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Involvement of dopamine in the regulation of dysfunctions related to depression in rodent models: motivational versus emotional symptoms

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TABLE OF CONTENTS

Extended summary

Depression is characterized by cardinal symptoms such as negative affect and depressed mood. However, this disorder also can include motivational symptoms such as anergia, fatigue and psychomotor slowing. These are highly resistant to treatment and often remain as residual symptoms after antidepressant drug therapy. In fact, the most common class of antidepressants, the serotonin (5-HT) transporter (SERT) inhibitors, are useful for treating mood and anxiety symptoms in depression, but it has been observed that they are relatively ineffective at treating motivational dysfunction, and in fact, they can exacerbate fatigue. It has been suggested that these effort-based symptoms are related to dysfunctions in mesolimbic dopamine (DA) (specifically in the nucleus accumbens (Nacb)) and related forebrain circuitry. Across multiple paradigms of effort-based decision-making in animal models, low doses of DA antagonists or Nacb DA depletions reduce the willingness to work for a high reward option and increase selection of low reward alternatives that require very little effort. Moreover, in depressed people, fatigue problems interfere with participation in exercise, and it has been observed that a lack of activity can contribute to the development of depression. Of course, engaging in voluntary physical activity is always undertaken in relation to the possible selection of other alternatives, such as sedentary behaviors or food consumption. Voluntary wheel running occurs spontaneously in mice of all strains, sexes, and ages. Thus, a recently developed 3-choice-T-maze task establishes voluntary running in a wheel as the high effort/highly preferred option competing with palatable food and a fruit odor. This test can be used to study spontaneous preferences for reinforcers that require vigorous activity, and to evaluate the impact of DA manipulations and the potential therapeutic effects of different type of antidepressants. This test can assess anergia in a non-stressful setting, while traditional rodent models for the study of antidepressant drugs, such as the forced swim test (FST), assesses behavioral activation induced by stressful conditions. Thus, in the present dissertation I characterized the impact of DA depletion in several animal models that evaluate behavioral activation to escape an aversive situation (in the FST) or to approach and vigorously interact with a highly preferred reinforcer (a running wheel in the 3-choice-T-maze) that competes with reinforcers by more passive behaviors. The effect of DA depletion also was studied in rodent models of anxiety-related behavior, another symptom common in some depressed people.

The first chapter of the present dissertation characterizes and validates this 3-choice-T-maze task as a model of preference for physical activity versus sedentary options. These studies employed a model of anergia induced by DA depletion, by using a VMAT-2 blocker (tetrabenazine, TBZ) to alter choice behavior, and comparing those results with the effects on the FST and also with other manipulations that change the homeostatic or emotional value of the reinforcers that are available in the T-maze task. In Chapter 2, bupropion, a catecholamine transport blocker that elevates extracellular DA levels and is used as an antidepressant, was administered to animals tested on the FST or the 3-choice-T-maze task. This antidepressant was administered on its own, and also in combination with TBZ. Metabotropic markers of DA receptor activation are used to assess the impact of these pharmacological manipulations in Nacb. In addition, since clinical practice has focus on pharmacological strategies that increase serotoninergic transmission via SERT inhibitors such as fluoxetine, Chapter 3 studies evaluated the impact of fluoxetine on behavioral activation in both paradigms; the FST and the 3-choice-T-maze task, and its capability to alleviate TBZ-induced anergia. DARPP-32 phosphorylation was used as a cellular marker related to DA activity. Finally, it is important to emphasize that little is known about the behavioral activation impairments in animal models using female subjects, despite the fact that depression is twice as common in women than in men, and the response to antidepressants differs between sexes. Thus, Chapter 4 of the present thesis focuses on the impact of DA depletion on behavioral activation comparing female and male mice in several tests of behavioral activation and anxiety. The therapeutic effect of both types of antidepressants (bupropion and fluoxetine) after DA depletion were also assessed using the FST.

Resumen

La depresión se caracteriza por un conjunto de síntomas fundamentales como el afecto negativo o el humor depresivo. Sin embargo, este trastorno también incluye síntomas motivacionales como la anergia, fatiga o enlentecimiento psicomotor. Estos síntomas son muy resistentes a tratamiento y con frecuencia permanecen como síntomas residuales tras la terapia farmacológica. De hecho, el tipo de antidepresivos más común, los inhibidores de la recaptación de serotonina (5-HT) son útiles para tratar síntomas emocionales o ansiosos que aparecen en la depresión, pero se ha observado que estos fármacos son relativamente ineficaces para tratar los síntomas motivacionales y, de hecho, pueden exacerbar la fatiga. Se sugiere que estos síntomas motivacionales están relacionados con alteraciones en la dopamina (DA) mesolímbica (específicamente en la zona núcleo accumbens (Nacb)) y con circuitos relacionados del prosencéfalo. A través de múltiples paradigmas animales de toma de decisiones basados en el esfuerzo, bajas dosis de antagonistas de DA o depleciones de DA en el Nacb reducen la disposición de trabajar para conseguir una elevada recompensa e incrementan las elecciones a alternativas con bajas recompensas pero que requieren poco esfuerzo para conseguirlas. Además, en personas con depresión, la fatiga interfiere con la participación en hacer ejercicio y se ha demostrado que una baja actividad física puede contribuir a desarrollar depresión. Por supuesto, la actividad física voluntaria siempre se realiza en relación con la posible selección de otras alternativas, como los comportamientos sedentarios o el consumo de alimentos. Correr de forma voluntaria en una rueda de actividad ocurre de forma espontánea en ratones de todas las cepas, sexos y edades. Por lo tanto, una tarea recientemente desarrollada de 3 opciones de laberinto en T establece la carrera voluntaria en una rueda como la opción de alto esfuerzo / altamente preferida que compite con alimentos sabrosos y un olor a frutas. Esta prueba se puede utilizar para estudiar las preferencias espontáneas para los reforzadores que requieren una actividad vigorosa, y para evaluar el impacto de las manipulaciones de DA y los posibles efectos terapéuticos de diferentes tipos de antidepresivos. Esta prueba puede evaluar la anergia en un entorno no estresante, mientras que los modelos tradicionales de roedores para el estudio de fármacos antidepresivos, como la prueba de natación forzada (FST), evalúa la activación conductual inducida por condiciones estresantes. Por lo tanto, en la presente tesis he caracterizado el impacto de la depleción de DA en varios modelos animales que evalúan la activación conductual para escapar de una situación aversiva (en el FST) o para

acercarse e interactuar vigorosamente con un reforzador altamente preferido (una rueda en movimiento opción-T-laberinto) que compite con los reforzadores en el que se requieren comportamientos más pasivos para conseguirlos. El efecto de la depleción de DA también se ha estudiado en modelos de ansiedad en roedores, otro síntoma común en algunas personas con depresión.

El primer capítulo de la presente tesis caracteriza y valida esta tarea de 3 opciones de laberinto en T como un modelo de preferencia por la actividad física frente a las opciones sedentarias. Estos estudios han empleado un modelo de anergia inducida por la depleción de DA, mediante el uso de un bloqueador VMAT-2 (tetrabenazina, TBZ) para alterar el comportamiento de elección, y comparando esos resultados con los efectos sobre el FST y también con otras manipulaciones que cambian el valor homeostático o emocional de los reforzadores que están disponibles en la tarea del laberinto en T. En el Capítulo 2, el bupropión, un bloqueador del transporte de catecolamina que eleva los niveles de DA extracelular y se usa como antidepresivo, se administró a los animales probados en el FST o en la tarea del laberinto en T de 3 opciones. Este antidepresivo se administró solo, y también en combinación con TBZ. Los marcadores metabotrópicos de la activación del receptor DA se utilizan para evaluar el impacto de estas manipulaciones farmacológicas en Nacb. Además, dado que la práctica clínica se centra en las estrategias farmacológicas que aumentan la transmisión serotoninérgica a través de los inhibidores de SERT como la fluoxetina, los estudios del Capítulo 3 evaluaron el impacto de la fluoxetina en la activación conductual en ambos paradigmas; el FST y la tarea de 3 opciones de laberinto en T, y su capacidad para aliviar la anergia inducida por TBZ. La fosforilación de DARPP-32 se utilizó como marcador celular relacionado con la actividad de DA. Finalmente, es importante enfatizar que se sabe poco sobre los impedimentos de activación conductual en modelos animales que usan roedores femeninos, a pesar de que la depresión es dos veces más común en mujeres que en hombres, y la respuesta a los antidepresivos difiere entre sexos. Por lo tanto, el Capítulo 4 de la presente tesis se centra en el impacto de la depleción de DA en la activación conductual comparando ratones machos y hembras en varias pruebas de activación conductual y ansiedad. El efecto terapéutico de ambos tipos de antidepresivos (bupropión y fluoxetina) después de la depleción de DA también se evaluó utilizando el FST.

General Introduction

1. The activational component of motivation.

Motivation is defined as a set of processes that allow organisms to regulate the probability, proximity and availability of significant stimuli (Salamone and Correa, 2002; 2012; Salamone et al, 2018, 2016). The ability of organisms to interact with the environment in order to obtain significant stimuli such as water, food and sex is critical for survival and it requires to overcome environmental obstacles with the objective to gain these stimuli (Salamone et al, 2018, 2016). Therefore, motivated behavior is composed of several components that can be dissociated from each other, for instance; a directional component (preference for specific stimuli or activity) and an energetic or activational component (what are you willing to do in order to obtain those stimuli) (Salamone and Correa 2002, 2012; Kelley et al, 2005).

The directional component of motivation requires that the organism approaches selected positive stimuli, but also works to get away from negative stimuli (e.g. painful conditions, predators or stressors). The distinction between directional and activational components of motivation has been an important center of interest in psychology for many decades (Duffy, 1963; Cofer and Apley, 1964; Salamone 1997). The ability to produce vigorous and rapid responses and maintain them over time, is a fundamental and highly adaptive feature of motivational processes because these responses enable organisms to exert the effort necessary to reach the work-related limitations that separate them from important stimuli (Salamone et al, 2018).

Studies of behavioral activation are important for understanding some aspects of psychopathology. Thus, symptoms such as anergia, psychomotor retardation, apathy and fatigue refer to a lack of behavioral activation that can be seen in multiple psychiatric disorders such as depression and schizophrenia and also, in some neurological diseases such as Parkinson disease (Treadway et al, 2012; Yang et al, 2015; Gold et al, 2013; Chong et al, 2016). The severity of these behavioral activation impairments is highly correlated with problems in social function, employment and treatment response (Tylee et al, 1999; Stahl et al, 2002). The development of animal models of behavioral activation dysfunctions could enhance the understanding of the neurochemical basis of motivational symptoms in pathologies, as well as it would allow to develop efficient

psychopharmacology strategies to treat these motivational symptoms (Salamone et al, 2018, 2016).

2. Implication of dopamine and nucleus accumbens in behavioral activation and effort-related choice.

Usually, behavioral activation and effort-related decision making are evaluated using tasks that offer animals a choice between a preferred reinforcer that can only be obtained doing a high effort versus a lower effort/lower value option (Salamone et al, 2016, 2018). Operant behavioral methods such as fixed ratio (FR) or progressive ratio (PROG) schedules allow the study of response allocation under conditions in which animals have to choose to work for a preferred food by pressing a lever in the high-effort option or simply approach and consume a less preferred food as the low-effort option (Salamone et al, 1991; Randall et al, 2012; Sommer et al, 2014; Yohn et al, 2016a,b,c,d; Rotolo et al, 2019; Nunes et al, 2013).

Considerable evidences, from basic animal studies, indicate that dopamine (DA) in the nucleus accumbens (Nacb), is the central component that regulates behavioral activation and energy expenditure (Salamone and Correa, 2002, 2012; Salamone et al, 2016; Robbins and Everitt, 2007; Beller et al, 2012, 2015). DA depletions and injections of DA receptor antagonists into the Nacb impair these active behaviors that lead to obtaining palatable and preferred food, but, in parallel, they increase the consumption of the less preferred food that is available and does not require effort (Robbins and Koob, 1980; Wallace et al, 1983; Salamone et al, 1986; 1988; McCullogouh and Salamone, 1992). DA antagonism and Nacb DA depletions interact with operant ratio requirements. Thus, impairments are greater when the schedules require larger ratios (Aberman and Salamone, 1999; Caul and Brindle, 2001; Salamone et al, 2002; Ishiwari et al, 2004). In addition, in different type of effort-choice tasks, such as the T-maze procedure in which a barrier can be placed in the arm where there is a high quantity of food reinforcer (food pellets), and in the other arm of the maze there is no barrier but there are fewer reinforcers, DA depletion and DA antagonisms decrease choice for the high density arm and increase the selection of the low density arm with no barrier (Salamone et al, 1994; Cousins et al, 1996; Mott et al, 2009; Pardo et al, 2012).

Mesolimbic DA also regulates social behaviors that allow animals to approach or avoid social stimuli. Thus, dopaminergic transmission in the Nacb modulates social motivation (Achterberg et al, 2016; Robinson et al, 2011; Trezza et al, 2011, 2012) and tasks that evaluate and instigate the interaction between rodents can increase DA levels in the NAc (Pellis and Pellis, 2009; Manduca et al, 2016; Trezza et al, 2011, 2012).

Interference with Nacb DA does not impair food intake, preference for different types of food and does not reduce sucrose preference or hedonic reactivity as seen in free feeding preference studies (Salamone et al, 1991; Koch et al, 2000; Nunes et al, 2013; Pardo et al., 2015). Thus, dopaminergic manipulations than alter effort-related choice are not altering choice based on the quality of food (Salamone and Correa, 2002, 2012; Salamone et al, 2016, 2018).

Finally, although dopaminergic systems are involved in motor function, previous studies have indicated that changes in effort-related decision making produced by DA depletions are not depending of the total amount of lever presses the animal can generate because, when there is not free food available and the animal has no other choice, rats with moderate DA depletions still press the lever in a FR5 operant procedure (Salamone et al, 1994b). Thus, animals with impaired DA transmission are capable of responding, although they select the way that requires less effort.

3. Active reinforcers as a tool to evaluate behavioral activation in animal models.

Effort-related tasks are based upon the election of the animal to exert an active response by pressing a lever or climbing a barrier in order to obtain palatable food. In addition, some animal studies have developed other methods in which behavioral activation can be studied by using the possibility of engaging in vigorous activities which is a highly preferred reinforcer in rodents. Therefore, running on a running wheel (RW) appears to be motivationally regulated like other appetite behaviors (Mueller et al, 1997; Sherwin, 1998; Belke and Pierce, 2014). This reinforcer can be used as the motivational stimulus for the establishment of a conditioned place preference (Lett et al, 2000), and also as a reinforcer in operant-conditioning procedures (Premack and Premack, 1963; Belke and Pierce, 2014). Moreover, if a RW is present in a complex environment that offers other alternatives such as drugs of abuse, animals will spend a significantly amount of time

engaged in running on a RW (McMillan et al, 1995; Cosgrove et al, 2002), and rodents often choose running over food consumption when the choice between food and RW is available (Premack and Premack, 1963; Routtenberg, 1968; Mueller et al, 1997).

A novel T-maze task has been developed in order to assess preferences between active reinforcers versus sedentary ones (Correa et al, 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020). This T-maze task, in contrast to other effort-related tasks, does not involve lever pressing or climbing a barrier with the objective to get a reinforcer of which the animal is partially deprived (i.e food), but instead allows the possibility of freely engaging in consumption of reinforcers that are not present usually in their environment; wheel running, consuming palatable food or sniffing a non-social odor. Rodents do not need to be food deprived in order to consume the palatable food reinforcer that contains 50% carbohydrates, although they need to be exposed to this new test and in order to reduce neophobia (Correa et al., 2016). The non-social odor used for these type of studies is a fruit odor (strawberry) that has demonstrated to not generate avoidance as is the one that generates more exploration among several floral or fruit odors (López-Cruz et al., 2017). Finally, mice have a high preference for engaging in physical activities, and specially running on a voluntary RW (Routtenberg et al, 1968; Correa et al, 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020). Moreover, this rodent task offers the opportunity to evaluate changes in preferences between these concurrently presented reinforcers, as measured by time interacting with each stimuli (Correa et al., 2016). Thus, this task could be used to evaluate the brain mechanisms involved in the decision-making processes that are involved in the spontaneous preference for active reinforcers and sustaining vigorous behaviors over time.

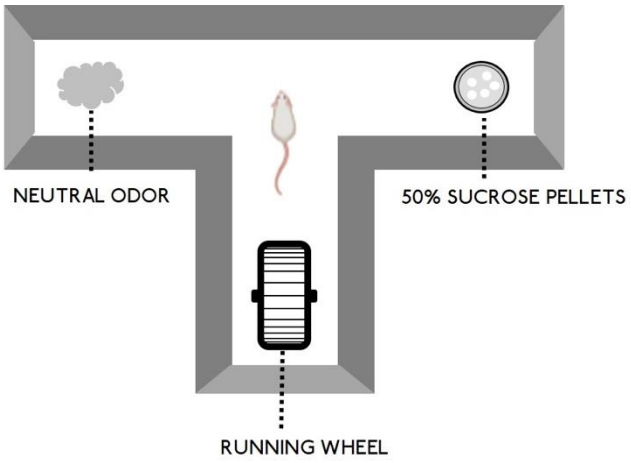

Figure 1. Schematic representation of the 3-choice T-maze task settings.

4. DARPP-32 phosphorylation at threonine 34 and 75 as an indicator of DA receptor D1 and D2 function.

As described before, multiple behavioral studies have been performed in order to characterize the role of DA as a modulator of effort-related decision making tasks (Nunes et al, 2013; Randall et al, 2014; Yohn et al, 2014, 2015a,b). In addition, NAcb DA release, tissue content and electrophysiological properties have been studied in relation to those behaviors (Segovia et al., 2011; Nunes et al, 2013; Floresco, 2007; Smith-Roe and Kelley, 2000, Seamans and Phillips, 1994; Salamone, 1996). Finally, other studies assessing effort-based decision making have specifically focused on DA in NAcb at the metabotropic intracellular level (Nunes et al, 2013; Segovia et al., 2011; Randall et al, 2014; Mai et al, 2012; Valjent et al, 2005; Svenningson et al, 2004).

Dopamine- and cAMP-regulated phosphoprotein, Mr 32 KDa (DARPP-32) is highly present in medium spiny neurons (MSNs) in the ventral and dorsal striatum projection neurons (Greengard et al, 1999; Ouimet et al, 1998). MSNs are divided into two subtypes which selectively express one of two peptides: enkephalin or dynorphin. MSNs than contain dynorphin, which also express the peptide substance P, principally express dopamine D1 receptors while enkephalinergic MSNs mainly express dopamine D2 receptors (Nishi and Shuto, 2017; Svenningson et al, 2005; Valjent et al, 2005). In addition, when D1 and D2 receptors are activated, DARPP-32 phosphorilates and this can be interpreted as an index of DA receptor activation (Svenningson et al , 2004, Nunes et al, 2013). The function of DARPP-32 depends on its relative state of phosphorylation at two main regulatory sites: threonine 34 and 75 (Thr34 and Thr75). When DARPP-32 phosphorilates at Thr34 by the action of the protein kinase A (PKA), DARPP-32 inhibits the protein phosphatase 1 (PP-1) leading to the phosphorylation of several classes of effector proteins, including tanscription factors, ionotropic receptors and ion channels (Greengard et al, 1999). However, DARPP-32 produces the opposite effect when its phosphorylation occurs at Thr75: DARPP-32 inhibits the PKA signaling and allows the activation of the PP-1 protein (Bibb et al, 1999; Svenningson et al, 2004). Therefore, the phosphorylation of DARPP-32 at Thr34 or Thr75 seems to be directly related with the activation of DA D1 or D2 receptors (Nunes et al, 2013; Randall et al, 2015; López-Cruz et al, 2018; Svenningson et al, 2004) **(Figure 2).**

Activation of D1 receptors increases the activity of adenylyl cyclase and this increase in turn activates cyclic AMP-dependent protein kinase (cAMP-PK) resulting an increase of the phosphorylation of DARPP-32 at Thr34. For instance, administration of DA D1 agonists such as SKF 81297 increases pDARPP-32(Thr34) but this effect is blocked after the administration of the D2 agonist quinpirole (Svenningson et al, 1999). Moreover, the administration of D2 antagonists such as haloperidol or eticlopride increases pDARPP-32(Thr34) and this action is blocked by the action of D1 antagonists (Svenningson et al, 1999). In addition, activation of D2 receptors decreases cAMP levels and produces, as a result, the increase of DARPP-32 at Thr75, although there is less information about this intracellular pathway (Greengard et al, 1999). Therefore, the opposite modulation in D1 and D2 receptors produced by antagonists or agonists contributes to the knowledge that DARPP-32 phosphorylation is taking place in different populations of neurons (Svenningson et al, 2004; Bateup et al, 2008; Nunes et al, 2013).

