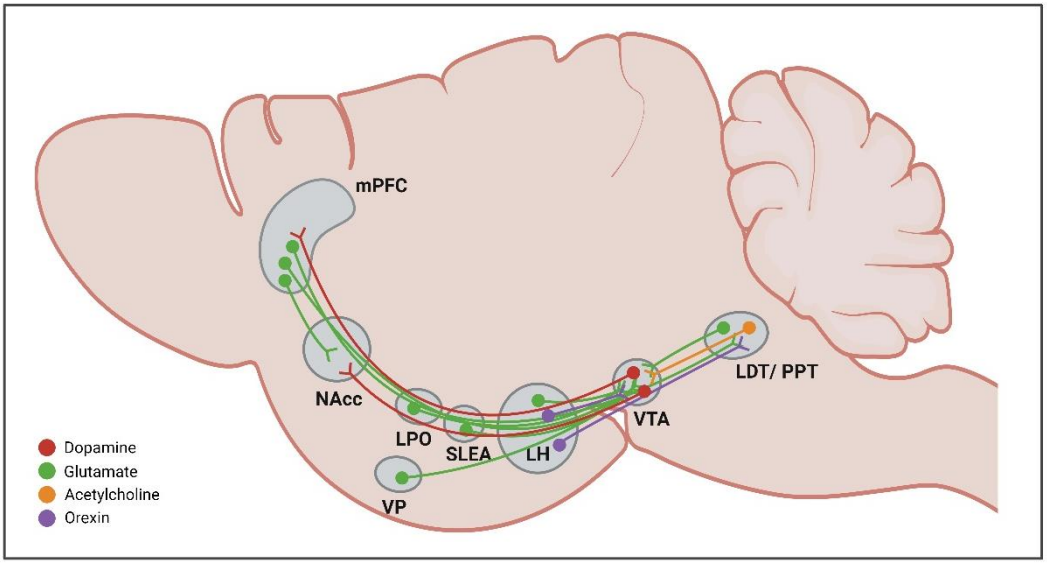


Figure 13. Sagittal view of pathways involved in MFB-LH ICSS rewarding effect. Abbreviations: LDT: laterodorsal tegmental area; LH: lateral hypothalamus; LPO: lateral preoptic area; mPFC: medial prefrontal cortex; NAcc: nucleus accumbens; PTT: caudal pedunculopontine tegmental area; SLEA: sub-lenticular extended amygdala; VP: ventral pallidum; VTA: ventral tegmental area. Adapted from Shizgal (2012).

In conclusion, these studies elucidate a complex system through which ICSS provokes its rewarding effects, highlighting the need for additional research. The dopamine system emerges as a crucial factor contributing to the rewarding effects of ICSS, although it is essential to acknowledge the involvement of other

neurotransmitters. Figure 13 provides a visual representation of the aforementioned systems.

3.3 ICSS effects on memory

ICSS has proven to be an effective treatment to improve different types of learning processes and memories in a wide variety of experimental paradigms.

One of the most remarkable effects of ICSS is its ability to reverse the detrimental effects caused by lesions in brain areas related to learning and memory. For instance, ICSS fully reverses the damaging effects of a complete BLA lesion in a 2-AA task (Segura-Torres et al., 2010). Remarkably, rats with BLA lesions, when treated with ICSS, not only outperformed control rats, but also maintained this enhanced performance for ten days. Moreover, ICSS counteracts the damaging effects of bilateral LA lesions in a 2-AA task (Kádar et al., 2014) or the memory disturbances of an Alzheimer's disease rat model in the MWM (Puig-Parnau et al., 2023). Similar results have been reported regarding lesions in the fornix (Yoganarasimha & Meti, 1999).

Additionally, MFB-ICSS has proven beneficial for both implicit and explicit learning tasks. Post-training ICSS improves accuracy of performance in a T-maze (Soriano-Mas et al., 2005) and in a MWM task (Chamorro-López et al., 2015; García-Brito et al., 2020; Ruiz-Medina et al., 2008). Regarding non-emotional implicit tasks, post-training ICSS also improves the acquisition and retention of a visual-discrimination task (García-Brito et al., 2017).

Both pre- and post-training ICSS positively affect the acquisition of emotional implicit tasks, but different mechanisms are suggested to underlie their positive effects. On the one hand, post-training ICSS reinforces the association between stimuli and response by strengthening the synaptic connections previously created. On the other hand, pre-training ICSS increases general arousal and alertness, which could facilitate the accelerated learning of the associations required by the task.

Thus, administration of ICSS improves conditioning of the 2-AA task both before (Segura-Torres et al, 1988) and after the training (Segura-Torres et al., 1991).

Moreover, ICSS administered after one acquisition session improves the retention of a 2-AA task tested 48 h afterwards (Aldavert-Vera et al., 2013). This treatment also equalizes the performance of poor and good learners in a 2-AA task (Aldavert-Vera et al., 1996), and facilitates the acquisition of a 2-AA task in 18-month old rats, although this facilitation was not maintained long-term (Aldavert-Vera et al., 1997). Moreover, ICSS administered non-task contingent also facilitates the performance on the acquisition and retention sessions of a 2-AA task and their effects are long-lasting (Huguet et al., 2020).

Interestingly, ICSS administered immediately after two extinction sessions of a 2-AA task improved the extinction of the avoidance response of males in the second session, although these effects were dissipated when tested in a spontaneous recovery test at 28 days later (Huguet et al., 2017).

3.4 ICSS effects at a molecular level

The observed effects of ICSS on learning and memory suggest that post-training and non-contingent MFB-ICSS enhance both implicit and explicit task learning, reduce performance disparities between poor and good learners and restore learning and memory functions following damage to specific brain areas. These outcomes indicate that ICSS could enhance memory through molecular mechanisms beyond simply increasing arousal levels.

MFB-ICSS increases levels of several neurotransmitters, including NA, DA, Glu as well as activity of the enzyme acetylcholinesterase (AChE) in the HPC (Shankaranarayana Rao et al., 1998), potentially strengthening synapses. Additionally, the generalized arousal state induced by MFB-ICSS (Nieh et al., 2016; Wright & Craggs, 1977) can activate a wide range of brain structures due to its connection between the forebrain, midbrain and hindbrain (Bielajew & Harris, 1991). Considering that adequate arousal states positively modulate memory

acquisition and consolidation (Cahill & McGaugh, 1998), ICSS could enhance memory through this mechanism.

MFB-ICSS has also been shown to promote structural changes in plasticity-related areas, such as the hippocampus. This is made evident by faster and better learning in a spatial Morris water maze, increased size and branching complexity of apical CA1 dendritic arborization, and enhanced spine density in the CA1 field (Chamorro-López et al., 2015). Moreover, MFB-ICSS facilitation of avoidance response was accompanied by an increase in mossy fiber sprouting in the oriens stratum of CA2 and CA3 (Huguet et al., 2017). This suggests that ICSS reactivates specific neurons by strengthening the synapses and increasing spine sizes formed during learning of each task.

In addition to morphological plasticity, ICSS seems to enhance other parameters of structural changes, namely neurogenesis. Three-day MFB-ICSS treatment increases cell proliferation in the hippocampal DG of mice and rats (Takahashi et al., 2009), while 10-day MFB-ICSS increases the number of DCX-positive cells in the granular layer of the DG (Huguet et al., 2020).

Moreover, MFB-ICSS influences hippocampal gene expression, upregulating the expression of c-Fos in CA3 and DG, as well as the expression of 62 hippocampal genes at 70 min after a single ICSS session. Importantly, 10 of the upregulated genes, including *Cart*, *Adcyap1*, *Sgk*, *Ret* and *c-Fos* are related to either learning and memory or neural plasticity (Huguet et al., 2009). Additionally, a microarray analysis showed that MFB-ICSS upregulated the expression of CREB-dependent synaptic plasticity related genes (*c-Fos, Arc, Bdnf, Ptgs-2, Crem* and *Icer*) in a time-dependent manner following administration (Kádar et al., 2013).

Taken together, these data support the hypothesis that MFB-ICSS influences the synaptic plasticity mechanisms, potentially contributing to enhanced learning and memory. For instance, post-training MFB-ICSS facilitates the 48 h retention in a 2-AA task and increases c-Fos and Nurr1 positive cells in several brain regions (Aldavert-Vera et al., 2013). Similarly, ICSS-treated animals that perform

significantly better in the retention of a 2-AA task also show a significantly higher density of c-Fos positive cells in the retrosplenial cortex (Kádar et al., 2016). In addition, ICSS also promotes the expression of Arc-related synaptic plasticity response in several brain areas including the hippocampus, the retrosplenial cortex, the amygdala and the dorsolateral thalamus (Kádár et al., 2018).

In summary, ICSS modulates a wide variety of neural mechanisms at different levels, collectively contributing to its positive effects on learning and memory processes.

4. Sex and sexual hormones

4.1 Introduction

In recent decades, the inclusion of female rodents in research has brought to light important sex differences at genetic, physiological, developmental and functional levels. As a result, it is crucial to consider and study sex differences whenever possible.

However, before delving further, it is essential to clarify the distinction between the terms sex and gender. In human studies, these terms have often been used interchangeably. Gender refers to the social, environmental, cultural and behavioral constructs that define self-identity. While historically only two genders were distinguished, contemporary understanding acknowledges the existence of multiple genders. On the other hand, according to the National Institute of Health, sex is a biological component determined by sex chromosomes. Individuals with two X chromosomes are classified as female, while those with one X chromosome and one Y chromosome are classified as male. All sex differences emerge from this genetic inequality in sex chromosomes. Although other combinations of sex chromosomes exist and are worthy of further research (Clayton & Tannenbaum, 2016), they are beyond the scope of this work.

Specific genes located in the Y chromosome (*Sry*) or the X chromosome (*Xist*) promote differential development affecting genes, brain, cell functioning, and behavior. The presence of two (or more) X chromosomes initiates the expression of *Xist* from one X chromosome, leading to the silencing of that chromosome and resulting in only one active sexual chromosome. This mechanism serves to equalize the genetic load between females and males (Furlan & Galupa, 2022). Conversely, the Y-encoded *Sry* gene promotes irreversible and permanent masculine differentiation leading testis development. Thus, the presence or absence of *Sry* determines the sexual differentiation of the gonads (Arnold, 2017; McCarthy & Arnold, 2011).

Sex-specific hormones contribute to two types of differentiation. Firstly, they exert organizational effects by permanently and irreversibly defining tissues, sex-specific gonads and specific wiring patterns and brain structures development. Secondly, they have reversible activational effects by modulating physiology and brain function depending on their cyclic or sustained presence. Cycling hormones produce activational effects throughout the body (Sherwin & Henry, 2008).

As discussed, sex and sex hormones play a key role in development and cell functioning, potentially impacting cognition and emotional responses. Therefore, it is imperative for research in neuroscience to account for these factors in order to yield reliable results with high translational power.

4.2 Estrous cycle

Female rodents, much like women, experience cyclic expression of sex-specific hormones known as the estrous cycle. It is crucial to grasp the fundamental characteristics of this cycle, as scientific literature highlights its significant impact on different levels of behavior and cognition studies.

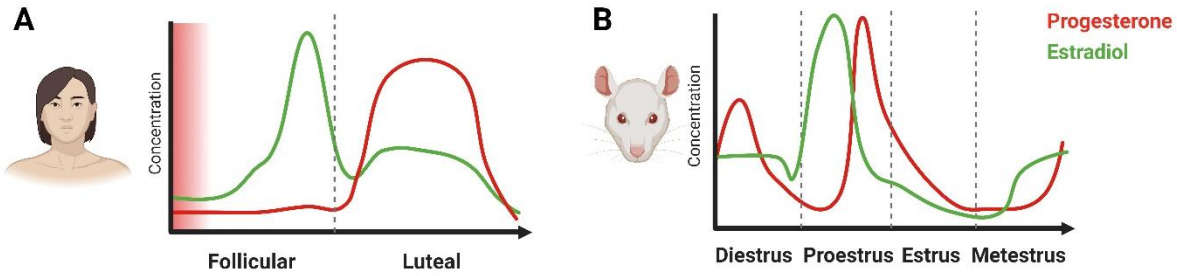


Figure 14. Menstrual and estrous cycle. **A**. Hormone fluctuations during the menstrual cycle. The menses are depicted with a red gradient. **B**. Hormone fluctuations during the rat estrous cycle.

Sex hormone levels are regulated by the hypothalamic-pituitary-gonadal axis. It starts with the hypothalamus releasing Gonadotropin-releasing hormone (GnRH), which stimulates the pituitary glands gonadotroph cells to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Buffet et al., 1998). During the follicular phase, estradiol levels peak and trigger LH and FSH release, leading to ovulation. During the luteal phase, LH and FSH decrease while estradiol moderately increases again along with progesterone. Menstrual bleeding or menses occurs

between the end of the luteal phase and the beginning of the next follicular phase, when all hormone levels are low (Dorsey et al., 2021; Mihm et al., 2011). Women have a short period of high sexual fertility after the follicular phase, known as estrous (Gangestad & Thornhill, 2008).

In rodents, these cyclic hormonal and anatomical changes are referred to as the estrous cycle. The average length of the estrous cycle in female rats is 4 days (Mandl, 1951), making rats an ideal model for studying these changes. The rodent estrous cycle can be divided into four distinct phases; estrus, proestrus, diestrus and metestrus (see Figure 14B). Ovulation occurs at the beginning of the proestrus stage and lasts until the end of the estrus stage. Prolactin, LH and FSH are low throughout the estrous cycle and increase in the afternoon of proestrus. Estradiol gradually increases starting in the metestrus, peaks during proestrus, and returns to baseline levels in estrus. Progesterone follows estradiol patterns, increasing during metestrus and peaking at the end of the proestrus (Marcondes et al., 2002; Smith et al., 1975; Sportnitz et al., 1999).

Men also experience cyclic hormone fluctuations, mainly regulated by testosterone. However, their cycles are shorter, fluctuating throughout the day (Barberia et al., 1973). In addition, men have lower estrogen and progesterone levels compared to women (Macdonald et al., 1979).

4.3 Determination of the estrous cycle

The estrous cycle in rodents can be determined through direct or indirect measurements. Direct measurement involves blood collection to assess hormone levels in blood (Florido et al., 2021). In males, blood collection can also be used to assess testosterone levels. A widely used indirect method is vaginal smear cytology, which determines the stage of the estrous cycle by analyzing the proportion of different types of cells in the smear. For males, an indirect measurement of testosterone levels changes is the confrontation tube test, which determines social dominance, correlating with testosterone levels (Florido et al., 2021).

Vaginal smear cytology involves observing four types of cells: neutrophils or leukocytes, small nucleated epithelial cells, large nucleated epithelial cells, and anucleated keratinized epithelial cells. The stage of the estrous cycle can be determined based on the proportion of these cells (see Figure 15). Neutrophils are very small, round and fine, making them likely to be ruptured during collection. Small nucleated epithelial cells are nonkeratinized, small, round or oval, with a high nuclear to cytoplasmic ratio. Large nucleated epithelial cells are round to polygonal, can have irregular or angular borders and a lower nuclear to cytoplasmic ratio. Anucleated keratinized epithelial cells lack a nucleus and have angular edges, forming elongated structures called "keratin bars".

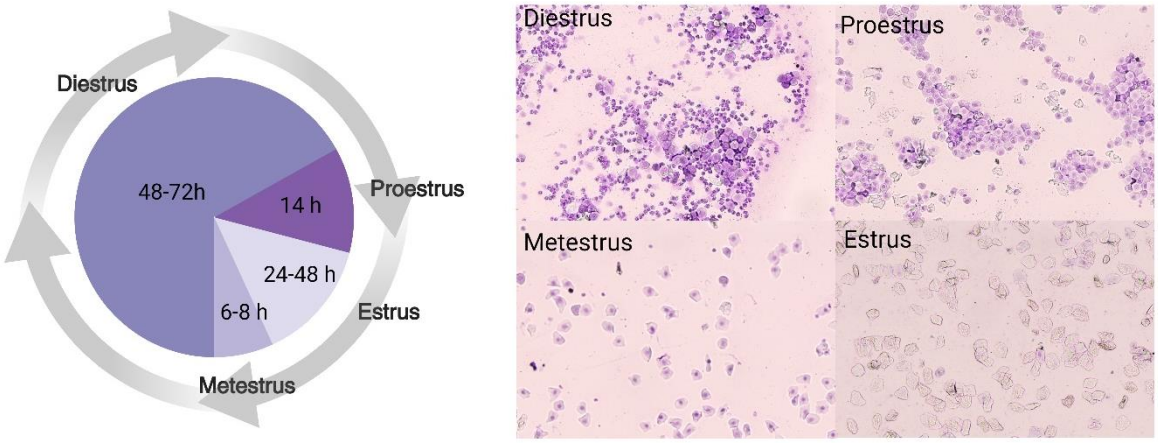


Figure 15. **Phases of the estrous cycle**. **Left**. illustration depicting the four phases of the estrous cycle along with their approximate duration in rats. **Right**. Pictures showing the type of cells observed in each phase of the rat estrous cycle.

In rats, proestrus lasts an average of 14 h in rats and it is characterized by the presence of small nucleated epithelial cells with uniform size that tend to form cohesive clusters (also called grape clusters). Estrus lasts between 24 to 48 h and it is characterized by the presence of anucleated keratinized epithelial cells. Metestrus lasts between 6 to 8 h, and it is characterized by the presence of anucleated keratinized epithelial cells and neutrophils. Finally, diestrus is the longest phase ranging from 48 to 72 h and it is characterized by low amounts of anucleated keratinized epithelial cells, along with a combination of neutrophils and small and large nucleated epithelial cells (Cora et al., 2015).

4.4 Estrogen effects on cognition and behavior

Sex steroids are hormones produced by the sexual glands (ovaries or testes) and include estrogens, androgens and progestogens. Estrogen, discovered as a female sex hormone by Marrian and Butenandt (1931), has three major forms of endogenous estrogens (estrone (E1), 17β-estradiol (E2), and estriol (E3)), with 17β-estradiol (E2) being the most potent and prevalent form of estrogen found in non-pregnant women. The effects of estrogens are mediated by two types of receptors, ERα and ERβ, which are widely expressed throughout the brain, in areas such as the amygdala, the hippocampal formation, cerebral cortex, basal forebrain, cerebellum, locus coeruleus, midbrain raphe nuclei, glial cells and central gray matter (Ciocca & Vargas, 1995; Gasbarri et al., 2012; Sherwin & Henry, 2008). This widespread distribution could explain estrogen's influence on emotion, well-being, and cognition.

Hormonal changes during the menstrual and estrous cycle affect various other aspects beyond the anatomy of the gonads and the activation of hormonal-specific pathways. These changes impact physiology, cognition, and mood, although some studies have failed to detect these differences (Zeng et al., 2023). Research methods to assess the effects of estrogen on behavior and cognition include: (1) tracking variables across natural estrous cycle stages, (2) administering estradiol to ovariectomized (OVX) rodents for hormone replacement therapy, and (3) studying aging animals in menopause, which can also be mimicked using aging OVX rodents (Spencer et al., 2008).

Estradiol's effects on cognition vary depending on the brain region involved in each task. It can impair striatum-dependent tasks (Davis et al., 2005; Korol, 2004) and facilitate prefrontal-dependent tasks (Luine et al., 2003; Rapp et al., 2003) or hippocampus-dependent tasks (Daniel et al., 1997; Korol & Kolo, 2002; Lacreuse et al., 2002; Li et al., 2004; Luine et al., 1998; Rapp et al., 2003; Sandstrom & Williams, 2004; Xu & Zhang, 2006). Moreover, impairments on object recognition memory in OVX rats, a task dependent on the prefrontal cortex and the hippocampus (Luine et al., 2003; Wallace et al., 2006), are reversed with estradiol replacement (Korol,

2004). In a plus maze task, rats in proestrus show hippocampus-dependent strategies, while those in estrus use striatum-dependent strategies (Korol et al., 2004). Similarly, verbal memory performance in women fluctuates across the menstrual cycle and positively correlates with estrogen levels (Rosenberg & Park, 2002).

Furthermore, estrogen's effects on cognition involve multiple systems and levels, including changes in neuroanatomy, excitability, neurotransmitter release and signaling pathways functionality. Estrogen directly affects the hippocampus, promoting dendritic plasticity and increasing the density of dendritic spines in hippocampal pyramidal neurons, which in turn improves performance of hippocampus-dependent tasks. This effect is evident in OVX rats showing decreased density levels of dendritic spines in the CA1 stratum radiatum, which was restored by estradiol replacement (Gould et al., 1990; Wolley & McEwen, 1992; Woolley et al, 1990). Additionally, rats in late proestrus display 30% higher spine density in CA1 and more "mushroom" shaped spines, compared to rats in late estrus (González-Burgos et al., 2005). Estrogen also enhances CA1 neurons excitability, promoting LTP (Spencer et al., 2008). Furthermore, estrogen influences the activity of neurotransmitters systems in both humans and animals, including Ach (Norbury et al., 2007), catecholamines, serotonin (Amin et al., 2006), and gamma-aminobutyric acid (GABA) (Spencer et al, 2008) as their receptors are distributed extensively throughout the brain (Gasbarri et al., 2012; Spencer et al., 2008).

Testosterone also plays an important role in cognition, seemingly improving task performance. Circulating levels of testosterone predict performance of visuospatial tasks (Cartier et al., 2024; Falter et al., 2006; Goyette et al., 2012; Kocoska-Maras et al., 2011; Moè, 2011), and a single dose of testosterone significantly improves visuospatial ability in women (Aleman et al., 2004). Perhaps even more strikingly, levels of testosterone found in patients with dementia (Bowen et al., 2000) and in men with Alzheimer's disease (Hogervost et al., 2001) are lower in comparison to healthy controls.

4.5 Sex differences on fear and anxiety

Anxiety and fear-related disorders affect women and men disproportionately. Studies suggest that women are twice as likely as men to suffer from PTSD, with an estimated lifetime prevalence of 7.8% (Breslau, 2009; Kessler et al., 1995; Kinrys & Wygant, 2005). Moreover, women have double the probability of experiencing panic attacks and social phobias (Gradus et al., 2017; Jonge et al., 2016; Steel et al., 2014; Wardenaar et al., 2017). These disparities in the prevalence of mental disorders may stem from complex interactions among neurobiological, physiological and sociocultural factors (Tolin & Foa, 2006).

One potential factor contributing to these differences is sex hormones. Research indicates that estradiol significantly impacts the emotional processing of fear-related experiences (Zeidan et al., 2011), a topic that will be explored below.

4.5.1 Non-conditioned fear and anxiety

As previously mentioned, women are more prone to anxiety disorders compared to men, highlighting the importance of using rodent models to better understand these sex differences (Toufexis et al., 2006).

Studies using rodent models have revealed that females tend to show less fear and anxious behaviors than their male counterparts (Fernandes et al., 1999; Johnston & File, 1991; Steenbergen et al., 1990). For instance, female rats spend more time in the open arms of the EPM in comparison to male rats, suggesting lower anxiety levels in females (Johnston & File, 1991).

Estradiol has proven therapeutic effects on anxiety symptoms (Arpels, 1996; Best et al., 1992), and the influence of sexual hormones has also been explored in research. Interestingly, the tendency of female rats to exhibit more or less fear- and anxiety-like symptoms seems to depend on the stage of the estrous cycle. Rats in proestrus and estrus spend more time in the open arms of the EPM compared to those in diestrus and metestrus (Díaz-Veliz et al., 1997). Similarly, in a study by Marcondes et al., (2001), female rats in proestrus spend significantly more time in

the open arms compared to those in diestrus. Thus, higher hormonal levels positively impact anxiety levels by decreasing them. Moreover, estradiol treatment reverses these effects in rats in diestrus, achieving similar levels as those in proestrus. Additionally, OVX rats treated with estrogen spend more time in the center of the OF arena compared to untreated OVX and OVX rats treated with both estrogen and progesterone (Hiroi & Neumaier, 2006). This suggests that estrogen reduces anxiety, while progesterone opposes this effect. Furthermore, administration of 17β-estradiol in the dorsal hippocampus of OVX rats decreases anxiety and depressive behavior in standard laboratory tests, such as the open field test, the elevated plus maze and the forced swim test (Walf & Frye, 2006).

The effects of sexual hormones on emotional processing may stem from their ability to modulate neurotransmitters synthesis and/or their influence over receptor activity involved in anxiogenic and anxiolytic actions (Genazzani et al., 2007).

4.5.2 Conditioned fear

Female and male rats exhibit differences in fear conditioning levels during classical conditioning tasks. In contextual FC, findings are mixed. Some studies report reduced contextual FC in females, indicated by decreased freezing during retrieval tests (Clark et al., 2019; Daviu et al., 2014; Gresack et al., 2009; Maren et al., 1994; Ribeiro et al., 2010; Wiltgen et al., 2001), while others find stronger contextual FC in females (Keiser et al., 2017; Moore et al., 2010). However, results from cued FC fail to report sex differences (Baker-Andresen et al., 2013; Baran et al., 2009; Chen et al., 2014; Clark et al., 2019; Fenton et al., 2014; Maes et al., 2002; Markus & Zecevic, 1997). The only notable difference observed is that female rats perform more active behaviors, such as darting response, during test sessions (Colom-Lapetina et al., 2019; Gruene et al., 2015a).

Sexual hormone levels results are more cohesive. In naturally cycling females, those in proestrus, characterized by high levels of E2, show lower levels of context FC (Cushman et al., 2014), although these findings are not always replicated (Keiser et al., 2017). Moreover, ovariectomy (OV) increases contextual fear conditioning and

estrogen replacement reverses these effects (Gupta et al., 2001). The effects of estradiol are dose-dependent, with long-term estrogen treatment enhancing contextual FC in OVX mice. Interestingly, this effect is associated with increased corticotropin-releasing hormone mRNA expression in the CeA (Jasnow et al., 2006). Mixed results are found regarding cued FC, with some studies showing no effects of hormones (Chen et al., 2014; Milad et al., 2009b) and others reporting enhanced cued fear response with estradiol administration in OVX mice (Jasnow et al., 2006). Moreover, gonadectomy increases cued fear expression in males which is reversed by testosterone administration (Chen et al., 2014).

In terms of the extinction of fear memories, females tend to express more fear during extinction in comparison to males, showing resistance to extinction (Baker-Andresen et al., 2013; Baran et al., 2009; Fenton et al., 2014), which is related to persistent PrL activity (Fenton et al., 2014). Moreover, females exhibit more spontaneous recovery in cued and contextual fear (Fenton et al., 2014; Fenton et al., 2016; Matsuda et al., 2015). These sex-dependent differences on fear extinction could be due to gonadal hormones. Impaired extinction is observed in female rats during metestrus, when estrogen and progesterone levels are low (Milad et al., 2009b), and during diestrus (Blume et al., 2017). Other studies report improved extinction of female rats in proestrus (Rey et al., 2014; Gruene et al., 2015b). Moreover, estrogen and progesterone injections before extinction facilitate extinction consolidation of rats in metestrus, whereas the blockade of estrogen and progesterone receptors during proestrus disrupts extinction consolidation (Milad et al., 2009b). Similarly, immediate post-extinction ERβ agonist administrations facilitate the extinction of a fear conditioning task (Zeidan et al., 2011). Along the same lines, ERβ agonist administration enhances extinction of contextual fear conditioning in OVX rats (Chang et al., 2009). Another study found that estradiol administration during cued fear extinction enhances fear extinction recall, while the effects of progesterone vary depending on the time of the administration in relation to the extinction session (Graham & Daher, 2016). In males, the stimulation of testosterone and estradiol release by a GnRH agonist enhances extinction recall 24 h later, whereas the inhibition of estradiol synthesis impairs fear extinction (Maeng et al., 2017).

Taken together, these findings suggest a protective role of sexual hormones in mitigating fear responses.

4.5.3 Avoidance behavior

There are striking differences between sexes in operant conditioning tasks. Female rats tend to acquire active avoidance behavior faster than males (Beatty & Beatty, 1970; Denti & Negroni, 1975; Scouten et al., 1975), while males typically outperform females in passive avoidance tasks (Denti & Epstein, 1972). In both one- and two-way active avoidance tasks, females learn to escape sooner than males during the initial trials of the task (Dalla et al., 2008; Shors et al., 2007), with some males failing to learn the task altogether (Dalla et al., 2008). These differences in performance of operant tasks could be due to different factors.

Firstly, increased movement during footshock presentation may lead to quicker avoidance responses. Female rats are more active in comparison to male rats, as shown in the OF test and in running wheels (Beatty & Fressler, 1976; Dawson et al., 1975; Hyde & Jerussi, 1983; Slob et al., 1981). On the other hand, testosterone in males might serve as an anxiolytic promoting passive strategies in response to a footshock (Toufexis et al., 2006). In addition, when female rats are treated with testosterone during adulthood, they perform male-like responses (Beatty & Beatty, 1970). Secondly, the strategy used by each sex could be a determining factor for operant learning. Female rats respond actively to aversive stimulation, whereas male rats respond passively by freezing (Beatty & Beatty, 1970; Kirk & Blampied, 1985; Steenberg et al., 1990; van Haaren et al., 1990). Thirdly, pain and nociception could influence animals' responses and adaptation to the environmental cues. Female rats are more sensitive to shocks than male rats (van Haaren et al., 1990; Kosten et al., 2005), although in some studies the higher intensities are enough to dilute these differences (Beatty & Beatty, 1970). Last but not least, hormonal levels may contribute to female and male performance differences in active avoidance paradigms, although findings are mixed. Some studies suggest that mean avoidances are facilitated during diestrus and disrupted during proestrus, and that OVX rats outperform intact females (Díaz-Veliz et al., 1989). Others, however, show that

female rats perform better than male rats in a 2-AA task when they are in proestrus (Sfikakis et al., 1978). Moreover, low administration doses of E2 on OVX rats impair the acquisition of active avoidance behavior (Díaz-Veliz et al., 1991; Singh et al., 1994), and estradiol administration reverses this impairment (Singh et al., 1994). Interestingly, gonadectomy in adulthood does not prevent the observed sex differences in operant conditioning (Beatty & Beatty, 1970; Scouten et al., 1975; Dalla et al., 2008). Other studies have shown a facilitation of operant conditioning learning by castration of adult males (Dalla et al., 2008).

In terms of extinction of active avoidance, female rats tend to exhibit slower extinction in comparison to males (Beatty & Beatty, 1971; Ikard et al., 1972; Van Haaren et al, 1990). Sexual hormone levels also influence the extinction of operant conditioning. Estrogen replacement in OVX female rats delayed extinction in a one-way avoidance task (Telegdy & Stark, 1973), while estrogen and testosterone administration in male rats facilitated extinction in a shuttle passive avoidance task (Rivas-Arancibia & Vazquez-Pereyra, 1994).

The diversity of these findings may be attributed to the effects of sexual hormones, as well as the neural circuits underlying behavioral expression of fear (Bauer, 2023).

4.6 Sex and orexins

The expression of hypocretins is sex dimorphic, with female rats showing higher levels of *prepro*-hypocretin mRNA in comparison to male rats (Jöhren et al., 2002). This leads to elevated concentrations of Ox-A, specifically in the lateral and posterior hypothalamus of female rats (Taheri et al., 1999; Dorsey et al., 2021). Moreover, the orexinergic system is sensitive to estradiol levels, since estradiol increases c-Fos expression in orexin neurons in the hypothalamus, promoting arousal and wakefulness (Deurveilher et al., 2008). Additionally, the production of orexins and its receptors fluctuates across the estrous cycle of female rats, with peak levels of Ox-A, Ox-B and OX1R protein expression during the proestrus phase, when progesterone and estradiol concentrations are highest (Porkka-Heiskanen et al., 2004; Silveyra et al., 2007). Notably, OVX female rats exhibit lower OX1R levels in

the hypothalamus, which are restored upon estradiol administration (Silveyra et al., 2007, 2009). These fluctuations in the orexinergic system throughout the estrous cycle impact arousal, feeding, reward processing and stress response (Kim et al., 2023). Understanding these fluctuations is essential for developing effective treatments in clinical settings.

Furthermore, male hormones also influence Ox signaling. Gonadectomy reduces OX1R expression in the anterior hypothalamus, whereas testosterone administration restores receptor expression (Silveyra et al., 2009).

4.7 Sex and ICSS

Mixed findings exist regarding the rate of response to ICSS between female and male rats. One early study found that male rats performed more response rates in comparison to females (Velley & Cardo, 1977). However, another study found the opposite result, with females showing higher response rates (Cohen & Lieblich, 1981). Thus, no solid conclusions can be reached (Stratmann & Craft, 1997).

It seems that sexual hormones' cyclic changes modulate the neural circuits underlying ICSS behavior. The female sexual hormones tune the mesolimbic DA system' activity to naturally rewarding stimuli (Becker, 1999). Specifically, estrogen induces changes in neuronal excitability by acting on membrane receptors in striatal GABAergic neurons and DA terminals, leading to increased DA release (Becker, 1999). Interestingly, ICSS behavior varies throughout the estrous cycle, with response rates peaking when estrogen levels are high, particularly during the night between the proestrus and the estrous stages (Meyerson et al., 1969; Prescott, 1966; Quadagno et al., 1972; Steiner et al., 1980). In fact, the frequency of responses per session is the best predictor of changes related to the estrous cycle (Steiner et al., 1982). These estrogen-induced effects on ICSS behavior may involve an enhancement of the rewarding value of the electrical stimulation or an increase in general motor activity (Bless et al., 1997).

HYPOTHESIS AND OBJECTIVES

1. Hypothesis

Orexins play an important role in emotional processes and emotional memory, and OX1R has been proven to be implicated in the acquisition and extinction of classical fear-related paradigms. Differences between male and female rats have been described in classical and instrumental conditioning of emotional responses. Therefore, we hypothesize that OX1R could also be involved in the acquisition and extinction of active avoidance behavior in a sex-dependent manner.

2. Objectives

2.1. General objective

The general objective of the thesis is to study active avoidance behavior, focusing particularly on the involvement of the orexinergic system - specifically, OX1R receptors - and sexual differences, as well as explore a potential facilitating treatment of the extinction behavior.

2.2. Specific objectives

1. To explore sexual differences in the acquisition, retention, and short and long-term extinction of two-way active avoidance conditioning (2-AA) (**Experiment 1**).
2. To study the effects of OX1R blockade, through intracerebroventricular administration of SB334867, on the acquisition of 2-AA conditioning in male (**Experiment 2**) and female rats (**Experiment 3**).
3. To examine the effects of SB334867 administration (icv) on the activation of the brain's extinction circuit by c-Fos protein expression following the spontaneous recovery test conducted 28 days after extinction, in male and female rats (**Experiment 4**).
4. To determine whether the estrous cycle influences the acquisition and extinction of 2-AA conditioning (**Experiment 5**).
5. To compare the effects of icv administration of SB334867 on the extinction of 2-AA conditioning between sexes (**Experiment 6**).

6. To assess the effect of icv administration of SB334867 on several non-conditioned emotional tests, in males and females (**Experiment 7**).
7. To investigate whether the stimulation of the brain's reinforcement system through ICSS could potentially enhance the extinction of 2-AA conditioning in those subjects who exhibit slower extinction, as typically observed in female rats (**Experiment 8**).

MATERIALS AND METHODS

In this chapter, a common methodology used in all experiments with particular specifications for each of them is presented.

