Federal University of Rio Grande do Norte
Brain Institute

Effects of Paroxetine during Elevated Plus Maze Test and Retest

Master's Thesis

Neuroscience Graduate Program

Izabela Lima Paiva
Advisor: Prof. Adriano B.L. Tort
Natal – 2022

IZABELA LIMA PAIVA

Efeitos da Paroxetina Durante Sessões de Teste e Reteste no Labirinto em Cruz Elevado

Dissertação apresentada ao Programa de Pós-Graduação em Neurociências da Universidade Federal do Rio Grande do Norte como requisito parcial para a obtenção do título de Mestre em Neurociências.

Orientador: Prof. Dr. Adriano B. L. Tort

NATAL/RN
2022

Universidade Federal do Rio Grande do Norte - UFRN
Sistema de Bibliotecas - SISBI
Catalogação de Publicação na Fonte. UFRN - Biblioteca Setorial Árvore do Conhecimento - Instituto do Cérebro - ICe

Paiva, Izabela Lima.
Effects of Paroxetine during Elevated Plus Maze Test and Retest / Izabela Lima Paiva. - Natal, 2022.
51f.: il.

Dissertação (mestrado em Neurociências) - Universidade Federal do Rio Grande do Norte, Instituto do Cérebro, 2022.
Orientador: Adriano B. L. Tort.

1. Ansiedade. 2. Paroxetina. 3. Camundongos. I. Tort, Adriano B. L. II. Título.

RN/UF/BSICe-UFRN CDU 616.89-008.441

Elaborado por ISMAEL SOARES PEREIRA - CRB-15/741

Título: Efeitos da Paroxetina Durante Sessões de Teste e Reteste no Labirinto em Cruz Elevado

Autora: Izabela Lima Paiva

Data da defesa: 30/06/2022

Banca Examinadora:

Prof. Dr. Flávio Freitas Barbosa

Universidade Federal da Paraíba

Prof. Dr. Hindiael Aeraf Belchior

Universidade Federal do Rio Grande do Norte

Prof. Dr. Adriano B. L. Tort

Universidade Federal do Rio Grande do Norte

Index

Figure Index

Agradecimentos

À Universidade Federal do Rio Grande do Norte (UFRN) e à Universidade de Uppsala que proporcionaram a possibilidade de agregar conhecimento através de vivências acadêmicas formais e não formais. Ao CNPq e à CAPES. A todos os integrantes do Instituto do Cérebro da UFRN e da Universidade de Uppsala e demais departamentos dessas instituições com os quais tive a oportunidade de aprender e que me ajudaram nesse processo de formação.

Agradeço especialmente ao meu orientador Prof. Adriano B. L. Tort e à Dra. Martina Blunder por me receberem como discente, por me acompanharem e orientarem no processo de produção desse trabalho e pela vivência como discente de mestrado. Às pós-doutorandas Diana A. Nôga e Marina P. Reis por me receberem e por todo o ensinamento compartilhado. Ao Prof. Hindiael Aeraf Belchior por aceitar me orientar durante o estágio à docência.

Aos meus companheiros e companheiras de departamento e pesquisa: Ana Luiza, Eduardo, Bruno, Clara, Yu-Fang, Millos, Lucas Caiã, Joseph, Ingrid, Bárbara, Tawã e Annie, pela partilha diária nessa jornada científica, por todas as conversas e cafezinhos que me fortaleceram durante esse processo.

Às professoras Janine Inez Rossato e Katarina Leão por participarem do meu comitê de acompanhamento de mestrado e qualificação, e por todas as sugestões e ideias que fizeram esse trabalho mais completo. Aos professores Hindiael Aeraf Belchior e Flávio Freitas Barbosa por aceitarem integrar meu comitê de defesa.

Aos meus amigues e pilares de sustentação Marcel, Bruno, Micarla, Diana, Eduardo, Clara, Elisa, Elleonora, Otaciana, Esther, Michel, Ana Luíza, Viviane, Caio, Lucas Caiã, Lucas Barbosa, Allana, Mônica, Natália, Bruno, Vítor, Rodrigo Santiago, Joseph, Alan, Lívia, Étore, Joyce e Joachim por estarem ao meu lado em tantos momentos decisivos acolhendo minhas dúvidas e fazendo a vida mais leve. Ao meu terapeuta Lucas Menezes por todo aconselhamento, suporte e gentileza. Aos meus pais e família, que sempre estiveram ao meu lado me amparando de maneira incondicional.

Efeitos da paroxetina durante sessões de teste e reteste no labirinto em cruz elevado

Resumo

Ansiedade e medo mediam respostas de luta e fuga, que são essenciais para a sobrevivência. Como patologia, no entanto, a ansiedade é caracterizada pela supervalorização de ameaças e preocupação sustentada. Tal condição impacta fortemente a vida cotidiana, podendo resultar em disfunções sociais. Segundo uma estimativa da Organização Mundial de Saúde, os transtornos de ansiedade afetam cerca de 3,6% da população mundial, e esse número aumentou drasticamente após a pandemia de COVID-19. O labirinto em cruz elevado (LCE) é a tarefa mais utilizada para avaliar o comportamento do tipo ansioso em roedores. Esta tarefa é baseada em um conflito entre evitação e abordagem gerado pelo instinto natural de roedores em explorar um novo ambiente e sua aversão a espaços abertos e iluminados. Curiosamente, quando os animais são reexpostos ao labirinto, pode ocorrer um fenômeno de tolerância, denominado em inglês como one-trial tolerance (OTT). Neste fenômeno, drogas que têm efeito ansiolítico quando administradas no primeiro dia de exposição ao LCE tendem a não ser ansiolíticas quando administradas no segundo dia. É importante ressaltar que o OTT tem implicações para a translação da pesquisa pré-clínica para a clínica, uma vez que o LCE é frequentemente usado como um modelo animal agudo de ansiedade na investigação de possíveis tratamentos. No presente estudo, investigamos os efeitos da administração sistêmica de paroxetina, um inibidor seletivo da recaptação de serotonina amplamente utilizada no tratamento de transtornos de ansiedade, durante uma sessão de teste (primeira exposição) e reteste (segunda exposição) no LCE ocorrendo em dias consecutivos. Dado que os transtornos de ansiedade apresentam diferenças de gênero, sendo mais prevalentes no sexo feminino, estudamos tanto camundongos machos quanto fêmeas da linhagem C57BL/J6, e os resultados obtidos foram analisados combinados e separadamente. Nossos resultados mostram que a paroxetina é de fato ansiolítica durante a sessão de teste no LCE. No entanto, observamos o fenômeno de OTT em animais tratados com veículo na sessão de teste e com paroxetina 24 horas depois na sessão de reteste, caso em que a paroxetina deixou de ter efeito ansiolítico. Curiosamente, também descobrimos que o tratamento com paroxetina na primeira sessão de LCE leva a um efeito ansiolítico a longo prazo; ou seja, os animais posteriormente injetados com veículo na sessão de reteste exibiram menos comportamento do tipo ansioso do que os animais tratados com veículo nas sessões de teste e reteste no LCE. Outros protocolos experimentais revelaram que a paroxetina administrada após a primeira exposição ao LCE também foi associada a um efeito ansiolítico de longo prazo na segunda exposição ao LCE 24 horas depois. Os efeitos da paroxetina foram similares em machos e fêmeas, embora diferenças tenham sido encontradas para alguns comportamentos específicos. Ao todo, concluímos que a paroxetina tem efeitos diferentes durante as sessões de teste e reteste no LCE.

Palavras-chave: ansiedade, paroxetina, ISRS, LCE, OTT, camundongo.

Effects of Paroxetine during Elevated Plus Maze Test and Retest.

Abstract

Anxiety and fear mediate critical fight-and-flight responses, which are essential to survival. As a pathology, anxiety is characterized by the overvaluation of potential threats and sustained worry. Such a condition strongly impacts daily life, resulting in a social dysfunction that can trigger other mood disorders such as depression and bipolar disorder. According to a World Health Organization estimative, anxiety disorders affect around 3.6% of the world population, and this number increased drastically after the COVID-19 pandemic. The elevated plus-maze (EPM) is the most used task for assessing anxiety-like behavior in rodents. This task is based on an avoidance-approach conflict generated by the natural behavior of rodents to explore a new environment and its aversion to open and light spaces. Interestingly, when the animals are re-exposed to the maze, a phenomenon called one-trial tolerance (OTT) may occur. In this phenomenon, drugs that have an anxiolytic effect when administered on the first day of EPM exposure tend not to be anxiolytic when administered on the second day. Of note, OTT has implications for translatability from preclinical research since the EPM is often used as an acute task during screening for anxiety treatments. In the present study, we investigated the effects of the systemic administration of paroxetine, a selective serotonin reuptake inhibition widely used to treat anxiety disorders, during a test (first exposure) and a retest (second exposure) session in the EPM taking place in consecutive days. Given that anxiety disorders exhibit gender differences since they are more prevalent in females, we studied cohorts of both male and female C57BL/J6 mice, which were analyzed either combined or separately. Our results show that paroxetine is indeed anxiolytic during the test session in the EPM. Nevertheless, we observed the OTT phenomenon in animals treated with vehicle in the test session and with paroxetine 24 hours later in the retest session, in which case paroxetine no longer had an anxiolytic effect. Curiously, we also found that paroxetine treatment in the first EPM session leads to a long-term anxiolytic effect; namely, animals later injected with vehicle in the retest session exhibited a lower anxiety-like behavioral profile than animals treated with vehicle in both the test and retest sessions. Further experimental protocols revealed that paroxetine administered after the first EPM exposure was also associated with a long-term anxiolytic effect in the second EPM exposure 24 hours later. Most of our results held true when analyzing males and females separately, though specific sex differences in some behaviors could also be found. In all, we conclude that paroxetine has different effects during the test and retest sessions in the EPM.

Keywords: anxiety, paroxetine, SSRI, EPM, one-trial tolerance, mouse.

1. Introduction

1.1 – Anxiety: General Overview

Anxiety and fear in humans mediate critical fight-and-flight responses, which are essential to survival (LeDoux and Pine, 2016). Anxiety as a pathological condition has been overgrowing and is widespread globally. It is characterized by the overvaluation of potential threats and sustained worry (American Psychiatric Association, 2013). Anxiety can arise due to a specific situation or generalized without relation to an event. This state can evoke peripheral and cortical responses in humans, including sweating, increased heart rate, breathing rate changes, insomnia, and learning deficits.

Such a condition strongly impacts people's daily lives, resulting in a social dysfunction. According to a WHO estimative, anxiety disorders affect around 3.6% of the world population, increasing drastically after the COVID-19 pandemic. This pathological condition can be classified into four subclasses: Generalized Anxiety Disorder (GAD), Social Anxiety Disorder, Panic Disorder, and Phobias. Yet it can appear associated with other mental disorders like depression and tinnitus (Tiller, 2012; Pattyn et al., 2016).

Anxiety has a higher predominance in women than in men (Jalnapurkar, Allen and Pigott, 2018). The causes of it have strong participation of societal and cultural dynamics. However, the prevalent anxiety disorders diagnosis in women also has a biological component depending mainly on the different levels of the reproductive hormones, estrogen and progesterone, during the reproductive life of women (Altemus, Sarvaiya, and Epperson, 2014). Moreover, the testosterone hormone in men induces a less anxious profile (McHenry et al., 2014). The progesterone levels can induce modulation of brain areas that are related to anxiety, such as the medial prefrontal cortex, hippocampus and amygdala (Lebron-Milad and Milad, 2012). The biological evidence supports the idea that anxiety disorders might be sustained by partially or totally different physiological mechanisms between men and women, but the pathophysiological mechanism is still not clear.

Even though anxiety is prevalent and has a high impact on human mental health,

the physiological mechanisms behind its expression and the role of the neurotransmission systems are not precisely defined. Therefore, the establishment of a therapeutic protocol is challenging. Commonly the treatment of pathological anxiety involves the association of psychotherapy and the prescription of antidepressant drugs such as serotonin reuptake inhibitors, serotonin-norepinephrine reuptake inhibitors, benzodiazepines, and in some cases, tricyclic antidepressants (Murrough et al., 2015).

1.2 – Animal behavior and anxiety

Anxiety and fear are crucial for mediating fight-and-flight responses not only in humans but also in several other animal species under danger. Indeed, since this type of response is highly conserved among species, it allows for the study, in animal models, of the pathophysiological mechanisms involved in anxiety as well as of potential pharmacological treatments (Carobrez and Bertoglio, 2005). Rodents are widely used in these studies; they exhibit the so-called anxiety-like behavior that can be identified through the use of fear conditioning tasks such as the four-plate test, conditioned taste aversion, active/passive avoidance, or based on survival instincts tasks such as free exploration, open field, zero-maze, and elevated plus-maze (Rodgers et al., 1997).

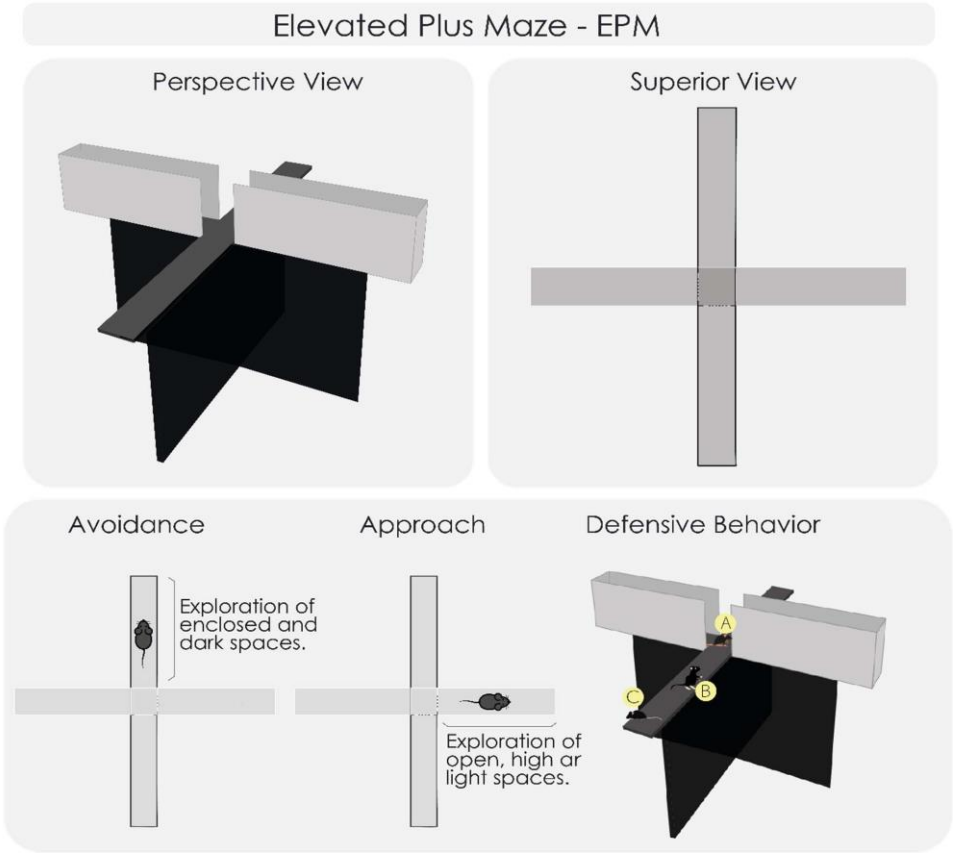

Figure 1 – Anxiety-like behavior in the elevated plus-maze (EPM). *Upward to the left is a perspective view the EPM, and to the right is a superior view. At the bottom left and middle panels are examples of avoidance the open arms) and approach behaviors. An approach-avoidance conflict drives maze exploration during the first exposition to the EPM. On the right side are some typical defensive behaviors: (A) risk assessment, (B) rearing, and (C) nose dip.*

The elevated plus-maze (EPM), which comprises two closed arms perpendicularly crossed by two open arms (Figure 1), is the most used task for assessing rodent anxiety-like behavior (Walf and Frye, 2007). It is based on the approach-avoidance conflict generated in naive animals by the instinct to explore new environments and their natural avoidance of open and light spaces (Carobrez and Bertoglio, 2005). The open arms approach is sensitive to anxiolytic drugs such as benzodiazepines, GABA receptor ligands, and selective serotonin reuptake inhibitors (SSRIs) (Dawson and Tricklebank, 1995).

During the EPM task, several metrics are taken to reflect the level of anxiety-like behavior of the animals, including measurements of the number of open arm entries, percentual of open arm entries, percentual of time spent in the open vs. closed arms, closed arm entries, and the presence of defensive behaviors, such as freezing, defecation, and stretched attend postures (Rodgers et al., 1997; Arabo et al., 2014).

Throughout the drug screening in the EPM, animals who receive only vehicle injection tend to take refuge in closed arms and perform few and short expeditions to open arms. In contrast, those treated with anxiolytic drugs tend to explore open arms more (Dawson and Tricklebank, 1995). Interestingly, for some anxiolytic drugs, animals that were previously exposed to the EPM (even without drug treatment) exhibit a significant reduction in drug effect when re-exposed to the EPM, which can be seen by a decrease in the exploration of open arms during this second EPM session when treated with the anxiolytic drug (Figure 2). This phenomenon is called one-trial tolerance (OTT) (Schneider et al., 2011).

The OTT phenomenon is not entirely understood and is usually considered a disadvantage to be overcome in the EPM experiments; due to its occurrence, researchers usually avoid retesting animals in this task (Schneider et al., 2011). One of the hypotheses about the OTT is that this phenomenon is due to the formation of a memory associated with maze exploration (Schneider et al., 2011). Therefore, based on this hypothesis, retesting animals in the EPM would be a way to access cognitive aspects of anxiety-like behavior, such as memory, in addition to the assessment of the natural defensive behavior

evoked by the single exposition to the maze.

