HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

Athens International Master's Programme in Neurosciences

University of Crete, Biology Department

Laboratory of Neurophsyiology and Behavior

RESEARCH THESIS PROJECT

SEXUAL DIMORPHIC PERFORMANCE IN THE TEMPORAL ORDER RECOGNITION TASK AND ENHANCED SYNAPTIC PLASTICITY IN FEMALE C57/BL6 MICE AFTER WORKING MEMORY TRAINING.

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May, 2022

Sexual dimorphic performance in the temporal order recognition task and enhanced synaptic plasticity in female C57/BL6 mice after working memory training.

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CONTENTS

Summary

Episodic-like memory, which recollects the "what," "where," and "when" components, and working memory, which is ‘‘active and relevant only for a short period of time’’, are two commonly studied memory systems, which have been thoroughly assessed in male subjects. The major aim of this study was to enrich the inadequate literature on the performance of female mice in tasks that assess the “when” ELM component and WM. The first approach was to compare the general prefrontal cortex function and temporal memory capacity of male and female C57/BL6 mice through the analysis of their PFC-regulated habituation and temporal order object recognition task performance, respectively. Males traveled more in the periphery of the box than females, while there were no differences in the TOR performance. Given that only a few studies have ever suggested that long-term synaptic changes could be associated with some aspects of WM, the second objective was to determine how working memory training in the delayed alternation task alters hippocampal fepsp potentiation after LTP induction. Electrophsyiological recordings in a different cohort of female C57/BL6 mice revealed that fepsp potentiation was greater in non-adaptive but longer-lasting in fully-adaptive mice.

Περίληψη

Η επεισοδιακή μνήμη, η οποία αναφέρεται στις συνιστώσες "τι", "πού" και "πότε" μιας προσωπικής εμπειρίας, και η μνήμη εργασίας, η οποία είναι "ενεργή και σχετική μόνο για σύντομο χρονικό διάστημα", είναι δύο συστήματα μνήμης που έχουν μελετηθεί ευρέως και έχουν αξιολογηθεί διεξοδικά σε ασθενείς και πειραματόζωα ανδρικού φύλου. Ο κύριος στόχος αυτής της μελέτης ήταν να εμπλουτίσει την ανεπαρκή βιβλιογραφία σχετικά με τις επιδόσεις των θηλυκών ποντικών σε πειραματικές διεργασίες που αξιολογούν τη συνιστώσα "όταν" της επεισοδιακής μνήμης και τη μνήμη εργασίας. Η αρχική προσέγγιση ήταν να συγκρίνουμε τη γενική λειτουργία του προμετωπιαίου φλοιού και την ικανότητα χρονικής μνήμης αρσενικών και θηλυκών ποντικών C57/BL6, το οποίο επιτεύχθηκε μέσω της ανάλυσης της εξοικείωσης τους με την πειραματική συσκευή και των επιδόσεων τους στο πείραμα αναγνώρισης αντικειμένων με χρονική σειρά (TOR), αντίστοιχα. Τα αρσενικά μετακινούνταν σημαντικά περισσότερο στην περιφέρεια του κουτιού απ’ ότι τα θηλυκά, ενώ δεν υπήρχαν διαφορές στην επίδοση τους στο κυρίως συμπεριφορικό πείρμαμα (TOR). Δεδομένου ότι ένας μικρός αριθμός μελετών έχει προτείνει ότι οι μακροπρόθεσμες συναπτικές αλλαγές θα μπορούσαν να συσχετιστούν με ορισμένες πτυχές της μνήμης εργασίας, ο δεύτερος στόχος ήταν να προσδιοριστεί πώς η εκπαίδευση της μνήμης εργασίας μέσω της «δοκιμασίας εναλλαγής βραχίονα με καθυστέρηση» μεταβάλλει τις συναπτικές ιδιότητες του ιπποκάμπου σε θηλυκά C57/BL6ποντίκια μετά την επαγωγή μακροπρόθεσμης συναπτικής ενδυνάμωσης (LTP). Οι ηλεκτροφυσιολογικές καταγραφές αποκάλυψαν ότι η συναπτική ενδυνάμωση ήταν σημαντικά αυξημένη στην ομάδα των μερικώς προσαρμοσμένων ζώων, αλλά διατηρούνταν περισσότερο στα πλήρως προσαρμοσμένα ποντίκια.

Highlights

PFC-regulated habituation analysis revealed that female mice exhibited lower locomotion levels in the open-field apparatus compared to male subjects.

In TOR, both male and female mice had comparable results in time of exploration and discrimination index of the “old familiar” object.

Following the DAT performance, LTP was induced in hippocampal slices of adaptive and non-adaptive females.

Keywords

female C57/BL6 mice; PFC-regulated habituation; Locomotion; Temporal order memory; working memory; LTP

Λέξεις-κλειδιά

θηλυκά ποντίκια C57/BL6, εξοικείωση ρυθμιζόμενη από τον προμετωπιαίο φλοιό, Κινητικότητα, Χρονική μνήμη, Μνήμη εργασίας, μακροπρόθεσμη συναπτική ενδυνάμωση

Abbreviations

DAT=Delayed alternation task, **EM**= Episodic memory, **ELM**=Episodic-like memory, **fepsp**= field excitatory postsynaptic potential, **HPC**=hippocampus, **LTP**=Long –term potentiation, **OF**=open-field, **PFC**=prefrontal cortex, **TOM**=Temporal order memory, **TOR**=Temporal order object recognition task, **WM**=working memory, **WT**=wild-type

Introduction

Episodic memory is defined as the ability to recollect the three components of a unique personal experience: “what” happened, “where” and “when” it occurred (Belblidia *et al.*, 2015). In the past, the existence of episodic memory in non-human species was controversial; although associative accounts of animal learning recognized that behavior can change in response to single events, this did not necessarily imply that animals need or are later able to recall representations of unique events at a different spatiotemporal context (Morris, 2001). The groundbreaking study of Clayton and Dickinson first indicated the recall of specific experiences in animals (Clayton and Dickinson, 1998), in which scrub jays were trained to find a variety of foods according to their specific locations and the time they were encountered. With this combination of what-where-when components, the authors functionalized the components of episodic memory into observable behavioral terms in an animal model and called it "episode-like memory” (ELM).

Since then, a plethora of tasks have been developed to study the ELM in rodents; most of these procedures involve undergoing extensive training and food deprivation, while others focus on a spontaneous behavior of rodents, the innate tendency to seek novelty, in order to study the *"what", "where" and "when"* components of the ELM (Belblidia *et al.*, 2015).

A well-established paradigm of a training-free animal model is *spontaneous object exploration* in rodent studies of recognition memory. This test is built on the natural tendency of many species to explore novel stimuli and can be used to assess the memory of "when", among the other components of ELM (Chao *et al.*, 2020). The *temporal order memory* task, in particular, evaluates the ability of rodents to discriminate the relative recency of stimuli/events (Hannesson, Howland and Phillips, 2004). This task is a three-trial procedure, composed of two sample phases, during which two copies of a novel object are presented, and the test phase, in which the animal encounters one "old familiar" object from trial one and another "recent familiar" object from trial two (Dere, Huston and De Souza Silva, 2005a; Chao *et al.*, 2020). Recency discrimination has been proven to critically rely on the PFC as indicated by cross-species studies in which damage to the PFC impairs recency discrimination performance ((for studies in rodents, see (Hannesson, Howland and Phillips, 2004; Gareth R.I. Barker and Warburton, 2011; Barker *et al.*, 2017), for human patients see (Bertossi *et al.*, 2016)). The hippocampus also plays a key role in the processing of memory for object recency and/or temporal order recognition, as is strongly indicated by lesion studies (Mitchell and Laiacona, 1998; DeVito and Eichenbaum, 2010; Allen *et al.*, 2020; Shandilya and Gautam, 2020a), and the presence of specialized neuronal cells ("time" and "sequence" cells) that are reliably activated at specific moments during gaps between stimuli (Pastalkova *et al.*, 2008; Allen *et al.*, 2016) and between items presented in the correct or incorrect sequential position, respectively (MacDonald *et al.*, 2013).

The first goal of this study was to evaluate the PFC function and compare the performance of male and female C57/BL6 mice in the temporal order object recognition task (TOR), in order to enrich the scarce findings of the existing literature. There have been only a few studies on female mice (Velli *et al.*, 2021), none of which, to my knowledge, addressed the innate temporal order memory capacity of untreated C57/BL6 females.

In addition to being associated with temporal order recognition, the interconnected hippocampus and medial prefrontal cortex areas have been shown to mediate another crucial form of memory known as *working memory*. WM has originally been defined as memory ''active and relevant only for a short period of time'' and is essentially a system of short-term storage and processing of external and internal representations in order to link remote spatiotemporal information (Goldman-Rakic, 1995). In rodents, "working memory is a representation of an object, stimulus, or spatial location that is typically used within a testing session, but not between sessions, to guide behavior" (Dudchenko, 2004). The pioneering work of Olton and

Honig in the 1970s first established this term to describe the animal's short-term storage of information; in their study, they established the radial arm maze in which the rat is placed on the central platform, and a food reward is available at the ends of each arm. They observed that rats would retrieve the food from each arm, and quickly learned to visit all the arms without re-entering a previously visited one (Dudchenko, 2004). In a later study, the researchers introduced the idea that (spontaneous or rewarded) alternation in the T and Y mazes is also suggestive of working memory function, based on the assumption that rats should remember the last arm visited, avoiding crossover of spatial information from within-session consecutive trials (Olton, Collison and Werz, 1977). A well-studied paradigm of awarded alternation based on this hypothesis is the *delayed alternation task in the T maze,* which can be traced back to a study by Carr (Carr, 1917). The basic principles of the task are the following: the animal is first placed at the base of the T, runs up the stem and enters one of the arms of the T, at the end of which it can receive a reward. It is then picked up by the experimenter and repositioned at the base of the T. On the next trial, the animal will typically run up the stem and enter the other arm of the maze (*alternation*), while inserting a delay between the first and second runs renders this a delayed alternation task (Dudchenko, 2004).

Neuronal networks in various brain regions, including the prefrontal cortex (PFC), parietal and temporal cortical regions, and the hippocampus, have been shown to support WM (Constantinidis and Procyk, 2004; Tamura *et al.*, 2017; Boku *et al.*, 2018). Persistent activity during the delay period of WM tasks in primates, which has also been observed in rodent studies, is one of the main cellular mechanisms proposed to sustain WM function (Liu *et al.*, 2014; Kamigaki and Dan, 2017; Missaire *et al.*, 2021), while a synaptic theory of WM based on short-term pre-synaptic plasticity was also recently proposed (Mongillo, Barak and Tsodyks, 2008; Mi, Katkov and Tsodyks, 2017). However, only a small number of studies have ever suggested that long-term synaptic changes involving post-synaptic modifications could be linked to some aspects of WM (Konstantoudaki *et al.*, 2018; Chalkiadaki *et al.*, 2019; Missaire *et al.*, 2021; Stavroulaki *et al.*, 2021), most of which involve male participants.

As a result, the *second goal* of this study was to shed light on the effects of spatial WM training in the T-maze delayed alternation task on the LTP in the hippocampus of female C57/BL6 mice. To ensure that our findings are the result of WM task training, we used two types of controls: (a) a passive control group that remained in the homecage and (b) an active control group that performed an alternation task without any delays.