1. Animals

All the animals used in the experiments of this thesis were sexually mature adult Wistar rats (between three and six months old) from our laboratory stock. The rat colony is renewed once a year to avoid distancing from the genetic line. Animals were kept under conditions of controlled temperature (20-24 ºC) and humidity (40-70%), and subjected to an artificial 12-hour light/dark cycle (light on at 08:00). All behavioral tests took place during the first 6 h of light. All subjects were kept in an ad libitum regime of food and water. All procedures were carried out in compliance with the Directive 2010/63/EU and were approved by the institutional animal care committee 10515.

2. Stereotactic surgery

Stereotaxic surgeries were performed to chronically implant either a cannula or an electrode, or both. Under general anesthesia of 5% isoflurane (IsoFlo (Aesica Queenboough Limited, Kent, United Kingdom)) and maintained at 2.5% isoflurane. All rats received a subcutaneous injection of 2mg/kg Metacam ® (Boehringer Ingelheim, Kiel, Germany) during the intervention. Once the cannula or/and electrode were placed, they were anchored to the skull jeweler's screws and dental cement. After the stereotaxic procedure, animals were weighed and handled daily during the post-surgery recovery period (7 days).

2.1 Cannula implantation

Rats were chronically implanted with a 7.5 mm infusion guide cannula (Plastics One®, Raonoke, VA, US, purchased through Bilaney consultants, Düsseldorf, Germany; ref: C315G/PK/Spc) into the left lateral ventricle (LV), according to coordinates from the stereotaxic atlas of Paxinos and Watson at anterior: -0.7 mm from bregma, lateral: 1.6 mm and ventral: -4.0 mm. A dummy cannula (Plastics One®; ref: C315DCN/ Spc) filled the infusion guide cannula and expanded 0.5 mm into the parenchyma. The cannula was fixed in position using an auto-polymerizing acrylic resin. The cannula was anchored to the skull with jeweler's screws and dental cement.

2.2 Electrode implantation

In experiments using intracranial self-stimulation, rats were also chronically implanted with a monopolar stainless steel electrode (150 μm in diameter) aimed at the right LH into the fibers of the MFB, according to coordinates from the stereotaxic atlas of Paxinos and Watson, anterior: −1.8 mm from bregma, lateral: 2.0 mm (right hemisphere) and ventral: −8.5 mm with the cranium surface as the dorsal reference (Vila-Solés et al., 2022).

3. Intracerebroventricular injection of SB-334867

The selective OX1R antagonist SB334867 was dissolved in dimethyl sulfoxide (DMSO), aliquoted in tightly sealed vials and stored at -20ºC for up to 30 days. The SB group received a total volume of 2μL/5μL of SB-334867 and the Vehicle group received 2μL of 100% DMSO into the LV at a rate of 1μL/minute. At the time of infusion, animals were gently restrained with a cloth while their dummies were removed and replaced with a 26-gauge injector extending 0.5 mm below the cannula tip. The injector was connected by polyethylene tubing to a single 10μL syringe that was placed in the infusion pump. After finishing the infusion, the injectors were left in place for 1 minute in order to avoid regurgitation of the liquid when removing the injector (García-Brito et al., 2018).

4. Two-way active avoidance task (2-AA task)

4.1 Apparatus

The active avoidance conditioning was conducted in a 50 x 24 x 23 cm automated two-way shuttle-box (Shuttle Flex Fusion, AccuScan Instruments Inc. Columbus, Ohio, USA) enclosed in a sound-attenuating box ventilated by an extractor fan. The box was controlled by Fusion (v. 3.8) software in a training schedule of 1 min (± 10 s), in a no-door version. The CS was a 80dB and 1kHz tone of 3 s duration and a red light. The US was a 0.6 μA electrical footshock, presented for 10 s at maximum. Both stimuli ceased when the rat crossed to the other compartment, or after the total 13s of presence of CS and US. For the extinction and spontaneous recovery sessions, the

CS was presented in the absence of the US. Avoidance responses, latency of response, non-responses and intertrial crossings were recorded for further analysis.

4.2 Behavioral procedure

The habituation session of the 2-AA task consisted of placing the animal for 5 min into the Shuttle box without any stimuli to reduce emotional reactivity and increase familiarity of the learning environment. Locomotor behavior (intertrial crossings) was recorded for further analysis.

A distributed model of acquisition was performed using 10 trials per day for 5 consecutive days, or a massed model using 50 trials per day for 3 consecutive days. The retention sessions were identical as the acquisition sessions but consisted of 20 or 30 trials per session depending on the experiment. The performance of these sessions ensured the consolidation of acquisition learning to start the extinction process.

The extinction sessions consisted of 50 trials each, but without the presence of the US. A massed extinction protocol was used based on previous experiments (Tapias-Espinosa et al., 2014). The use of massed extinction sessions increases the difficulty of the extinction process, which is an advantage in studying the recovery of this learning. Finally, the spontaneous recovery test was performed 28 days after the last extinction session, and consisted of 50 trials without the US, identical to the extinction sessions.

During the acquisition, retention, extinction sessions and the spontaneous recovery test, the behavioral outcome was extracted for further analysis. Specifically, the variables mean avoidances, latencies of response, non-responses and intertrial crossings were analyzed.

5. Non-conditioned fear and anxiety tests

5.1 Elevated Zero Maze

5.1.1 Apparatus

The elevated zero maze was conducted in a 51 cm elevated circular arena with two closed arms (295 mm height) and two open arms. The animal behavior was analyzed by a video tracking system (Smart v3.0) (Fig.16).

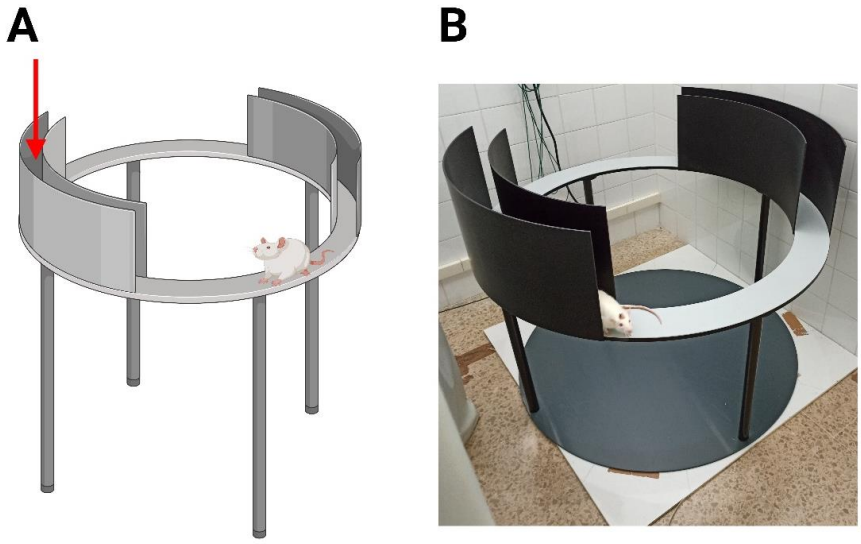


Figure 16. **Elevated Zero Maze**. **A**. Illustration of the elevated zero maze. Red arrow indicates the location in which the animal is placed at the beginning of the test. **B**. Picture of the apparatus used in this thesis.

5.1.2 Procedure

The procedure consisted of placing the animal in the apparatus for 5 min to analyze its behavior. The variables assessed were the following: percentage of time spent in the closed and open arms, rearings and groomings performed in the closed and open arms, headings (introduction of the head until the ears to the other compartment), transitions between the two compartments, and micturitions and defecations performed in the two compartments. One rearing was counted each time the rat stood up. If the animal stood up twice consecutively in the same spot, it was counted as one rearing behavior.

5.2. Light/dark test

5.2.1 Apparatus

The light/dark test was conducted in a box of Perspex with two compartments; a 20 × 31 × 24 cm black compartment with a 40 W red bulb above it and a 31 x 31 x 24 cm white compartment with a 100 W white bulb light above it. The two compartments were separated by a 100 x 100 mm gate. Both bulbs were 370 mm above the floor of the box. The animal behavior was analyzed by a video tracking system (Smart v3.0) (Fig.17).

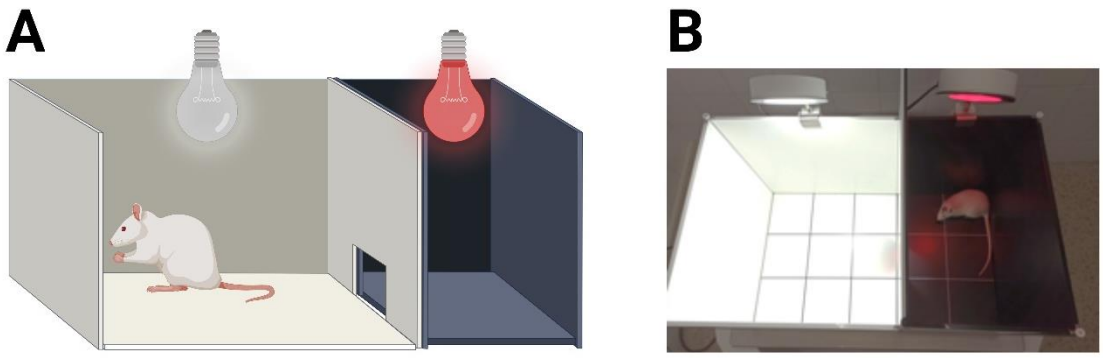


Figure 17. **Light/dark box**. **A**. Illustration of the light/dark box. The location of the rat depicts the place and position in which the animal was placed at the beginning of the test. **B**. Picture of the apparatus used in this thesis.

5.2.2 Procedure

The dark/light test consisted of placing the animal in the apparatus facing the extreme of the light compartment and allowing it to freely explore the box for 5 min. The assessed variables were the following: percentage of time spent in the light and dark zone, latency of the first entry to the dark zone (s), headings (introduction of the head until the ears to the other compartment), number of transitions performed between the two compartments, groomings, rearings, micturitions and defecations performed in the two compartments.

5.3 Open field

5.3.1 Apparatus

The open field test was conducted in a white circular arena of 80 cm of diameter, surrounded by 34 cm high walls (Fig.18). The animal behavior was analyzed by a video tracking system (Smart v3.0).

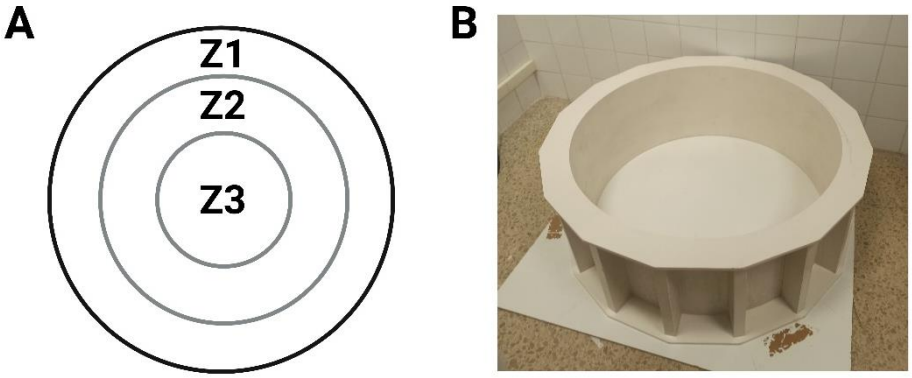


Figure 18. **Open field**. **A**. Illustration depicting the virtual zones used to assess subjects' behavior during the test. **B**. Picture of the open field apparatus used in this thesis.

5.3.2 Procedure

Rats were placed in the center of the arena and allowed to explore for 10 min. After each test, the box was cleaned and deodorized with 75% alcohol solution to avoid olfactory cues. The time spent in the three virtual zones was recorded and the number of rearings, groomings, micturitions and defecations were assessed.

5.4 Marble burying test

5.4.1 Apparatus

The marble burying test was conducted in a black wooden box (42 × 31 × 40 cm) filled with 5 cm height sawdust. Twenty marbles (12 mm of diameter) were located in 5 rows of four marbles maintaining equidistance between them (Fig.19).

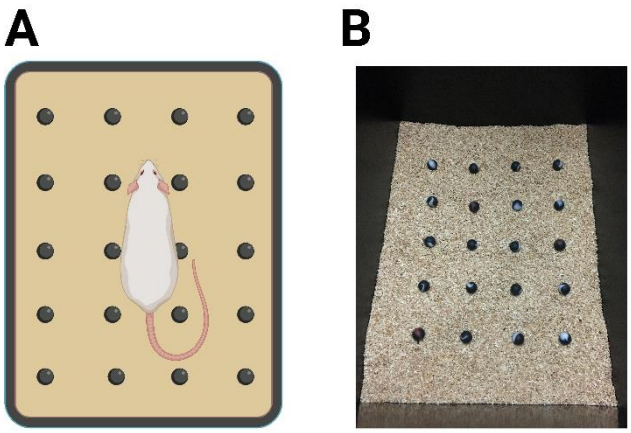


Figure 19. **Marble burying test**. **A**. Illustration depicting the marble burying test apparatus. **B**. Picture of the Marble burying apparatus used for this thesis.

5.4.2 Procedure

Rats were placed in the center of the box and allowed to explore for 20 min. The variables assessed were the following: number of marbles buried, rearings, groomings and latency to bury first marble. A marble was considered buried when a minimum of two thirds of the sphere was under the bedding material.

5.5 Freezing response to inescapable footshock

5.5.1 Apparatus

The freezing response to inescapable footshock was conducted in a 50 x 24 x 23 cm automated Shuttle-box (Shuttle Flex Fusion, AccuScan Instruments Inc. Columbus, Ohio, USA), enclosed in a sound-attenuating box ventilated by an extractor fan. The box was controlled by Fusion software in a no-door version.

5.5.2 Procedure

Subjects were placed inside the Shuttle box. After an initial period of habituation to the chamber of 60 s, a light and a tone of 1000 Hz appeared for 10 s. Immediately after their ceasing, an inescapable footshock of 0.7 mA appeared for 20 s. There was an ITI of 30 s between the four times that the appearance of the stimuli happened (Fig.20).

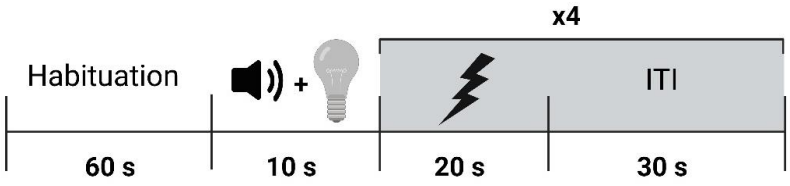


Figure 20. **Descriptive timeline of inescapable footshock test**. Abbreviations: ITI: intertrial interval.

6. Intracranial self-stimulation treatment

6.1 ICSS behavior shaping and optimal intensity search

Rats were taught to self-stimulate by pressing a lever in a conventional Skinner box (25 × 20 × 20 cm^3). Electrical brain stimulation consisted of 0.2 s trains of 50 Hz sinusoidal waves at intensities ranging from 40 to 180 μA.

After shaping ICSS behavior, rats performed an optimal intensity (OI) search session to establish the current intensity that would be applied to each rat during the treatment sessions. Increasing current intensities of 5 μA were applied every 2 min and the number of lever presses were recorded. OI was defined as the current intensity at which animals perform the maximum number of responses. For further details, see Vila-Solés et al. (2022).

6.2 ICSS treatment

ICSS rats were placed in a self-stimulation box and were free to press the lever to self-administer electrical stimulation at the previously established OI. The treatment finished once rats achieved 2500 trains of stimulation. Rats that did not receive ICSS treatment were placed in a Skinner box for 45 min. For further details, see Vila-Solés, et al. (2022).

7. Estrous cycle assessment

Vaginal cytologies were performed every day at the same time (between 12:00 pm and 13:00 pm) always after behavioral testing and at the same place. Samples were collected by introducing and rubbing a cotton swab into the vaginal entry and rubbed onto microscope slides. Violet cresyl staining was performed soaking the slides in 0.5% cresyl violet acetate stain solution for 5 min and two consecutive

baths on distilled water for 1 minute each. Pictures of the cytologies were captured at 10x using a BX-41 Olympus microscope attached to an Olympus DP-70 digital camera (Japan).

8. c-Fos study

8.1 Tissue collection

Thirty min after the end of the spontaneous recovery session, animals received a Dolethal (Vetoquinol, S.A, Lure, France) overdose (150 mg/Kg, i.p) and were transcardially perfused with a solution of 0.1M of phosphate buffer saline (PBS), pH 7.4, followed by a solution of 4% paraformaldehyde in PBS. Brains were then removed and post-fixed in 4% paraformaldehyde in PBS, then cryoprotected in 15% and 30% sucrose in PBS and stored at -80ºC (García-Brito et al, 2018).

8.2 Immunohistochemistry

Free-floating coronal sections of 30 µm were incubated with 0.3% H2O2 in Tris-buffered saline (TBS) for 30 min and in 1% Bovine serum albumin (BSA) in 0.3% Triton X-100 in TBS for 30 min. Sections were incubated in primary antibody rabbit anti-cFos (Santa Cruz Biotechnology Inc. Santa Cruz, CA, US; ref.: sc-166940) at 1:500 in 0.5% BSA in 0.3% Triton X-100 in TBS for 60 min at room temperature and then overnight at 4 °C. The next day samples were incubated with biotinylated goat anti-Mouse-IgG secondary antibody (Jackson Immunoresearch Inc., USA; ref.: 115-065-166) at 1:500 in 0.5% BSA in 0.3% Triton X-100 in TBS for 60 min and subsequently treated with Streptavidin-HRP (Pelkin Elmer ® Life Science Inc., USA) at 1:3600 in 0.3% Triton X-100 in TBS, followed by treatment with 3, 3′-Diaminobenzidine (DAB) solution (Vector ®) for 7 min. Negative controls without primary or secondary antibodies were included. See *Supplementary Material* section for the detailed immunohistochemistry protocol.

8.3 Image acquisition and analysis

Using a Zeiss microscope (Zeiss Group, Oberkochen-Germany) attached to a DSLR digital camera with a magnification of 10x (EOS_6D_Mark II, Canon Europe, Amsterdam, The Netherlands), microphotographs of the following brain regions

were captured: PrL, IL, medial septum (MS), diagonal band of Broca (DBB), BNST, dorsal and ventral hippocampus (dHPC and vHPC), and LA, CeA and BLA. The image analysis software Image-J 1.43 (http://rsb.info.nih.gov/ij/) was employed to bilaterally count the number of c-Fos immunostained nuclei using regions of interest (ROIs). See Figure 21 for the location of the ROIs.

Images were corrected for brightness and contrast parameters, and were treated with a Gaussian Blur filter. An appropriate gray threshold was set for each area. Finally, images were converted to binary and a Watershed filter was applied to facilitate cell counting where high concentrations of c-Fos immunopositive neurons were located. All these parameters were maintained for all subjects and they were adjusted according to the area studied. Images were analyzed automatically using the Macro function of ImageJ. Three histological sections were counted and averaged for all areas. The coordinates for each area were matched for each group in order to reduce variability between samples (AP: PrL and IL: 3.72 to 2.70, MS, DBB and BNST: 1.20 to -0.36, dCA1, dCA2, dCA3, DG, LA, BLA, and CeA: -2.52 to -3.60, and vCA1 and vCA3: -5.20 to -6.04).

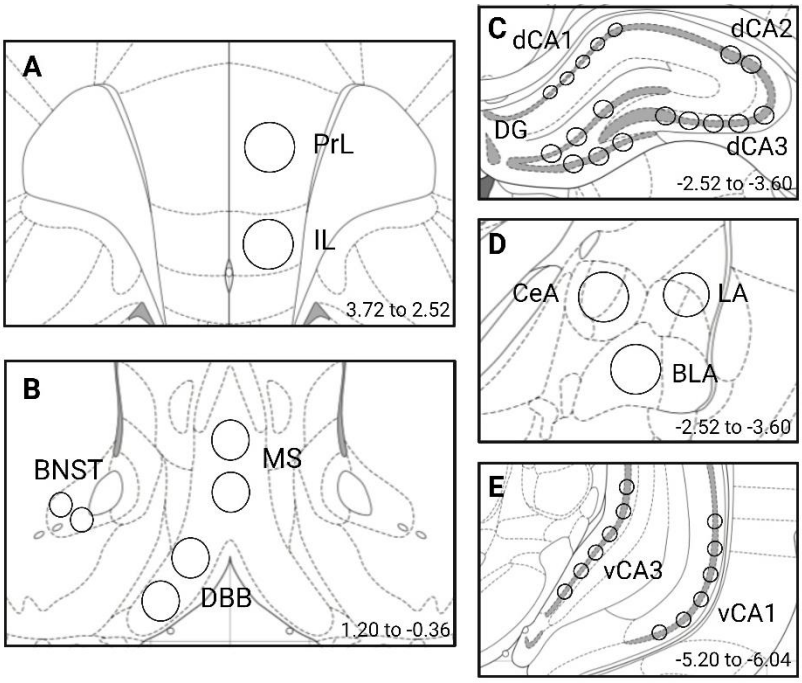


Figure 21. **Regions of interest (ROIs) for the quantification of c-Fos immunopositive nuclei**. **A**. PFC: (PrL and IL). **B**. Septal area: MS, DBB and BNST. **C**. dHPC: (dCA1, dCA2, dCA3, and DG). **D**. Amygdala (AMY): (CeA, LA and BLA). **E**. cHPC: (vCA1 and vCA3).

9. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 25. Normality analyses were performed using the Shapiro-Wilk normality test. For the analysis of 2-AA task performance, a GLM for repeated measures was conducted using factor GROUP, SESSION or BLOCK of the session, and GROUP × SESSION. In one experiment, the analysis of the slopes was performed. In experiments with more than two groups, contrasts analysis was performed to assess between which groups the differences existed. When two independent variables were used, a 2 × 2 GLM was conducted. For the analysis of two independent variables in a non-repeated measures model, a two-way ANOVA analysis was used to assess the factors SEX and SB for independent sample results. Mann-Whitney U test was used for non-parametric comparisons. A Breslow survival analysis was conducted to study the progression of the extinction curves of the experimental groups. Cohen's d effect size analysis was performed to study the effect of sexual hormones on behavior and neuronal activation when the sample size of each group was very low. Correlations between samples were estimated using the Spearman's correlation test. The relationship between areas analyzed by c-Fos immunopositive neurons for each sex was studied using a principal components analysis (PCA) with oblimin rotation. Statistically significant results were considered when $p < .05$ using 95% confidence interval.

RESULTS

STUDY I. Do males and females learn active avoidance behavior equally?

This section presents and discusses the results from Study I, aimed at addressing the first objective of this thesis. The goal was to establish a paradigm enabling the differentiation of avoidance behavior between males and females within our experimental conditions.

Experiment 1. Sex differences on the acquisition, retention, extinction and spontaneous recovery of a 2-AA task.

In experiment I, sex differences were assessed in a 2-AA task under the specific experimental conditions employed in our laboratory. This was done to corroborate the results found in the existing literature and to acknowledge the baseline behavior of our animals, to then modulate the action of OX1R.

Rats underwent a handling and a habituation session on day 1. Five acquisition sessions of 10 trials each were performed from days 7 to 11. A retention session consisting of 30 trials took place on day 14. Two extinction sessions, each comprising 50 trials, were conducted on days 15 and 16. Finally, 28 days after the last extinction session, animals underwent a spontaneous recovery test which involved 50 trials. The final sample consisted of 18 rats (Female= 9; Male = 9).

A comprehensive timeline outlining the behavioral procedure of this experiment is depicted in Figure 22.

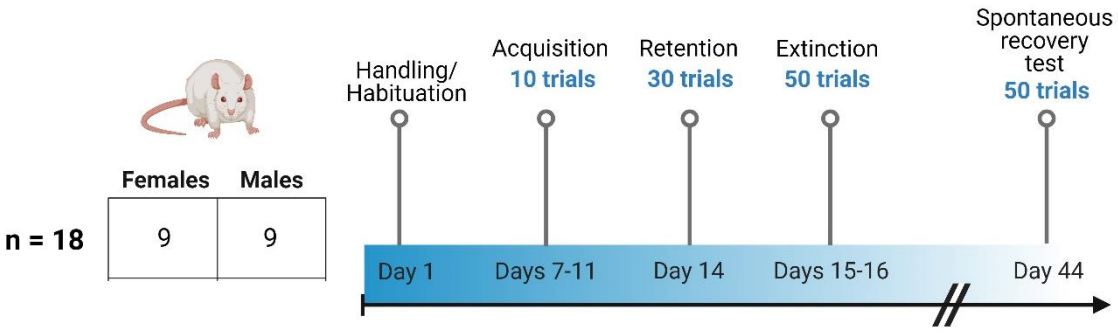


Figure 22. Timeline of the experimental procedure used in Experiment 1. Timeline of the experiment with every test performed. The number of trials performed in each session are depicted using blue letters.

The final sample consisted of 18 Wistar rats; 9 female rats with a mean age of 216.84 days (SD = 65.24) and a mean weight of 240.86 g (SD = 25.62) and 9 male rats with a mean age of 216.80 days (SD = 62.25) and a mean weight of 459.16 g (SD = 64.77), as shown in Figure 22.

1.1 Acquisition

Mean avoidances, latencies of response and non-responses during the acquisition sessions are depicted in Fig.23 A/D/G.

Regarding the mean avoidances, although factor SEX was significant ($F_{1,16}$= 12.322, P = .003), a significant interaction between SEX × SESSION was found ($F_{4,64}$= 4.925, P = .002) (Fig.23A). Simple effects study revealed differences between the two groups in A2 (P = .003), A3 (P = .028), A4 (P = .000) and A5 (P = .010), but not in A1, indicating that both groups started diverging from session two onwards (Fig.23A).

Female rats performed a higher number of avoidance responses during task acquisition compared to male rats. Latencies of response results supported the mean avoidances findings, with a significant effect of SEX ($F_{1,16}$= 10.017, P = .006) independently of the session (SEX × SESSION: $F_{4,64}$= 1.960, P = .141) (Fig.23D), revealing that female rats showed significantly lower latencies of response than male rats. Male rats exhibited a tendency to perform more non-responses ($F_{1,16}$= 3.642, P = .074), irrespective of the session (SEX × SESSION: $F_{4,64}$= 2.397, P = .100) (Fig.23G). Supporting this trend, significant differences between groups emerged in A3 (P = .045), indicating that male rats had more non-responses in this session compared to female rats.

1.2 Retention

Mean avoidances, latencies of response and non-responses during the retention session are depicted in Fig.23 B/E/H.

Analyzing mean avoidances, despite the factor SEX being significant ($F_{1,16}$= 8.098, P = .012), a significant interaction SEX × BLOCK was found ($F_{2,32}$= 4.503, P = .019), with differences observed between males and females specifically in R1.1 (P = .002) (Fig.23B). No differences were observed between groups in the later blocks of the retention. Regarding the latencies of response, factor SEX was significant ($F_{1,16}$= 6.946, P = .018) depending on the block of trials (SEX × BLOCK: $F_{2,32}$= 4.599, P = .018) (Fig.23E). Females showed lower latencies than males only in R1.1 (P = .004). Concerning non-responses, no significant differences between groups were found (SEX: $F_{1,16}$= 1.000, P = .332; SEX × BLOCK: $F_{2,32}$= 1.000, P = .332). This may be due to the low number of this type of responses in this phase of conditioning, as observed in Figure 23H.

1.3 Extinction

Mean avoidances, latencies of response and non-responses during the two extinction sessions are depicted in Fig.23 C/F/I.

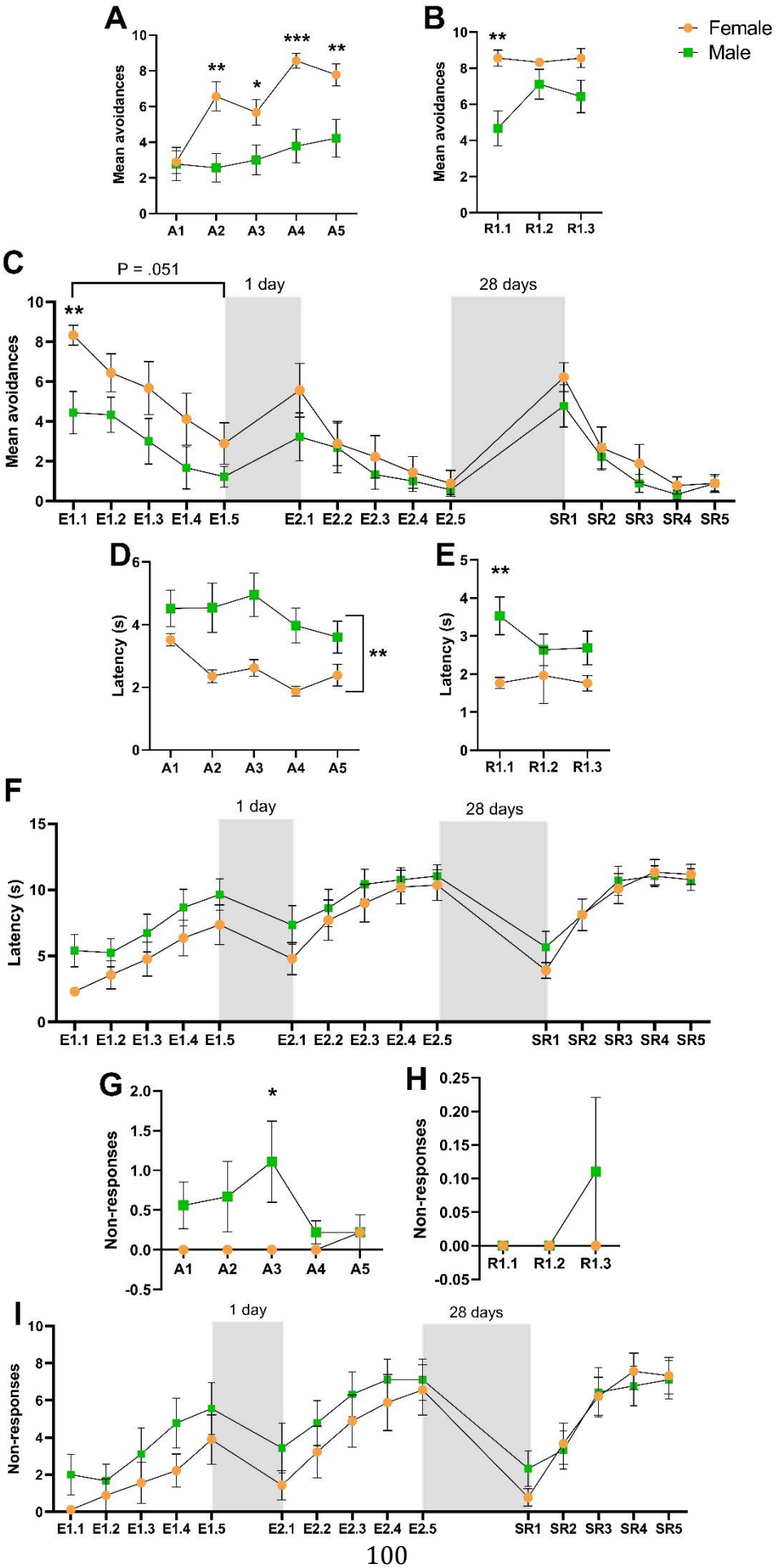

A
B
Female
Male
Mean avoidances
A1 A2 A3 A4 A5
R1.1 R1.2 R1.3
C
P = .051
1 day
28 days
E1.1 E1.2 E1.3 E1.4 E1.5 E2.1 E2.2 E2.3 E2.4 E2.5 SR1 SR2 SR3 SR4 SR5
D
E
Latency (s)
F
G
H
Non-responses
I

Figure 23. **Behavioral outcome from the 2-AA task performed in Experiment 1**. **A**. Mean avoidances for the five acquisition sessions (A1 to A5). **B**. Mean avoidances for the retention session divided into three 10-trial blocks. **C**. Mean avoidances for the two extinction sessions (E1 and E2) and for the spontaneous recovery test (SR) divided into five 10-trial blocks per session. **D**. Latencies of response for the five acquisition sessions. **E**. Latencies of response for the retention session divided into three 10-trial blocks **F**. Latencies of response for the two extinction sessions (E1 and E2) and for the spontaneous recovery test (SR) divided into five 10-trial blocks per session. **G**. Non-responses for the five acquisition sessions. **H**. Non-responses for the retention session divided into three 10-trial blocks. **I**. Non-responses for the two extinction sessions (E1 and E2) and for the spontaneous recovery test (SR) divided into five 10-trial blocks per session. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Data are expressed as mean ±SEM.

Regarding mean avoidances from the first extinction session (E1), the factor SEX showed a clear tendency toward significance ($F_{1,16}$= 4.432, P = .051) independent of the session ($F_{4,64}$= .924, P = .455). Specifically, females displayed a higher number of avoidance responses, reaching significance in E1.1 (P = .005). No differences between groups were found regarding latencies (SEX × BLOCK: $F_{4,64}$= .282, P = .764; SEX: $F_{1,16}$= 2.308, P = .148) (Fig.23F) and non-responses (SEX × BLOCK: $F_{4,64}$= .411 , P = .708; SEX: $F_{1,16}$= 1.706, P = .210)(Fig.23I).

No differences were found in the mean avoidances (SEX × BLOCK: $F_{4,64}$= 1.227, P = .305; SEX: $F_{1,16}$= .530, P = .477), latencies (SEX × BLOCK: $F_{4,64}$= .984, P = .389; SEX: $F_{1,16}$= .567, P = .462) (Fig.23F), and non-responses (SEX × BLOCK: $F_{4,64}$= .411 , P = .708; SEX: $F_{1,16}$= .701, P = .415) of the second extinction session (E2) (Fig.23I).

1.5 Intertrial crossings

Intertrial crossings performed in the acquisition, retention, extinction sessions and spontaneous recovery test are depicted in Fig.24 A-E.

No differences between male and female were observed in the intertrial crossings during the habituation session (t_{16} = .-.252, P = .804) (Fig.24A). In the acquisition sessions, differences between groups were observed ($F_{1,16}$= 10.583, P = .005) depending on the session (SEX × SESSION: $F_{4,64}$= 6.024, P = .004). Female rats performed more crossings in A4 (P = .002) and A5 (P = .000) (Fig.24A). In the retention session, female rats performed more crossings ($F_{1,16}$= 14.453, P = .002)

regardless of the block studied (SEX × BLOCK: $F_{2,32}$= 1.349, P = .274) (Fig.24B). In contrast, no differences were detected between males and females in the extinction sessions, either in E1 (SEX: $F_{1,16}$= 1.255, P = .279; SEX × BLOCK: $F_{4,64}$= .479, P = .562) (Fig.24C) or in E2 (SEX: $F_{1,16}$= 0.52, P = .823; SEX × BLOCK: $F_{4,64}$= .810, P = .524) (Fig.24D). Instead, a significant interaction SEX × BLOCK was found in the spontaneous recovery test ($F_{4,64}$= 3.651, P = .021; SEX: $F_{1,16}$= .859, P = .368), with females performing a higher number of crossings than males, specifically in SR1 (P = .026) (Fig.24E).