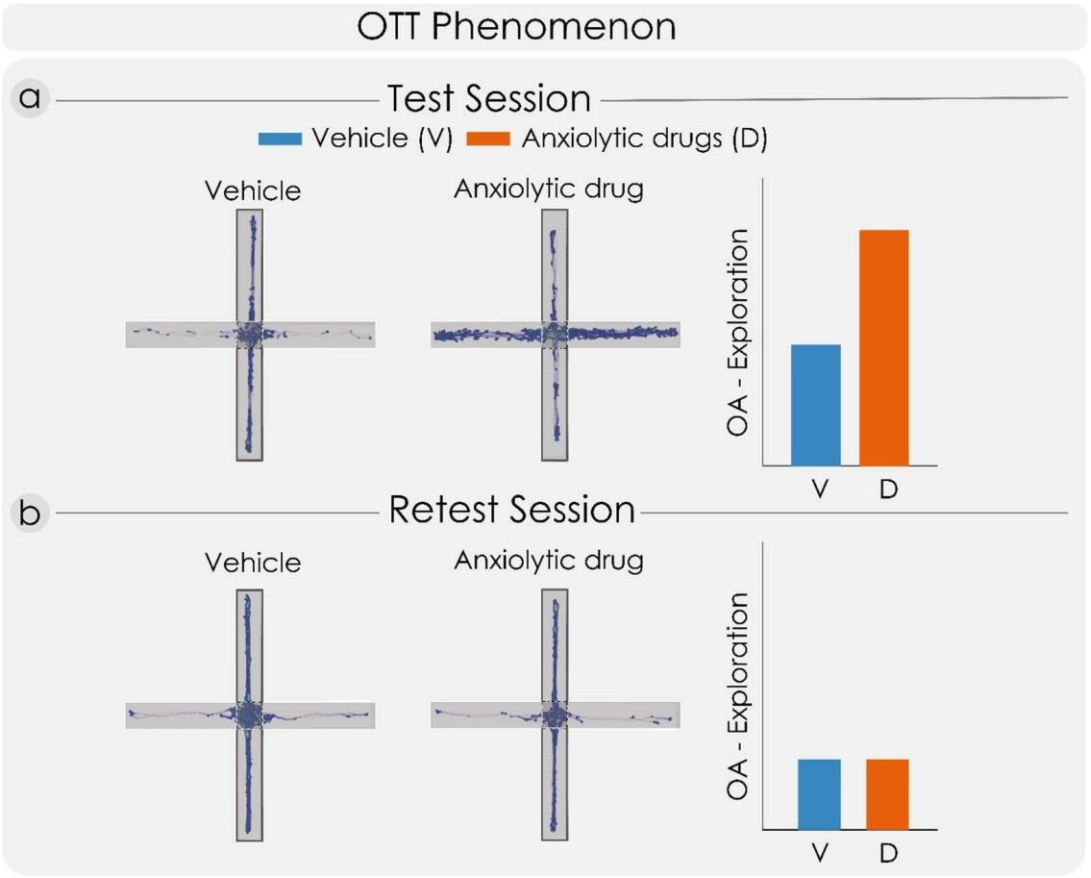

Figure 2 – Representative scheme of the one-trial tolerance (OTT) phenomenon. *Top view of the apparatus and an example of exploration trajectory exhibited by a mouse during exposure to the elevated plus maze (EPM). Right, bar graph representing the pattern of EPM exploration by mice after intraperitoneal injection of vehicle (V) or an anxiolytic drug (D). The horizontal axis depicts the pharmacological treatment according to the experiment day, and the vertical axis shows the number of open arms (OA) exploration for each case. Note that the anxiolytic drug has no effect during the second EPM exposure in animals previously treated with vehicle in the first EPM exposure, which characterizes the OTT phenomenon. This figure was made with our own data.*

1.3 – Neurobiological and pharmacological aspects of anxiety

Studies have shown that anxiety can induce significant changes in cortical and limbic areas and the autonomic system (Carlisi and Robinson, 2018). The central cortical region involved is the prefrontal, which directly modulates the amygdala, hippocampus, and other subcortical areas and has direct connections to the brainstem structures, raphe's dorsal and medial nuclei, and locus coeruleus (Garakani, 2006).

The prefrontal cortex establishes bidirectional connections with the olfactory system, which receives and sends dense projections to the piriform cortex and other olfactory regions (Salimi et al., 2019). These regions also send projections to the amygdala and

entorhinal cortex. Through these connections, the olfactory system also impacts the modulation of the fear response in cortical areas and possibly during anxiety-like behaviors expression. The medial prefrontal cortex (mPFC) sends projections to both the dorsal raphe nucleus and the basolateral nucleus of the amygdala (Liu et al., 2020).

The hippocampus presents a structural and functional differentiation along the dorsoventral axis that allows participation in the different cognitive processes through the connections it establishes with different areas of the brain. It plays a role in acquiring new memories, spatial navigation, and regulation of fear response or anxiety-like behaviors. Through the central portion of the hippocampus, projections are sent to the mPFC and amygdala (Padilla-Korean et al., 2016). Lesions performed in the ventral hippocampus induce an anxiolytic effect with a low impact on spatial learning, while the opposite happens for the dorsal portion of the hippocampus. Once injured, there is a loss in the acquisition and evocation of spatial memories.

The serotonergic system is widely distributed among brain areas and is associated with an extensive range of cognitive processes in the brain as memory formation and emotions (Gordon and Hen, 2004). It has emerged such as an essential neurotransmission system in contexts of fear and anxiety. The release of serotonin mediated by the raphe's nucleus is directedly modulated by projections sent from the mPFC to the dorsal raphe nucleus, which in turn sends projections back to the mPFC that sends projections to the basolateral nucleus of the amygdala, as shown in Figure 3. Together, those findings highlight the importance of the serotoninergic system to the modulation of fear and anxiety-like responses.

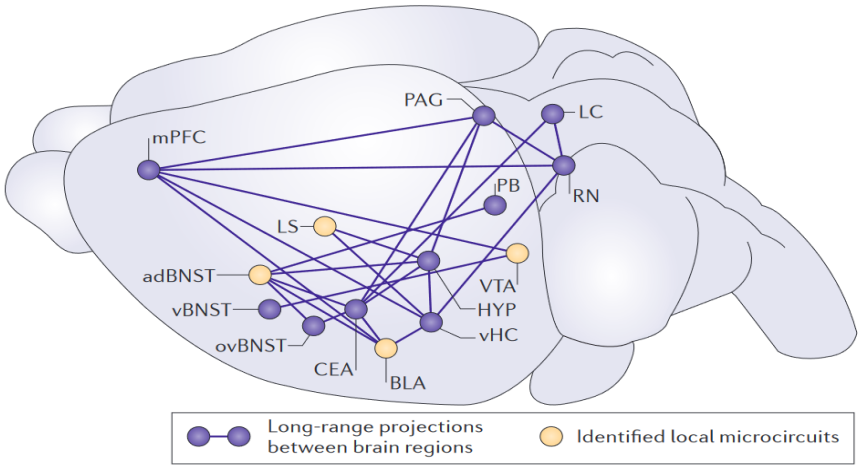

Figure 3 – Schematic representation of brain areas involved in the anxiety-like behavior: *Basolateral amygdala (BLA); Central amygdala (CEA); bed nucleus (BNST); ventral hippocampus (vHC); entorhinal cortex (EC); ventral tegmental area (VTA); hypothalamus (HYP); locus coeruleus (LC); lateral septum (LS); medial prefrontal cortex (mPFC); periaqueductal grey (PAG); parabrachial (PB); raphe nuclei (RN). Figure from: Tovote et. Al, 2015.*

The memory function and anxiety interplay in a complex manner. Anxiety disorders are often associated with some traumatic and harmful situation as happens with panic disorders (Kalueff, 2007). On other side therapeutic technics based on the rescue of those memories, memory recall, and the reassociation, memory reconsolidation, of that context or memory with harmless association has been a powerful therapeutic approach (Barad, 2005). Such evidence supports the idea that anxiety disorders might rise from a maladaptive in mnemonical process. Yet has been shown that interfering with memory consolidation after an aversive experience can decrease the level of anxiety-like response in rodents exposed to a retest session (Escarabajal, Torres, and Flaherty 2003). Interestingly, the memory consolidation process is influenced by different neuromodulators; they can act either in an early phase, that can last up to six hours, or in a late phase, that can last up to days (Rossato et al. 2009).

1.4 – Project overview and justification

In the present work, we sought to investigate further the OTT phenomenon in mice exposed and re-exposed to the EPM (Andreatini and Bacellar, 2000). The motivation for such is several-fold: for one, there is currently a critique toward the translatability from preclinical research, which is due to the fact that the EPM is often used as an acute task to investigate anxiety-like behavior during drug screening for anxiety treatment (Korte and De Boer, 2003). Therefore, we consider the investigation of animals repeatedly exposed to an anxiogenic stimulus, and repeatedly treated with a potential anxiolytic drug, a necessary step to start circumventing such a gap. Secondly, is paroxetine, a classical SSRI, also subjected to OTT? Surprisingly, even though paroxetine is a widely used anxiolytic drug, it has never been tested for OTT to the best of our knowledge. Thirdly, from a pharmaceutical point of view, the OTT protocols classically comprise Vehicle (at a first exposition)-Drug (at a second exposition), and Vehicle-Vehicle animal groups. But what about the behavior of animals receiving drugs in the first exposition? That is, Drug-Vehicle and Drug-Drug groups? This question is of interest to address potential "state"-dependencies of the behavioral effects. Moreover – and fourthly – to test for potential drug effects over memory consolidation, we also performed experimental protocols with animals treated after being experienced in the EPM.

It is well-known that anxiety disorders exhibit significant gender differences; for instance, they are more prevalent in females (World Health Organization, 2017). Therefore, in the current work, we also sought to investigate whether there is sex- specific behavioral profiles in mice subjected to the OTT protocols and have thus worked with cohorts of both female and male animals. Finally, as additional sets of experiments, we sought to gain insight into the brain regions involved and potential physiological mechanisms of OTT. To this end, we performed behavioral and electrophysiological recordings (preliminary) in animals subjected to the same EPM-OTT protocols.

2. Aims

2.1 General

To evaluate the effects of paroxetine on anxiety-like behavior of female and male mice exposed twice to the elevated plus-maze (EPM).

2.2 Specific

- To verify if the acute paroxetine administration has anxiolytic effect in the first exposure in the EPM.

- To investigate if the classical OTT phenomenon takes place in animals undrugged in the first EPM exposition (“test session”) and treated with paroxetine in the second EPM exposition (“retest session”).

- To investigate if the administration of paroxetine in the test session influences the behavior of the animals treated with paroxetine or vehicle in the retest session.

- To investigate the effect of paroxetine over the retest session when injected immediately and 6 hours after the test session.

- In all protocols, to investigate if there are any sex differences in the test and retest sessions.

3. Methods

Animals:

We used male and female mice (Mus musculus) of the C57BL/6J strain, 8-12 weeks of age, weighing approximately 20-35g, kept under controlled temperature (24 ± 1ºC), on a 12-hour light-dark cycle, and distributed in groups of five animals accommodated on polyethylene cages with water and food ad libitum.

The animals were treated with either vehicle (solution with 10% DMSO, 5% Tween 80®, 85% saline) or paroxetine (solution with 10% paroxetine 10 mg/kg, 5% Tween 80®, 85% saline). Both the vehicle and paroxetine solutions were prepared right before the experiment and injected with a volume of 10 ml/kg. The animals were kept in a vivarium at the Uppsala University. All procedures were approved by the Animal Ethics Committee of Uppsala, Sweden (12149/2020).

Elevated Plus Maze:

The elevated plus-maze consisted of a plastic cross-shaped apparatus, elevated 50cm above the ground, with two opposing open arms and two opposing closed arms (Figure 1). The floor of the arms was made of gray plastic, 37 cm long and 5.4 cm wide, and was connected to a central platform of 5.5 x 5.5 cm. The walls of the closed arms were made of gray plastic, 15 cm high. At the beginning of each session, the animals were placed in the experimental room during 30 minutes for habituation. The EPM was cleaned with a 10% ethanol solution between trials of animals of the same sex and 70% ethanol between trials of animals of different sex to reduce odors. All the animals were placed at EPM facing one of the open arms for free exploration. Images were captured by a high-resolution camera with a sampling rate of 30 frames per second throughout the 10 minutes of the task. The animal tracking was made by EthoVision XT15 software (Noldus).

We performed three groups of experiments (Pharmacological Protocol 01, 02 and 03, described below) using independent animal cohorts. The interval of 24 hours between the sessions took in account the paroxetine half-life of approximately 21 hours. Figure 4 provides an overview of the behavior-pharmacological protocols. The time between test and retest to access the OTT effect could variate from hours to days in accordance with the hypothesis in discussion ((Gazarini, Stern, and Bertoglio 2011; Escarabajal, Torres,

and Flaherty 2003). For example, to access the impact of memory impairment during acquisition phase Gazarini, et al 2011, exposed the animals to a retest session 3 hours after the test session.

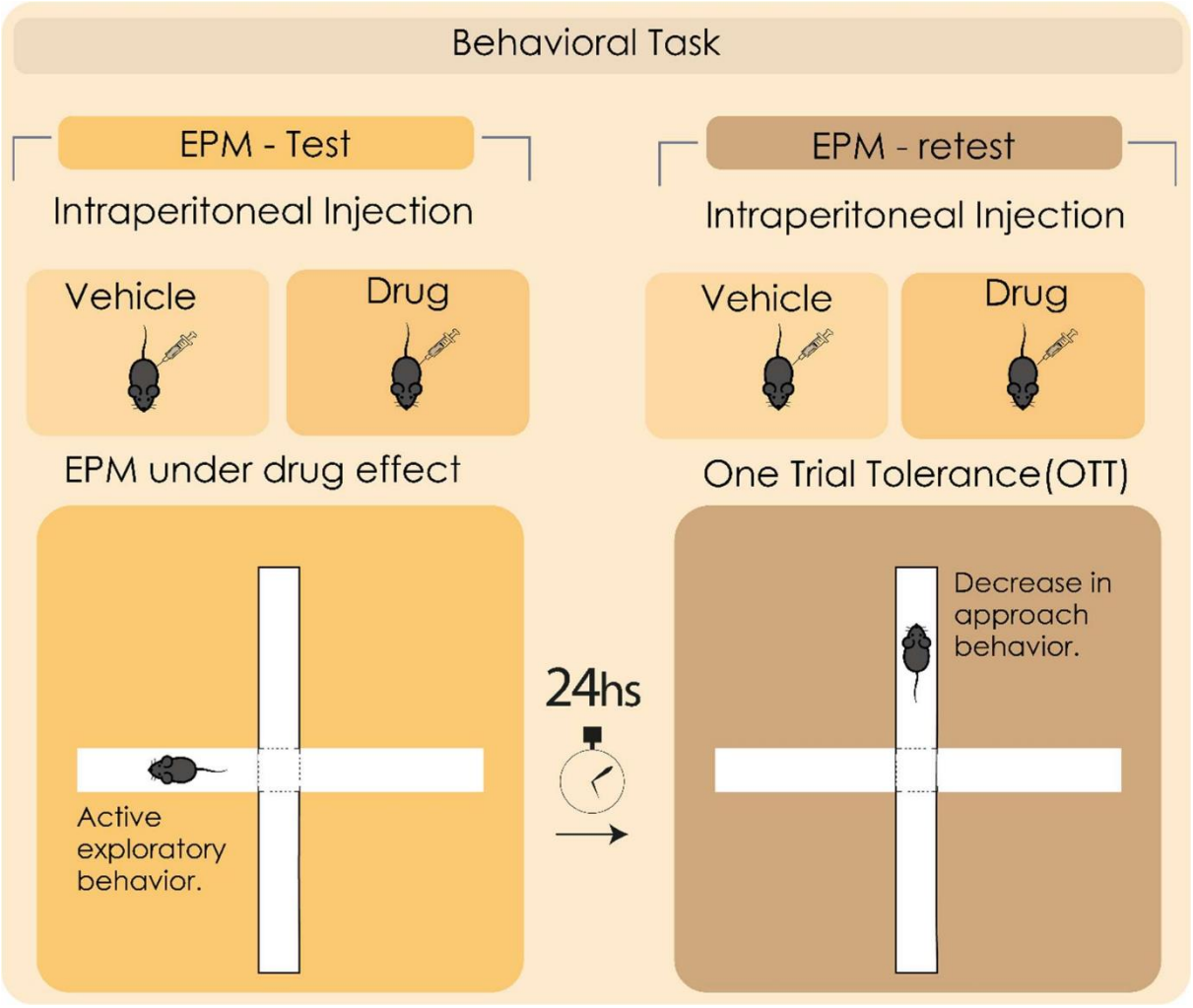

Figure 4 – Schematics of the behavior-pharmacological protocols. *Mice were subjected to a test and a retest session in the EPM. Different groups of animals receive different treatments (vehicle or paroxetine) associated with each experimental session, which were administered either 1 hour before, immediately after, or 6 hours after maze exploration. Particular focus was paid to investigating if animals exhibited the one-trial tolerance (OTT) phenomenon in the retest session.*

Pharmacological Protocol 01: Paroxetine or vehicle injection 1 h before 10-min EPM expositions:

The animals were divided into two experimental groups on the first day: vehicle and paroxetine. They received an intraperitoneal injection with the respective solution 1 hour before the 10-minute exposure to the EPM. Then the animals were removed from the maze and left in their home cages for 24 hours. Afterward, they were re-exposed to

the maze for 10 minutes and further sub-divided into four final groups depending on the drug treatment administered 1 hour before: vehicle-vehicle; vehicle-paroxetine; paroxetine-vehicle; and paroxetine-paroxetine.

Pharmacological Protocol 02: Paroxetine or vehicle injection immediately after the first 10-min EPM exposition:

In this protocol, the animals were treated with either vehicle or paroxetine right after the first 10-minute exposure to the EPM; 24 hours later, they were re-exposed to the maze for 10 minutes without any drug treatment.

Pharmacological Protocol 03: Paroxetine or vehicle injection 6 hs after the first 10-min EPM exposition and 1 h before the second 10-min EPM exposition:

This protocol is similar to the first behavioral task, except that, instead of vehicle or paroxetine being injected before the first 10-min EPM exposition, they were injected 6 hours afterward. As in the first protocol, animals were treated with vehicle or paroxetine 1-hour before the second 10-min EPM exposition, thus comprising 4 final groups: vehicle-vehicle; vehicle-paroxetine; paroxetine-vehicle; and paroxetine-paroxetine. In addition, this experiment has a batch of animals that received paroxetine 1 hour before the first 10-min EPM exposition, followed by either vehicle or paroxetine 1 hour before the second 10-min EPM exposition. These two additional groups thus mirror the protocol of behavioral task 01 and served as a positive control group for the effects of paroxetine.

Statistical analysis:

For all the pharmacological protocols, the comparison between the vehicle and the paroxetine groups in the test session was made by means of the Student t-test. For the multiple comparisons among groups in the retest session, we used one-way Anova followed by Dunnett's post-hoc test. Two-way ANOVA was applied to compare male and female data under different treatments. A p-value lower than 0.05 was considered statistically significant.