Methods

Animals

All experiments were conducted in adult (>3-month-old) C57BL/6 male and female mice. Mice were bred in the animal facility of the Department of Biology, University of Crete, housed in same-sex groups (3–4 per cage), and provided with standard mouse chow and water ad libitum, under a 12-hr light/dark cycle (light on at 7:00 a.m.) with controlled temperature (23 ± 1°C). All procedures were performed according to protocols approved by the Research Ethics Committee of the University of Crete and followed the European Union ethical standards outlined in the Council Directive 2010/63/ EU of the European Parliament on the protection of animals used for scientific purposes.

Experimental design

A. Male and female mice first performed the 3-day habituation in the open-field apparatus and then the TOR task.

B.A different cohort of C57BL/6 adult female mice first conducted the Delayed Alternation Task (DAT) (2-day habituation in the elevated plus-maze apparatus, then 9-day training). 1–3 days following the end of the last behavioral session, the mice brains were processed for in vitro brain slice electrophysiology recordings.

All the behavioral experiments took place in the same period during the day, between 9 a.m. and 13 p.m., mice were initially handled by the experimenter for 7 (Habituation, TOR) to 10 days (DAT) before proceeding to their respective behavioral task.

Setting up the behavioral experiments

The devices were placed in the centre of the room and the experiments were conducted under low light (Habituation, Temporal order object recognition task) or under strong illumination from the LED lamps on the ceiling (Delayed alternation task). The temperature was kept constant at ±24 ^{o}C. The experimental apparatus and the objects used were thoroughly cleaned with paper and 70% ethanol solution before and after each use to exclude object discrimination based on olfactory cues. During the behavioral tasks, the subjects' movements were recorded through a camera device connected to a computer, placed in such a position where every part of the apparatus was visible while ensuring that the camera itself was not visible to the animal. The recording of the animals was carried out using a "Camera" application. Open-field data analysis was completed using the monitoring program ToxTrac (Rodriguez *et al.*, 2018). The analysis of TOR phases was conducted through Jwatcher, a widely-used tool for the quantitative analysis of behavior, while DAT measurements were done manually through the video recordings.

Habituation in the open-field apparatus

Prior to the TOR task, C57BL/6 animals were placed in the open-field arena (four

walls made of plexiglass, area dimension 45 × 45 × 45cm) devoid of any object, where their spontaneous locomotor response to the novel environment was measured for 15 minutes for 3 consecutive days (habituation). The habituation was later analyzed in 5-minute bins, during which the locomotion and thigmotaxis (the tendency to remain close to the walls) were both measured as the total distance traveled in the whole arena and near the walls, respectively.

Temporal order object recognition task

The TOR task is used to assess the recency memory of mice. After habituating in the open-filed apparatus for 3 days,on test day, the animals were subjected to two sample trials and one test trial with an inter-trival interval of 25 min, as described in **Fig.1**. During the waiting period between the trials, the animals were removed from the arena and returned to a temporary cage within the behavior room. Object exploration was defined by the direction of the nose towards the object at a distance of ≤ 2 cm. Any other behavior, such as wandering around or resting on the object, was not considered explorative. The time that mice explored the two different objects was measured and two indices were calculated: the discrimination index (exploration time of old familiar object - exploration time of recent familiar object / total object exploration time) and the exploration index (exploration time of old familiar object + exploration time of recent familiar object / total recording time).

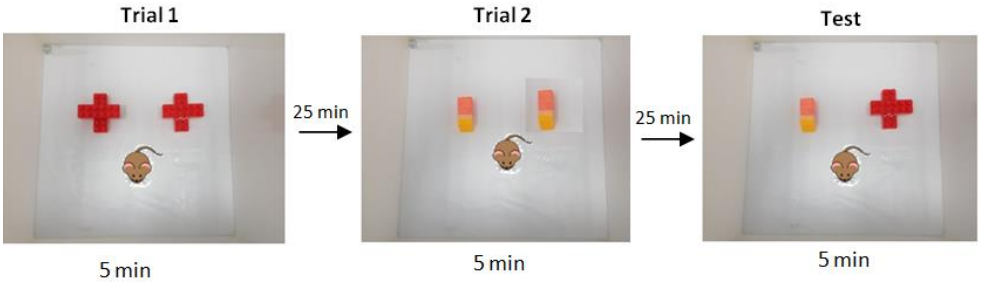


Figure 1: Temporal order object recognition task

The experiment includes a total of three phases; two sample phases and one test phase. In each sample phase, mice explore two identical, non-familiar objects, which differ in each phase but are located in specific and identical positions in the open-field arena. In the third phase (test) an object from the first phase (old familiar) and an object from the second phase (recent familiar) are placed in the arena, in the same positions as in the previous phases. The three phases are performed 25 minutes apart, and in each phase, the mouse is allowed to explore the apparatus for 5 minutes.

Delayed alternation task

C57/BL6 adult female (n=11) mice performed the delayed alternation task, the duration of which was 11 days regardless of the outcome. The aim of the first 2 days was to familiarize the mice with the T-maze apparatus. During the following 9 days, the main experiment for working memory training was carried out. Mice were

initially handled by the experimenter for 10 days. Since day 3 of handling, the animals were food restricted so that they maintained 85%–90 of their initial weight and then habituated to the T-maze apparatus.

a. Habituation in the T-maze

After being familiarized with the experimenter for 10 days, the animals went through 2-day habituation, a process critical to reducing the subjects' stress levels prior to the main experiment, as it is closely engaged with memory and learning.

On *day 1,* the T-maze apparatus was cleaned with 70% diluted ethanol and small pieces of reward food (chocolate-flavored cereal) were scattered all over it. Then all the mice that took part in the experiment (adaptive and non-adaptive) were placed together in the maze 3 times for a 10-min period each time, with a 10 min break in-between. During the break, ethanol was used to clean up food debris and feces. While the mice were in the maze, the food was replenished as needed.

On *day 2,* the same procedure was followed for each mouse separately, except that the food was placed only on the 2 arms-targets (left and right arm of the device, see **Fig.2**). During the last 10-min in the device, each mouse was placed on the opening arm and the time it took to select an arm was recorded, then it was placed back on the starting arm. The procedure was repeated until the end of the 10-min period. If the mouse needed less than 30 seconds to select a target arm, it was considered ready to participate in the experiment.

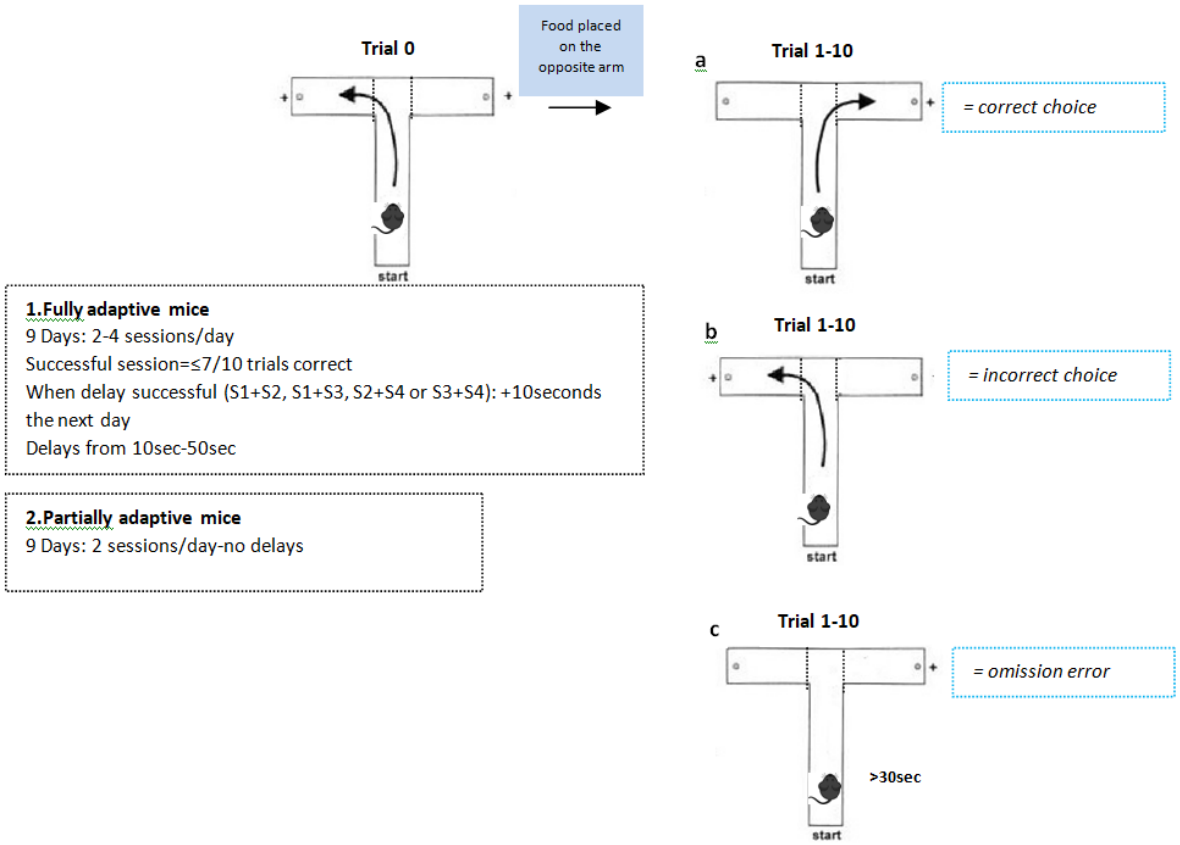


Figure 2: Delayed Alternation Task-Experimental configuration

DAT took place in the T-maze apparatus (includes a start arm and two goal arms, 45 × 5 cm each). The basics of the experimental procedure were kept unchanged for the *fully adaptive* and the *partially adaptive* group. Starting with the fully trained group **(1)** each animal was subjected daily to 2 - 4 sessions of 11 (1 starting trial +10) trials, depending on their performance in each session. A *trial* was defined as the path that the mouse took from the start arm to the goal arm, i.e. the selection arm. For the selection to be valid, the mouse should have passed the trial line (vertical dashed line) with its tail as well. In the 1st trial, i.e. *trial 0* the food was placed on both arms of the maze, thus the subject had to make a random arm choice and consume the food there. In the next trial, i.e. *trial 1*, the food was placed on the opposite arm from the previous choice. **a.** If the mouse found the food, the choice was recorded as *correct;* **b.** on the opposite case, the choice was recorded as *incorrect*, **c.** if the choice was delayed for more than 30 seconds, it was recorded as an *omission error*. In every incorrect choice, the food remained in the same arm until the correct one was made. After each correct choice, the reward was placed in the opposite arm. **(2)**The partially trained group (non-adaptive mice) underwent 2 sessions daily without any delays regardless of the result.

b. Main experiment-DAT

After the habituation was successfully completed, the delayed alternation started and was recorded with a camera. Starting with the fully trained group (fully adaptive mice), each animal was subjected daily to 2 - 4 sessions of 11 (1 starting trial +10) trials, depending on their performance in each session. The basic experimental design for the fully adaptive and non-adaptive mice is thoroughly explained in Fig.2. The control group (naïve mice) did not take part in the experiment. They remained inside the cage and were fed or subjected to the handling procedure to maintain familiarity with the experimenter.