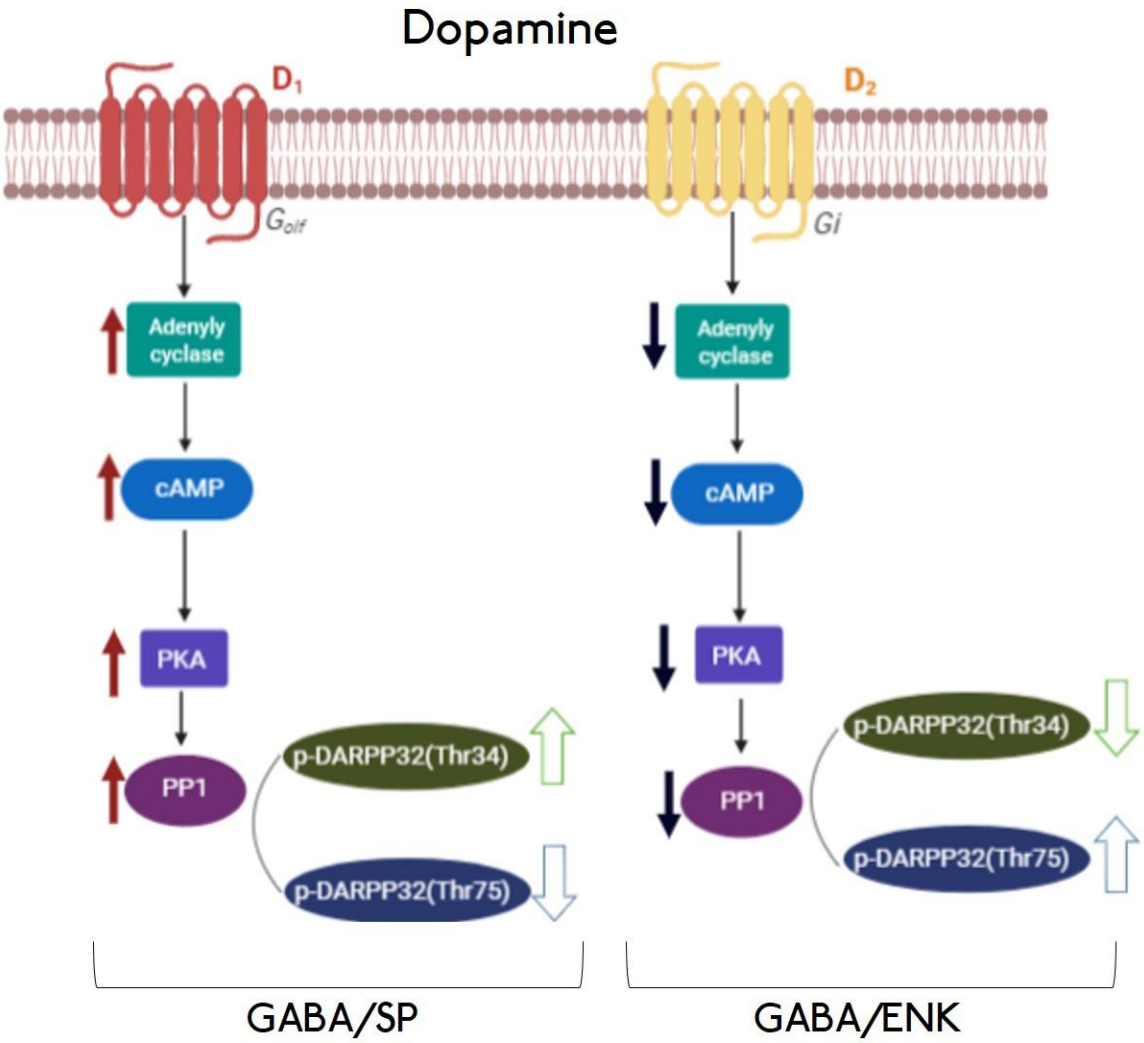

Figure 2. Diagram showing the intracellular cascade in the D_1 and D2 receptor and effect of DA on DARPP-32 phosphorylation. D1 receptor stimulation increases c-AMP production and PKA activity, which phosphorylates DARPP-32 to yield pDARPP-32(Thr34). D2 receptor stimulation decreases c-AMP production and PKA activity, which increases the phosphorylation of pDARPP-32(Thr75) expression (for details, see Svenningson et al, 2004; Bateup et al, 2008; Nunes et al, 2013; López-Cruz et al, 2018).

5. Tetrabenazine as a tool to induce behavioral activation impairments in animal models: impact of DARPP-32 phosphorylation patterns.

Reduced selection of high-effort choices can be induced in rodents by multiple conditions such as stress (Shafei et al, 2012), administration of DA antagonisms as we described before (Salamone et al, 1994b; Pardo et al, 2012) or injections of proinflammatory cytokines (Nunes et al, 2014; Yohn et al, 2015a). In addition, the decrease of the high-effort election can be produced by the administration of Tetrabenazine (TBZ) a DA depleting agent.

TBZ inhibits the vesicular monoamine transporter type-2 (VMAT-2), reducing the vesicular storage and leading to a depletion of monoamines. TBZ at low doses exerts its greatest effects depleting striatal DA (Pettibone et al, 1984; Tanra et al, 1995; Nunes et al, 2013). Usually, TBZ is used for treating the hyperkinetic movements in Hungtington's disease, but it has reported to induce depressive symptoms including fatigue as major side effects in people that have used it chronically (Frank, 2009; Guay et al, 2010; Chen et al, 2012). TBZ has been used in classical animal models of depression (Preskorn et al, 1984; Kent et al, 1986; Wang et al, 2010; Carratalá-Ros et al, 2020) and also in effort-related decision-making tasks in rats (Nunes et al, 2013; Yohn et al, 2015a, 2016; Randall et al, 2014; Pardo et al., 2015; Rotolo et al, 2019) where the administration of low doses of TBZ alters effort-related choice behavior as assessed in FR/choice and PROG/choice tasks. This VMAT-2 blocker shift behavior of rodents towards the election of less-costly but less-valued reinforcers however, it does not alter the preference for food (Nunes et al, 2013) or sucrose (Pardo et al., 2005). Moreover, TBZ does not produce effects on reinforcer devaluation like those induced by pre-feeding (Randall et al, 2012, 2014; Pardo et al, 2015) and it does not alter hedonic reactions to sucrose (Pardo et al, 2015). In mice, TBZ decreases the preference for active reinforcers, increasing time doing more sedentary activities as seen in the 3-choice-T-maze task (López-Cruz et al, 2018; in the present dissertation Carratalá-Ros et al, 2020).

In addition, it has been observed that TBZ reduces DA content and release in NAcb core assessed by microdialysis (Randall et al, 2013) or tissue punctures of ventral striatum (López-Cruz et al, 2018). TBZ also affects DA-related signal transduction which results in a consistent reduction of NAcb D1 and D2 receptor transmision (Robertson and Fibiger, 1992; Bateup et al, 2008). TBZ reduces C-fos expression in NAcb core and shell indicating a reduction in D2 transmission (Robertson and Fibiger, 1992). Moreover, immunohistochemical studies in rats have observed that administration of TBZ produces a significant increase of DARPP-32 expression phosphorylated at Thr34 and at Thr75 in NAcb (Nunes et al., 2013). Results from previous studies suggest that TBZ-induced increases in DARPP-32(Thr34) would indicate a reduction of the D2 receptor transmission while the increase of DARPP-32(Thr75) would mark a reduced transmission of D1 receptors (Svenningsson et al, 2004; Bateup et al, 2008; Nunes et al, 2013; Yger and Girault, 2011) **(Figure 3).** Therefore, TBZ has demonstrated to be connected with DA transmission, in particular, with NAcb DA considered the main key to regulate motivational processes. Moreover, TBZ is a useful pharmacological tool to evaluate pharmacological strategies in order to restore these effort-based behavioral impairments.

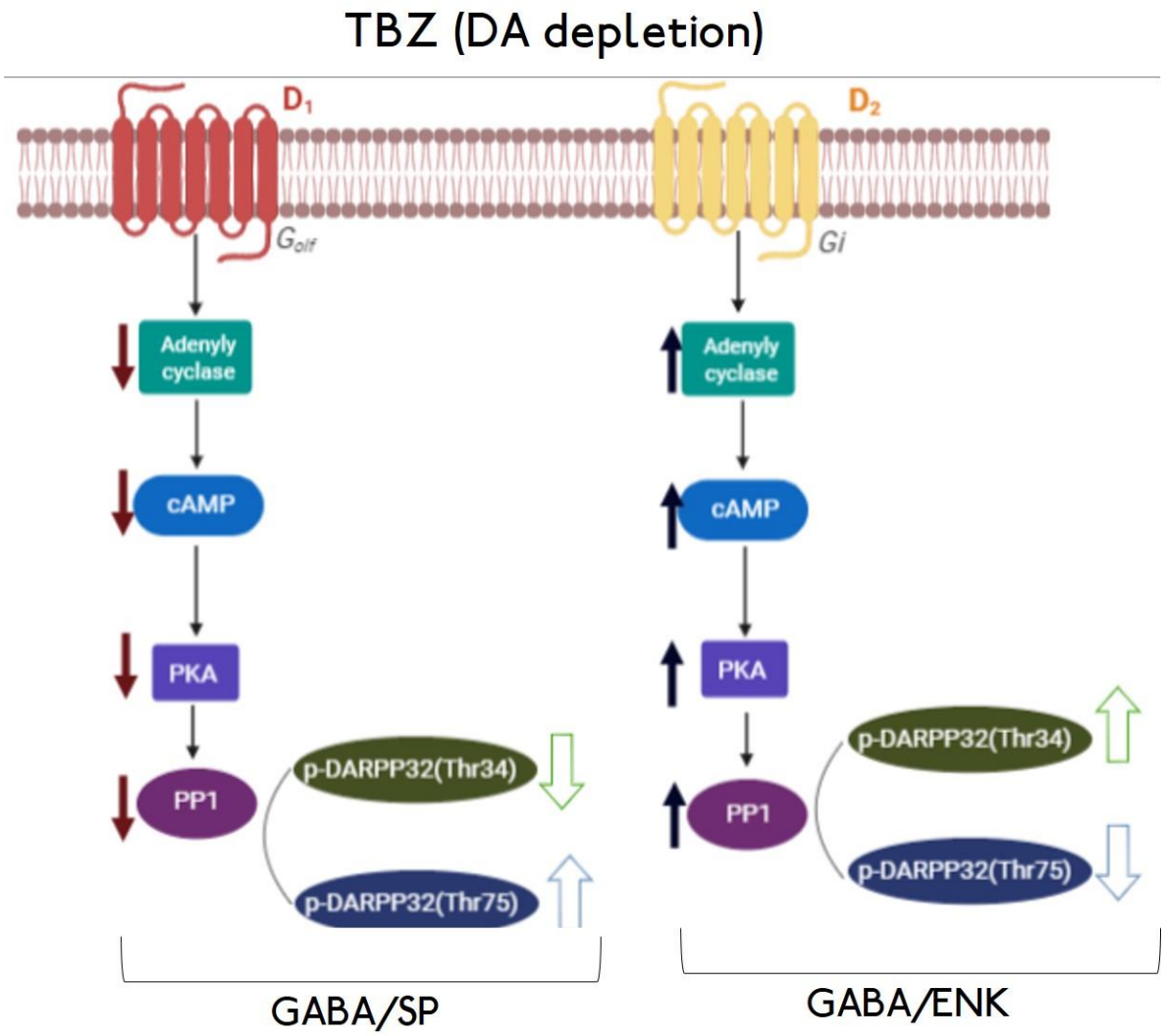

Fig 3. TBZ, which depletes DA, is hypothesized to have the opposite effect of DA, increasing pDARPP-32(Thr75) in substance P-positive neurons and pDARPP-32(Thr34) in enkephalin-positive neurons (For more details: Nunes et al, 2013; López-Cruz et al, 2018).

6. Two categories of antidepressants with different mechanisms of action differ in their therapeutical impact on anergia.

Behavioral activation and effort-related processes are important aspects of normal motivation. The impairment of these aspects, leads to disabling features of human psychopathology, specifically, in depressed people (Tylee et al, 1999; Salamone et al, 2006, 2015; Treadway and Zald, 2011). The need to develop pharmacological strategies in order to alleviate these motivational symptoms should be taken into consideration in preclinical research.

Serotonin (5-HT) selective uptake inhibitors (SSRIs) are the most commonly used antidepressants. However, it has been observed that motivational symptoms such as fatigue and anergia are resistant to SSRI treatment, and in fact, this type of antidepressant can make these symptoms worse (Sthal, 2002; Cooper et al, 2014; Fava et al, 2014; Rosthchild et al, 2014). In animal models of effort-related decision making, the use of fluoxetine, a SSRI, fails to increase behavioral activation on their own. For instance, fluoxetine (SERT blocker) and atomoxetine (NET blocker) do not increase PROG lever pressing across a wide range of doses (Yohn et al, 2016c). Atomoxetine also does not improve performance on a ratio-discounting task (Hosking et al, 2015). Moreover, these type of drugs are not able to alleviate the effort-related impairment produced by TBZ on FR5/chow-feeding tasks (Yohn et al, 2016b).

On the other hand, catecholamine uptake inhibitors seem to treat efficaciously behavioral activation impairments among people with depression (Fabre et al, 1983; Rampello et al, 1991; Pae et al, 2007; Cooper et al, 2014). This type of drugs has been tested in animal models and they result in an effective attenuation of the effort-related actions of TBZ. One of the catecholamine uptake inhibitors most used in the clinical field is bupropion also known as Wellbutrin commercially. Several studies have reported that this antidepressant is more effective than the SSRIs at treating fatigue symptoms in depression (Papakostas et al, 2006; Cooper et al, 2014). Bupropion is able to reverse the effects produced by TBZ in FR5/choice tasks (Nunes et al, 2013; Yohn et al, 2016a), PROG/choice tasks (Randall et al, 2015a) and in T-maze barrier tasks (Yohn et al, 2015a). On its own, bupropion is also able to increase the selection of high effort options in order to obtain high value reinforcers assessed in PROG/choice tasks. Moreover, it has been demonstrated that this catecholamine uptake inhibitor increases DA in Nacb and DARPP-32 immunoreactivity at both phosphorylation sites; Thr34 and Thr75 (Randall et al, 2014b).

Taking these studies into consideration, it seems that drugs acting on DA transmission appear to be effective at reversing effort-related impairments produced by TBZ and also enhances the behavioral output by its own while 5-HT inhibitors do not produce these effects (Yohn et al, 2016a).

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Objectives

Since the clinical literature seems to indicate that different classes of antidepressants that act as monoamine uptake inhibitors, differ in their therapeutical improvement of motivational symptoms, such as fatigue and psychomotor slowing, frequently seen in people with depression, the present group of studies will assess and compare the effect of two of the most used antidepressants; fluoxetine and bupropion, an SSRI and a DAT inhibitor.

We will evaluate the impact of these drugs on a mouse model of preference for voluntary running as a reinforcer that has to compete with other more sedentary options concurrently presented in the recently developed 3-choice-T-maze task (López-Cruz et al, 2018). The present dissertation studies, not only the role of these antidepressants on their own, but also in animals that had previously received a DA depleting agent. TBZ is used to induce anergia, thus mimicking this symptom present in some depressed patients.

The 3-choice-T-maze task allows the assessment of spontaneous preferences between reinforcers with different effort demands in a setting that does not involve stressful conditions. The results in this T-maze task will be compared with the results in a classical animal model to assess the therapeutic properties of antidepressants the Forced Swim Test (FST). The FST evaluates the persistence in trying to escape a stressful situation, thus it also assesses behavioral activation as an activity that is negatively reinforced. In addition, we will evaluate behavioral activation and anxiety related parameters in classical rodent models for the evaluation of anxiety (another important symptom in some depressed people).

Because DA in Nacb has been demonstrated to be a key player in behavioral activation and in effort-based decision making, phosphorylation of DARPP-32 will be studied to understand the impact of these treatments in Nacb function.

Finally, we will evaluate potential differences between male and female mice in these behavioral paradigms and in response to these drugs. Moreover, we will evaluate these sex differences in another rodent species; rats. The choice-T-maze task will be used for these rat studies.

These objectives will be treated in different chapters:

Chapter 1 studies the effect of a wide range of TBZ doses, which is known to induce depressive symptoms in humans, in measures of behavioral activation, depressive-like behaviors and anxiety. In addition, this chapter explores how the effects produced by TBZ on behavioral activation can be mimicked by manipulating reinforcement value under multiple conditions; food deprivation, aversive conditions, increase in force required, etc. This chapter has already been published: Carratalá-Ros C, López-Cruz L, SanMiguel N, Ibáñez-Marín P, Martínez-Verdú A, Salamone JD, Correa M. (2020). Preference for exercise versus more sedentary reinforcers: validation of an animal model of tetrabenazine-induced anergia. **Frontiers in Behavioral Neuroscience.** 13: 289. doi: 10.3389/fnbeh.2019.00289

Chapter 2 evaluates the impact of the catecholamine uptake inhibitor bupropion by its own and given after the DA depleting agent TBZ on behavioral activation, depressive-like behaviors and anxiety. Intracellular markers of DA receptor activity are also evaluated after the administration of TBZ-Bupropion.

Chapter 3 explores the effect of the serotonin uptake inhibitor fluoxetine on its own, or in combination with TBZ on depressive-like behaviors, behavioral activation and anxiety. In addition, this chapter also studies if the effect produced by fluoxetine on the T-maze task can be mimicked by changing the value of food. Intracellular markers of DA receptor activity will be also evaluated.

Chapter 4 compares the impact of DA depletion induced by TBZ in male and female mice on behavioral activation, depressive-like behaviors, anxiety and social interaction. In addition, this chapter also explores the effectivity of bupropion and fluoxetine in alleviating behavioral activation impairments induced by TBZ in male and female mice.

Appendix I uses the T-maze choice task in male and female rats and compares the impact of fluoxetine and a selective 5HT-2C receptor antagonists on preference for active reinforcers.

CHAPTER I

Preference for exercise versus more sedentary reinforcers: validation of an animal model of tetrabenazine-induced anergia.

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Abstract

Physical activities can have intrinsic motivational or reinforcing properties. The choice to engage in voluntary physical activity is undertaken in relation to the selection of other alternatives, such as sedentary behaviors, drugs or food intake. The mesolimbic dopamine (DA) system plays a critical role in behavioral activation or exertion of effort, and DA antagonism or depletion induces anergia in effort-based decision-making tasks. However, little is known about the neural mechanisms underlying the decision-making processes that establish preferences for sedentary versus activity-based reinforcers. In the present work with male CD1 mice, we evaluated the effect of tetrabenazine (TBZ), a DA depleting agent, on a 3-choice-T-maze task developed to assess preference between reinforcers with different behavioral activation requirements and sensory properties (i.e., a running wheel (RW) vs. sweet pellets or a neutral non-social odor). We also studied the effects of TBZ on the forced swim test (FST), which measures climbing and swimming in a stressful setting, and on anxiety tests (dark-light box; DL, and elevated plus maze; EPM). In the 3-choice task, TBZ reduced time running in the wheel but increased time spent consuming sucrose, thus indicating reduced activation but relatively intact sucrose reinforcement. The effect of TBZ was not mimicked by motivational manipulations that change the value of the reinforcers, such as making the RW aversive or harder to move, food-restricting the animals, inducing a binge-like eating pattern, or introducing social odors. In the FST, TBZ decreased time climbing (most active behavior), and increased immobility, but did not affect anxiety in the DL or EPM. These results indicate that the 3-choice-T-maze task could be useful for assessing DA modulation of preferences for exercise based on activation and effort requirements, differentiating those effects from changes in preference produced by altering physical requirements, food-restriction state, and stress during testing.

Dopamine, Accumbens, voluntary exercise, reward, depression, binge eating, aversion.