4. Results

4.1 Pharmacological Protocol 01: Paroxetine or vehicle injection 1 h before 10-min EPM expositions

Results from behavioral task 01 demonstrate that paroxetine indeed shows acute anxiolytic properties in mice. Namely, during the test session, animals treated with paroxetine 1 hour before exhibited more entrances (Figure 5A, $T(91) = 8.37$, $p < 0.0001$, t-test) and spent more time (Figure 5B, $T(91) = 8.65$, $p < 0.0001$, t-test) in the open arms of the EPM when compared with the vehicle group. Moreover, animals treated with paroxetine also presented a higher traveled distance (Figure 5C, $T(91) = 3.14$, $p = 0.0023$, t-test).

In the retest session, animals that received vehicle in the test and paroxetine in the retest exhibited the OTT effect, meaning the lack of anxiolytic effect after exposure to the EPM (Figure 5A and B, OA Entries: $F(3,89) = 15.31$, $p < 0.0001$, VEH-VEH x VEH-PAR $p = 0.99$; % Time in OA: $F(3,89) = 7.77$, $p = 0.0001$, VEH-VEH x VEH-PAR $p = 0.99$, one-way ANOVA followed by Dunnett's post hoc).

Interestingly, animals treated with paroxetine in the test session, independently of the treatment in the retest session, showed a lower anxiety-like behavior pattern than the control group (Figure 5A and B, OA Entries: $F(3,89) = 15.31$, $p < 0.0001$, VEH-VEH x PAR-VEH $p = 0.0002$, VEH-VEH x PAR-PAR $p < 0.0001$, % Time in OA: $F(3,89) = 7.77$, $p = 0.0001$, VEH-VEH x PAR-VEH $p = 0.0029$, VEH-VEH x PAR-PAR $p = 0.0015$, one-way ANOVA followed by Dunnett's post hoc; see Figures 7 and 8 for more details). Furthermore, the animals treated with paroxetine in the test session, independently of the treatment in the retest session, also displayed higher locomotion, as inferred by the traveled distance in the maze (Figure 5C, Distance: $F(3,89) = 47.90$, $p < 0.0001$, VEH-VEH x PAR-VEH $p < 0.0001$, VEH-VEH x PAR-PAR $p < 0.0001$, one-way ANOVA followed by Dunnett's post hoc).

When analyzing males and females separately, we observed a similar response to paroxetine for the number of entries and the time spent in the open arms (Figure 6A and B) in the test session. A two-way Anova showed effect of treatment (OA - Entries: $F(1,89) = 74.90$, $p<0.0001$, Males: $p<0.0001$, Females: $p<0.0001$ and % Time OA: $F(1,89) = 76.30$, $p<0.0001$, Males: $p<0.0001$, Females: $p<0.0001$) but not of sex (OA Entries:

F(1,89) = 3.94, p=0.05, % Time OA: F(1,89) = 2.15, p=0.15) or interaction (OA Entries: F(1,89) = 2.10, p = 0.15, % Time OA: F(1,89) = 0.44, p=0.51).

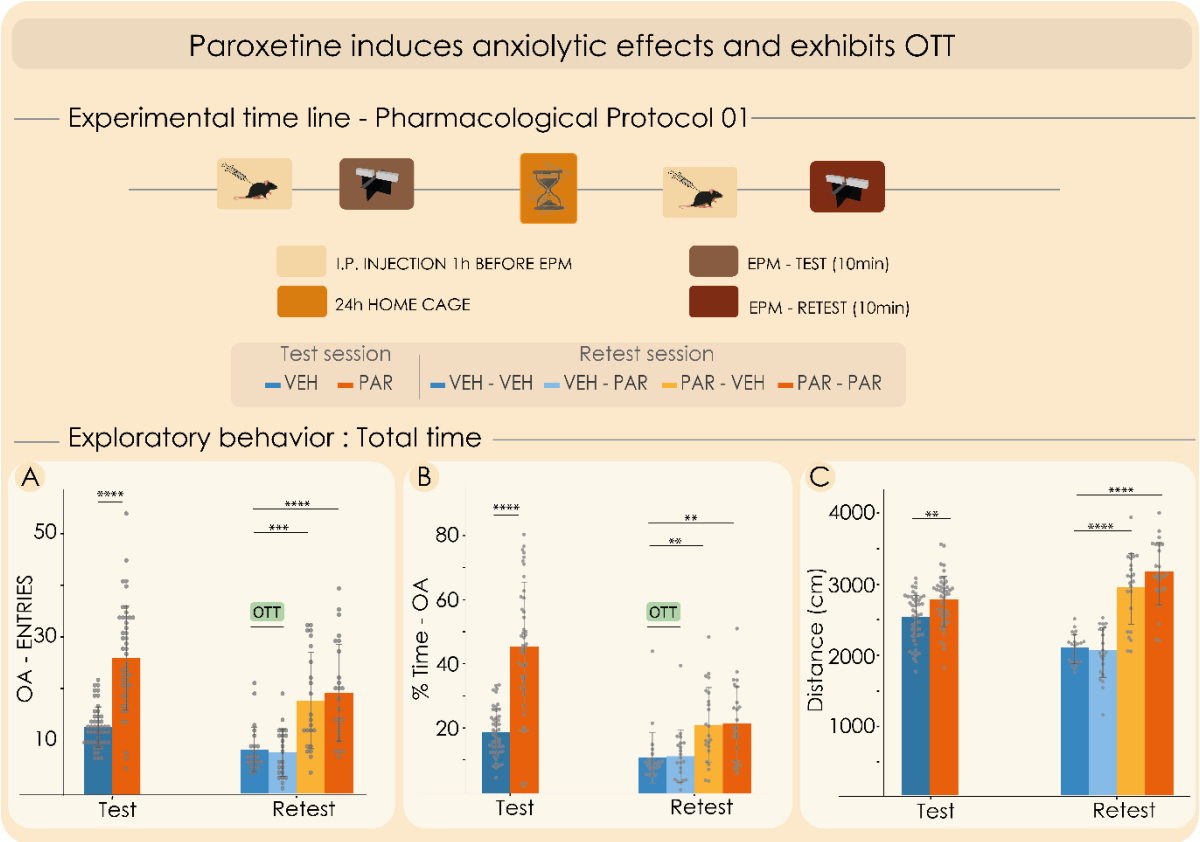

Figure 5 – Paroxetine induces anxiolytic effects and exhibits OTT. *On the top, the experimental time line of the behavioral task 01. On the bottom, the EPM statistics for males and females analyzed together. (A) Open Arms (OA) Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. Data shown as mean ± SEM. ** p < 0.01, *** p < 0.001, **** p < 0.0001, t-test (A) or one-way ANOVA followed by Dunnett's post hoc (B and C). VEH = 48, PAR = 45, VEH-VEH=23, VEH-PAR = 22, PAR-VEH = 23, PAR-PAR = 25.*

Also, during the test session, paroxetine-treated males showed an increase in the locomotory pattern while the females did not (Figure 6C, Distance: F (1,42) = 9.71, Males: p = 0.03 and Females: p = 0.11, two-way ANOVA). The difference between paroxetine and vehicle-treated animals starts from the first minutes of the task, with paroxetine-treated animals exploring more the open arms than vehicle-treated animals (Figure 6D and E).

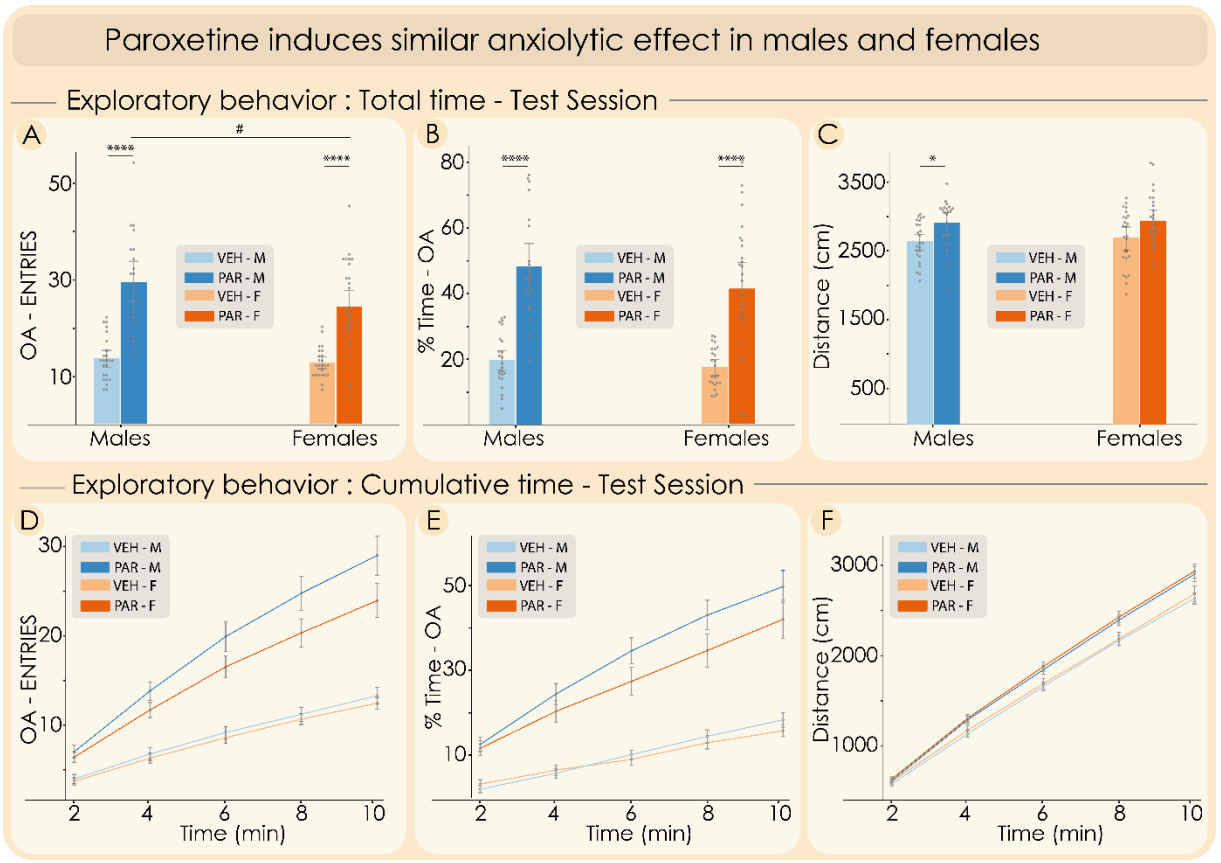

Figure 6 – Paroxetine induces a similar anxiolytic effect in males and females. *On the top, the average of the exploratory behavior in the total time. (A) OA Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. On the bottom, is the average of the exploratory behavior for each two minutes along the 10 minutes session. (D) OA Entries, (E) Percentage of time in OA, (F) Distance traveled in the apparatus. Data are shown as mean ± SEM. * $p \leq 0.05$, **** $p < 0.0001$, # $p = 0.0501$, two-way ANOVA; Males: VEH = 24, PAR = 21; Females: VEH = 24, PAR = 24.*

Animals treated with paroxetine in the test session exhibited a lower level of anxiety-like behavior in the retest session when compared with the animals who received the vehicle in both sessions (Figure 5). When analyzing females and males separately (Figures 7A and B), a two-way ANOVA showed effect of treatment (OA Entries: F(1,42) = 18.86, p<0.0001, Males: p=0.0078, Females: p=0.0071, % Time – OA: F (1,42) = 11.86, p=0.0013, Males: p=0.0499, Females: p=0.0283) but not of sex (OA Entries: F(1,42) = 2.38, p=0.13, % Time – OA: F(1,42) = 2.86, p=0.098) or interaction (OA Entries: F(1,42) = 0.0045, p = 0.95), % Time – OA: F(1,42) = 0.0086, p = 0.93). In the retest session, males and females from the group PAR-VEH showed a higher locomotory pattern (Figure 7C). A two-way ANOVA showed effect of treatment (Distance: F(1,42) = 51.94, p<0.0001, Males: p<0.0001, Females: p<0.0001) but not of sex (Distance: F(1,42) = 0.025, p=0.876) or interaction (Distance: F(1,42) =7.7×10^{-6}, p =0.998).

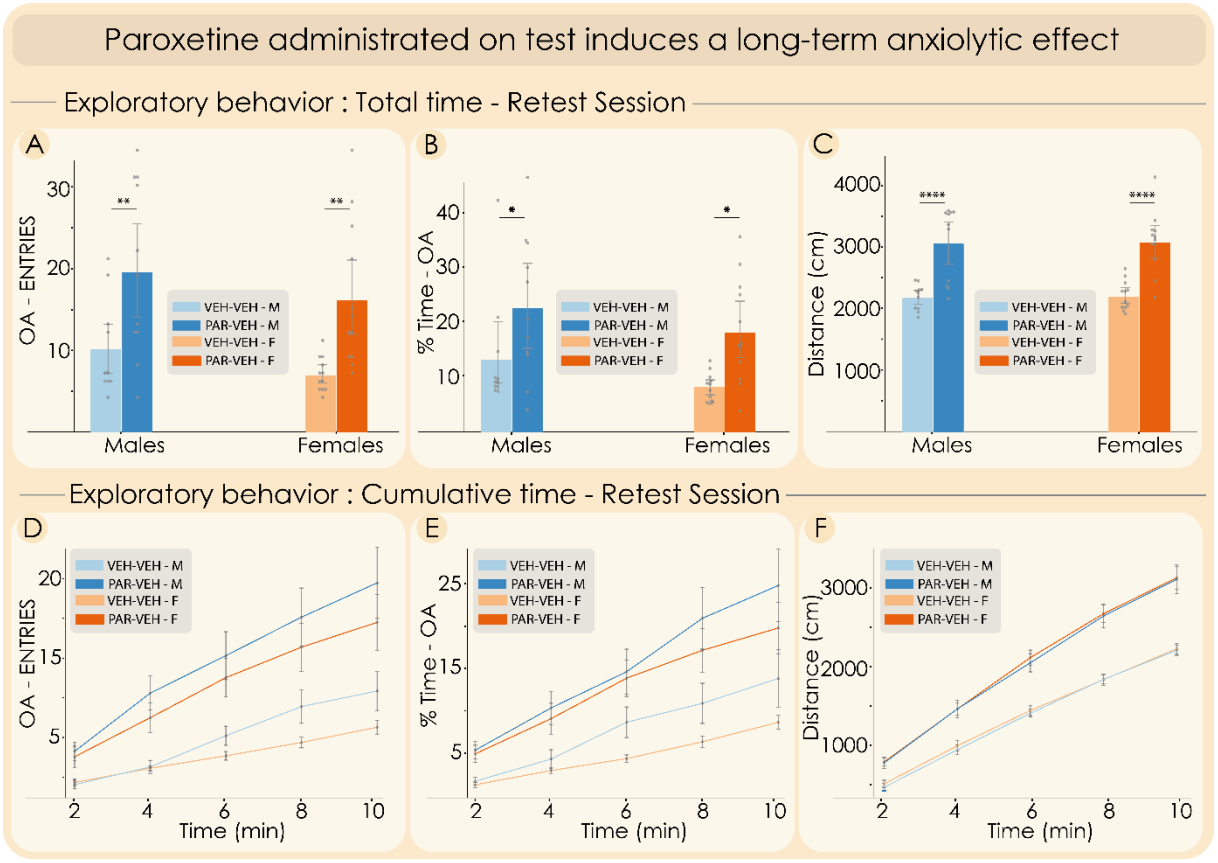

Figure 7 – Paroxetine induces a long-term anxiolytic effect. *On the top, the average of the exploratory behavior in the total time. (A) OA Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. On the bottom, the average of the exploratory behavior for each two minutes along the 10 minutes session. (D) OA Entries, (E) Percentage of time in OA, (F) Distance traveled in the apparatus. Data shown as mean ± SEM. ** p < 0.01, *** p < 0.001, **** p < 0.0001, two-way* ANOVA*; Males: VEH-VEH = 11, PAR-VEH = 11; Females: VEH-VEH = 12, PAR-VEH = 12.*

Considering the existence of the OTT phenomenon, we looked at the animals treated with paroxetine in the retest session (Figure 8). A two-way ANOVA showed effect of treatment for open arms entries ($F(1,43) = 27.75$, $p < 0.0001$), percentage of time in open arms ($F(1,43) = 11.73$, $p = 0.0014$) and distance traveled ($F(1,43) = 51.94$, $p < 0.0001$), as well as an effect of sex for open arms entries ($F(1,42) = 5.74$, $p = 0.02$) and percentage of time in open arms ($F(1,42) = 7.57$, $p = 0.0086$). There was no effect of sex for distance traveled ($F(1,43) = 0.77$, $p = 0.38$), or interaction for any of the metrics (OA Entries: $F(1,43) = 0.52$, $p = 0.48$, % Time – OA: $F(1,43) = 0.11$, $p = 0.74$, Distance: $F(1,43) = 2.413$, $p = 0.13$).

During the retest session, the animals treated with paroxetine in the test session explored more the open arms than those treated with vehicle in the test session (Figure

8A and B, OA Entries: males $p < 0.0001$, Males: $p = 0.0003$, Females: $p = 0.004$; % Time – OA: Males: $p = 0.024$, Females: $p = 0.062$). Males and females exhibited the OTT phenomenon when treated with vehicle in the test session and paroxetine in the retest session (Figure 8A and B). Interestingly, males from the group VEH-PAR explored more the open arms than the females of the same group (Figure 8A and B, OA Entries: $p < 0.0001$, Males: $p = 0.0003$, Females: $p = 0.004$; % Time – OA: Males: $p = 0.024$, Females: $p = 0.062$). Finally, males and females from the group PAR-PAR showed higher locomotion in the retest session (Figure 8C); a two-way ANOVA showed effect of treatment (Distance: $F(1,43) = 51.94$, $p<0.0001$, Males: $p<0.0001$, Females: $p<0.0001$) but not of sex (Distance: $F(1,43) = 0.77$, $p=0.38$) or interaction (Distance: $F(1,43) = 2.41$, $p=0.13$).

