Mild maternal separation in mice of both sexes: impact in adulthood on vigor to approach or to escape motivational stimuli and interaction with dopamine depletion

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ABSTRACT

Early-life stress affects brain development and can lead to psychiatric disorders such as depression later in life. Little is known about the effect of early-life stress on motivational processes such as effort-based decision-making, which could be impaired in people with depression. Mesolimbic dopamine (DA) regulates behavioral activation and effort in motivated behaviors, and stress produces opposite effects on DA release depending if it is acute or chronic. Maternal Separation (MS) could act as an early-life stressor depending on its duration and intensity. Using CD1 male and female mice, we evaluate the impact of early but mild MS (PND3-5, 90 min), on selection of effortful responses in adulthood under positive or aversive conditions. In a three-choice-T-maze, mice preference for active reinforcers such as a running wheel (RW) versus sedentary ones was evaluated, and in a forced swim task (FST), time dedicated to escape or passively floating was measured. In addition, we studied if MS interacts with DA depletion in adulthood, administering tetrabenazine (TBZ), a VMAT-2 inhibitor that induces fatigue and anergia. Males and females do not differ; they spend more time in the RW and less eating or sniffing a neutral odor in the T-maze, independently of separation. However, separated mice of both sexes spent more time in the RW, and climbing in the FST compared to non-separated mice. Only among males, TBZ reduced time in RW, increased time eating, reduced climbing and increased immobility. Females were not affected by DA depletion. Anxiety was evaluated in a dark-light box, and separated males where less anxious than non-separated, but TBZ did not affect either sex.

Keywords: Early-life stress, maternal separation, dopamine, behavioral activation, sex differences.

INTRODUCTION

Stress is a physical, physiological and social environmental change experienced by an organism (Habib et al., 2001). The hypothalamic-pituitary-adrenal (HPA) axis mediates the neuroendocrine stress response (Smith and Vale, 2006; Herman et al., 2016), including central changes in the concentration of neurotransmitters such as dopamine, noradrenaline or serotonin (Cabib and Puglisi-Allegra, 1996; Jacobs, 1994; Moret and Briley, 2011).

Early life stress produces short- and long-term effects in brain development that can lead to psychiatric disorders such as depression or anxiety later in life (Lupien et al., 2009; Dallé and Mabandla, 2018). In order to study early life stress in the laboratory is common to use animal models of maternal separation (MS) in which the pups suffer the stress of been separated from the mother for a period of time (Lupien et al., 2009; Dallé and Mabandla, 2018). Duration of separation in a single session, and repetition of separation across consecutive days are important factors that can lead to different effects on the behavioral outcome (Tractenberg et al., 2016). Some studies show that repeated MS decreases the monoaminergic response, producing similar effects as major depression (Amos-Kroohs el al., 2016; Ohta et al., 2014), while a single exposition to MS increased the response on that system (Llidó et al., 2016). Thus, short-term MS is used to evaluate the protective response to stressors and long-term separation evaluate the environmental factors affecting normal neurobiological development (Roman and Nylande, 2005). Another important aspect to take into account is the moment of separation: the two first weeks of live (PND 1 to 14) are considered a period of hipoactivity of the HPA axis, while the next two weeks (PND 14 to 21) are considered a period of hyperactivity (Tractenberg et al., 2016).

Studies have shown that early life stress could affect synaptic plasticity and brain development, decreasing levels of neural cell adhesion molecules (NCAM) in different areas, such as substantia nigra or ventral tegmental area (Chocyk et al., 2010), areas that are the main source of dopamine in the central nervous system. In addition, it has been demonstrated that early life stress can disturbe the mesolimbic and mesocortical dopaminergic systems, producing a decrease in dopamine synthesis (Thierry et al., 1968; Lupien et al., 2009, Hemmerle et al., 2012).

The mesolimbic dopaminergic system, specifically the nucleus accumbens (Nacb), plays an important role in the activational component of motivated behavior, in energy expenditure and in effort-based decision making (Salamone and Correa, 2002, 2012). Motivated behavior is directed towards positive stimuli or away from aversive stimuli, but in both cases is also

characterized by a high degree of activity (Salamone and Correa 2002, 2012). Moreover, the activational component of motivation is involved in the cost/benefit analyses in which the effort, vigor, persistence or endurance is required to get the reinforcer and the value of that stimulus is relative to others that require less behavioral activation (Salamone and Correa, 2002, 2012; Salamone et al. 2007). Activation-or effort-related dysfunctions, such as anergia or fatigue are common and debilitating set of symptoms present in many psychopathologies like major depression (Caligiuri and Ellwanger, 2000; Demyttenaere et al., 2005; Kluger, 2017; Salamone and Correa, 2012; Tellez et al., 2005; Tylee et al., 1999).

Stress has been found to have a potential effect on cost-benefit (effortful) decision-making. Some studies show that restrain stress impairs effort in an operant lever-pressing task (Bryce and Floresco, 2016; Shafei et al., 2012). Stress in adult life can modulate mesolimbic dopamine levels in different ways, for instance depending on whether it is acute or chronic and if the animal can escape from it or not (Cabib and Puglisi-Allegra, 1996). Acute stress using a mild foodshock or brief restraint experiences increases dopamine release in Nacb (Roth et al., 1988; Imperato et al., 1989, 1991, 1993). However, chronic stress decreases dopamine and its metabolites, dihydroxyphenylacetic (DOPAC) and homovanillic acid (HVA) on Nacb (Imperato et al., 1993). In humans, a study using positron emission tomography (PET) revealed that long-term exposure to psychosocial adversity was associated with dampened striatal dopaminergic function (Bloomfield et al., 2019).

The incidence of depression in women is higher than in men (Kesskerm 2003; Hankin et al., 2007, 2009; Parker and Brotchie, 2010; Silverstein et al., 2012). Also, the typology of the symptoms differs due to gender. Somatic symptoms like fatigue are more common in women than in men (Bjornelv et al., 2011; Dekker et al., 2007), while the difficulty of men are related to social relationships (Poutanen et al., 2009; Breslin et al., 2009). These differences in the type of symptoms start at adolescence and their prevalence increases with age (Silverstein et al., 2002). Furthermore, experiments in which animals were evaluated in the forced swim test (FST), a paradigm useful to evaluate behavioral activation in response to aversive stimuli, showed that females were more active than males, increasing the time climbing and decreasing immobility time (Alonso et al., 1991; Barros and Ferigolo, 1998; Brummelte et al., 2006; Simpson et al., 2012; Fuentes et al., 2014).

In order to assess behavioral activation in a context in which all stimuli are positive our laboratory and others have developed different behavioral procedures in rodents based on the

preference for different reinforcers, including tasks that give animals the option of vigorously working (lever pressing or climbing a barrier) to obtain access to more highly valued reinforcers vs. approaching and consuming a less preferred reinforcer (Cousins et al., 1994; Salamone and Correa, 2002; Salamone et al., 2016; Mott et al., 2016; Mai et al., 2012; Pardo et al., 2012; 2015; Randall et al., 2012; Sommer et al., 2014; Yohn et al., 2015a, 2016b; Correa et al., 2016; López-Cruz et al., 2018; SanMiguel et al., 2018). In these tasks, conditions that alter dopamine transmission, such as administration of dopamine antagonists or dopamine depleting agents such as tetrabenazine (TBZ), can alter behavioral activation and reduce selection of high-effort choices in rats and mice (Nunes et al., 2013; Randall et al., 2014; Hosking et al., 2015; Pardo et al., 2012; 2015; Yohn et al., 2015a, 2016b; Rotolo et al., 2019; Correa et al., 2016; 2018; 2020; López-Cruz et al., 2018; Carratalá-Ros et al., 2020). TBZ acts inhibiting the vesicular monoamine transporter-type 2 (VMAT-2), which leads to a blockade of vesicular storage and a depletion of monoamines, with its greatest effects al low doses being on striatal dopamine in rats and mice (Pettibone et al., 1984; Nunes et al., 2013; López-Cruz et al., 2018). In humans, TBZ is used to treat Huntington's disease, but major side effects include depressive symptoms, such as fatigue and depression (Frank, 2009; Guay, 2010; Chen et al., 2012). In fact, TBZ administered chronically in a mouse model of Huntington's disease (Wang elt al., 2010) improved motor defcits but increased depression-like measures in the FST.