Introduction

Motivated behavior is characterized by a high degree of behavioral activation, as demonstrated by the speed, vigor or persistence seen in the instigation and maintenance of instrumental responding (Salamone and Correa, 2002, 2012; Robbins and Everitt, 2007; Mai et al, 2012; McGinty et al, 2013; Floresco, 2015). Animal research has demonstrated that this activational aspect of motivation is partly regulated by the mesolimbic dopamine (DA) system (Salamone et al., 2006, 2016, 2018). In particular, nucleus accumbens DA and related neural systems have been implicated in motivational dysfunctions such as anergia and fatigue seen in many neurological and psychological pathologies (Stahl 2002; Salamone and Correa, 2002, 2012; Treadway et al, 2012).

Diverse tasks have been used in rodents for evaluating behavioral activation and effort-related decision making, including tasks that give animals the option of vigorously working (lever pressing or climbing a barrier) to obtain access to more highly valued reinforcers versus approaching and consuming a less preferred reinforcer (Cousins et al, 1994; Salamone et al, 2002, 2016; Mott et al, 2009; Pardo et al, 2012, 2015; Randall et al, 2012; Mai et al, 2012; Sommer et al, 2014; Yohn et al, 2015a,b; SanMiguel et al., 2018; Correa et al., 2018). In these tasks, conditions that alter DA transmission, such as administration of DA antagonists or tetrabenazine (TBZ), can alter behavioral activation and reduce selection of high effort choices in rats (Nunes et al, 2013; Randall et al, 2014; Pardo et al, 2015; Yohn et al, 2015a,b, 2016a; Hosking et al, 2015; Contreras-Mora et al, 2018; Rotolo et al, 2019). TBZ acts by 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 at low doses being on striatal DA 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, including 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 et al., 2010) improved motor deficits, but increased depression-like measures in the forced swim test (FST).

The FST is the classical rodent task for assessing the antidepressant properties of many substances. This test is based on the observation that rodents exposed to a stressful non-escapable situation such a deep tank full of water initially try to escape in a vigorous way, but eventually they will cease these vigorous attempts and will instead passively float in the chamber (Porsolt et al, 1977). Thus, immobility is the classical parameter that is typically measured, and antidepressant drugs have repeatedly been shown to reduce immobility time (Porsolt et al, 1977; Armario et al, 1988; Costa et al, 2013). However, escape-related mobility such as climbing or struggling are active behaviors that also are modified by antidepressant drugs (Armario et al, 1988; Lucki 1997). These active behaviors are more likely to be affected by DA manipulations than the traditional immobility measure (Gil and Armario, 1998; Costa et al, 2013). Thus, drugs that affect dopaminergic transmission such as haloperidol have been shown to decrease climbing or struggling behavior in rats (Gil and Armario, 1998). However, TBZ has not been assessed in relation to active behaviors such as climbing or struggling in the FST.

Recently, a T-maze choice task has been developed that assesses the impact of drugs on behavioral activation and effort-related choice (Correa et al, 2016; López-Cruz et al, 2018). This task allows the animal to freely choose between running on a wheel, consuming sucrose pellets, or sniffing a neutral non-social odor. Although foods high in sucrose are generally highly preferred, inducing strong hedonic reactivity in rodents (Berridge, 2000; Levine et al, 2003), voluntary wheel running occurs in mice of all strains, sexes, and ages (Walker and Mason, 2018). Previous studies in our laboratory have demonstrated that adult male CD1 mice strongly preferred the RW in the T-maze choice task, and that DA antagonism or DA depletion partially shifted choice behavior, reducing time running but increasing time eating sucrose (Correa et al, 2016; López-Cruz et al, 2018). This T-maze task does not involve the use of food restriction, as is the case with other effort-based choice tasks like the T-maze-barrier-climbing task for food reinforcement (Pardo et al., 2012; Correa et al., 2018), and does not involve stressful conditions, such as the FST. Moreover, since mice have a high preference for engaging in physical activities (Routtenberg et al, 1968; Correa et al, 2016; López-Cruz et al, 2018) the present task offers a good test for the evaluation of the brain mechanisms involved in the decision-making processes that establish spontaneous preference for active and vigorous behaviors.

In the present work, we explored the impact of a broad range of TBZ doses that have been demonstrated to deplete accumbens DA in mice (López-Cruz et al, 2018), comparing their impact on different measures of behavioral activation induced by stressful conditions (FST), or by spontaneous preference for physical activity in the T-maze-choice task. In addition, we explored if the ability of TBZ to reduce RW preference and increase palatable food preference can be mimicked by manipulating reinforcement value of the various conditions. Thus, we did a series of behavioral manipulations that attempted to increase the value of the sweet pellets by food restricting the animals prior to the T-maze tests or, in another group of animals, by inducing a binge-like eating pattern after randomly presenting sweet pellets during short sessions several days a week previously to the T-maze sessions. We also increased the potential value of the odor stimulus by using conspecific same or different sex odors. Finally, we reduced RW value increasing the resistance of the wheel or introducing an anxiogenic light over the RW. The effect of TBZ on anxiety paradigms (dark/light box, DL; and elevated plus maze, EPM) was also assessed in order to identify anxiolytic-like actions that could be affecting the results in the T-maze and the FST.

Materials and Methods

Animals. CD1 adult male mice (N=166) purchased from Janvier, France S.A. were 8-10 weeks old (30-40 g) at the beginning of the study. Mice were housed in groups of three or four per cage, with standard laboratory rodent chow and tap water available ad libitum. The colony was kept at a temperature of 22 + 2 ºC with lights on from 08:00 to 20:00 h. All procedures were covered by a protocol approved by the Institutional Animal Care and Use committee of 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 Behavioral 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, CIMYT Quimica SL, Spain) was dissolved in a vehicle solution of 0.9% saline (80%) plus dimethylsulfoxide (DMSO 20%, final pH 5.5), and administered 120 minutes before testing. Time elapsed after injection

and range of doses was selected based on previous work (López-Cruz et al., 2018; Correa et al., 2018) and pilot studies demonstrating that in mice this is an optimal dose and time lead to deplete DA. DMSO (20% v/v) was used as the control group. TBZ and DMSO was administered intraperitoneally (IP).

Testing procedures.

All behavioral procedures started two hours after the light period onset. The behavioral test room was illuminated with a soft light, and external noise was attenuated.

T-maze RW-sucrose-odor choice task. The T-maze apparatus consisted of a central area that leads to 3 arms (see figure 1). In one of them, sucrose pellets (TestDietTM, 50% sucrose, 45 mg 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). This concentration of sucrose in the pellets was selected after piloting different concentrations (i.e. 100% sucrose pellets generate avoidance), and the non-social odor used for the present studies (strawberry) was the one that generated more exploration among non-social odors in previous studies (López-Cruz et al, 2017). In general, mice were allowed to freely explore and interact with the stimulus during 15 minutes sessions, once a day, 5 days a week. Training phase 1: to avoid neophobia to the sweet tasting pellets, animals were enclosed in that arm with the food during 5 sessions (no exploration of the other arms was allowed during this phase). Training phase 2: during 2 weeks animals had free access to the three stimuli until a stable baseline was obtained. Test phase: This phase lasted during 4 more weeks. For each week, there were 4 baseline drug free sessions plus a testing session in which animals received TBZ or were tested under different behavioral manipulations. The day before the test for the behavioral manipulation was considered as the baseline (BL) for those experiments. Sessions were videotaped and a trained observer manually registered several parameters. 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 Rachlin, 1969). Entries into the arms and time spent in the arms of the T-maze were also simultaneously recorded. All these measures were taken based on previous studies (Correa et al., 2016; López-Cruz et al., 2018).

Forced swim test (FST). This paradigm is considered to be a model of behavioral despair and is used as a test for assessing depressive-like states (Porsolt et al, 1977). Naïve mice were placed in a transparent cylindrical glass tank (26 cm high and 18 cm diameter) filled with water (14 cm) and maintained at a temperature of 25ºC. Water was changed between animals. During the 6-minute 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 to balance the body and keep the head above the water. In addition, we also assessed escape-related mobility behavior such climbing or struggling (Armario et al, 1988). 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 a box with absorbing paper under a warming light, and were monitored for 10 minutes.

Dark and light box (DL). The DL test is based on the conflict between the tendency to explore a novel environment and the avoidance of a brightly lighted open area (Blumstein and Crawley 1983). The DL apparatus consisted of a polypropylene chamber divided in two compartments by a partition containing a small opening (5 cm H x 5 cm W). The light compartment (25 cm W x 25 cm H x 25 cm L) was open, painted in white and illuminated with an intense light (335 lx), while the dark compartment (25 cm W x 25 cm H x 18 cm L) was painted in black and had a removable ceiling to close it (Kulesskaya and Voikar, 2014). To start the test session, mice were individually placed in the dark chamber facing one corner. Test sessions were videotaped. Total number of crosses between compartments as an index of locomotion, and total time spent in the lit chamber, as an index of anxiogenesis were recorded for 5 min (procedure is based on López-Cruz et al., 2014).

Elevated plus maze (EPM). After being in the DL box for 5 minutes, animals were placed in the EPM for 5 more minutes. The EPM consists of two open and two enclosed arms (65 cm L x 5 cm W) arranged in a plus configuration with an intersection in a central platform. It was made of black polypropylene and is elevated 50 cm above the floor. The open arms had a 1 cm border around their perimeter and the closed arms had a 20 cm translucent wall. This anxiety paradigm measures the avoidance that rodents show to

elevated open spaces (Lister 1987; Walf and Frye 2007). Under normal conditions, mice spend more time and make more entries into the closed arms of the maze. Animals were placed in the central platform with their head pointing at one enclosed arm, and they were assessed during 5 minutes. Sessions were videotaped and a trained observer registered total time spent in the open arms, and total entries in the 4 arms as an index of locomotion. An entry into an arm was recorded when the animal crossed with all four legs the line that connected that arm with the central platform (procedure is based on López-Cruz et al., 2014).

Experiments.

Table 1 summarizes the experiments conducted, the experimental design and number of mice used in each experiment.

Pharmacological or Behavioral manipulation	Experimental design	Control condition
TBZ effect:		
T-maze	N=13. Within groups	Vehicle day
FST.	N=47. Between groups	Vehicle group
DL, EPM	N=41. Between groups	Vehicle group
Change in relative value of sweet pellets:		
Standard food restriction	N=8. Within groups	BL with no food restriction
Binge-eating	N=25. Between groups	Group exposed to standard food
Change in the relative value of the odor: Social odors	N=10. Within groups	BL with non-social odor
Change in the relative value of the RW:		BL with no extra weight

Increase in RW resistance	N=8. Within groups	BL with no light over RW
Light over the RW	N=14. Within groups	

Table 1. Experimental design and number of mice used in each experiment. (FST, Forced swim test; DL box, Dark/Light box; EPM: elevated plus maze).

Experiment 1. Effect of TBZ on preference for active reinforcers as measured in the 3-choice-T-maze task. After reaching a stable baseline level of performance in the T-maze, animals (n=13) received vehicle or the DA depleting agent TBZ (vehicle, 4.0, 6.0 and 8.0 mg/kg) 120 min before the test began. Animals received one dose of the drug every week in a randomly varied order. In previous work from our laboratory using rats and mice, we have observed that TBZ does not produce sensitization or tolerance when administered once a week; animals recover their behavioral baseline performance after few hours of the acute intraperitoneal injection. The T-maze paradigm requires a baseline performance of two weeks before tests start, and that performance is maintained across weeks, thus allowing a repeated measures design.

Experiment 2. Manipulations that change the relative value of the sweet pellets.

Experiment 2.1. Effect of standard food restriction in the home cage. Animals (n=8) were trained and, after reaching stable levels, they were food restricted to 4.0 mg of standard chow the night before the test, in order to increase their appetite for the sucrose pellets. This amount of standard food was selected based on previous studies using food restriction (Pardo et al, 2012; Yang et al, 2019). Baseline with *ad libitum* food was used as the control condition.

Experiment 2.2. Binge-eating like consumption of sweet pellets. Before the T-maze testing took place, during 4 weeks, at intermittent random days (3 days a week in average), non-food restricted mice (n=12) were placed individually in standard home cages with free *ad libitum* access to sweet pellets (50% sucrose) and water for one hour during the light cycle (based on Murphy et al., 2018). The control group **(n=13)** was also placed in the same type of cages for an hour with standard food and water. After these 4 weeks, mice were trained in the T-maze.

Experiment 2.3. Effect of TBZ and of increasing food value on the amount of sweet pellets consumed in the T-maze. The effect of the highest dose of TBZ (8.0 mg/kg) vs. vehicle from experiment 3 was used in order to make comparisons with the effects of food restriction and binge-like eating exposure on milligrams of sucrose pellets consumed on the test day. The effects of food restriction and binge-like eating exposure were compared to their respective control groups. Thus, all these data were obtained from experiments 3, 5.1, and 5.2.

Experiment 3. Manipulations that change the relative value of the odor: effect of social odors. Animals (n=10) were trained as in previous experiments and on the test day, a social odor (bedding from male or from female conspecifics) replaced the strawberry odor during two consecutive days: in the first session, male conspecific odor was used and, the following day, female mice odor replaced the male odor. Estrous cycle of the females was not controlled. Baseline was taken with the non-social odor and was used as the control condition.

Experiment 4. Manipulations that change the relative value of the RW.

Experiment 4.1. Effect of increasing RW resistance. Mice (n=8) were trained as in previous experiments. The test was performed during 2 consecutive days: in the first day weights were attached to the wheel so that the resistance increased 75%, and for the second day additional weights increased resistance to 95%. Before increasing the resistance of the wheel, behavior of animals in the T-maze was established as baseline and used as control condition.

Experiment 4.2. Effect of an intense light over the RW. Mice (n=14) were trained normally in the T-maze and for the test day a bright light; like the one used in the DL box) was placed over the RW in order to add an aversive component. Baseline with standard test lighting conditions was used as the control condition.

Experiment 5. Effect of TBZ on depressive-like behaviors assessed in the FST. Naïve animals (n=47) received one dose of TBZ or vehicle (vehicle, 4.0, 6.0 or 8.0 mg/kg) and 120 min after the injection were placed in the FST. Mice were exposed only once to the FST since behavioral habituation develops in one session.

Experiment 6. Effect of TBZ on anxiety parameters as measured in the DL and EPM paradigms. Mice (n=41) received one dose of TBZ (0, 4, 6 or 8 mg/kg) 120 min before the test began. Animals were first place in the DL box for 5 minutes, and immediately after this test, they were placed in the EPM for 5 more minutes. Mice were exposed only once to both paradigms, since behavioral habituation develops in one session.

Statistical Analyses

Normally distributed and homogenous data (according to Kolmogorov-Smirnov test) for the FST, DL, and EPM experiments employed a between groups design and data were analyzed by one-way repeated measures ANOVA. Data for the key dependent variable in the T-maze experiments**;** time with the three reinforcers, was analyzed by a MANOVA in previous work (Correa et a., 2016). Because the three-way interaction was significant, univariate ANOVAs were conducted for each reinforcer in all the dependent variables. Since, in the present work baselines are consistent among the 3 stimuli and they are also consistent across experiments, we performed univariate ANOVAs. Thus, **n**ormally distributed data in the T-maze experiments followed a within groups design with the exception of the binge-eating experiment, which used two different groups of animals. Thus, when more than 2 experimental conditions were used such as in the TBZ experiment, the experiment with increasing RW resistance, and the social odors experiments, data were analyzed by repeated measures ANOVA. When the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used to compare each treatment with the vehicle control group (Keppel, 1991). For these comparisons, α level was kept at 0.05 because the number of comparisons was restricted to the number of treatments minus one. The effects of intense light over the RW, and food restriction, were evaluated by Student's t-test for dependent samples. Binge-like eating of sweet food was analyzed by Student's t-test for independent samples. All data were expressed as mean ± SEM, and significance was set at $p<0.05$. STATISTICA 7 software was used.

Results

Experiment 1. Effect of TBZ on preference for active reinforcers as measured in the 3-choice-T-maze task. Repeated measures ANOVA showed that total time spent sniffing

the neutral odor was not significant (F(3,36)=0.29, p=0.83). However, TBZ produced a significant effect on time spent eating (F(3,36)=3.46, p<0.05), and time spent running in the RW (F(3,36)=2.99, p<0.05) (Figures 2A-C). Planned comparisons revealed that all doses of TBZ produced a significant increase in time consuming sucrose pellets in comparison with the vehicle group (p<0.05 for 4.0 and 6.0 mg/kg, and p<0.01 for 8.0 mg/kg). Planned comparisons also showed that mice treated with 8.0 mg/kg TBZ spent significantly less time running in the RW compared to vehicle group (p<0.01). Repeated measures ANOVA showed that total time spent in the RW compartment (F(3,36)=0.98, p=0.40), the food compartment (F(3,36)=1.11, p=0.35), and the neutral odor compartment (F(3,36)=0.89, p=0.45) were not significant (Figure 2D). Finally, TBZ did not produce significant differences in the number of entries into the RW compartment (F(3,36)=0.98, p=0.40), food compartment (F(3,36)=0.70, p=0.55), and neutral odor compartment (F(3,36)=0.46, p=0.71) (data shown in Figure 2E).

FIG 2

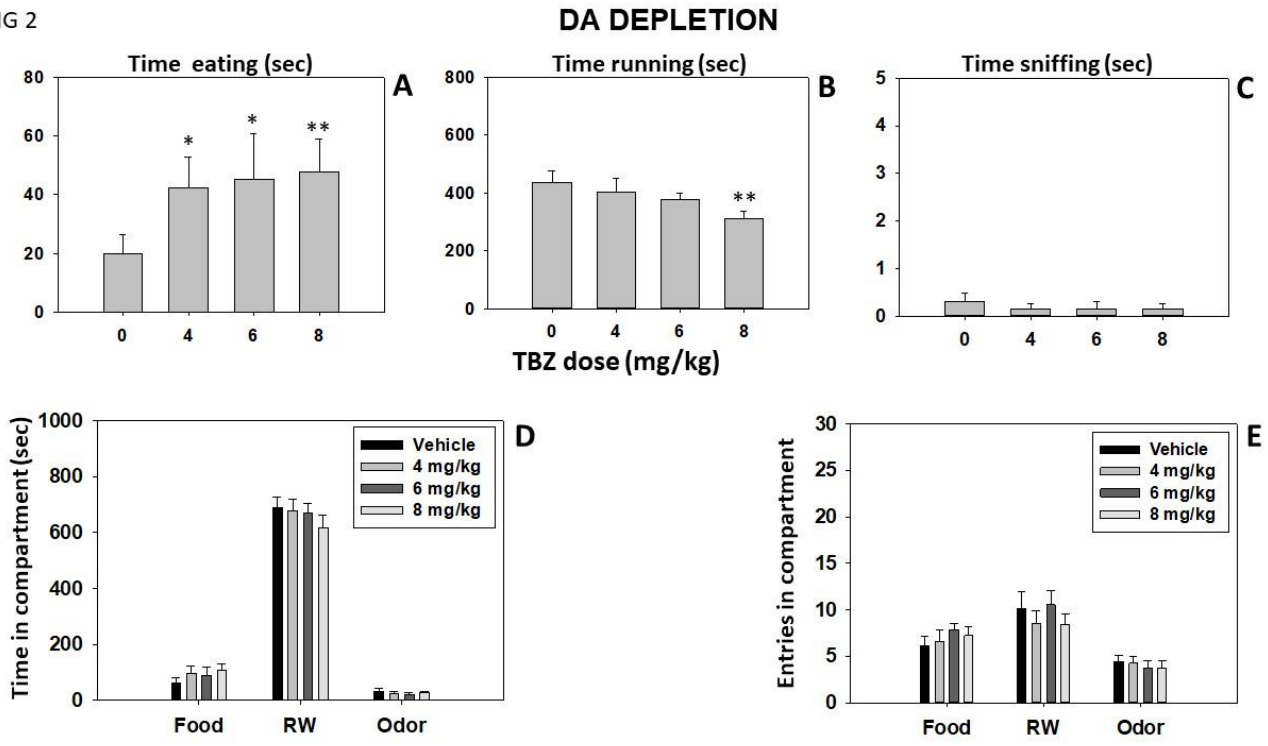


Figure 2. Effect of tetrabenazine (TBZ) (Vehicle, 4, 6 and 8 mg/kg) on time eating (A), time running (B), time sniffing (C), time spent in each compartment (D) and entries into compartments (E) in the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. *p<0.05, **p<0.01 significantly different from vehicle.

Experiment 2. Manipulations that change the relative value of the sweet pellets.