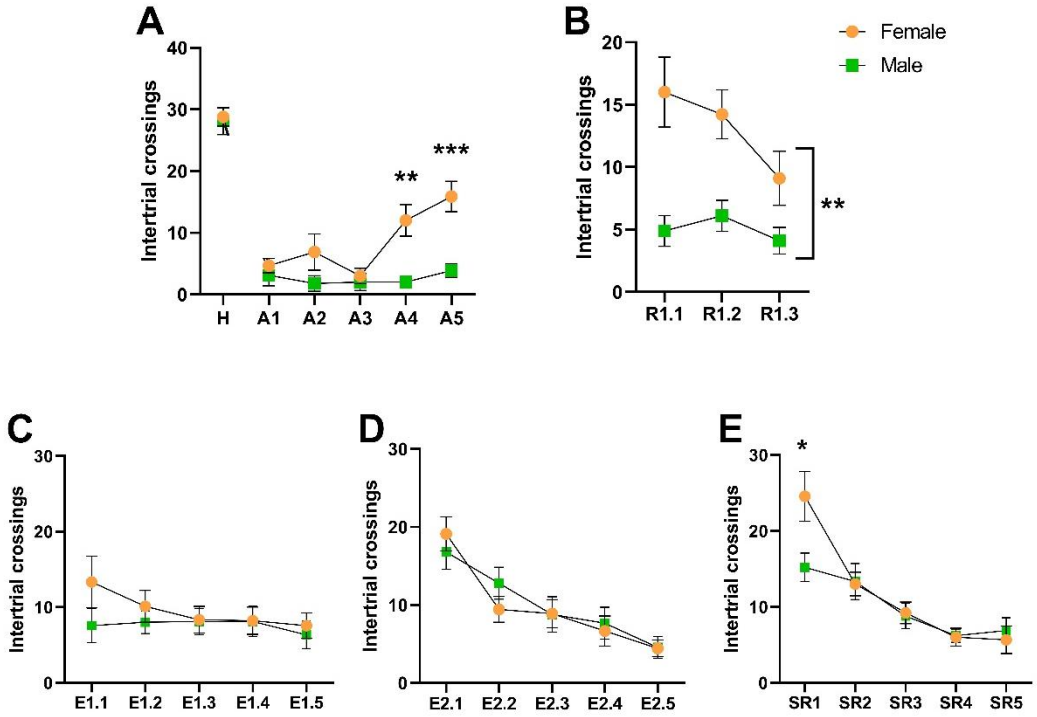


Figure 24. **Intertrial crossings throughout the 2-AA task in Experiment 1**. **A**. Intertrial crossings for the five acquisition sessions. **B**. Intertrial crossings for the retention session divided into three 10-trial blocks. **C**. Intertrial crossings for the first extinction sessions divided into five 10-trial blocks. **D**. Intertrial crossings for the second extinction session divided into five 10-trial blocks. **E**. Intertrial crossings for the spontaneous recovery test divided into five 10-trial blocks (SR). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Data are expressed as mean ± SEM.

1.6 Discussion

The findings of this experiment show that female rats demonstrate a superior performance in the conditioning of a 2-AA task, as shown by higher conditioning levels during the acquisition and retention sessions. Moreover, female rats exhibit a greater initial difficulty to extinguish the task.

Male and female rats acquire avoidance behavior at different rates

The first noteworthy result of this study reveals that female rats learn the task faster, as shown by the mean avoidances and latencies during the acquisition and retention sessions. Consequently, females acquire a robust level of conditioning with fewer trials, while males only reach the levels achieved by females by the end of the retention session, after having undergone a higher number of trials.

Our findings align with previous studies showing that female rats learn the 2-AA task faster than males. This is evidenced by the higher number of avoidance responses performed by females (Beatty & Beatty, 1970; Denti & Epstein, 1972; Scouten et al., 1975), regardless of the shock intensity (Beatty & Beatty, 1970) or training conditions (Beatty & Beatty, 1970; Denti & Epstein, 1972). The lower number of avoidance responses observed in males towards the end of the acquisition sessions suggest potential difficulty for males in acquiring this behavior. In fact, it has been reported that some male subjects never learn to escape and avoid a footshock (Dalla et al., 2008). However, our results show that males did eventually learn avoidance behavior at a level consistent with previous experiments (Aldavert-Vera et al., 1997; Kadar et al., 2014). The use of a moderate footshock in a distributed training protocol, such as the one used in our study, likely contributed to a decrease in non-responses.

In the RT-3d, both male and female rats maintain the conditioning levels achieved during the acquisition sessions. Thus, female rats consistently outperform males in the first 10-trial block of the retention. The first trials are considered to measure recall (Roozendaal & McGaugh, 2011), while as the retention session progresses, the animal is subjected to overtraining. In the latter blocks of the retention, that are considered the stages in which re-learning happens. However, by the end of the retention session, males manage to reach the avoidance levels displayed by females, having undergone more training trials.

No clear sex-differences are evidenced in extinction of avoidance behavior

Although our results do not clearly demonstrate differences between sexes in the extinction phase, a general trend emerges wherein females exhibit a higher number of avoidances during the first extinction session, particularly in the early trials. These results agree with previous studies indicating that female rats are slower to extinguish the conditioned response compared to males (Beatty & Beatty, 1971; Ikard et al., 1972; Van Haaren et al, 1990).

One potential explanation for the slower extinction in females may be attributed to their tendency to respond in earlier trials, leading to challenges in learning the new contingencies during extinction learning (Beatty & Beatty, 1971; Ikard et al., 1972; Van Haaren et al., 1990). The short latencies of response observed in females during the initial 10-trial block support this notion. However, it is possible that the lower conditioning levels observed in males during the acquisition sessions, as well as at the beginning of the retention session could facilitate the extinction process. Thus, while both groups achieve similar performance levels by the end of the retention, the overall lower number of correct – avoidance – responses in males may suggest weaker conditioning learning, whereas female rats present a strong conditioning from the beginning of the learning sessions. Both of these explanations are plausible and not mutually exclusive.

These results support the two main theories explaining the nature of extinction. On the one hand, the fewer avoidance responses by males throughout the entire training would have yielded a weaker association of this learning, making the extinction training more effective in attenuating or "erasing" this association. On the other hand, the reduced occurrence of avoidance responses by males during training might facilitate the successful competition between new associations and pre-existing ones.

While existing research literature has noted that females exhibit more spontaneous recovery during cued and contextual fear conditioning (Fenton et al., 2014, 2016; Matsuda et al., 2015), our results indicate similar performance between males and females for all assessed variables. Thus, no sex-differences in extinction memory

recall are revealed when tested long-term. The disparities observed in our experiment and those reported in other studies could be attributed to the specific task employed.

Different strategies are used by each sex to perform the task

The experimental data indicate that females tend to employ active coping mechanisms to learn a task whereas males adopt passive coping mechanisms (Dawson et al., 1975; Beatty & Fressler, 1976; Slob et al., 1981; Hyde & Jerussi, 1983; Toufexis et al., 2006). Specifically, in a 2-AA task, the use of active coping mechanisms has been associated with faster and better conditioning (Beatty & Beatty, 1970; Kirk & Blampied, 1985; Steenberg et al., 1990; Haaren et al., 1990).

The present experiment's results show that female rats perform significantly more intertrial crossings at the end of the acquisition and in the retention sessions. Although intertrial crossings could be considered a form of unspecific locomotor activity (Brush et al., 1979; Guenaire and Delacour 1983), they often reflect the coping strategies employed to learn the task (Wilcock et al., 1981; Boix et al., 1988; Gendron & Brush 1996; Aguilar et al., 2004; see review by Fernández-Teruel et al., 2019) and frequently correlate with avoidance responses. Notably, the greatest disparities in intertrial crossings between females and males in this experiment are observed when females exhibit the highest levels of conditioning. Conversely, there are no discernible differences in locomotion levels during the habituation phase preceding conditioning. These results support the idea that females' superior performance of the task is not solely attributed to higher activity levels, but rather reflects their use of a more effective strategy to solve the task.

Conclusions

In conclusion, our experimental setup enables us to distinguish between sex-based variations in avoidance conditioning. Additionally, it supports the likelihood of males attaining a high level of conditioning with sufficient training.

STUDY II. Is OX1R involved in the active avoidance behavior of males and females?

The orexin system, specifically the OX1R, is known to play a crucial role in the acquisition and extinction of fear memories. While several studies have highlighted the involvement of orexins in classical conditioning paradigms or passive avoidance tasks, there is a notable gap in the existing literature regarding the effects of orexins in active avoidance paradigms.

The evidence obtained from Experiment I suggests a clear advantage for female rats in avoidance conditioning along with a tendency to exhibit slower extinction than males.

This section introduces the results from Study II, which address the second, third, fourth and fifth aims of this thesis. Study II investigates the impact of OX1R selective blockade on both the acquisition and extinction phases of a 2-AA task.

The effects of OX1R selective blockade on the acquisition of a 2-AA task are analyzed in both males (Experiment 2) and in females (Experiment 3). Subsequently, following the spontaneous recovery test, the neuronal activation of extinction circuits was assessed in both males and females (Experiment 4). In Experiment 5, the influence of the estrous cycle on a 2-AA task, as well as on non-conditioned fear and anxiety is studied. Finally, the effects of OX1R selective blockade on the extinction of a 2-AA task are evaluated in male and female rats (Experiment 6).

Experiment 2. Effects of OX1R blockade on the acquisition of the 2-AA task in male rats.

Experiment 2 addresses the second aim of this thesis. Post-acquisition OX1R blockade (SB334867) effects are assessed in a 2-AA task in male rats.

Animals underwent surgery for cannula implantation on day 1. Subjects had a handling and a handling/habituation session on days 11 and 12. The acquisition sessions consisted of 10-trials per session for 5 consecutive days and took place on days 15 to 19. SB334867 or DMSO were administered immediately after each acquisition session. The first retention session (RT-3d) consisted of 20 trials performed on day 22, and the second retention session (RT-10d) consisted of 30 trials performed on day 29. Between the two retentions, two non-conditioned fear and anxiety tests (EZM and LD tests) were performed on days 25 and 26, respectively, 30 min after the infusion was administered. Two extinction sessions of 50 trials each were performed on days 30 and 31. Finally, 28 days after the last extinction session, the spontaneous recovery test, consisting of 50 trials, was performed.

A comprehensive timeline outlining the behavioral procedure of this experiment is shown in Figure 25.

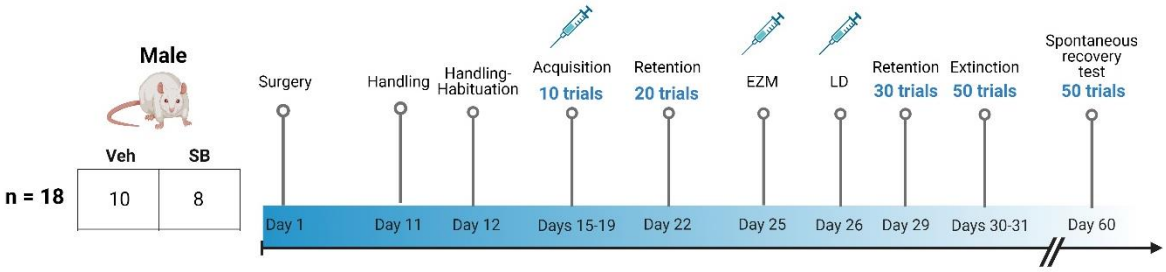


Figure 25. Timeline of the experimental procedure used in Experiment 2. Timeline of the experiment with every test performed. The number of trials performed in each session are depicted using blue letters. SB or vehicle administrations are represented with a syringe.

The final sample consisted of 18 male rats; 10 Vehicle rats with a mean age of 106.00 days (SD = 11.52) and a mean weight of 399.37 g (SD = 36.43) and 8 SB rats with a mean age of 104.12 days (SD = 3.75) and a mean weight of 405.58 g (SD = 27.48), as shown in Figure 25. Six subjects were excluded from the statistical analysis for the following reasons: the cannula placement was incorrect in 3 rats (see Figure 26), one rat exhibited a significant brain deformity due to the infusions into the ventricle, and technical issues with the software controlling the shuttle box prevented the retrieval of behavioral data from two rats.

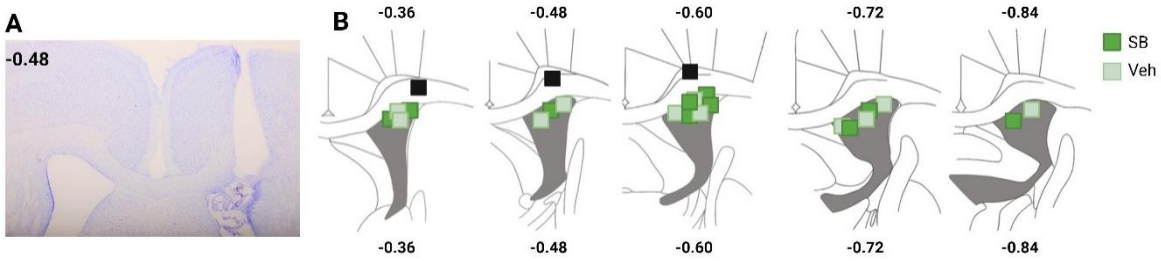


Figure 26. **Cannula location of subjects from Experiment 1**. **A**. Representative slice with correct cannula placement. **B**. Illustration depicting cannula placement for all subjects. Black squares depict incorrect cannula placements. Cannula placement was assessed between the coordinates -0.36mm to -0.84mm from Bregma (Paxinos & Watson, 2007).

2.1 Weight

Subject's weight was controlled throughout the experiment to assess possible effects of the specific OX1R blockade on food intake. There was no statistically significant effect of SB infusion on subjects' weight over time (SB: $F_{1,16}$= .932, P = .349; SB × SESSION: $F_{13, 208}$= 1.080, P = .372) (Fig.27).

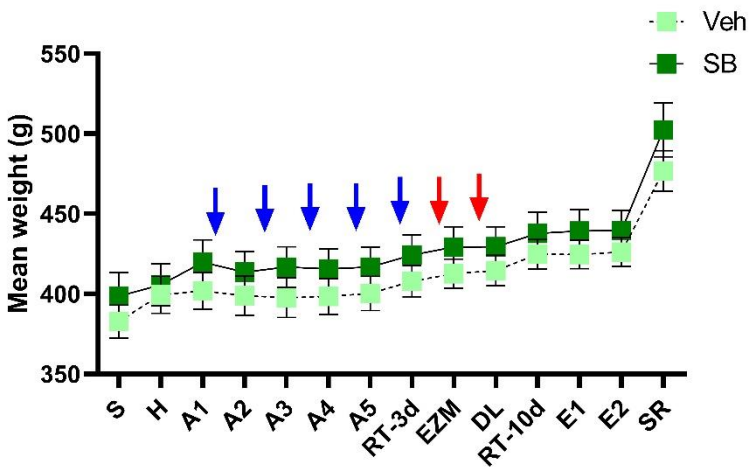


Figure 27. **Evolution of the weight throughout the experimental sessions in Experiment 2**. Each point in the X axis represents a session from the experimental procedure. Note that the time intervals between sessions may vary. Arrows indicate SB334867 or Veh infusions. Blue arrows indicate a post-session infusion while red arrows indicate a pre-session infusion. S = surgery, H = habituation, A1 to A5 = acquisition sessions, respectively 1, RT-3d = first retention session 1, EZM = elevated zero maze, DL = dark/light test, RT-10d= second retention session, E1 = extinction session 1, E2 = extinction session 2, and SR = spontaneous recovery test performed 28 days after the second extinction session. Data are expressed as mean ±SEM.

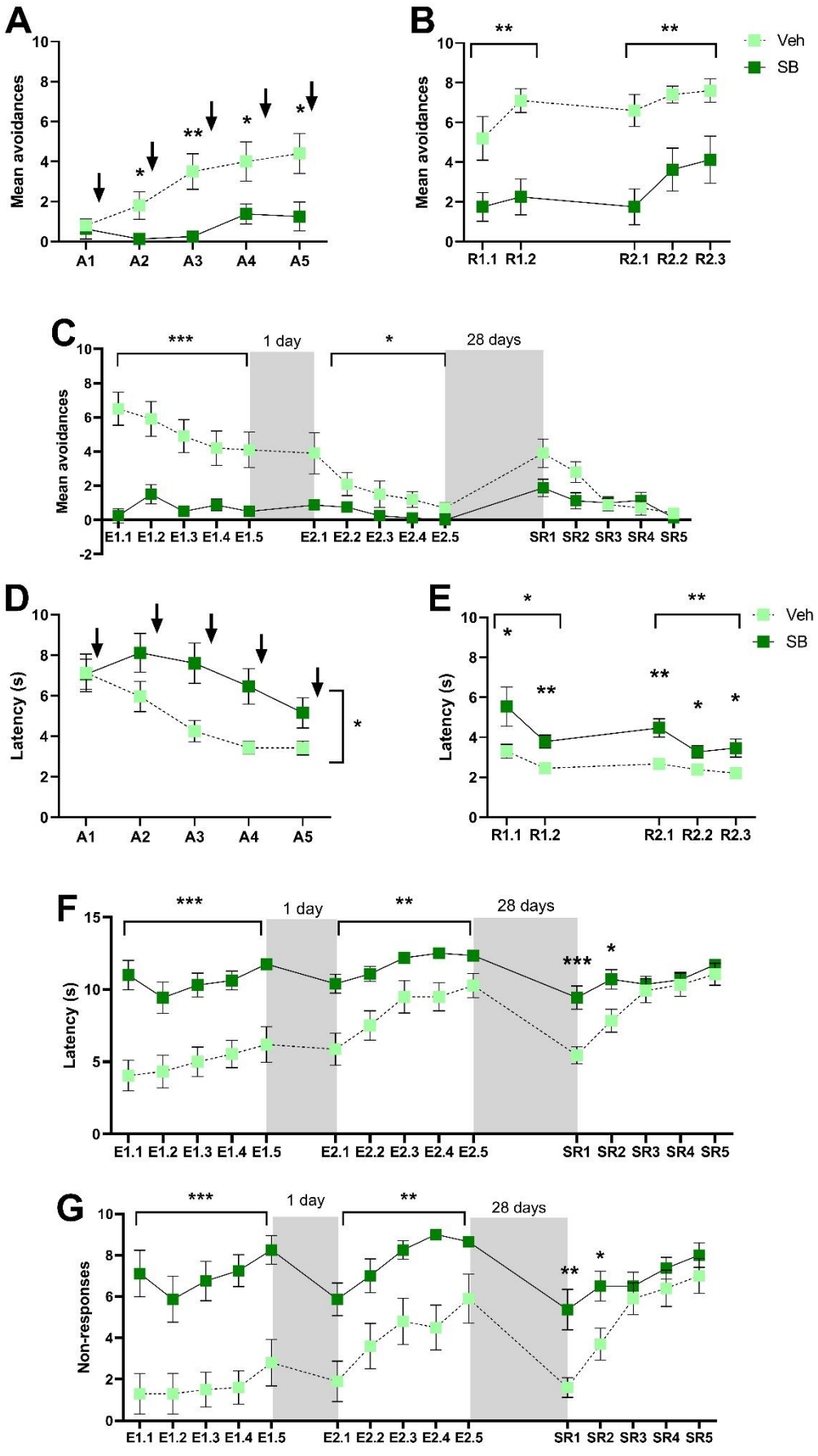

A
Mean avoidances
A1
A2
A3
A4
A5
B
Mean avoidances
R1.1 R1.2
R2.1 R2.2 R2.3
Veh
SB
C
Mean avoidances
1 day
28 days
E1.1 E1.2 E1.3 E1.4 E1.5
E2.1 E2.2 E2.3 E2.4 E2.5
SR1 SR2 SR3 SR4 SR5
D
Latency (s)
A1
A2
A3
A4
A5
E
Latency (s)
R1.1 R1.2
R2.1 R2.2 R2.3
Veh
SB
F
Latency (s)
1 day
28 days
E1.1 E1.2 E1.3 E1.4 E1.5
E2.1 E2.2 E2.3 E2.4 E2.5
SR1 SR2 SR3 SR4 SR5
G
Non-responses
1 day
28 days
E1.1 E1.2 E1.3 E1.4 E1.5
E2.1 E2.2 E2.3 E2.4 E2.5
SR1 SR2 SR3 SR4 SR5

Figure 28. **Behavioral outcome of the 2-AA task from Experiment 2**. Mean avoidances during the **A**. acquisition sessions, the **B**. two retention sessions, and the **C**. two extinction sessions and the spontaneous recovery test. Latencies of response during the **D**. acquisition sessions, the **E**. two retention sessions, and the **F**. two extinction sessions and the spontaneous recovery test. **G**. Non-responses performed in each 10-block of the two extinction sessions and the spontaneous recovery test. Arrows indicate post-training SB334867 or DMSO infusions. A = acquisition sessions. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Data are expressed as mean ±SEM.

2.2 Acquisition

Mean avoidances and latencies of response during the acquisition sessions are depicted in Fig.28 A/D.

A repeated measures GLM revealed disruptive effects of SB334867 on the acquisition of the task. Regarding mean avoidances, although factor SB was significant ($F_{1,16}$= 6.994, P = .018), a significant interaction SB × SESSION ($F_{4,64}$= 3.847, P = .029) (Fig.28A) was found. Simple effects showed that the differences between groups started to be noticeable in A2, which is the first session after the first infusion. Significant differences were found between SB and Veh groups in A2 (P = .0.50), A3 (P = .006), A4 (P = .043) and A5 (P = 0.027) sessions. The analysis of the slopes of the two groups showed that the Veh group number of mean avoidance was different from 0 (P < .001), with a significant linear tendency (P= .001), and a significant increase from session A1 to A5 (P = .001), whilst the SB group presented a constant not different from 0 (P= .254), with a non-significant linear tendency (P= .320), and a non-significant increase from A1 to A5 (P= .514). Regarding latencies of response, factor SB was significant (SB: $F_{1,16}$= 7.044, P = .017) regardless of the session (SB × SESSION: $F_{4,64}$= 2.960, P = .065), with SB subjects displaying a worse performance in the acquisition of the task (Fig.28A). The values of non-responses were so low that neither the results of the analysis nor graphs are presented for the acquisition or the retention sessions.

2.3 Retention

Mean avoidances and latencies of response of the retention sessions are depicted in Fig.28 B/E.

SB also affected the retention of the 2-AA task. The SB group showed a lower number of avoidance responses compared to the Veh group in both the RT-3d and RT-10d ($F_{1,16}$= 14.840, P = .001; $F_{2,32}$= 16.891, P = .001, respectively), regardless of the block ($F_{1,16}$= 4.356, P = .266; RT-10d: $F_{2,32}$= .808, P = .455, respectively) (Fig. 28B). Latencies of response were also affected in the retention sessions. Factor SB was significant for RT-3d ($F_{1,16}$= 8.004, P = .012) and RT-10d ($F_{1,16}$= 13.903, P = .002), regardless of the block (RT-3d: $F_{1,16}$= 1.610, P = .223; RT-10d: $F_{2,32}$= 2.021, P = .149) (Fig.28E).

2.4 Extinction

Mean avoidances, latencies of response and non-responses of the extinction sessions are depicted in Fig.28 C/F/G.

Regarding mean avoidances, differences between groups were observed in both E1 ($F_{1,16}$= 19.674, P = .000) and E2 ($F_{1,16}$= 4.612, P = .047), regardless of the block (E1: $F_{4,64}$= 1.793, P = .141; E2: $F_{4,64}$= 2.254, P = .115), with the SB group showing a lower number of avoidances - a better extinction - than the Veh. As expected, the latencies of response results align with the avoidances results. Regarding latencies of response, factor SB was significant for both extinction sessions (E1: $F_{1,16}$= 23.598, P = .000; E2: $F_{1,16}$= 9.848, P = .006) regardless of the block (E1: $F_{4,64}$= .687, P = .536; E2: $F_{4,64}$= 1.650, P = .206) (Fig.28F). Regarding non-responses, factor SB was also significant for both extinction sessions (E1: $F_{1,16}$= 24.870, P < .001; E2: $F_{1,16}$= 11.736, P = .003) irrespective of the block (E1: $F_{4,64}$= .267, P = .817; E2: $F_{4,64}$= .788, P = .471) (Fig.28G).

A survival analysis was carried out to see if SB had an effect on the time - or number of trials - required by the animals to display a stable extinction response, defined as 5 non-responses in consecutive trials (Fig.29A and 29B).

Significant differences were found between the two curves (SB and Veh) in a Breslow comparison analysis for E1 (Breslow: X^2 = 9.521, df = 1, P = .002) (Fig.29A) and E2 (Breslow: X^2 = 4.367, df = 1, P = .037) (Fig.29B). By the time 100% of SB

subjects had achieved the condition, in E1 only 30% of Veh subjects had, rising to 50% in E2.

2.5 Spontaneous recovery test

Mean avoidances, latencies of response and non-responses during the spontaneous recovery test are depicted in Fig.28 C/F/G.

Regarding mean avoidances, differences between groups varied depending on the block (SB × BLOCK: $F_{4,64}$= 5.146, P = .001), showing a tendency towards significance during the first blocks of the session (SR1: P = .071; SR2: P = .055) (Fig.28C). These results are supported, with greater statistical power, by the analysis of latencies of response, in which differences between groups varied according to the block (SB × BLOCK: $F_{4,64}$= 6.599, P < .001; SB: $F_{1,16}$= 4.024, P = .062). Significant differences were found between groups in SR1 (P = .001) and SR2 (P = .018) (Fig.28F). Regarding non-responses, despite groups behaving differently during the whole session (SB: $F_{1,16}$= 4.696, P = .046), these differences varied depending on the block of the session (SB × BLOCK: $F_{4,64}$= 3.536, P = .011). Simple effects analysis showed once again that these differences were found in the first two blocks of the session (SR1: P = .002; SR2: P = .020) (Fig.28G). In summary, SB subjects have higher levels of extinction (lower recovery of the CR) than Veh subjects, specifically in the initial trials of the session.

After reviewing the behavioral data from the 2-AA task, we questioned whether the SB group presented the same relapse effect in the spontaneous recovery test. To analyze this, we compared their performance between the last 10-trial block of E2 and the first 10-trial block of the SR. We found significant differences in mean avoidances between these two blocks (BLOCK: $F_{1,16}$= 30.285, P < .001) regardless of the specific block studied (SB × BLOCK: $F_{1,16}$= 2.064, P = .170), although the SB factor itself was significant (SB: $F_{1,16}$= 4.581, P = .048). Similar outcomes were found in analyzing latencies of response (BLOCK: $F_{1,16}$= 68.119, P < .001; SB × BLOCK: $F_{1,16}$= 4.217, P = .057; SB: ($F_{1,16}$= 12.767, P = .003) and non-responses (BLOCK: $F_{1,16}$= 47.046, P < .001; SB × BLOCK: $F_{1,16}$= 1.928, P = .184; SB: $F_{1,16}$= 10.629, P = .005).

A survival analysis was carried out to examine whether SB influenced the time or number of trials needed for the animals to establish a stable extinction response, defined as 5 non-responses occurring in consecutive trials (Fig.29C). The analysis revealed that SB animals required fewer trials to demonstrate stable extinction behavior after relapse induced by new exposure to the context and the conditioned stimulus (Breslow: $X^2 = 4.049$, df = 1, P = .044) (Fig.29C). None of the Veh subjects had achieved the criterion by the time 45% of SB subjects had.

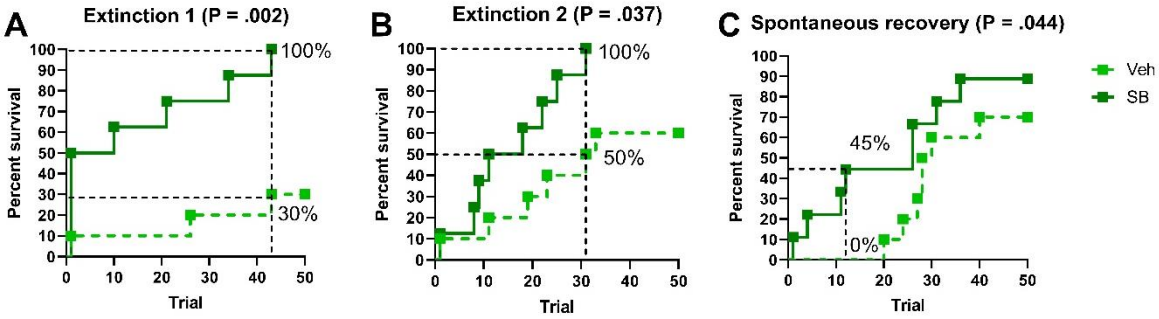


Figure 29. **Survival curves assessing extinction evolution in Experiment 2**. **A**. First extinction session. **B**. Second extinction session. **C**. Spontaneous recovery test. The curves represent the percentage of subjects from each experimental group that accomplish the extinction criterion (5 consecutive trials performing a non-response).

2.6 Intertrial crossings

Intertrial crossings performed in the acquisition, retention, extinction sessions and spontaneous recovery test are depicted in Fig.30 A-E.

No differences were observed regarding the locomotor exploratory activity of animals during the habituation session prior to the administration of SB ($t_{16} = .150$, P = .882). SB did not have any significant effect on intertrial crossings across any session of the experiment (A1-A5: SB $F_{1,16}= 1.094$, P = .311; SB × SESSION: $F_{4,64}= .544$, P = .600; R1: $F_{1,16}= 1.952$, P = .181; SB × BLOCK $F_{1,16}= .177$, P = .679; R2: SB: $F_{1,16}= 1.464$, P = .244; SB × BLOCK: $F_{1,16}= .923$, P = .408; E1: SB: $F_{1,16}= 1.339$, P = .264; SB × BLOCK: $F_{1,16}= .275$, P = .822; E2: SB $F_{1,16}= .010$, P = .922; SB × BLOCK $F_{1,16}= 1.466$, P = .245; SR: SB $F_{1,16}= .615$, P = .445; SB × BLOCK $F_{1,16}= 1.110$, P = .360) (Fig.30A-E).

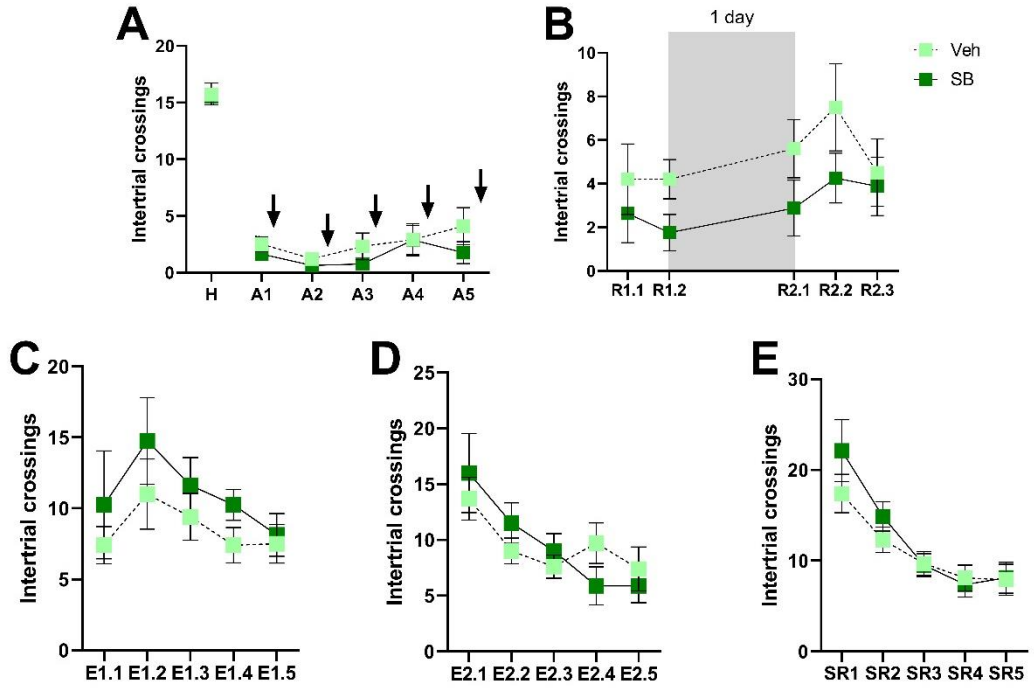


Figure 30. **Intertrial crossings of the 2-AA task from Experiment 2**. **A**. Acquisition sessions. **B**. Two retention sessions. **C**. First extinction session. **D**. Second extinction session. **E**. Spontaneous recovery test. Arrows indicate post-training SB334867 or DMSO infusions. H = habituation, A = acquisition sessions, R = retention sessions, E = extinction sessions, and SR = spontaneous recovery sessions. Arrows indicate post-training SB334867 or DMSO infusions. Data are expressed as mean ±SEM.

2.7 Non-conditioned fear and anxiety tests

2.7.1 Elevated Zero Maze (EZM)

In all variables assessed in the EZM, the Veh group performed a significantly higher number of groomings in the closed arms than the Veh group (U = 20.00, p = .024) (Fig.31E).

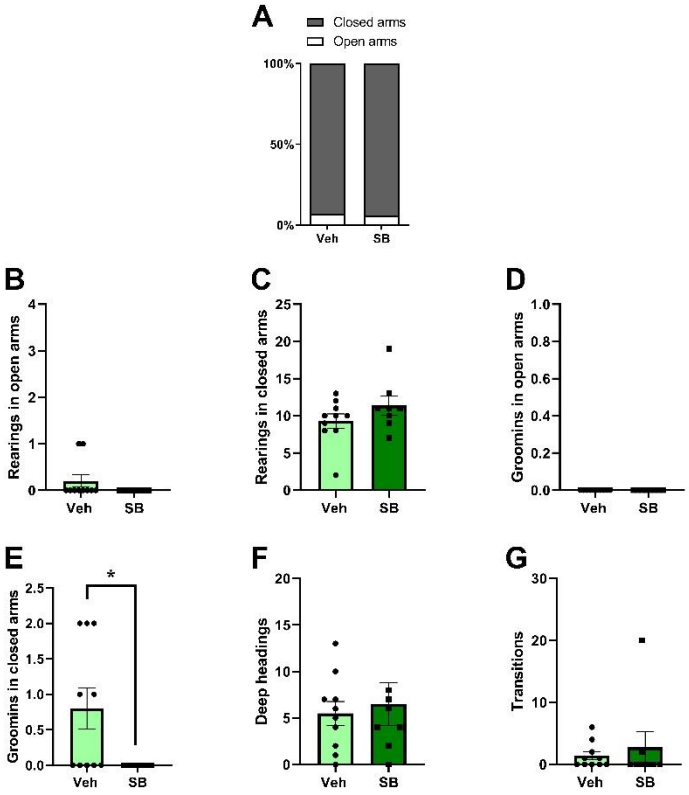


Figure 31. **Effects of SB334867 on the Elevated Zero Maze in male rats**. **A**. Time spent in open and closed arms (%). **B**. Rearings in open arms. **C**. Rearings in closed arms. **D**. Groomings in open arms. **E**. Groomings in closed arms. F. Deep headings between the open and closed arms. **G**. Number of transitions between the open and closed arms. * P < 0.05. Data are expressed as mean ±SEM.