Recently, a choice-T-maze task has been developed in different versions (Correa et al., 2016; 2020; López-Cruz et al., 2018; Carratalà-Ros et al., 2020). This is in general a behavioral paradigm in which animals have to choose freely between different reinforcers that require different behavioral activation (run in a running wheel (RW), eat sucrose pellets or sniffing a neutral odor). In this paradigm, TBZ produces a shift in the relative preferences, decreasing the time animals spend running in a RW but increasing the time spend consuming sucrose pellets, indicating reduced activation but relatively intact sucrose reinforcement (López-Cruz et al., 2018; Carratalà-Ros et al., 2020).

Taking all this information into account, the aim of the present group of studies is to assess the effect of early life stress using early mild-MS in female and male mice in an effort-based decision making paradigm (Three-choice-T-maze Task) established to study relative preference for active reinforcers versus more sedentary ones. Moreover, we study the potential interaction of MS early in life with the effect on adult life of TBZ, to see if separated animals were more vulnerable to the anergia inducing effects of dopamine depletion in this behavioral paradigm. In addition, we also assessed these manipulations in the FST in which behavioral activation is induced by stressful conditions. The effect of TBZ on anxiety as measured in the

dark and light (DL) box was also assessed in order to identify anxiolytic or anxiogenic-like actions that could be affecting the results in the T-maze and the FST of these manipulations.

METHODS

Animals.

Pregnant CD1 female mice were obtained from Janvier, France S.A. and pups were born 19 weeks after arrival in the animal colony at Universitat Jaume I. The day of birth was considered as postnatal day 0 (PND 0). Pups in the experimental group were separated from their mothers for 90 minutes daily from PND3 to PND5. While the dam was removed to an individually cage, pups were grouped in an incubator to maintain body temperature and at the end of the separation period pups were returned to the home cage with the dams. Control animals were left undisturbed with their dams. At PND21 animals were housed in groups of three or four per cages, with standard laboratory rodent chow and tap water available *ad libitum*. The colony was kept at a temperature of 22 ± 2ºC with lights on from 08:00 to 20:00 h.

The training period in the T-maze started al PND45, and baseline was recorded on PND60. The pharmacological tests in the T-maze started on PND64. The FST was performed on PND49 and the DL on PND56 (Fig. 1).

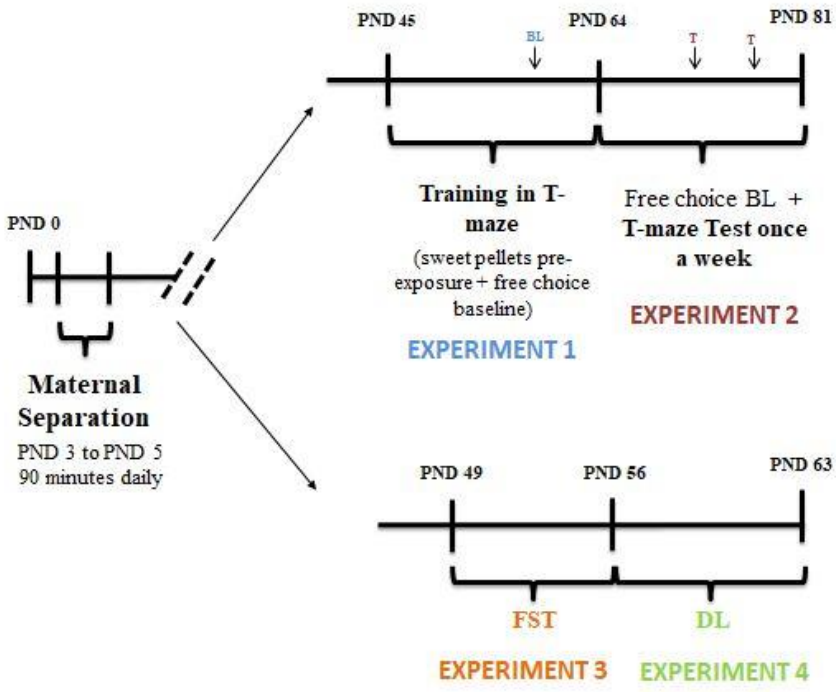

Fig. 1: Timeline for maternal separation and for the behavioral experiments.

All animals were under a protocol approved by the Institutional Animal Care and Use committee from Universitat Jaume I. All experimental procedures complied with directive 2010/63/EU of the European Parliament and of the Council, and with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research", National Research Council 2003, USA. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Pharmacological Agents

Tetrabenazine (TBZ, Cymit Quimica SL, Spain) was dissolved in a vehicle solution of 0.9% saline (80%) plus dimethyl sulfoxide (DMSO 20%, final pH 5.5) and administered 2 hours before testing. The dose of 8 mg/kg of TBZ was selected based on previous studies demonstrating that, in mice, this dose is the most efficient at depleting dopamine in ventral striatum and at inducing effects in these paradigms (López-Cruz et al., 2018; Carratalá-Ros et al., 2020). Vehicle and TBZ were administered intraperitoneally (IP).

Apparatus and testing procedures.

T-maze RW-Sucrose-Odor Choice Task. The T-maze apparatus consisted of a central area that leads to three arms (Fig. 2). In one of them, sucrose pellets (TestDietTM, 50% sucrose, 45mg each) were available; in another arm, there was a RW; and in the third arm there was a hole with a cotton ball socked with a fruit odor (based on López-Cruz et al 2018, and Carratalà-Ros et al 2020).

Each session started introducing the animal in the center of the maze, always facing the longest wall of the "T". Mice were allowed to freely explore and interact with the stimulus during 15-minutes sessions, once a day, five days a week. Animals were trained during two weeks and at the end of the second week, one session of baseline (BL) was recorded. One week after BL, test sessions took place once a week in two consecutive weeks in which all mice received the dose of TBZ or vehicle in a random varied order. Sessions were videotaped, and a trained observer blind to the experimental conditions evaluated the number of crossings into each compartment, the time spend in each compartment, and the time interacting with each

stimulus (running in the RW, consuming the sucrose pellets, or sniffing the hole). Time interacting with the stimulus was selected as the main dependent measure because it allowed for the evaluation of the three stimuli with the same units. Time allocation is a useful measure of preference, relative reinforcement value, and response choice (Baum and Rachelin, 1969).

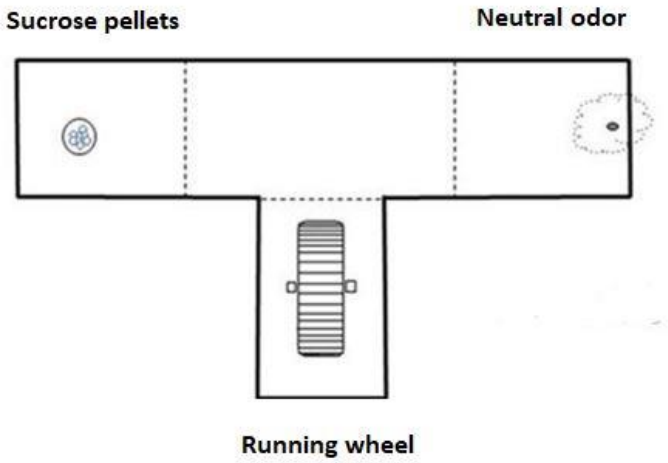

Fig. 1: Schematic representation of the three-choice-T-maze Task.

Forced Swim Test (FST). The apparatus consists in a transparent cylindrical glass tank (26 cm high and 18 cm in diameter) filled with water (14 cm) and maintained at a temperature of 25ºC. Water was changed between animals. During de 6-min test, mice were videotaped from the side, and struggling/climbing, immobility, and swimming were later measured by an observer unaware of the experimental condition. Immobility was defined as a period when the animal remained motionless, making only minor movements. Climbing is defined as any energetic and vertical movement of all four limbs against the wall of the tank. Swimming was recorded when animals carried out horizontal movements with their forepaws leading to the displacement of the body throughout the swim chamber (Armario et al., 1988). After the test, mice were dried with a soft towel, put back in the box with absorbing paper under a warming light, and monitored for 10 minutes. This paradigm is considered to be a model of behavioural despair and is used as a test for assessing depressive-like behaviors (Porsolt et al., 1977).