Experiment 2.1. Effect of standard food restriction in the home cage. Food restriction significantly decreased time in RW, and significantly increased time eating compared with BL condition as showed by Student's t-test for dependent samples ($t=4.95$, $p<0.01$ and $t=-4.24$, $p<0.01$ respectively). However, there was no significant difference between conditions on time spent sniffing the neutral odor ($t=-0.42$, $p=0.68$) (Figures 3A-C). Student's t-tests for time in each compartment showed significant differences between both conditions in time spent in food compartment ($t=-3.10$, $p<0.01$), and RW compartment ($t=3.22$, $p<0.01$), but no significant effect on time in the odor compartment ($t=1.50$, $p=0.17$) (Figure 3D). Finally, there were no significant differences in total entries to the food compartment ($t=-0.84$, $p=0.42$), to the RW compartment ($t=0.30$, $p=0.76$), and to the odor compartment ($t=2.19$, $p=0.06$) (Figure 3E).

FIG 3

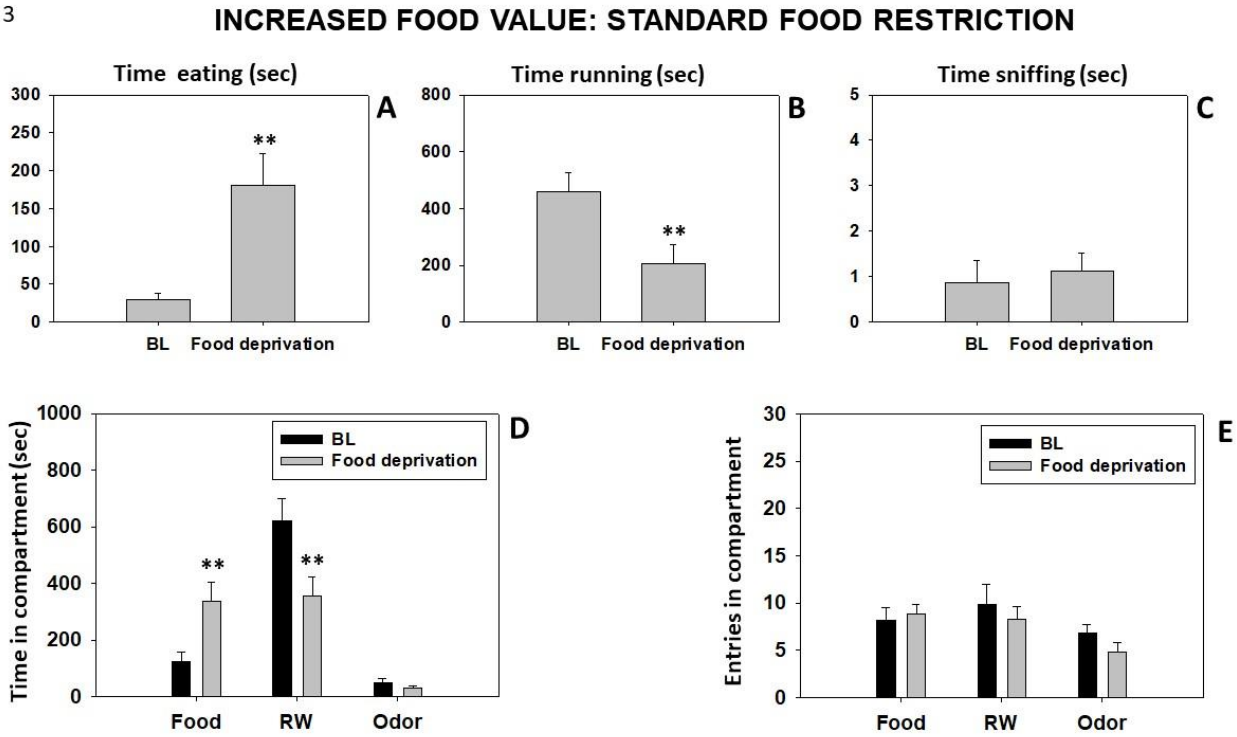


Figure 3. Effect of home food restriction in mice behavior in the T-maze task. Bars represent mean (±SEM) of time (seconds) spent eating (A), running (B), and sniffing the neutral odor (C), time in different compartments (D) and entries (E) during a 15 minutes session. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. **p<0.01 significantly different from baseline (BL).

Experiment 2.2. Binge-like consumption of sweet pellets. A Student's t-test for independent samples showed a significant increase in time eating sucrose pellets ($t=-5.60$, $p<0.01$), and time sniffing the neutral odor ($t=-4.68$, $p<0.01$), but a significant decrease in time running in the RW ($t=3.92$, $p<0.05$) compared with the BL condition (Figures 4A-C). Moreover, Student's t-test for time in each compartment showed significant differences between both conditions for time spent in the food compartment ($t=5.60$, $p<0.01$), the RW compartment ($t=5.99$, $p<0.01$) and the odor compartment ($t=4.68$, $p<0.01$) (Figure 4D). Finally, there were no significant differences in total entries to the food compartment ($t=-1.88$, $p=0.08$), and the RW compartment ($t=1.06$, $p=0.30$), but there was a significant increase in total entries to the odor compartment ($t=-2.75$, $p<0.01$) (Figure 4E).

FIG 4

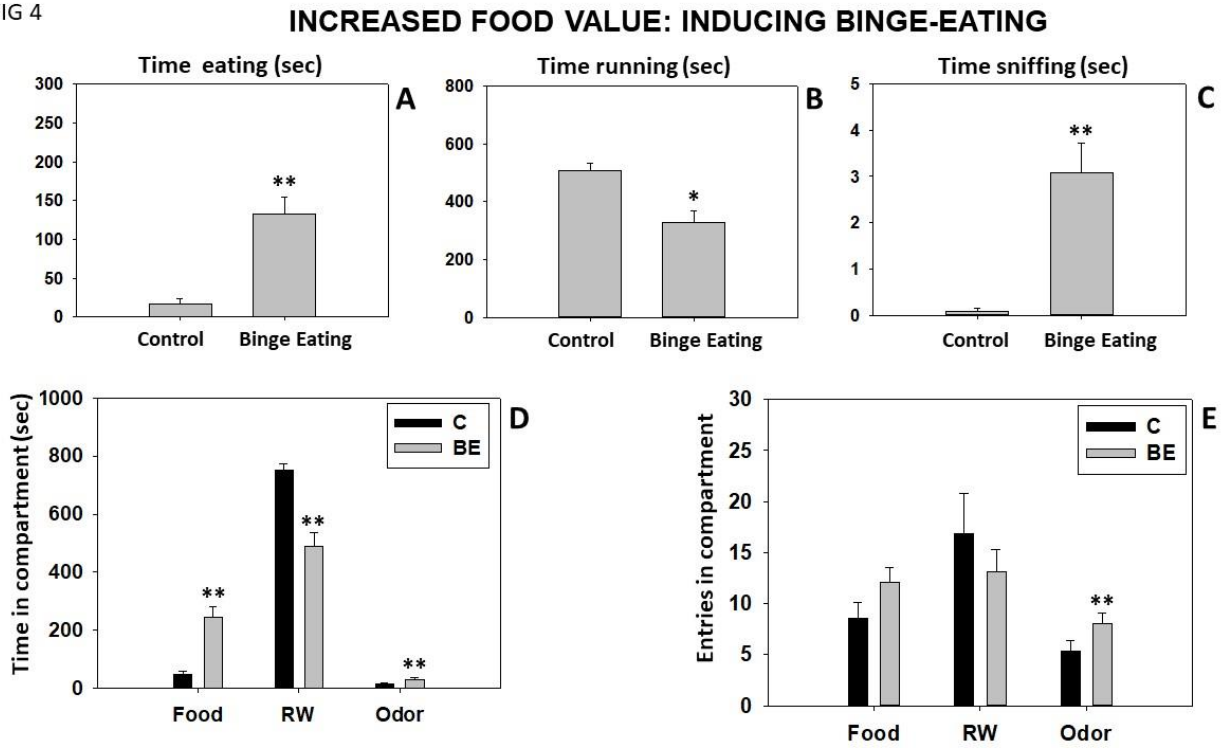


Figure 4. Effect of binge eating pattern for sucrose pellets on time eating (A), time running (B), time sniffing (C), time spent in each compartment (D) and compartment entries (E) in the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. *p<0.05, **p<0.01 significantly different from control group.

Experiment 2.3. Effect of TBZ and changes in food value on the amount of sweet pellets consumed in the T-maze. The Student's t-test for dependent samples did not show significant differences between the vehicle condition and the TBZ 8.0 mg/kg condition ($t=-0.75$, $p=0.46$). However, Student's t-test for dependent samples showed that there were significant differences among conditions in the experiment in which animals were food deprived ($t=3.05$, $p<0.01$). Similarly, the Student's t test for independent samples showed significant differences between the control group and the binge-like eating group ($t=-3.84$, $p<0.01$). Data are shown in Table 2.

	Pellet intake (mg)	
	Control	**Experimental condition**
TBZ (Vehicle and 8.0 mg/kg)	169.6 ± 48.1	228.4 ± 50.5
Food restriction	157.7 ± 32.9	736.1 ± 235.8**
Binge-eating	301.1 ± 61.4	684.6 ± 82.5**

Table 2. Effect of tetrabenazine (TBZ) and behavioral manipulations that increased food value on pellets consumed in the T-maze. Mean (± SEM) of milligrams consumed. **p<0.01 significantly different from their corresponding control condition.

Experiment 3. Manipulations that change the relative value of the odor: effect of social odors. Repeated measures ANOVA showed an overall effect of odor type on time sniffing ($F(2,18)=85.98$, $p<0.01$). Planned comparisons revealed a significant increase in time sniffing the same-sex conspecific and female odors (both $p<0.01$) (Figure 5C). The ANOVA also yielded an overall effect on time running in the RW ($F(2,18)=7.34$, $p<0.01$), and the planned comparisons showed a significant difference between BL and the female odor condition ($p<0.01$) (Figure 5B). However, there was not an effect of type of odor on time spent eating ($F(2,18)=0.61$, $p=0.55$) (Figure 5A). For the time spent in the various compartments, the repeated measures ANOVA showed an overall effect on time in the odor compartment ($F(2,18)=23.45$, $p<0.01$), and time in the RW compartment ($F(2,18)=8.99$, $p<0.01$). Planned comparisons demonstrated a significant increase in time spent in the odor compartment when the odor was male or female ($p<0.05$, and $p<0.01$ respectively), and there was a significant decrease in time spent in the RW compartment

only when the odor was female ($p<0.01$). There was not an effect of odor type on time spent in the food compartment ($F(2,18)=2.08$, $p=0.15$) (Figure 5D). Finally, repeated measures ANOVA showed an overall effect of social odors on entries to the RW compartment ($F(2,18)=4.31$, $p<0.05$), entries to the food compartment ($F(2,18)=4.50$, $p<0.05$), and entries to the odor compartment ($F(2,18)=6.35$, $p<0.01$). Planned comparisons showed an increase in entries to the food compartment when the male or the female odors were used as compared with the BL ($p<0.05$), and the same was observed for the compartment where the odor was present ($p<0.05$ for the male odor, and $p<0.01$ for the female odor both compared to BL). However, the increase in total entries to the RW compartment increased only when the female odor was present in comparison with the neutral odor condition ($p<0.05$) (Figure 5E).

FIG 5

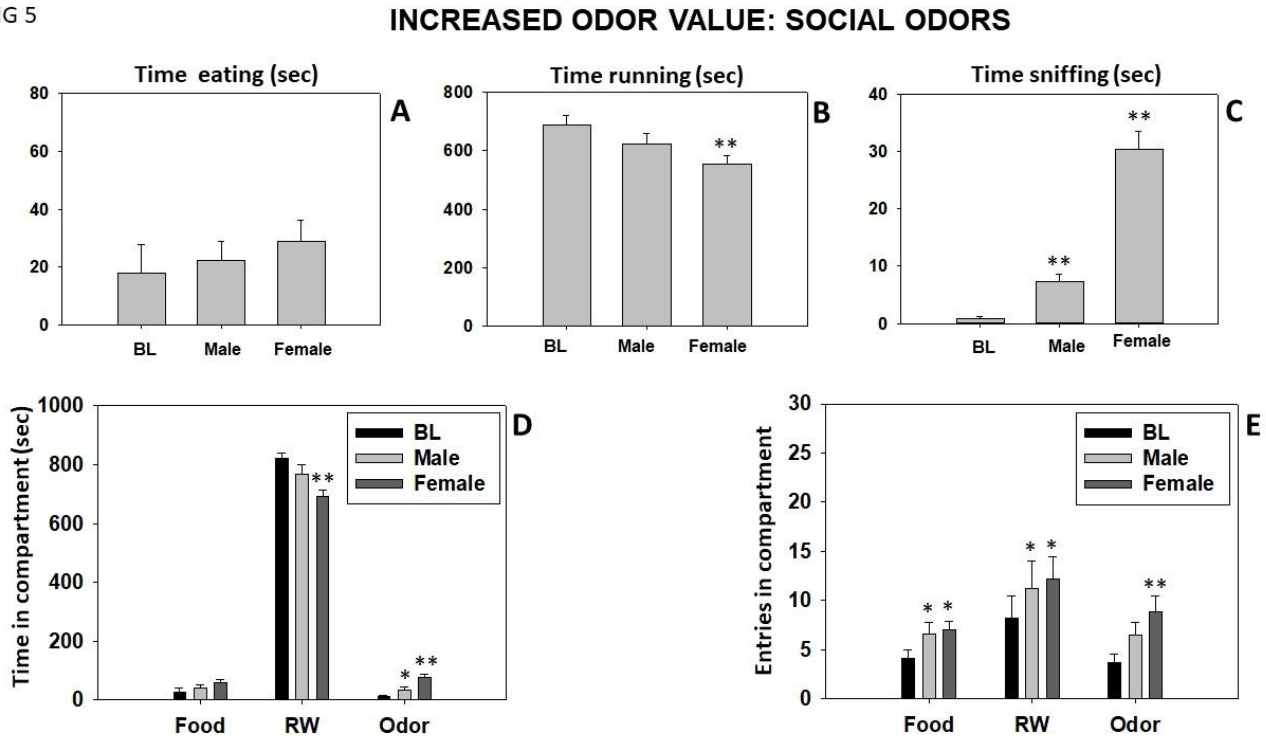


Figure 5. Effect of changing from fruit odor to social odors in the odor compartment of the T-maze task on; time eating (A), time running (B), time sniffing (C), time spent in each compartment (D) and entries into compartments (E) in the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. *$p<0.05$, **$p<0.01$ significantly different from baseline (BL).

Experiment 4. Manipulations that changed the relative value of the RW.

Experiment 4.1. Effect of increasing RW resistance. Repeated measures ANOVA did not show a significant effect of increasing RW resistance on time eating ($F(2,10)=1.03$, $p=0.38$), or time sniffing the neutral odor ($F(2,10)=0.40$, $p=0.68$). However, RW resistance produced a significant effect on time running in the RW ($F(2,10)=5.12$, $p<0.05$). Planned comparison showed a decrease in time running in the RW when the resistance was the highest (95%) in comparison with BL condition ($p<0.01$) (Figure 6A-C). Increasing the RW resistance did not yield a significant effect on time spent in the food compartment ($F(2,10)=3.71$, $p=0.06$), time spent in the RW compartment ($F(2,10)=2.12$, $p=0.17$), or time spent in the odor compartment ($F(2,10)=2.64$, $p=0.11$) (Figure 6D). Although repeated measures ANOVA did not show an overall effect of increasing RW resistance on total entries to the RW compartment ($F(2,10)=0.96$, $p=0.40$), it did yield a significant effect on entries to the food compartment ($F(2,10)=4.89$, $p<0.05$), and to the odor compartment ($F(2,10)=10.88$, $p<0.01$). Planned comparisons showed a decrease in total entries after applying 75% more resistance to the RW in comparison with the BL condition in the odor compartment (Figure 6E).

FIG 6

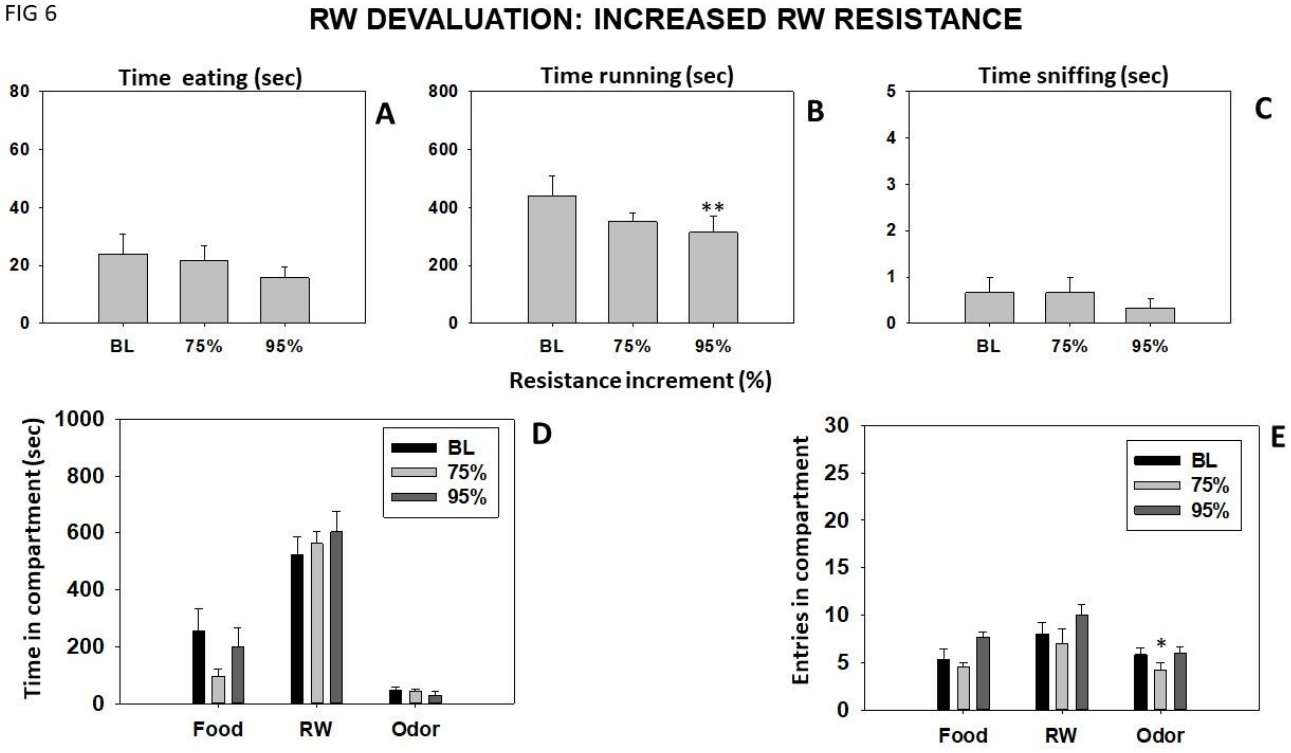
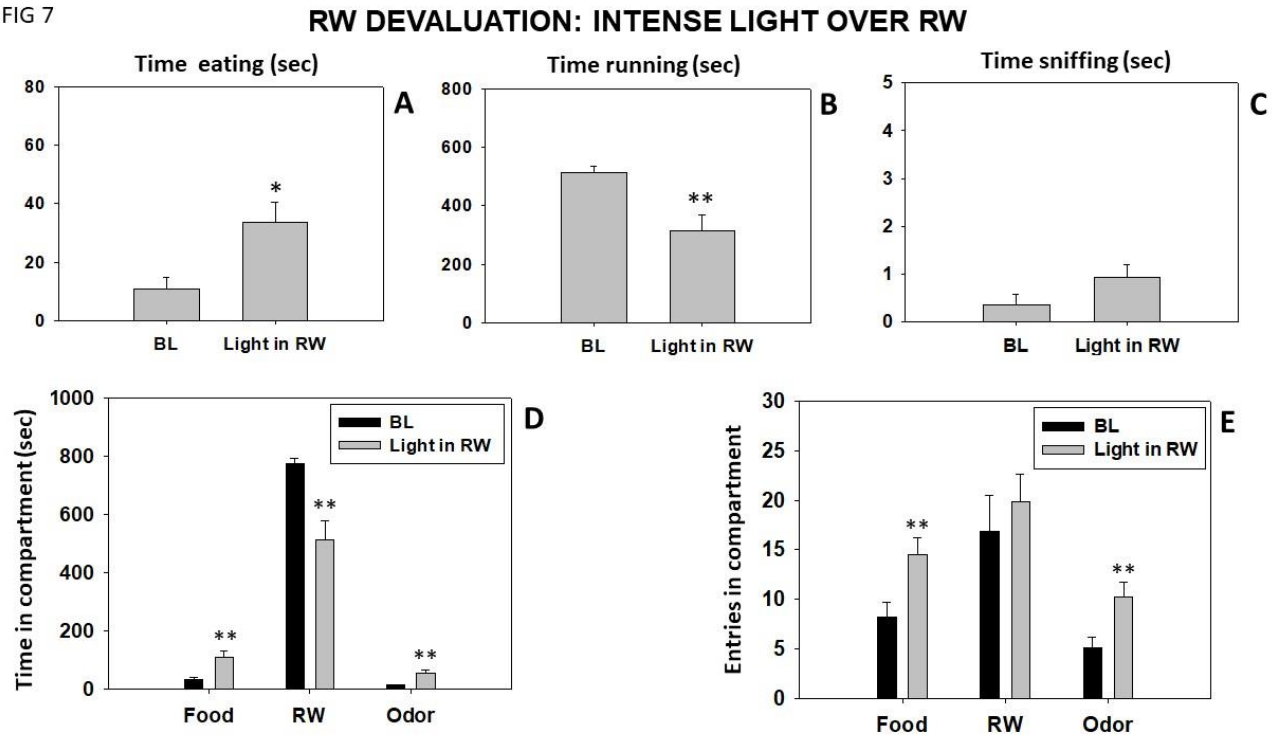


Figure 6. Effect of increasing RW resistance on mice behavior in the T-maze task. Bars represent mean (±SEM) of time (seconds) eating (A), running (B), and sniffing the neutral odor (C), time spent in each compartment (D) and compartment entries (E) during a 15 minutes session. Bars

represent mean ± S.E.M. of accumulated seconds or number of entries. *p<0.05, **p<0.01 significant different from baseline (BL).