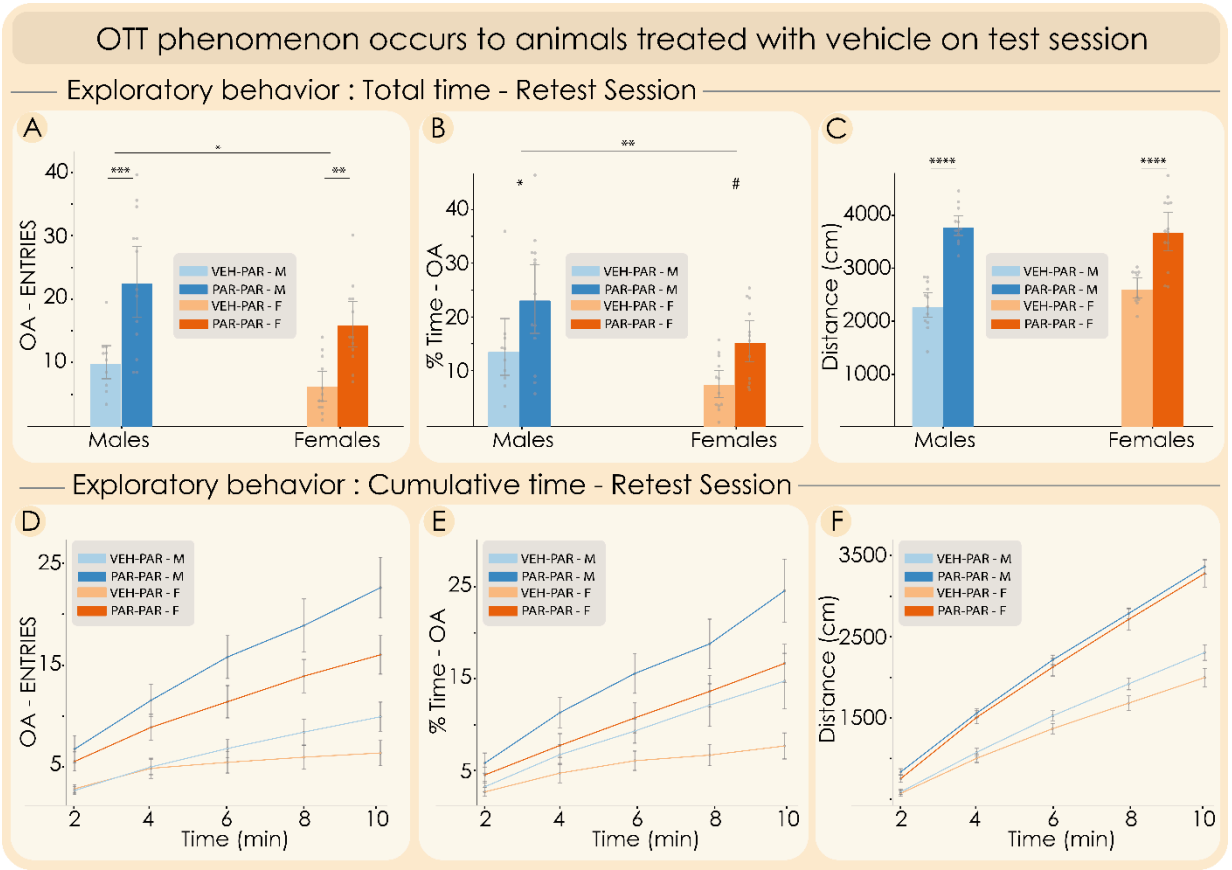

Figure 8 – OTT phenomenon occurs to animals treated with vehicle on the test session. *On the top, the average of the exploratory behavior in the total time. (A) OA Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. On the bottom, the average of the exploratory behavior for each two minutes along the 10 minutes session. (D) OA Entries, (E) Percentage of time in OA, (F) Distance traveled in the apparatus. Data are shown as mean ± SEM. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p = 0.062$, two-way Anova; Males: VEH-VEH = 10, PAR-VEH = 13; Females: VEH-VEH = 12, PAR-VEH = 12.*

4.2 Pharmacological Protocol 02: Paroxetine or vehicle injection immediately after the first 10-min EPM exposition

We hypothesized two explanations for the "long-term" effect of paroxetine observed in the group PAR-VEH in the retest session (see Figures 6 and 8). On one hand, paroxetine could be changing the way animals experience the apparatus on the first exposure, possibly reducing the aversive valence of the experience. On the other hand, paroxetine could be interfering with the formation of the memory associated with the EPM exposure. To test these hypotheses, we then administered paroxetine or vehicle right after the first EPM exposure. We reasoned that if the "long-term" effect remained, the first hypothesis could be discarded and paroxetine would probably be interfering with memory formation.

During the test session, animals expressed similar levels of open arms exploration and locomotion, which was expected since they had not received any treatment (Figure 9A-C, OA Entries: $T(46) = 0.99$, $p = 0.33$; % Time in OA: $T(46) = 0.50$, $p = 0.62$, Distance: $T(46) = 0.74$, $p = 0.46$, t-test). Interestingly, the animals treated with paroxetine right after the first EPM exposure presented a decrease in the anxiety-like behavior levels during the retest session compared with the vehicle group (Figure 9A and B, OA Entries: $T(46) = 3.00$, $p = 0.004$; % Time in OA: $T(46) = 2.94$, $p = 0.005$, t-test). Moreover, the administration of paroxetine right after the first exposure to the maze induced an increase in locomotion in the retest session (Figure 9C, Distance: $T(46) = 5.16$, $p < 0.0001$, t-test). Therefore, these results indicate that the long-term anxiolytic effect of paroxetine remains even when it is administered after the first EPM exposure (c.f. Figures 5 and 9).

Naïve males and females had a similar exploratory pattern during the test session (Figure 10A-C). When analyzing females and males separately, a two-way ANOVA showed no effect of treatment (OA Entries: $F(1,44) = 0.96$, $p = 0.33$, Males: $p = 0.98$, Females: $p = 0.41$, % Time – OA: $F(1,44) = 0.25$, $p = 0.62$, Males: $p = 0.72$, Females: $p = 0.29$, Distance: $F(1,44) = 0.59$, $p = 0.45$, Males: $p = 0.12$, Females: $p = 0.65$), no sex difference (OA Entries: $F(1,44) = 0.74$, $p = 0.39$, % Time – OA: $F(1,44) = 0.0003$, $p=0.99 = 9868$, Distance: $F(1,44) = 1.78$, $p = 0.19$) and no interaction (OA Entries: $F(1,44) = 0.54$, $p = 0.46$, % Time – OA: $F(1,44) = 2.36$, $p = 0.13$, Distance: $F(1,44) = 3.80$, $p = 0.058$).

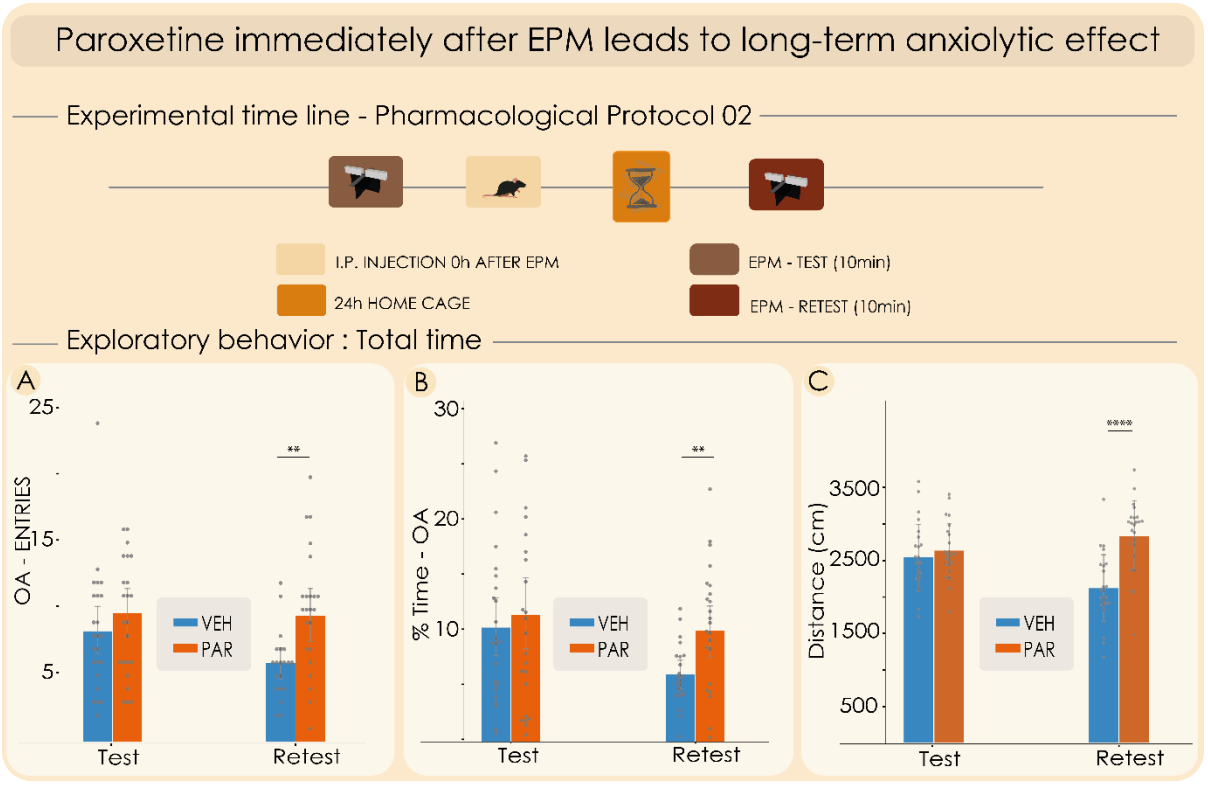

Figure 9 – Paroxetine immediately after EPM leads to long-term anxiolytic effect. *On the top, the experimental time line of the behavioral task 02. On the bottom, the EPM statistics for males and females analyzed together. (A) OA Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. Data shown as mean ± SEM. ** p < 0.01, **** p < 0.0001, t-test. VEH = 24, PAR = 24.*

During the test session, females that would receive paroxetine immediately after the EPM exposition already presented a tendency to explore more the open arms, which could induce a bias in the results for the retest session. In this sense, it would be necessary to repeat this experiment to confirm the results obtained.

In the retest session, the females, but not the males, treated with paroxetine immediately after the test session exhibited a lower level of anxiety-like behavior when compared with the vehicle group. They presented more entries and spent more time in the open arms (Figure 11A and B); when analyzing females and males separately, a two-way ANOVA showed effect of treatment (OA Entries: $F(1,44) = 9.00$, $p = 0.004$, Males: $p = 0.34$, Females: $p = 0.01$, % Time – OA: $F(1, 44) = 8.83$, $p = 0.005$, Males: $p = 0.51$, Females: $p = 0.006$) but no sex difference (OA Entries: $F(1,44) = 0.82$, $p = 0.37$, % Time–OA: $F(1,44) = 0.62$, $p = 0.43$) and no interaction (OA Entries: $F(1,44) = 1.25$, $p = 0.27$, % Time – OA: $F(1,44) = 2.23$, $p = 0.14$).

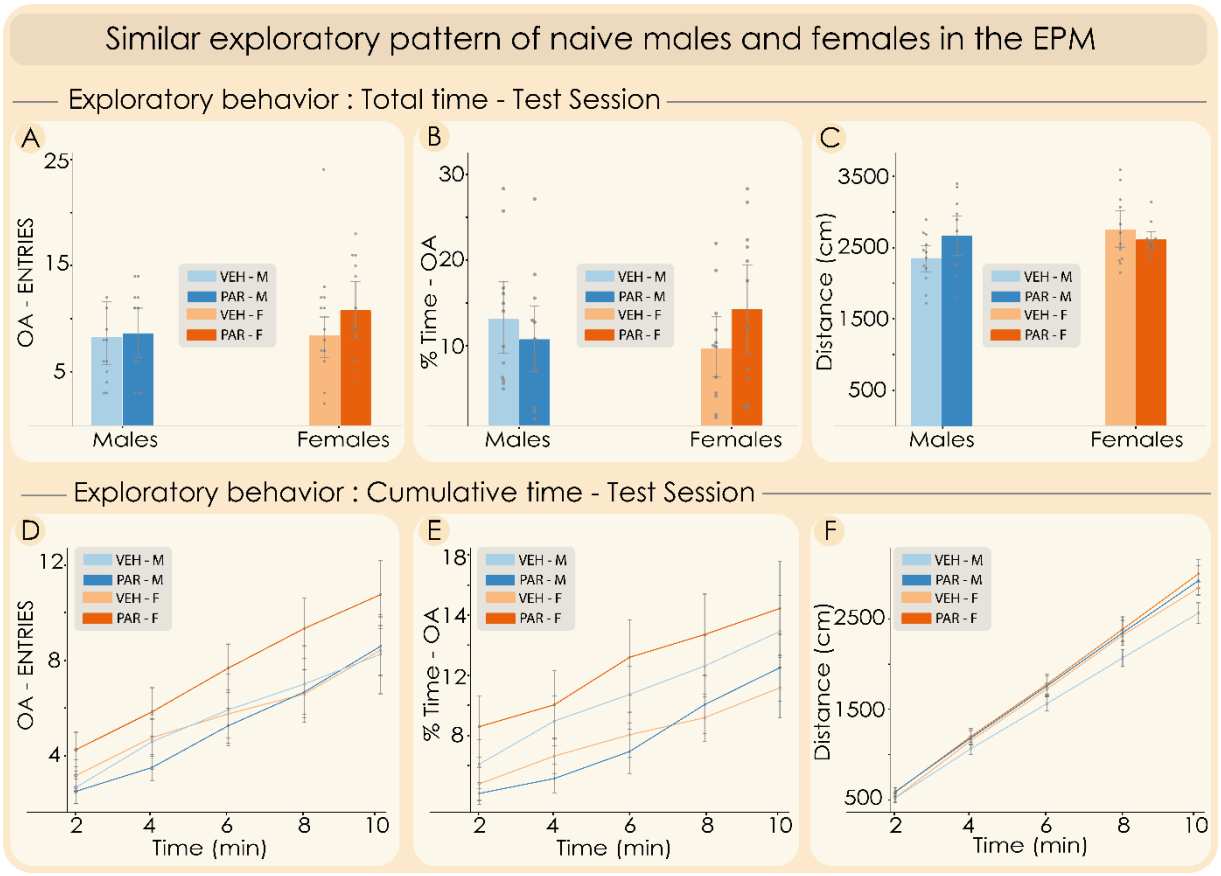

Figure 10 – Similar exploratory pattern of naïve males and females in the EPM. *On the top, the average of the exploratory behavior in the total time. (A) OA Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. On the bottom, the average of the exploratory behavior for each two minutes along the 10 minutes session. (D) OA Entries, (E) Percentage of time in OA, (F) Distance traveled in the apparatus. Data shown as mean ± SEM, two-way ANOVA. Males: VEH = 12, PAR = 12; Females: VEH = 12, PAR = 12.*

It is important to note that these results could be related to the behavioral bias mentioned before. Moreover, males and females treated with paroxetine showed increased locomotion in the retest session (Figure 11C); a two-way ANOVA showed of treatment (Distance: $F(1,44) = 28.35$, $p < 0.0001$, Males: $p = 0.0016$, Females: $p = 0.0006$) with a sex difference (Distance: $F(1,44) = 4.85$, $p = 0.033$) but no interaction (Distance: $F(1,44) = 0.057$, $p = 0.81$). Meanwhile, during the test session naive animals did not exhibit any difference between the pharmacological groups in the traveled distance, as expected since in this protocol the treatment administration was performed after this first EPM session (Figure 10C, Distance: $F(1,44) = 0.59$, $p = 0.45$, Males: $p = 0.12$, Females: $p = 0.65$).

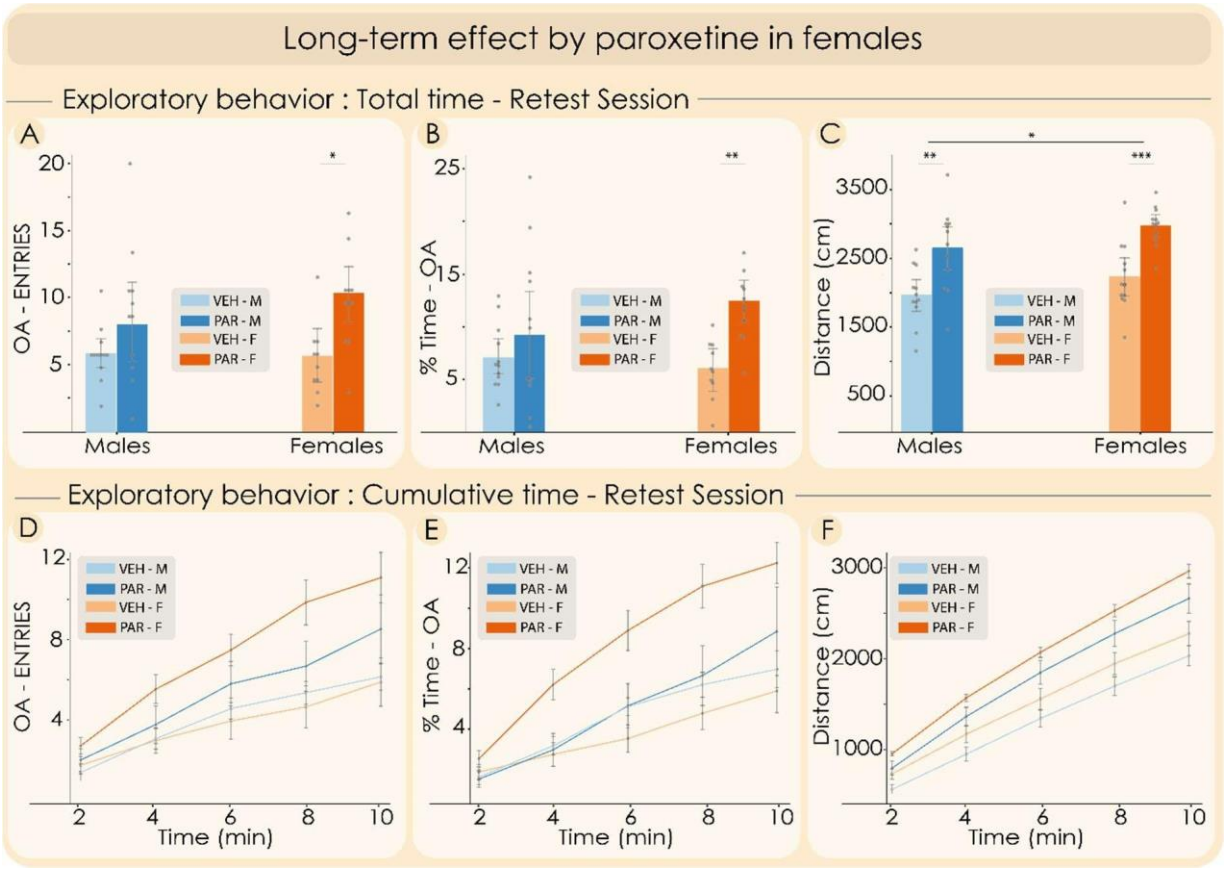

Figure 11 – Long-term effect by paroxetine in females.*. On the top, the average of the exploratory behavior in the total time. (A) OA Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. On the bottom, the average of the exploratory behavior for each two minutes along the 10 minutes session. (D) OA Entries, (E) Percentage of time in OA, (F) Distance traveled in the apparatus. Data are shown as mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, two-way ANOVA; Males: VEH = 12, PAR = 12; Females: VEH = 12, PAR = 12.*

4.3 Pharmacological Protocol 03: Paroxetine or vehicle injection 6 hs after the first 10-min EPM exposition and 1 h before the second 10-min EPM exposition

The results from females and grouped data on behavioral task 02 suggest a possible effect of paroxetine on the consolidation of the EPM memory. In order to evaluate if this effect is time-dependent, we injected paroxetine or vehicle six hours after the first EPM exposure. We also repeated the groups that received paroxetine 1h before the test and vehicle or paroxetine 1h before the retest (PAR-VEH and PAR-PAR) to confirm if the "long-term" effect of paroxetine was reproducible.