Dark and light (DL) box. The DL apparatus consists of a polypropylene box divided into two compartments separated by a small opening. The light compartment is open, painted in white, and illuminated with a bright light. The dark compartment is black and has a removable roof to

close it. This behavioral paradigm is based on the conflict between the natural tendencies of mice to explore novel environments and the avoidance of open and illuminated areas (Blumstein and Crawley, 1983). To start the test session, animals were individually placed in the dark compartment and videotaped for 5 minutes. Total of crosses between compartments as an index of locomotion and total time spend in the lit chamber as an index of anxiety, were recorded.

Statistical analyses

Normally distributed data for the BL preferences in the T-maze experiment (experiment 1) employed a between-groups design for sex and for separation condition, and data were analysed by a two-way factorial ANOVA. In addition, a two-way factorial ANOVA with a between factor (separation condition) and a within factor (TBZ treatment) was used in experiment 2. Normally distributed data separated by sex in the FST (experiment 3) and DL test (experiment 4) were analysed by a two-way factorial ANOVA with a between factor (separation condition) and a within factor (TBZ treatment). Sidak test was used for *post hoc* comparisons ($\alpha = 0.05$). All data were expressed as mean ± SEM, and significance was set at $p<0.05$. Prism 8 software was used.

Experiments

Experiment 1. Differences in spontaneous preferences in male and female mice in the Three-Choice-T-maze Task: Impact of mild maternal separation. Control and separated male and female mice (N=48; 24 male and 24 females) were trained during two weeks in the T-maze and BL was recorded on PND 60, during the third week of training (18 days after training started).

Experiment 2. Effect of dopamine depletion on preference for active reinforces in male and female mice: Impact of mild maternal separation. One week (7 days) after the BL session, control and separated male and female mice received TBZ (vehicle and 8 mg/kg) 120 minutes before the test began, and they were placed in the T-maze for 15 minutes. Animals received either vehicle or TBZ (in a randomly varied order) once a week in consecutive weeks. These animals were the same as in experiment 1.

Experiment 3. Effect of dopamine depletion on scaping behavior assessed in the FST in male and female mice: Impact of mild maternal separation. Control and separated male (n=37) and female mice (n=37) received TBZ (vehicle or 8 mg/kg), their behavioral output was measured in

the FST during 6 minutes. Mice received one of the doses, and were exposed only once to the paradigm since behavioral habituation develops in one session.

Experiment 4. Effect of dopamine depletion on anxiety parameters as measured in the DL paradigm in male and female mice: Impact of mild maternal separation. Control and separated male (n=40) and female mice (n=37) received TBZ (vehicle or 8 mg/kg) and after 120 minutes were placed in the DL box for 5 minutes. Mice were exposed only once to the paradigm since behavioral habituation develops in one session. These animals were the same as in experiment 3.

RESULTS

Experiment 1. Differences in spontaneous preferences in male and female mice in the Three-Choice-T-maze Task: Impact of mild maternal separation.

To assess the effects of mild maternal separation and sex in spontaneous preferences in the Three-Choice-T-maze Task, independent two-way ANOVAs were used. The paradigm had three different reinforcers: food, RW, and neutral odor. For each reinforcer we evaluated 3 different dependent variables:

1.1. Time spend in interaction with each reinforcer is the variable that offers a more direct evaluation of stimulus preference. There was a significant main effect of sex ($F(1, 44)=9.79$, $p=0.003$), and a significant interaction ($F(1, 44)=10.89$, $p=0.001$), but no main effect of maternal separation ($F(1, 44)=1.45$, $p=0.230$) on time spend eating. Sidak's multiple comparisons revealed that separated female eat more than separated males ($p=0.001$), and also more than control females ($p=0.015$) (Fig. 3A). The two-way ANOVA for time spent running in the RW showed that there was a significant effect of separation ($F (1, 44)=6.33$, $p=0.016$), but no main effect of sex ($F(1,44)=0.10$, $p=0.749$), and no interaction ($F(1,44)=0.05$, $p=0.824$) were found (Fig. 3B). The two-way factorial ANOVA revealed no significant effect of separation ($F(1,44)=0.68$, $p=0.414$), and sex ($F (1,44)=1.20$, $p=0.278$), but a significant interaction ($F (1,44)=4.23$, $p=0.045$) on time sniffing the neutral odor. However, Sidak's multiple comparisons revealed no differences between groups (Fig. 3C). Thus, both separated male and female spend more time running in the RW and separated females eat more than the other animals.

3-CHOICE-T-MAZE TASK: TIME INTERACTING WITH STIMULI

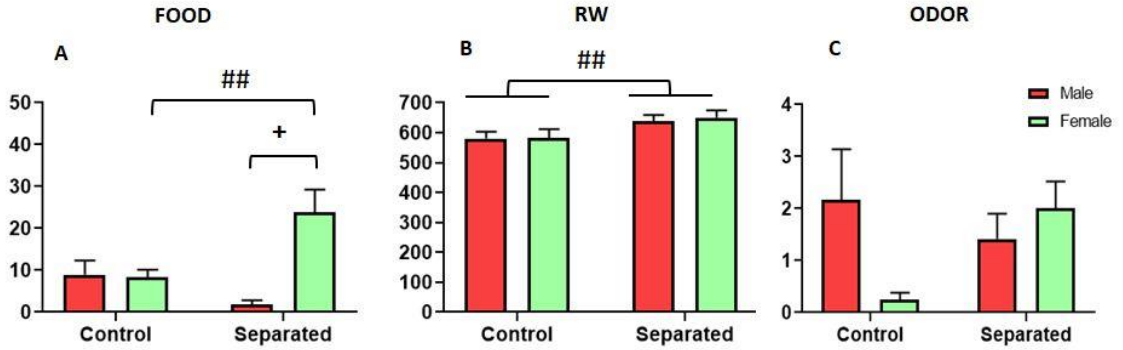

Fig. 3: Effect of separation and sex on time in interaction with each stimulus, A) food, B) running wheel, and C) odor in the T-maze task assessed during 15 min. Data are expressed as mean ± S.E.M. of accumulated seconds. ## p<0.01 significant differences between control and separated mice. + p<0.05 significant differences between male and female separated mice.

1.2. We also assessed time spend in each compartment as a way to evaluate potential place preferences induced by the stimuli. The two-way ANOVA for time in the food compartment revealed a statistically significant main effect of sex ($F(1,44)=15.90$, $p=0.001$), no effect of separation ($F(1,44)=1.06$, $p=0.308$), and no significant interaction ($F(1,44)=0.76$, $p=0.387$) (Fig. 4A). For the time spend in RW compartment, the two-way ANOVA showed a significant main effect of sex ($F(1,44)=12.16$, $p=0.001$), no effect of separation ($F(1,44)=0.20$, $p=0.654$), and no interaction ($F(1,44)=0.11$, $p=0.737$) (Fig. 4B). Finally, the two-way ANOVA for time in odor compartment showed no main effect of sex ($F(1,44)=0.05$, $p=0.826$), of separation ($F(1,44)=0.74$, $p=0.395$), and no significant interaction ($F(1,44)=0.17$, $p=0.679$) (Fig. 4C). Thus, female mice, independently if they were separated or not, spent more time than males in the food compartment, and less time than males in the RW compartment.

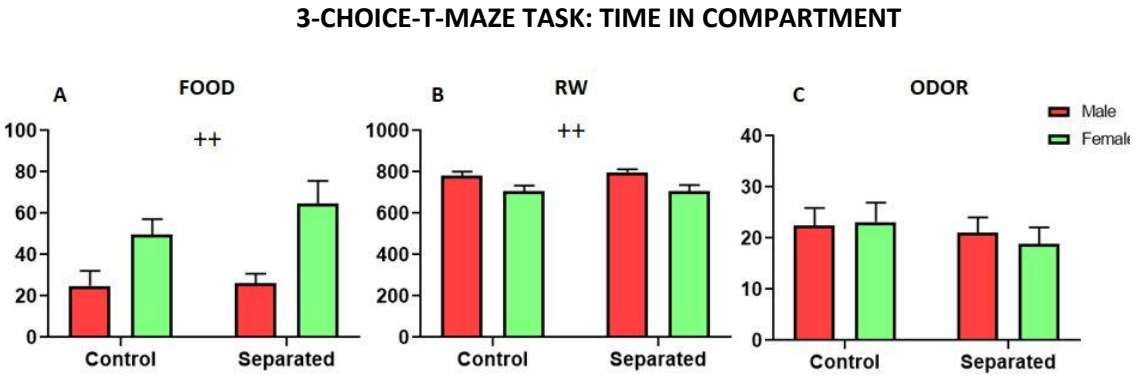

Fig. 4: Effect of separation and sex on time in different compartments, A) food compartment, B) running wheel compartment, and C) odor compartment during a 15-min session. Data are expressed as mean ± S.E.M. of accumulated seconds. ++ p<0.01 significant differences between sexes.