Electrophysiological recordings

Field excitatory postsynaptic potentials (fEPSPs) were recorded in the hippocampus brain slice preparation. Mice were decapitated under halothane anesthesia. The brain was removed immediately and placed in ice-cold, oxygenated (95% O2/5% CO2) artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 3.5 KCl, 26 NaHCO3, 1 MgCl2, and 10 glucose (pH 7.4, 315 mOsm/L). The brain was blocked and glued onto the stage of a vibratome (Leica, VT1000S, Leica Biosystems GmbH, Wetzlar, Germany). Brain slices (400 μm thick) containing the hippocampus were obtained and transferred to a submerged chamber, which was continuously super-fused with oxygenated (95% O2/5% CO2) aCSF containing (mM): 125 NaCl, 3.5 KCl, 26 NaHCO3, 2 CaCl2, 1 MgCl2, and 10 glucose (pH 7.4, 315 mOsm/L) in room temperature. The slices were allowed to equilibrate for at least an hour in this chamber before experiments began. Slices were then transferred to a submerged recording chamber, which continuously super-fused oxygenated (95% O2/5% CO2) aCSF containing (in mM): 125 NaCl, 3.5 KCl, 26 NaHCO3, 2 CaCl2, 1 MgCl2 and 10

glucose (pH 7.4, 315 mOsm/L) in room temperature. Recording and stimulating electrodes were placed on the stratum radiatum layer of the CA1 region of the hippocampus, about 300 µm apart. Responses were amplified using a Dagan BVC-700A amplifier (Dagan Corporation, Minneapolis, MN, USA) and digitized using the ITC-18 board (Instrutech, Inc) on a PC using custom-made procedures in IgorPro (Wavemetrics, Inc, Lake Oswego, OR, USA). Data were acquired and analyzed using custom-written procedures in IgorPro software (Wavemetrics, Inc, Lake Oswego, OR, USA). The electrical stimulus consisted of a single square waveform of 100 µs duration given at intensities of 0.05–0.3 mA generated by a stimulator equipped with a stimulus isolation unit (World Precision Instruments, Inc). The fEPSP amplitude was measured from the minimum value of the synaptic response (4–5 ms following stimulation) compared to the baseline value prior to stimulation. Both parameters were monitored in real-time in every experiment. Baseline stimulation parameters were selected to evoke a response of 1 mV. The paired-pulse protocol consisted of two pulses at baseline intensity separated by 100, 50, and/or 20 ms. LTP was induced using theta-burst stimulation (5 pulses at 100 Hz x 4 times at theta-rhythm (every 200 ms)). This stimulation was repeated twice with an inter-stimulus interval of 20 s. Synaptic responses were normalized to the average 10 min pre-stimulus (tetanus or theta-burst).

Statistics

Data analysis was performed with Microsoft Excel and statistical analysis with Graphpad Prism 8. The significance level (type-I error threshold) was set at 5%. A comparison was reported as statistically significant if the p-value<0.05. The data were tested for the presence of outliers before being tested for normality (D'Agostino-Pearson omnibus and Kolmogorov–Smirnov normality test). For the locomotion analysis, one-way or two-way ANOVA with repeated measures (mixed-effects model) and Post-hoc comparisons (Sidak's multiple comparisons test) were applied, while for the TOR task analysis, one-way ANOVA and unpaired t-tests with Welch's correction were used. For the comparison of fepsp values, repeated measures ANOVA and Post-hoc comparisons (Tukey's test) were applied.

Results

Both male and female mice exhibited increased locomotion in the periphery during habituation in the OF box, with male mice showing the highest level.

Before the TOR task, C57/BL6 animals of both sexes were habituated in the open-field apparatus for three days, during which their locomotion was measured as the total distance (in mm) traveled in the three distinct zones of the box, as defined by the experimenter (**Fig.1**).

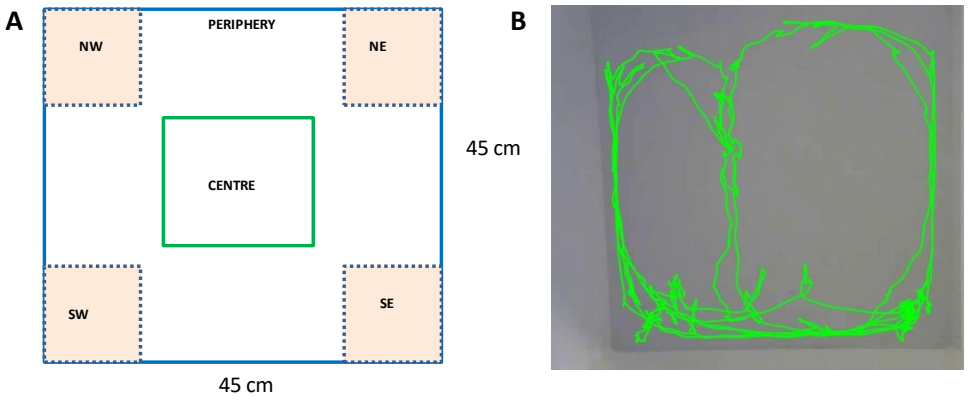


Fig.1| Schematic design of OF zones and trajectory of animal's motion

A.Graphical representation of distinct zones designed in the open-field apparatus for locomotion analysis; center (green solid line), periphery (blue solid line), and four wall edges (dotted lines). *NW=NorthWest, NE=NorthEast, SW=SouthWest, SE=SouthEast,* **B.**Representative example of the animal's movement, where the trajectory is depicted as a continuous, green trail in *Toxtrac* graphical outputs.

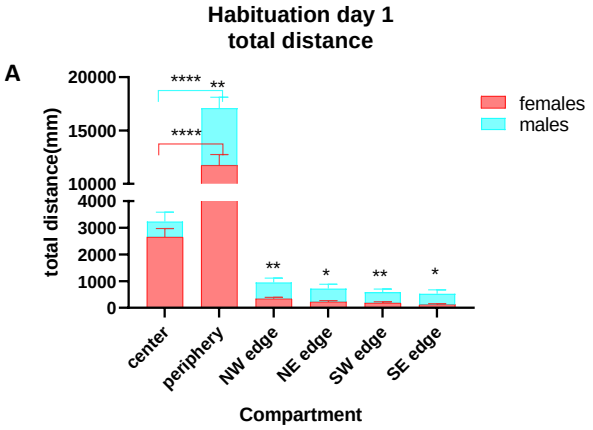
Males exhibited increased locomotion in all three zones of the OF compared to females, with the highest locomotion levels found in periphery.

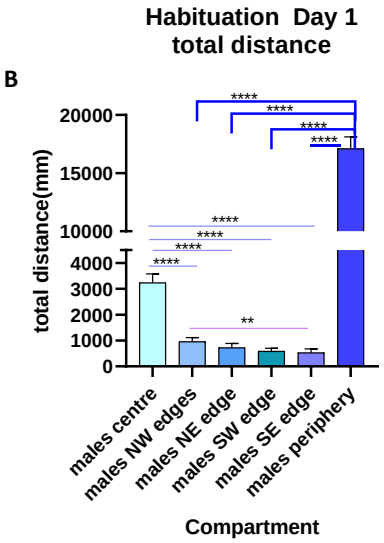
Habituation Day 1

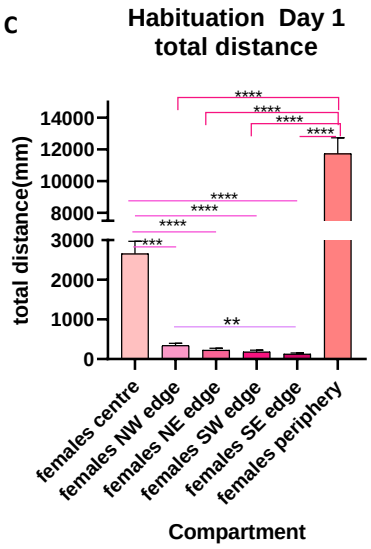
The three-day habituation of male and female mice was first analyzed. Total analysis of *Habituation Day 1* with repeated measures two-way ANOVA revealed that both sexes traveled a greater distance in the peripheral zone than in the center, while males exhibited significantly greater peripheral and wall locomotion levels compared to females **(Fig.2A).**Intra-sex analysis indicated statistically higher central and peripheral levels compared to the wall area, whereas sstatistical differences between wall edges were discovered in both sexes (**Fig.2B, Fig.2C**).

The first habituation day was subsequently analyzed into three 5-min time intervals, with no major differences observed in the locomotion pattern of the two sexes throughout the time bins. Males appeared to travel more in the open-field box than

females, although the differences did not reach a significant level. Within-males statistics revealed higher peripheral and central levels compared to the wall area, while females' displacement in the box followed the same pattern. Only in male mice did the locomotion around the edges vary (Supplementary Fig.1).







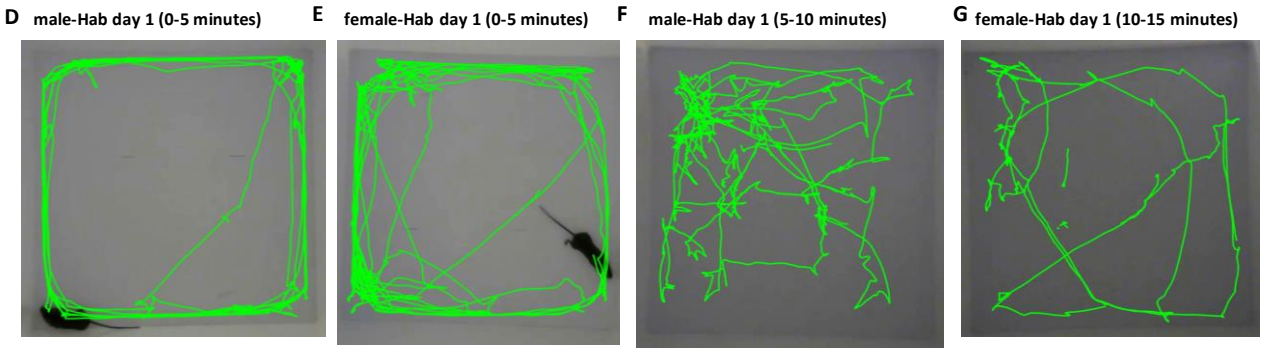


Fig.2| Total distance of males and females traveled in the open-field box during the 15 minutes of habituation day 1. A.Stacked bar graphs showing the total distance (in mm) of both males and females in the distinct areas of the open-field box, during the total 15 minutes of habituation on day 1. Two-way ANOVA with repeated measures analysis showed that there was a significant effect of area ($F(1.246, 110.1) = 292.1$, $p < 0.0001$) and sex ($F(1,118) = 14.12$, $p = 0.0001$), as well as of area*sex interaction ($F(5,442) = 9.292$, $p < 0.0001$) in the locomotion. Post hoc analyses revealed that male mice displayed greater locomotion levels in the peripheral ($p=0.0016$) and wall area ($p_{NW}=0.0023$, $p_{NE}=0.0277$, $p_{SW}=0.0094$, $p_{SE}=0.0470$), compared to females. The intra-sex analysis showed that both sexes travelled significantly more in the periphery compared to the center of the box ($p_{males}<0.0001$, $p_{females}<0.0001$), **B.** One-way repeated measures ANOVA for the intra-male analysis revealed significant differences between the areas ($F (1.325, 55.67) = 243,7$, $p<0.0001$). Post-hoc analyses showed that male mice traveled significantly more in the peripheral and central area compared to the wall edges (for center vs edges: $p<0.0001$, for periphery vs edges: $p<0.0001$), whereas the locomotion levels among the edges also differed (for NW vs SE edge: $p=0.0092$), **C.** One-way repeated measures ANOVA revealed a similar pattern in the locomotion levels of female mice throughout the arena (repeated measures: $F (1.167, 54.40) = 92.41$, $p<0.0001$. For center vs edges: $p<0.0001$, for periphery vs edges: $p<0.0001$, for NW vs SE edge: $p=0.0012$). **D-G**. Representative locomotion trajectories of both sexes throughout the three time bins of habituation analysis. *Vertical line above each bar represents the SEM (n=17 for males-center and periphery, n=20 for males-walls, n= 21 for females center and periphery, n=25 for female mice-walls). *p< 0.05, **p < 0.01 and ***p = 0.001, ****p<0.0001. SEM: Standard error of the mean. *NW=NorthWest, NE=NorthEast, SW=SouthWest, SE=SouthEast.*

Habituation Day 2

During the fifteen minutes of *Habituation Day 2*, subjects of both sexes traveled more in the periphery compared to the center. No significant variations in the locomotion level derived from the inter-sex comparison with repeated measures two-way ANOVA (**Fig.3A**). The intra-sex analysis with one-way repeated measures ANOVA revealed statistically higher peripheral and central levels compared to the wall edges for both sexes (**Fig.3B,3C**).