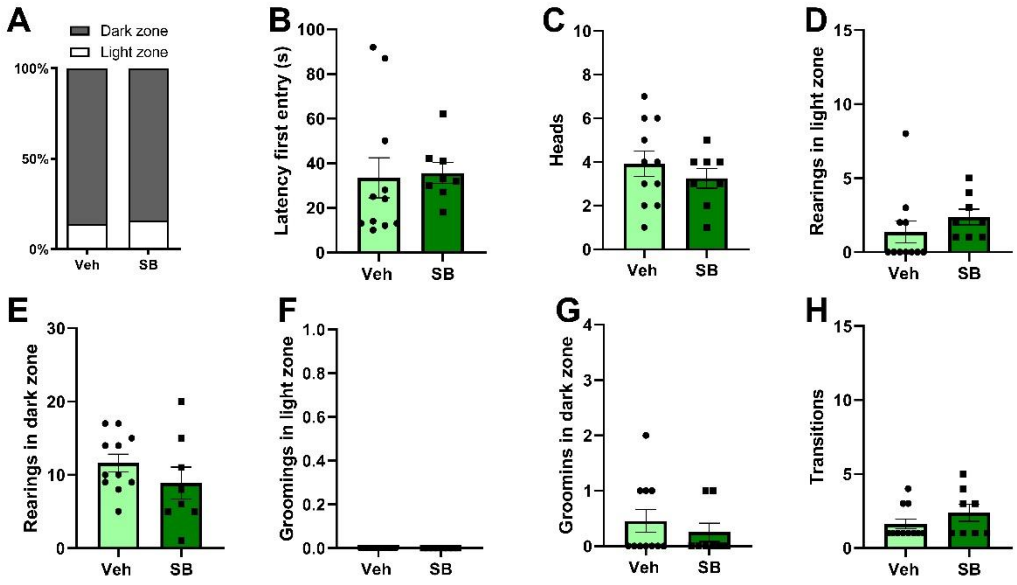


Figure 32. **Effects of SB334867 on the Light/dark test in male rats**. **A**. Time spent in the light and dark zone (%). **B**. Latency of the first entry to the dark zone (s). **C**. Headings between the light and dark zone. **D**. Rearings in the light zone. **E**. Rearings in the dark zone. **F**. Groomings in the light zone. **G**. Groomings in the dark zone. **H**. Transitions between the light and dark zones. Data are expressed as mean ±SEM.

2.7.2 Light/dark box test (L/D)

No differences were found between the SB and VEH groups in any of the variables assessed in the light/dark test (Fig.32 A-H).

2.8 Discussion

The findings of this experiment indicate that the administration of the OX1R selective blockade immediately after each acquisition session impairs the 2-AA task conditioning in male rats. Moreover, differences between groups were evident in the retention, extinction and spontaneous recovery test.

<u>OX1R selective blockade disrupts the consolidation of a 2-AA conditioning</u>

In this experiment, the administration of SB led to impaired performance in the 2-AA task across all acquisition sessions after the first administration. Notably, SB animals failed to learn avoidance behavior, as evidenced by the consistent lack of avoidances and the absence of improvement. However, increasing training through retention sessions enabled the SB subjects to eventually achieve the conditioning levels of Veh subjects by the end of the acquisition sessions. This finding contradicts the notion of a proactive effect of SB administration on conditioning, evident at three and seven days after the last microinfusion.

Currently, there is a lack of studies examining the effects of the OX1R selective blockade in rats using a 2-AA task. However, existing research indicates that orexins play an important role in the acquisition of several fear conditioning tasks in rodents. This is supported by the fact that blocking OX1R or reducing Ox-A availability hinders this learning process, while administering Ox-A enhances it. Specifically, it has been reported that orexin deficient female mice (orexin/ ataxin-

3 (O/A3)) exhibit a recall deficit in a 2-AA task, which can be rescued by Ox-A infusions (Mavanji et al., 2017). Similarly, icv infusions of Ox-A have been shown to improve retention in a T-maze footshock paradigm assessing active avoidance behavior (Jaeger et al., 2002). Consistent with these findings, classical conditioning paradigms reveal that pre-administration of SB334867 impairs the acquisition of fear memories (Sears et al., 2013). Moreover, impaired freezing is observed in both contextual and cued FC in OX1R-/- mice (Soya et al., 2013), and in mice receiving systemic SB334867 administration (Flores et al., 2014).

The severe impairment observed in SB subjects does not seem to be due to locomotor disruptions, since intertrial crossings in this experiment were not affected. This outcome is consistent with previous studies reporting no effects of OX1R on locomotor activity (Johnson et al., 2010b). Additionally, it is unlikely that SB detrimental effects on 2-AA conditioning stem from alterations of fear and anxiety levels, as pre-infusions of SB did not significantly modify male rats' fear or anxiety levels in neither the elevated zero maze nor the light/dark test. However, studies assessing the effects of orexins on non-conditioned fear and anxiety yield mixed results, reporting anxiolytic (Khalil & Fendt, 2017; Pan et al., 2020; Han et al., 2021; Kordijaz et al., 2017), anxiogenic (Suzuki et al., 2005; Avolio et al., 2011; Li et al., 2010; Lungwitz et al., 2012) or no effects (Steiner et al., 2012; Staples & Cornish, 2014) caused by changes in orexinergic systems, possibly due to variations in methodologies, animal models and tests employed.

Rather, OX1R blockade could have interfered with synaptic plasticity mechanisms, potentially serving as the primary cause of the impaired conditioning. In support of this notion, there are studies demonstrating that acute (Akbari et al., 2011) or chronic (Gao et al., 2023) blockade of OX1R impairs hippocampal long-term potentiation (LTP), a major mechanism involved in synaptic plasticity related to memory consolidation. Although the task used in this experiment (2-AA task) is an amygdala-dependent task, it involves the implication of the hippocampus.

OX1R selective blockade during the acquisition impacts performance in the extinction of a 2-AA task

In this experiment, SB animals display a rapid extinction of the conditioned response. This could be due to a weaker association between US-CS and instrumental responses, rather than the establishment of new contingencies.

Interestingly, SB subjects present a similar relapse as Veh subjects in the spontaneous recovery test. Despite displaying a low number of CR during the extinction, its spontaneous recovery suggests that the low conditioning levels attained during the retention sessions were enough to produce a relapse of the CR when exposed to the conditioning context and stimulus again. This finding contradicts the possibility of a proactive effect of SB on subsequent learning phases of the behavioral procedure.

Conclusions

In conclusion, this experiment demonstrates that contingent administration of SB after the acquisition of a 2-AA task impairs the learning of avoidance behavior, and may hinder future learning opportunities. Moreover, the experimental design does not allow for the direct examination of the effects of SB on other learning stages, such as the extinction.

Experiment 3. Effects of OX1R blockade on the acquisition of the 2-AA task in female rats.

Evidence from Experiment 2 shows that OX1R blockade administration after acquisition sessions severely impairs 2-AA conditioning in male rats. Together with the fact that female rats achieved higher conditioning levels on the acquisition sessions of the 2-AA task (see Experiment 1), it prompts consideration of whether the OX1R selective blockade has similar effects in females.

Experiment 3 follows the same experimental procedure as Experiment 2 (see *Experiment 2*), with the exception that female rats are used.

A comprehensive timeline outlining the behavioral procedure of this experiment is shown in Figure 33.

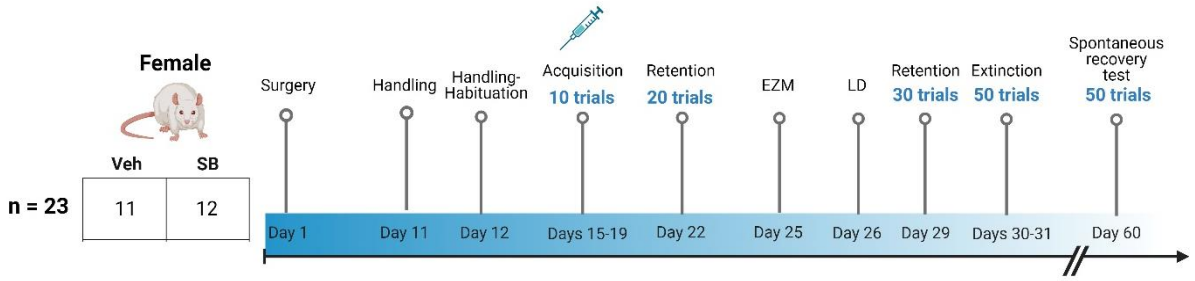


Figure 33. **Timeline of the experimental procedure used in Experiment 3**. Timeline of the experiment with every test performed. The acquisition sessions consisted of 10-trials per session for 5 consecutive days. SB334867 or DMSO were administered immediately after each acquisition session, and it is represented in the timeline with a syringe.

The final sample consisted of 23 female rats: 11 rats in the Vehicle group, with a mean age of 106.81 days (SD = 4.83) and a mean weight of 240.00 g (SD = 14.63), and 12 rats in the SB group, with a mean age of 108.08 days (SD = 4.83) and a mean weight of 239.69 days (SD = 16.46), as shown in Figure 33. One subject was excluded from the statistical analysis due to respiratory issues, leading to being euthanized. All animals had correctly implanted cannulas and were included in the statistical analysis, as it depicted in Figure 34.

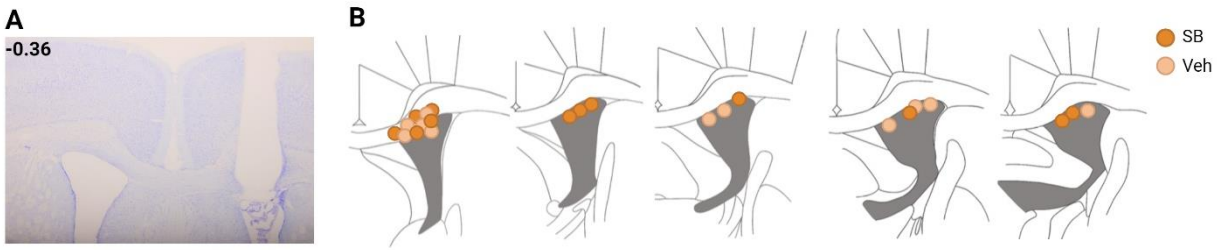


Figure 34. **Cannula location of subjects from Experiment 3**. **A**. Representative slice with correct cannula placement. **B**. Illustration depicting cannula placement for all subjects. Cannula placement was assessed between the coordinates -0.36mm to -0.84mm from Bregma (Paxinos & Watson, 2007).

3.1 Weight

No statistically significant interaction between treatment and weight evolution was found (SB × SESSION: $F_{13, 260}$= .398, P = .866) (Fig.35). Moreover, the factor GROUP was not significant (GROUP: $F_{1,20}$= .300, P = .590), indicating that SB334867 did not appear to affect either food intake or animals' body weight.

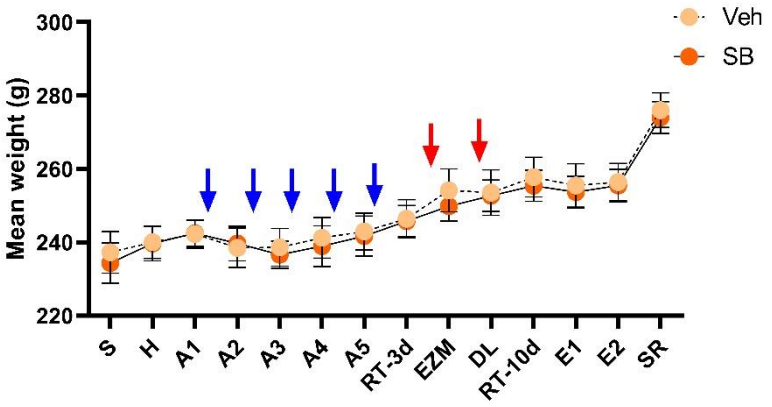


Figure 35. **Evolution of the weight throughout the experimental sessions in Experiment 3**. Each point in the X axis represents a day from the experiment. Note that the time intervals between sessions may vary. Arrows indicate SB334867 or DMSO infusions. Blue arrows indicate a post-session infusion while red arrows indicate a pre-session infusion. S = surgery, H = habituation, A1 to A5 = acquisition sessions, respectively 1, RT-3d = first retention session 1, EZM = elevated zero maze, DL = dark/light test, RT-10d= second retention session, E1 = extinction session 1, E2 = extinction session 2, and SR = spontaneous recovery test performed 28 days after the second extinction session. Data are expressed as mean ±SEM.

3.2 Acquisition

Mean avoidances and latencies of response during the acquisition sessions are depicted in Fig.36 A/D.

No statistically significant effects of SB were observed in the acquisition phase for mean avoidances (SB × SESSION: $F_{4,84}$= 1.755, P = .146; SB: $F_{1,21}$= .060, P = .809) (Fig.36A) or latencies of response (SB × SESSION: $F_{4,84}$= 1.949, P = .134; SB: $F_{1,21}$= .230, P = .637) (Fig.36D). Since the values of non-responses were either zero or close to zero, the statistical analysis of this variable was not conducted for the acquisition and retention sessions.

3.3 Retention

Mean avoidances and latencies of response of the retention sessions are depicted in Fig.36 B/E.

SB did not have any effect on the variables assessed during the two retention sessions. Specifically, there were no differences between groups in mean avoidances, for the whole session (RT-3d: $F_{1,21}$= .126 P = .726; RT-10d: $F_{1,21}$= .321, P = .557), or their evolution throughout the blocks (SB × BLOCK RT-3d: $F_{1,21}$= 1.141, P = .298; RT-10d: $F_{2,42}$= 1.142, P = .320) (Fig.36B). Similarly, the latencies of response remained equal across groups during both the entire session (SB RT-3d: $F_{1,21}$= .552, P = .466; RT-10d: $F_{1,21}$= .219, P = .644) and their evolution across blocks (SB × BLOCK RT-3d: $F_{1,21}$= .764, P = .171; RT-10d: $F_{2,42}$= .1.074, P = .333) (Fig.36 E).

3.4 Extinction

Mean avoidances, latencies of response and non-responses during the extinction sessions are depicted in Fig.28 C/F/G.

SB did not affect the performance of the subjects during the extinction sessions for any variable assessed either. Specifically, regarding mean avoidances, neither the SB factor nor the SB × BLOCK interaction factor were significant in mean avoidances during any of the two extinction sessions (E1: SB: $F_{1,21}$= .036, P = .851; SB × BLOCK: $F_{4,84}$= .821, P = .464; E2: SB: $F_{1,21}$= .042, P = .840; SB × BLOCK: $F_{4,84}$= 1.106, P = .359) (Fig.28C). Similarly, there were no significant differences in latencies of response (SB: E1: $F_{1,21}$= .087, P = .771; E2: $F_{1,21}$= .001, P = .979; SB × BLOCK: E1: F4,84= .232, P = .919; E2: F4,84= 1.390, P = .244) (Fig.28F) and non-responses (SB: E1: $F_{1,21}$= .594, P = .449; E2: $F_{1,21}$= .001, P = .970; SB × BLOCK: E1: $F_{4,84}$= .253, P = .816; E2: $F_{4,84}$= 1.783, P = .140) (Fig.28G).

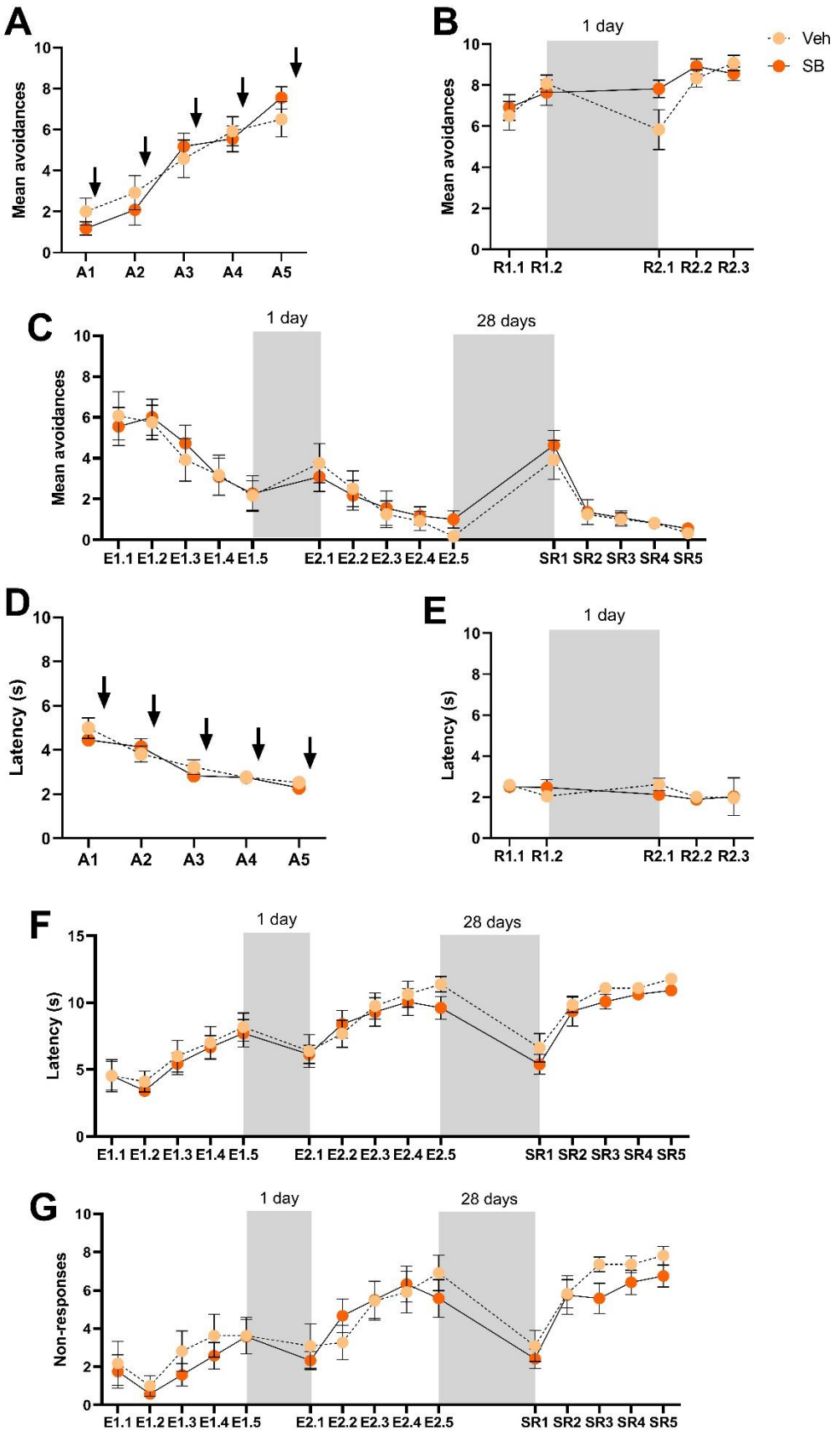


Figure 36. Behavioral outcome of the 2-AA task from Experiment 3. Mean avoidances for **A.** the acquisition sessions, **B.** two retention sessions, and **C.** the two

extinction sessions and spontaneous recovery test. Latencies of response for **D**. acquisition sessions, **E**. the two retention sessions, and **F**. the two extinction sessions and spontaneous recovery test. **G**. Non-responses performed in each 10-block of the two extinction sessions and spontaneous recovery test. Arrows indicate post-training SB334867 or DMSO infusions. Data are expressed as mean ±SEM.

3.5 Spontaneous recovery test

Mean avoidances, latencies of response and non-responses during the spontaneous recovery test are depicted in Fig.28 C/F/G.

Similar to what was observed in the previous phases of the experiment, SB did not affect his spontaneous recovery (mean avoidances; SB: $F_{1,21}$= .015, P = .905; SB × BLOCK: $F_{4,84}$= .037, P = .959)(Fig.28C), latencies of response; SB: $F_{1,21}$= .635, P = .434; SB × BLOCK: $F_{4,84}$= .171, P = .874)(Fig.28F) and non-responses; SB: $F_{1,21}$= 1.332, P = .261; SB × BLOCK: $F_{4,84}$= .719, P = .581)(Fig.28G).

3.6 Intertrial crossings

Intertrial crossings performed in the acquisition, retention, extinction sessions and spontaneous recovery test are depicted in Fig.37 A-E.

No differences between groups were observed regarding the number of crossings during the habituation session (t_{21} = 1.009, P = .325). Similarly, SB infusion did not affect the intertrial crossings performed in the acquisition sessions (SB: $F_{1,21}$= .129, P = .723; SB × BLOCK: $F_{4,84}$= 1.058, P = .267) (Fig.37A), for RT-3d (SB: $F_{1,21}$= .260, P = .615; SB × BLOCK: $F_{1,21}$= .014, P = .908), for the RT-10d (SB: $F_{1,21}$= .766, P = .391; SB × BLOCK: $F_{2,42}$= .114, P = .893) (Fig.37B), for the E1 (SB: $F_{1,21}$= .159, P = .694; SB × BLOCK: $F_{4,84}$= .117, P = .937) (Fig.37C), for the E2 (SB: $F_{1,21}$= .250, P = .622; SB × BLOCK: $F_{4,84}$= .154, P = .924) (Fig.37D), and SR (SB: $F_{1,21}$< .001, P = .994; SB × BLOCK: $F_{4,84}$= 1.017, P = .387) (Fig.37E).

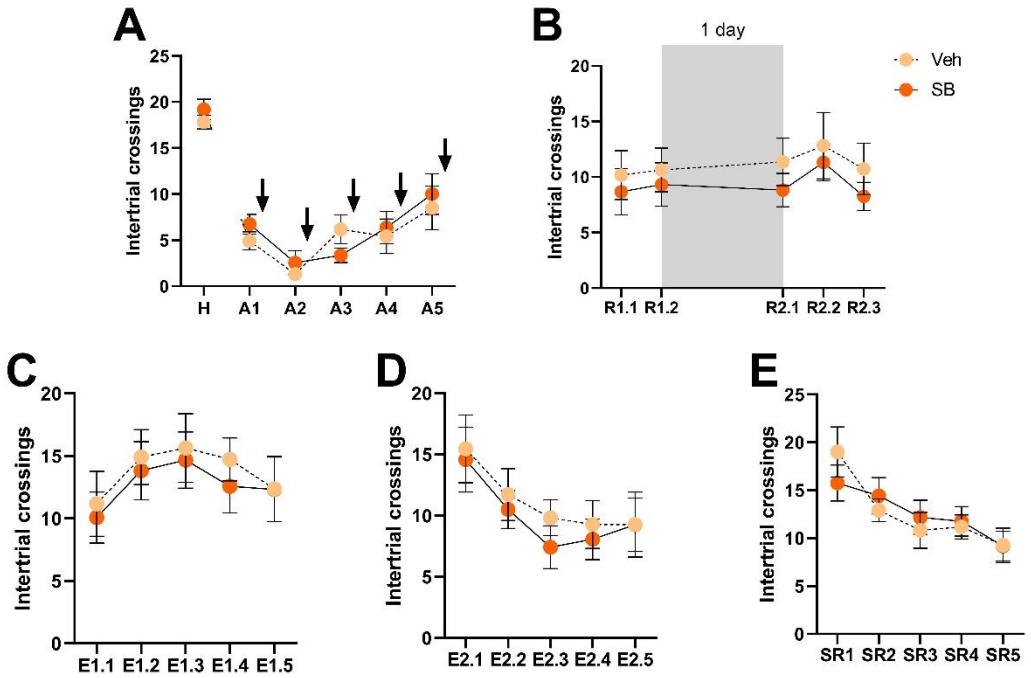


Figure 37. **Intertrial crossings of the 2-AA task for Experiment 3**. **A**. Acquisition sessions. **B**. Two retention sessions. **C**. First extinction session. D. Second extinction session. **E**. Spontaneous recovery test. Arrows indicate post-training SB334867 or DMSO infusions. H = habituation, A = acquisition, R = retention, E = extinction, and SR = spontaneous recovery session. Arrows indicate post-training SB334867 or DMSO infusions. Data are expressed as mean ±SEM.

3.7 Non-conditioned fear and anxiety tests

3.7.1 Elevated Zero Maze (EZM)

No differences were found between groups for any variable assessed in the EZM (Fig.38 A-G).

3.7.2 Light/dark box test (L/D)

No differences were found between the SB and Veh group in any variables assessed in the L/D test (Fig.39 A-H).

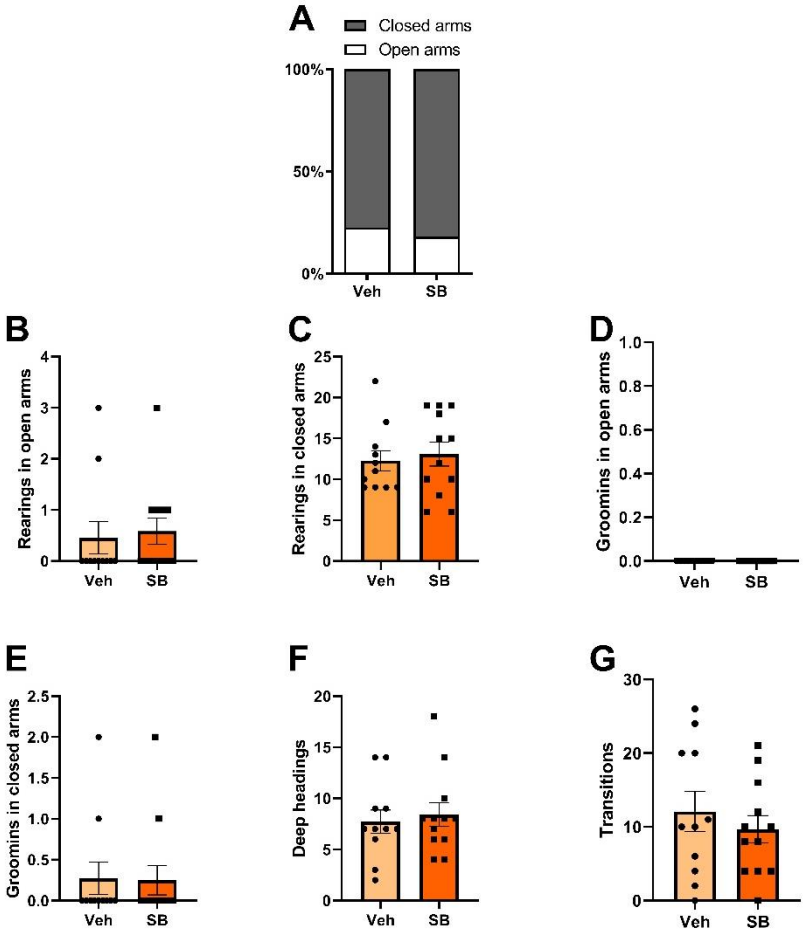


Figure 38. **Effects of SB334867 on the Elevated Zero Maze in female rats**. **A**. Time spent in open and closed arms (%). **B**. Rearings in open arms. **C**. Rearings in closed arms. **D**. Groomings in open arms. **E**. Groomings in closed arms. **F**. Deep headings. **G**. Number of transitions between the open and closed arms. Data are expressed as mean ±SEM.

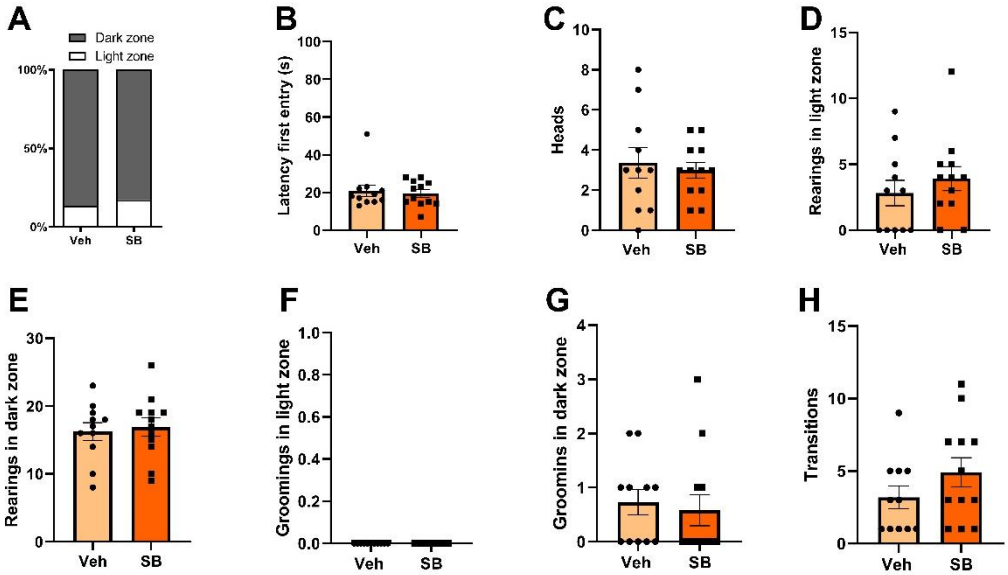


Figure 39. **Effects of SB334867 on the Light/dark test in female rats**. **A**. Time spent in the light and dark zone (%). **B**. Latency of the first entry to the dark zone (s). **C**. Headings between the light and dark zone. **D**. Rearings in the light zone. **E**.

Rearings in the dark zone. **F**. Groomings in the light zone. **G**. Groomings in the dark zone. **H**. Transitions between the light and dark zone. Data are expressed as mean ±SEM.

3.8 Discussion

The findings of this experiment show that the OX1R specific blockade does not affect the acquisition of a 2-AA task in females, unlike what was observed in males (Experiment 2). Moreover, in females, there were no differences in any subsequent phase of the behavioral procedure.

OX1R selective blockade does not impair the acquisition of a 2-AA task in females

The experiment revealed that blocking OX1R did not hinder learning of the 2-AA task in females. This finding contrasts with the results from Experiment 2, which showed that icv infusions with SB334867 drastically impaired the acquisition of the 2-AA task in males. This is the first instance where the effect of OX1R selective blockade on a 2-AA task has been tested in female rats. To our knowledge, there is only one study demonstrating that orexin deficient female mice (OA/3) encountered difficulties with the 2-AA conditioning, which were alleviated by Ox-A infusion into the CA1 (Mavanji et al., 2017). However, there are notable procedural differences between our experiment and the one carried out by Mavanji et al., (2017) which could account for the contradictory findings. Firstly, our experiment employed a delay procedure of the 2-AA task, while in the study by Mavanji et al., (2017) utilized a hippocampus-dependent trace procedure. It is plausible that the orexinergic system could have different implications in brain circuits involved in each of these procedures (Noorani et al., 2021; Selbach et al., 2010). Another explanation could be the degree of orexinergic system impairment induced by both procedures. In the OA/3 mouse model, there is a progressive loss of Ox neurons, reaching up to 99% at 12 weeks of age, whereas our experiment used a non-selective antagonist, with a transient effect.

Furthermore, it is puzzling why SB did not have effects on the 2-AA task in females, but did so in males (Experiment 2). One possible explanation is the sex dimorphic

expression of orexins. Female rats have higher preprohypocretin mRNA and Ox-A levels, specifically in the lateral and posterior hypothalamus (Jöhren et al., 2002; Taheri et al., 1999; Dorsey et al., 2021), and these neurons project to multiple regions, including areas related to learning, memory, and motivation (Elahdadi Salmani et al., 2022). Therefore, elevated basal levels of orexins in females might positively modulate plasticity mechanisms in learning and memory areas, potentially explaining their superior performance in the 2-AA task. Additionally, hypothalamic orexinergic projections activate the mesolimbic reinforcement system (Peyron et al., 1998; Nambu et al., 1999; Korotkova et al., 2003; Borgland et al., 2006), which may favor the reinforcement of the instrumental response in the 2-AA task.

Another factor to consider is that SB is a non-competitive selective antagonist of OX1R, allowing for simultaneous binding of SB and Ox-A to the receptor (Malherbe et al., 2009). Since females have higher levels of Ox-A, the antagonist's effects could be more easily compensated, reducing or even neutralizing its functional effects. Moreover, it is still unclear whether females exhibit a greater expression of OX1R in areas of the active avoidance circuit. These aspects could be addressed in future studies, for instance, by employing higher doses of the antagonist or determining receptors' concentrations between sexes.

Conclusions

In conclusion, this experiment marks the first instance where post-acquisition infusions of a OX1R selective antagonist did not impair the acquisition of a 2-AA task in female rats. Future research should focus on the sex-specific effects of SB to elucidate the underlying neurobiological mechanisms responsible for these differences.

Experiment 4. Activation of extinction brain circuits in male and female rats.

Results from Experiments 2 and 3 show a sex-specific effect of the OX1R blockade on active avoidance conditioning. Moreover, we observed differences between SB and Veh in the spontaneous recovery test in males. Experiment 4, aimed to investigate whether these behavioral differences were mirrored in the neuronal activation of extinction-related brain areas, evaluated by c-Fos expression.

The investigation into neuronal activity following the spontaneous recovery test used the male rats from Experiment 2 and the female rats from Experiment 3. In these experiments' subjects received SB or Veh infusions after each acquisition session. Animals were euthanized within 15 to 30 min after completing the spontaneous recovery test.

4.1 Effects of OX1R blockade (SB) on c-Fos expression

For each region, the SB factor, the HEMISPHERE factor and the SB x HEMISPHERE interaction were analyzed. It is noteworthy that these two last factors were not statistically significant in any of the areas evaluated. Therefore, the graphical representation of the number of c-Fos positive neurons/mm2 corresponds to the average of both hemispheres.

Prefrontal Cortex

In both IL and PrL, there were no observed differences between SB and Veh groups neither in females (IL: U = 46.00, P = .526; Fig.40A; PrL: t_{18} = -1.350, P = .194; Fig.40C) nor in males. (IL: t_{15} = -.536, P = .600; Fig.40B; PrL: t_{15} = .250, P = .806; Fig.40D).

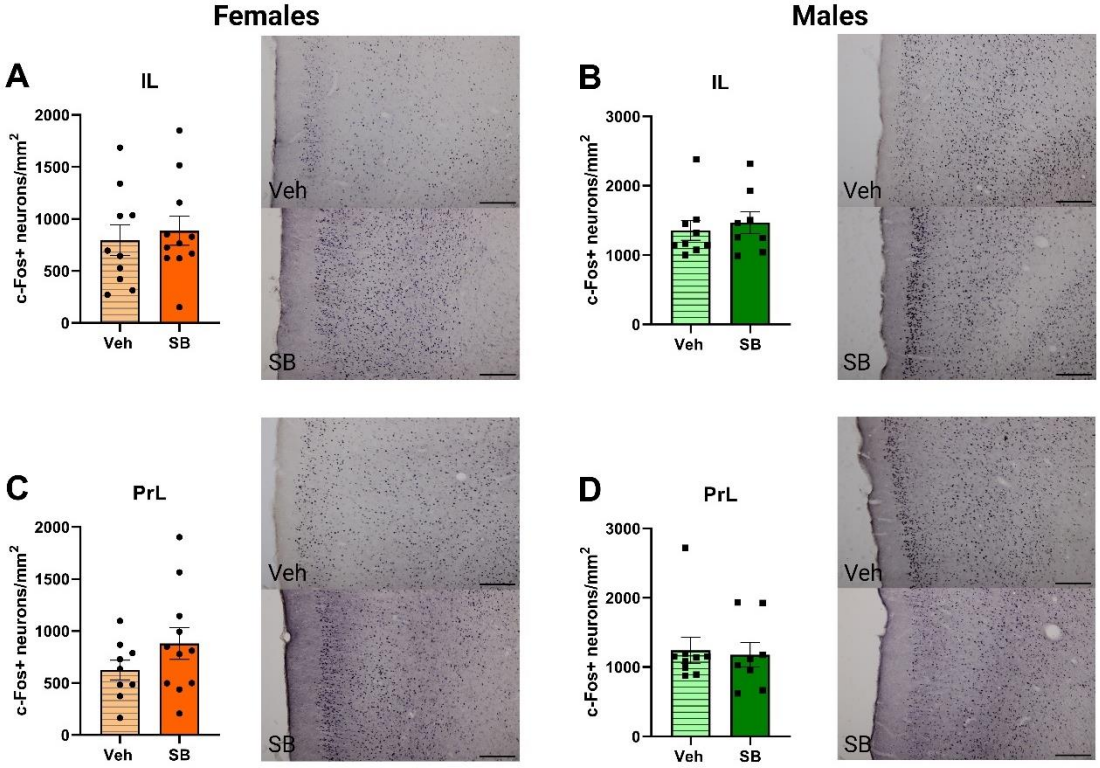


Figure 40. **Effects of SB on c-Fos expression in the prefrontal cortex**. c-Fos+/mm2 neurons in the IL cortex of **A**. female and **C**. male rats. c-Fos+ neurons in the PrL cortex of **B**. female and **D**. male rats. Microphotographs of each area are found to the right of each corresponding graph. Scale bar = 200 µm.