During the test session, we could not observe differences between animals that would receive vehicle or paroxetine 6 hours after, as expected (Figure 12A and B, Test session: OA Entries: T (94) = 0.73, p = 0.47, % Time – OA: T(94) = 0.24, p = 0.81, t-test). On the other hand, animals that received paroxetine 1h before the test session

showed an anxiolytic profile characterized by increased entries and percentage of time spent inside the open arms (Figure 10A and B, right side inset panel: OA Entries: T(94) = 8.47, p < 0.0001, % Time – OA: T(94) = 7.79, p < 0.0001, t-test), thus confirming our previous results (Figure 5).

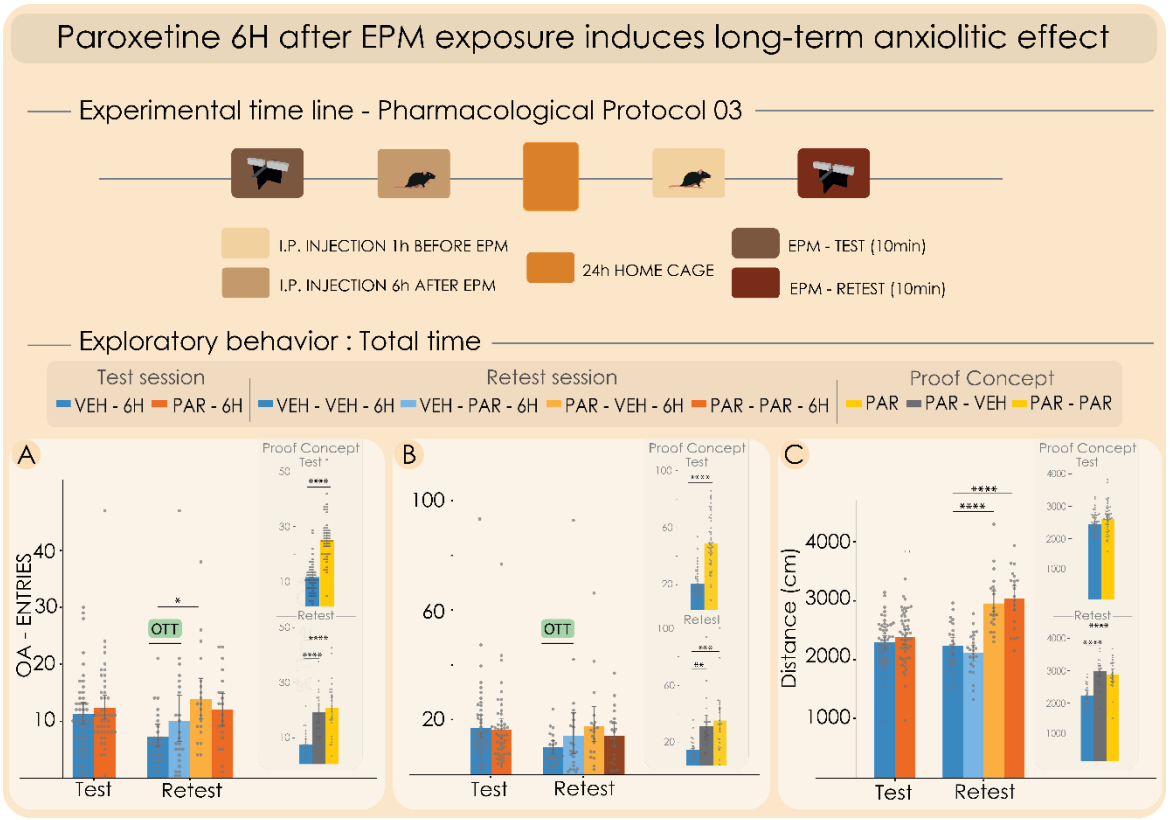

Figure 12 – Paroxetine 6H after EPM exposure induces long-term anxiolytic effect. *On the top, the experimental time line of the Behavioral Task 03. On the bottom, the EPM statistics for males and females analyzed together. (A) OA Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. Data shown as mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, t-test or one-way ANOVA followed by Dunnett's post hoc. VEH-6H = 48, PAR-6H = 48, PAR =48, VEH-6H-VEH =24, VEH-6H-PAR = 24, PAR-6H-VEH = 20, PAR-6H-PAR = 20, PAR-VEH=24, PAR-PAR=24.*

During the retest session, the animals treated with paroxetine six hours after the test session and vehicle in the retest session showed more entrances to the open arms (Figure 12A; Retest session: OA Entries: F (3,84) = 2.95, p=0.037 = 0371, VEH-6H-VEH x PAR-6H-VEH p = 0.017, one-way ANOVA followed by Dunnett's post hoc). Intriguingly, the animals treated with paroxetine in the two sessions or the ones treated with vehicle in the test session and paroxetine in the retest session showed the OTT phenomenon when compared with the vehicle group (Figure 12A and B; Retest session: OA Entries: F(3,84) = 2.95, p=0.037 = 0371, VEH-6H-VEH x VEH-6H-PAR, p = 0.47,

VEH-6H-VEH x PAR-6H-PAR p = 0.11; %Time in OA: F(3,84) = 1.147, p = 0.34, VEH-6H-VEH x VEH-6H-PAR, p = 0.61, VEH-6H-VEH x PAR-6H-PAR p = 0.62, one-way ANOVA followed by Dunnett's post hoc). In addition, all the animals treated with paroxetine six hours after the test session exhibited an increase in the distance traveled in the maze during the retest session (Figure 12C, Distance: F (3,84) = 26.64, p < 0.0001, VEH-6H-VEH x PAR-6H-VEH p < 0.0001, VEH-6H-VEH x PAR-6H-PAR p < 0.0001, one-way ANOVA followed by Dunnett's post hoc). Of note, as shown before, animals that received paroxetine 1h before the test showed a lower level of anxiety-like behavior in the retest session irrespective of the treatment received on retest and exhibited higher locomotion (Figure 12A-C, right side inset panel; Retest session: OA Entrance: F(2,68) = 17.39, p < 0.0001; VEH-6H-VEH x PAR-VEH p < 0.0001; VEH-6H-VEH x PAR-PAR p < 0.0001; % Time – OA: F(2,68) = 9.62, p = 0.0002; VEH-6H-VEH x PAR- VEH p = 0.0025; VEH-6H-VEH x PAR-PAR p = 0.0002; Distance: F(2,68) = 34.52 p < 0.0001; VEH-6H-VEH x PAR-VEH p < 0.0001; VEH-6H-VEH x PAR-PAR p <0.0001).

The males from the paroxetine group 6h after the test session and vehicle 1h before the retest session showed lower levels of anxiety-like behavior with a higher number of entries and spent more time in the open arms when compared with the vehicle group (Figure 13A and B); a two-way ANOVA showed effect of treatment (OA Entries: F(1,40) = 14.05, p = 0.0006, Males: p = 0.0032, Females: p = 0.14, % Time – OA: F(1,40) = 6.38, p = 0.016, Males: p = 0.0476, Females: p = 0.44) with a sex difference for the open arms entries but no difference for the time spent in the open arms (OA Entries: F(1,40) = 4.46, p = 0.04, % Time – OA: F(1,40) = 1.98, p = 0.17) and no interaction (OA Entries: F(1,40) = 1.67, p = 0.20, % Time – OA: F(1,40) = 0.94, p = 0.34). The distance traveled of this group was also different from the group treated with vehicle in the two sessions, independently of the sex (Figure 13C); a two-way ANOVA showed effect of treatment (Distance: F(1,40) = 28.56, p < 0.0001, Males: p = 0.0028, Females: p = 0.0003) and no sex difference (Distance: F(1,40) = 0.91, p = 0.34) nor interaction (Distance: F(1,40) = 0.053, p = 0.82).

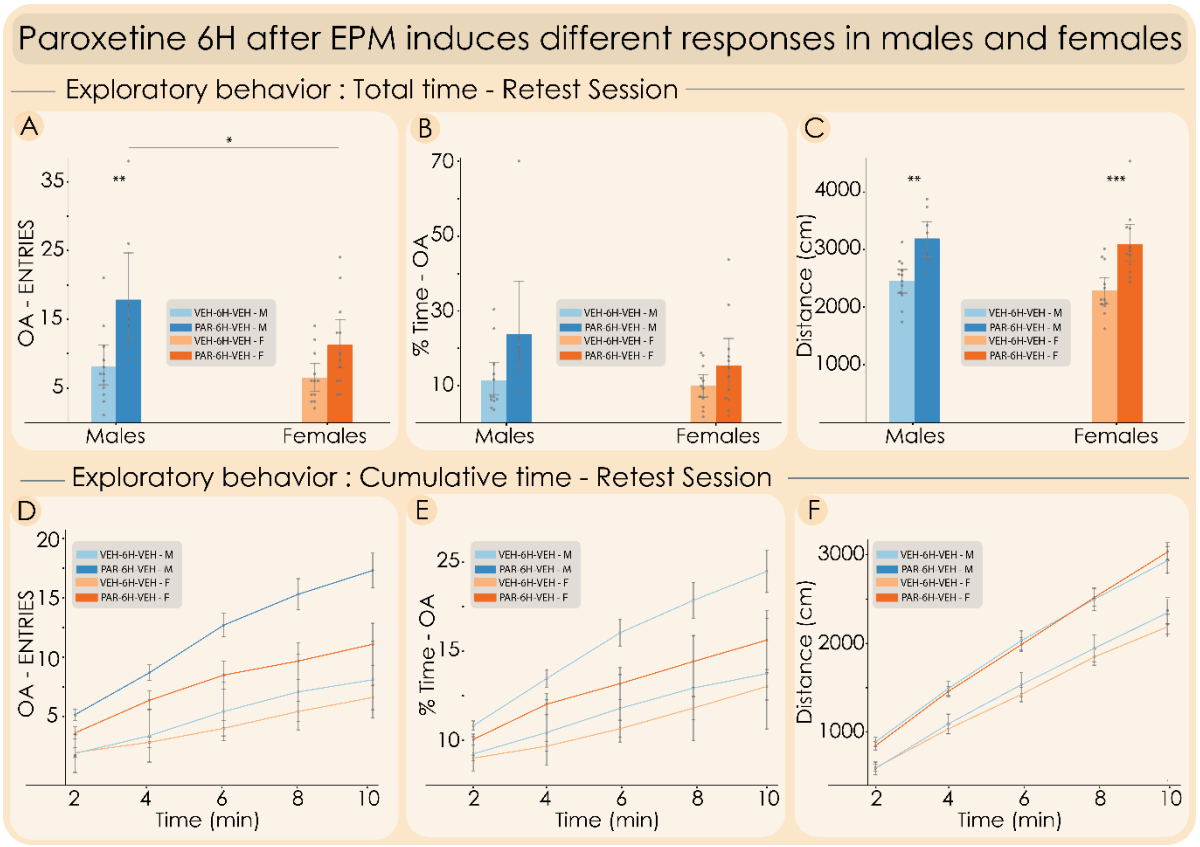

Figure 13 – Paroxetine 6H after EPM induces different responses in males and females. *On the top, the average of the exploratory behavior in the total time. (A) OA Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. On the bottom, the average of the exploratory behavior for each two minutes along the 10 minutes session. (D) OA Entries, (E) Percentage of time in OA, (F) Distance traveled in the apparatus. Data shown as mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, two-way ANOVA; Males: VEH-6H-VEH = 12, PAR-6H-VEH = 8; Females: VEH-6H-VEH = 12, PAR-6H-VEH = 12.*

Interestingly, there was a sex-difference in the animals treated with vehicle six hours after the test session and paroxetine one hour before the retest session. Males exhibited a higher number of open arms entries and spent more time in the open arms during the retest session (Figure 14A and B); when analyzing females and males separately, a two-way ANOVA showed effect of treatment (OA Entries: $F(1,40) = 1.025$, $p = 0.32$, Males: $p = 0.999$, Females: $p = 0.24$, % Time – OA: $F(1,40) = 0.018$, $p = 0.89$, Males: $p = 0.83$, Females: $p = 0.66$) with a sex difference (OA Entries: $F(1,40) = 8.12$, $p = 0.0069$, % Time – OA: $F(1,40) = 4.24$, $p = 0.046$) and no interaction (OA Entries: $F(1,40) = 1.13$, $p = 0.29$, % Time – OA: $F(1,40) = 0.91$, $p = 0.35$). Locomotion was higher for those treated with paroxetine in the test and retest sessions when compared with those from the vehicle group irrespective of gender (Figure 14C); when analyzing females and males separately, a two-way ANOVA showed effect of treatment (Distance: $F(1,40) = 55.51$, $p < 0.0001$,

Males: $p < 0.0001$, Females: $p < 0.0001$) and no sex difference (Distance: $F(1,40) = 3.36$, $p = 0.074$) or interaction (Distance: $F(1,40) = 0.36$, $p = 0.55$).

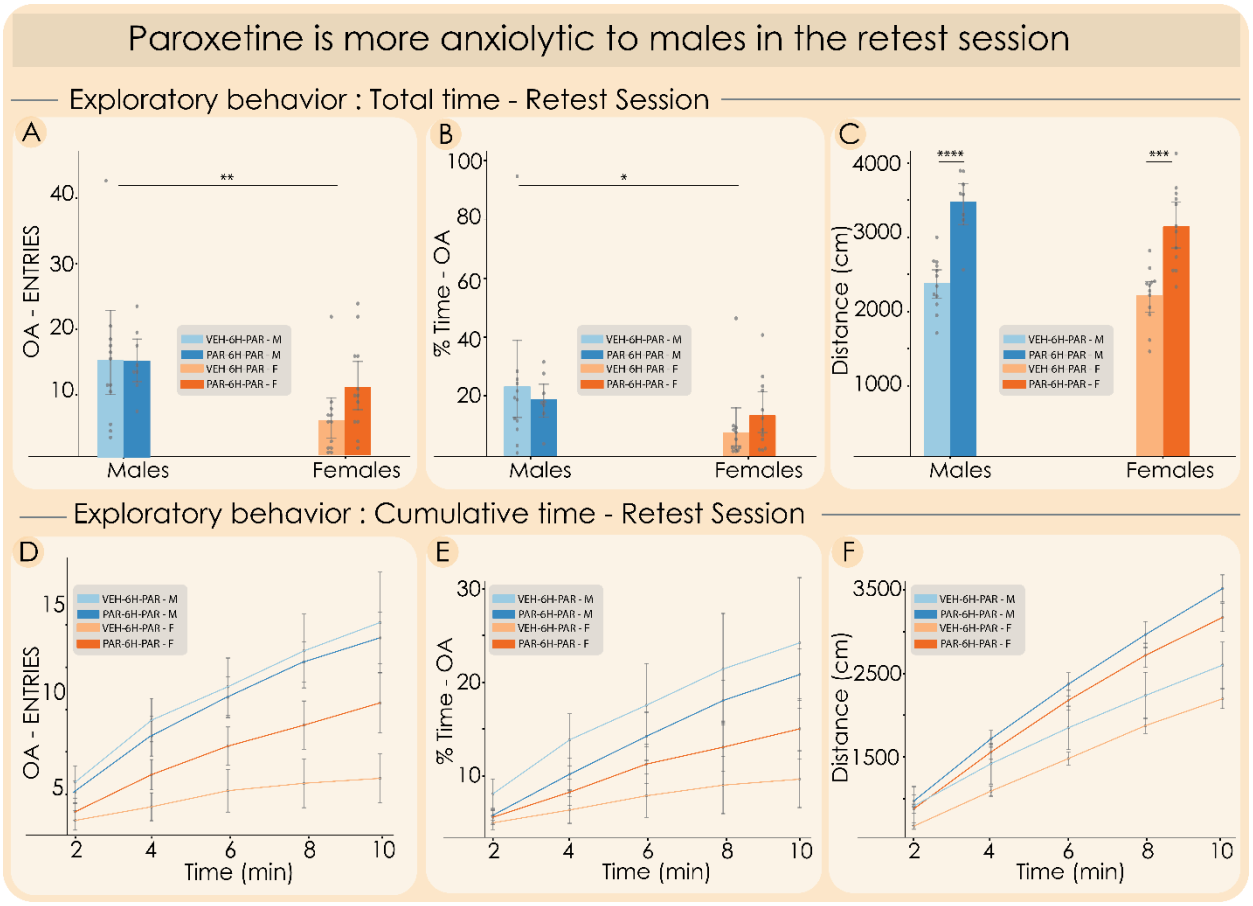

Figure 14 – Paroxetine induces higher locomotory pattern during retest session. *On the top, the average of the exploratory behavior in the total time. (A) OA Entries, (B) Percentage of time in OA, (C) Distance traveled in the apparatus. On the bottom, the average of the exploratory behavior for each two minutes along the 10 minutes session. (D) OA Entries, (E) Percentage of time in OA, (F) Distance traveled in the apparatus. Data shown as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA; Males: VEH-6H-PAR = 12, PAR-6H-PAR = 8; Females: VEH-6H-PAR = 12, PAR-6H-PAR = 12.*

5. Discussion

In the present work, we used the elevated plus-maze (EPM) with two expositions, test and retest sessions, to evaluate the effects of paroxetine on cognitive aspects of anxiety-like behavior in female and male mice. The EPM is the most common test used to induce acute anxious states and to evaluate the effects of pharmacological agents on anxiety-like behaviors in rodents (Carobrez and Bertoglio 2005; Dawson and Tricklebank 1995; Asth et al. 2012), thus allowing the screening of potential treatments for anxiety (Bourin 2015). But even though the EPM is a widely used task, there is currently a discussion in the literature about retesting animals in the maze (Andreatini and Bacellar 2000; Bourin 2018; 2019). Usually, this task is applied in one single exposition, probably because the re-exposure of animals to the maze tends to induce an increase in the avoidance pattern to the open arms. This finding suggests that, during first exposure to the EPM, the animals create a memory with spatial and emotional components derived from the experience in a novel environment where they faced a conflict between exploration and the aversion induced by the open and light condition in the open arms.