Experiment 4.2. Effect of an intense light over the RW. The bright light condition significantly decreased time that animals spent running in the RW (t=2.98, p<0.01), and increased time eating the sucrose pellets compared with the BL condition (t=-2.38, p<0.05) as shown by the Student's t-test for dependent samples. However, the increase in sniffing time did not reach statistical significance (t=-1.37, p=0.19) (Figures 7A-C). Student's t-test for time in these compartments showed a significant decrease in time spent in the RW compartment (t=3.44, p<0.01), and a significant increase in time spent in the food (t=-2.89, p<0.01), and in the odor (t=-3.60, p<0.01) compartments (Figure 7D). Finally, the t-tests for dependent samples showed an increase on entries to the food compartment (t=-4.03, p<0.01), and to the odor compartment (t=-3.14, p<0.01), although there were no significant differences in entries to the RW compartment compared with the BL condition (t=-0.83, p=0.41) (Figure 7E).

FIG 7



Figures 7. Effect of placing an intense light over the RW on time eating (A), time running (B), time sniffing (C), time spent in each compartment (D) and entries into compartments (E) in the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. *p<0.05, **p<0.01 significantly different from baseline (BL).

Experiment 5. Effect of TBZ on depressive-like behaviors assessed in the FST. The ANOVA for time spent swimming did not show significance ($F(3,43)=1.90$, $p=0.14$). However, TBZ produced a significant effect on immobility ($F(3,43)=4.02$, $p<0.01$), and on climbing ($F(3,43)=3.86$, $p<0.01$). Planned comparisons revealed that the groups that received the two highest doses of TBZ (6.0 and 8.0 mg/kg) displayed significantly more time immobile ($p<0.05$ and $p<0.01$, respectively) and less climbing ($p<0.01$) in comparison with the vehicle group (Figure 8).

FIG 8

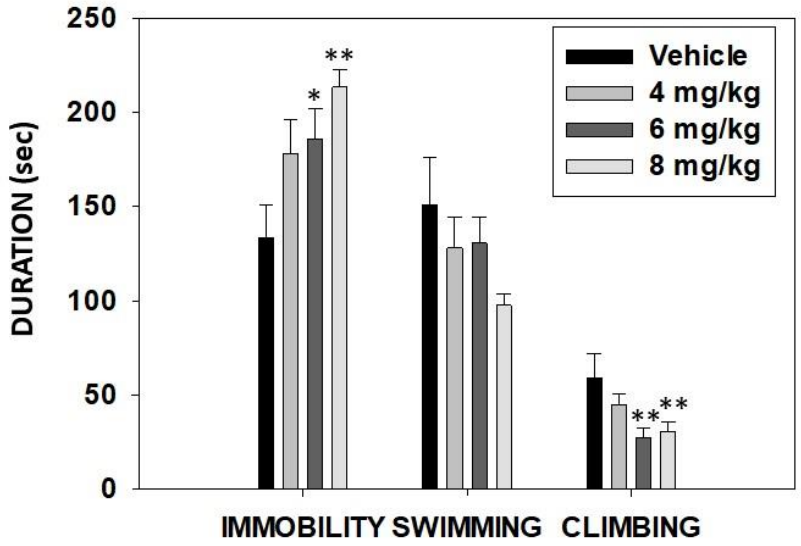


Figure 8. Effect of tetrabenazine (TBZ) (Vehicle, 4, 6 or 8 mg/kg) on duration of immobility, swimming and climbing behavior in the FST assessed during 6 minutes. Bars represent mean ± S.E.M. of accumulated seconds. *$p<0.05$, **$p<0.01$ significantly different from vehicle.

Experiment 6. Effect of TBZ on anxiety parameters as measured in the DL and EPM paradigms. The ANOVAs did not show any significant effects on time spent in the illuminated arena of the DL box ($F(3,37)=2.22$, $p=0.10$), or time spent in the open arms ($F(3,38)=0.68$, $p=0.56$) of the EPM, both of which are indices of anxiety. However, TBZ did have a significant effect on the total number of crosses between compartments in the DL box ($F(3,37)=5.69$, $p<0.01$), and total number of crosses to all arms in the EPM

(F(3,38)=6.38, p<0.01). Planned comparisons revealed that the groups that received TBZ 6.0 and 8.0 mg/kg displayed significantly fewer crosses than vehicle group (p<0.01) in the DL box, but only the group that received the highest dose of TBZ (8.0 mg/kg) displayed fewer crosses (p<0.01) than vehicle group in the EPM. The total number of crosses assessed in the DL box and EPM can be considered as an index of locomotion. Data are shown in Table 3.

		TBZ (mg/kg)		
	Vehicle	**4**	**6**	**8**
DL				
Time in light compartment (sec)	125.6 ± 6.1	116.8 ± 8.9	84.5 ± 13.5	117.1 ± 11.4
Crosses between compartments	23.6 ± 1.6	20.2 ± 1.8	17.2 ± 4.1**	12.6 ± 1.4**
EPM				
Time in open arms (sec)	79.2 ± 13.7	75.2 ± 13.9	82.2 ± 8.5	95.9 ± 12.6
Crosses between arms	19.1 ± 1.0	15.2 ± 2.1	18.9 ± 1.5	11.2 ± 1.4**

Table 3. Effect of tetrabenazine (TBZ) on mice behavior in the Dark and Light (DL) Box and Elevated Plus Maze (EPM). Every test lasted 5 minutes. Mean (± SEM) seconds or frequency. ** p<0.01 significantly different from vehicle.

Discussion

The present group of studies evaluated the impact of the VMAT-2 inhibitor TBZ on behavioral activation as assessed in a 3-choice-T-maze task that evaluates preference for vigorous physical activity vs. other sources of reinforcement that could be obtained with little effort. These experiments also studied how TBZ can affect behavioral activation induced by stressful conditions such as the non-escapable FST.

In the 3-choice-T-maze task, mice freely distribute their time performing an effortful activity (running in a RW) or doing more sedentary activities (eating sucrose pellets or sniffing a fruit odor). Under basal conditions, mice spent a large part of the time running, some time consuming sucrose, and very little time sniffing a non-social odor. Consistent with this finding, previous studies have shown that running has a high motivational value, because animals work to unlock a RW (Belke et al., 2005, 2014; Collier et al., 1990). Also, the RW can be used as a motivational stimulus for inducing conditioned place preference (Lett et al., 2000; Trost and Hauber, 2014; Basso and Morrell 2015). In our study, TBZ decreased time spent running but increased time eating, with no change in time interacting with the neutral odor. These results are consistent with previous studies in our laboratory using lower doses of TBZ (López-Cruz et al, 2018), and also using haloperidol (D2 receptor antagonist), in a simpler version of the T-maze (Correa et al, 2016). In addition, in the present study we further analyzed other behavioral variables such as time spent in the different compartments or total number of entries into each compartment. TBZ produced no significant change in these measures of place preference and exploration, indicating that mice did not avoid being in close proximity to the RW and showed normal motor exploration of the T-maze. A further statistical comparison of vehicle versus the highest dose of TBZ with a Student t-test for related samples did not show a significant effect on these two variables either. Thus, by depleting DA (López-Cruz et al, 2018), these doses of TBZ seem to be producing anergia or reduced behavioral activation rather than affecting the primary reinforcing effects of sucrose, since time spent eating sucrose actually increased.

However, in order to test potential alternative explanations, we manipulated the reinforcing value of these 3 reinforcers. Thus, we addressed potential factors that could be increasing food value. In one of the experiments, animals were exposed to the T-maze

after having gone through home food restriction, in order to increase homeostatic value for food and food seeking. In this experiment, time spent eating was significantly increased almost ten times, and time running decreased by half the baseline values. Thus, this manipulation profoundly changed baseline preferences, and it was the only manipulation that made RW and food equally preferred (mice spent around 200 seconds interacting with each one of them). In a different experiment, animals exposed daily to an unlimited amount of sweet food for a limited time demonstrated a binge eating pattern increasing about ten times their time eating in the T-maze compared to control animals, almost to the level of animals in the previous experiment restricted of standard food. In addition, binge eating animals not only showed reduced time spent running, but they also showed increased time sniffing the strawberry odor. This pattern of results was parallel to results for the variable time in the compartment, and for entries in the compartments, although only entries in the odor compartment increased significantly. These results indicate that a binge-like-eating pattern produces a shift in relative preferences towards sedentary stimuli (odor and food). However, when we analyzed the amount of pellets consumed after DA depletion and after food manipulations (Table 2), we observed that animals significantly consumed more grams of sucrose pellets only after food restriction and after stablishing a binge-like pattern of intake compared to their respective control conditions. Although TBZ did not significantly change total grams of pellets consumed, it did significantly increase time spent eating at the highest dose. This is important because previous behavioral studies indicate that time allocated to a particular activity is a fundamental marker of reinforcement value (Baum and Rachlin, 1969). Previous studies have shown that administration of TBZ had no significant effect on free food intake both in mice and rats (Correa et al, 2018; López-Cruz et al, 2018; Pardo et al, 2015; Nunes et al, 2013), and did not induce changes in hedonic taste reactivity to sucrose (Pardo et al, 2015). In rats, responding on effort-based choice tasks involving lever pressing, TBZ-induced decreases in lever pressing are actually accompanied by increased intake of the concurrently available chow (Nunes et al, 2013; Yohn et al, 2016a). Taken together, these data are consistent with other studies indicating that the TBZ-induced relative shifts in choice behavior do not appear to be due to changes in primary food motivation (Nunes et al, 2013; Randall et al, 2012, 2014). Moreover, considerable evidence indicates that nucleus accumbens DA depletions do not impair the unconditioned reinforcing properties of food (Koob et al, 1978; Salamone et al, 1991; Kelley et al, 2005; Salamone and Correa, 2002, 2012; Salamone et al, 2016). Thus, the T-maze choice paradigm appears to be

useful for exploring changes in patterns of food consumption (rather than total food consumed), as well as changes in preferences after conditions that impair voluntary exercise.

We also tried to alter the value of the least preferred reinforcer, the fruit odor. There is considerable evidence showing an increase in sexual motivation of male rodents when a female odor is present (Trezza et al., 2011; Portillo and Paredes, 2004). In rodents, olfactory stimuli from conspecific estrous females can be conditional cues that induce incentive motivation and seeking behavior parallel to a significant increase in accumbens DA (Fujiwara and Chiba, 2018). Thus, we increased the value of the odor by using social odors (conspecific male and conspecific female). All the subjects were male, and the male odor significantly increased time sniffing the cotton ball, but did not produce a significant reduction in time running. However, when the cotton contained a female odor, male mice increased sniffing time almost ten-fold, and this increase was paralleled by a significant reduction in time spent running, but not time eating. These results were parallel for the time-in-compartment variable. However, the presence of these social odors made animals more active in exploring the T-maze: mice increased entries in all the compartments when conspecific odors substituted the fruit odor. Thus, in the present results, male mice showed a decrease in the relative preference for the running wheel when a female odor was present, making food and opposite sex odor equally preferred (around 30 seconds of interaction with each stimuli).

Finally, in order to reduce time in the RW, we tried to devalue the RW stimulus in different ways. Devaluing the RW by making it more resistant to turn and thus, increasing muscular effort, produced a very different pattern of effects compared to TBZ. Animals reduced time running but there was no compensatory increase in time eating. In fact, animals tended to increase time spent in the RW compartment indicating no avoidance of the RW, and no increased interest in the other stimuli. Thus, force requirement does not seem to be the key factor underlying the effects of TBZ on RW preference in the present experiments. This result is similar to previous experiments using weights attached to a lever to increase resistance in an operant setting (Ishiwari et al., 2004). In that experiment, rats with a local Nacb DA depletion were relatively insensitive to different force requirements, but were very sensitive to temporal or rate components of work requirements as compared to controls.

The term fatigue has been used for many different aspects of human performance. Fatigue includes a range of effects that can vary between reductions in physical and cognitive function that extend from an exercise-induced impairment of motor performance, to the sensations of tiredness and weakness that accompany some clinical conditions (Enoka and Duchateau 2016). In order to assess all these components, most authors have stablished a dichotomy between central and peripheral fatigue or perceived fatigability and performance fatigability (Enoka and Stuart 1992; Enoka and Duchateau 2016). Among perceived fatigability, psychological factors, and in particular motivational factors, play a role in healthy individuals (McCormick et al., 2015), but they play an even more prominent role in the type of fatigue reported in various clinical conditions, such as in Parkinson's disease (Kluger et al., 2013), and in depressed patients (Caligiuri and Ellwanger, 2000; Demyttenaere et al., 2005; Fava et al., 2014). Interestingly, this type of fatigue in Parkinson's disease is significantly correlated with level of depression (Kluger et al., 2013). In summary, it seems that the type of fatigue generated by DA depletion, rather than a decline in muscle force, is more likely to reduce the capacity to sustain high levels of voluntary activation.

The FST, is the classical animal test to evaluate antidepressant actions of different type of drugs (Armario et al., 1988; Lucki 1997). This test, traditionally, provides information about passive behaviors such as immobility (Porsolt et al, 1977), as a measure of behavioral despair or “giving up”, but it also can provide information about behaviors directed to the maintenance of vigorous and persistent active responding in order to escape (Gil and Armario, 1998). In fact, this test provides information about different parameters of behavioral activation such as climbing and swimming (Armario et al., 1988; Gil and Armario, 1998; Slattery and Cryan, 2012). In the present setting, the administration of a broad range of TBZ doses that has previously been shown to reduce DA levels in ventral striatum of mice (López-Cruz et al, 2018), dose dependently produced a significant decrease in climbing (active behavior), and also increased time spent immobile, in the FST (Figure 8). Previous studies have shown that after chronic oral administration of TBZ by putting it in the food, the immobility of mice in the FST increased significantly (Wang et al, 2010). Moreover, D2 receptor antagonists not only increased immobility in rats, but also concurrently decreased active climbing (Gil and Armario, 1998; Li et al, 2005). Thus, DA depletion in the T-maze RW test (a test of

preference for rewarding stimuli with different vigor requirements), and also in the FST (a test of unescapable stress) (Armario and Nadal 2013), reduced behavioral activation.

In addition, because aversive states such as stress and anxiety could be factors that play a role in T-maze procedures, and since it has been previously demonstrated that, in operant paradigms of effort-based choice in rats, acute stress decreased preference for the high-effort reward (Shafiei et al., 2012), we performed a T-maze experiment in which we reduced the value of the RW by placing over it an intense light, like the one used in anxiety paradigms, thus making it more aversive. Indeed, animals spent less time running, and tended to spend more time engaging with the other two stimuli, although only eating was statistically significant. However, the pattern of results for the other two variables was quite different from the results found with TBZ: animals significantly reduced time spent in the RW compartment, and increased time spent in the other two compartments. They also increased entries into those other two compartments. Thus, animals not only reduced significantly the act of running, they also spent less time in close proximity of the RW, and explored the other two stimuli (not only the food) more. Moreover, TBZ did not produce any significant effect on anxiety-related parameters, such as time spent in the lit chamber of the DL box, or time spent in the open arms of the EPM paradigm (Table 3). These results are in accordance with previous studies in which the administration of a high dose of TBZ (8.0 mg/kg) did not affect anxiety in the DL box (Correa et al, 2018), and administration of the D2 antagonist haloperidol did not produce any anxiogenic effect in mice assessed in the EPM paradigm (Pail et al, 2015). However, in the present studies, the administration of the two highest doses of TBZ (6.0 and 8.0 mg/kg) significantly decreased the total number of crosses between compartments in the DL box, and total arm entries in the EMP paradigm. These are parameters that, while affected by the aversive nature of some areas in the paradigm, are considered to be more related to spontaneous locomotion.

In summary, the present work confirms that the 3-choice T-maze task, in which rodents show a high preference for exercise, is sensitive to pharmacological and motivational manipulations, and that mice allocate their behavior from one stimulus to another depending on a number of different conditions. TBZ administration induces changes in preferences based on effort requirements, and those effects do not closely resemble changes in preference produced by altering the force requirements or enhancing the reinforcing value of food. While making the RW more aversive by shining a light on it

produced some effects that are similar to TBZ (e.g. reducing time spent running and increasing eating), these manipulations do not produce the same effects on other measures (e.g. time in RW compartment). TBZ also reduced climbing in the FST, indicating that TBZ generally reduces highly active behaviors. Numerous studies have reported that TBZ and DA antagonists affect effortful behaviors in rodents when animals have to work (lever pressing or climbing a barrier) in order to obtain a high-value reinforcer when they concurrently have a free low-value reinforcer (Nunes et al, 2013; Randall et al, 2014; Pardo et al, 2012, 2015; Yohn et al, 2015a,b, 2016a,b; Contreras-Mora et al, 2018; Correa et al, 2018; Rotolo et al, 2019; Yang et al, 2019). In conclusion, the 3-choice-T-maze task can evaluate preference for exercise and anergia induced by DA impaired function, and this task could have potential clinical relevance for modeling the psychomotor retardation, anergia, fatigue and the low effort bias seen in some human psychopathologies.

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CHAPTER II

Bupropion for the treatment of motivational disfunctions related to depression as measured in a three-choice task for mice: modulation of DARPP-32

Abstract

Nucleus Accumbens (Nacb) dopamine (DA) depletion induces anergia and fatigue in effort-based decision tasks in humans and animals. These motivational symptoms are seen in depression and are highly resistant to treatment. In these studies, we evaluated the effect of the catecholamine uptake blocker bupropion on its own, and after the administration of tetrabenazine (TBZ), a VMAT2 blocker that depletes DA, and induces depression in humans. These drugs were evaluated in male CD1 mice on a 3-choice-T-maze task developed to assess preference between a reinforcer that requires voluntary behavioral activation (running wheel, RW) or other more sedentary reinforcers (sweet food pellets and a neutral non-social odor), and also in the forced swim test (FST), which measures behavioral activation in a stressful setting. Anxiety was also evaluated in the dark-light box (DL), and in the elevated plus maze (EPM). Bupropion increased selection of RW activity on the T-maze. TBZ reduced time running, but increased time-consuming sucrose, indicating an induction of fatigue but not an effect on primary sucrose motivation. In the FST, bupropion reduced immobility, increasing swimming and climbing, and TBZ produced the opposite effects. Bupropion reversed the effects of TBZ on the T-maze, on the FST, as well as the effects on pDARPP32-Thr34 and pDARPP32-Thr75 in Nacb core as assessed with immunohistochemistry and western blotting. None of these manipulations affected anxiety-related parameters. Thus, bupropion shows antidepressant properties in traditional animal models like the FST, but also in the T-maze task, which could be useful for assessing preferences based on effort requirements.