Basal Forebrain

No differences between groups were found in the septal area and the BNST in female rats (MS: t_{15} = -1.075, P = .299; DBB: t_{14} = -.645, P = .530; BNST: t_{15} = -1.309, P = .210) (Fig.41 A/C/E) or male rats (MS: U = 33.50, P= .810; BDB t_{15} = -.389, P = .703; BNST: t_{14} = .758, P = .461) (Fig.41 B/D/F).

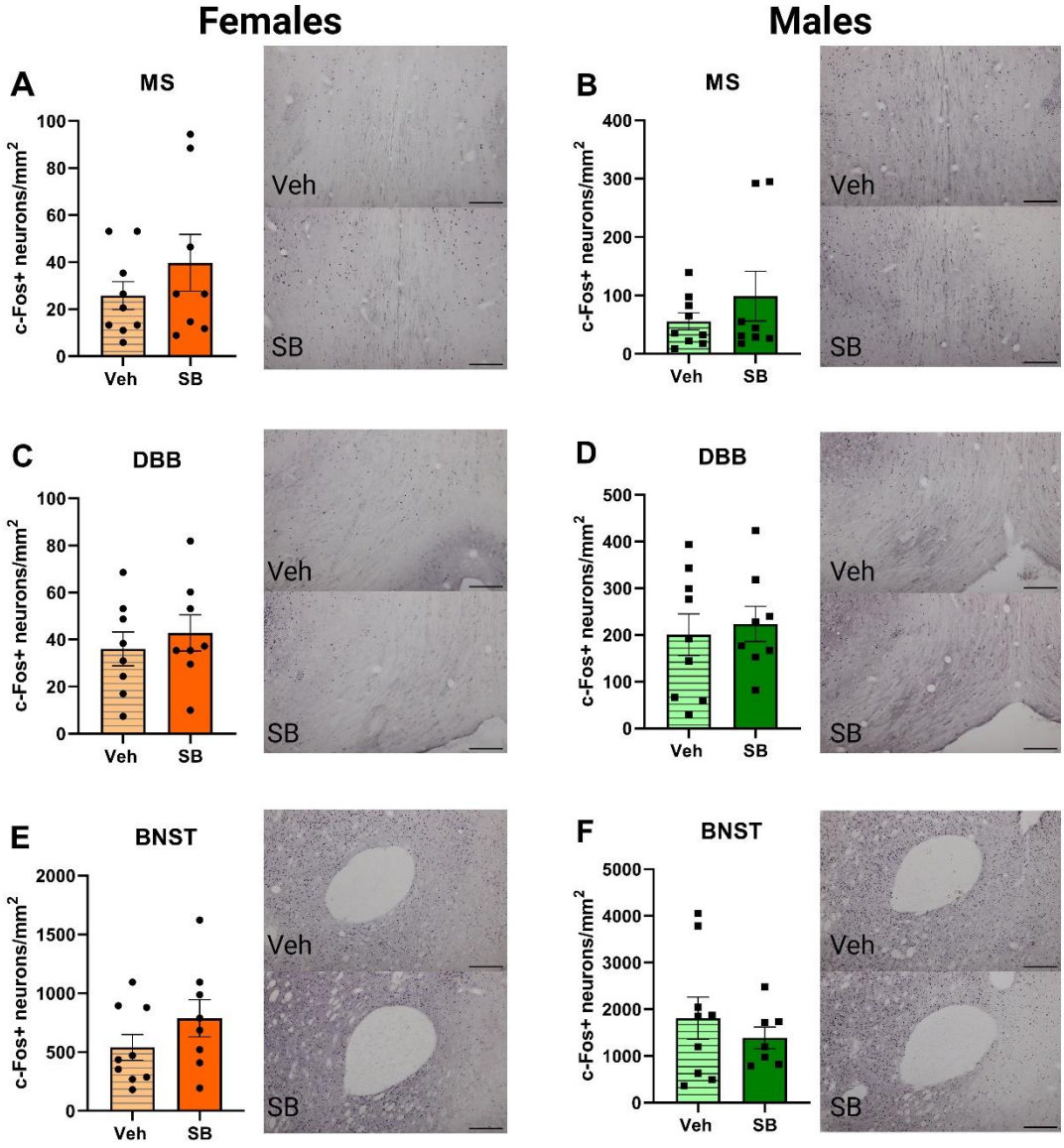


Figure 41. **Effects of SB on c-Fos expression in the basal forebrain**. c-Fos+/mm2 neurons in the MS, Diagonal band of Broca (DBB) and bed nucleus of the stria

terminalis (BNST). c-Fos+ neurons in the MS of **A**. female and **B**. male rats. c-Fos+ neurons in the BDB of **C**. female and **D**. male rats. c-Fos+ neurons in the BNST of **E**. female and **F**. male rats. Microphotographs of each area are found to the right of each corresponding graph. Scale bar = 200 µm.

Amygdala

No differences of c-Fos expression were found between groups in any region studied in the amygdala for female (LA: U = 25.50, P= .602; BLA: t_{11} = .028, P = .979; CeA: t_{14} = -.052, P = .959) (Fig.42 A/C/E) and male rats (LA: t_{15} = -.757, P = .461; BLA: t_{14} = 1.533, P = .147; CeA: t_{14} = -.052, P = .959) (Fig. 42 B/D/F).

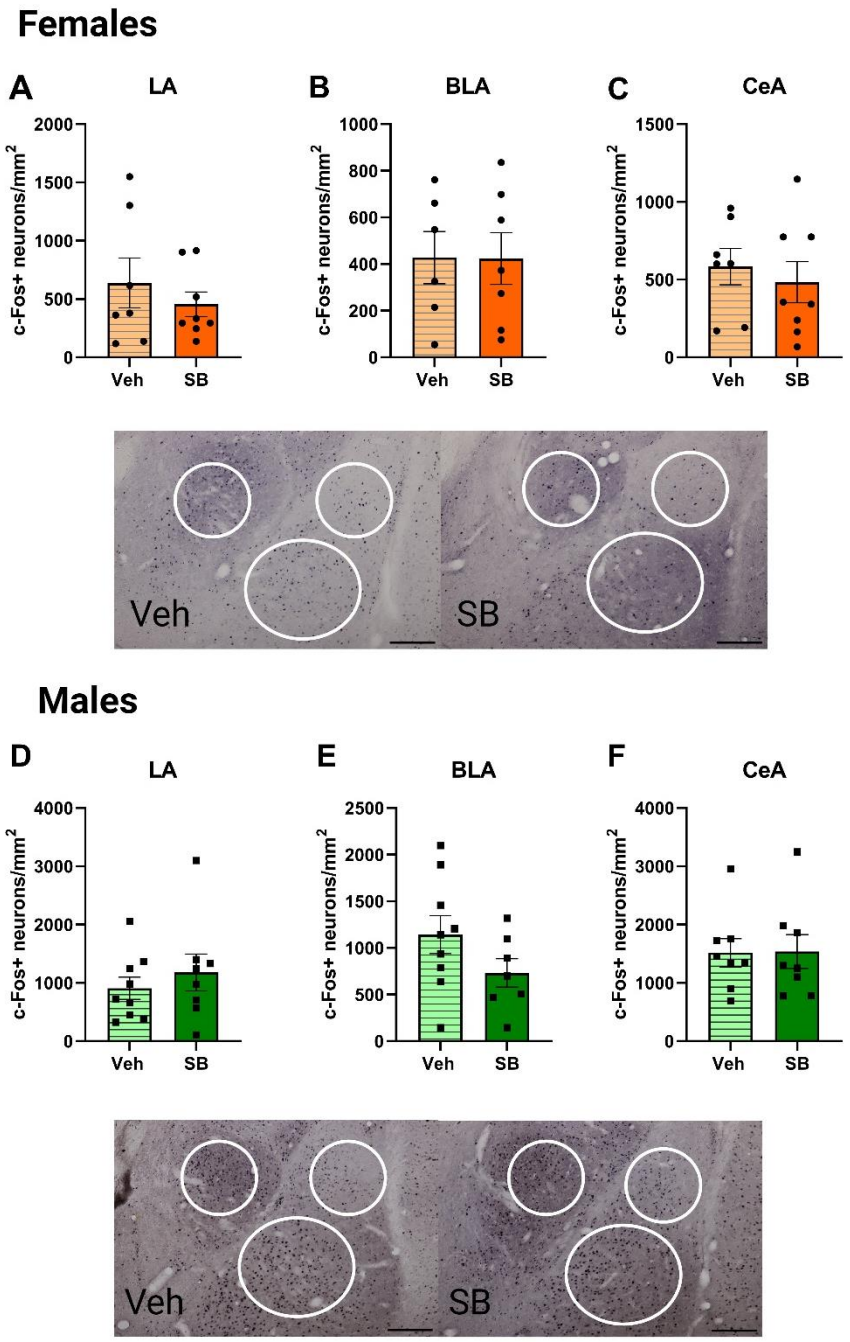


Figure 42. **Effects of SB on c-Fos expression in the amygdala**. c-Fos+/mm2 neurons in the lateral amygdala (LA) in **A**. females and **D**. males, basolateral amygdala (BLA) for females **B**. and **E**. males, and central amygdala (CeA) for **C**. females and **F**. males. Microphotographs of each area are found to the right of each corresponding graph. Scale bar = 200 μm.

Hippocampus

No differences between groups were found regarding c-Fos+ expression in the hippocampus in female (dCA1: U = 15, P= .132; dCA2: t_{12} = -.848, P = .413; dCA3: t_{11} = -.025, P = .980; DG: t_{16} = .601, P = .557) (Fig.43 A/C/E/G) or male rats (dCA1: t_{16} = .775, P = .450; dCA2: t_{13} = -.505, P = .622; dCA3: t_{15} = .223, P = .827; DG: t_{15} = 1.068, P = .302) (Fig.43 B/D/F/H).

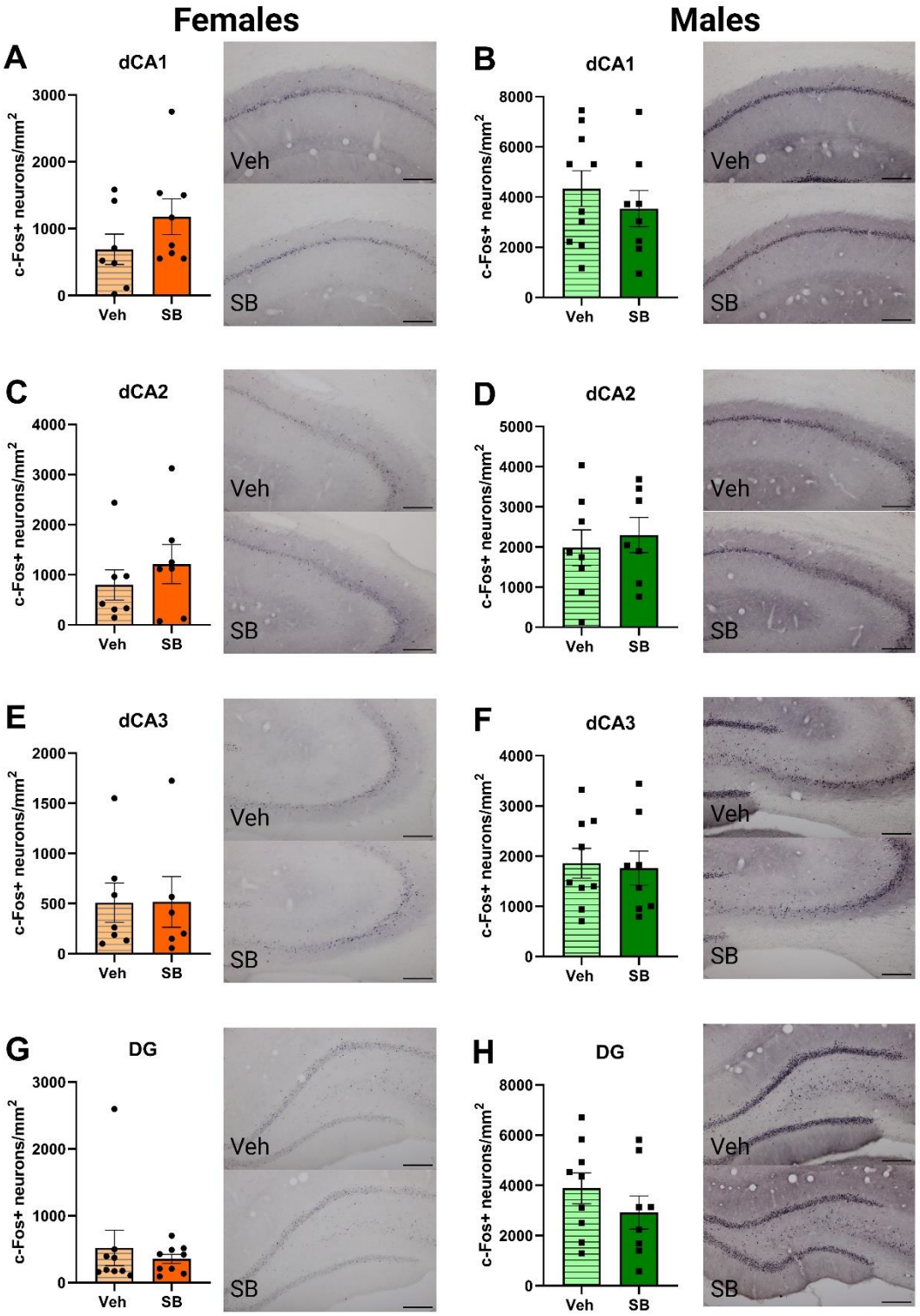


Figure 43. Effects of SB on c-Fos expression in the dorsal hippocampus. c-Fos+/mm2 neurons in the dorsal cornus ammonis 1 (dCA1), dorsal cornus ammonis 2 (dCA2), dorsal cornus ammonis 3 (dCA3), and dentate gyrus (DG). Microphotographs of each area are found to the right of each corresponding graph. Scale bar = 200 µm.

Finally, no differences between groups were found regarding c-Fos expression in the ventral hippocampus in female rats (vCA1: t_{16} = -.879, P = .392; vCA3: t_{14} = .499, P = .626) (Fig. 44 A/C) or in male rats (vCA1: t_{16} = -.1662, P = .116; vCA3: t_{16} = -.692, P = .499) (Fig. 44 B/D).

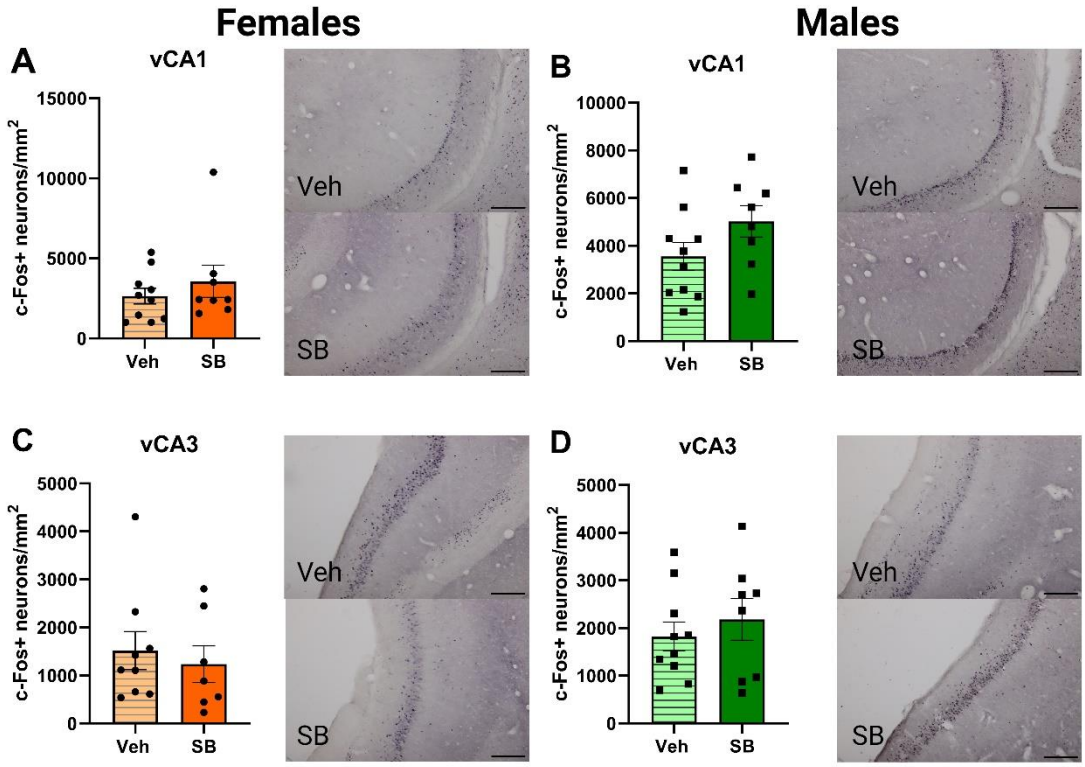


Figure 44. **Effects of SB on c-Fos expression in the ventral hippocampus**. c-Fos+/mm2 neurons in the cornus ammonis 1 (vCA1) for **A**. female and **B**. male rats, and ventral cornus ammonis 3 (vCA3) in **C**. female and **D**. male rats. Microphotographs of each area are found to the right of each corresponding graph. Scale bar = 200 µm.

4.2 Relations among c-Fos expression in the brain circuit involved in active avoidance extinction in males and females

Due to the observed differences in active avoidance behavior between sexes, it is reasonable to explore whether there are functional differences in the brain circuit underlying the extinction of active avoidance between males and females.

To compare the relationship between brain areas underlying 2-AA extinction across sexes, we conducted a principal components analysis (PCA, oblimim rotation) using c-Fos+ immunohistochemistry outcomes. To streamline the analysis, we only included regions with highly correlated neural activity between them. The final

items included in the PCA were the prefrontal cortex (average of PrL and IL c-Fos+ expression), septal area (septum and BDB), BNST, dorsal hippocampus (dCA1, dCA2, dCA2 and DG), ventral hippocampus (vCA1 and vCA3) and amygdala (LA, BLA and CeA).

In order to assess similarity or difference between the components obtained by males and females, we calculated Tucker's congruence coefficient. For example, comparing the first components obtained by males with the first component obtained by females. Values higher than 0.80 indicate analogous components, whereas values under 0.30 suggest non-analogous components.

Males

Bartlett's sphericity test was statistically significant (P = .002). A 3-components model explained 80.35 percent of the variance. Component 1 included two items: septal area and dHPC. Component 2 consisted of one item: the PFC. Component 3 included three items: BNST, AMY and vHPC (Fig.45A/C).

Females

Barlett's sphericity test was statistically significant (P = .006). A 3-components model explained 83.35 percent of the variance. Component 1 included two items: septal area and BNST. Component 2 consisted of two items: PFC and dHPC. Component 3 included two items: AMY and vHPC (Fig.45A/C).

Tucker's congruence coefficient of the first and second components in females and males (C1m-C1f = 0.41; C2m-C2f = -0.74) concluded that these two components were not analogous. Conversely, the Tucker's congruence coefficient for the third component (C3m-C3f = 0.80) demonstrated that these circuits were functionally similar (Fig. 45B).

A

Males

	Component		
	1	2	3
PFC	-0.01	0.95	0.01
Septal area	0.96	-0.09	-0.11
BNST	-0.03	-0.36	0.68
dHPC	0.85	0.09	0.14
AMY	0.46	0.16	0.64
vHPC	-0.02	0.13	0.85

Females

	Component		
	1	2	3
PFC	-0.01	-0.98	-0.07
Septal area	0.76	-0.13	0.05
BNST	0.95	0.08	-0.02
dHPC	0.01	-0.87	0.10
AMY	-0.03	0.06	0.97
vHPC	0.07	-0.11	0.79

B

	C1m-C1f	C2m-C2f	C3m-C3f
Tucker's congruence coefficient	0.41	-0.74	**0.80**

C

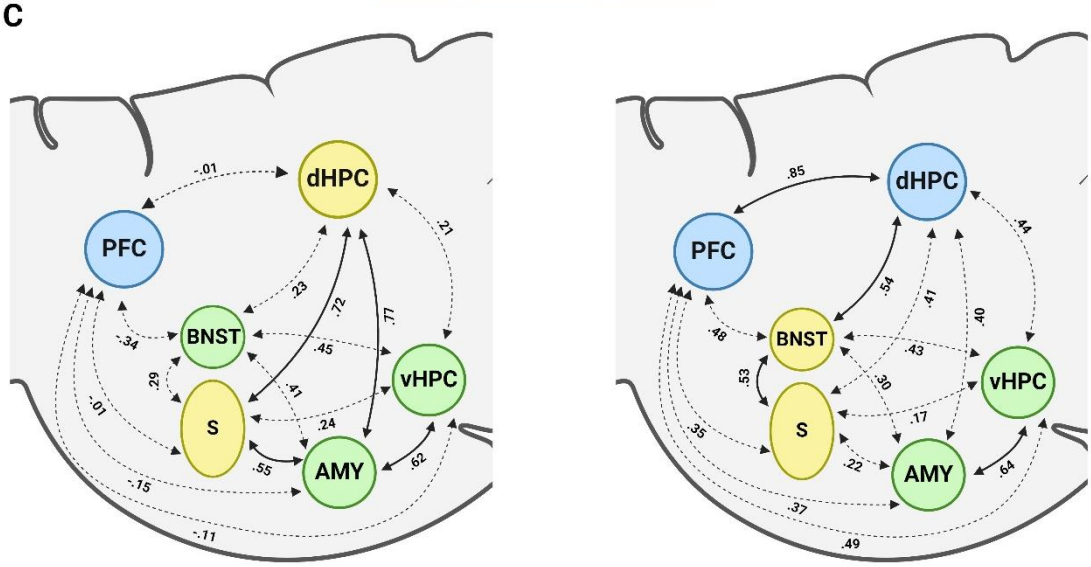


Figure 45. Sex differences on neuronal activation patterns in extinction-related brain areas. **A**. Components for male and female rats. Colors correspond to each of the three components obtained in the PCA. **B**. Tucker's congruence coefficient values for the three components of male and female rats (C1m-C1f, C2m-C2f, C3m-C3f). **C**. Anatomical representation showing correlation values between the different areas according to the observed c-Fos protein expression after the RS test. p <0.05 depicted with a bold line, p>0.05 depicted in a dashed line. Abbreviations: AMY: amygdala; BNST: bed nucleus of the stria terminalis; dHPC: dorsal hippocampus; PFC: prefrontal cortex; S: septum; vHPC: ventral hippocampus.

Statistics analysis of the brain circuits activation among sexes reveals differences between them. Interestingly, the component showing a high index of similarity between sexes corresponds to brain areas associated with emotional responses (AMY and vHPC). However, differences arise when assessing the similarity of circuits of contextual control.

4.3 Correlations between the spontaneous recovery test performance and c-Fos immunohistochemistry outcomes

Males. Among Veh males, a significant positive correlation was found between the c-Fos+ neurons in the BNST and the mean avoidances performed in the spontaneous recovery test ($r(7) = .790$, $P = .011$) (Fig.46B). Conversely, in SB males, negative correlations were observed between latency in the first 10-block trial of the SR, and c-Fos levels in the BNST ($r(5) = -.964$, $P < .001$) (Fig.46C).

Females. Due to the lack of significant differences in SR test between the SB and Veh groups, the study of correlations was carried out for the entire sample. In female rats, a significant negative correlation was found between the expression of c-Fos+ neurons in the BNST and the avoidances performed in the first 10-block of the spontaneous recovery session ($r(15) = -.484$, $P = .049$) (Fig.46A).

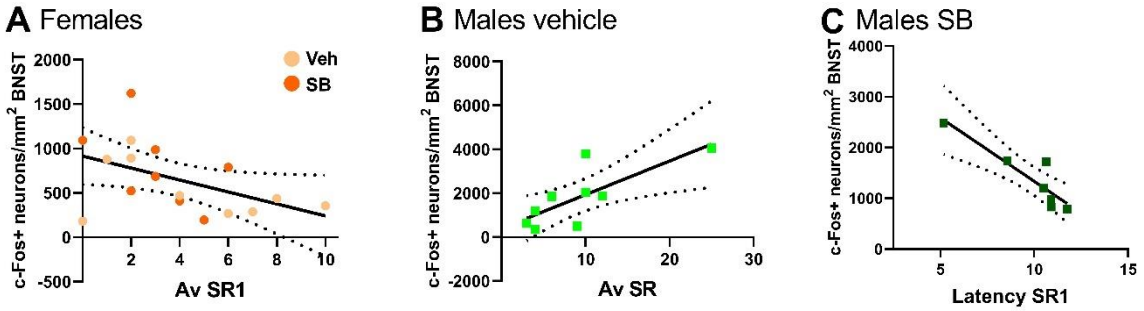


Figure 46. Scatterplots depicting correlations between c-Fos+ neurons/mm2 and behavioral variables of the spontaneous recovery test. **A**. BNST and mean avoidances in the first 10-trial block for both female groups. **B**. BNST and mean avoidances for the SR session for vehicle males. **C**. BNST and latencies for the first 10-trial block of the SR for SB males.

4.4 Discussion

The results of this experiment show that OX1R selective blockade during the acquisition of a 2-AA task did not affect c-Fos expression in extinction brain circuits, assessed after the remote spontaneous recovery test, in either males or females.

No differences in c-Fos expression between SB and Veh groups

Since no behavioral differences were found between the female SB and Veh groups in the spontaneous recovery test (Experiment 3), we did not expect to find differences in neuronal activation either. Regarding males, despite exhibiting behavioral differences at the beginning and during the progression of the spontaneous recovery test, no differences in c-Fos expression were observed.

There are no experimental precedents assessing the effects of contingent SB administration on the acquisition of the 2-AA task and the neuronal activation following this session. Nevertheless, existing research has looked into the role of OX1R in fear conditioning and c-Fos expression in the locus coeruleus (LC), which is heavily innervated by orexin neurons and is where the strongest OX1R expression is found, specifically in NA^{LC} neurons (Mieda et al., 2011; Marcus et al., 2001). Studies have highlighted the involvement of OX1R in the LC in fear expression, fear memory consolidation, and the maintenance of behavioral fear (Sakurai et al., 2014; Soya & Sakurai, 2020). Preceding research has demonstrated that SB administration before training reduces the effects of excitatory optical stimulation in the LC, albeit without observable effects on a neuronal level (Sears et al., 2013). It has also been reported that c-Fos expression is significantly lower in the NA^{LC} neurons of OX1R-/- mice after performing cued and contextual FC tests (Soya et al., 2013). Furthermore, pharmacogenetic and optogenetic inhibition of NA^{LC} neurons have been proven to impair fear learning and decrease c-Fos expression in the LC, while optogenetic excitation induces opposite effects, with SB mitigating the effects of optogenetic stimulation (Soya et al., 2017). In this experiment, c-Fos expression in the LC was not assessed, since our main goal was studying the activity of extinction-related brain circuits. However, in future studies investigating the involvement of OX1R in the acquisition of a 2-AA task, it would be pertinent to evaluate LC activity to validate findings from fear conditioning paradigms.

One possible explanation for the lack of differences in c-Fos expression in extinction-related areas could be that males did not undergo a true extinction process. Their good performance in the extinction is more likely due to a low level of conditioning

rather than the acquisition of a new contingency competing with the previous one. Consequently, it is not surprising that differences in neuronal activation in brain areas involved in extinction learning were not detected.

Lastly, while we cannot completely rule out methodological issues in the immunohistochemistry study, the results obtained from the principal components analysis demonstrate sufficient congruence and coherence, mitigating concerns regarding this possibility.

Differences between sexes in the functional components of extinction brain circuits

The principal components analysis revealed that the extinction of the 2-AA task involves one of the three components obtained, similarly for both males and females. Specifically, this component is composed of the amygdala and ventral hippocampus, areas associated with emotional response (Martínez-Rivera et al., 2019; Rosas-Vidal et al., 2018). On the other hand, the component composed of the septal area is the most dissimilar between sexes. In males, a strong relationship is observed between the activity of the septal area and the dorsal hippocampus, whereas in females, a strong relationship is found between the septal area and the BNST. Another non-analogous component between males and females is the one formed by the prefrontal cortex, which is related to the inhibitory regulation of the amygdala (Bravo-Rivera et al., 2014; Jiao et al., 2015; Tapias-Espinosa et al, 2018). However, unlike the septal area component, this component demonstrates a Tucker's coefficient that closely approaches the analogy criterion. In males, this component is composed of the prefrontal cortex, whereas in females, it includes both the prefrontal cortex and the dorsal hippocampus.

Therefore, similarities between sexes are observed regarding the components related to emotion and the cholinergic component of learning. However, sex differences are most evident in the differential involvement of two brain areas: the dorsal hippocampus and the BNST.

The dorsal hippocampus is a structure involved in conditioned fear memory (Maren et al., 2013; Phillips & LeDoux, 1992) and contextual control of extinction retrieval (Bouton et al., 2006; Corcoran & Maren, 2004). In males, the dorsal hippocampus is part of the same circuit as the septal area, composed of the medial septum and the diagonal band of Broca. The cholinergic input from the MS and the DBB to the dHPC is the exclusive source of acetylcholine in the HPC (Mesulam et al., 1983; Woolf et al., 1983), and it is critical for the Pavlovian contextual fear conditioning and its extinction (Knox & Keller, 2016; Tronson et al., 2009). Thus, the circuit involving the dorsal hippocampus and septal area in males serves as the necessary cholinergic input to the HPC in extinction learning mediation. Conversely, in females, the dorsal hippocampus is located within the same circuit as the prefrontal cortex. It is not surprising to observe a functional relationship between the prefrontal cortex and the dorsal hippocampus in the extinction of fear memories, since the scientific literature suggests that extinction requires a tripartite neural circuit involving the amygdala, the prefrontal cortex and the hippocampus (Bouton et al., 2021).

The differential implication of the dorsal hippocampus in males and females suggests that contextual control could have different implications depending on sex. Indeed, studies conducted in contextual FC exhibit differences between sexes, even yielding contradictory results. Some studies indicate that females achieve higher levels of conditioning (Keiser et al., 2017; Moore et al., 2010), while others suggest that they attain lower levels of conditioning (Maren et al., 1994; Wiltgen et al., 2001; Gresack et al., 2009; Ribeiro et al., 2010; Daviu et al., 2014, Clark et al., 2019). Future studies should aim to determine the sex-specific contextual implication in the extinction of fear memories, specifically in the 2-AA task.

The second brain area that shows higher functional differences between sexes is the BNST, part of the extended amygdala and a sexually dimorphic structure (Bauer, 2023). It is involved in long-term, unpredictable sustained anxiety (Avery et al., 2016). The BNST serves as an interface between the prefrontal cortex, the amygdala, hypothalamus and hippocampus, integrating information and contributing to autonomic, endocrine and behavioral responses (Goode & Maren, 2017). Furthermore, it plays a role in discriminating between safe and unsafe

environments, indicating its influence over contextual information processing (Alvarez et al., 2011).

In our experiment, the BNST was found to be functionally involved with the septal area in females, and with the amygdala in males. The correlation analysis supports this differential role of the BNST in the two sexes. In males, high levels of c-Fos in the BNST correlate with a worse performance in the spontaneous recovery test, characterized by increased avoidance responses and reduced latencies. This suggests that the functional similarity between the BNST and the amygdala impedes an effective extinction when emotional activation is high. In fact, extinction deficits are observed when inactivating the IL, a brain region that inhibits amygdala's activity during extinction training (Sierra-Mercado et al., 2011). Conversely, in females, high c-Fos levels correlate with a better extinction performance, and interestingly, the BNST shares the same component and direction as cholinergic regions of the basal forebrain. The synergic action of these regions could explain the enhanced extinction learning in females (Knox & Keller, 2015).

The regions of the BNST analyzed in this thesis included the anterior and ventral portions of the medial division and the ventral portion of the lateral division. The anterior BNST region is involved in fear and anxiety and it is the main termination zone of CeA axons (Gungor & Paré, 2016; Krettek & Price, 1978). Given that the anterior BNST can be divided in three sectors (anterolateral, anteromedial and anteroventral) and that they receive distinct inputs and contribute to differential projections, their distinct role in extinction of avoidance behavior should be considered in future studies.

While the ideas presented here offer insights into qualitative differences in the functioning of extinction brain circuits between males and females, caution is warranted in interpreting these findings. Comparing the expression of c-Fos between males and females would be of great interest, as male rats obtained a higher number of c-Fos positive neurons in all areas studied in absolute terms. Unfortunately, methodological limitations of the experiment prevent us from making a direct comparison between males and females, including differences in

experimental timelines and immunohistochemistry procedures conducted in separate weeks due to the large number of samples collected and the various areas studied.

Conclusions

In conclusion, this experiment shows that the selective blockade of OX1R during the acquisition did not affect c-Fos expression in brain extinction circuits. However, it suggests the possibility of functional sexual differences within these circuits.

Experiment 5. Effects of the estrous cycle on avoidance behavior.

Previous experiments have revealed that acquisition and neuronal activation levels in extinction-related circuits of female rats remained unaffected by OX1R selective blockade. However, it is well-established that female hormonal levels can influence fear response. Despite this knowledge, little is known about how these hormonal fluctuations impact active avoidance behavior. In this experiment we aim to evaluate the influence of the estrous cycle on both the behavior outcomes and the expression of c-Fos in female rats from Experiment 3.

Before starting the behavioral procedure (two days post surgery until three days before starting handling sessions), we assessed the estrous cycle of the experimental subjects in order to confirm the validity of the cytology method. The behavioral timeline mirrors that of Experiment 3, as shown in Figure 49, but it also depicts the collection of vaginal smears (as shown by the purple segment between surgery and handling sessions).