1.3. The last variable evaluated in this experiment was the number of entries in each compartment as a measure of general exploratory ambulation. The two-way ANOVA on number of entries in the food compartment indicated a significant effect of separation ($F(1,44)=17.67$, $p=0.001$), and sex ($F(1,44) = 5.23$, $p=0.027$), but no significant interaction ($F(1,44)=0.76$, $p=0.387$) (Fig. 5A). As for the number of entries in the RW compartment, the ANOVA only revealed a significant effect of sex ($F(1,44)=8.89$, $p=0.004$), but no effect of separation ($F(1,44)=2.47$, $p=0.123$), and no interaction ($F(1,44)=0.03$, $p=0.874$) (Fig. 5B). Finally, the two-way ANOVA show no main effect of sex ($F(1,44)=1.717$, $p=0.196$), or separation ($F(1,44)=0.254$, $p=0.616$), an no interaction ($F(1,44)=0.06$, $p=0.815$) on number of entries into the odor compartment (Fig. 5C). Thus, separated animals entered less into the food compartment than control animals. Females enter more times in the food and in the RW compartment than males, indicating a higher degree of exploration than males.

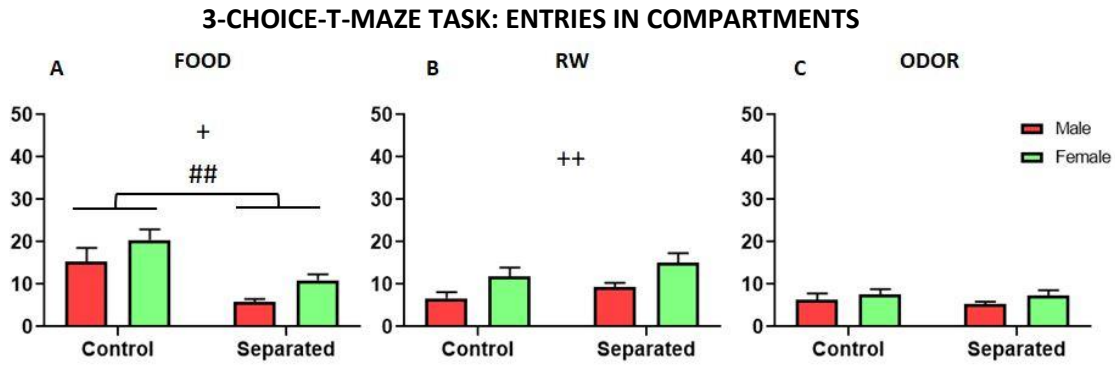

Fig. 5: Effect of separation and sex on the number of entries in A) food compartment, B) running wheel compartment, and C) odor compartment during a 15-min session. Data are expressed as mean ± S.E.M. of number of entries. ## p<0.01 significant differences between control and separated animals. ++p<0.01, + p<0.05 significant differences between sexes.

Experiment 2. Effect of dopamine depletion on preference for active reinforces in male and female mice: Impact of mild maternal separation.

A series of two-way ANOVAs were used to assess the effect of dopamine depletion induced by the administration of TBZ at a dose of 8 mg/kg, and mild maternal separation on preference for active reinforcers. In this experiment data from male and female mice were analysed separately. The same three variables in the T-choice-T-maze Task were evaluated for both sexes.

2.1. For the variable time interacting with the reinforcers in males, two-way ANOVA showed a significant effect of drug treatment ($F(1,21)=5.05$, $p=0.035$), but no significant effect was found for separation ($F(1,21)=3.31$, $p=0.083$), and no interaction ($F(1,21)=0.42$, $p=0.526$) on time eating (Fig. 6A). As for the time running, the two-way ANOVA revealed a significant effect of treatment ($F(1,21)=5.27$, $p=0.032$), and separation ($F(1,21)=10.29$, $p=0.004$), but no significant interaction ($F(1,21)=0.16$, $p=0.689$) (Fig. 6B). For time sniffing the neutral odor, no main effects were found for treatment ($F(1,21)=0.43$, $p=0.519$), or separation ($F(1,21)=3.55$, $p= 0.074$), and no interaction ($F(1,21)=0.04$, $p=0.849$) (Fig. 6C). These data suggest that dopamine depletion decreases time males spend running and increases time they spend eating. In addition, as demonstrated in experiment 1.1., separated males spend more time running than control males.

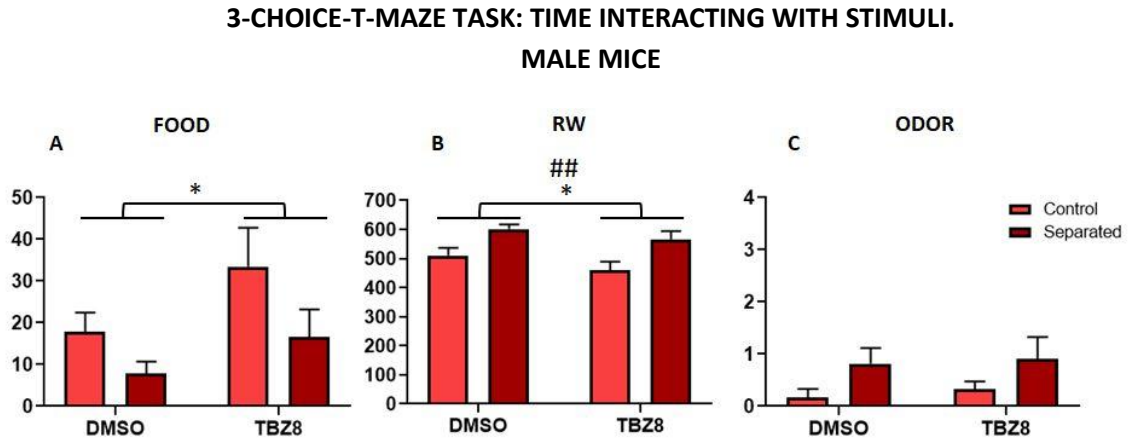

Fig. 6: Effect in males of TBZ (8mg/kg) and separation on time spend A) eating, B) running, and C) sniffing in the T-maze assessed during 15 min. Data are represented as mean ± S.E.M of accumulated seconds. ## p<0.01 significant differences between control and separated mice. * p<0.05 significant treatment differences.

2.2. The two-way ANOVAs for time in each compartment showed no main effect of treatment ($F(1,21)=0.99$, $p=0.331$), separation ($F(1,21)=0.24$, $p=0.629$), and no interaction ($F(1,21)=0.01$, $p=0.919$) on time spend in food compartment (Fig. 7A). For the time spend on RW

compartment there were no significant effects of treatment (F(1,20)=0.48, p=0.496), separation (F(1,20)=0.33, p=0.571), and no interaction (F(1,20)=0.05, p=0.824) (Fig. 7B). Finally, there was no significant effect of treatment (F (1,20) = 2.31, p = 0.146), separation (F (1,20) = 2.43, p = 0.135), and no significant interaction (F(1,20)=0.95, p=0.342) on time spend in odor compartment (Fig. 7C). Thus, neither dopamine depletion nor mild maternal separation affect the time males spend in each compartment. Thus, these two manipulations do not produce increase in preference or avoidance for the space in which the reinforcer is present.

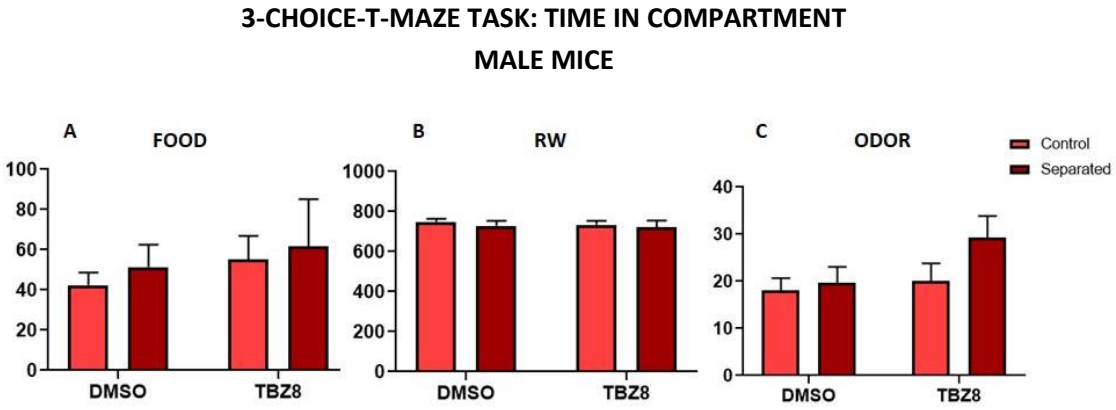

Fig. 7: Effect of TBZ (8 mg/kg) and separation on time in A) food compartment, B) running wheel compartment, and C) odor compartment during a 15-min session in males. Data are expressed as mean ± S.E.M. of accumulated seconds.