Throughout the separate time intervals, males and females demonstrated elevated peripheral locomotion levels compared to central, as well as central and peripheral compared to the wall area (Supplementary Fig.2).

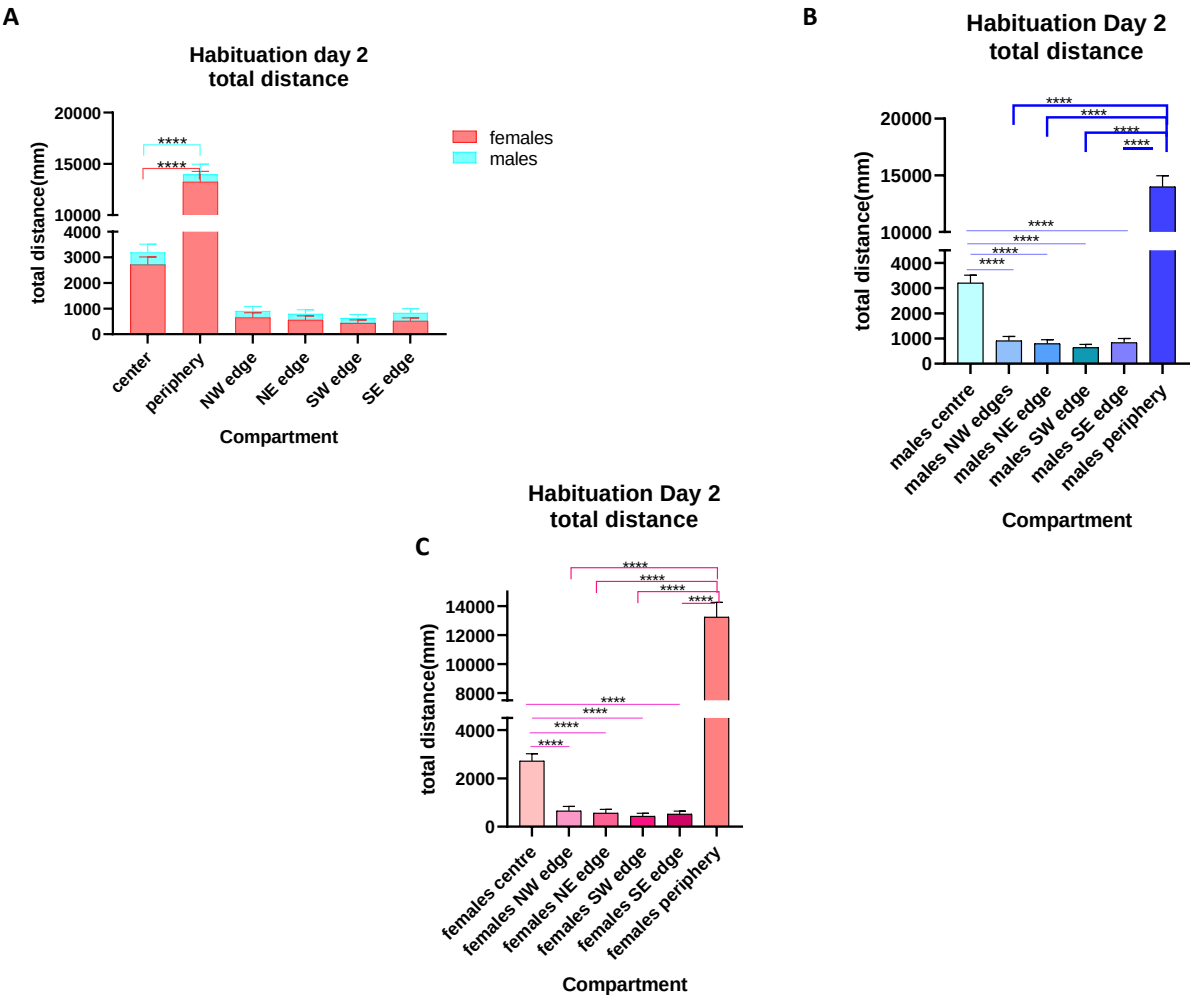


Fig.3| Total distance of males and females traveled in the open-field box during the 15 minutes of habituation day 2. A.Stacked bar graphs showing the total distance (in mm) of both males and females in the distinct areas of the open-field box, during the total 15 minutes of habituation on day 2. Two-way ANOVA with repeated measures analysis showed that there was a significant effect of area ($F(1.289, 137.7) = 319.8$, $p < 0.0001$), albeit no of sex ($F(1,114) = 1.704$, $p = 0.1944$) nor of area*sex interaction ($F(5,534) = 0.1125$, $p = 0.9896$) in the locomotion. Post hoc analyses revealed no differences between the two sexes in the total distance travelled throughout the box. The intra-sex analysis showed that both sexes travelled significantly more in the periphery compared to the center of the box ($p_{males}<0.0001$, $p_{females}<0.0001$), **B.** One-way repeated measures ANOVA for the intra-male analysis revealed significant differences between the areas ($F (1.318, 71.16) = 180.7$, $p<0.0001$). Post-hoc analyses showed that male mice traveled significantly more in the peripheral and central area compared to the wall edges (for center vs edges: $p<0.0001$, for periphery vs edges: $p<0.0001$), **C.** One-way repeated measures ANOVA revealed a similar pattern in the locomotion levels of female mice throughout the arena (repeated measures: $F (1.278, 69.00) = 146.8$, $p<0.0001$. For center vs edges: $p<0.0001$, for periphery vs edges: $p<0.0001$). *Vertical line above each bar represents the SEM (n=19 for males-center and periphery, n=20 for males-walls, n= 19 for females center and periphery, n=19 for female mice-walls). *p< 0.05, **p < 0.01 and ***p = 0.001, ****p<0.0001. SEM: Standard error of the mean.[*] NW=NorthWest, NE=NorthEast, SW=SouthWest, SE=SouthEast*.

Habituation Day 3

Subjects of both sexes traveled a greater distance in the peripheral zone than in the center of the box during the fifteen minutes of Habituation Day 3, while males moved significantly more in the NE wall edge of the apparatus compared to females

(Fig.4A). The intra-sex analyses showed statistically higher central and peripheral levels compared to the wall area, whereas both sexes displayed statistical differences in their locomotion around the edges (**Fig.4B, 4C**).

Throughout the distinct time intervals, males and females demonstrated elevated locomotion levels in the periphery versus the center, as well as in both zones compared to the wall area (Supplementary Fig.3). Only during the first period was a statistically higher peripheral locomotion level of males compared to females observed(Supplementary Fig.3A).

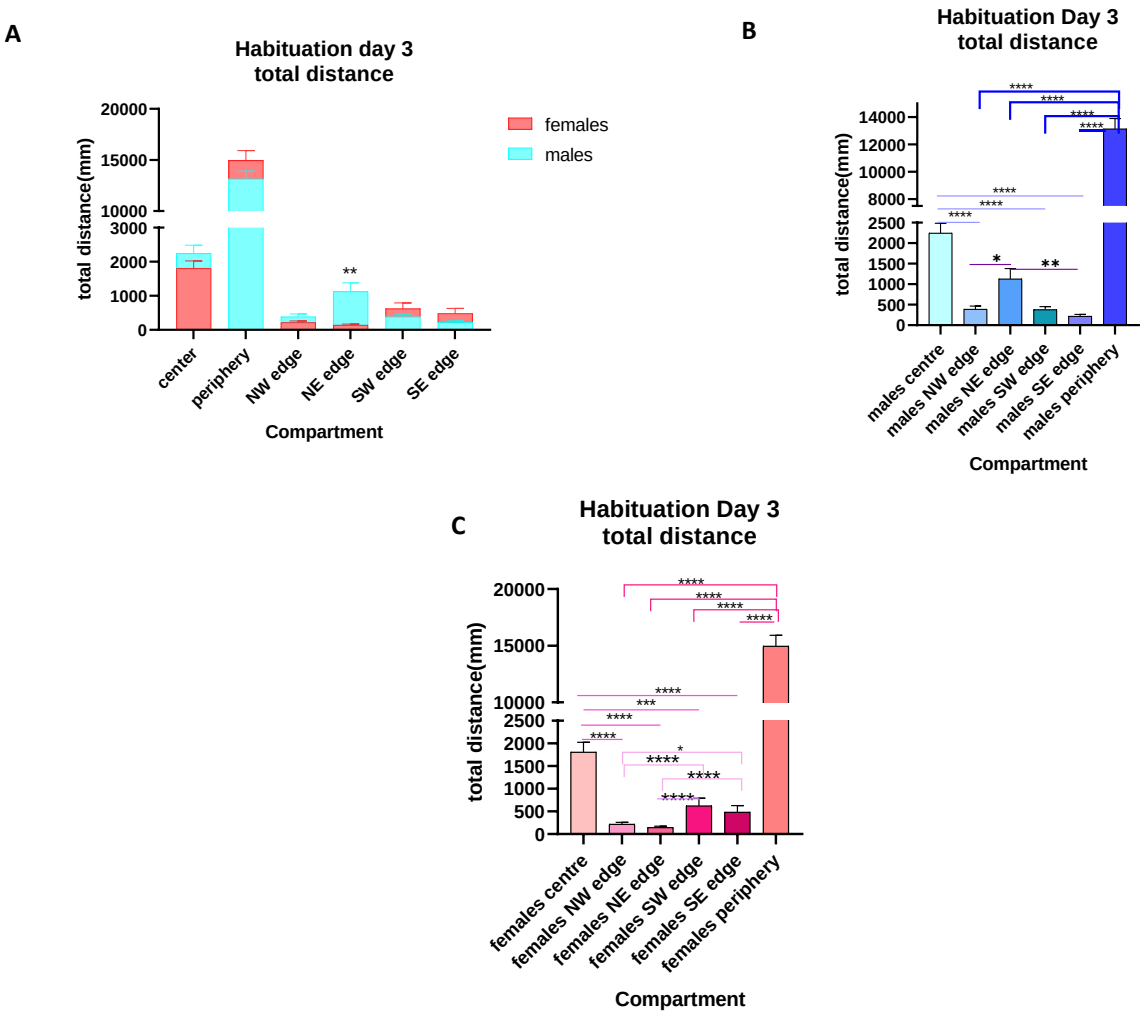


Fig.4| Total distance of males and females traveled in the open-field box during the 15 minutes of habituation day 3. A.Stacked bar graphs showing the total distance (in mm) of both males and females in the distinct areas of the open-field box, during the total 15 minutes of habituation on day 3. Two-way ANOVA with repeated measures analysis showed that there was a significant effect of area ($F(1.220, 86,14) = 296.3$, $p < 0.0001$), albeit no of sex ($F(1,100) = 0.1496$, $p = 0.6998$) nor of area*sex interaction ($F(5,353) = 2.112$, $p = 0.0634$) in the locomotion. Post hoc analyses revealed a significant difference between the two sexes in the total distance travelled around the NE edge ($p=0.0028$). The intra-sex analysis showed that both sexes travelled significantly more in the periphery compared to the center of the box ($p_{males}<0.0001$, $p_{females}<0.0001$), **B.** One-way repeated measures ANOVA

for the intra-male analysis revealed significant differences between the areas (F (1.399, 44.78) = 180.4, p<0.0001). Post-hoc analyses showed that male mice traveled significantly more in the peripheral and central area compared to the wall edges (for center vs NW,SW and SE edges: p<0.0001, for periphery vs all edges: p<0.0001, for NW vs NE edge:p=0.0472), **C.** One-way repeated measures ANOVA revealed a similar pattern in the locomotion levels of female mice throughout the arena (repeated measures: F (1.151, 44.87) = 149.4, p<0.0001. For center vs NW, NE and SE edges: p<0.0001, center vs SW edge:p=0.0006, for periphery vs all edges: p<0.0001).*Vertical line above each bar represents the SEM (n=19 for males-center and periphery, n=20 for males-walls, n= 19 for females center and periphery, n=19 for female mice-walls). *p< 0.05, **p < 0.01 and ***p = 0.001, ****p<0.0001. SEM: Standard error of the mean.* NW=NorthWest, NE=NorthEast, SW=SouthWest, SE=SouthEast*.