Intriguingly, the re-exposure of animals to the EPM under the treatment of anxiolytic drug results in a decrease in the anxiolytic response induced by the drug when compared to its effect on first exposure to the maze; alternatively, such an effect can also be operationally described as lack of difference in anxiety-like behavior between drug-treated and control vehicle-treated animals (Figure 2). This phenomenon is known as the one-trial tolerance (OTT) effect (File, Mabbutt, and Hitchcott, 1990). Studies suggest that the OTT occurrence is associated with a memory formation process (Holmes and Rodgers 1998; Carobrez and Bertoglio 2005; Roy et al. 2009). Somewhat paradoxically, however, prolonged exposure to the open arms induces an increase in stress and corticosterone, and increased levels of corticosterone block the induction of long-term potentiation which can interfere with the learning process (File et al. 1994; Rodgers et al. 1999). Yet, Bertoglio and Carobrez (2000) elegantly showed that the anxiety-like behavior of animals upon a re-exposure to the EPM depends on how the animals experienced the maze in the first exposure. Namely, the authors used different versions of the EPM protocol in the first session, in which the animals could freely explore the open and closed arms in the usual way, or else the animals were forced to always stay in either the open or closed arms (Bertoglio and Carobrez 2000). Interestingly, animals that could choose between the

aversive (open arms) and safe (closed arms) EPM zones (i.e., trained in the usual way) displayed an increased avoidance of the open arms upon re-exposure, which is indicative of proper maze learning. On the other hand, animals confined to either the closed or the open arms in the first session did not display such an increase in open arm avoidance in the re-exposure session (Bertoglio and Carobrez 2000).

Noteworthy, in a subsequent study, Bertoglio and Carobrez (2002) demonstrated that the OTT phenomenon to a benzodiazepine drug (midazolam) only occurs for animals that first experience the EPM in the usual way (i.e., freely exploring all arms). In contrast, the drug is still effective (anxiolytic) in the retest session for animals confined to either the closed or open arms in the test session (Bertoglio and Carobrez 2002). And in yet another follow-up study, Bertoglio and Carobrez (2004) showed that an amnesic drug (scopolamine) injected prior to the test session (called Trial 1 in that study) could prevent the appearance of the OTT phenomenon to either midazolam or memantine in the retest session (Trial 2). Additionally, Gazarini et al. (2011) showed that dorsal hippocampal infusions of anysiomicin before the EPM exposure induced an anxiolytic effect of the midazolam in the retest session. Vargas et al. (2006) showed that a 1-minute exposition to the EPM, which usually does not cause OTT, would induce the phenomenon when memory enhancers where administered. Consistently, memory learning impairment induced by propranolol in different doses before the maze exposure also hindered the OTT phenomenon showed by midazolam in the retest session (Stern, Carobrez, and Bertoglio 2008); moreover, the anxiolytic effect of midazolam displayed a positive correlation with propranolol doses (Stern, Carobrez, and Bertoglio 2008).

Further studies have demonstrated that the expression of the OTT can be associated with serotonergic activity depending on the subtype of receptors and the brain area expressing them, and also if the animal was previously exposed or not to the EPM (Canto-de-Souza, Nunes-de-Souza, and Rodgers 2002; Nunes-de-Souza, Canto-de-Souza, and Rodgers 2002; Guimarães, Carobrez, and Graeff 2008; Handley and McBlane 1993). For example, the local agonism of 5-HT1A receptors in the dorsal hippocampus induced an anxiogenic response, while the agonism of those receptors in the dorsal raphe nucleus induced an anxiolytic response. Yet, animals with previous exposure to the EPM showed an anxiolytic response under 5-HT1A agonism (Canto-de-Souza, Nunes-de-Souza, and Rodgers 2002; Nunes-de-Souza, Canto-de-Souza, and Rodgers 2002). These findings highlight the complexity of the serotonergic system in modulating anxiety and anxiolytic

responses. Of note, although the serotoninergic system is likely to play a role in OTT expression, the presence of this phenomenon under systemic administration of paroxetine, a classical SSRI drug, had not been previously shown. In fact, the therapeutical strategies and the physiological mechanisms behind anxiety expression are still poorly understood and challenging. The use of SSRIs has emerged as an efficient approach to treating anxiety disorders but their mechanism to induce anxiolysis remains unclear (Garakani et al. 2020). Curiously, SSRIs can also induce anxiogenic states (Bagdy et al. 2001; Nutt 2005).

Recently, LeDoux and Pine (2016) highlighted that, in humans, anxiety has cortical and non-cortical responses, but the animal models we currently use fail to assess the cortical responses. Therefore, considering that OTT seems to be associated with cortical processes like learning and memory, we believe that retesting animals in the EPM is a way to access cortical responses to anxiety-like behaviors and that exploring how different drugs behave in this model could help to fill some gaps in our understanding of anxiety.

Our results have shown that, during the test session, males and females injected with paroxetine one hour before the EPM exhibited an acute anxiolytic response (Figures 5 and 6). This result is consistent with previous literature showing that paroxetine can induce an acute anxiolytic state in rodents (Pádua-Reis et al. 2021). Thus, using rodents as a pre-clinical model to investigate the neurophysiological and behavioral effects of paroxetine on anxiety is valid. Yet, an interesting fact is that the opposite of what happens to rodents has been reported in humans; namely, paroxetine, as the other SSRIs, does not induce an anxiolytic state acutely but only after a period of prolonged treatment with the drug (Wilde et al. 1993). These findings indicate that both rodents and humans are sensitive to serotonergic modulation but with different response curves: rodents respond in an acute manner and humans after a prolonged exposition. This difference might be due to some process of receptors adaptation (Gray et al. 2013). Nevertheless, it should be noted that the neurobiological circuits responsible for processing fear and anxiety are highly conserved among mammalians (Rodgers et al. 1997).

To the best of our knowledge, our results are the first to show that the OTT phenomenon does occur for paroxetine (Figure 5). Namely, male and female mice under vehicle condition in the test session and paroxetine injected one hour before the retest

session showed the presence of OTT, in which their avoidance of the open arms was lower than that of paroxetine-treated animals in the first session and similar to the vehicle-injected animals in the retest session, thus displaying lack of an anxiolytic effect (Figure 5). These findings highlight the complexity of the serotonergic system during the modulation of anxiety and the anxiolytic responses and are compatible with the idea that memory acquisition and consolidation might be important for the expression of the OTT phenomenon in the EPM, as reviewed above (Bertoglio and Carobrez, 2000; Bertoglio and Carobrez, 2002; Bertoglio and Carobrez, 2004; Stern et al., 2008).

Of note, the animals that received paroxetine injection one hour before EPM exposure during the test session showed the presence of a long-term anxiolytic effect during the retest session when injected with vehicle. That is, the "paroxetine-vehicle" animals explored more the open arms in the retest session when compared to the "vehicle-vehicle" animals (see Figures 5 and 7), even though both groups of animals received the same treatment (i.e., vehicle) in the retest session. Nevertheless, it should be noted that such a long-term anxiolytic effect was lower in magnitude than the acute anxiolytic effect of paroxetine observed in the first EPM exposure (for instance, in Figure 5 compare the levels of open-arm exploration exhibited by "paroxetine" animals in the test session vs. "paroxetine-vehicle" animals in the retest session). Curiously, similar levels of anxiolysis were observed between the "paroxetine-vehicle" and the "paroxetine-paroxetine" animal groups in the retest session (Figure 5), suggesting the possibility that the "paroxetine-paroxetine" animals were also subjected to OTT. Accordingly, under this scenario, the classical display of OTT, in which the drug-treated animals do not differ in the level of open-arm exploration from the vehicle-treated animals (vehicle-vehicle group), would not be observed since it becomes masked by the presence of the long-term anxiolytic effect of the drug. In this sense, the lack of an anxiolytic effect in the retest session would only be possible to be identified when directly comparing the "paroxetine-vehicle" and the "paroxetine-paroxetine" groups since both were subjected to the long-term effect.

Related to our results, Escarabajal et al. (2003) have previously shown that animals treated with either the benzodiazepine chlordiazepoxide or buspirone in the test session and later treated with chlordiazepoxide in the retest session do exhibit an anxiolytic effect of chlordiazepoxide in the latter session. In contrast, rats injected with vehicle in the test session and subsequently with chlordiazepoxide in the retest session display the classical OTT phenomenon (Escarabajal, Torres, and Flaherty 2003). Therefore, together with our

results, these findings suggest that the anxiolytic effect in the retest session depends on the drug state of the animal in the test session. Further interestingly, animals treated with chlordiazepoxide in the test session and subsequently with vehicle in the retest session displayed greater open-arm exploration in the retest session than vehicle-vehicle treated animals (see Figure 2 in Escarabajal et al., 2003), therefore akin to the long-term anxiolytic effect of paroxetine observed here (our Figure 7).

We hypothesized that paroxetine could be either changing the way animals experience the apparatus in the first exposure, possibly reducing the aversive valence of the experience, or else it could be interfering with the formation of the memory associated with the first EPM exposure (Cano-Colino, Almeida, and Compte 2013; Švob Štrac, Pivac, and Mück-Šeler 2016). Therefore, in the second set of experiments, we injected paroxetine right after the first EPM exposure. Under this protocol, if the long-term effect of paroxetine remained, this might be indicative that the first hypothesis could be discarded and that the paroxetine effect is probably associated with interference with memory formation. When analyzing males and females together, the results of this protocol have shown that indeed paroxetine exhibits a long-term anxiolytic effect when injected immediately after the test session (Figure 9), in which animals previously treated with paroxetine explored more the open arms in the retest session than animals that received vehicle after the test session. (Note that under this protocol the animals are not treated prior to the retest session). Of note, a long-term effect of paroxetine in increasing the distance traveled in the maze could also be observed (Figure 9), similarly to what has been found in our first experimental protocol (Figure 5). Nevertheless, when analyzing male and female mice individually, the long-term anxiolytic effect was only statistically significant in females (Figure 11). Male animals, on the other hand, did not show a significant reduction in anxiety-like behavior (as assessed by increased open-arm exploration) during the retest session; even though their group means were greater than the means of vehicle-treated animals (see Figure 11), such a difference did not reach statistical significance. Whether this result is due to a lack of statistical power or else a true gender difference remains to be further explored. Noteworthy, the long-term effect of paroxetine in increasing locomotion in this protocol was statistically significant for both female and male mice (Figure 11C and F).

Another aspect already mentioned in the Results section is that, in these experiments, during the test session females from the paroxetine group exhibited a

tendency to explore more the maze than the females from the vehicle group (Figure 10), even though no difference between groups were expected prior to the injection of either paroxetine or vehicle (which, under this protocol, took place after the test session). This could have introduced a behavioral bias into the retest session since the females of the paroxetine group tended to be naturally more active. In any event, however, the statistical tests showed no significant difference between the two groups in the test session.

Considering that paroxetine might indeed have different effects in males and females leads to the conclusion that both hypotheses framed above are viable. Accordingly, the results for the females in this protocol support the hypothesis that paroxetine can interfere with the memory formation process that takes place after EPM exposure, while the results observed in males reinforce the hypothesis that paroxetine might act during the acquisition phase, reducing the aversiveness associated to the maze. Of note, during the collection of our behavioral data from females, the cycle was not controlled or monitored, thus our results could also be resulting of hormonal bias. Therefore, more studies with monitored female cycles need to be carried out to clarify the nature of this response to paroxetine.

The female results and the combined data from males and females indicate the possibility of paroxetine interfering with the memory formation process. A myriad of studies has shown that, following new learning, molecules (and drugs) act (interfere) in different phases of the memory consolidation process, that is, at different time points. In particular, protein synthesis inhibitors such as azinomycin act in a short consolidation window of six hours (Kwapis et al. 2011; Jobim et al. 2012), whereas BDNF and some cannabinoids have an effect in a later consolidation window (Rossato et al. 2009). Taking these studies into consideration, we decided to investigate the effects of paroxetine injected in a later time window, set as six hours after the first EPM exposure.

The results of this third protocol suggest that paroxetine injected six hours after the first EPM exposure might act in a late phase of memory consolidation (Figure 12). Notably, males treated with paroxetine exhibited a long-term anxiolytic effect with a higher exploration of the open arms (Figure 13). Would this indicate that in males the effect of paroxetine is associated with a late phase of memory consolidation and in females with an early phase? To clarify this possibility, complementary studies are needed.

Noteworthy, as in all protocols above, both male and female animals exhibited a clear long-term effect of paroxetine in increasing locomotion (assessed by the traveled distance in the maze) in the retest session (Figures 12, 13 and 14). As a matter of fact, the paroxetine-induced increase in locomotion was the most robust effect we observed across all experiments (note the existence of this effect in all figures from the Results section, from Figure 5 to Figure 14).

Measuring locomotory activity was not the primary goal of our study; it was included to assess if paroxetine would induce any kind of sedative effect and generate an anxiolysis-like response in the animals. The robustness of our findings called our attention and suggested to us that paroxetine could be leading to a long-term effect in the locomotion in all cases studied for both male and female. That is, under this perspective, the absence of statistical significance for open-arm exploration for some of the cases studied here would be due to lack of statistical power along with a large variability of the anxiety-related metrics across animals in the same group, which contrasted with the much lower variability of locomotion levels within groups. Alternatively, such increased locomotion effect could be sustained by the direct serotonergic innervation of neurons in the brainstem and spinal cord, important areas to coordinate the locomotory centers (Flaive et al. 2020), and not necessarily be directly related by anxiety levels. In this case, males and females having different responses to anxiety-like behavior suggest that they could also exhibit differences in serotonergic modulation.

In summary, paroxetine induces an acute anxiolytic-like response in male and female black C57/J6 mice when injected one hour before EPM exposure. In addition, a first exploration of the EPM leads to the presence of the OTT phenomenon for paroxetine administered in the retest session. Paroxetine also induces an increase in the exploration of the open arms in the retest session when injected one hour before in both sexes, even for animals treated with vehicle in the retest session, a phenomenon we referred to as a “long-term” effect. In females, this effect might be resulting from interference with the formation of the memory associated with the experience in the earlier consolidation phase after EPM exposure; this is because paroxetine administration immediately after the maze induced a long-term anxiolytic response, while six hours afterward it did not induce a significant decrease in anxiety-like behaviors. On the other hand, the results for the males indicate that the paroxetine effect might be associated with a late consolidation phase: while paroxetine injection right after the EPM exposure did not induce a long-term

anxiolytic effect, the administration six hours afterward led to such an effect. Due to these contrasting results, however, additional studies need to be conducted, especially because we do not discard the possibility of large data variability leading to a lack of statistical power underlying some of the negative findings reported here. Though not the primary outcome investigated in our study, a robust observation across all experimental protocols and sexes was that paroxetine administration was associated with increased locomotion in the maze. Whether such a finding relates to anxiety levels or is purely of motor origin remains to be investigated. In all, our results highlight the importance of implementing studies with both sexes to better understand how male and female subjects respond to anxiety tasks and anxiolytics.

6 – Appendix: In vivo recordings

6.1 – Brain oscillations and anxiety

Neuronal oscillations can be identified by recording local field potentials (LFP). These recordings can be performed through implants positioned adjacent to the brain tissue, through techniques such as EEG, or invasively through the implantation of electrodes into the tissue. The formation of the potentials is the result of the electrophysiological activity of a population of neurons near the electrode contact that, when going through processes of hyperpolarization and depolarization (such as during synaptic activity), also influence the amount of charges in the extracellular medium, generating variations of its potential difference when compared to a reference electrode.

Neuronal oscillations can be decomposed into different frequency bands, and these, in turn, can be related to varying states of brain functioning and behavior. The most investigated frequency ranges in animal models are slow oscillations (~1 Hz), delta (~1.5 - 4 Hz), theta (~8 - 12 Hz), and gamma (~30 - 160 Hz), see Figure 15 (Buzsáki & Draguhn, 2011). It is believed that low-frequency neuronal oscillations (<30Hz) can transmit information through different regions of the brain and that high-frequency oscillations (>30Hz) act in the processing of local information.

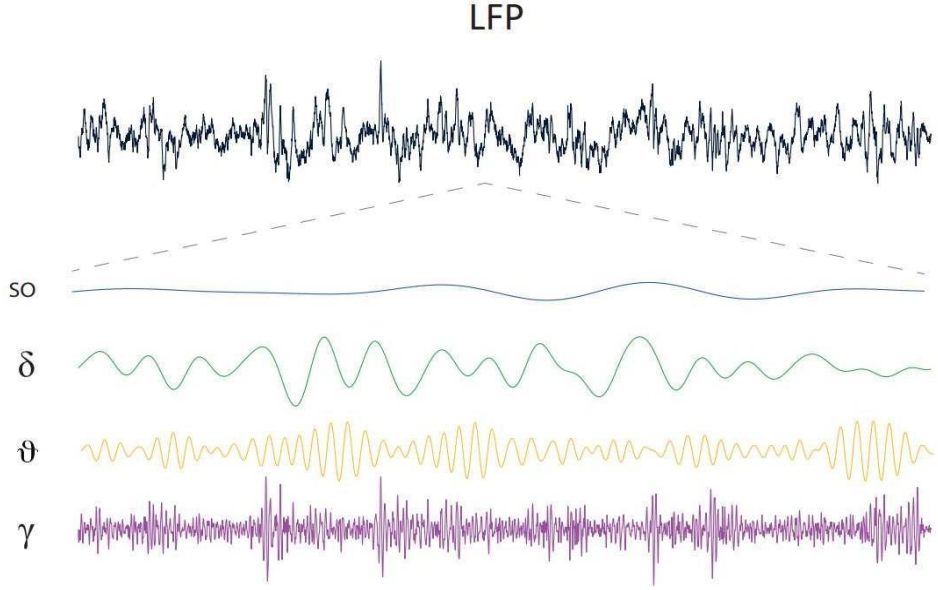

Figure 15 – Representation of LFP and main analyzed frequencies. SO = Slow Oscillations; δ = Delta; Θ = Theta; γ = Gamma. The signal was sampled on a 5 second time scale; a.u. = arbitrary units.