Bupropion, dopamine, motivation, effort, depression, nucleus accumbens, rodent

Introduction

Psychiatric pathologies such as depression are characterized not only by emotional and cognitive symptoms, but also by motivational deficiencies that lead to manifestations such as psychomotor symptomatology including anergia, psychomotor slowing, fatigue and lassitude (Stahl, 2002; Demyttenaere et al, 2005; Treadway and Zald, 2011; Fava et al, 2014; Barch et al, 2016; Salamone et al, 2016c). Many times, these symptoms are not considered central for depression, but clinical data shows how their severity limits long term functional outcomes, producing problems with employment and social functions (Fava et al, 2014; Rostchild et al, 2014; Salamone et al, 2007; Chong et al, 2016). People with major depression have deficits in the exertion of effort, reward seeking and effort-related decision making that do not change the experience of pleasure in response to primary stimuli (Treadway and Zald, 2011; Argyropoulos and Nutt, 2013; Pizzagalli, 2014; Knowland and Lim, 2018).

There are many evidences pointing to the implication of the mesolimbic dopamine (DA) circuit, in particular nucleus accumbens (Nacb), as the neural basis of effort-related dysfunctions in depression (Caligiuri and Ellwagner, 2000; Schmidt et al, 2001; Volkow et al, 2001; Salamone et al, 2007, 2016c, 2018). In animal models, the administration of the vesicular monoamine transporter type-2 (VMAT-2) blocker and DA depleting agent tetrabenazine (TBZ) produces motivational anergia, which shifts behavior from high demanding options to obtain highly preferred reinforcers, towards low-effort ones, obtaining in consequence, less valued reinforcers (Nunes et al, 2013; Randall et al, 2012; Pardo et al., 2015a; Yohn et al, 2015a, 2016a; Correa et al., 2018; López-Cruz et al, 2018; Rotolo et al, 2019). Moreover, TBZ has been reported to induce depressive symptoms in humans (Frank, 2009; Guay et al, 2010), and has also been used in classical animal models of depression (Jang et al, 2009; Wang et al, 2010; Carratalá-Ros et al, 2020).

Moreover, it has been shown that blockade of the DA transporter (DAT), that leads to the increase of extracellular DA, can improve motivational symptoms in depressed patients (Patkar et al, 2006; Goss et al, 2013; Malhi et al, 2016). Thus, the catecholamine uptake inhibitor bupropion has demonstrated efficacy to treat depression (Feighner et al, 1986; Kiev et al, 1994; Weihs et al, 2000; Pae et al., 2007; Papakostas et al, 2006; Cooper et al, 2014). Its main mechanism of action is to block DAT, and it shows low potential to produce abuse in humans (Nutt et al 2007; Learned-Coughlin et al, 2003; Sthal et al,

2004), and in rodents (Mori et al, 2013). In addition, it has been shown that this DAT blocker increases extracellular DA and DA-singal transduction in Nacb (Randall et al, 2014a). The enhancement of DA transmission can reverse effort-decision making impairments (Salamone et al, 2007; Floresco et al, 2008; Mai et al, 2012; Hosking et al, 2015; Salamone et al, 2016b; Salamone et al, 2016c; Yohn et al, 2015a,b; Yohn et al, 2016a,b; Rotolo et al, 2019). Thus, in PROG/chow or FR5/chow feeding tasks, bupropion was able to alleviate and reverse the effects produced by TBZ, increasing the effort to work in order to obtain a preferred food (Randall et al, 2014a; Yohn et al, 2015a,b, 2016a). Moreover, this DAT inhibitor has been also used in classical paradigms to assess antidepressant effects of drugs, such as the forced swim test (FST; Porsolt et al., 1977). This paradigm requires a stressful and non-escapable setting in which naïve rodents at first try to actively scape, but after a while they give up and remain immobile, mainly flouting. All these behaviors can be improved by different categories of antidepressants drugs (Porsolt et al, 1977; Armario et al, 1988; Lucki, 1997). It seems that active behaviors are more likely to be affected by DA manipulations than the traditional immobility measure (Gil and Armario, 1998; Costa et al, 2013). Thus, bupropion has anti-immobility effects in mice and rats (Yamada et al, 2004; Kitamura et al, 2010; Yuen et al, 2017), and also increases swimming and climbing (Yuen et al, 2017; Hayashi et al, 2011; Rénéric and Lucki, 1998).

In evaluating effort-based decision-making processes in humans and in rodent models, lever pressing is the most commonly used work requirement (Treadway et al, 2012; Randall et al, 2014b; Yohn et al, 2015; 2016a). However, more hole-body physical behaviors such as rearing, barrier climbing or wheel running can be used as the requirement to get access to the most preferred reinforcer, or even as the more preferred activity (Correa et al, 2016). Physical activities can require high levels of performance and endurance, but they also can have intrinsic motivational properties (Belke et al, 2005; Belke and Pierce, 2014). One of the most commonly studied voluntary physical activities in rodent models is wheel running, which can be performed under non-stressed conditions, and according to the rhythmicity of the animal. Mice run spontaneously when given access to running wheels and, depending on the strain, they can run for a total distance of up to 20 km per day and a total activity time of around 3 to 7 hours a day (Manzanares et al, 2018). Thus, wheel running can be used as the highly preferred and highly demanding choice in relation to other less preferred but less effort demanding

alternatives, such as consumption of palatable food or drugs of abuse (Premack and Premack, 1963; McMillan et al, 1995; Mueller et al, 1997; Cosgrove et al, 2002; Correa et al, 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020; Presby et al, 2020). However, little is known about the neural mechanisms involved in the decision-making processes that establish those preferences for vigorous physically demanding behaviors.

In view of these premises, the present study explores the impact of the DAT inhibitor, bupropion, administered alone or in combination with the DA depleting agent TBZ, in the 3-choice T-maze task which uses voluntary exercise in a running wheel as the most preferred but high effort demanding option in competition with less preferred but more sedentary options such as consumption of high-sucrose containing food or sniffing floral odors (Correa et al, 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020). We compare the impact of bupropion alone or after TBZ administration, on spontaneous preference in non-deprived animals and under non-stressful conditions, with the impact of the same doses of bupropion on the FST. Moreover, we assess if the present pharmacological manipulations have an impact on anxiety-related behaviors in classical rodent models (dark and light box; DL box and the elevated plus maze: EPM). Finally, an additional experiment studies how the most effective dose of bupropion on these behaviors, affects DA receptor dependent metabotropic markers in one of the main sub-regions of Nacb (core) using immunochemistry and western blotting methods.

Materials and Methods

Animals. CD1 adult male mice (N=102) purchased from Janvier, France S.A. were 8-14 weeks old (40-50 g) at the beginning of the study. Mice were housed in groups of three or four per cage, with standard laboratory rodent chow and tap water available *ad libitum*. The colony was kept at a temperature of 22 $^{\pm}$ 2 ºC with lights on from 08:00 to 20:00 h. All animals were under a protocol approved by the Institutional Animal Care and Use committee of 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 Behavioral Research", National Research Council 2003, USA. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Pharmacological agents. The catecholamine uptake inhibitor bupropion hydrochloride (Alfa Aesar, Spain) was dissolved in 0.9% saline, which also served as the vehicle control for these studies. Bupropion was administered 30 minutes before the test started. Doses and times were selected from previous work in our laboratory (Randall et al, 2014a,b; Yohn et al, 2015a) and from studies with the forced swim test (Yamada et al, 2004; Kitamura et al, 2010). The VMAT-2 inhibitor tetrabenazine (TBZ, CIMYT Quimica SL, Spain) was dissolved in a solution of 0.9% saline (80%) plus dimethylsulfoxide (DMSO 20%, final pH 5.5) and administered 120 min before testing. The vehicle solution of 20% DMSO and saline was used as the vehicle control for TBZ. Time and doses were selected based on previous work (López-Cruz et al, 2018; Correa et al, 2018; Carratalá-Ros et al, 2020). All drugs were administered intraperitoneally (IP).

Testing procedures

All behavioral procedures started two hours after light period onset. The behavioral test room was illuminated with a soft light, and external noise was attenuated.

T-maze RW-sucrose-odor choice task. The T-maze apparatus (25 cm L x 11 cm W x 30 cm H) consisted of a central area that lead to 3 arms (based on Correa et al, 2016). Each arm provided of different type of stimuli. In one of them, sweet pellets (TestDietTM, 50% sucrose, 45 mg 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 neutral non-social odor (López-Cruz et al, 2018; Carratalá-Ros et al, 2020). Training as well as test sessions lasted 15 minutes. Mice were trained 5 days a week. Training phase 1: to avoid neophobia to the sweet tasting pellets, animals were enclosed in that arm with the food during 5 sessions. Training phase 2: during 2 more weeks animals were exposed, one 15 min session a day to the T-maze with free access to the three stimuli. Test phase: This phase lasted during 4 or 5 more weeks depending on the experiment. For each week there were 4 baseline days plus a testing session in which animals received drug injections. Test sessions were videotaped and a trained observer manually registered several parameters. Time interacting with each of 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 Rachlin, 1969). Entries into the arms and time spent in the arms of the T-maze were simultaneously recorded. All these measures were taken based on previous studies (Correa et al., 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020).

Forced swim test (FST). This paradigm is considered to be a model of behavioral despair and is used as a test for assessing depressive-like states and for evaluating drugs with potential as antidepressants (Porsolt et al, 1977). Immobility was defined when the animal remained motionless, making only minor movements to balance the body and keep the head above the water. In addition, we also assessed escape related mobility behavior such climbing or struggling (Armario et al, 1988). Climbing is defined as any energetic and vertical movement of all four limbs against the wall of the tank. Mild 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). Naïve mice were placed in a transparent cylindrical glass tank (26 cm high and 18 cm diameter) filled with water (14 cm) and maintained at a temperature of 25ºC. Water was changed between animals. During the 6 minutes test, mice were videotaped from the side, and climbing, immobility and mild swimming were later measured by an observer unaware of the experimental condition. After the test, mice were dried with a soft towel, put back in a box with absorbing paper under a warming light, and were monitored for 10 minutes.

Dark and light box (DL). The DL test is based on the conflict between the tendency to explore a novel environment and the avoidance of a brightly lighted open area (Blumstein and Crawley 1983). The DL apparatus consisted of a polypropylene chamber divided in two compartments by a partition containing a small opening (5 cm H x 5 cm W). The light compartment (25 cm W x 25 cm H x 25 cm L) was open, painted in white and illuminated (335 lx), while the dark compartment (25 cm W x 25 cm H x 18 cm L) was painted in black and had a removable ceiling to close it. To start the test session, mice were individually placed in the dark chamber facing one corner. Test sessions were videotaped, and the total number of crosses and the total time spent in the lit chamber were recorded for 5 min (procedure is based on López-Cruz et al, 2017).

Elevated plus maze (EPM). After being in the DL box for 5 minutes, animals were placed in the EPM for 5 more minutes. The EPM consists of two open and two enclosed arms (65 cm L x 5 cm W) arranged in a plus configuration and intersecting in a central platform. It is made of black polypropylene and is elevated 50 cm above the floor. The open arms have a 1 cm border around their perimeter and the closed arms have a 20 cm

translucent wall. This anxiety paradigm measures the avoidance that rodents show to high open spaces. Under normal conditions, mice spend more time, and make more entries into the closed arms of the maze (Lister, 1987). Animals were placed in the central platform with their head pointing at one enclosed arm, and they were assessed during 5 minutes. Sessions were videotaped and a trained observer registered total time spent in the open arms and total number of entries in the four arms as an index of locomotion. An entry into an arm was recorded when the animal crossed with all four legs the line that connected that arm with the central platform (procedure is based on López-Cruz et al, 2017).

Immunohistochemistry for phosphorylated DA and c-AMP related phosphoprotein-32 kDaltons (pDARPP-32). After drug treatments, animals were anesthetized with CO2 and transcardially perfused with 0.9% physiological saline for 5 min, followed by perfusion with 3.7% formaldehyde for 5 min and brains were extracted. Tissue was fixed in 3.7% formaldehyde overnight and moved to 30% sucrose cryo-protectant. Then 40-um sections were cut using a cryostat. To measure the immunoreactivity to phosphorylated DA and c-AMP-regulated phosphoprotein 32kDa (pDARPP-32) nonspecific binding sites were blocked with a solution of 3% H202 for 30 min at room temperature following then with 1% bovine serum albumin and 0.1% Triton X-100 in PBS for 1 hour at room temperature. Phosphorilated DARPP-32 immunoreactivity was visualized with a polyclonal rabbit antibody for pDARPP-32 phosphorilated at threonine 34 residue (Thr34, 1:1000; Santa Cruz Biotechnology) or polyclonal rabbit antibody for pDARPP-32 phosphorilated at threonine 75 residue (Thr75, 1:500, Santa Cruz Biotechnology). These antibodies were dissolved in solutions that also contained 0.1% bovine serum albumin and 0.1 Triton X-100 in PBS for 24 h incubation on a rotating shaker at 4ºC. After the primary antibody treatment, the sections were rinsed in PBS (3 times for 5 min) and incubated in the secondary antibody, anti-rabbit HRP conjugate envision plus (DAKO) for 1hour and 30 minutes on a rotating shaker at room temperature. Finally, sections were washed and rinsed for 3-5 min in 3,3’ diaminobenzidine chromagen. The sections were then mounted to gelatin-coated slides, air dried and coverslipped using Eukitt quick-hardening (Sigma-Aldrich) as a mounting medium. The tissue was then examined by light microscopy.

Quantification of the number of cells that express immunoreactivity for pDARPP-32 (Thr34) and pDARPP-32 (Thr75) in the Nacb was performed by photographing the

sections with a 20X (0.125 mm^2 / field) objective (Eclipse E600; Nikon) upright microscope equipped with a Leica DFC 450C camera (Leica Microsystems) and captured digitally using LASX software. Cells that were positively labeled for pDARPP-32 (Thr34) and pDARPP-32 (Thr75) were quantified with ImageJ software (version 1.51) and a macro written to automate particle counting within the region of interest. The size of the region of interest was 1000 x 1000 um. For each animal, cell counts were at levels that correspond to 0.70-1.70 mm anterior to bregma (Paxinos and Watson, 1997) bilaterally from at least three sections and counts were averaged across slides and sections.

Immunofluorescence double-labeling studies for pDARPP-32(Thr75) and enkephalin. Free floating coronal sections of brains (40 um) were serially cut using a cryostat and rinsed in 0.01M PB. To measure the immunoreactivity to pDARPP-32, nonspecific binding sites were blocked and cells were permeabilized in a solution containing 0.3% T.X and 5% of goat serum in PBS for 30 min at room temperature on a rotating platform before primary antibody incubation. pDARPP-32 immunoreactivity was visualized with a polyclonal rabbit antibody for pDARPP-32(Thr75) (1:200; Aviva Systems). pDARPP-32(Thr75) was double labeled with a primary antibody for enkephalin (goat polyclonal, 1:800; Origene). These antibodies were dissolved in solutions that also contained 5% goat serum and 0.3% T.X in PBS for 24h incubation on a rotating shaker at 4ºC. After the primary antibody treatment, the sections were rinsed in PBST (6 times for 10 min) and incubated in the secondary antibody solution containing donkey anti-rabbit Alexa Fluor 488 (1:350, Life Technologies) or donkey anti-guinea pig Alexa 647 (1:350, Abcam). These antibodies were dissolved in solutions that also contained 5% goat serum and 0.3% T.X in PBS for 2h incubation on a rotating shaker at room temperature. The sections were then mounted to gelatin-coated slides, dried and coverslipped using Mowiol (Abcam). Immunofluorescence staining was visualized for high-resolution observation on a confocal microscope (Leica DMI8, Leica Microsystems CMS GmbH, Wetlar Germany).

Western Blotting for phosphorylated DA and c-AMP related phosphoprotein-32 kDaltons (pDARPP-32). After drug treatments, animals were anesthetized with CO2, decapitated and brains were extracted. Fresh striatal tissue samples were homogenized in ice-cold lysis buffer (130Mm NaCl, 20Mm Tris-Hcl at Ph 8.0, and 1% Nonidet P40), containing protease inhibitors (10 ug/ml of aprotinin, 20ug/ml of leupetine, and 1Mm

PMSF) as well as phosphatase inhibitors (10 Mm NaF, 1Mm sodium orthovanade and 10Mm DTT). Homogenates were centrifuged at 13000 rpm for 15 min at 4ºC. Aliquots of supernatants were collected and used Bradford quantification of total protein. Every sample was boiled for 3 min for protein denaturation. Equal amounts (30ug) of striatum protein samples were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membrane (Bio-rad) for 90 min at 30V. Filtering membranes were incubated in 5% of Bovine Serum Albumin (BSA, Sigma-Aldrich) dissolved in TBS-T (Tris Base 200Mm and 5M NaCl) containing 0.1% Tween 20, and then incubated overnight at 4ºC with polyclonal rabbit anti-DARPP32-Thr75 antibody (1:500, Cell Signaling). Membranes were probed with Anti-actin polyclonal antibody (1:500, Abcam), as an internal standard for protein quantification. After rinses with TBS-T buffer, membranes were incubated with goat anti-rabbit IG secondary antibody coupled to HRP (1:20000, Bio-rad) during 1h and developed by enhanced chemiluminescence system (1:40, ThermoFisher Scientific). The membranes were exposed to Image Quant LAS 4000 (Leica). The relative densities of brands were analyzed using ImageJ software. Every sample was replicated at least twice to ensure the reproducibility of the method.

Experiments

Experiment 1. Effect of bupropion on preference for reinforcers as measured in the 3-choice-T-maze task. After reaching stable baseline level of performance in the T-maze task, animals (N=14) received a dose of BUP (0, 5, 10 and 15mg/kg) and were placed to the paradigm during 15 minutes. Animals received one dose of the drug every week in a randomly varied order. The T-maze paradigm requires a baseline performance of two weeks before tests start, and that performance is maintained across weeks, thus allowing a repeated measures design.

Experiment 2. Effect of bupropion in combination with TBZ on preference for reinforcers as measured in the 3-chgoice-T-maze task. A new group of mice (N=13) received TBZ (veh, 8 mg/kg) and 90 minutes later (30 minutes before they were placed in the T-maze during 15 minutes), they received a dose of bupropion (5, 10 and 15 mg/kg). Animals received one drug combination (veh-veh, TBZ-veh, TBZ-5, TBZ-10 and TBZ-15 mg/kg) every week during 5 weeks in a random varied order.

Experiment 3. Effect of bupropion on FST performance. Animals (N=40) received one dose of bupropion (vehicle, 5, 10 or 15 mg/kg) and 30 min after the injection were placed in the FST during 6 minutes. Mice were exposed only once to the FST since behavioral habituation develops in one session.

Experiment 4. Effect of bupropion in combination with TBZ on FST performance. Three groups of naïve mice (N=35) received a combination of TBZ (8 mg/kg, a dose that had demonstrated to be effective in the FST) (Carratalá-Ros et al, 2020) plus vehicle or the most effective dose of bupropion (10 mg/kg) or the combination of both vehicles. Bupropion was given 90 minutes after TBZ and 30 minutes before animals were placed in the FST.

Experiment 5. Effect of bupropion on anxiety parameters as measured in the DL and EPM paradigms. Mice (N=40) received one dose of bupropion (vehicle, 5, 10 or 15 mg/kg) and 30 min after injection were first placed in the DL box for 5 minutes. Immediately after this test, they were placed in the EPM for 5 more minutes. Mice were exposed only once to both paradigms, since behavioral habituation develops in one session.

Experiment 6. Effect of bupropion in combination with TBZ on anxiety parameters as measured in the DL and EPM paradigms. Three groups of naïve mice (N=35) received a combination of TBZ (8 mg/kg) plus vehicle or the most effective dose of bupropion (10 mg/kg) or the combination of both vehicles. Bupropion was given 90 minutes after TBZ and 30 minutes before animals were placed in the DL box for 5 minutes, and immediately after this test, they were placed in the EPM for 5 more minutes.

Experiment 7. Effect of bupropion in combination with TBZ on DARPP-32 phosphorylation patters in Nacb as measured by immunohistochemistry. Mice (N=16) received veh-veh, TBZ-veh, or TBZ-10 mg/kg, 2 hours before perfusion. Coronal sections were analyzed by immunochemistry for pDARPP-32 (Thr34) and pDARPP-32 (Thr75).

Experiment 8. Effect of TBZ on pDARPP-32(Thr75) levels in ventral striatum as measured by western blot. Mice (N=18) received DMSO or 8 mg/kg TBZ 120 min

before brain extraction. Striatum samples were analyzed by western blotting for pDARPP-32(Thr75).