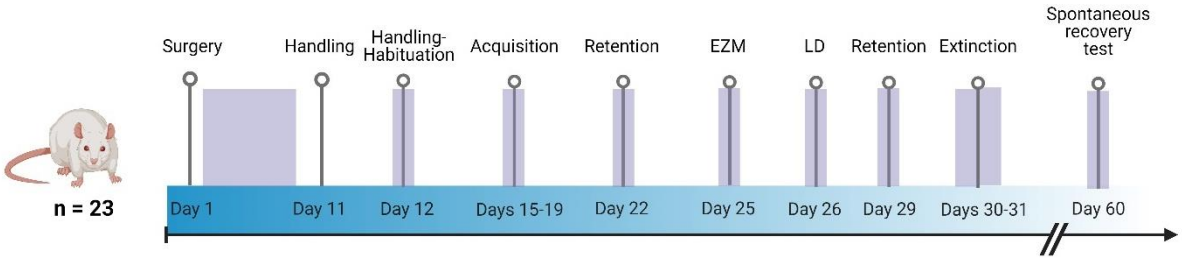


Figure 47. **Timeline of the experimental procedure with vaginal smears study**. Timeline of the experiment with every test performed. All the purple segments depict the days in which vaginal smears were performed. A total of 20 samples were obtained from each subject. The two forward slashes represent the 28 days elapsed between the last extinction session and the performance of the spontaneous recovery test.

The total sample consisted of 23 female Wistar rats, corresponding to the subjects from Experiment 3.

Some difficulties arose during the assessment of the phase of the estrous cycle due to the small sample size, which resulted in non-homogeneous groups for the four stages of the estrous cycle (estrous, proestrus, diestrus, and metestrus). Consequently, the five standardized categories used to classify the stages of the estrous cycle were grouped according to high or low hormone levels: metestrus and diestrus taken together (Met/Diest), estrous and metestrus (Estrus/Met), estrous, the end of estrous and beginning of proestrus (Estrus/Proestrus) and proestrus (see Table 4).

The results obtained on Experiment 3 showed that SB did not affect the 2-AA task. Consequently, the influence of the ESTROUS (high or low hormone level) was studied considering all subjects without dividing the sample into SB and Veh groups.

Estrous cycle phases	Final classification
Metestrus/Diestrus	Low hormone levels
Estrus-Metestrus	
Estrus	High hormone levels
Estrus-Proestrus	
Proestrus	

Table 4. Classification of the stages of the estrous cycle in groups for statistical analysis.

5.1 Acquisition and retention of the 2-AA task

Each of the rats were classified according to the phase of their estrous cycle on the first day of acquisition of conditioning (ESTROUS-A1: HIGH or LOW hormonal levels). As depicted in Fig. 48 A/D, hormonal levels did not have any effect on the performance of the acquisition sessions regarding mean avoidances (ESTROUS-A1: $F_{1,21}$= .178, P = .678; ESTROUS-A1 × SESSION: $F_{4,84}$= .702, P = .593), or latencies of response (ESTROUS-A1: $F_{1,21}$= .981, P = .981; ESTROUS-A1 × SESSION: $F_{4,84}$= .814, P = .489).

Each of the rats were classified according to the phase of their estrous cycle on the day of the RT-3d and the RT-10d (ESTROUS-RT-3d: HIGH or LOW hormonal levels, and ESTROUS-RT-10d: HIGH or LOW hormonal levels).

Similarly, no significant effect of ESTROUS-RT-3d was observed on the two retention sessions. As depicted in Fig. 50 B/E, no differences between groups were found in the RT-3d retention session regarding mean avoidances (ESTROUS-RT-3d: $F_{1,21}$= .186, P = .671; ESTROUS-RT-3d × BLOCK: $F_{1,21}$= .320, P = .578) or latencies of response (ESTROUS-RT-3d: $F_{1,21}$= .205, P = .656; ESTROUS-RT-3d × BLOCK: $F_{1,21}$= 1.123, P = .301). In addition, no differences between groups were found in the RT-10d regarding mean avoidances (ESTROUS-RT-10d: $F_{1,19}$= .073, P = .974; ESTROUS-

RT-10d × BLOCK: $F_{6,38}$= .320, P = .923) or latencies of responses (ESTROUS-RT-10d: $F_{1,19}$= .247, P = .862; ESTROUS-ac1 × BLOCK: $F_{6,38}$= .310, P = .868).

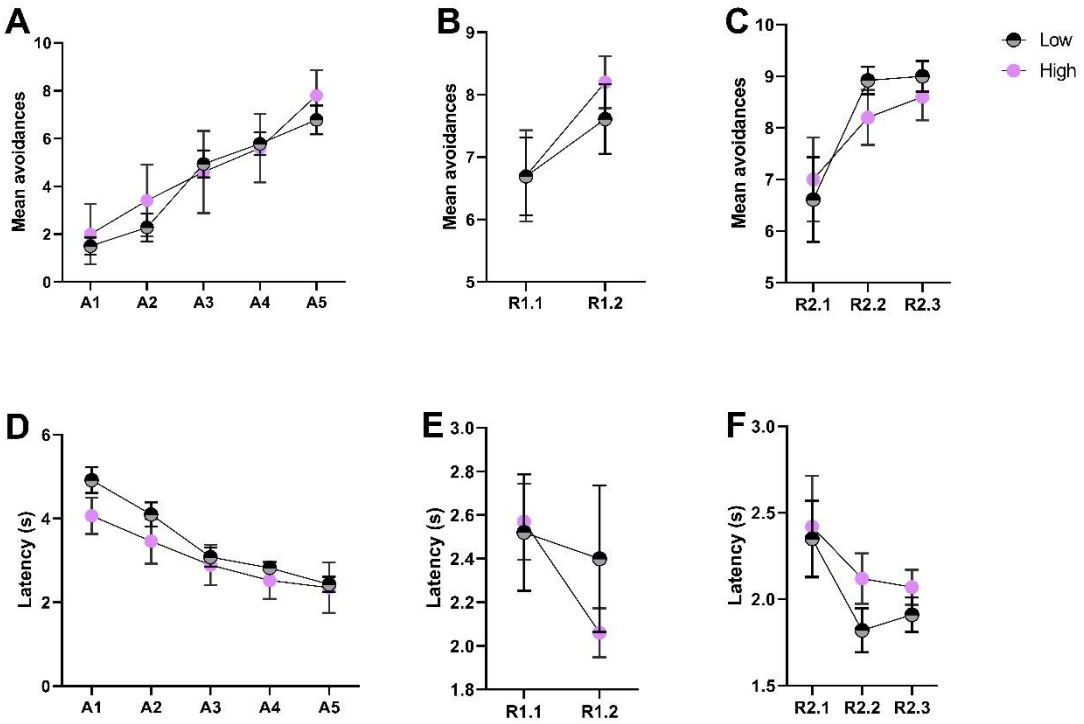


Figure 48. **Estrous cycle effects on the acquisition and retention of the 2-AA task**. Mean avoidances regarding hormonal levels in the Acquisition 1 session for the **A**. five acquisition sessions, **B**. the first retention session and **C**. the second retention session. Latencies of response regarding hormone levels for the **D**. five acquisition sessions, **E**. the first retention session and **F**. the second retention session. The data from the acquisition session was analyzed according to the hormone levels of A1, while the data from the two retention sessions was analyzed according to the hormone levels of each day (RT-3d and RT-10d).

5.2 Extinction of the 2-AA task

Interestingly, hormonal levels present on the day of the first extinction session (ESTROUS-E1) seemed to affect the performance of the extinction phase. As shown in Fig. 49 A/B/C, when rats were classified according to the phase of their estrous cycle on the first day of extinction (ESTROUS-E1), females with high hormone levels performed significantly less avoidances in E1 and E2 than females with low levels (ESTROUS-E1: $F_{1,21}$= 4.476, P = .046), regardless of the block (ESTROUS-E1 × BLOCK: $F_{9,189}$= 1.041, P = .396), as well as higher latencies of response (ESTROUS-E1: $F_{1,21}$= 5.148, P = .034; ESTROUS-E1 × BLOCK: $F_{9,189}$= 1.733, P = .141) and more non-responses (ESTROUS-E1: $F_{1,21}$= 7.099, P = .015; ESTROUS-E1 × BLOCK: $F_{9,189}$=

2.067, P = .080). No effects on the extinction performance were observed when analyzing the behavioral outcomes based on the hormone levels of the second extinction day (E2).

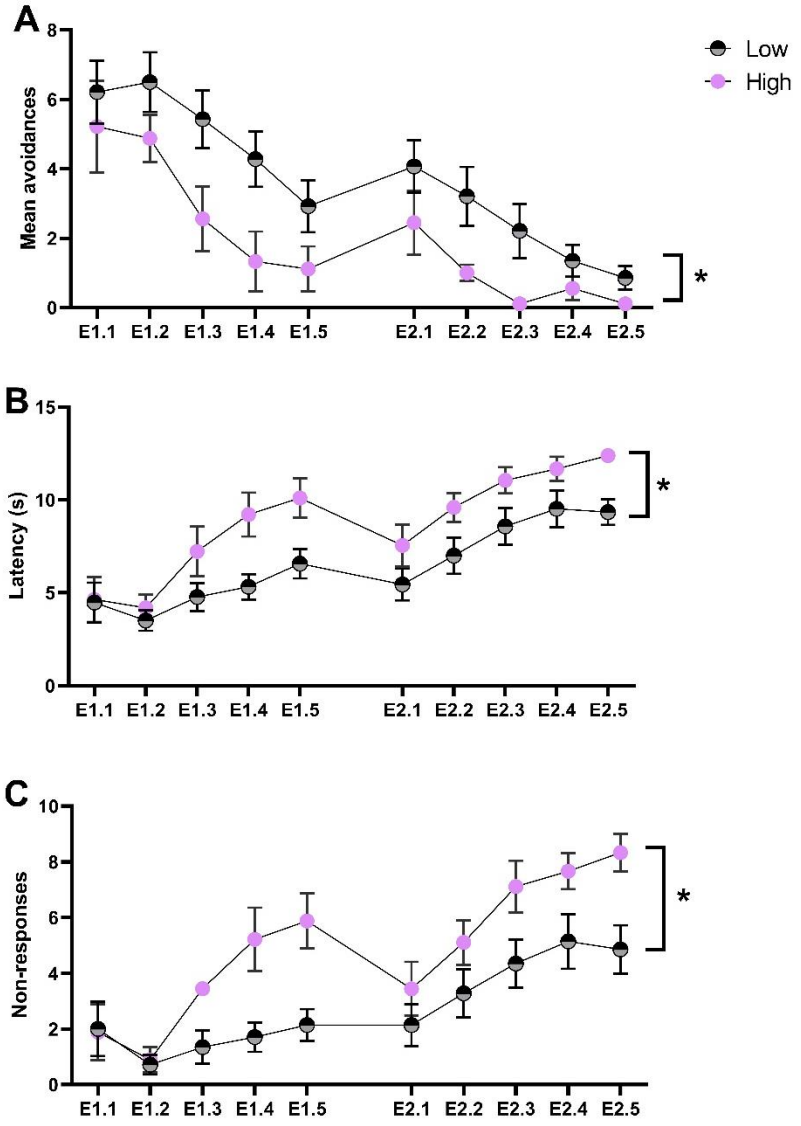


Figure 49. **Estrous cycle effects on the extinction of the 2-AA task**. **A**. Mean avoidances, **B**. latencies of response and **C**. non-responses shown by females with high and low hormonal levels over the course of the 10-trial blocks comprising the two extinction sessions, E1 and E2.

5.3 Spontaneous recovery test of the 2-AA task

As shown in Fig. 50 A-C, hormonal levels on the day of the test did not affect their performance regarding mean avoidances (ESTROUS-SR: $F_{1,21}$= .007, P = .933; ESTROUS-SR × BLOCK: $F_{4,84}$= .396, P = .666), latencies of response (ESTROUS-SR:

$F_{1,21}$= .614, P = .442; ESTROUS-SR × BLOCK: $F_{4,84}$= .330, P = .753) or non-responses (ESTROUS-SR: $F_{1,21}$= 1.174, P = .291; ESTROUS-SR× BLOCK: $F_{4,84}$= .312, P = .869).

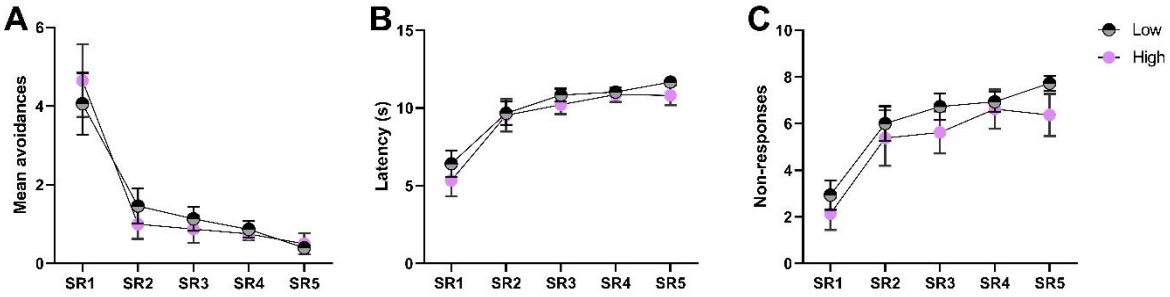


Figure 50. **Estrous cycle effects on the spontaneous recovery of the 2-AA task**. Hormonal levels differences regarding **A**. mean avoidances, **B**. latencies of response and **C**. non-responses shown by females with high and low hormonal levels over the course of the 10-trial blocks comprising the SR session.

5.4 Neuronal activation after the spontaneous recovery test

Some effects of the hormonal levels on the spontaneous recovery test day (ESTROUS-SR) on the neuronal activation at the end of the session. As depicted in Fig. 51 A-H, female with high hormone levels presented higher neuronal activation in PrL (d = -.68) and in IL cortex (d = -.75), as well as in BDB (*U*= 11.50, P = .034), MS (d = -.60), BNST (d = -.58), DG (-.78), dCA2 (d = -.68) and dCA3 (d = -1.03) compared to females with low hormone levels. No differences regarding c-Fos expression in any region of the amygdala were found between females with low and high hormone levels.

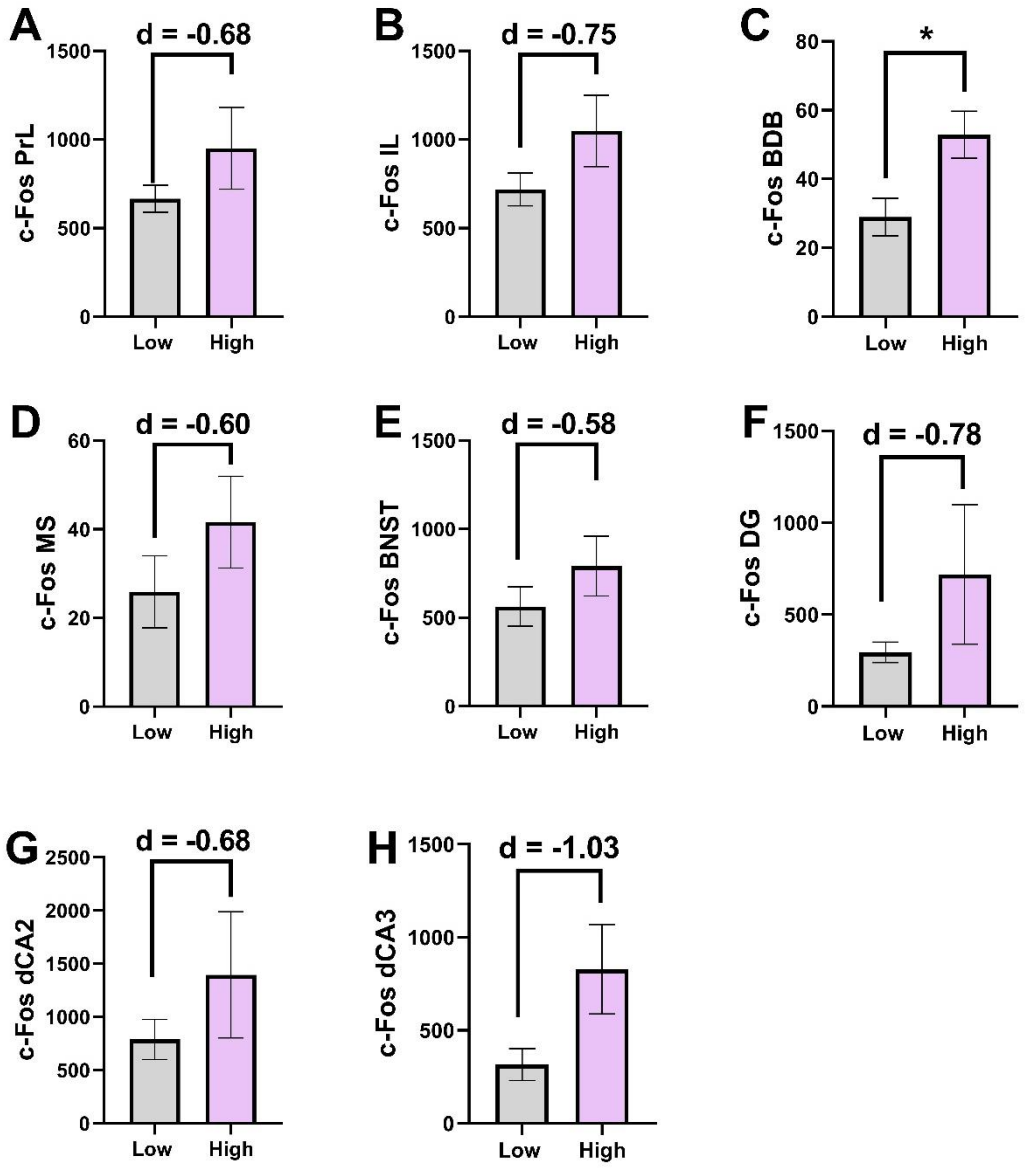


Figure 51. **Estrous cycle effects on c-Fos expression**. c-Fos+ neurons/mm2 for the **A**. prelimbic cortex, **B**. infralimbic cortex, **C**. Broca's diagonal band, **D**. medial septum, **E**. bed nucleus of the stria terminalis, **F**. dentate gyrus, **G**. dorsal CA2 and **H**. dorsal CA3. d = Cohen's d analysis. Values around 0.5 represent a medium effect size and values greater than 0.8 represent a large effect size.

5.5 Non-conditioned fear and anxiety

Some differences between females with low and high hormonal levels were observed in some variables assessed in the EZM when using effect size analysis. The value obtained from Cohen's d analysis represents the interpretation of the effect size. Values around 0.2 represent a small effect size, values around 0.5 represent medium effect size, and values around 0.80 or higher represent large effect size.

Females with higher hormone levels on the day they performed the EZM spent more time in the open arms (d = -0.73) (Fig.52A), performed more rearings in the open arms (d = -.74) (Fig.52B), and performed more deep headings (d = -.62) (Fig.52C) in comparison females with lower hormonal levels. These results are obtained from effect size analysis, so they should be taken with extreme caution. Further studies with higher sample sizes for each group should explore these effects in depth.

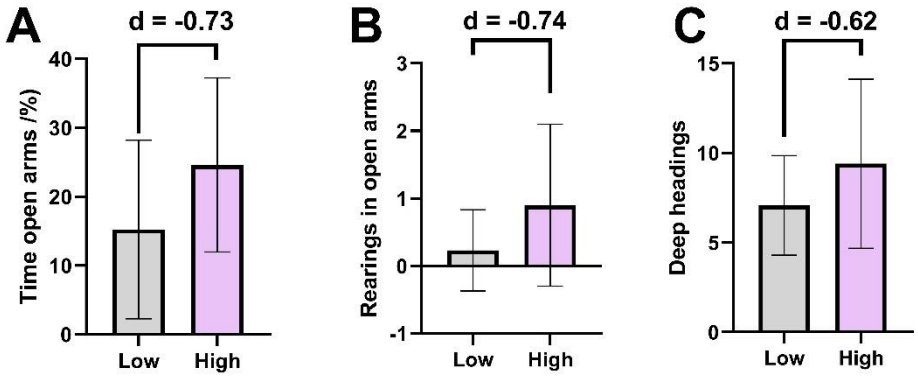


Figure 52. **Effects of the Estrous cycle effects stage on performance on the elevated zero maze.** **A**. Percentage of time spent in the open arms. **B**. Rearings performed in the open arms. **C**. Deep headings performed. Cohen's d value is depicted in each graph.

No differences between the groups with low hormonal levels and high hormonal levels were observed regarding the variables assessed for the LD test.

5.6 Discussion

The results of this experiment demonstrate that sexual hormone levels in females modulate the extinction of a 2-AA task and the expression of non-conditioned fear and anxiety.

Estrous cycle does not affect the acquisition and retention of the 2-AA task

The results obtained seem to indicate that the sexual hormone levels on the first day of the acquisition do not influence the conditioning of the 2-AA task using a distributed procedure. These results do not align with findings of other studies where the conditioning of an active avoidance task is affected by the estrous phase

of the animals. However, the relationship between hormonal levels and the level of conditioning remains unclear, as one study observes better performance when animals are undergoing diestrus (Díaz-Veliz et al., 1989), while another study observes it in proestrus (Sfikakis et al., 1978). Remarkably, these studies involve massed procedures of training, subjecting animals to a high number of trials on the same day of hormone levels determination. Therefore, the low number of trials in the first session of the distributed training used in our experiment could explain the lack of estrous cycle effects on performance. Future studies could consider increasing the sample size and implementing a daily collection of vaginal samples throughout all the acquisition sessions to explore this further.

Differences in retention levels based on hormone levels are also not observed. The fact that there is a high positive correlation between performance in the last acquisition session and the retention sessions (Spearman correlation: $r > .88$, $P < .001$, data not shown) indicates that retention levels primarily depend on the levels achieved during acquisition rather than the stage of the estrous cycle during retention.

<u>High sexual hormone levels facilitate the extinction of a 2-AA task</u>

The present results demonstrate that females undergoing proestrus on the first extinction session exhibit better extinction. These findings align with other studies indicating improved extinction of conditioned fear during the proestrus phase in mice (Rey et al., 2014; Gruene et al., 2015b) and extend these observations to a more complex task, such as 2-AA.

These outcomes corroborate the proven effects of estradiol on cognition. Estradiol facilitates hippocampus and prefrontal cortex dependent tasks, which correspond to an extinction training, and impair the performance of striatum-dependent tasks, which correspond to acquisition (Davis et al., 2005).

Multilevel effects of high sexual hormone levels

Sexual hormone levels have also shown to affect other aspects in this experiment apart from avoidance behavior.

Firstly, high sexual hormone levels appear to decrease anxiety levels, as observed in the variables "time spent in open arms, rearings in open arms and deep headings" performed in the EZM. These results align with the scientific literature where estradiol has demonstrated anxiolytic effects (Best et al., 1992; Arpels, 1996). Furthermore, the obtained results mimic those in animal research, where rats in proestrus spend more time in the open arms of the EPM (Díaz-Veliz et al., 1997; Marcondes et al., 2001). Additionally, other studies have corroborated these findings using estradiol administration, which counteracts the anxiogenic effects of phases with lower hormone levels (Hiroi & Neumaier, 2006; Walf & Frye, 2006). It has been hypothesized that the effects of sex hormones on anxiety could be explained by the modulation of neurotransmitter synthesis and the activity of receptors involved in anxiolytic effects (Genazzani et al., 2007).

Secondly, high sexual hormone levels seem to increase neuronal activity in the extinction brain circuit, as shown by higher c-Fos expression in prefrontal cortex, septal area and dorsal hippocampus. However, it should be noted that the sample size has been a significant limitation in this experiment. In some cases, effect size analyses had to be employed to analyze the data. In future studies, it would be beneficial to address this limitation, and to consider the estrous cycle stage of the experimental subjects. This would allow researchers to determine the optimal timing for each phase of the task, aiming to achieve homogeneous sample sizes across the four estrous cycle phases and enable more conclusive statistical analyses. However, the use of the 2-AA task complicates these methodological modifications, as it is a long and multi-session learning task.

If confirmed in larger samples as well as in humans, these results could inform personalized exposure or desensitization therapies in the clinical field, taking into consideration sexual hormone levels.

Conclusions

In conclusion, the results of this study highlight the significance of the hormone levels in female rats for the expression of non-conditioned fear and anxiety, as well as for the extinction of an instrumental task involving emotional memory.

Experiment 6. Effects of OX1R blockade on the extinction of a 2-AA task in males and females.

Results from Experiment 2 and Experiment 3 indicate that the blockade of the OX1R has different effects depending on sex; it disrupts the acquisition of male rats, whereas no effects are observed in female rats.

It remains unknown whether SB specifically affects extinction in active avoidance. Thus, in the present experiment, the effects of immediate OX1R blockade following extinction sessions on the extinction process and subsequent spontaneous recovery of the 2-AA task were assessed in males and females.

Animals underwent a handling and habituation session to the shuttle box and to the microinfusion pump on day 1. The acquisition sessions consisted of 50 trials per session for 3 consecutive days that took place on days 3 to 5. In this experiment, massed training with a higher number of trials was chosen to ensure good conditioning levels before performing extinction training. Subjects perform two extinction sessions of 50 trials for 2 consecutive days on days 8 to 9. The OX1R blockade drug (SB334867) or vehicle were administered immediately after each extinction session. Twenty-eight days after the last extinction session, rats performed the spontaneous recovery test, which consisted of 50 trials.

A comprehensive timeline outlining the behavioral procedure of this experiment is shown in Figure 53.

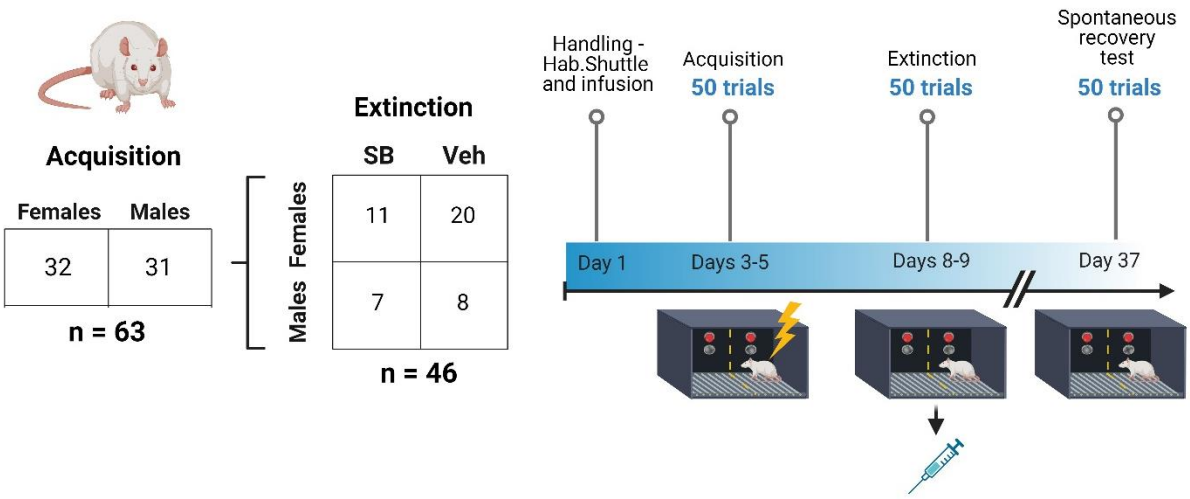


Figure 53. Timeline of the experimental procedure used in Experiment 6. The sample used for the analysis of the acquisition and the extinction sessions and SR test are different since the animals that presented problems with the cannula were eliminated from the analysis of the extinction and subsequent phases. Note that post-training infusions of SB33487 were performed after each extinction session and microinfusions are depicted with a syringe in the timeline.

The final sample consisted of 63 Wistar rats, 32 female rats with a mean age of 221.84 days (SD = 75.16) and a mean weight of 253.11 g (SD = 19.47) and 31 male rats with a mean age of 214.71 days (SD = 78.27) and a mean weight of 406.42 g (SD = 46.75), as shown in Figure 53.

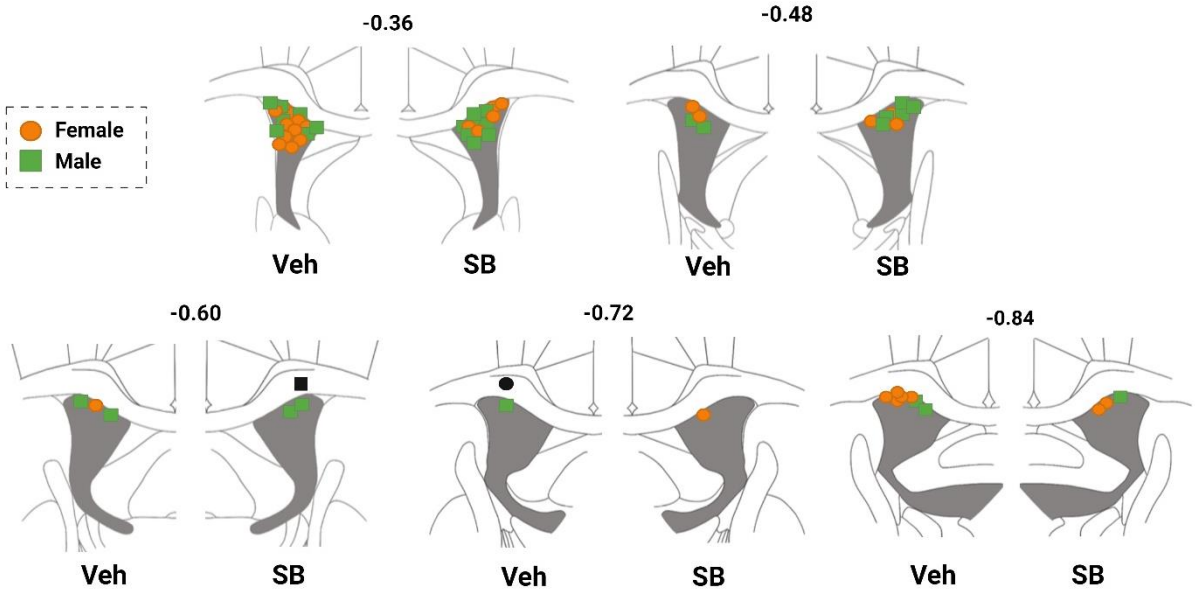


Figure 54. Cannula location of subjects from Experiment 6. Illustration depicting cannula placement for all subjects. Black squares or black circles depict wrong cannula placements. Cannula placement was assessed between the coordinates -0.36mm to -0.84mm from Bregma (Paxinos & Watson, 2007).

6.1 Weight

Differences were found between groups (GROUP: $F_{1,59}$= 66.635, P < .001) that were dependent on the session (GROUP × SESSION: $F_{18,354}$= 11.208, P < .001) (Fig. 55). Bonferroni post-hoc analysis revealed no differences between Fem-Veh and Fem-SB (P = 1.000) or Mal-Veh and Mal-SB (1.000), but differences were found between sexes. All the comparisons between sexes were significant (P < .001), with male rats exhibiting higher weight in comparison to female rats, as expected.

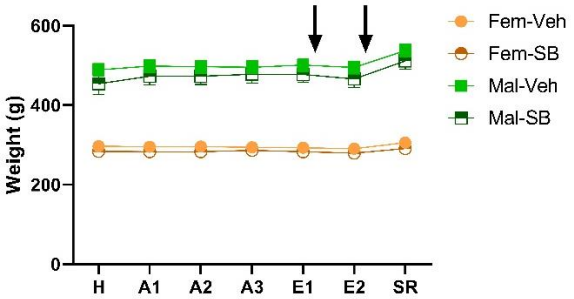


Figure 55. Evolution of the weight throughout the experimental sessions in Experiment 6. H= habituation, A1 = first acquisition session, A2 = second acquisition session, A3 = third acquisition session, E1 = first extinction session, E2= second extinction session, SR = spontaneous recovery test. Black arrows indicate post-extinction microinfusions of SB. Data are expressed as mean ±SEM.

6.2 Behavioral outcome before OX1R selective blockade infusion (Acquisition sessions and E1)

Mean avoidances and latencies of response during the acquisition sessions are depicted in Fig.56 A-B.

The analysis performed for the 2-AA task before the infusion of SB showed clear sex differences (SEX: $F_{1,61}$= 40.73, P < .001; SEX × SESSION: $F_{3,183}$= 7.349, P < .001). The interaction of factors exhibited a quadratic trend (SEX × SESSION: $F_{1,61}$= 19.455, P < .001), indicating that female rats present a significantly more pronounced evolution in comparison to males. The latencies of response outcomes support the results regarding mean avoidances, showing sex differences (SEX: $F_{1,61}$= 28.77, P < .001) regardless of the session (SEX × SESSION: $F_{3,183}$= .960, P = .387).

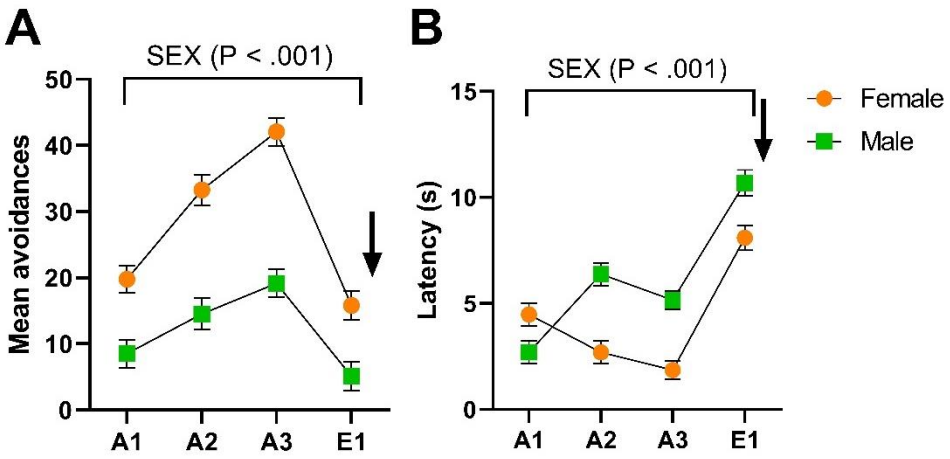


Figure 56. **Sex effects on the acquisition of the 2-AA task in Experiment 6**. **A**. Mean avoidances and **B**. Latencies performed in the acquisition sessions (A1-A3) and in the first extinction session. Black arrows represent post-extinction microinfusions. Data are expressed as mean ±SEM.

6.3 Behavioral outcome after OX1R selective blockade infusion (E2 and SR)

Some subjects were excluded from the analysis of the second extinction session and the spontaneous recovery test. Two animals were excluded due to cannula misplacement (see Figure 54). Moreover, we deemed that animals that did not achieve a minimum level of acquisition were not suitable for assessing the effects of OX1R selective blockade on the extinction of the task. Therefore, subjects that did

not achieve a 50% of avoidances in the third acquisition session (25 avoidance responses) were excluded from further behavioral analysis. Consequently, fifteen male rats were excluded due to not attaining a minimum conditioning level during the acquisition sessions. The total sample used for analyzing the effects of SB on the second extinction and the spontaneous recovery test (sessions after the microinfusion) consisted of 46 Wistar rats (Fem-SB = 11; Fem-Veh = 20; Male-Veh = 7; Male-SB = 8), as shown in Figure 53.

Mean avoidances and latencies of response during the extinction sessions are depicted in Fig.57 A-C.

No differences between groups were found in the second extinction session and the spontaneous recovery test regarding mean avoidances (GROUP: $F_{3,42}$= .366, P = .778; GROUP × SESSION: $F_{3,42}$= 1.736, P = .174), latencies of response (GROUP: $F_{3,42}$= .366, P = .778; GROUP × SESSION: $F_{3,42}$= 3.997, P = .014), and non-responses (GROUP: $F_{3,42}$= .279, P = .840; GROUP × SESSION: $F_{3,42}$= 3.166, P = .034).