During fear or anxiety-like response, changes in these oscillatory patterns occur due to the integration of the input from the environmental cues and the risk assessment of a specific situation. The bidirectional connection between the ventral hippocampus (vHC) and the medial prefrontal cortex (mPFC) is pointed as crucial to the sustainment of this behavior and it is marked by increased synchrony in the theta range (Adhikari et al., 2010). The ventral portion of the hippocampus is responsible to sustain this synchrony. The interruption of the projections sent by the vHC to the mPFC induces a drastic reduction in the synchrony between these two areas (Neill et al., 2013). Another interesting fact is that the inhibition of vHC and mPFC communication does not affect locomotory patterns observed in the EPM. A study has also shown that vHC cells selectively fire during exposure to aversive contexts (Jimenez et al., 2019). Optogenetic results obtained by Padilla-korean et al. (2016) showed that the bilateral inhibition of the vHC terminals in the mPFC increases the exploration of the open arms in the EPM and also decreases the aversion of mice to the central zone of the open field. As a result of this inhibition, a decrease in the synchrony in the frequency of theta between vHC-mPFC was reported. These results indicate that vHC induces an excitatory effect in mPFC cells during situations that evoke anxiety-like behavior; they also highlight the relationship between the hippocampus and prefrontal cortex as essential for the proper processing of anxious-like conditions, and the synchronization in the theta frequency range would be one of the mechanisms.

The deficit in the functioning of the serotonergic system may induce changes in the synchrony between the mPFC and HC in anxiety-like behaviors. During EPM exploration, mice with knockout 5-HT1A receptors exhibited high synchrony in the theta frequency range between these two regions (Adhikari et al., 2010). In addition to the synchrony present between vHC and mPFC, an increase in theta power in the vHC and dHC was also observed (Adhikari et al., 2010). Previous work has also demonstrated that, during anxiogenic experiences, cells from the hippocampus tend to have selective firing patterns (Moita et al., 2004).

It has been shown that during freezing induced by exposure to aversive odors, an oscillatory rhythm in the 4 Hz range coupled with respiration emerges in the prelimbic mPFC (Moberly et al., 2018). Also, studies have shown that respiration, though olfactory bulb projections, can entrain oscillatory patterns in cortical and limbic structures such as the amygdala (Karalis and Sirota, 2018; Tort et al., 2018). However, the link between

anxiety and respiratory frequencies remains unclear.

These findings support the idea that anxious states are highly linked to changes in the oscillatory pattern among different brain areas. Anxiety-like behaviors encompass and integrate different sensorial inputs and cognitive processes once they are correlated to the safety evaluation and as a consequence of a maladaptive process. However, the sustainment of these states remains unclear.

6.2 – Methods: Electrophysiological recordings

Surgical procedure

For electrophysiological recordings of LFP and respiration, the animals were implanted with arrays of electrodes (made with tungsten wires of 70 micrometers in diameter) coupled to thermoelectric sensors (Ômega®, insp#33559) and a pair of screws in contact with the cerebrospinal as ground. During the implant surgery, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg), xylazine (4 mg/kg) and atropine (0.04 mg/kg). Then the animals were positioned in the stereotaxic and an incision was made to expose the skull; after the incision, a craniotomy was performed to open two windows for electrode implantation in the following coordinates (in mm): AP: 2.3; ML: 0.50; DV: -1.5 for medial prefrontal cortex, AP: -3.65; ML: 2.8; DV: -3.65 for the ventral hippocampus. Yet, a pair of thermosensors was placed at the coordinates: AP: 11; ML: 0.5. At the end of the surgery, extra screws and a layer of acrylic was placed to guarantee the fixation of the implant on its place.

In-vivo recordings protocol

LFP recordings: In order to perform the electrophysiological recordings, a setup was implemented. The implementation of the setup was divided in four axes: 1 – recording implants design, manufacture and test; 2 – implant surgery, 3 – acquisition system (Open Ephys® and tracking) implementation and synchronization, and 4 – post experimental procedures (craniotomy, electrodes position checking). The animals were recorded for 10 minutes in their own cages for baseline signal acquisition. Then, exposed to the elevated plus maze for also 10 minutes. The electrophysiological signal was transmitted from the implants through an Intan 1832 plate and amplified at a rate of

30,000 kHz and sent to the acquisition system of the Open Ephys® system.

Video recordings: Images of the animals during the recordings were captured by a high-resolution camera with a sampling rate of 30 frames and sent to the Ethovision® animal tracking software.

6. 3 – Preliminary results

To address how brain connectivity and respiratory patterns are modulated by anxiety-like behavior induced by the EPM, and how it can be affected by systemic administration of paroxetine, we planned to perform electrophysiological recordings from the medial prefrontal cortex and ventral hippocampus together with the respiratory signal. Unfortunately, due to several methodological issues we faced upon following this path, along with the time constraint to finish this Masters dissertation, the *in vivo* recordings had to be temporally interrupted in favor of the behavioral-pharmacological experiments and their data analysis. However, advances were made in the implementation of the recording implants design, manufacture and test, implant surgery, acquisition system (Open Ephys® and tracking) implementation and synchronization, and post experimental procedures (craniotomy, electrodes position marking and verification), which we let registered here.

A total of 14 animals was submitted to the surgery. Due to several methodological issues, including the adjustment of the mPFC coordinates, only two animals were successfully submitted to the recording protocols. In any event, the figures below illustrate a summary of the achieved research steps, namely: electrodes array design and manufacture (Figure 16), electrophysiology setup and recordings (Figure 17), electrodes array implant surgery (Figure 18), *in-vivo* recordings (Figure 19), and electrodes position verification (Figure 20).

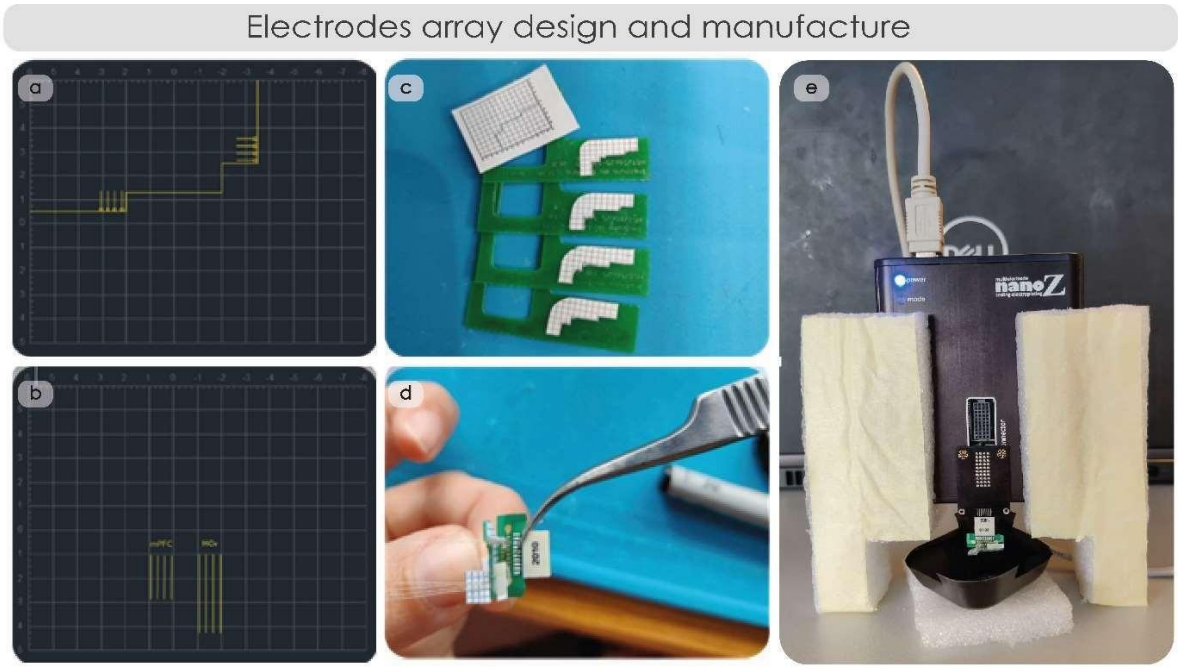

Figure 16 – Electrodes array design and manufacture. *A - AutoCAD® guide made by the recreation of the Paxinos axes at anteroposterior and medium lateral coordinates for the mPFC and vHC; B – the dorsoventral coordinates both for the mPFC and vHC; C – picture showing the printed guide and the PCB pieces used to build the support; D – Electrodes array; E – Electrodes array impedance test and plating with nanoZ®.*

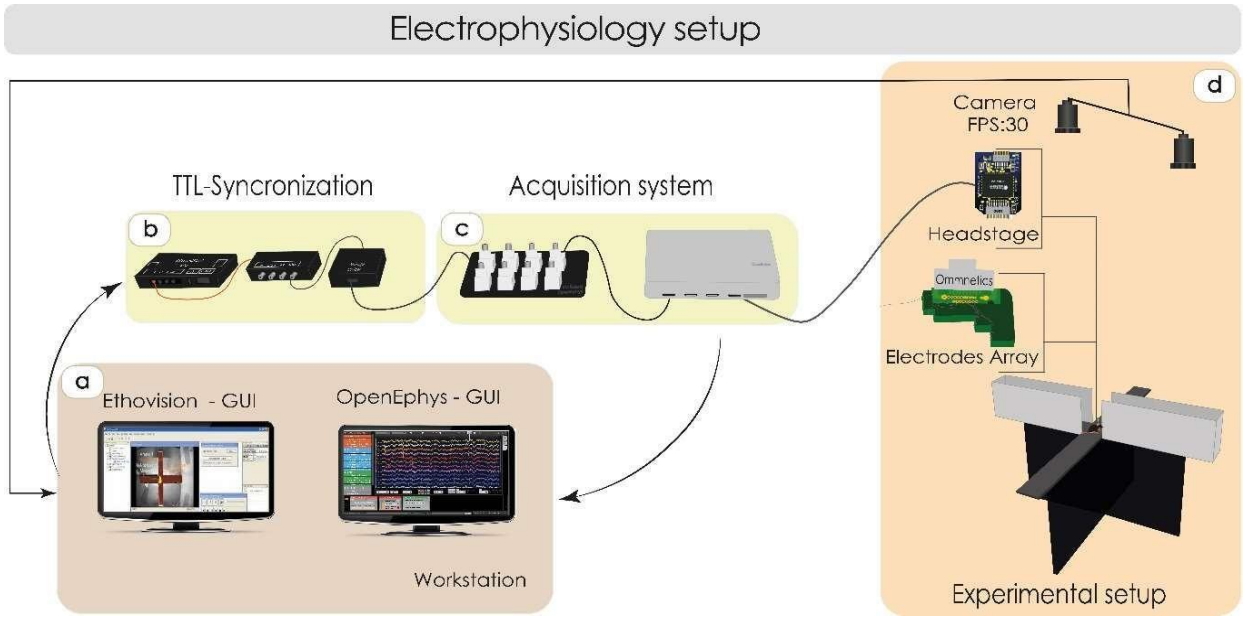

Figure 17 – Electrophysiology setup. *Schematic representation of the recording setup established at the Neuroscience Department of Uppsala University. A – Workstation with the Ethovision® and Open Ephys® user interface to process and store the LFP and video signals; B – TTL-Synchronization system, from the left to the right side, an extension of the Ethovision® system with a TTL generator and a self-made voltage divider; C – Open Ephys® Acquisition system, from the left to the right side an auxiliary board to receive the TTL signal, and an acquisition board to the LFP; D – Experimental setup, from the top to the bottom, two high-resolution cameras, headstage connector and amplifier, schematic representation of the implant and mice placed at EPM, and the EPM.*

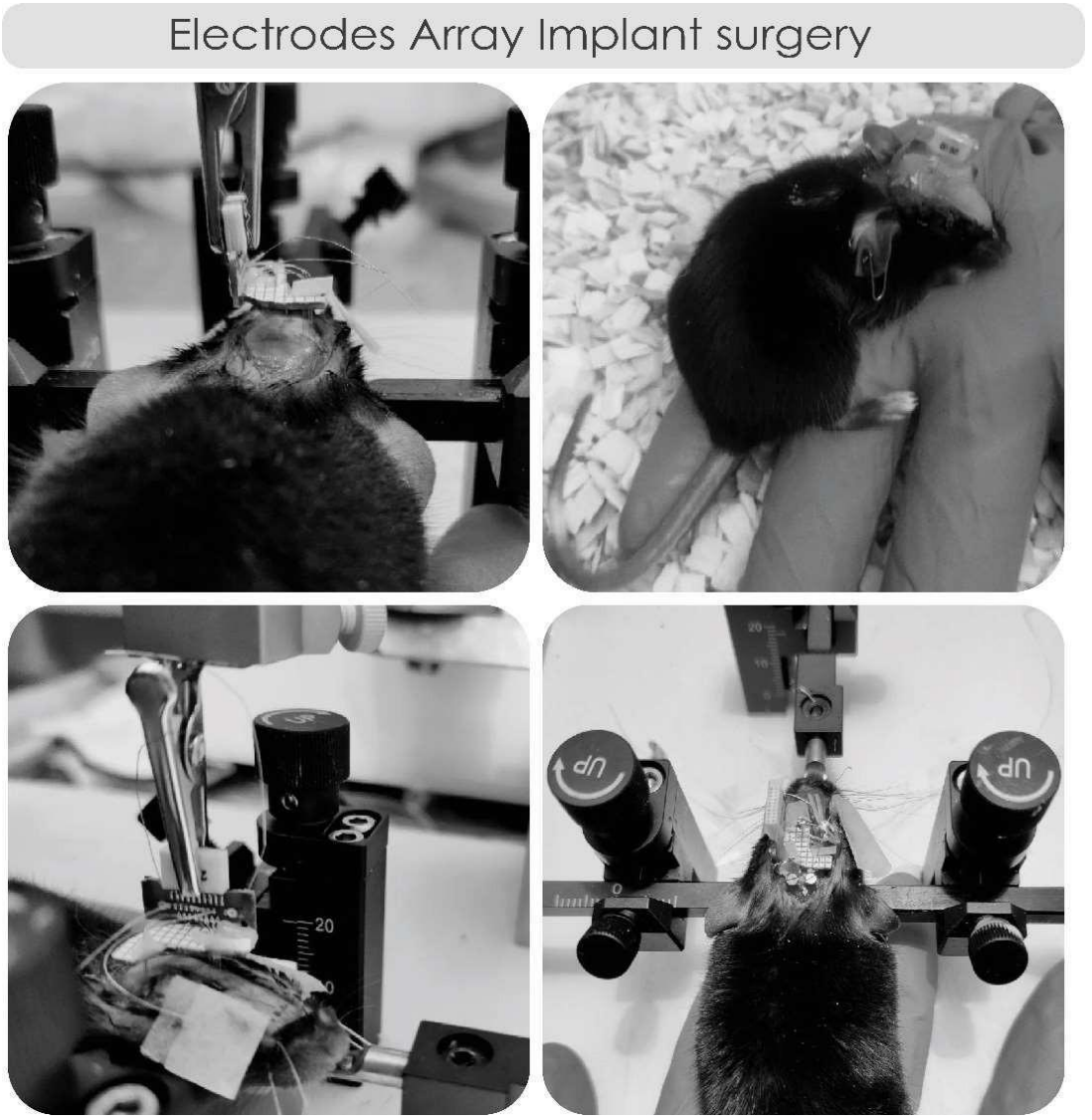

Figure 18 – Electrodes array implant surgery. *Upward to the left an anterior view of the surgery, at the bottom, a lateral view of the surgery. Upward to the right is a picture of one animal recovering from anesthesia; at the bottom is a superior view after the placement of the electrode array and nasal thermosensor.*

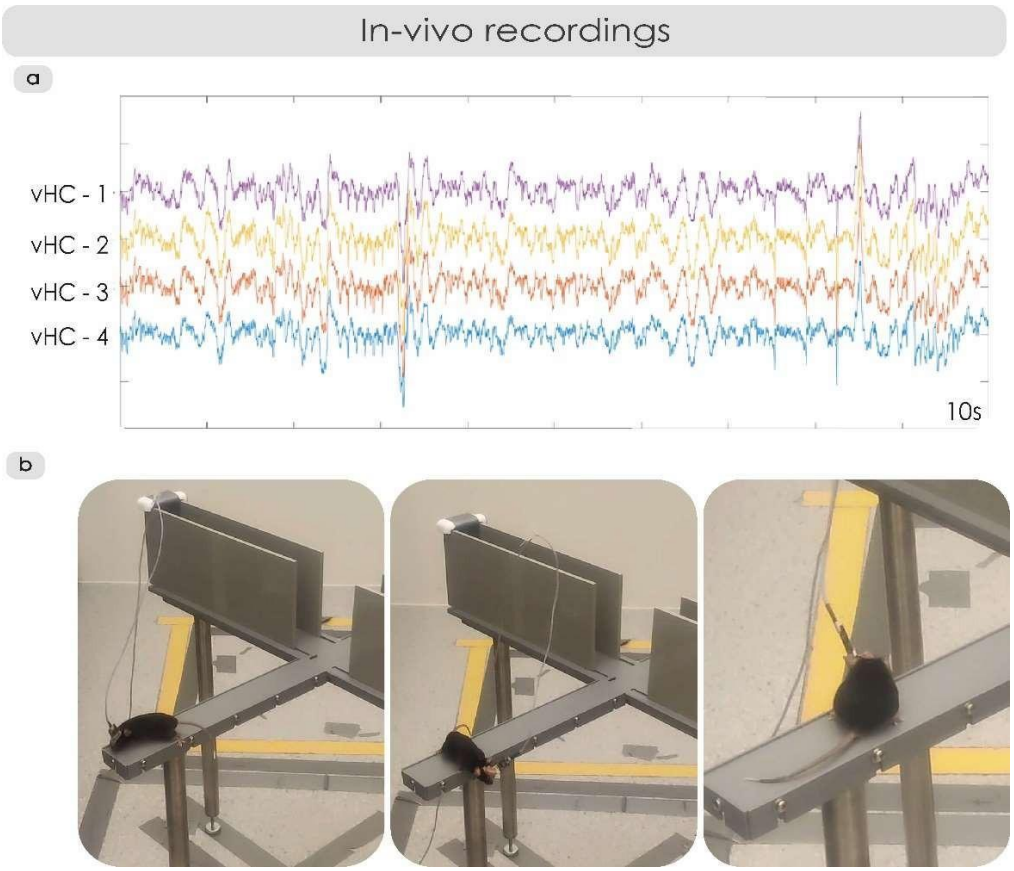

Figure 19 – In-vivo recordings. *A – LFP signal from the vHC. Due to methodological issues, it was not possible to obtain mPFC and respiratory signals. B – Pictures of a pilot recording to test the signal acquisition, noise, and synchronization of an animal performing the EPM task while recorded.*