Statistical Analyses. Normally distributed data (according to Kolmogorov-Smirnov test) in the T-maze experiment followed a within groups design. Thus, in the bupropion experiment, data were analyzed by repeated measures ANOVA. Normally distributed and homogenous data for the FST, DL, and EPM experiments employed a between groups design and data were analyzed by one-way ANOVA. Immunohistochemical studies were analyzed by one-way ANOVA. When the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used to compare each treatment with the vehicle control group (Keppel, 1991). For these comparisons, α level was kept at 0.05 because the number of comparisons was restricted to the number of treatments minus one. Western blotting data were analyzed by a Student t-test analysis for independent samples. All data were expressed as mean ± SEM, and significance was set at $p<0.05$. STATISTICA 7 software was used.

Results

Experiment 1. Effect of bupropion on preference for reinforcers as measured in the 3-choice-T-maze task. The repeated measures ANOVA showed that bupropion did not produce any significant effect on time spent eating the sucrose pellets ($F(3,39)=2.38$, $p=0.08$), or time sniffing the neutral odor ($F(3,39)=0.45$, $p=0.71$) (Fig 1A and 1C). However, bupropion produced a significant effect on time running in the RW ($F(3,39)=3.26$, $p<0.05$). Planned comparisons revealed that all doses of bupropion (5, 10 and 15 mg/kg) significantly increased time running in the RW ($p<0.05$ for the two lower doses and $p<0.01$ for the highest one) compared to the vehicle group (Figure 1B). Moreover, repeated measures ANOVA also showed a significant effect of bupropion on time spent in the RW compartment ($F(3,39)=4.35$, $p<0.01$), and time spent in the food compartment ($F(3,39)=3.92$, $p<0.01$). However, there was no significant effect of bupropion on time spent in the neutral odor compartment ($F(3,39)=1.78$, $p=0.16$) (Figure 2E). Planned comparisons showed that all doses of bupropion increased time spent in the RW compartment in comparison with the vehicle group (10 mg/kg $p<0.05$, and 5 and 15 mg/kg $p<0.01$)**.** Similarly, bupropion-treated mice spent less time in the sucrose pellets

compartment in comparison with the vehicle group (10 mg/kg $p<0.05$, and 5 and 15 mg/kg $p<0.01$). Finally, the catecholamine blocker did not produce significant differences in the number of entries into the food compartment ($F(3,39)=1.39$, $p=0.42$), entries to the RW compartment ($F(3,39)=1.39$, $p=0.25$), or entries to the neutral odor compartment ($F(3,39)=0.13$, $p=0.93$) (Figure 1D).

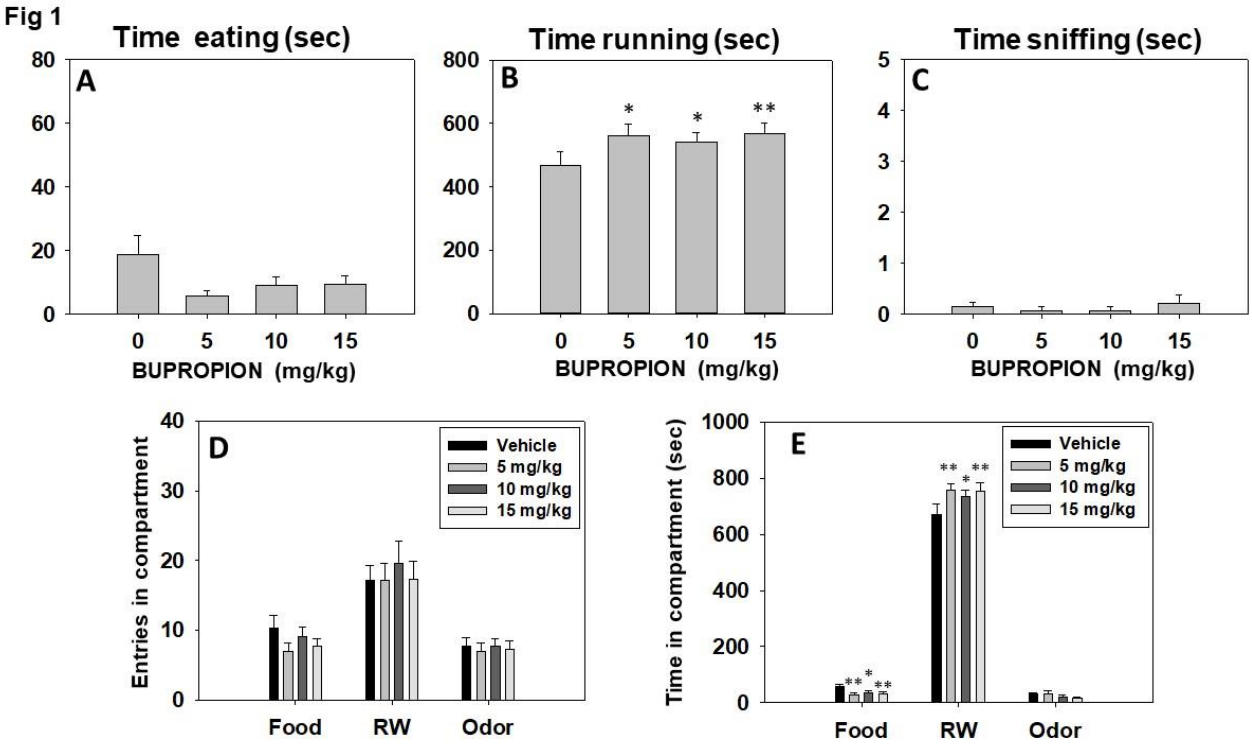

Figure 1. Effect of bupropion (vehicle, 5, 10 and 15 mg/kg) on time eating (A), time running (B), time sniffing (C), entries into compartments (D), and time spent in each compartment (E) of the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. *$p<0.05$, **$p<0.01$ significantly different from vehicle.

Experiment 2. Effect of bupropion in combination with TBZ on preference for reinforcers as measured in the 3-choice-T-maze task. Repeated measures ANOVA resulted in a significant effect of treatment on time spent eating the sucrose pellets ($F(4,48)=4.60$, $p<0.01$). Planned comparisons revealed the TBZ plus vehicle increased the time animals spent eating sucrose pellets ($p<0.05$) compared to vehicle, and all doses of bupropion were able to decrease significantly time consuming the food ($p<0.01$) in comparison with the TBZ-vehicle condition (Figure 2A). In addition, repeated measures ANOVA also show a significant effect of treatment on time running in the RW

($F(4,48)=3.06$, $p<0.05$). Planned comparisons revealed that the TBZ-vehicle condition significantly reduced time running in the RW ($p<0.05$) in comparison with the vehicle-vehicle condition, but 10 mg/kg of bupropion reversed that effect, increasing time running in the RW when compared to the TBZ-treated condition ($p<0.01$) (Figure 2B). However, the repeated measures ANOVA did not produced a significant effect on sniffing the neutral odor $F(4,48)=0.54$, $p=0.70$) (Figure 2C).

Repeated measures ANOVA did not show a significant effect of treatment on time spent in the RW compartment ($F(4,48)=1.93$, $p=0.11$), or time spent in the neutral odor compartment ($F(4,48)=0.91$, $p=0.46$) (Data are shown in figure 2E). However, there was a significant effect on time spent in the food compartment ($F(4,48)=4.04$, $p<0.01$). Planned comparisons showed TBZ-vehicle treated mice spent more time in the food compartment in comparison with the vehicle-vehicle group ($p<0.05$), and all doses of bupropion (5, 10 and 15 mg/kg) reversed this effect when compared with the TBZ-vehicle treatment ($p<0.01$)**.** Finally, repeated measures ANOVA analyzing the dependent variable entries in compartment did not show a significant effect of treatment on total number of entries to the food compartment ($F(4,48)=1.48$, $p=0.22$), or to the neutral odor compartment ($F(4,48)=2.48$, $p=0.06$) (Figure 2D). However, there was a significant effect of bupropion and TBZ combination on total entries to the RW compartment ($F(4,48)=2.92$, $p<0.05$), and planned comparisons showed that TBZ-10 mg/kg of bupropion treated mice decreased number of entries to the RW compartment in comparison with the vehicle-vehicle treatment ($p<0.01$).

Fig 2

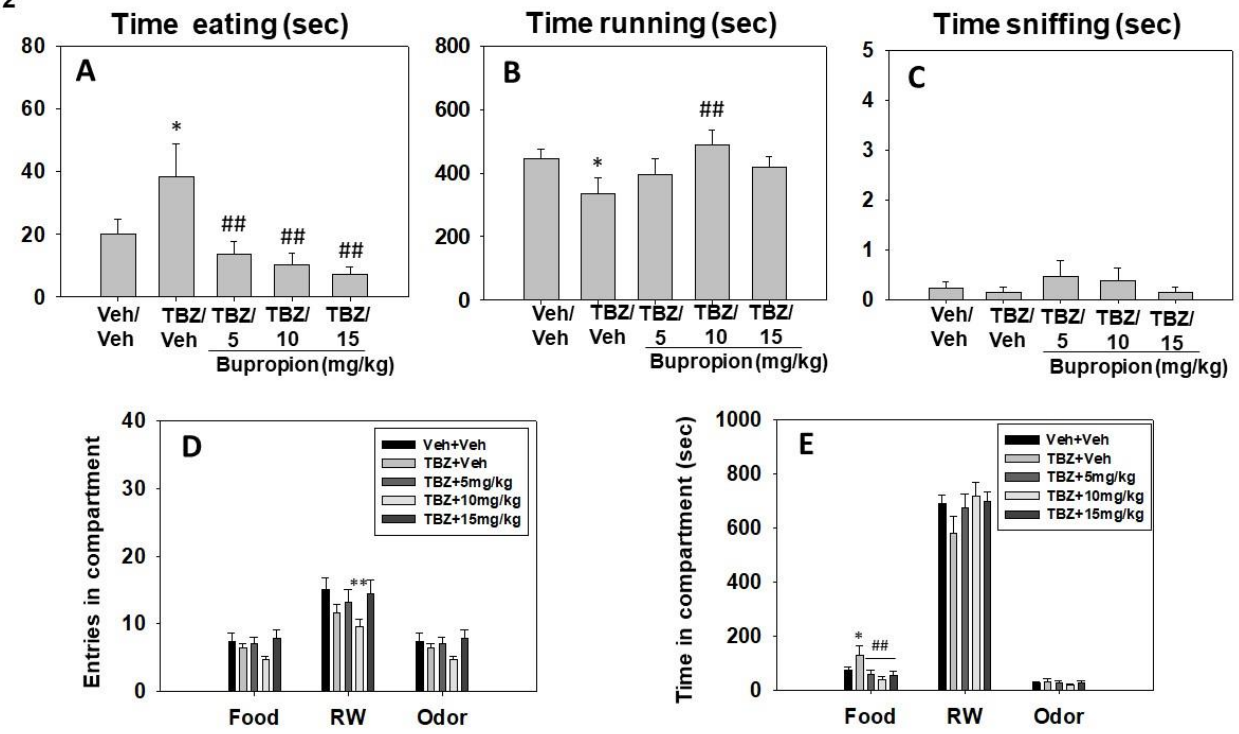

Figure 2. Effect of TBZ (vehicle or 8 mg/kg) plus bupropion (vehicle, 5, 10 and 15 mg/kg) combination on time eating (A), time running (B), time sniffing (C), entries into compartments (D), and time spent in each compartment (E) of the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. *p<0.05, **p<0.01 significantly different from Vehicle-Vehicle. ##p<0.01 significantly different from TBZ-Veh.

Experiment 3. Effect of bupropion on FST performance. The one-way ANOVA's for the different dependent variables showed a significant effect on time spent immobile ($F(3,36)=7.27$, $p<0.01$), time spent swimming ($F(3,36)=5.47$, $p<0.01$), and time spent climbing ($F(3,36)=2.77$, $p<0.05$) in the FST. Planned comparisons revealed that all the groups that received bupropion (5, 10 and 15 mg/kg) displayed significantly less time immobile ($p<0.01$), and spent more time swimming ($p<0.05$ for 10 mg/kg and $p<0.01$ for 5 and 15 mg/kg) in comparison with the vehicle group. However, only the group that received 10 mg/kg of bupropion displayed more time climbing than the vehicle group ($p<0.01$) (Fig. 3A).

Experiment 4. Effect of bupropion in combination with TBZ on FST performance. A series of one-way ANOVA's showed a significant effect of bupropion on immobility ($F(2,32)=13.98$, $p<0.01$), swimming ($F(2,32)=7.65$, $p<0.01$), and climbing ($F(2,32)=4.56$, $p<0.01$). Planned comparisons revealed that TBZ-treated mice spent more time immobile ($p<0.01$), and less time swimming and climbing ($p<0.05$) in comparison with the vehicle group. Moreover, bupropion was able to reverse these effects, and mice that received the combination of TBZ (8 mg/kg) and bupropion (10 mg/kg) spent less time immobile, increased time swimming and climbing (all $p<0.01$) in comparison with TBZ-treated mice (Fig. 3B).

Fig 3

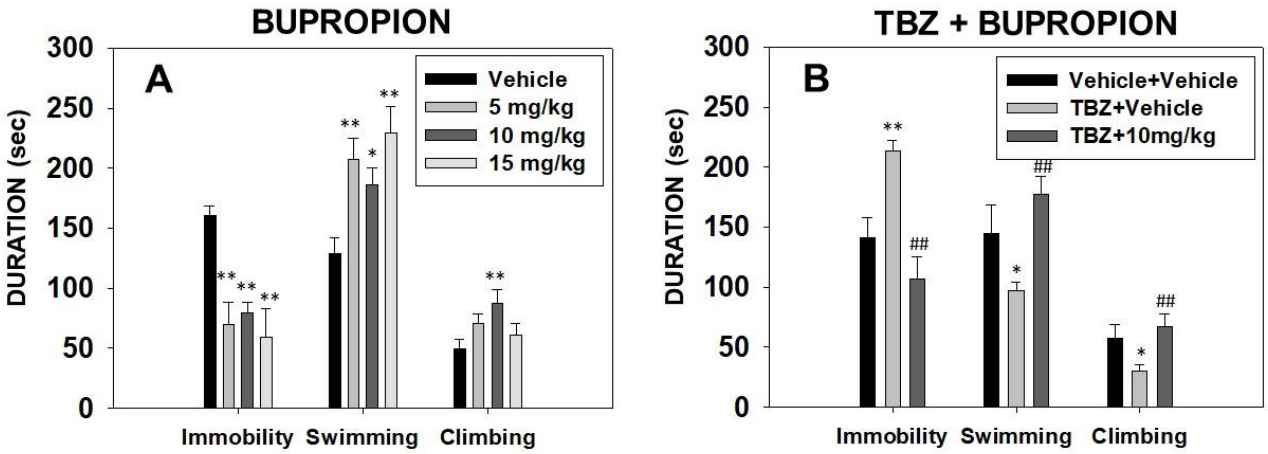

Figure 3. Effect of bupropion (vehicle, 5, 10 or 15 mg/kg) (A) and TBZ plus bupropion combination (Vehicle+Vehicle, 8 mg/kg TBZ+Vehicle, or 8 mg/kg TBZ and 10 mg/kg bupropion) (B) on duration of immobility, swimming and climbing behavior in the FST assessed during 6 minutes. Bars represent mean ± S.E.M. of accumulated seconds. *$p<0.05$, **$p<0.01$ significantly different from vehicle or vehicle-vehicle. ##$p<0.01$ significantly different from TBZ-Veh.

Experiment 5. Effect of bupropion on anxiety parameters as measured in the DL and EPM paradigms. The one-way ANOVA's for the different dependent variables in the anxiety paradigms did not show significant effects of bupropion on time spent in the illuminated arena ($F(3,37)=0.74$, $p=0.53$) or total of entries between the lit chamber and the dark chamber ($F(3,37)=0.27$, $p=0.84$) of the DL box (Table 1). Moreover, bupropion did not affect significantly time spent in the open arms of the EPM paradigm ($F(3,33)=2.34$, $p=0.09$), although there was a significant effect on total number of entries to all arms ($F(3,33)=6.23$, $p<0.01$) in the EPM. Planned comparisons showed that the highest dose of bupropion (15 mg/kg) increased significantly total of entries to all arms of the EPM ($p<0.01$) in comparison with the vehicle group.

	Bupropion (mg/kg)			
DL box	**Vehicle**	**5**	**10**	**15**
Time in the lit chamber (sec)	128.8 ± 8.6	117.8 ± 13.1	116.9 ± 10.1	106.3 ± 12.8
Crosses between compartments	27.0 ± 2.3	25.4 ± 2.8	24.8 ± 4.0	28.9 ± 4.5
EPM				
Time in open arms (sec)	83.6 ± 11.8	49.3 ± 10.8	82.8 ± 9.8	60.5 ± 7.4
Entries to all arms	17.6 ± 2.3	13.0 ± 2.2	17.5 ± 2.5	27.0 ± 1.1**

Table 1. Effect of bupropion on mice behavior in the Dark and Light Box (DL) and Elevated Plus Maze (EPM). Every test lasted 5 minutes. Mean (± SEM) seconds or frequency. **$p<0.01$ significantly different from vehicle.

Experiment 6. Effect of bupropion in combination with TBZ on anxiety parameters as measured in the DL and EPM paradigms. The one-way ANOVA's did not show any significant effect of the treatment on time spent in the lit chamber of the DL box ($F(2,28)=0.35$, $p=0.70$), but the treatment produced a significant effect on the total of crosses between compartments ($F(2,28)=5.57$, $p<0.01$) of the DL box. Planned comparisons revealed that TBZ treatment decreased significantly the total of entries

between compartments in the DL box both in the TBZ-vehicle ($p<0.01$) and in the TBZ-bupropion (10 mg/kg) condition ($p<0.05$) in comparison with the vehicle-vehicle group (Table 2).

The one-way ANOVA's for the variables evaluated in the EPM showed that the treatment produced no significant effect on time spent in the open arms ($F(2,26)=2.70$, $p=0.08$), although it produced a significant effect on the total number of entries to all arms ($F(2,26)=6.67$, $p<0.01$). Planned comparisons indicated that both groups treated with TBZ showed reduced locomotion; TBZ plus vehicle ($p<0.01$) and TBZ plus10 mg/kg bupropion ($p<0.05$) in comparison with the vehicle-vehicle group.

DL box	Vehicle/Vehicle	TBZ/ Vehicle	TBZ/ Bupropion 10 mg/kg
Time in the lit chamber (sec)	125.6 ± 6.2	117.1 ± 11.5	111.3 ± 13.8
Crosses between compartments	23.6 ± 1.7	12.6 ± 1.5**	17.2 ± 2.2*
EPM			
Time in open arms (sec)	73.3 ± 17.4	100.5 ± 14.3	61.0 ± 8.7
Entries to all arms	19.0 ± 1.4	10.6 ± 1.6**	13.9 ± 1.3*

Table 2. Effect of TBZ and BUP combination on mice behavior in the Dark and Light Box and Elevated Plus Maze. Every test lasted 5 minutes. Mean (± SEM) seconds or frequency. *$p<0.05$, **$p<0.01$ significantly different from vehicle.

Experiment 7. Effect of bupropion in combination with TBZ on DARPP-32 phosphorylation patters in Nacb as measured by immunohistochemistry. One way ANOVA's showed an overall effect of treatment on pDARPP-32(Thr34) in Nacb core ($F(2,11)=18.35$, $p<0.01$). Planned comparisons revealed that the administration of 8 mg/kg TBZ increased significantly phosphorylation of DARPP-32(Thr34) in Nacb core ($p<0.01$) in comparison with the vehicle-vehicle group. TBZ plus bupropion (10 mg/kg) was significantly different from the TBZ-vehicle group ($p<0.05$) (Figure 4). However,

there was no significant effect of treatment on pDARPP-32(Thr75) ($F(2,12)=1.31$, $p=0.30$) (Figure 4).