2.3. Finally, the number of entries of male mice in each compartment was analysed using two-way ANOVA's, showing no significant effect of treatment (F(1,20)=0.01, p=0.906), separation (F(1,20)=0.01, p=0.906), and no interaction (F(1,20)=0.14, p=0.712) on the number of entries in food compartment (Fig. 8A). The results of number of entries into the RW compartment showed no significant effect of treatment (F(1,20)=0.04, p=0.847), separation (F (1,20)=0.13, p=0.129), and no interaction (F(1,20)=0.15, p=0.70) (Fig. 8B). The same pattern was seen for number of entries into the odor compartment, there was no effect of treatment (F(1,20)=2.23, p=0.151), separation (F(1,20)=0.02, p=0.884), or interaction (F(1,20)=0.24, p=0.631) (Fig. 8C). Thus, dopamine depletion and mild maternal separation do not affect exploration of the T-maze compartments in males.

3-CHOICE-T-MAZE TASK: ENTRIES IN COMPARTMENTS
MALE MICE

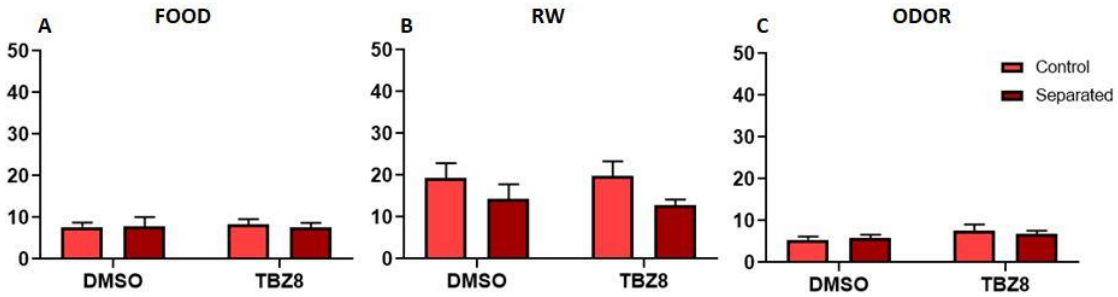

Fig. 8: Effect of TBZ (8 mg/kg) and separation on the T-maze task for the number of entries in A) food, B) running wheel, and C) odor compartments during a 15-min session in males. Data are expressed as mean ± S.E.M. of number of entries.

2.4. The same parameters and analyses were used for females. The two-way ANOVA for time of interaction with each reinforcer in females showed no significant effect of treatment ($F(1,21)=1.84$, $p=0.189$), separation ($F(1,21)=0.16$, $p=0.693$), and no interaction ($F(1,21)=5.52$, $p=0.999$) on time eating (Fig. 9A). For the time running in the RW, the ANOVA revealed a main effect of separation ($F(1,21)=9.12$, $p=0.006$), but non-significant effect of treatment ($F(1,21)=0.34$, $p=0.564$), and no significant interaction ($F(1,21)=3.13$, $p=0.092$) were found (Fig. 9B). Moreover, there was a significant effect of treatment ($F(1,21) = 5.49$, $p = 0.029$), and a significant interaction ($F(1,21)=5.49$, $p=0.029$), although no effect of separation ($F(1,21)=1.37$, $p=0.255$) on time females spend sniffing the neutral odor (Fig. 9C). Sidak's multiple comparisons revealed that separated female spend less time sniffing the neutral odor than control females ($p=0.031$), and TBZ decreased the time control females spend sniffing the neutral odor, but TBZ did not affected separated females ($p=0.005$).

3-CHOICE-T-MAZE TASK: TIME INTERACTING WITH STIMULI
FEMALE MICE

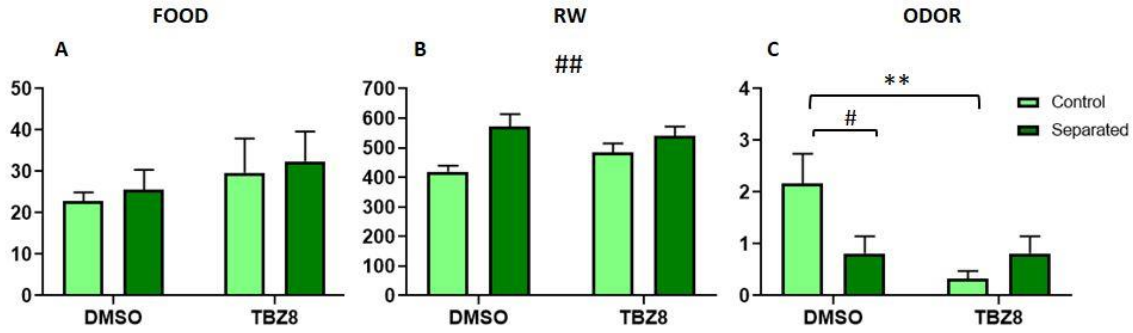

Fig. 9: Effect of TBZ (8 mg/kg) and separation on time spend A) eating, B) running, and C) sniffing during the T-maze task assessed during 15 min in females. Data are expressed as mean ± S.E.M. of accumulated seconds. # p<0.05, ## p<0.01 significant differences between control and separated animals. **p<0.01 significant differences due to the treatment.

2.5. The time females spend in each compartment were evaluated by two-way ANOVAs, and the results showed that there was no effect of treatment ($F(1,21)=1.52$, $p=0.231$), separation ($F(1,21)=0.03$, $p=0.875$), and no interaction ($F(1,21)=0.92$, $p=0.349$) on time females spend in food compartment (Fig. 10A). For the time spend in the RW compartment, the two-way ANOVA revealed no effects of main factors treatment ($F(1,21)=2.32$, $p=0.143$), and separation ($F(1,21)=0.09$, $p=0.766$), and no significant interaction ($F(1,21)=0.75$, $p=0.395$) (Fig. 10B). Also, no main effects were found for treatment ($F(1,21)=3.30$, $p=0.084$), separation ($F(1,21)=1.14$, $p=0.297$), or interaction ($F(1,21)=0.07$, $p=0.795$) on time in odor compartment (Fig. 10C). Thus, there are no differences between control and separated females. Moreover, TBZ did not affect the time females spend in each compartment. As in the case of males, dopamine depletion and mild maternal separation did not produce increase in preference or avoidance to the compartment where the reinforcers are present.

3-CHOICE-T-MAZE TASK: TIME IN COMPARTMENT
FEMALE MICE

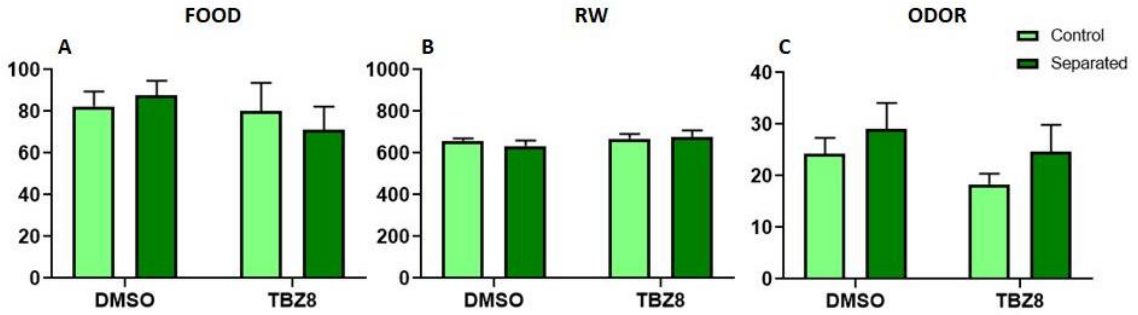

Fig. 20: Effect of TBZ (8 mg/kg) and separation on time spend in each compartment: A) food, B) running wheel, and C) odor in T-maze task during 15 min in females. Data are expressed as mean ± S.E.M. of accumulated seconds.