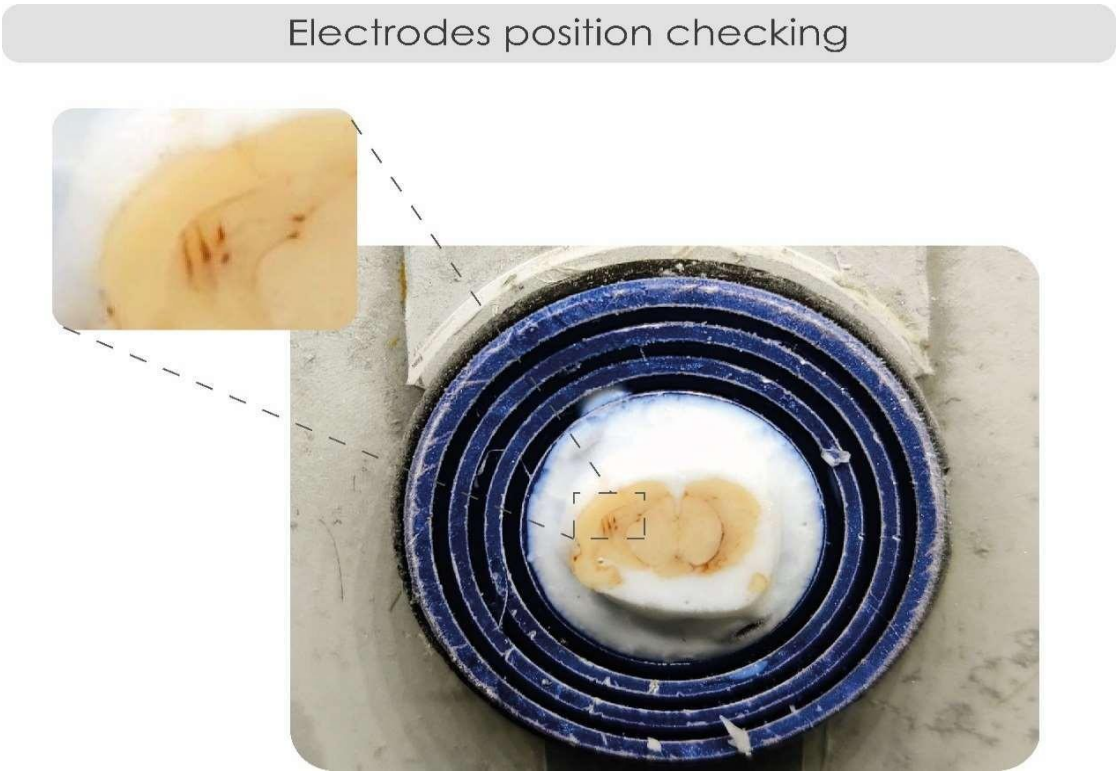

Figure 20 – Electrodes position verification. *Picture of a brain after implementation of the tissue marker and the positions of the electrodes in the vHC.*

7 – References

Abela AR, Browne CJ, Sargin D, Prevot TD, Ji XD, Li Z, Lambe EK, Fletcher PJ (2020) Median raphe serotonin neurons promote anxiety-like behavior via inputs to the dorsal hippocampus. Neuropharmacology 168:107985.

Albert PR, Vahid-Ansari F, Luckhart C (2014) Serotonin-prefrontal cortical circuitry in anxiety and depression phenotypes: pivotal role of pre- and post-synaptic 5-HT1A receptor expression. Front Behav Neurosci 8.

Altemus, Margaret, Nilofar Sarvaiya, and C. Neill Epperson. 2014. "Sex Differences in Anxiety and Depression Clinical Perspectives." *Frontiers in Neuroendocrinology* 35 (3): 320–30.

American Psychiatric Association ed. (2013) Diagnostic and statistical manual of mental disorders: DSM-5, 5th ed. Washington, D.C: American Psychiatric Association.

Andreatini R, Bacellar LFS (2000) Animal Models: Trait or State Measure? The Test-Retest Reliability of the Elevated Plus-Maze and Behavioral Despair. Prog Neuropsychopharmacol Biol Psychiatry:549–560.

Arabo A, Potier C, Ollivier G, Lorivel T, Roy V (2014) Temporal analysis of free exploration of an elevated plus-maze in mice. J Exp Psychol Anim Learn Cogn 40:457–466.

Asth, Laila, Bruno Lobão-Soares, Eunice André, Vanessa de Paula Soares, and Elaine Cristina Gavioli. 2012. "The elevated T-maze task as an animal model to simultaneously investigate the effects of drugs on long-term memory and anxiety in mice". Brain Research Bulletin 87 (6): 526–33.

Bagdy, Gyorgy, Marton Graf, Zsuzsanna E. Anheuer, Edit A. Modos, and Sandor Kantor. 2001. "Anxiety-like Effects Induced by Acute Fluoxetine, Sertraline or m-CPP Treatment Are Reversed by Pretreatment with the 5-HT2C Receptor Antagonist SB-242084 but Not the 5-HT1A Receptor Antagonist WAY-100635". The International Journal of Neuropsychopharmacology 4.

Barad, Mark. 2005. "Fear Extinction in Rodents: Basic Insight to Clinical Promise." Current Opinion in Neurobiology 15 (6): 710–15. https://doi.org/10.1016/j.conb.2005.10.005.

Bertoglio, L. J., and A. P. Carobrez. 2004. "Scopolamine given Pre-Trial 1 Prevents the One-Trial Tolerance Phenomenon in the Elevated plus-Maze Trial 2": Behavioural Pharmacology 15 (1): 45–54.

Bertoglio, Leandro J, and Antonio P Carobrez. 2000. "Previous Maze Experience Required to Increase Open Arms Avoidance in Rats Submitted to the Elevated Plus-Maze Model of Anxiety". Behavioural Brain Research 108 (2): 197–203.

Bertoglio, L.J, and A.P Carobrez. 2002. "Anxiolytic Effects of Ethanol and Phenobarbital Are Abolished in Test-Experienced Rats Submitted to the Elevated plus Maze". Pharmacology Biochemistry and Behavior 73 (4): 963–69.

Bourin, Michel. 2015. "Animal models for screening anxiolytic-like drugs: a perspective". Dialogues in clinical neuroscience 17 (3): 295.

Bourin, Michel. 2018. "The test-retest model of anxiety: An appraisal of findings to explain benzodiazepine tolerance". Pharmacology Biochemistry and Behavior.

Bourin, Michel. 2019. "The Test Retest Model of Anxiety: An Appraisal of Findings to Explain Benzodiazepine Tolerance". Pharmacology Biochemistry and Behavior 178 (março): 39–41.

Carobrez AP, Bertoglio LJ. 2005. Ethological and temporal analyses of anxiety-like behavior: The elevated plus-maze model 20 years on. Neurosci Biobehav Rev 29:1193–1205.

Carlisi CO, Robinson OJ. 2018. The role of prefrontal–subcortical circuitry in negative bias in anxiety: Translational, developmental and treatment perspectives. Brain Neurosci Adv 2:239821281877422.

Cano-Colino, Maria, Rita Almeida, and Albert Compte. 2013. "Serotonergic Modulation of Spatial Working Memory: Predictions from a Computational Network Model". Frontiers in Integrative Neuroscience 7.

Canto-de-Souza, Azair, Ricardo Luiz Nunes-de-Souza, and Robert John Rodgers. 2002. "Anxiolytic-like Effect of Way-100635 Microinfusions into the Median (but Not Dorsal)

Raphe Nucleus in Mice Exposed to the plus-Maze: Influence of Prior Test Experience". Brain Research, 10.

Dawson, Gerard R., and Mark D. Tricklebank. 1995. "Use of the Elevated plus Maze in the Search for Novel Anxiolytic Agents". Trends in Pharmacological Sciences 16 (2): 33–36.

Dawson GR, Tricklebank MD. 1995. Use of the elevated plus maze in the search for novel anxiolytic agents. Trends Pharmacol Sci 16:33–36.

Escarabajal, M.D, C Torres, and C.F Flaherty. 2003. "The Phenomenon of One-Trial Tolerance to the Anxiolytic Effect of Chlordiazepoxide in the Elevated plus-Maze Test Is Abolished by Previous Administration of Chlordiazepoxide or Buspirone". Life Sciences 73 (8): 1063–74.

File, Sandra E., Peter S. Mabbutt, and Paul K. Hitchcott. 1990. "Characterisation of the Phenomenon of “One-Trial Tolerance” to the Anxiolytic Effect of Chlordiazepoxide in the Elevated Plus-Maze". Psychopharmacology 102 (1): 98–101.

File, Sandra E., Helio Zangrossi, Fiona L. Sanders, and Peter S. Mabbutt. 1994. "Raised Corticosterone in the Rat after Exposure to the Elevated Plus-Maze". Psychopharmacology 113 (3–4): 543–46.

Flaive, Aurélie, Maxime Fougère, Cornelis Immanuel van der Zouwen, and Dimitri Ryczko. 2020. "Serotonergic Modulation of Locomotor Activity From Basal Vertebrates to Mammals". Frontiers in Neural Circuits 14 (novembro): 590299.

Garakani A. 2006. Neurobiology of Anxiety Disorders and Implications for Treatment. 73:9.

Gordon JA, Hen R. 2004. The serotonergic system and anxiety. Neuromolecular Med 5:27–40.

Garakani, Amir, James W. Murrough, Rafael C. Freire, Robyn P. Thom, Kaitlyn Larkin, Frank D. Buono, and Dan V. Iosifescu. 2020. "Pharmacotherapy of Anxiety Disorders: Current and Emerging Treatment Options". Frontiers in Psychiatry 11: 1412.

Gray, Neil A., Matthew S. Milak, Christine DeLorenzo, R. Todd Ogden, Yung-yu Huang,

J. John Mann, and Ramin V. Parsey. 2013. “Antidepressant Treatment Reduces Serotonin-1A Autoreceptor Binding in Major Depressive Disorder.” Biological Psychiatry 74 (1): 26–31.

Guimarães, Francisco Silveira, Antonio Pádua Carobrez, and Frederico Guilherme Graeff. 2008. "Chapter 4.3 Modulation of Anxiety Behaviors by 5-HT-Interacting Drugs". Em Handbook of Behavioral Neuroscience, edited by Robert J. Blanchard, D. Caroline Blanchard, Guy Griebel, and David Nutt, 17:241–68. Handbook of Anxiety and Fear. Elsevier.

Handley, S. L., and J. W. McBlane. 1993. "An Assessment of the Elevated X-Maze for Studying Anxiety and Anxiety-Modulating Drugs". Journal of Pharmacological and Toxicological Methods 29 (3): 129–38.

Hensler JG. 2006. Serotonergic modulation of the limbic system. Neurosci Biobehav Rev 30:203–214.

Holmes, A, e R. J. Rodgers. 1998. "Responses of Swiss–Webster Mice to Repeated Plus-Maze Experience Further Evidence for a Qualitative Shift in Emotional State?" Pharmacology Biochemistry and Behavior 60 (2): 473–88.

Jalnapurkar I, Allen M, Pigott T. 2018. “Sex Differences in Anxiety Disorders: A Review.” Psychiatry, Depression & Anxiety 4 (July): 1–9.

Jobim, Paulo F.C., Thiago R. Pedroso, Raissa R. Christoff, Aline Werenicz, Natasha Maurmann, Gustavo K. Reolon, and Rafael Roesler. 2012. "Inhibition of MTOR by Rapamycin in the Amygdala or Hippocampus Impairs Formation and Reconsolidation of Inhibitory Avoidance Memory". Neurobiology of Learning and Memory 97 (1): 105–12.

Kalueff, Allan V. 2007. “Neurobiology of Memory and Anxiety: From Genes to Behavior.” Neural Plasticity 2007: 1–12. https://doi.org/10.1155/2007/78171.

Karalis N, Sirota A. 2018. Breathing coordinates limbic network dynamics underlying memory consolidation. :29.

Kwapis, Janine L., Timothy J. Jarome, Janet C. Schiff, and Fred J. Helmstetter. 2011. "Memory Consolidation in Both Trace and Delay Fear Conditioning Is Disrupted by Intra-Amygdala Infusion of the Protein Synthesis Inhibitor Anisomycin". Learning &

Memory 18 (11): 728–32.

Korte SM, De Boer SF. 2003. A robust animal model of state anxiety: fear-potentiated behaviour in the elevated plus-maze. Eur J Pharmacol 463:163–175.

Liu W-Z, Zhang W-H, Zheng Z-H, Zou J-X, Liu X-X, Huang S-H, You W-J, He Y, Zhang J-Y, Wang X-D, Pan B-X. 2020. Identification of a prefrontal cortex-to-amygdala pathway for chronic stress-induced anxiety. Nat Commun 11:2221.

LeDoux JE, Pine DS. 2016. Using Neuroscience to Help Understand Fear and Anxiety: A Two-System Framework. Am J Psychiatry 173:1083–1093.

Lebron-Milad, Kelimer, and Mohammed R Milad. 2012. “Sex Differences, Gonadal Hormones and the Fear Extinction Network: Implications for Anxiety Disorders.” Biology of Mood & Anxiety Disorders 2 (1): 3.

Liu W-Z, Zhang W-H, Zheng Z-H, Zou J-X, Liu X-X, Huang S-H, You W-J, He Y, Zhang J-Y, Wang X-D, Pan B-X. 2020. Identification of a prefrontal cortex-to-amygdala pathway for chronic stress-induced anxiety. Nat Commun 11:2221.

McHenry, Jenna, Nicole Carrier, Elaine Hull, and Mohamed Kabbaj. 2014. “Sex Differences in Anxiety and Depression: Role of Testosterone.” Frontiers in Neuroendocrinology 35 (1): 42–57.

Moita, M. A., Rosis, S., Zhou, Y., LeDoux, J. E., and Blair, H. T. 2004. Putting fear in its place: remapping of hippocampal place cells during fear conditioning. J Neurosci, 24(31), 7015-7023.

Murrough JW, Yaqubi S, Sayed S, Charney DS. 2015. Emerging drugs for the treatment of anxiety. Expert Opin Emerg Drugs 20:393–406.

Pattyn T, Van Den Eede F, Vanneste S, Cassiers L, Veltman DJ, Van De Heyning P, Sabbe BCG. 2016. Tinnitus and anxiety disorders: A review. Hear Res 333:255–265.

Nunes-de-Souza, Ricardo Luiz, Azair Canto-de-Souza, and Robert John Rodgers. 2002. "Effects of Intra-Hippocampal Infusion of WAY-100635 on plus-Maze Behavior in Mice Influence of Site of Injection and Prior Test Experience". Brain Research, 10.

Nutt, David J. 2005. "Overview of Diagnosis and Drug Treatments of Anxiety Disorders". CNS Spectrums 10 (1): 49–56.

Pádua-Reis, Marina, Diana Aline Nôga, Adriano B. L. Tort, and Martina Blunder. 2021. "Diazepam Causes Sedative Rather than Anxiolytic Effects in C57BL/6J Mice". Scientific Reports 11 (1): 9335.

Rodgers, R. J., J. Haller, A. Holmes, J. Halasz, T. J. Walton, and P. F. Brain. 1999. "Corticosterone Response to the Plus-Maze: High Correlation with Risk Assessment in Rats and Mice". Physiology & Behavior 68 (1): 47–53.

Rodgers, R.J., B.-J. Cao, A. Dalvi, and A. Holmes. 1997. "Animal Models of Anxiety: An Ethological Perspective". Brazilian Journal of Medical and Biological Research 30 (3): 289–304.

Rossato, Janine I., Lia R. M. Bevilaqua, Iván Izquierdo, Jorge H. Medina, and Martín Cammarota. 2009. "Dopamine Controls Persistence of Long-Term Memory Storage". Science 325 (5943): 1017–20.

Roy, Vincent, Pierre Chapillon, Mustapha Jeljeli, Jean Caston, and Catherine Belzung. 2009. "Free versus Forced Exposure to an Elevated Plus-Maze: Evidence for New Behavioral Interpretations during Test and Retest". Psychopharmacology 203 (1): 131–41.

Rodgers RJ, Cao B-J, Dalvi A, Holmes A. 1997. Animal models of anxiety: an ethological perspective. Braz J Med Biol Res 30:289–304.

Schneider P, Ho Y-J, Spanagel R, Pawlak CR. 2011. A Novel Elevated Plus-Maze Procedure to Avoid the One-Trial Tolerance Problem. Front Behav Neuroscience.

Singewald N, Salchner P, Sharp T. 2003. Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs. Biol Psychiatry 53:275–283.

Salimi M, Ghazvineh S, Zare M, Parsazadegan T, Dehdar K, Nazari M, Mirnajafi-Zadeh J, Jamaati H, Raoufy MR. 2019. Distraction of olfactory bulb-medial prefrontal cortex circuit may induce anxiety-like behavior in allergic rhinitis. PloS One 14:e0221978.

Smith KS, Engin E, Meloni EG, Rudolph U. 2012. Benzodiazepine-induced anxiolysis

and reduction of conditioned fear are mediated by distinct GABAA receptor subtypes in mice. Neuropharmacology 63:250–258.

Stern, C, A Carobrez, and L Bertoglio. 2008. "Aversive Learning as a Mechanism for Lack of Repeated Anxiolytic-like Effect in the Elevated plus-Maze". Pharmacology Biochemistry and Behavior 90 (4): 545–50. https://doi.org/10.1016/j.pbb.2008.04.013.

Švob Štrac, Dubravka, Nela Pivac, and Dorotea Mück-Šeler. 2016. "The Serotonergic System and Cognitive Function". Translational Neuroscience 7 (1): 35–49.

Tiller JWG. 2012. Depression and anxiety. Med J Aust 1:28–31.

Tort AB, Brankačk J, Draguhn A. 2018. Respiration-entrained brain rhythms are global but often overlooked. Trends Neurosci 41:186–197.

Wilde, J., R. Spiers, C. Mertens, F. Bartholomé, G. Schotte, and S. Leyman. 1993. "A Double-Blind, Comparative, Multicentre Study Comparing Paroxetine with Fluoxetine in Depressed Patients". Acta Psychiatrica Scandinavica 87 (2): 141–45.

Walf AA, Frye CA. 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc 2:322–328.

World Health Organization WH. 2017. Depression and other common mental disorders: global health estimates. World Health Organization.