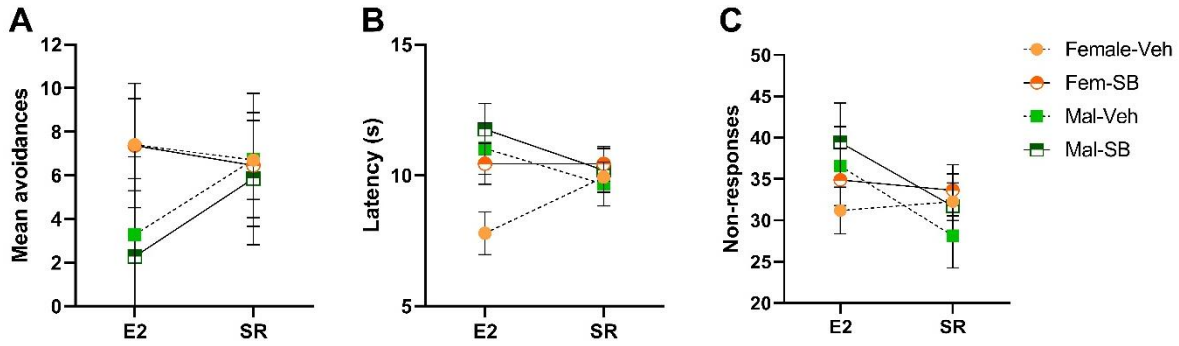


Figure 57. Behavioral outcome of the 2-AA task after SB334867 microinfusions. **A.** Mean avoidances performed during E2 and in the SR. **B.** Latencies of response performed during E1 and SR. **C.** Non-responses performed during E1 and the SR. E2 = extinction session 2, SR = spontaneous recovery test. Data are expressed as mean ±SEM.

6.5 Intertrial crossings

Intertrial crossings performed during the acquisition, extinction sessions and spontaneous recovery test are depicted in Fig.58 A-F.

No differences were observed regarding the locomotor exploratory activity between sexes during the habituation session, before the administration of SB (t_{61} = .611, P = .543). Differences between sexes were found in the intertrial crossings performed in the sessions performed before the microinfusion procedure (SEX: $F_{1,61}$= 6.946, P = .011), regardless of the session (SEX × SESSION: $F_{3,183}$= 2.672, P = .064). No differences were found regarding the second extinction session and the spontaneous recovery test, the sessions performed after the microinfusion procedure (GROUP: $F_{1,42}$= .140, P = .936; GROUP × SESSION: $F_{3,42}$= 4.381, P = .009).

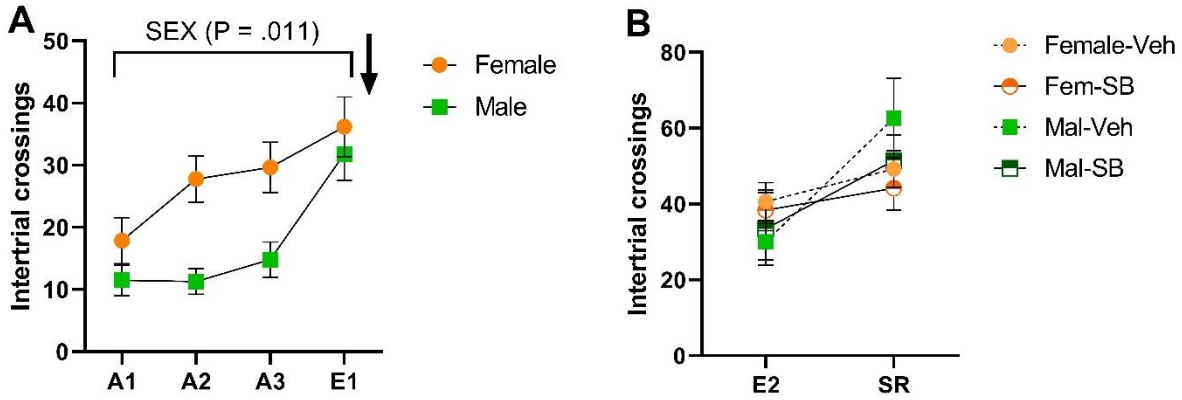


Figure 58. Intertrial crossings of the 2-AA task from Experiment 6. **A**. Intertrial crossings during the acquisition sessions (A1-A3) and the first extinction session (E1). **B**. Intertrial crossings during the second extinction session (E2) and the spontaneous recovery test (SR). Black arrows represent post-extinction microinfusions. Data are expressed as mean ±SEM.

6.6 Discussion

The results of this experiment reveal sex differences in the acquisition of a 2-AA task following a distributed learning protocol and indicate no effects of the OX1R selective blockade on the extinction of a 2-AA task.

<u>Corroboration that female and male rats learn a 2-AA task differently</u>

Results from the acquisition sessions reveal that female rats learn active avoidance behavior more rapidly than males under the same learning conditions. Moreover, the use of a massed learning protocol significantly impedes conditioning in males.

The purpose of employing a massed protocol involving a total of 150 trials of conditioning was to ensure optimal levels of learning before conducting extinction. However, we observed that male rats finished the third acquisition session with an average of 20 avoidances out of 50 possible.

OX1R selective blockade does not affect the extinction of 2-AA task

To investigate the potential effects of OX1R selective blockade, we focused on analyzing the second extinction session, as post-extinction infusions were performed after the first extinction session. Analysis of the second extinction session and spontaneous recovery test reveals that SB had no effect on the acquisition or consolidation of extinction learning under the conditions utilized.

There is a significant lack of literature regarding the role of orexins in the extinction of instrumental tasks such as two-way active avoidance or passive avoidance. However, studies have examined the involvement of orexins in the extinction of classical conditioning tasks, such as fear conditioning. These studies demonstrate that SB334867 facilitates the extinction of contextual and cued fear conditioning, rescues the extinction of an inbred mouse strain characterized by impaired extinction (Flores et al., 2014), reduces freezing by increasing activation of two pro-extinction brain areas (BLA and IL cortex) (Flores et al., 2017). Similarly, a study administering Ox-A infusions reported impaired fear extinction (Ten-Blanco et al., 2022). These studies suggest that the Ox-A/OX1R pathway is relevant for the consolidation of recent fear memory extinction (Shi L et al., 2018).

The absence of SB effects in this experiment may be due to the training protocol used. Future studies could investigate whether using a distributed learning protocol allows for the observation of facilitating effects of SB on active avoidance extinction, as previous studies suggest.

Furthermore, future research in this line should prioritize facilitating the acquisition of the 2-AA task in males if the goal is to observe the effects of OX1R selective

blockade on extinction learning. Therefore, consideration could be given to implementing a distributed protocol of learning during acquisition, along with the possibility of increasing learning sessions until a minimum level of learning is achieved for those rats requiring it.

Conclusions

In conclusion, the results of this experiment corroborate, on the one hand, the sex differences in the performance of the two-way active avoidance task, and on the other hand, show that under the experimental conditions used, SB does not affect the extinction of the 2-AA task.

STUDY III. Is OX1R involved in non-conditioned fear and anxiety in males and females?

This section presents the results from Study III, which addresses the sixth aim of this thesis proposed to assess the effects of the OX1R selective blockade on non-conditioned fear and anxiety tests.

Experiment 7. Effects of orexin-1 receptor blockade on emotional reactivity in males and females

Evidence from the literature has shown that the Ox-A/OX1R pathway is involved in fear and anxiety. The scope of this thesis is the study of active avoidance behavior which involves fear and anxiety, specially in the early stages of the learning process. Thus, the study of OX1R selective blockade was assessed in non-conditioned fear and anxiety tests to assess how this pathway might be involved in such processes in male and female rats, and assess if the effects of the OX1R selective blockade on the acquisition and extinction of a 2-AA task could be mediated by the potential effects on non-conditioned fear and anxiety.

Animals underwent surgery for cannula implantation on day 1 and a handling and handling/habituation session on day 5. Animals performed the open field (OF) test on day 10. One hour after the OF, either the OX1R blockade (SB334867) or vehicle were administered. 30 min later, the animals performed the light/dark test (LD). Two days later, on day 12, animals received a microinfusion of the OX1R blockade or vehicle performed the marble burying test and 30 min later. On day 13, animals also received a microinfusion of OX1R blockade or vehicle and 30 min later they underwent the inescapable footshock test. The three non-conditioned fear and anxiety tests were organized following a rationale to use the less noxious tests at the beginning of the experiment and progress to more aversive tests toward the end of the experiment.

A comprehensive timeline outlining the behavioral procedure of this experiment is shown in Figure 59.

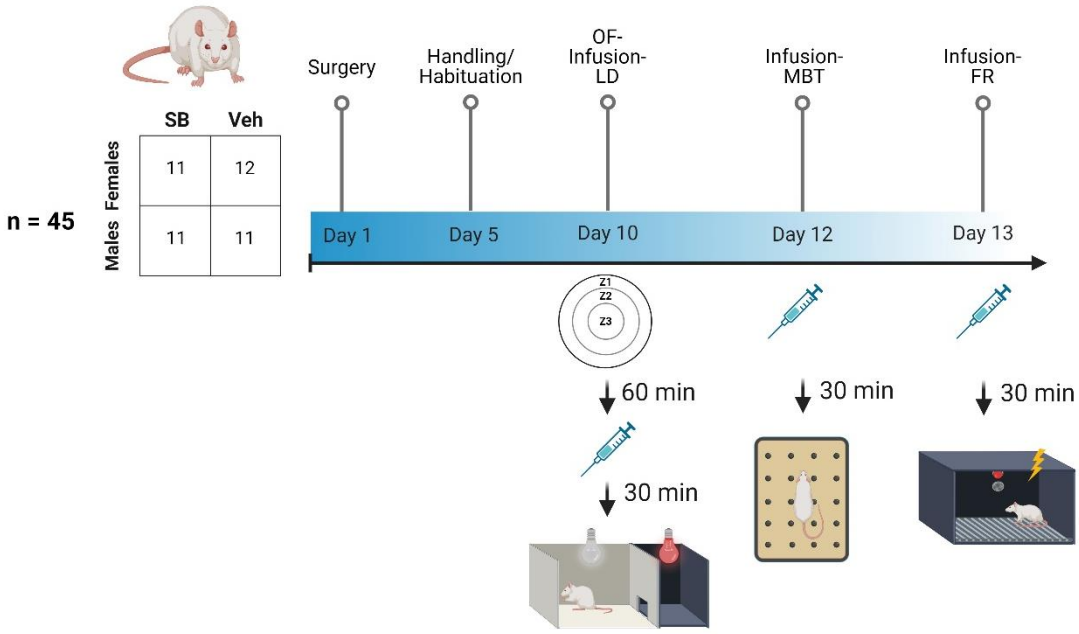


Figure 59. **Timeline of the experimental procedure used in Experiment 7**. SB or vehicle administrations are represented with a syringe.

The final sample consisted of 45 Wistar rats; 11 Female+SB rats with a mean age of 97.00 days (SD = 6.73) and a mean weight of 265.23 g (SD = 107.24), 12 Female+Veh rats with a mean age of 97.16 days (SD = 4.80) and a mean weight of 237.60 g (SD = 19.45), 11 Male+SB rats with a mean age of 97.00 days (SD = 5.00) and a mean

weight of 406.55 g (SD = 31.77) and 11 Male+Veh rats with a mean age of 106.81 days (SD = 33.22) and a mean weight of 412.26 g (SD = 34.58), as shown in Figure 59. Three subjects were excluded from the analysis because of cannula misplacement, as shown in Figure 60. Micturitions and defecations performed during the L/D test were excluded from statistical analysis due to their values being near zero. Note that the variable "Groomings in light zone" from the light/dark test is absent because all the values for all groups were 0.

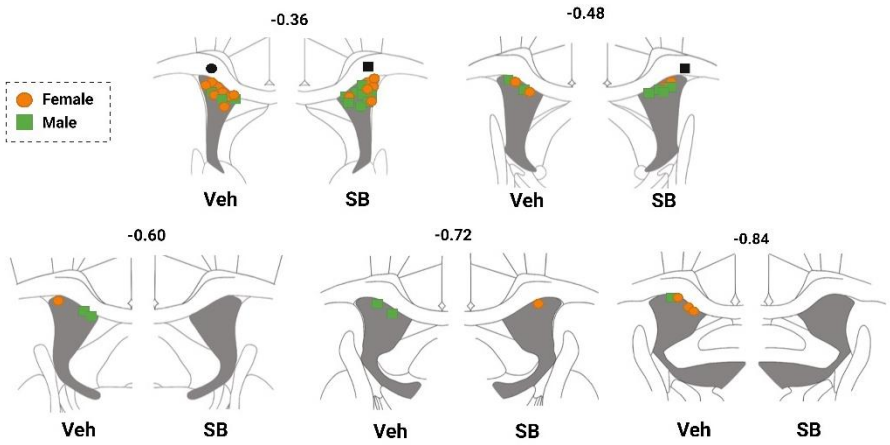


Figure 60. **Cannula location for Veh and SB groups from Experiment 7**. Female subjects are depicted using orange circles and male subjects are depicted using green squares. Black squares or black circles correspond to animals with incorrect cannula location.

7.1 Open Field test

Although no microinfusion treatment was applied before or after the open field test, a two-way ANOVA was performed for SB factor to address experimenter bias. That analysis showed no statistical differences between groups in the open field outcomes. Thus, the data from the OF test was analyzed considering factor SEX, and simple comparisons analysis between females and males were conducted.

Significant differences between the two sexes were found in two of the variables studied. First, male rats spent significantly more time in zone 1, which corresponds to the area near the walls, in comparison to female rats (U = 157, P = .029) (Fig.61A). Second, male rats performed significantly more micturitions in comparison to female rats (U = 158.5, P = .026) (Fig. 61F).

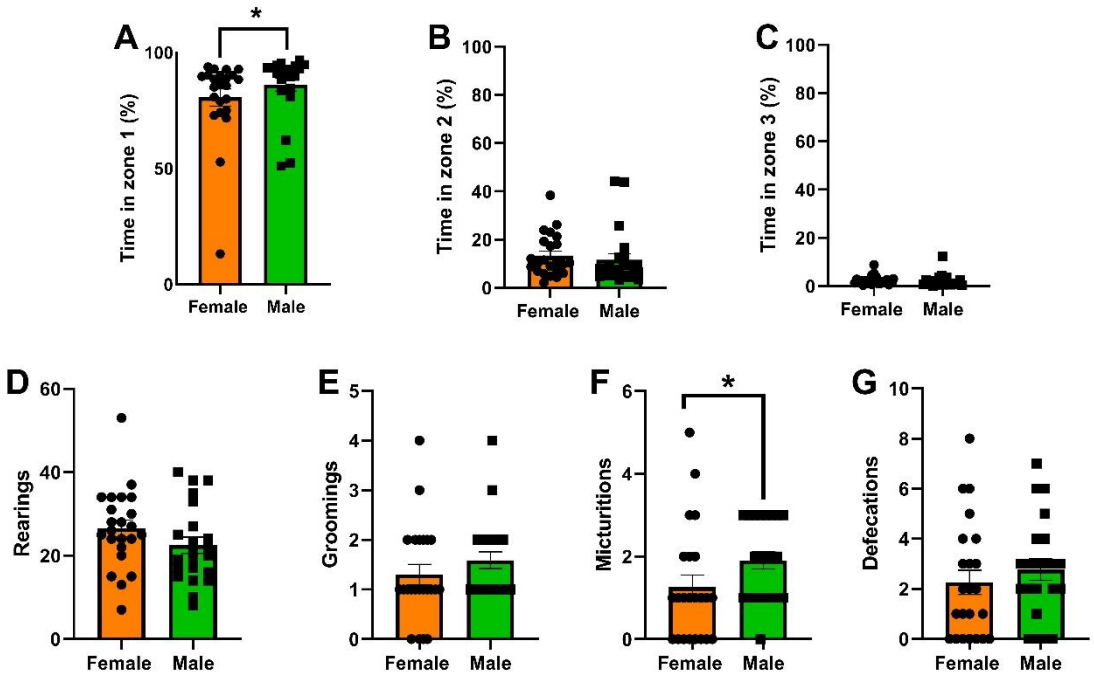


Figure 61. **Open field test variables assessed in females and males in Experiment 7**. **A**. Percentage of time spent in zone 1. **B**. Percentage of time spent in zone 2. **C**. Percentage of time spent in zone 3. **D**. Rearings performed. **E**. Groomings performed. **F**. Micturitions. **G**. Defecations. Female and male subjects are separated in different groups in the graphs. Female = orange with circles; Male = green with squares.

7.2 Light/dark test

A significant interaction between the factors SEX and SB was found for the percentage of time spent in the light zone (SEX × SB: $F_{1,41} = 7.720$, P = .008). Simple effects analysis showed that vehicle female rats spent significantly more time in the light zone in comparison to vehicle male rats (*t*= 2.294, P= .027), although the differences disappear when comparing SB groups (Fig.62A). Moreover, SB reduced the time spent in this zone in female rats (*t*= 2.833, P = .007) but not in SB males (*t*= 1.115, P = .271).

A significant interaction was also found regarding transitions performed between the light and dark compartments (SEX × SB: $F_{1,41} = 5.496$, P = .024). Simple effects analysis revealed that vehicle female rats performed significantly more transitions in comparison to vehicle male rats (*t*= 3.099, P= .004), but no differences were

observed between sexes when comparing SB groups (*t*= 0.247, P= .806) (Fig.62D). Additionally, SB reduced the number of transitions in female rats (*t*= 3.477, P= .001) but not in male rats (*t*= .123, P= .902).

Finally, a significant interaction was found regarding rearings performed in the light zone (SEX × SB: $F_{1,41} = 4.400$, P = .042). Similarly to previous results, a difference was found between vehicle female rats in comparison to vehicle male rats, with female rats performing more rearings (*t*= 2.783, P= .008), but these differences were not found when comparing SB groups (*t*= .211, P= .834) (Fig.62F). Furthermore, SB reduced the rearings in female rats (*t*= 2.711, P= .010) but not in male rats (*t*= .281, P= .780).

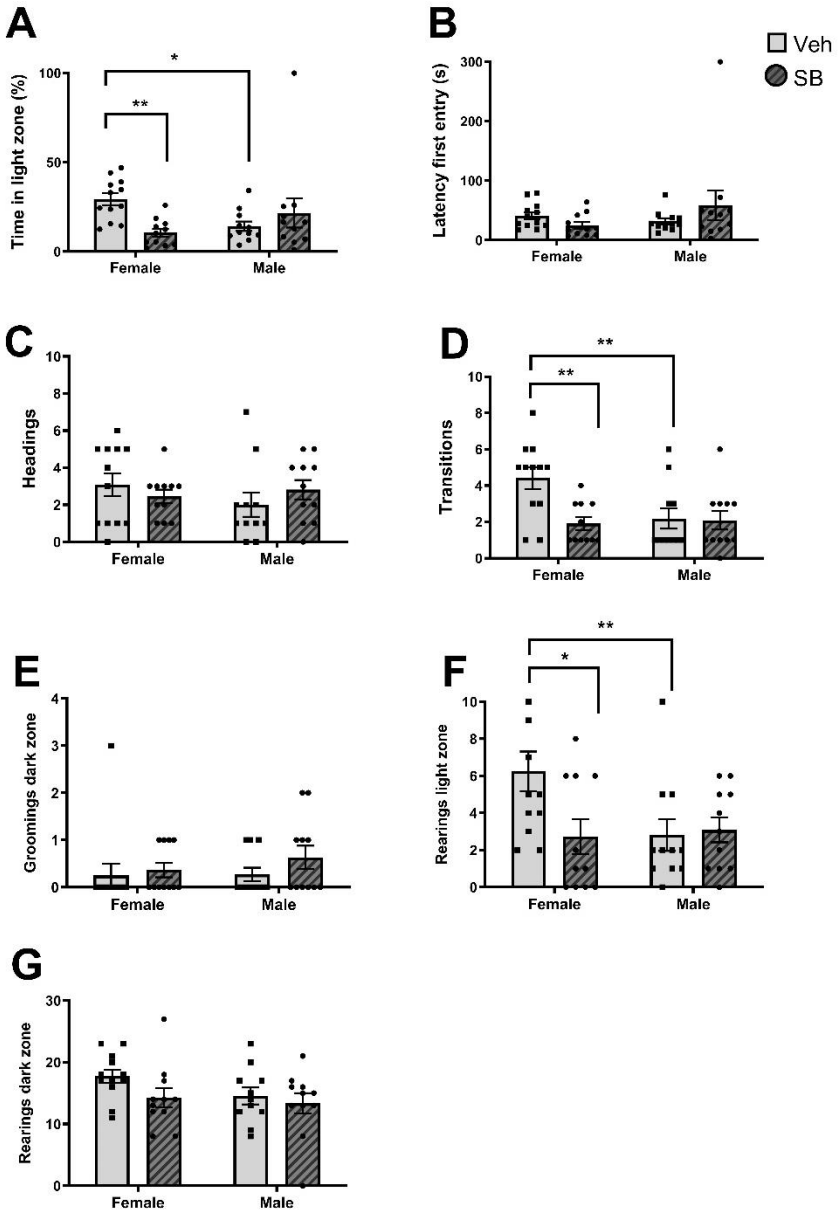


Figure 62. **Effects of SB334867 on the light/dark test in Experiment 7**. **A**. Percentage of time spent in the light zone. **B**. Latency of the first entry to the dark compartment (s). **C**. Headings between the two compartments. **D**. Transitions between the two compartments. **E**. Groomings in the dark zone. **F**. Rearings in the light and **G**. dark zone. *P ≤ 0.05, **P ≤ 0.01.

7.3 Marble burying test

The factor SEX was significant regarding the amount of marbles buried (SEX: $F_{1,41}$= 6.438, P= .015), the number of marbles that are completely buried (SEX: $F_{1,41}$= 10.450, P= .002) and the latency of the first marble buried (SEX: $F_{1,41}$= 6.186, P= .017) (Fig.63A-B). Female rats buried significantly less marbles in comparison to male rats and they took longer to bury the first marble, regardless of the treatment group (SEX × SB: $F_{1,41}$= .598, P= .444; SEX × SB: $F_{1,41}$= .216, P= .645; SEX × SB: $F_{1,41}$= 3.456, P= .070, respectively). This last trend toward statistical significance in the interaction between factors regarding the latency of the first marble buried shows, similarly to the L/D test results, that SB equalizes female's performance to males, and simple effects analysis corroborate this effect (*t*= 3.146, P= .003). A representative end result for each experimental group of the MBT is shown in Figure 64.

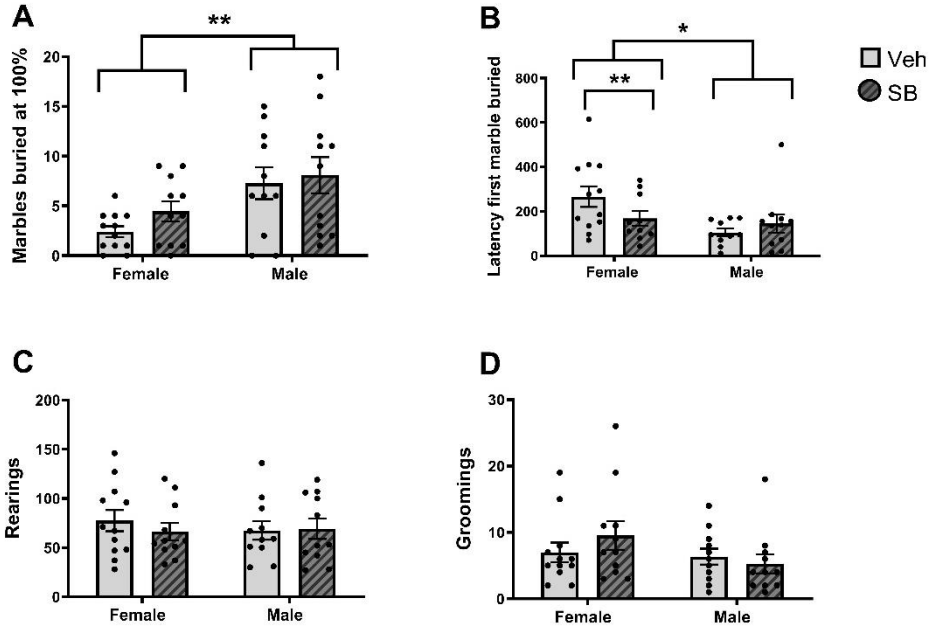


Figure 63. **Effects of SB334867 on the marble burying test in Experiment 7**. **A**. Number of marbles completely buried. **B**. Latency of the first marble buried (s). **C**. Rearings performed. **D**. Groomings performed.

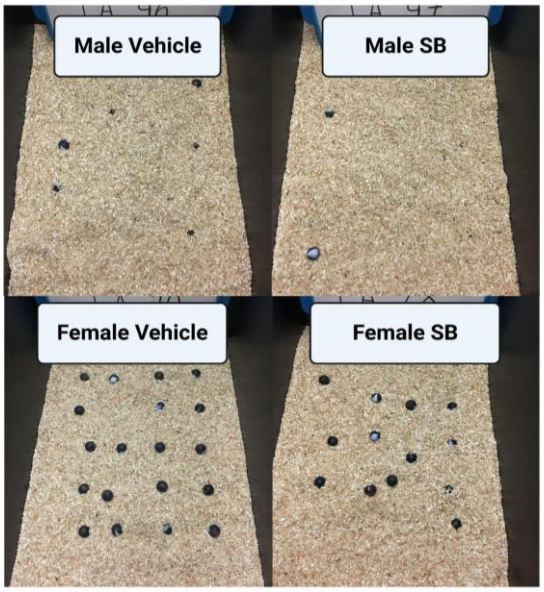


Figure 64. Representative images from each experimental group of the MBT end result.

7.4 Freezing response to inescapable footshock

A univariate GLM showed a significant interaction between factors only for the second ITI (SEX × SB: $F_{1,41}$= 6.964, P= .012). In this regard, vehicle male rats performed significantly more freezing during this period of time in comparison to vehicle female rats (*t*= 3.196, P= .003), and SB only increased the freezing behavior in females (*t*= 3.362, P= .002) (Fig.65E).

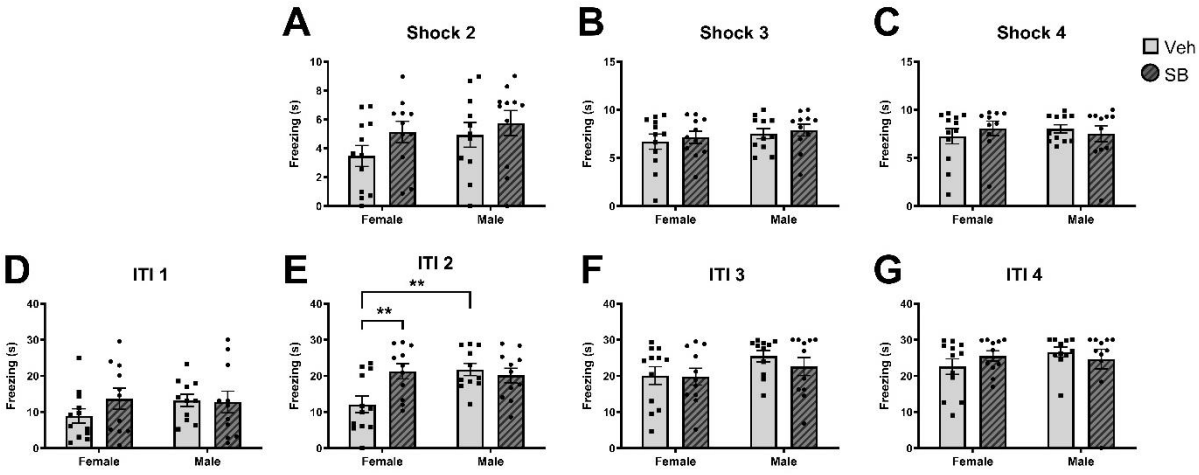


Figure 65. Effects of SB334867 on the freezing response during the footshocks and in the ITI. Freezing response during the **A**. second, **B**. third, and **C**. fourth shock. Freezing response during the **D**. first, **E**. second, **F**. third, and **G**. fourth ITI.

Having results from the open field test without any application of treatment provided a good opportunity to assess whether the behavior of animals in that test correlated with their behavior in subsequent tests performed in this experiment.

Regarding vehicle males, a negative correlation was found between the percentage of time spent in zone 1 of the OF and the percentage of time spent in the light zone of the light/dark test ($rs(9) = -.609$, $p = .047$) (Fig.68A). This correlation shows that the more time animals spent near the walls in the OF test, indicative of anxiety, the less time spent in the light zone of the LD test, which also corresponds to high anxiety levels. A positive correlation was also found between the rearings performed in the OF and the marbles buried in the MBT ($rs(9) = .753$, $p = .007$) (Fig.68B). This correlation shows that the animals that tended to move and explore more in the OF, also exhibited the same pattern of behavior in the MBT. Finally, a positive correlation was found between the percentage of time spent in zone 1 and the freezing during the second shock in the inescapable footshock test ($rs(9) = .727$, $p = .011$) (Fig.68C).

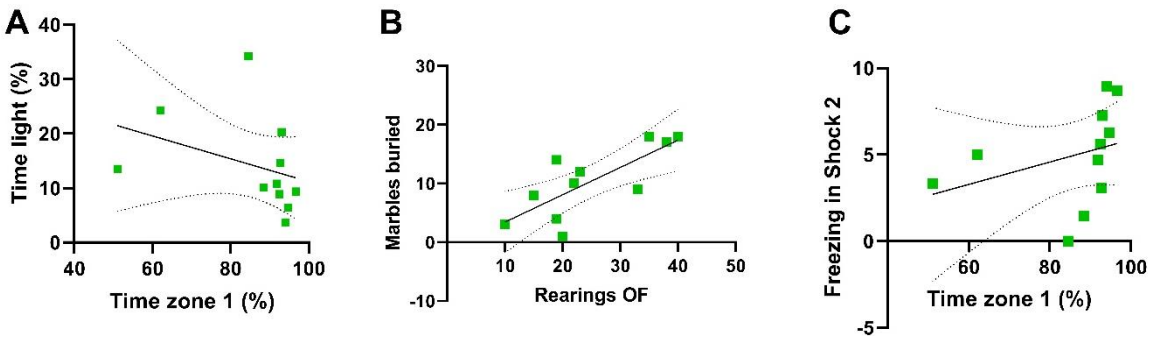


Figure 66. Scatterplots depicting correlations between open field test variables and other non-conditioned anxiety and fear tests in Experiment 7.

7.5 Discussion

The results of this experiment indicate that male rats display more fear and anxiety related behaviors than females, and the selective OX1R blockade eliminates these differences.

Sex differences are evident in non-conditioned fear and anxiety levels

Male rats show more fear and anxiety-related behaviors in comparison to female rats, as evidenced by variables assessed in the open field test, the light/dark test, marble burying test and the inescapable footshock. Specifically, males spend more time near the walls in the OF test, less time spent in the light zone, lower number of transitions and rearings in the light zone in the L/D test, higher number of marbles buried and lower latency to bury the first marble in the MBT (Bourin & Hascoët, 2003; Prut & Belzung, 2003; Santana-Santana et al., 2021). Moreover, males also display a higher freezing response in the ITI 2 of the inescapable footshock test. The correlations analysis between variables assessed in the different tests indicates the presence of some convergent construct validity among them.

These findings are consistent with previous literature indicating that males tend to exhibit more anxious and fear-related behaviors (Fernandes et al., 1999; Johnston & File, 1991; Steenbergen et al., 1989; Steenbergen et al., 1990). The anxiolytic effects of estradiol in females may contribute to these sex differential effects (Díaz-Veliz et al., 1997; Marcondes et al., 2001). Additionally, females perform more active behaviors in comparison to males, as shown by more rearings in the light zone in the L/D, which is consistent with studies reporting a natural tendency of females to perform such behaviors (Gruene et al., 2015a; Colom-Lapetina et al., 2019).

Moreover, no differences between sexes are observed regarding exploratory behavior, assessed by rearings in the OF, the MBT, or in the dark zone of the L/D test.

OX1R selective blockade increases anxiety in females

The results of the present experiment demonstrate that sex differences disappear when OX1R selective blockade is administered, as male and female SB subjects do not show differences in any assessed variable. The administration of SB did not cause any effects on males but it increased anxiety-related behaviors in females, making them different from female vehicle subjects.

One potential explanation for these differential results between sexes is that, given their higher baseline levels of anxiety and fear, the anxiogenic effects of SB may be more challenging to demonstrate in males than in females. This would be consistent with the lack of effects of OX1R selective blockade in males (Staples & Cornish, 2014), or by oral administration of almorexant (Steiner et al., 2012). However, one study reported that SB334867 reduced anxiety levels in males when administered intraperitoneally in Sprague-Dawley rats, while no effects on females were observed (Blume et al., 2018). Another possibility is that there may be a different function of the orexinergic system in non-conditioned fear and anxiety responses between sexes.

A limitation of this experiment is that the estrous cycle of females was not accounted for. Several studies have suggested that sexual hormone levels may modulate non-conditioned fear and anxiety, which are assessed in the open field test (Miller et al., 2021), EPM (Marcondes et al., (2001) and burying activity in the MBT (Shneider & Popik, 2007).

These findings have potential implications for human clinical conditions related to anxiety- or fear-related disorders, and future studies should investigate whether the administration of Ox-A or stimulation of the orexinergic system through other methods could reduce non-adaptive anxiety and fear responses.

Conclusions

In conclusion, the results of this experiment reveal clear sex differences and a sex-differential effect of OX1R selective blockade in non-conditioned fear and anxiety tests.

STUDY IV. Can extinction of the 2-AA task be improved in females?

This section presents the results from Study IV, which addresses the eighth aim of this thesis proposed to determine the effects of Intracranial Self-Stimulation (ICSS) on the extinction of a 2-AA task in females.

Experiment 8. Effects of medial forebrain bundle self-stimulation (MFB-ICSS) on the extinction of the 2-AA in female rats.

Female rats exhibit higher levels of conditioning and a slower rate of extinction compared to male rats in the two-way active avoidance task.

Intracranial Self-Stimulation (ICSS), a method of electrical stimulation of brain areas of reinforcement, improves the acquisition and retention of different types of learning tasks. In this experiment, we study whether MFB-ICSS is also effective in facilitating the extinction process of active avoidance conditioning in female rats.

This experiment aims to study the effects of medial forebrain bundle intracranial self-stimulation (MFB-ICSS), a treatment known for consistently facilitating learning and memory, on the extinction process in female rats. The rats used in this experiment were the same as those utilized in Experiment 1. Two weeks after finishing the spontaneous recovery session of the first experiment, they underwent a stereotaxic intervention to chronically implant an electrode in the fibers of the MFB, in the region of the lateral hypothalamus. After a post-surgical recovery period of 4 weeks, subjects underwent a new acquisition - or reacquisition- session. One week later, the animals were subjected to 3 extinction sessions of 30 trials (1 daily) and one session after 28 days to evaluate spontaneous recovery.

After re-acquisition, the female rats were distributed into two groups: the Female-ICSS group, which received the MFB-ICSS treatment (2500 stimulation trains) immediately after each of the 3 extinction sessions, and the Female-Sham group. The use of the same animals allowed for a comparative study between sexes regarding the flexibility of reacquiring and extinguishing the conditioning again. Additionally, we anticipated that female rats would exhibit very high levels of conditioning, allowing us to test the effectiveness of the MFB-ICSS treatment on extinction and the subsequent spontaneous recovery.