Total locomotion analysis through all habituation days

As illustrated in **Fig.5**, the analysis of the total locomotion of mice in the whole open-field box throughout the three habituation days with repeated measures two-way ANOVA (independent factors: habituation day and sex) revealed significantly increased male locomotion levels only on the first habituation day, as well as statistically higher levels of both sexes on the first habituation day compared to the following two.

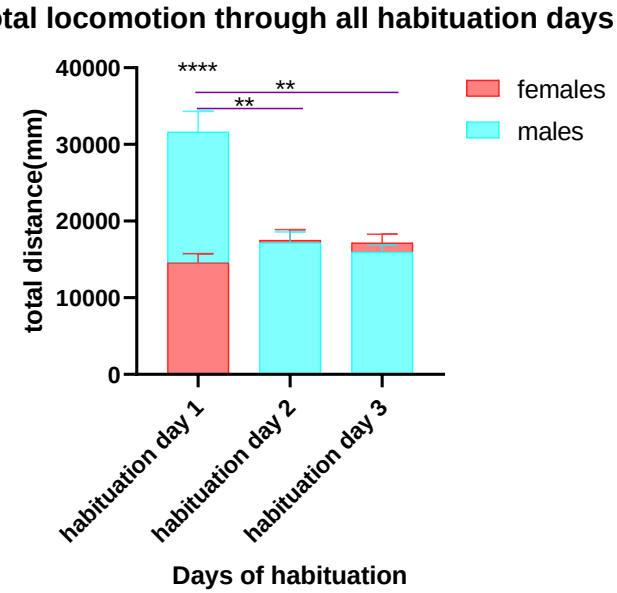


Fig.5| Total locomotion levels of both sexes in all zones of the open-field box during the three habituation days. Two-way ANOVA with repeated measures analysis revealed a significant effect of habituation day (F (1.719, 180.5) = 13.39, p < 0.0001) and sex (F (1,122) = 9.818, p=0.0022), as well as of day*sex interaction (F(2,210) = 26.46, p < 0.0001) in the total locomotion levels. Post-hoc comparisons detected significantly higher periphery levels in males compared to females (p<0.0001) on the first habituation day and statistically elevated total locomotion levels on habituation day 1 compared to the following two (for hab day 1 vs hab day 2:p=0.0082, for hab day 1 vs hab day 2:p=0.0059). When each sex was tested

separately, significantly increased levels of male locomotion levels were observed on habituation day 1 compared to the following two (for hab day 1 vs hab day 2:p<0.0001, for hab day 1 vs hab day 2:p<0.0001, for hab day 2 vs hab day 3:p=0.7524), while females did not alter their locomotion across days (for hab day 1 vs hab day 2:p=0.3163, for hab day 1 vs hab day 2:p=0.2991, for hab day 2 vs hab day 3:p=0.9676). *Vertical line above each bar represents the SEM (n=19 for males-center and periphery, n=20 for males-walls, n= 20 for females center and periphery, n=23 for female mice-walls). *p< 0.05, **p < 0.01 and ***p = 0.001, ****p<0.0001. SEM: Standard error of the mean.*

Temporal order object recognition memory task: male and female mice displayed similar exploration time and discrimination index of the "old familiar" object.

As illustrated in **Fig. 6,** both sexes spent similar amounts of time exploring the "old familiar" and "recent familiar" objects during the test phase of TOR (**Fig.6A).** They also had equal discrimination and exploration indices of the "old familiar" object **(Fig.6B, 6C**). Finally, the sum of exploration indices of all TOR phases is collectively presented in **Fig.6D**. The only significant difference was detected in male mice, which tended to explore the objects more in trial 1 compared to the test phase.

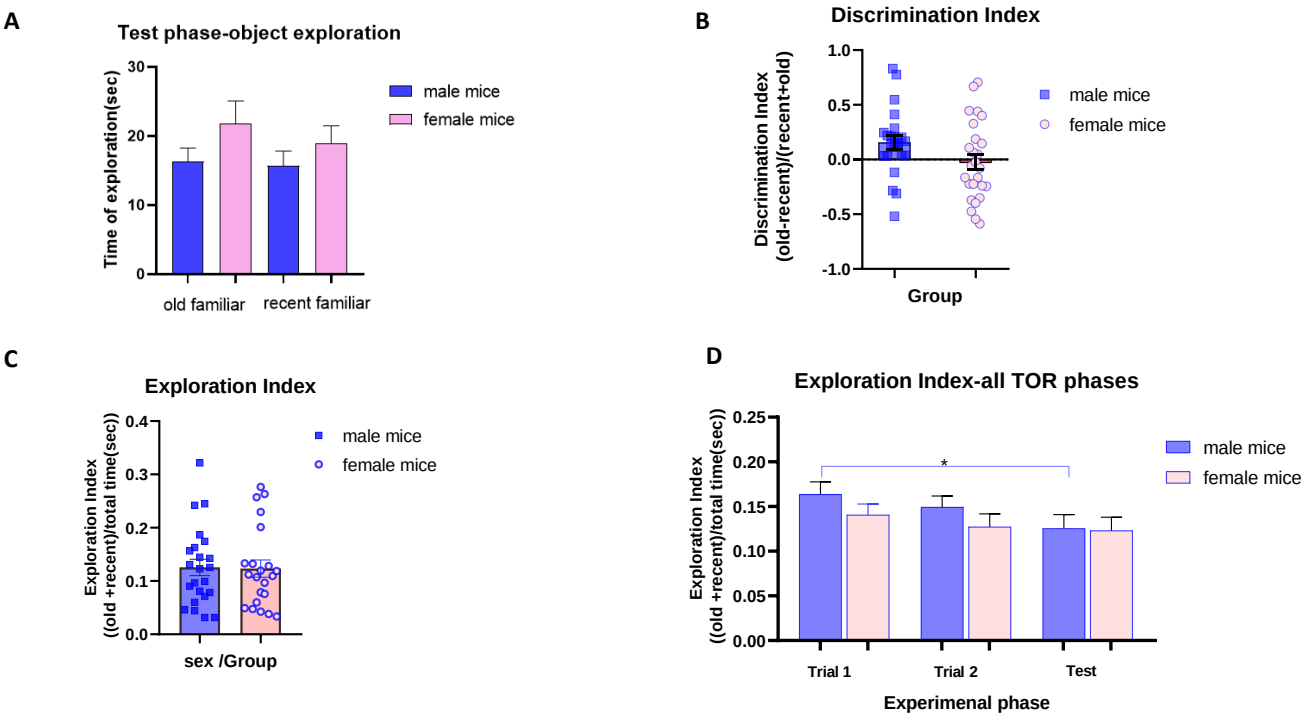


Fig.6| Male and female mice displayed similar exploration time and discrimination index of the "old familiar" object in the TOR task. A. Bar graph showing the exploration time (in sec) of the "old familiar" object (firstly introduced in trial 1) and the "recent familiar" object (firstly introduced in trial 2) during the test phase of the TOR task. One-way ANOVA test (Brown-Forsythe and Welch's analysis) did not reveal any significant difference in the exploration time of male mice compared to females nor within each group (Brown-Forsythe: F (3.000,77.44)=1.233, p=0.3035. Welch's ANOVA: F (3.000, 49.94)=1.041,

p=0.3824), **B.** Unpaired t-test with Welch's correction revealed no significant variation in the discrimination index of the "old familiar" object between the two sexes (t=1.874, df=46.97, p=0.0672), **C.** No significant differences were revealed between the exploration indices of the two groups (unpaired t-test with Welch's correction: t=0.0995, df=42.77, p=0.9212), **D.**Bar graphs showing the object exploration tendency of both male and female mice in all phases of the TOR task. Two-way ANOVA with repeated measures analysis showed that there was a significant effect of the TOR phase (F (1.435, 68.14) = 3.535, p=0.0492), albeit no of sex (F (1,48) = 0.9272, p = 0.3404) nor of TOR phase*sex interaction (F(2,95) = 0.7393, p = 0.4802) in the exploration index. Post hoc analyses revealed significantly higher levels of exploration in males during Trial 1 compared to Test phase (p=0.0290). No significant differences were revealed between the sexes (for Trial 1:p=0.5258, for Trial 2:p=0.5878, for Test phase:p=0.9993). *Vertical line above each bar represents the SEM (n= 23 for male mice, n=27 for female mice.*p< 0.05, **p < 0.01 and ***p = 0.001, ****p<0.0001. SEM: Standard error of the mean.*

LTP induction in hippocampal slices of adaptive and non-adaptive females following delayed alternation task performance.

Behavioral changes in PFC and HPC-mediated functions, such as the working memory training in the T-maze delayed alternation task, are primarily supported by the underlying neuronal properties. In vitro electrophysiological recordings were thus performed to further study the change in neuronal properties of females undergoing the DAT (see **Table 1** for DAT performance of fully-adaptive and non-adaptive females). The ability of the stratum radiatum layer of the CA1 hippocampal region to exhibit long-term potentiation (LTP) in response to tetanic stimulation (5 pulses at 100 Hz x 4 times, every 200 ms) was specifically investigated. In fully-adaptive female mice, which performed DAT with increasing delays in the start arm, tetanic stimulation resulted in increased fepsp peak amplitude which lasted for 50 minutes (**Fig.7B).** LTP was also induced in non-adaptive mice (**Fig.7C**), although the elevated peak values gradually returned to lower levels about 30 minutes post-tetanus. In naïve mice, tetanic stimulation did not induce any significant fepsp peak potentiation (**Fig.7D**). When non-adaptive females were compared to naive and fully-adaptive mice, their fepsp responses were significantly higher (**Fig.7E**). The post-tetanic change in synaptic plasticity was also reflected by the alteration in fepsp slope values (**Fig.8)**. Tetanic stimulation was found to induce an increased, sustained potentiation in fully adaptive mice (**Fig.8A**) and shorter-lived, enhanced potentiation levels in non-adaptive females (**Fig.8B**), while the slope values in naïve females remained unchanged (**Fig.8C**). The fepsp responses were significantly increased in non-adaptive females compared to fully-trained and naïve mice (**Fig.8D**).