2.6. The number of entries of females in each compartment were evaluated using a series of two-way ANOVAs, and they revealed that there was a significant effect of treatment ($F(1,21)=8.16$, $p=0.009$), but not of separation ($F(1,21)=0.00$, $p=0.981$), and no significant interaction ($F(1,21)=0.02$, $p=0.883$) on number of entries in food compartment (Fig. 11A). The analyses for the number of entries in RW compartment showed no main effects of treatment ($F(1,21)=2.61$, $p=0.121$), separation ($F(1,21)=0.69$, $p=0.417$), and no interaction ($F(1,21)=0.28$, $p=0.603$) (Fig. 11B). Finally, the ANOVA revealed no significant effects of treatment ($F(1,21)=0.63$, $p=0.437$), separation ($F(1,21)=2.79$, $p=0.109$), and no interaction ($F(1,21)=0.15$, $p=0.706$) on number of entries in odor compartment (Fig. 11C). Thus, TBZ decreased the number of entries in food compartment for both, control and separated females.

3-CHOICE-T-MAZE TASK: ENTRIES IN COMPARTMENTS
FEMALE MICE

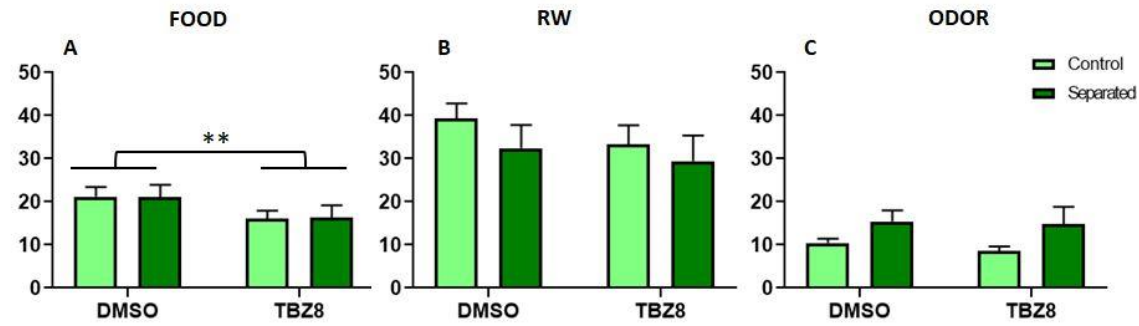

Fig. 11: Effect of TBZ (8 mg/kg) and separation on number of entries in A) food, B) running wheel, and C) odor compartment in T-maze task during 15 min in females. Data are expressed as mean ± S.E.M. of number of entries. **p<0.01 significantly different from vehicle.

Experiment 3. Effect of dopamine depletion on scaping behavior assessed in the FST in male and female mice: Impact of mild maternal separation.

In order to assess the effect of dopamine depletion in the FST for control and separated male and female mice separated two-way ANOVAs were used.

For males, the statistical analysis revealed a significant effect of treatment ($F(1,36)=9.44$, $p=0.004$), no effect of separation ($F(1,36)=0.24$, $p=0.626$), and no significant interaction ($F(1,36)=1.61$, $p=0.212$) for immobility time (Fig. 12A). Also, time spend swimming was affected by treatment ($F(1,36)=6.64$, $p=0.014$), but not by separation ($F(1,36)=2.95$, $p=0.944$), and there was no interaction between the two main factors ($F(1,36)=0.53$, $p=0.470$) (Fig. 12B). Finally, the two-way ANOVA for time spend climbing showed a significant effect of treatment ($F(1,36)=4.98$, $p=0.032$), and also of separation ($F(1,36)=10.06$, $p=0.003$), although no significant interaction ($F(1,36)=3.10$, $p=0.087$) (Fig. 12C). These data suggest that TBZ increased the time males stay immobile and decrease the time they spend swimming or climbing. Furthermore, in general separated males spend more time climbing than control males.

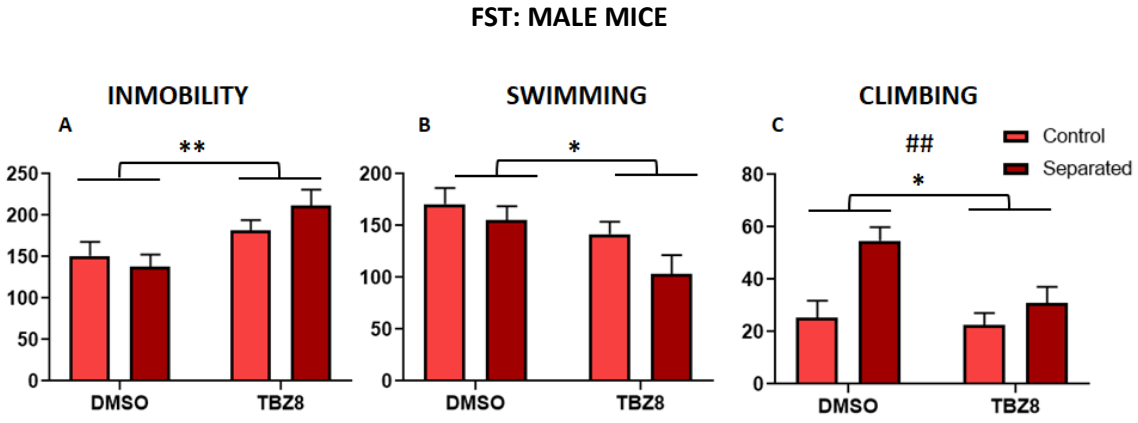

Fig. 13: Effect of TBZ (8 mg/kg) on duration of immobility (A), swimming (B), and climbing (C) in the FST assessed during 6 min in males. Data are expressed as mean ± S.E.M. of accumulated seconds. *p<0.05, **p<0.01 significant differences between vehicle and TBZ. ##p<0.01 significant differences between control and separated animals.

The same analyses were used for females. The two-way ANOVA revealed no significant effect of treatment ($F(1,37) = 1.24$, $p = 0.273$), or separation ($F(1,37) = 1.25$, $p = 0.270$), and no interaction ($F(1,37) = 3.19$, $p = 0.082$), on immobility time (Fig. 13A). Time spend swimming was not significantly affected by treatment ($F(1,37) = 1.85$, $p = 0.182$), separation ($F(1,37) =$

1.77, p = 0.192), and there was no significant interaction (F (1,37) = 2.09, p = 0.157) either (Fig.13B). For the most active variable (climbing), there was no effect of TBZ treatment (F (1,37) = 0.68, p = 0.416), and no effect of maternal separation (F (1,37) = 0.45, p = 0.505), but there was a significant interaction (F (1,37) = 4.14, p = 0.049) on time spend climbing (Fi. 13C). Sidak's multiple comparisons revealed no significant differences between groups. However, unpaired t-test comparing the two vehicle groups revealed that separated females spend more time climbing than non-separated females (p = 0.008).

Thus, it seems that females are not sensitive to TBZ at the dose of 8 mg/kg in the FST, a paradigm that evaluates behavioral activation under stressful conditions.

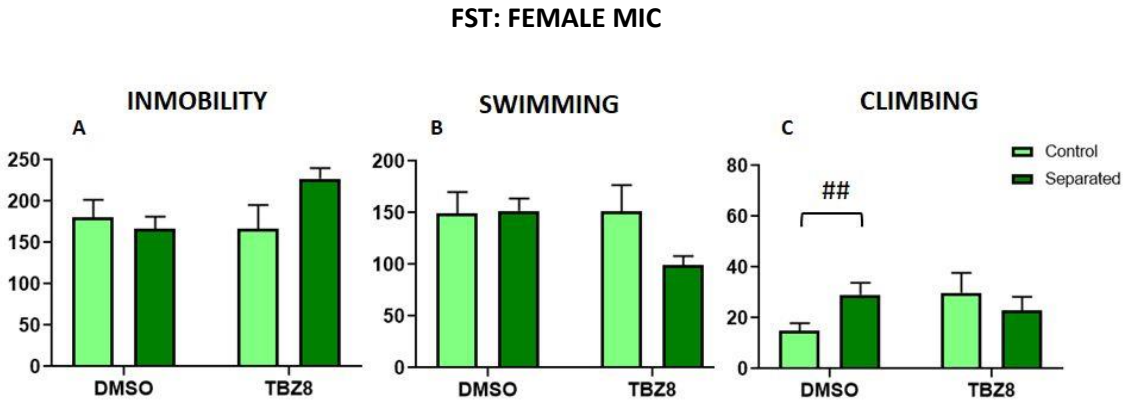

Fig. 13: Effect of TBZ (8 mg/kg) on duration of immobility (A), swimming (B), and climbing (C) in the FST assessed during 6 min in females. Data are expressed as mean ± S.E.M. of accumulated seconds. ## p<0.01 significant differences between separated and no separated females among the vehicle condition.