A comprehensive timeline outlining the behavioral procedure of this experiment is shown in Figure 67.

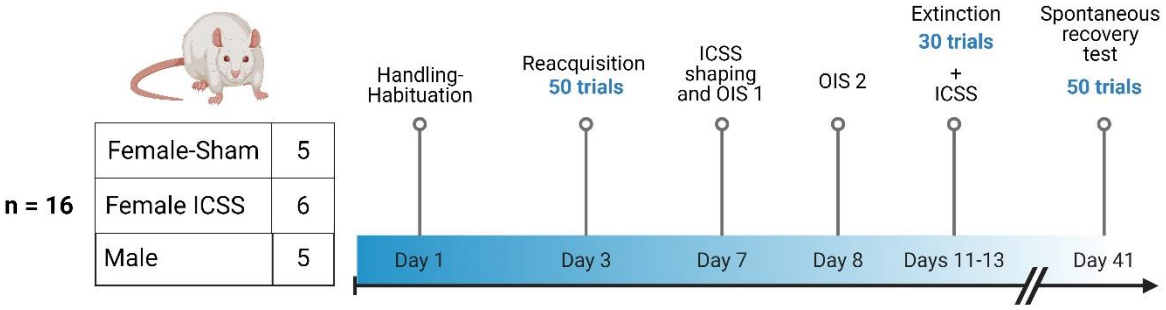


Figure 67. Timeline of the experimental procedure used in Experiment 8. Rats underwent surgery for electrode implantation two weeks after the end of Experiment 1 (four weeks before the beginning of Experiment 8).

The final sample consisted of 16 Wistar albino rats, approximately six months old: 11 female rats with a mean weight of 250.72 g (SD = 20.81) and 5 male rats with a mean weight of 396.42 g (SD =27.14), as shown in Figure 67. No subjects were excluded because of electrode misplacement, as shown in Figure 68.

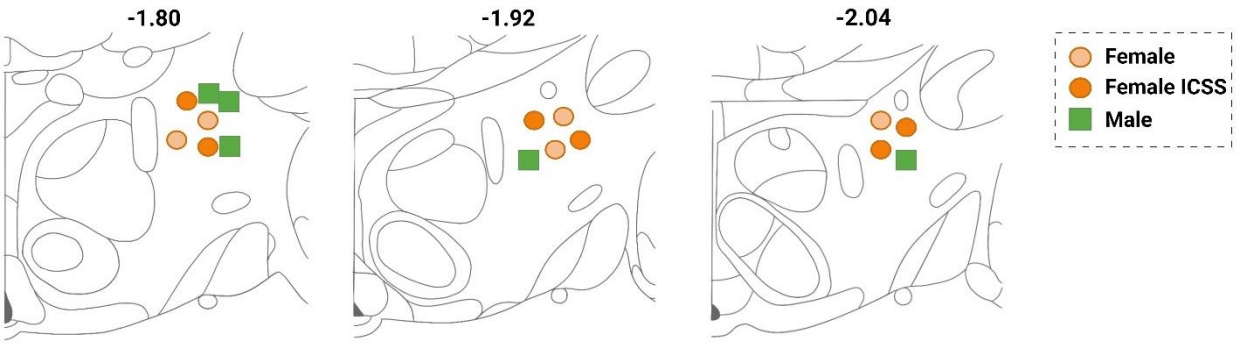


Figure 68. Electrode location of subjects from Experiment 8. Illustration depicting electrode tip placement for all subjects. Electrode placement was assessed between the coordinates -1.80mm to -2.04mm from Bregma (Paxinos & Watson, 2007).

8.1 Reacquisition

The study of the reacquisition session, subdivided in 5 10-trial blocks, revealed significant differences between groups regarding the mean avoidances (GROUP: $F_{1,13}$= 10.095, P = .002), regardless of the block (GROUP × BLOCK: $F_{8,52}$= .746, P = .651) (Fig.70A). Bonferroni post-hoc analysis revealed that Male group performed a lower number of avoidance than either group of females (Female-Sham: P = .006, Female-ICSS: P = .005), previous to the ICSS treatment. Similar results were found regarding latencies of response (GROUP: $F_{1,13}$= 7.309, P = .007; GROUP × BLOCK: $F_{8,52}$= .345, P = .853) (Fig.70C). Once again, these differences were found between the Female-Sham and Male groups (P= .018), and between the Female-ICSS and Male groups (P= .015).

8.2 Extinction

Regarding mean avoidances, differences between groups were observed in the first extinction session (GROUP E1: $F_{1,13}$= 6.955, P = .009; GROUP × BLOCK: $F_{4,26}$= .953, P

= .423) (Fig.70B). Female-Sham performed more avoidances than the Male group (P= .008). However, the differences between groups disappeared in the second and third extinction sessions (E2: GROUP: $F_{1,13}$= 1.350, P = .293; GROUP × BLOCK: $F_{4,26}$= .281, P = .852; E3: GROUP: $F_{1,13}$= 1.520, P = .255; GROUP × BLOCK: $F_{4,26}$= .238, P = .914). The analysis of latencies did not show any differences between groups for E1 (GROUP: $F_{1,13}$= 3.207, P = .074; GROUP × BLOCK: $F_{4,26}$= 1.459, P = .264), E2 (GROUP: $F_{1,13}$= 1.312, P = .303; GROUP × BLOCK: $F_{4,26}$= .172, P = .951), and E3 (GROUP: $F_{1,13}$= 1.858, P = .195; GROUP × BLOCK: $F_{4,26}$= .207, P = .932) (Fig.70D). Finally, no differences between groups were found regarding non-responses for E1 (GROUP: $F_{1,13}$= 2.110, P = .161; GROUP × BLOCK: $F_{4,26}$= 1.302, P = .305), E2 (GROUP: $F_{1,13}$= 1.047, P = .379; GROUP × BLOCK: $F_{4,26}$= .430, P = .785), and the E3 (GROUP: $F_{1,13}$= 1.887, P = .191; GROUP × BLOCK: $F_{4,26}$= .396, P = .809) (Fig.70E).

In the latency and non-response graphs (Fig 70D and E), certain differences can be seen between females treated with ICSS and those that were not. The lack of significance could be attributed to the small sample size. For this reason, we did a complementary study comparing the groups within each block of the E3 and spontaneous recovery sessions. Dunnett's T analysis showed that the Female-ICSS group had higher latencies of response and more non-responses than the Female-Sham group in the third 10-trial block (E3.3) (P = .049; P= .048, respectively).

To corroborate the differences between Female-ICSS and Female-Sham groups, a survival analysis was carried out to see if ICSS affected the time - or number of trials - required by the animals to present a stable extinction response, defined as 5 non-responses in consecutive trials (Fig.69). A tendency to significance was found between these groups (X^2 = 3.156, df = 2, P = .076). By the time 80% of Female-ICSS rats achieved the condition of extinction, only 33% of Female-Sham did.

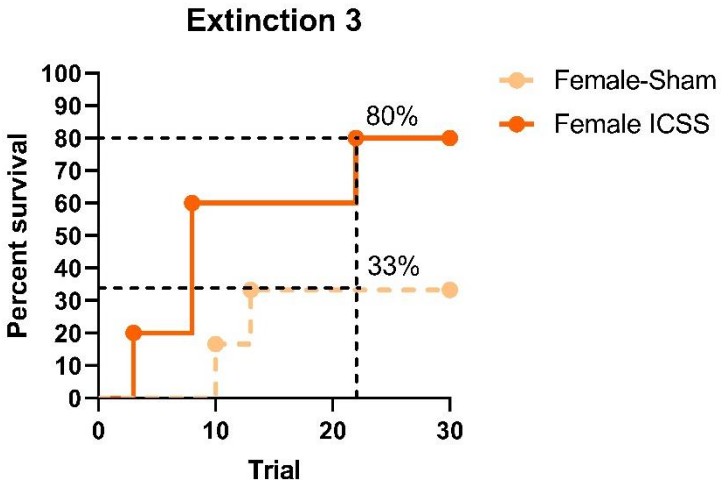


Figure 69. **Survival curves for the third extinction session of female rats in Experiment 8**. The curves represent the percentage of subjects from each experimental group that accomplish the extinction criterion (5 consecutive trials performing a non-response).

8.3 Spontaneous recovery test

No differences between groups were found for the spontaneous recovery test regarding mean avoidances (GROUP: $F_{1,13}$= 1.313, P = .302; GROUP × BLOCK: $F_{8,52}$= .752, P = .646), latencies of response (GROUP: $F_{1,13}$= 1.175, P = .340; GROUP × BLOCK: $F_{8,52}$= 1.453, P = .197) or non-responses (GROUP: $F_{1,13}$= .617, P = .555; GROUP × BLOCK: $F_{8,52}$= 1.406, P = .216). However, Dunnett's T showed that the Female-ICSS group performed more non-responses than the Female-Sham group in the third block of the session (SR3) (P = .048).

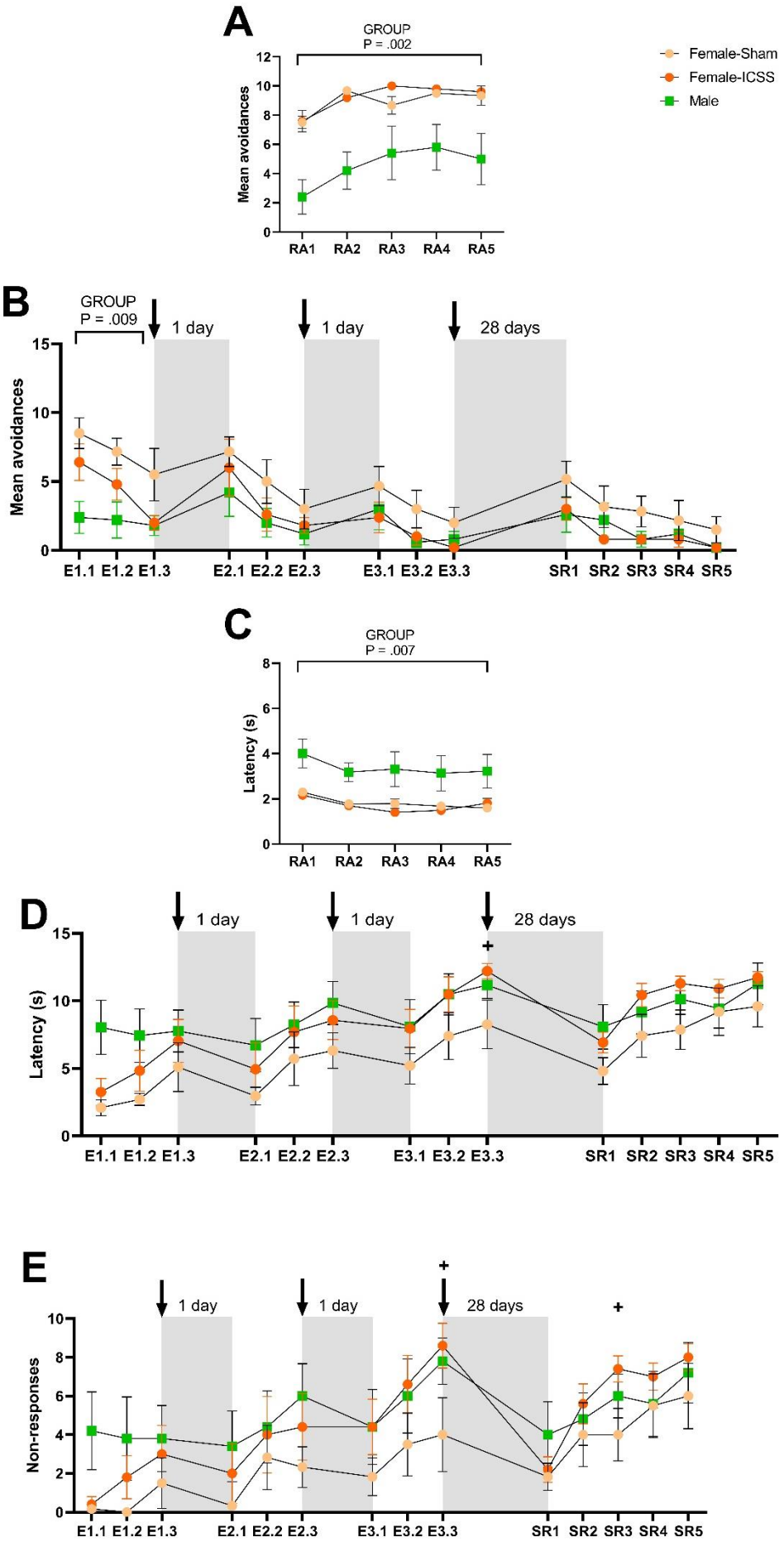

A
GROUP
P = .002
Mean avoidances
RA1 RA2 RA3 RA4 RA5
Female-Sham
Female-ICSS
Male
B
GROUP
P = .009
1 day
1 day
28 days
Mean avoidances
E1.1 E1.2 E1.3 E2.1 E2.2 E2.3 E3.1 E3.2 E3.3 SR1 SR2 SR3 SR4 SR5
C
GROUP
P = .007
Latency (s)
RA1 RA2 RA3 RA4 RA5
D
1 day
1 day
28 days
Latency (s)
E1.1 E1.2 E1.3 E2.1 E2.2 E2.3 E3.1 E3.2 E3.3 SR1 SR2 SR3 SR4 SR5
E
1 day
1 day
28 days
Non-responses
E1.1 E1.2 E1.3 E2.1 E2.2 E2.3 E3.1 E3.2 E3.3 SR1 SR2 SR3 SR4 SR5

Figure 70. **Behavioral outcome from the 2-AA task in Experiment 8**. Mean avoidances for the **A**. reacquisition session, and the **B**. three extinction sessions and the spontaneous recovery test. Latencies of response for the **C**. reacquisition session, and the **D**. three extinction sessions and the spontaneous recovery test. **E**. Non-responses performed in the three extinction sessions and the spontaneous recovery test. Arrows indicate post-training ICSS treatment. + differences between the Female-ICSS and Female-Sham groups. Data are expressed as mean ±SEM.

8.4 Intertrial crossings

No differences between groups were found in E1 (GROUP: $F_{1,13}$= .626, P = .550; GROUP × BLOCK: $F_{4,26}$= 1.011, P = .420), E2 (GROUP: $F_{1,13}$= .281, P = .760; GROUP × BLOCK: $F_{4,26}$= .274, P = .892), E3 (GROUP: $F_{1,13}$= 1.571, P = .245; GROUP × BLOCK: $F_{4,26}$= .262, P = .899) or SR (GROUP: $F_{1,13}$= .115, P = .892; GROUP × BLOCK: $F_{8,52}$= .551, P = .732) (Fig.71A-D).

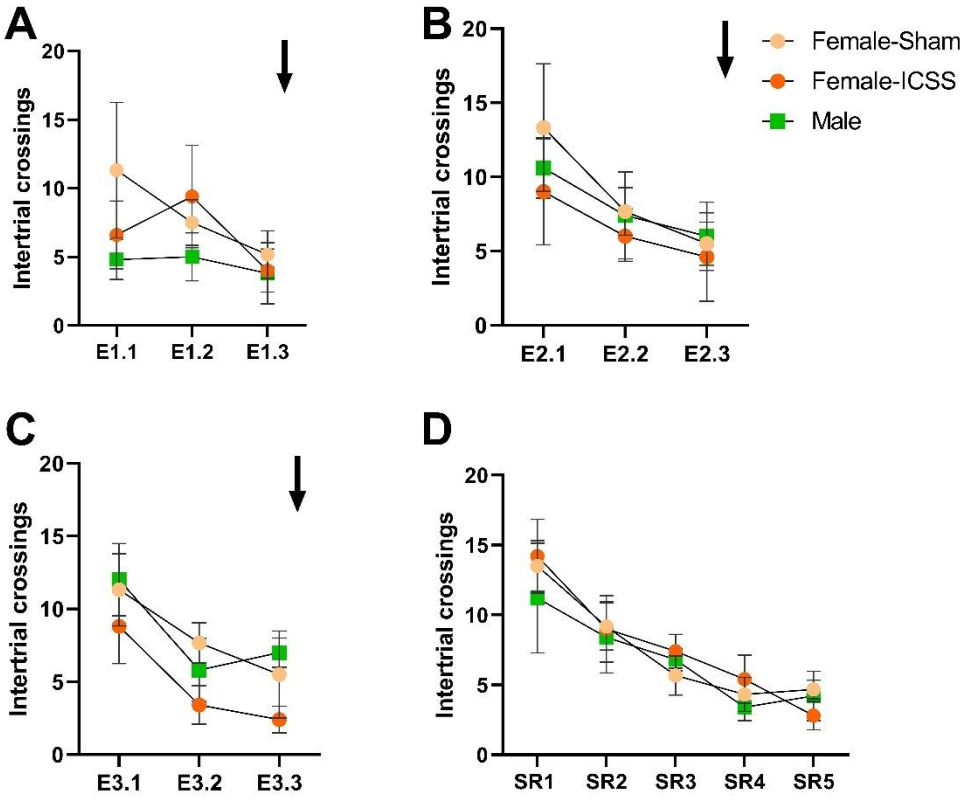


Figure 71. **Intertrial crossings through the extinction and spontaneous recovery test in Experiment 8**. Intertrial crossings during the **A**. first extinction session, the **B**. second extinction session, the **C**. third extinction session and the **D**. spontaneous recovery test. E = extinction sessions, and SR = spontaneous recovery sessions. Arrows indicate post-training ICSS treatment. Data are expressed as mean ±SEM.

8.5 Discussion

Results of this experiment reveal that female rats display higher levels of reacquisition than males, and suggest that MFB-ICSS could be used as a treatment to facilitate extinction in female rats.

Female rats perform better in the re-acquisition

One notable result of our experiment is the accelerated and robust re-acquisition of the 2-AA conditioning shown by the female rats, a month and a half after undergoing an extinction session of the previously acquired conditioning (Experiment 1). This rapid reacquisition in females aligns with findings of previous studies, where re-acquisition takes place in the same context in which the conditioned response was acquired and extinguished (Bouton, 1993; Delamater & Westbrook, 2014; Maren, 2015). However, even under identical contextual conditions and employing a distributed training protocol that typically favors male rats over massed training approach, females consistently display higher levels of performance compared to males. To our knowledge, this is the first study to compare the reacquisition of the 2-AA task between sexes.

ICSS may facilitate the extinction of 2-AA conditioning in females

During the first extinction session, Female-Sham rats exhibit a lower extinction response than males, consistent with findings in other studies noting sex-dependent differences in the early stages of extinction (Beatty & Beatty, 1971; Ikard et al., 1972; Van Haaren et al, 1990). However, no statistical differences were observed between the Female-ICSS and Male groups in this experiment. This observation is surprising given that both groups of females reach similarly high levels of conditioning in the re-acquisition phase, and the ICSS treatment is not administered until the completion of this first extinction session.

One potential explanation for this finding is that MFB-ICSS given during the shaping and OI sessions, was conducted three days prior to the first extinction session, and

might have had a proactive effect. Research suggests that the shaping of ICSS behavior can positively impact subsequent learning, particularly when this intervention is closely timed with the learning sessions (Ruiz et al., 2007). Moreover, it has been demonstrated that the non-contingent administration of ICSS alongside training can enhance future learning (Huguet et al., 2020; Velley & Cardo, 1976; Yoganarasimha et al., 1998). In the present experiment, the stimulation received in the ICSS behavior modeling sessions could predispose these animals to better extinction, perhaps by facilitating new contingencies.

In the third extinction session, female-ICSS rats exhibit better extinction than untreated females, and similar to that of males. Specific differences are noted between Females-ICSS and Female-Sham considering latencies and non-responses in one of the ten-trial blocks of the session. Although the effect of the ICSS on extinction in the present experimental conditions seems modest, increasing the sample size may reveal more significant effects. To our knowledge, only one study in male rats has demonstrated the facilitatory effect of ICSS treatment, administered after extinction sessions, on this process (Huguet et al., 2017). These facilitative effects of ICSS on extinction learning may be attributed to different underlying mechanisms. First, MFB-ICSS could be strengthening synapses by increasing release of different neurotransmitters related to learning and memory (Shankaranarayana Rao et al., 1998). Second, the enhanced extinction may result from an increase in arousal levels, leading to enhanced connection between brain areas (Wright & Craggs, 1977; Nieh et al., 2016). Third, MFB-ICSS could be producing plasticity-related changes, since it has been proven to increase the size and branching complexity of HPC neurons (Chamorro-López et al., 2015). Fourth, ICSS could be improving extinction training by upregulating genes related to learning, memory and synaptic plasticity (Huguet et al., 2009; Kádar et al., 2013).

Conclusions

In conclusion, this experiment sheds light on the facilitative effects of MFB-ICSS on the extinction of a 2-AA task in females, revealing previously unknown facilitative effects of ICSS on emotional learning.

GENERAL DISCUSSION

The primary objective of this thesis is to study acquisition and extinction of active avoidance conditioning, with a specific focus on sexual differences and the involvement of the OX1R. Additionally, we explore whether administering an ICSS treatment in the medial forebrain bundle could enhance extinction in female rats.

After characterizing sex differences in the 2-AA task using our behavioral procedure, we evaluated OX1R selective blockade on the acquisition and extinction of the task in male and female rats. We then assessed the neuronal activation levels and patterns of subjects after the spontaneous recovery test. Moreover, we analyzed the effects of female sexual hormones on 2-AA behavior and non-conditioned fear and anxiety. With these results, we proceeded to assess the possible anxiolytic or anxiogenic effects of OX1R selective blockade on female and male rats. Additionally, we evaluated whether the MFB-ICSS can improve the extinction of the 2-AA task in females.

Sex-differences in 2-AA behavior

The results of this thesis demonstrate that female Wistar rats learn more rapidly and achieve higher conditioning levels than male rats in the 2-AA task. This phenomenon has been observed in both distributed (Experiment 1) and massed training protocols (Experiment 6 and 8). Training sessions consisting of 50 trials distributed across 5 sessions of 10 trials each, with an additional retention session (30 trials), allowed male rats to reach performance levels similar to females (Experiment 1). Conversely, massed training (3 sessions of 50 trials each) increased the differences between males and females, with males displaying greater difficulty. In fact, at the end of the acquisition sessions, males continue to demonstrate much lower levels of conditioning than females (Experiment 1). It is noteworthy that in Experiment 6, half of the males exhibited a number of correct responses (avoidances) below 50% at the end of the acquisition sessions. Under these training conditions, our results align with those of Dalla et al. (2008), who reported that some male rats never learn to escape and avoid a footshock. Therefore, our results suggest that success in learning avoidance behavior depends not only on the total number of trials but also on their distribution over time, and sex.

The conditions employed in our experiments reveal that female rats acquire active avoidance behavior more easily than males, even when they are subject to the reacquisition of the previously extinguished response. They also show that the use of distributed training protocols favor conditioning in male rats.

Regarding extinction learning, female rats exhibited a slower extinction compared to male rats. The initial deceleration observed could be attributed to the higher number of avoidance responses displayed by females during the conditioning phase, which interferes in the establishment of the new contingency of extinction sessions (presence of CS and absence of US) necessary for CR extinction. These outcomes do not necessarily imply a specific difficulty for females in extinction learning, but rather a delay in engaging in escape or non-responses. Nonetheless, sex differences diminish over the course of extinction training, and female rats are able to achieve successful extinction after two extinction sessions of 50 trials each, and maintain this conditioning until the spontaneous recovery test, conducted 28 days after the last extinction session. Only minor sex differences in spontaneous recovery were found in Experiment 8, wherein subjects underwent reacquisition of avoidance behavior after having been previously extinguished. This result indicates that female rats have the cognitive flexibility necessary to achieve good extinction despite starting from very well-consolidated - or well-established - levels of conditioning.

Low levels of conditioning in males represent a methodological limitation when comparing extinction between males and females. Future studies should consider adding conditioning sessions for males to compensate for this deficit, thus allowing for a more accurate assessment of avoidance behavior extinction. Otherwise, we cannot genuinely discuss the results obtained regarding sex-based comparisons of extinction and spontaneous recovery in the 2-AA task.

The results of this thesis indicate that there are sex differences regarding anxiety levels and unconditioned fear (Fernandes et al., 1999; Johnston & File, 1991, 2001; Steenbergen et al., 1989; Steenbergen et al., 1990). It has been observed that male rats exhibit more fear and anxiety-related behaviors than females. These

findings could account for the difficulties male rats have in acquiring the 2-AA task in all experiments conducted. On the other hand, it could be argued that the low levels of anxiety and fear observed in females could have a positive impact on the conditioning of avoidance behavior.

The results of this dissertation also demonstrate that female sexual hormone levels positively modulate the extinction of active avoidance behavior. These findings are consistent with those obtained in other studies, wherein female rats in proestrus exhibit improved extinction of the 2-AA task (Sfikakis et al., 1978), and similarly, better extinction of fear conditioning (Rey et al., 2014; Gruene et al., 2015b). However, there are studies showing contradictory results, where low levels of female sexual hormones facilitate fear extinction (Díaz-Veliz et al., 1989) when using Sprague-Dawyley rats in a unique 50 trials acquisition sessions with 0.20 mA footshock. In addition to the effects of hormones on avoidance behavior, we have obtained results suggesting their modulatory effect on non-conditioned fear and anxiety levels. High levels of sexual hormones reduce anxiety and non-conditioned fear, as also shown by other studies (Arpels, 1996; Best et al., 1992; Díaz-Veliz et al., 1997; Hiroi & Neumaier, 2006; Marcondes et al., 2001; Walf & Frye, 2006). This could constitute another factor facilitating conditioning for avoidance behavior learning.

It should be noted that although there is a significant body of literature demonstrating effects of female sexual hormones, there are studies that do not find such effects (Emtyazi et al., 2022; Zeng et al., 2023). Therefore, the results obtained regarding the effects of estrous cycle on females' behavior should be corroborated with future studies, perhaps even by analyzing the stage of the cycle using direct measurements (Florido et al., 2021) or increasing the sample size.

Finally, the analysis of the neuronal activation differences enabled us to characterize the circuits mediating the extinction of the 2-AA task in males and females. Although the results are inconclusive due to the small sample size, the outcomes may suggest a different functional implication of dorsal hippocampus and BNST in male and female rats, which implies certain sex-dimorphic differences in

contextual conditioning and in coping strategies in situations of stress or anxiety (Bouton et al., 2021). On one hand, correlation analysis revealed that the greater the activation of the BNST, the better the extinction in female rats and SB male rats, who show better extinction evolution during the spontaneous recovery test. Therefore, these results corroborate data showing that the BNST is a brain area of special importance for active avoidance behavior (Guerra et al., 2023). On the other hand, the principal component analysis suggests that in males, the BNST is more related to the emotional regulation circuit whereas in females it seems to work more closely with the basal forebrain cholinergic structures related to the hippocampus. The results of the principal component analysis also suggest that the dorsal hippocampus would be part, along with the prefrontal cortex, of the control and behavioral inhibition circuit in females, while in males, it would be more functionally related to the septal circuit.

It is crucial to consider that all factors contributing to sex differences must be carefully analyzed, since behavior, including the avoidance response, is influenced by the interplay of multiple factors, such as genetic variability, sexual hormonal levels, individual tendency to anxious behaviors, individual susceptibility to epigenetics and environmental factors.

Involvement of OX1R in the two-way active avoidance task

The results of this thesis reveal a differential pattern of effects of OX1R blockade, depending on the sex and timing of administration relative to the conditioning phase.

Firstly, OX1R selective blockade impaired the acquisition of the 2-AA task only in males (Experiments 2 and 3). These sex differential effects, as previously discussed (Experiment 3), could be associated with the higher synthesis of orexins in females, which might require higher doses of SB334867 to functionally affect the orexinergic system. On the other hand, the high biological predisposition of females to perform this type of active responses could reduce the need —or even be independent— of the contribution of the orexinergic system to condition the response. Activation of the orexinergic system, related to both arousal and plasticity

(Sakurai, 2007; Wayner et al., 2004), could be more decisive in males, where it could generate a more motivated-active behavior and/or facilitate associations between stimuli and responses involved in this conditioning. We cannot rule out that in cases of very little training or some specific difficulty generated in females, the OX1R system could indeed have appreciable effects.

Secondly, no effects of OX1R selective blockade on the extinction of this task have been observed in both sexes. To our knowledge, this is the first time these effects have been reported in active avoidance behavior using both sexes. However, SB334867 has been used to study the OX1R implications on the extinction of other types of task. In this sense, SB334867 administered intraperitoneally facilitated the extinction of cued and contextual FC (Flores et al., 2014), and it increased the activity of two pro-extinction areas (BLA and IL cortex) (Flores et al., 2017). The different types of conditioning paradigms—classical vs. instrumental—as well as the route of administration of the blocker, could explain why our results do not coincide with those of previous studies. Although we did not observe statistically significant effects of SB334867 on the extinction of the 2-AA task, we did observe in absolute values that females who received the microinfusions exhibited similar latencies to males, as opposed to female-vehicle subjects. Thus, these data could suggest a mild facilitating effect of OX1R selective blockade in females. In future studies, sample size should be increased, the administered dose of SB334867 varied, or a different extinction paradigm employed to further investigate this possibility.

Moreover, an increase in non-conditioned anxiety and fear has been observed following the administration of SB334867 in female rats. (Experiment 7). However, the study of the effects of the OX1R selective blockade on tests of anxiety and unconditioned fear conducted in Experiments 2 and 3 show no effects. One explanation for these discrepancies is that in the first two experiments where we analyzed this effect, the procedure of the 2-AA task may have interfered with the baseline levels of anxiety and unconditioned fear in the subjects, thus making it difficult to observe the effects of OX1R blockade.

The lack of effects of SB334867 on anxiety levels observed in males agrees with previous studies (Staples & Cornish, 2014), although anxiolytic effects were

observed in Sprague-Dawley males in one study (Blume et al., 2018). On the other hand, Blume et al. (2018) reported that intraperitoneal administration of SB334867 did not modify anxiety in females. One aspect to consider is the age of animals. In that same study, the effects of SB334867 are age-dependent, and the age of adult rats they use is around 69 days old. In our study, we use 90-day old rats. Thus, the discrepancies in our experiment and in Blume et al. (2018) experiment could be due to age differences and types of administration.

This dissertation suggests a possible role of the orexinergic system in the acquisition of the 2-AA task in male rats, which is not necessarily mediated by its effects on non-conditioned anxiety and fear.

Factors that modulate extinction

The results of the experiments conducted in this thesis highlight several potential factors (training, physiological, or treatment-related) that should be considered when designing therapies aimed at extinguishing maladaptive emotional responses.

Firstly, we observed that the training protocol is a key factor in the consolidation of emotional learning. Specifically, a distributed protocol facilitates conditioning. Secondly, we found that sex hormones can modulate the extinction of emotional conditioned responses. Finally, MFB-ICSS treatment has a positive effect on extinction of active avoidance behavior. Specifically, we observed that ICSS enhances the extinction of the 2-AA task in females, as previously demonstrated in males in a prior study (Huguet et al., 2017). Additionally, ICSS has been shown to positively impact other types and phases of learning (Aldavert-Vera et al., 1996, 2013; García-Brito et al., 2017, 2020; Ruiz-Medina et al., 2008). ICSS is a type of deep brain stimulation (DBS). DBS has been used both in animal studies to improve spatial memory (Hescham et al., 2013, 2016) and in clinical settings to regulate the activity of dysfunctional circuits associated with neurological diseases such as Parkinson's disease or Alzheimer's disease (Lyons, 2011). This is achieved by targeting brain regions involved in memory (Aldehri et al., 2018). Additionally, reinforcing stimulation of the MFB concurrently activates circuits associated with

reinforcement and a diverse array of regions implicated in different types of memory, such as the septum-hippocampal circuit, the amygdala, specific thalamic nuclei or the prefrontal and retrosplenial cortices (Aldavert-Vera et al., 2013; Kádár et al., 2016; 2018; Morgado-Bernal & Segura-Torres, 2022).

The efficacy of electrical stimulation of the brain's reinforcement system in facilitating extinction suggests that activation of motivational and reinforcement systems, ideally through non-invasive methods, could complement existing extinction therapies for emotional traumatic memories in specific mental disorders.

CONCLUSIONS

The main conclusions of this thesis are as follow:

1. Female rats exhibit a superior capacity for active avoidance conditioning compared to male rats.
2. Female rats are slower to extinguish active avoidance conditioning, although they eventually manage a complete extinction of the response with enough training.
3. The proestrus phase, characterized by high levels of sex hormones, appears to be a facilitating factor for extinction and increases c-Fos expression in emotional control regions of the extinction brain circuitry.
4. Patterns of c-Fos expression suggest certain sex-dependent functional differences in extinction circuitry.
5. OX1R selective blockade impairs acquisition of the 2-AA conditioning in males but not in females.
6. OX1R selective blockade does not affect extinction of the 2-AA conditioned response in neither males nor females.
7. SB334867 infused after acquisition sessions does not affect c-Fos expression in extinction-related brain circuits following spontaneous recovery.
8. Female rats are less anxious than males in non-conditioned fear and anxiety tests. SB334867 causes anxiogenic effects in females, making them more similar to males.
9. MFB-ICSS treatment facilitates extinction of the 2-AA conditioning in females.

Therefore, the results support our hypothesis that orexins are involved in emotional processing and active avoidance acquisition, and corroborate sex differences in emotional learning. Moreover, our results shed light on Intracranial Self-Stimulation at the medial forebrain bundle as a potentially promising treatment for extinction in females.

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SUPPLEMENTARY MATERIAL

c-Fos immunohistochemistry protocol

- Remove the samples from the -80ºC freezer and add TBS.
- Incubate the sections for 30 min in 0.3% H2O2 in agitation.
- Rinse the sections for 15 min x3 (TBS-T/TBS/TBS-T) in agitation.
- Incubate the sections in 1% BSA TBS-T for 30 min in agitation.
- Incubate the sections in the primary antibody (anti-c-Fos mouse, Sant Cruz Biotechnology, USA, sc-166940; 1/500) in 0.5% BSA TBS-T overnight at 4ºC in agitation.
- Rinse the sections for 15 min x3 (TBS-T/TBS/TBS-T) in agitation.
- Incubate the sections in the secondary antibody (anti-mouse, Jackson Immunoresearch, Inc, USA; 115-065-166; 1/500) in 0.5% BSA TBS-T for 1 h at room temperature and in agitation.
- Rinse the sections for 15 min x3 (TBS-T/TBS/TBS-T) in agitation.
- Incubate the sections in SA-HRP (Perkin Elmer) 1/2500 in TBS-T for 2 h in room temperature in agitation.
- Rinse the sections twice for 15 min with TBS and TrisHCl (50mmol/L) ph 7,6.
- Add DAB solution in the sections and wait 4 min, inside the hood, in constant agitation and covered from the light.
- Rinse the sections four times with TBS.
- Mount the sections in TrisHCl and let them dry.
- Dehydrate the sections in increasing and successive baths of etanol solutions (50, 70, 96, 100%) and finishing with a bath of Histoclear (Laboratorios Conda, Madrid, Espanya).
- Cover the sections with Pertex (41-4011-00, Medite GmbH, Wollenweberstr).