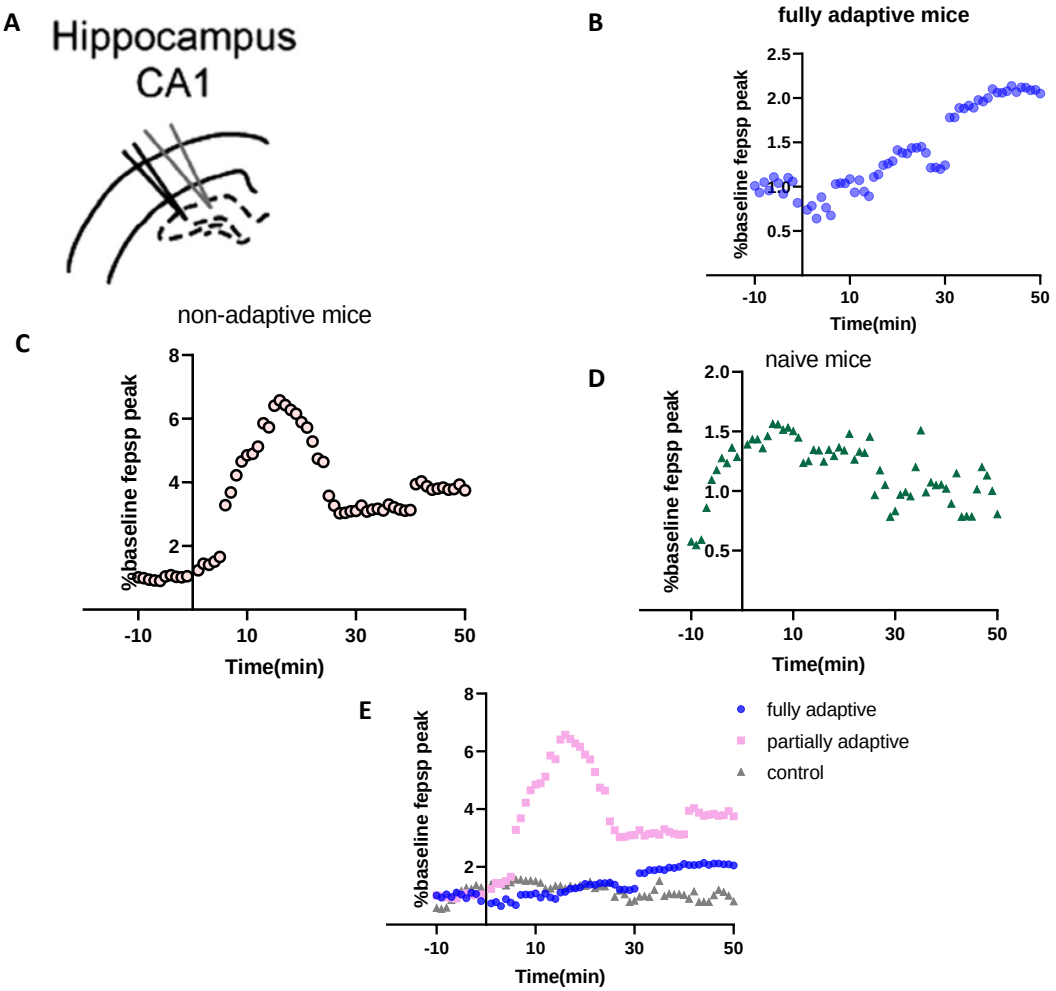


Fig.7| Long-term potentiation in the hippocampus of adaptive, non-adaptive, and naive groups, as measured by the change in normalized peak values. A. Graphical representation showing the position of the electrodes stimulating and recording electrodes (300 µm apart) in the hippocampus (*adaptation from Stavroulaki et al., 2021*), **B.**In fully-adaptive females, theta-burst stimulation induced significant potentiation in synaptic effectiveness that maintained stability for 50 minutes**, C.**In non-adaptive mice, the sharp increase in fepsp peak potentiation at 20 min post-tetanus eventually dropped at 30 minutes, **D.**A downward trend was observed in the fepsp levels of naïve (homecaged) mice after tetanic stimulation, E.The size of fepsp responses was significantly increased in fully and non-adaptive females compared to naïve mice (repeated measures ANOVA, $F(2,211) = 41.73$, $p < 0.0001$). Post-hoc comparisons showed a significant difference between the fully adaptive and the non-adaptive (Tukey's test, $p<0.0001$) and between non-adaptive and naïve mice (Tukey's test, $p<0.0001$), *n=2 fully adaptive, n=2 non-adaptive, n=3 control mice.*

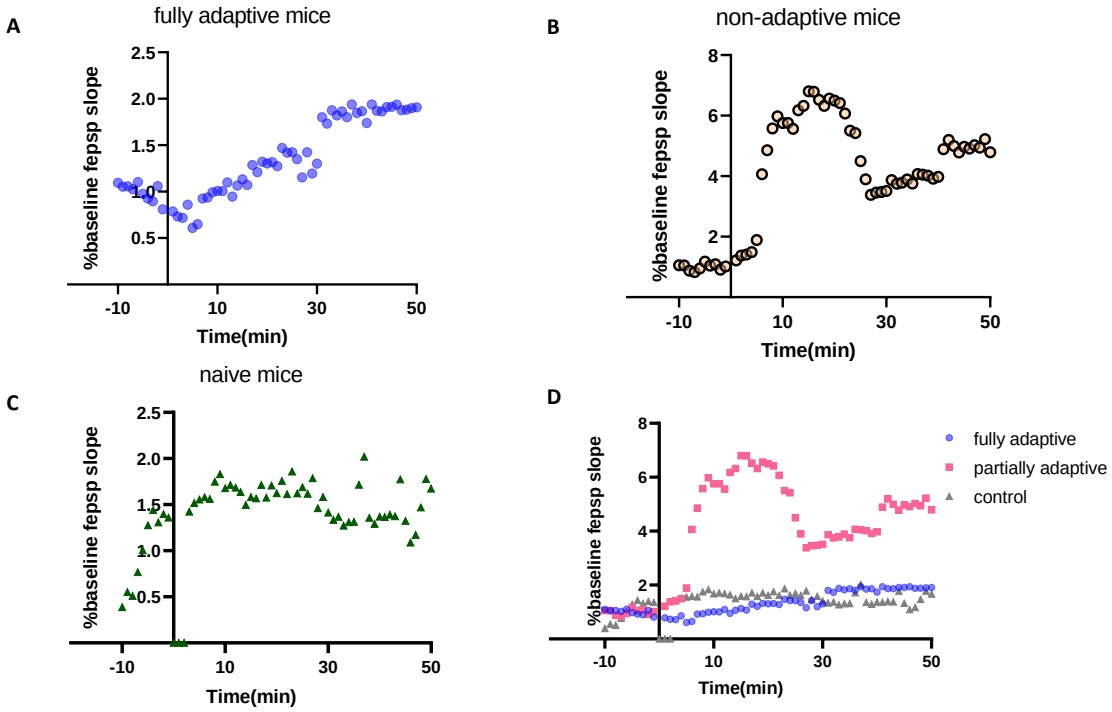


Fig.9| Long-term potentiation in the hippocampus of adaptive, non-adaptive, and naive groups, as measured by the change in normalized slope values. A. Fully-adaptive females displayed a gradual increase in slope fepsp values following tetanic stimulation (time=0 min) which persists throughout the 50 minutes of recording, **B.** In non-adaptive mice, the sharp increase in fepsp slope potentiation levels at 20 min post-tetanus eventually dropped at 30 minutes**, C.** The amplitude of fepsp in naive mice remained unaltered after stimulation, **D.** The fepsp responses were significantly increased in non-adaptive females compared to naïve and fully-adaptive mice (repeated measures ANOVA, $F(2,200) = 41.55$, $p < 0.0001$) Post-hoc comparisons showed a significant difference between the fully adaptive and the non-adaptive (Tukey's test, $p<0.0001$) and between non-adaptive and naïve mice (Tukey's test, $p<0.0001$), *n=2 fully-adaptive, n=2 non-adaptive, n=3 control mice.*

Table 1: ***DAT performance analysis.***

	Perseverance	%correct responses	# of sessions	Latencies for S1, S2 (s)
Adaptive 1	3±0.3	0.6±0.1	5±1	1.9±0.16
Adaptive 2	2±0.3	0.8±0.0	3±0	1.7±0.10
Adaptive 3	4±1.0	0.6±0.1	5±1	2.7±0.23
Adaptive 4	7±0.0	0.4±0.0	8±2	1.0±0.20
Adaptive 5	3±0.3	0.5±0.1	11±5	3.2±0.16
Adaptive 6	3±1.2	0.7±0.1	9±2	0.8±0.02
Adaptive 7	4±1.5	0.5±0.1	9±0	1.5±0.23
Adaptive 8	3±0.0	0.5±0.0	8±3	-
Adaptive 9	3±0.5	0.6±0.1	7±1	-
Non-adaptive 1	4±0.0	0.4±0.0	4±0	-
Non-adaptive 2	1±0.0	0.8±0.0	2±0	-
Non-adaptive 3	9±0.0	0.4±0.0	8±0	-
Non-adaptive 4	2±0.0	0.6±0.0	6±0	-
Non-adaptive 5	4±0.0	0.4±0.0	7±0	-

DAT behavioral measurements in 9 adaptive and 5 non-adaptive female mice. Perseverance stands for the number of consecutive mistakes made when entering each delay. % correct responses stands for the percent average of correct responses in the 2 first sessions of each delay, #of sessions represents the number of trials each animal needed to complete each entering delay. Latencies for S1, S2 (s) were measured as the time needed for each subjects to enter the selection arm (the 2 correct sessions) on the day of delay achievement. *Data are presented as mean±SE.*

Discussion

The purpose of this study was to compare general PFC function and temporal memory capacity in untreated C57/BL6 male and female mice, as well as to determine the working memory training effects in a different cohort of C57/BL6 females performing the DAT, as reflected by changes in hippocampal fepsp potentiation after LTP induction.

Male subjects displayed higher locomotion levels compared to females.

Habituation is a form of non-associative learning, which can be defined as the decline in the organism's tendency to respond to a stimulus following previous exposure (Yeap *et al.*, 2020). Spatial habituation has been studied in the open field (OF) since Hall's (1936)introduction of the paradigm and is since used in many laboratories worldwide.

According to previous research, a variety of brain regions underlie spatial habituation, namely the nucleus accumbens (Sargolini *et al.*, 1999; Coccurello *et al.*, 2000), CA1 (Yousefi *et al.*, 2013), basolateral amygdala (Mohammadi, Nasehi and Zarrindast, 2015), and importantly, the prelimbic area of PFC (Rinaldi *et al.*, 2007). The latter may also be implicated in the general activity of rodents in the open-field apparatus, as well as in the time-to-emerge and ambulation (Brito and Brito, 1990).

Although many parameters have been considered, ambulation is the most common proxy correlating with habituation (Paulus *et al.*, 1999; Tatem *et al.*, 2014). Exposure to the open-field for up to 15 minutes, as applied in this study, constitutes the short-term habituation and yields the most robust, reproducible, and reliable data (Wahlsten *et al.*, 2006). The total distance traveled in the central and peripheral zones of the OF box was recorded as a measure of locomotor activity to assess the three-day habituation of mice prior to the TOR task, while the distance travelled in the wall zone (along the four edges of the box) was recorded as a proxy for the animal's level of anxiety.