Experiment 4. Effect of dopamine depletion on anxiety parameters as measured in the DL paradigm in male and female mice: Impact of mild maternal separation.

A series of two-way ANOVAs were used to assess the effects of dopamine depletion on anxiety evaluated in the DL paradigm in control and separated male and female mice. The time spent in the lit compartment was used as the index of anxiety, and the total number of crosses between compartments were analysed as a marker of exploratory locomotion under anxiogenic conditions.

The two-way ANOVA for male mice showed a main effect of separation (F(1,35)=21.22, p=0.000), no effect of treatment (F(1,35)=0.51, p =0.482), and no significant interaction (F(1,35)=0.01, p=0.917), on the time mice spend in the lit compartment (Fig. 14A). For the total crosses between compartments, there were significant effects of treatment (F(1,35)=10.94,

p=0.002), and separation (F(1,35)=18.63, p=0.0001), and also a significant interaction (F(1,35)=0.027, p=0.027) (Fig. 14B). Sidak's multiple comparisons revealed that separated males crossed more between compartments than non-separated males under the vehicle condition (p=0.0002). Moreover, TBZ decreased the number of crosses among separated males (p< 0.0001), but not among the control ones.

Thus, separated males spend more time in the lit compartment than control males, indicating an anxiolytic effect. In addition, although separated males were more active than control animals under vehicle conditions, they were also more sensitive to dopamine depletion, reducing crosses between compartments.

DL: MALE MICE

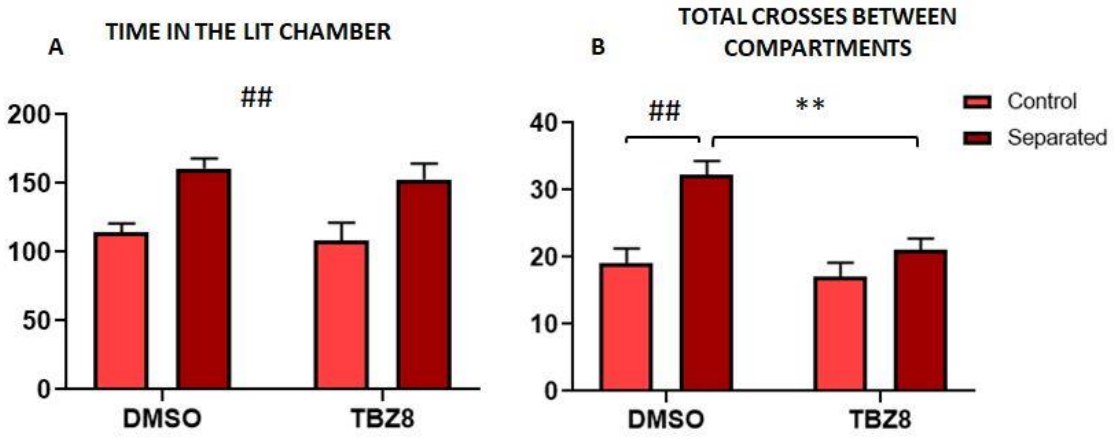

Fig. 14: Effect of TBZ (8 mg/kg) on A) time spend in the lit chamber and on B) the total number of crosses between compartments in the DL during 5 minutes in males. Data are expressed as mean ± S.E.M. of accumulated seconds and number of crosses. **p<0.01 significant differences due to treatment. ##p<0.01 significant differences between control and separated animals.

For females, the two-way ANOVA revealed no main effects of treatment (F(1,32)=0.10, p=0.756), or separation (F(1,32)=0.58, p =0.451), and no significant interaction (F(1,32)=2.14, p=0.154) on time spend in lit compartment (Fig. 15A). For total crosses between compartments there was a significant effect of separation (F(1,32)=5.34, p=0.028), but not of treatment (F(1,32)=1.57, p=0.219), and no interaction (F(1,32)=0.23, p=0.633) (Fig. 15B). These results suggest that mild maternal separation and dopamine depletion do not affect time spend in the lit compartment for females. Also, in general, separated females are more active than control females, since they cross more times between compartments.

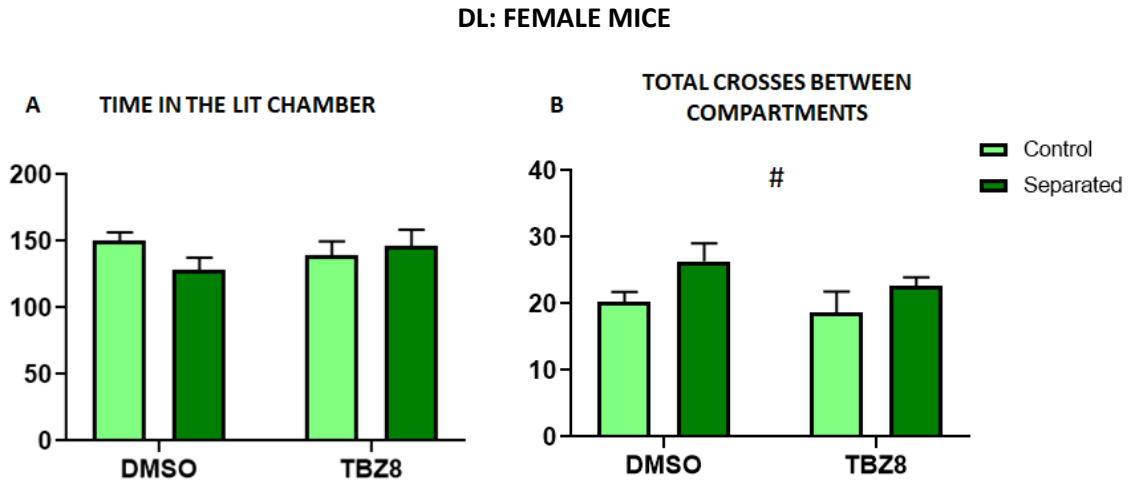

Fig. 15: Effect of TBZ (8 mg/kg) on A) time spend in the lit chamber and on B) total number of crosses between compartments in the DL during 5 minutes in females. Data are expressed as mean ± S.E.M. of accumulated seconds and number of crosses. #p<0.05 significant differences among control and separated animals.

DISCUSSION

The present group of studies assessed the impact of early-life stress via mild maternal separation and the effect of dopamine depletion, using TBZ, on behavioral activation measured on the three-choice-T-maze task. The 3-choice-T-maze paradigm evaluates preference for different reinforcers that required different levels of activation: a RW, sucrose pellets and non-social odor (Correa et al., 2016; López-Cruz et al., 2018). Moreover, the experiments also evaluated these manipulations in other behavioral paradigms: the FST, useful to evaluate the activation induced by a stressful or aversive situation, and the DL, a commonly used paradigm to assess anxiety-like behaviors.

In the first experiment, we evaluated spontaneous (baseline) preference in the T-maze of males and females, in separated and non-separated mice. All animals preferred to spend most of the time running in the RW and they spend less time eating or sniffing, independently of sex. These results in male were consistent with previous studies that use the paradigm in our laboratory (López-Cruz et al., 2018; Carratalà-Ros et al., 2020), and with other studies that had demonstrated that running has a high motivational value, animals work harder in order to unlock a RW (Collier et al., 1990; Belke et al., 2005; Belke and Pierce, 2014). Furthermore, we found that separated animals spend more time running than control, we hypothesize that the level of separation we use produce an increase in dopamine levels, like an acute stressor, and that increases in dopamine levels could produce this increase in active behaviors. However, in adult male rats acute restrain stress decreased the choice of high respond option (Shafei et al.,

2012), and an acute shock did not produce any effect on patterns of effortful choice behavior (Hart et al., 2017).