The open-field habituation analysis revealed a clear pattern of locomotor activity in which all subjects traveled more in the periphery than the center, as expected by untreated mice of this genetic background (Griebel *et al.*, 2000; Belzung and Griebel, 2001). On the first habituation day, males were revealed to travel more than females in all zones of the OF box apart from the central one, but the locomotion levels of both sexes eventually equalized on the following days. The intra-sex analysis showcased constantly greater peripheral and central locomotion levels compared to the wall area, while some variations among the edges were occasionally observed in both sexes. Finally, the across-habituation-days analysis in all zones highlighted the statistically increased male locomotion on the first habituation day compared to the following two, whereas females did not alter their locomoting behavior across days, a rather peculiar finding that will undoubtedly require experiments on a larger cohort of animals to be confirmed.

The inter-sex behavioral analysis results are consistent with only a subset of the literature findings; for example, C57/B6 males were found to locomote more than females in a dark open-field apparatus on the first day of 5-min habituation (Bolivar *et al.*, 2000), while in a recent study, female rats appeared less anxious than males in open-field tests (Börchers *et al.*, 2022). However, the majority of female rat studies indicate that they locomote more than males (Bernatova, Puzserova and Dubovicky, 2010; Domonkos *et al.*, 2017; Scholl *et al.*, 2019), a finding contradictory to the current results. Still, females are frequently overlooked due to concerns that behavioral variation during their reproductive cycle will confound the interpretation of experimental data (Scholl *et al.*, 2019), thus studies on female anxiety-like behavior remain variable and contradictory. For example, other findings show no implication of estrous cycle in rodent locomotion. Recent research on adult rats propose that estrous cycle is irrelevant to females' performance in the open-field (Scholl *et al.*, 2019; Lovick and Zangrossi, 2021), and emphasize the implication of exogenous interacting factors on this behavior (Miller *et al.*, 2021). Importantly, in a large scale research, Fritz, Amrein and Wolfer, 2017 retrospectively evaluated sex effects on coefficient of variation in 4,554 mice and found both sexes performing equally well in open-field exploration. These findings encourage females' inclusion in spatial memory, anxiety-related and locomotor behavioral tasks without testing for estrous cycle.

In line with the behavioral research, electrophysiological recordings in the prefrontal layers of male and female rodents are scarce in the literature, whereas none of them, to my knowledge, directly compare the innate neuronal activity of both sexes. So far, the studies most relevant to assessing the cognitive and general PFC neuronal functions often involve stress induction, either acute or chronic. A notable study in 2017 was the first to directly compare the age-and sex-dependent effects of repeated social stress on the cellular properties of rat mPFC Layer V neurons on different developmental points (Urban and Valentino, 2017), whereas in a recent study of our lab (Velli *et al.*, 2021), acute restraint stress was induced in adult mice to describe any sex-specific effects on the PFC function. fEPSPs recordings in PFC layer II revealed that LTP potentiation was significantly reduced in restraint male, but not female animals, compared to their respective control group. However, the lack of a direct comparison of LTP properties in males and females could be viewed as a limitation of this study. Due to inadequate research, a useful continuation of the currents findings would be to use in vitro electrophysiological recordings in mice to directly compare the sexual intrinsic neuronal properties regarding the general PFC functionality.

Male and female mice displayed equivalent performance in the TOR task.

TOR was performed by male and female adult C57/BL6 mice after the end of habituation. Both inter- and intra-sex analysis revealed that both sexes spent about the same time exploring the "old familiar" and "recent familiar" objects during the

test phase. Although it did not reach a significant level, the innate discrimination index of female mice tended to be slightly negative compared to males' (Fig.6B). According to relative rodent studies, intact "when" memory is reflected by a discrimination ratio greater than zero, indicating that an animal spends significantly more time exploring the "old familiar" object and has more contacts with it than with the "recent familiar" (DeVito and Eichenbaum, 2010; Zlomuzica, Dere and Dere, 2013). Studies that directly compare the episodic-like memory of male and female rodents are scarce, and most of them have been conducted in ovariectomized rats. A subset of them agree there is no sex difference in TOR performance (Rossetti *et al.*, 2018; Conner *et al.*, 2020), as also shown in human EM fMRI studies (Nyberg, Habib and Herlitz, 2000), whereas others propose that TOR performance improves during specific estral phases (Gresack and Frick, 2006; Tuscher *et al.*, 2015) or that female rodents have improved temporal memory in stress-related studies, even when estrous cycle has no effect (Wei *et al.*, 2014a; Velli *et al.*, 2021). The discrepancy among all these findings is most likely due to different experimental approaches, though the existence of sex differences in the underlying EM circuitry cannot be precluded. Another factor that could contribute to the findings' diversity is the wide range of inter-trial intervals used in the different TOR protocols worldwide, ranging from 30 minutes up to several hours, which may alter animals' temporal order memory in the test phase (Mitchell and Laiacona, 1998; DeVito and Eichenbaum, 2010; Belblidia *et al.*, 2015; Shandilya and Gautam, 2020b). In the current protocol, 25-min inter-trial interval was chosen based on experiments assessing temporal order memory for objects using rats (Dere, Huston and De Souza Silva, 2005b).

Theta-burst stimulation increases the size of postsynaptic response in non-adaptive mice more than in adaptive, though the duration is shorter.

Rewarded alternation in the T- maze indicates working memory function, based on the hypothesis that animals must remember the most recently visited arm, avoiding interference between spatial information from different successive trials within a session. This study revealed that WM training in DAT resulted in LTP induction following tetanic stimulation in fully-adaptive and non-adaptive females, though the fepsp potentiation lasted longer in fully-adaptive.

Because of the short-term nature of WM, studies focusing on short-term physiological and cellular mechanisms that may underpin WM have been conducted extensively in brain areas such as the HPC (Sloan, Döbrössy and Dunnett, 2006; Ainge *et al.*, 2007; Morellini, 2013; Boku *et al.*, 2018). Persistent activity (Goldman-Rakic, 1995) and short-term plasticity (Mongillo, Barak and Tsodyks, 2008) are traditionally thought as the cellular correlates of working memory, although long-term plasticity mechanisms have been recently implied in some WM aspects (Missaire *et al.*, 2021).

It should be noted that the current study of female DAT performance is a continuation of previous research from our lab (Stavroulaki *et al.*, 2021), in which training male C57/BL6 mice in DAT improved their performance in cognitive tasks

(e.g. reversal learning and AST), increased LTP in PFC and enhanced fEPSP response in HPC. The same protocol for inducing LTP in the CA1 *stratum radiatum* resulted in no significant difference in fepsp levels between adaptive, non-adaptive, and naive males (*Stavroulaki et al.,2021*: Fig.4C). In the current study, enhanced postsynaptic response was sustained the longest in fully adaptive females, even though non-adaptive females had significantly higher post-stimulus fepsp amplitude compared to naïve and fully adaptive.

Sexual dimorphic mechanisms on synaptic encoding processes could explain these differences; although the same actin regulation mechanisms for hippocampal LTP and memory have been documented in both sexes, the engagement of modulatory receptors (TrkB) and synaptic signaling intermediaries (Src, ERK1/2) requires neuron-derived estrogen and signaling through membrane-associated estrogen receptor (Wang *et al.*, 2018; Lu *et al.*, 2019; Gall, Le and Lynch, 2021; Uhl, Schmeisser and Schumann, 2022). Sex differences in NMDA subunit expression might play a role, since LTP in upper-layer synapses depends on NMDA receptor activation (Konstantoudaki *et al.*, 2016); for instance,GluN2A,GluN2B and NR2A,NR2B subunits are implicated in LTP (Sickmann *et al.*, 2014; Eyo *et al.*, 2018; Konstantoudaki *et al.*, 2018). If females have altered expression of either of these subunits, it might explain why non-adaptive subjects display higher fepsp potentiation. This sex dimorphic expression hypothesis has yet to be supported (Sickmann *et al.*, 2014), whereas the NR2A subunit was shown to increase in adult mice compared to adolescents, correlating with the enhanced LTP and faster DAT learning curve of the first group (Konstantoudaki *et al.*, 2018), but has yet to be studied in males vs females. No statistical differences were found in the perseveration index (average value of successive mistakes; Table 1)or the learning rate of DAT between the two groups of the current study. The inclusion of more animals in future experiments, however, is highly likely to interpret the behavioral and electrophysiological findings.

Future studies concomitantly assessing the DAT performance of both sexes are bound to rule out any differences in experimental procedure. It is also critical to broaden the evaluation of after-DAT LTP induction in the female PFC, a key-area implicated in WM and DAT (Constantinidis and Procyk, 2004; Spellman *et al.*, 2015; Tamura *et al.*, 2017; Boku *et al.*, 2018).

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Research proposal

Title: Exploration of the sexual differences in the neural circuits involved in the temporal order object recognition task in mice.

Project summary

Talk about the content, the research questions and the methods to use

Project description

Specific aims

Aim 1: Identification of the neuronal circuits supporting the temporal order recognition task (TOR) performance of male and female C57/BL6 mice.

Aim 2: Characterization of sexual differences in the strongly reciprocal connections of these neural circuits, such as PFC-thalamus and HPC–mPFC–PRH circuit.

Aim 3: Evaluate the effect of acute stress on the identified neuronal connections.

Introduction and significance

There are significant sex differences in the prevalence, type, and severity of symptoms in neuropsychiatric disorders. Females are more vulnerable to emotional disorders such as anxiety and depression (Kessler *et al.*, 2012), whereas males are more vulnerable to cognitive disruption-based disorders such as schizophrenia (Mendrek and Mancini-Marïe, 2016). Stress is a major precipitating factor in many of these psychiatric disorders (McEwen *et al.*, 2015), and its effects differ between men and women (Bangasser, Eck and Ordoñes Sanchez, 2019). Among the numerous brain regions involved in cognition and emotion, the prefrontal cortex (PFC) is a region that plays an important role in complex cognitive processes, particularly those involving executive functions, and is highly sensitive to the negative effects of stress (Musazzi *et al.*, 2019). Particularly, acute stress has been shown to have differential effects on PFC function, as both improvements (Yuen *et al.*, 2012; Musazzi *et al.*, 2019) and impairments in PFC function (Uribe-Mariño *et al.*, 2016) have been reported. The above-reported evidence have been conducted in models mainly developed and validated for male animals (Kokras and Dalla, 2014), while only a couple of studies in adolescent female rats showed that females respond different than males (Wei *et al.*, 2014b; Yuen, Wei and Yan, 2016). Published data from our lab demonstrate clearly that only male, acute-restrained mice had a significantly lower discrimination index in the temporal order object recognition task, implying that acute restrain stress only impairs recency memory in males. However, the molecular or neuronal substrate of the protective mechanisms supporting against the negative effects of acute stress in PFC-dependent cognitive procedures in females remain unexplored.

The link between stressor exposure and disorders that manifest differently in men and women has prompted researchers to look into sex differences in stress responses. Preclinical model data reveal sex differences in circuits, cells, and

molecules, which can lead to differences in how females and males respond to stress. For example, sexually dimorphic effects of freezing and darting have been reported in male and female rats, with darting occurring more frequently in females (Bangasser *et al.*, 2018). Learning is another factor generally influenced by stress; exposure to an acute stressor, for example, improves the acquisition of classical eye blink conditioning in male rats (Wood and Shors, 1998; Shors, 2001), while it impairs learning on this task in female rats (Wood and Shors, 1998). Thus, learning patterns differ between sexes and are, in fact, opposite after stressor exposure.