In the present experiment we also evaluate other behavioral variables like time spend in the different compartments or total number of entries into each compartment. MS did not produce any effect in these measures of place preference and exploration, except in the number of entries in food compartment. Separated animals did fewer entries in that compartment than controls. But these measures indicate that mice did not avoid being close to the RW and present a normal motor exploration of the paradigm. As for the sex-based differences, females spend more time in the food compartment and less in the RW compartment than males, but realize more entries in both compartments than males indicating that the motor exploration was undisturbed.

In the second part of the work, we used the dose of 8 mg/kg of TBZ because it had demonstrated to deplete dopamine levels in ventral striatum in mice (López-Cruz et al., 2018), and to produce anergia in the T-maze and in the FST (Carratalà-Ros et al., 2020) reducing behavioral activation rather than affecting the primary reinforcing effects of other stimuli (López-Cruz et al., 2018; Carratalà-Ros et al., 2020). Moreover, our results revealed that separated females eat more than the other females, and more than separated males. Previous studies suggest that MS had a lack of effect in reward seeking and consumption (sucrose solutions) (Shalev and Kafkafi, 2002), and early life stress induced by MS had no effect on motivation to obtain a reward (Stuart et al., 2019).

In the second experiment the behavioral paradigm used was the same than in the first, but in this case animals were divided by sex for assess their behavior. The other manipulation used for this experiment was the administration of TBZ, an VMAT-2 inhibitor. In the case of males, TBZ produced a change in the relative preference for the reinforcers. Animals spend less time running and increase the time they spend eating, with no differences with the time they spend sniffing the non-social odor. These results are consistent with previous studies in our laboratory using the same drug (López-Cruz et al., 2018; Carratalà-Ros et al., 2020). Although other drugs that also affect DA levels produce the same effect. Haloperidol, a D2 receptor antagonist, produced those changes in a simpler version of the T-maze (Correa et al., 2016). TBZ did not affect the time males spend in each compartment or the number of entries, so it did not affect place preference and exploration. Maternal Separation neither produced any effect in these two variables, indicating a normal motor exploration, although that

manipulation produced more activity in the RW, separated animals run more time than controls. In that experiment the behavior of females were also assessed, in the same paradigm and with the same dose of TBZ. Surprisingly, in this case TBZ did not produce any effect in behavior, females did not decrease the time spend running in the RW and increase the time they spend eating. But TBZ produced that control females spend less time sniffing. As for the time spend in each compartment and the number of entries, TBZ only affected the number of entries in food compartment, females with lower levels of DA did fewer entries to the food compartment. Maternal Separation only affected time females spend running, increasing the time as the same form than in males. Moreover, it produced a decrease in time sniffing the non-social odor in the vehicle condition but no when the TBZ was administered. The time spends in each compartment and the number of entries were not affected by separation, so in females maternal separation neither affect place preference or motor activity. These data suggest that probably a mild maternal separation produces high levels of behavioral activation.

In experiment three, animals were assessed in the FST, a classical paradigm useful for evaluate antidepressant effect of different types of drugs (Armario et al., 1988; Lucki, 1997). It provides information about active and passive behaviors: immobility is a measure of passive behavior, it measures behavioral despair or "giving up" (Porsolt et al., 1977), but there are other two behaviors that could be evaluated, swimming and climbing, parameters of behavioral activation (Armario et al., 1988; Gil and Armario, 1998; Slattery and Cryan, 2012). So, FST provide information about behaviors directed to the maintenance of vigorous and persistent active responding in order to escape from an aversive situation (Gil and Armario, 1998). In the present study we assessed the effect of TBZ, at dose that produce a depletion of DA levels in ventral striatum of mice (López-Cruz et al., 2018), and maternal separation in FST performance for male and female mice. In the present study, TBZ produce a significant increase in time spend immobile and a decrease in time spend swimming or climbing in males, but these results did not replicate in females. TBZ did not produce any effect in FST female behavior. The results of males are consistent with previous studies, TBZ decreases climbing and increase immobility (Wang et al., 2010; Carratalà-Ros et al., 2020), but there were no evidence for TBZ effect in females. As for maternal separation, these only affected the variable that require high levels of vigor, time climbing. In males, this manipulation affect independently of the treatment, and in females when the TBZ were not administered. So, early life stress induced by maternal separation increased, in both genders, active behaviors. Previous studies found that early-life stress produce a decrease in immobility and an increase in climbing or struggling in males and females rats, indicating an increased active coping (Rüedi-Bettschen et al., 2006;

Fuentes et al., 2014), but other studies did not observe changes (Wang et al., 2011; Clarke et al., 2013) or present increased passive coping strategies (Herofer et al., 2012; Lajud et al., 2012; Martisova et al., 2012; Amiri et al., 2016). Other studies affirm that the effect of early-life stress on the FST seems to be strain-dependent (Binder et al., 2011).

The last experiment assessed anxiety-like behaviors using a DL box, which is based on the conflict between the natural tendencies of mice to explore novel environments and the avoidance of open and illuminated areas (Blumstein and Crawley, 1983). As in the previous studies, animals of both sexes were administered with TBZ and the effect of maternal separation was assessed. In this case, TBZ did not produce any significant effect on the anxiety-related parameter, the time animals spend in the lit chamber, for both sexes. TBZ only affect the total number of crosses between compartments in separated males, no in females. Separated males did fewer entries than controls, but it is more related with spontaneous locomotion than with anxiety-like behaviors. This is consistent with previous studies, in which the same dose of TBZ (8mg/kg) did not produce any effect on the time spend in the lit compartment of DL box, so it did not produce any anxiogenic effect (Correa et al., 2018; Carratalá-Ros et al., 2020). The same occurred when a D2 antagonist (haloperidol) were administrated and used in other anxiety-like paradigm, the elevated plus maze (EPM) (Pail et al., 2015). Thus, DA depletion did not produce effect in anxious behavior. Furthermore, other studies found that TBZ decrease the total number of crosses between compartments (Carratalá-Ros et al., 2020); although in the present study it occurs only for separated males, no in control males. Regarding a maternal separation, it produced effect in vehicle condition of males and in all females in the total crosses between compartments. So, ELS induced by maternal separation produce an increase in locomotor activity. In females, MS did not produce any change in time spend in the lit compartment, but in males this factor increase the time they spend on it, indicating a possible anxiolytic effect in males. Although previous studies found that MS produce an increase in anxiety-like behaviors decreasing time in the lit compartment and exploratory behavior (Trujillo et al., 2015; Tractenberg et al., 2016; Jin et al., 2018). Probably, the differences between results remain in the type of MS, in the present study a mild maternal separation were used and in the other studies used longer ones.

The present work has potential clinical relevance, because DA has been implicated in anergia, psychomotor slowing, fatigue and lower energy, common symptoms of depression (Salamone et al., 2016a; Stahl, 2002; Treadway and Zald, 2011). Moreover, the incidence of depression in woman is higher than in males (Hankin et al., 2007, 2009). Some studies relate it with the higher level of activation of the HPA-axis in females (Oyola and Handa, 2017; Rincón-Cortés et

al., 2019). Thus, different levels of stress could influence in the activity of that axis and in the depressive symptoms (McEwan and Stellar, 1993; Fernandez-Guasti et al., 2012; Altemus et al., 2014; Oyola and Handa, 2017). So, the differences between males and females in the manipulation of DA levels and the HPA-axis reactivity could be an important factor to take into account, in order to study that kind of symptoms present in a wide variety in psychiatric disorders.

CONCLUSIONS

Our data highlight the importance of early-life experiences on establishing patterns of behavior later in life. In this case, mild maternal separation in the very early stages of development, potentially acting as a mild stressor, influences patterns of behavioral activation in different context, and with a positive outcome; animals seem more energetic or persistent. They increase their relative preference for activity-based reinforcers and also keep trying to escape an aversive situation. The time frame for this effect could change if this type of separation is experience later in development, and potentially with opposite effects. Thus, further studies should explore different times and duration of MS in order to better characterize this phenomenon and also in order to improve its translational potential.

Furthermore, the present results revealed differences between sexes in response to pharmacological manipulations, showing how in this case females seem less vulnerable to an agent that impairs exertion of effort in motivated behavior. This result further supports the importance of personalized treatments based on sex in parameters like anergia and fatigue, common symptoms of psychiatric disorders.

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