As for the brain areas implicated in the temporal order memory, they have been well delineated in male subjects (both animals and humans) in the past years. It has been proposed that regions such as the PFC and HPC play an important role in mediating various temporal memory parameters (Chao *et al.*, 2020), whereas the synaptic organization of the underlying circuits, or the dynamics that support ongoing communication between these areas and others have only recently started to be elucidated. A couple of studies have defined the cellular and synaptic properties of reciprocal circuits between the PFC and the thalamus. Specifically, L5 and L6 cortico-thalamic neurons mediate cortico-thalamic pathways by sending branching projections to mediodorsal and ventromedial thalamus. Similarly, thalamo-cortical pathways are divided into two projections, with MD strongly activating L2/3 CC neurons in the PFC and VM influencing multiple layers (Warburton and Brown, 2015; Collins *et al.*, 2018). Other studies have illustrated the role of the neuronal circuit between the perirhinal cortex and medial PFC on discriminating between familiar objects (G. R.I. Barker and Warburton, 2011; Warburton and Brown, 2015) and the contribution of hippocampal CA1 direct projections to the mPFC (Warburton and Brown, 2015). An innovative study of our group has recently highlighted the sex differences in the PFC c-Fos activation as a result of acute stress, which induced significantly higher c-Fos expression in stressed female mice compared to control groups, but not in stressed male mice (Velli *et al.*, 2021).

Based on the above-mentioned data, a primary goal of this proposal is to identify the neuronal circuits underlying the temporal order recognition task performance of male and female mice and characterize any sexual differences in the strongly reciprocal connections of these neuronal circuits, such as in the PFC-thalamus and HPC–mPFC–PRH circuit, as well as ultimately evaluating the effect of acute stress on the identified neuronal connections. This study is expected to significantly improve our understanding of the functional organization of the female brain and shed light on the mechanisms underlying female responses to stress factors, which could pave the way for female rodents to be included in preclinical pharmacological studies for neuropsychiatric disorders.

Research strategy

To in depth characterize the neuronal circuits underlying the TOR task and their potential sexual dimorphisms, we will apply an experimental strategy that will be organized as following:

1. Behavioral assessment of male and female mice in the TOR task after stereotaxic injection with a retrograde/anterograde tracer in the prefrontal, thalamic and hippocampal areas, as compared to sham-treated mice.

2. Simple optical microscope monitoring of the neuronal tracer injection point to confirm the correct brain coordinates and tracer dose.

3. Confocal microscope analysis of neuronal circuit activation in mice of both sexes after the TOR performance.

3. Induction of acute restraint stress in a different cohort of sterotaxic-injected and sham-treated male and female mice and repetition of the same protocol.

The entire project will last 24 months and will be carried out as meticulously described in the methodological sequence below:

First, all experiments will be carried out in C57/BL6 mice bred in our animal facility, a widely used inbred strain that is commonly used as a general-purpose strain, and all the behavioural experiments will take place in the same "dark" period during the day, between 9 a.m. and 13 p.m. The male and female adult subjects (>3 months-old) are going to be stereotaxically injected with the cholera toxin subunit B (CTB) (*Cholera Toxin Subunit B (Recombinant), Alexa Fluor™ 488 Conjugate*) into the mPFC, MD and VM thalamus, as well as in additional thalamic nuclei, and CA1 region of the hippocampus. It is crucial to use an experienced surgeon in this initial part of the project, in order to avoid animal fatalities and unwanted post-surgery effects on the subject's behavior.

Since its initial use as an axonal tracer, the subunit B of Vibrio cholerae toxin has primarily been used for retrograde labeling of neurons in the peripheral and central nervous systems, especially in the labeling of the highly bi-directional pathways between the cerebral cortex and the thalamus. Since it is also anterogradely transported with an extensive filling of axons and axon terminals, CTB allows for the identification of both afferents and efferents of the group of cells studied (Angelucci, Clascá and Sur, 1996). The previous discovery of a significant difference in anterograde and retrograde staining makes it useful for studying highly reciprocal neural connections other than those between the cerebral cortex and the thalamus, such as those between the PRH and mPFC, and CA1 and mPFC (Luppi, Fort and Jouvet, 1990).

In an initial trial, a couple of male and female brains will be cut into thin slices (40µm) using a vibratome (VT1000S, Leica Microsystems, Wetzlar, Germany) and slices containing the PFC, thalamus and hippocampus, as defined by the mouse brain atlas (Paxinos and Franklin, 2001) will be observed under the optical microscope, so as to locate the exact injection point of the CTB fluorescent tracer and readjust the bregma coordinates used in future stereotaxic surgeries, if needed. Preliminary data from our lab indicate that the injection of 0.5µl in 1:10 dilution is the ideal dose for the observation of neuronal circuits. After the correct brain injection coordinates

and fluorescent tracer are confirmed, we will proceed to the main experimental tasks.

Because the primary goal is to identify the neuronal circuits supporting male and female mice's temporal order recognition task (TOR) performance, the cholera-injected and sham-operated mice (which will undergo stereotaxic surgery without the CTB injection) will perform the temporal order memory task one week after the CTB injection. This time period has been shown to be sufficient for the animals to recover and be handled by the experimenter prior to the main task, while the fluorescent CTB tracer will not have lost its effectiveness (Bohland *et al.*, 2009; McSweeney and Mao, 2015). The TOR task is used to assess the recency memory of mice and will be organized as follows: three days prior to the main experimental day, the animals will be habituated in the open-field box for 15 minutes per day to eliminate any anxiety-related factors caused by the novel apparatus that could impede the main experimental procedure. On the main experimental day, the mice will be subjected to two sample trials and one test trial with an intertribal interval of 25 minutes; in each sample phase, mice will explore two identical, non-familiar objects, which will differ in each phase but will be located in specific and identical positions in the open-field arena. In the third phase (test) an object from the first phase (old familiar) and an object from the second phase (recent familiar) will be placed in the arena, in the same positions as in the previous phases.

A c-FOS immunofluorescence protocol frequently used in our lab will be applied to enable the microscopic observation of the neuronal circuits activated shortly after the TOR task (at 70-90 minutes). The immediate-early gene (IEG) c-Fos has been validated as a neuronal activation marker and, as a result, as a valuable tool for functional mapping of stress neurocircuitry (Kovács *et al.*, 2018). Mice will be killed and perfused transcardially with 4% paraformaldehyde, which is ideal for the preservation of most neural antigens. Post-fixed brains will then be cut into 40 μm coronal slices containing the areas of interest to conduct immunofluorescence, through which c-fos-expressing neurons will be labeled with the use of appropriate πριμαρυ mouse monoclonal c-fos antibody and secondary Goat Anti-Mouse IgG. The subsequent inspection of the slices under the confocal microscope is expected to provide the first significant indication of any sexual dimorphisms in the neural areas activated after TOR (c-fos labeled cells), as well as of the neuronal networks that support these regions (CTB- labeled neuronal somas and axons). The obtained images will then be analyzed with appropriate programs, namely Photoshop CS5 or ImageJ, so as to measure the density of c-fos and CTB labeled cells. One way ANOVA and post hoc analyses performed in Graphpad Prism 8 will detect any statistically significant sexual differences in the count of positive cells/area.

In the last part of the experimental procedure, male and female adult mice will be exposed to an acute stressor factor, namely acute restraint stress, as described in Velli et al. In further detail, mice will be placed in plastic cylindrical transparent restrainer tubes (12.3 cm). The tubes will have an airhole for ventilation and there

will be no room for mice to move. Each mouse will be placed in a single restrainer tube and left in a separate room from the no-restraint group for 2 hours, while the control group (NR) of male and female mice will be left in their home cage. The results of this research showcase the impaired performance of restraint (RS) male mice compared to RS females and the accompanying reduction in RS-induced c-Fos expression in the PFC (Velli *et al.*, 2021).

The final aim of this proposal is to investigate the effects of RS on additional brain areas such as the thalamus and the hippocampus, as well as to thoroughly characterize the stress implications on the neuronal circuits that support TOR performance. This experimental approach is expected to contribute significantly to our understanding of female neuronal network organization and to enrich our general knowledge on female brain and neurocircuitry anatomy, while the findings of this study could serve as a solid foundation for future research on stress-implicated neuropsychiatric disorders that remain untreated in female patients.

Budget

BUDGET		
Category		**Total in €**
Direct Costs Personnel		
Post-Doc Researcher(s)		**30000**
PhD Candidate(s)		**20000**
	Total Direct costs for Personnel	**50000**
Other Direct Costs	**Justification**	
6.1.2 Consumables	-Primary and secondary antibodies -other reagents for immunofluorescence protocol -cholera toxin subunit B -General chemicals -Plastics	**80000**
6.1.3 Travel	Registration and travel to at least 2 international scientific conferences	**5000**
6.1.4 Dissemination	Publication to scientific journal	**4000**
6.1.5 Use and/or Access to equipment etc.	Access to Confocal microscope facility of IMBB	**3000**
6.1.6 Equipment	-Behavioral apparatus in animal facility -Mouse stereotaxic frame (Cartesian Research) -Peristaltic pump for animal's perfusion -Vibratome -Optical and confocal microscope	**25000**
6.1.7 Other Costs	-	
6.1.8 Purchase of animals	Animal purchase form Jackson Laboratory	**3000**
	Total "other direct costs"	**120000**
	Total Direct Costs	**170000**
	Indirect Costs (Institution overhead, 10%)	**30000**
	Total Budget	**200000**

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Short CV

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Education:

- **Bachelor's Degree in Biology**
  National and Kapodistrian University of Athens, Greece
  Graduation degree: 8.73/10

  Bachelor's Thesis: "Acute stress effects on the structure and activation of limbic system's neural cells of male and female adult mice". Supervisor: Professor Sidiropoulou Kiki

  Experimental procedures:

  Animal handling, behavioral experiments (open-field anxiety test, Light-Dark test, temporal order recognition task), Golgi-staining, IF (Immuno-fluorescence).

- **Master's Degree in Neurosciences**
  National and Kapodistrian University of Athens, Greece
  Athens' International Master in Neurosciences

  Master's Thesis: "Sexual dimorphic performance in the temporal order recognition task and enhanced synaptic plasticity in female C57/BL6 mice after training in the delayed alternation task", Department of Biology, Unicersity of Crete, Neurophysiology and Behavior Laboratory
  Supervisors: Drs. Sidiropoulou Kiki and Stamatakis Antonis

  Experimental Procedures:
  Animal handling, behavioral experiments (open-field locomotion assessment, temporal order memory task, T-maze delayed alternation task), stereotaxic surgery, electrophysiological recordings.

- **English**: C1 level of proficiency, The University of Michigan
- **French**: C1 level, Sorbonne 1st degree, University of Sorbonne

Job Experience:

Employee at the molecular department of the diagnostic lab "A TO Z MEDICAL SOLUTIONS AIE"

Experimental procedures:
Handling Covid-19 samples for PCR extraction/Electrophoresis

Publications:

- **"Effects of working memory training on the synaptic properties of the prefrontal cortex and hippocampus: a study in female adult mice"**
  Lida Evmorfia Vagiaki, Vasiliki Stavroulaki, Maria Peteinareli, **Theodora Asimi**, Kyriaki Sidiropoulou
  Virtual Poster presentation in the *29th meeting of the Hellenic Society for Neuroscience.*

- Velli A, Iordanidou C, **Asimi T**, Vynichaki MI, Cholevas A, Mantouka AI, Nassens L, Chalkiadaki K, Sidiropoulou K. **Sexual dimorphic effects of restraint stress on prefrontal cortical function are mediated by glucocorticoid receptor activation.**
  Eur J Neurosci. 2021 Mar 24. doi: 10.1111/ejn.15203. Epub ahead of print. PMID: 33759255.

    - 08/10-10/10/2021: Virtual Poster presentation in the *29th meeting of the Hellenic Society for Neuroscience*,
    - Poster presentation in the *49th meeting of the European Brain and Behavior Society (EBBS),* Lausanne, Switzerland,2021