Fig 4

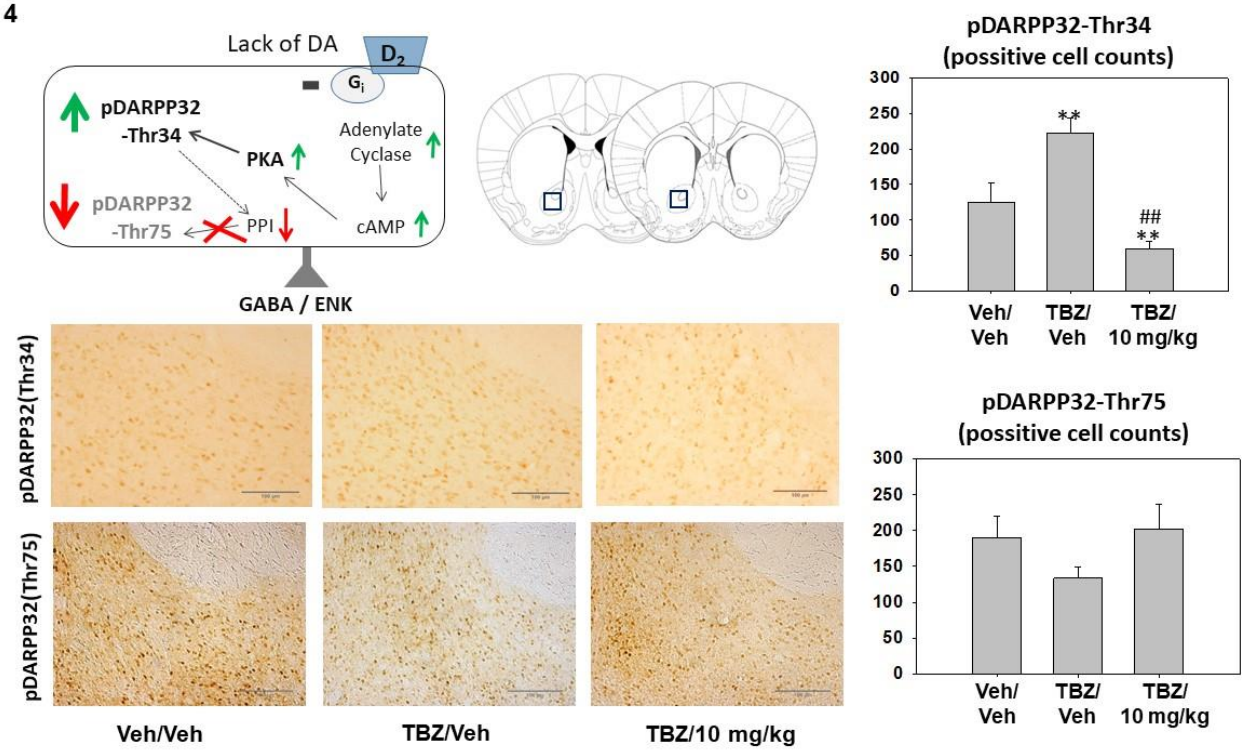

Figure 4. Effect of TBZ and bupropion combination (Vehicle+Vehicle, 8 mg/kg TBZ+Vehicle, 8 mg/kg TBZ+10 mg/kg bupropion) on number of cells counts positive for pDARPP-32(Thr34) and pDARPP-32(Thr75) immunoreactivity on Nacb core. Left: photomicrographs of individual representative brains. Right: mean ± S.E.M of number of pDARPP-32-(Thr34) counts in a $100um^2$ ROI. **p<0.01 significantly different from Vehicle+Vehicle. ##p<0.01 significant difference from TBZ-Vehicle.

Experiment 8. Effect of TBZ on pDARPP-32(Thr75) levels in ventral striatum as measured by western blot. The Student t-test for independent samples did not show a significant effect of treatment on pDARPP-32(Thr75) when analyzed by western blotting ($t(16)=1.27$, $p=0.22$) (Figure 6).

Fig 6

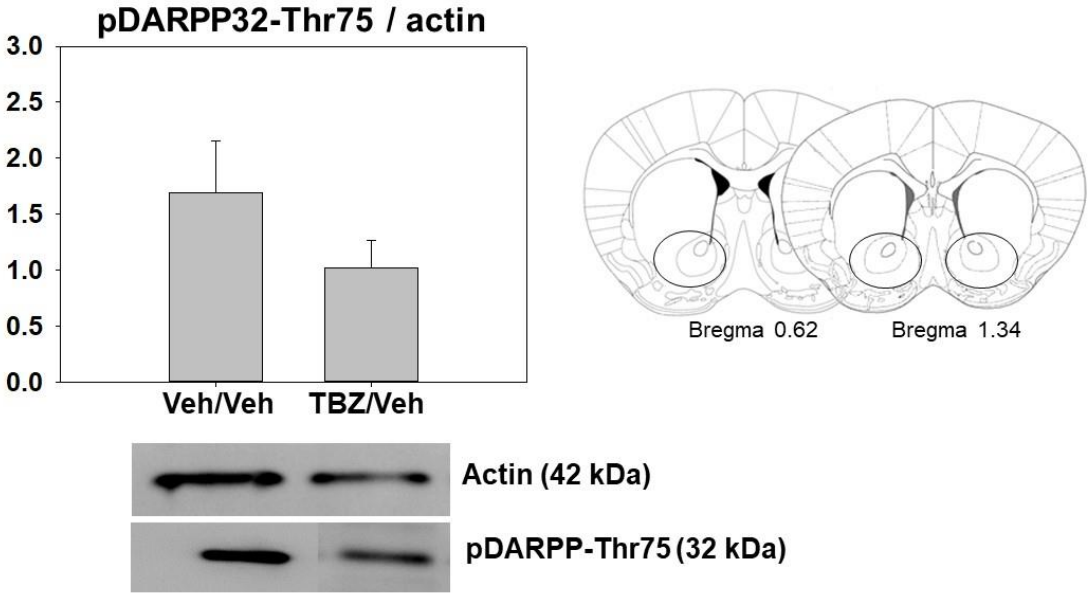

Figure 6. Effect of TBZ (Vehicle+Vehicle and 8 mg/kg TBZ+Vehicle) on pDARPP-32 (Thr75) protein level in ventral striatum. Data are expressed as mean ± S.E.M of density units of pDARPP-32 (Thr75). Lower part: representative western blots showing two bands corresponding to actin (molecular weight of 42 kDa), and pDARPP-32(Thr75) (molecular weight of 32kDa). Each line contains 30ug of ventral striatum homogenates taken as tissue punctures represented in the coronal brain sections.

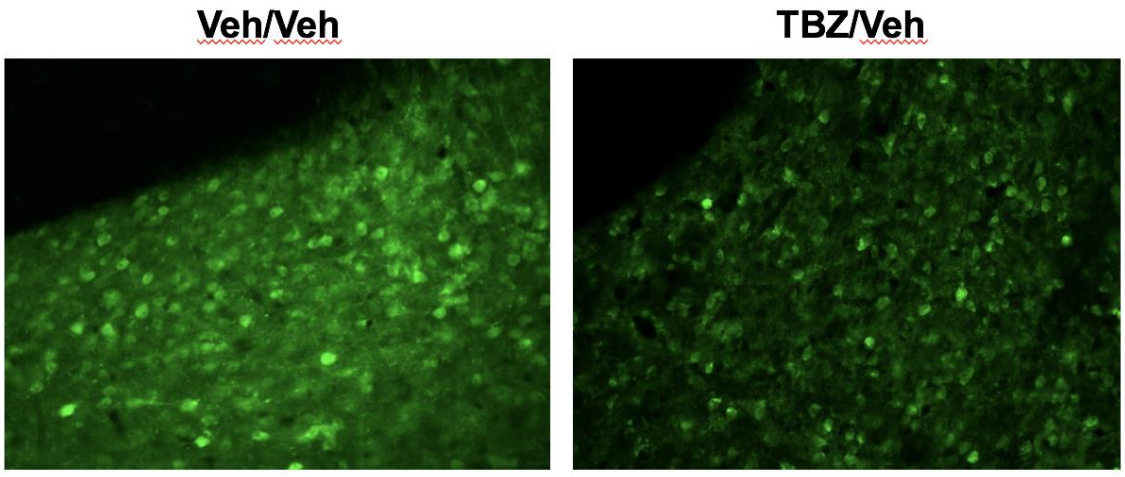

Figure 5. Top: Photomicrographs of pDARPP-32(Thr75) staining in nucleus accumbens core, showing representative mice treated with 8 mg/kg TBZ. Images were taken at 20x magnification.

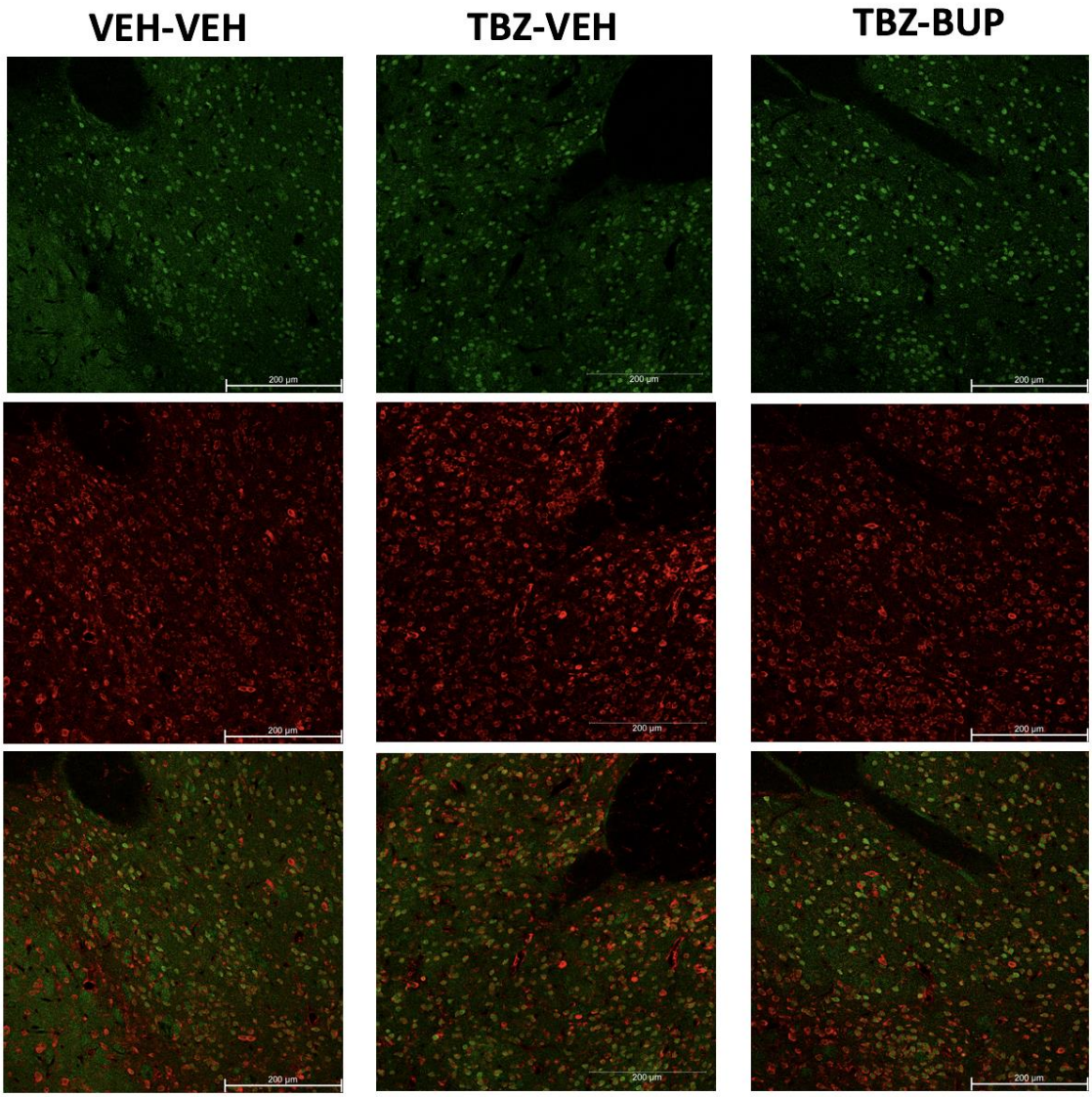

Figure 6. Top: Photomicrographs of pDARPP-32(Thr75) staining in nucleus accumbens core, showing representative mice after administration of different treatments. Images were taken at 20x. Scale bar: 200um.

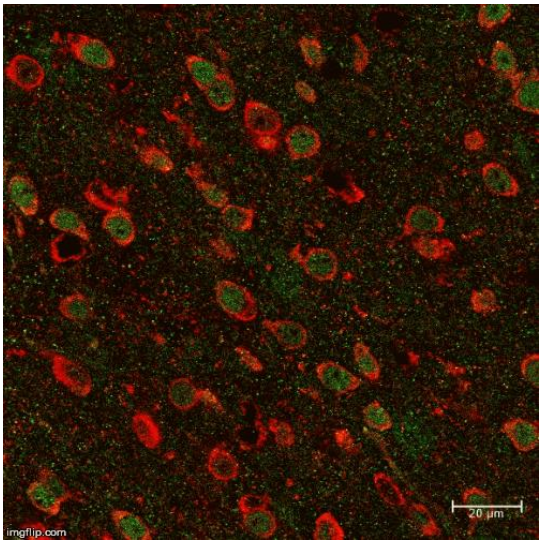

Figure 7. pDARPP-32(Thr75) is colocalized with enkephalin immunofluorescence activity. Images were taken at 40x. Scale bar: 20um.

Discussion

The current studies examined the ability of the DAT inhibitor bupropion to produce an effect on selection of active reinforcers assessed in a novel 3-choice-T-maze-task that provides information about the choice between highly and active physical activity vs. other sources of reinforcement that could be obtained with little effort. Those studies were compared with additional experiments that examined how bupropion was able to modulate behavioral activation in the FST, a classical paradigm that evaluates how antidepressant drugs potentiate behaviors to scape a stressful unescapable environment, interpreted as depression-like behaviors. Moreover, we also assessed if the same pharmacological manipulations have an impact on emotional parameters assessed in the DL box and EPM paradigm, used to evaluate anxiety-like behaviors. In addition, in order to know the ability of bupropion to alleviate fatigue and behavioral activation impairments we used TBZ (8 mg/kg), since this drug has been reported to induce motivational symptoms such as anergia or fatigue in people (Frank et al, 2009; Guay et al, 2010; Chen et al, 2012), as well as behavioral activation impairments in classical animal models of depression (Jang et al, 2009; Wang et al, 2010; Carratalá-Ros et al, 2020), and in effort-related decision making tasks (Nunes et al, 2013; Randall et al, 2014a; Yohn et al, 2016a, López-Cruz et al, 2018; Rotolo et al, 2019).

Bupropion is a catecholamine uptake inhibitor mostly prescribed in the clinical field as an antidepressant (Dwoskin et al, 2006; Papakostas et al, 2006; Nutt et al, 2007). By blocking DAT and increasing extracellular DA levels (Hudson et al, 2012; Randall et al, 2014a), bupropion is able to alleviate motivational symptoms such as anergia, fatigue and psychomotor retardation commonly seen in depression (Rampello et al, 1991; Sthal et al, 2002; Demyttenaere et al, 2005; Pae et al, 2007).

In our experiments, mice were introduced to the T-maze task, a paradigm that allows animals to freely choose between non-aversive stimuli; animals can choose between a highly preferred reinforcer that requires a high degree of vigor (running in a RW), or a palatable reinforcer (50% sucrose pellets) that is also preferred in contrast to other types of foods (Nunes et al, 2013), or doing more sedentary activities such as sniffing a fruit odor (López-Cruz et al, 2018; Carratalá-Ros et al, 2020). Under basal conditions, mice usually spent most of the time in the preferred reinforcer which is the RW, secondly spent their time eating the sucrose pellets, and the least preferred reinforcer is always the non-

social odor (López-Cruz et al, 2018; Carratalá-Ros et al, 2020). In the present studies, although the baseline of preference for the RW was already high, the administration of bupropion was able to increase significantly time spent running in comparison with the vehicle group, and also time spent in the RW compartment. This effect was paralleled by a mild reduction in preference for sucrose that was significant for the variable time spent in the food compartment (figure 1E), but there was no impact on the least preferred reinforcer; the odor. These doses of bupropion did not seem to affect general ambulation in the T-maze as there was no change in number of crosses between compartments. Our results are similar to previous studies where the administration of different doses of this DAT inhibitor in rats shifted behavior increasing the tendency to work by lever pressing for food in a highly demanding task; progresive ratio (Randall et al, 2014b; Bruijnzeel and Markou, 2003). Rats increase work output in order to obtain the same type of palatable food used in our T-maze, and they decreased the consumption of concurrently available chow that is much less preferred (Randall et al, 2014a,b). Other more selectively DAT inhibitors such as GBR12909, PRX-14040, lisdexamfetamine, MRZ-9547 and S-CE-123 also produced a significant increase on lever pressing in PROG/chow tasks and decreased free, but less preferred, chow (Sommer et al, 2014; Yohn et al, 2016a,b,c,d; Rotolo et al, 2019). In addition, mice lacking DAT, also increased the tendency to work for a food reward in a concurrent task (Cagniard et al, 2006).

Bupropion also decreased passive behaviors and increased active behaviors such as swimming and climbing assessed in the FST. Previous studies have reported similar effects of bupropion in mice and rats on traditional paradigms for the assessment of antidepressant drugs such as the tail suspension test and the FST (Bourin et al, 2005; Kitamura et al, 2008, 2010; Yamada et al, 2004; Hufgard et al, 2017). However, in very few of them climbing (or struggling to do so in order to scape a stressful and unescapable situation) has been assessed for bupropion previously (Hayashi et al, 2011; Rénéric and Lucki, 1998). The maintenance of vigorous and persistent active responding (Gil and Armario, 1998) in order to escape is related with DA transmission, and studies that use DA antagonists or DA reuptake inhibitors show a decrease or increase respectively on the output of active behaviors in the FST (Li et al, 2015; Kitamura et al, 2010; Yamada et al, 2004).

Previous studies from our laboratory have demonstrated the anergia inducing effects of the DA depleting agent TBZ in effort-based decision-making tasks such as the present T-maze RW-choice task (López-Cruz, 2018; Carratalá-Ros et al, 2020), the T-maze barrier-choice task (Yohn et al, 2015a,b; Correa et al, 2018), and also in operant procedures with different ratio requirements (PROG/chow tasks and FR5/chow) (Nunes et al, 2013; Randall et al, 2014a,b; Yohn et al, 2015a, 2016b, Rotolo, 2019). Those effects were not due to changes in appetite, food preference or food reactivity (Nunes et al, 2013; Randall et al, 2014a; Pardo et al, 2015a,b; Yang et al, 2019; Correa et al, 2018, Carratalá-Ros et al, 2020).

In the present study, we used a dose of TBZ (8 mg/kg) that had demonstrated to be effective in mice for depleting ventral striatum DA (López-Cruz, 2018) and shifted behavior towards sedentary sources of reinforcement (Carratalá-Ros et al, 2020). TBZ-treated mice reduced significantly time running in the RW and shifted their behavior towards spending more time doing sedentary activities (consuming sucrose pellets) than under the vehicle condition. Bupropion, was able to reverse TBZ-induced anergia. In fact, co-administration of 10 mg/kg of bupropion to TBZ-treated animals produced a significant reversal of the effects of the DA depleting agent, as indicated by an increase, equal to control levels, on time running in the RW, and a decrease on time consuming sucrose. These results are in accordance with previous studies done in our laboratory in which the administration of different doses of bupropion to rats alleviated TBZ-induced anergia in operant procedures such as PROG/chow tasks and FR5/chow tasks (Yohn et al, 2015a, 2016a; Randall et al, 2014a; Nunes et al, 2013).

In addition, the most effective dose of bupropion (10 mg/kg) in the T-maze task, and the one that had improved climbing in the FST, also reversed immobility induced by TBZ, and restored swimming and climbing to normal levels in TBZ treated mice. According with the present results, bupropion was able to decrease immobility time in VMAT-2 mutant mice evaluated in the FST (Fukui et al, 2007).

None of these “therapeutic” effects of bupropion on its own or in TBZ treated animals, seem to be due to anxiolytic actions, since there were no changes in anxiety measures in the DL box or in the EPM (Table 1 and 2). Similar doses of bupropion in adult mice had previously demonstrated not to have an impact on emotional parameters evaluated in the EPM paradigm (Carrasco et al, 2013, 2004; Redolat et al, 2005a), but these doses do have

effects on motor activity in mice (Redolat et al, 2005b), and because the EPM has demonstrated to be sensitive to the motor actions of bupropion (Carrasco et al, 2004), we also analyzed crosses between compartments in the DL box and entries to all arms in the EPM, as indices of changes in locomotion. Thus, the highest dose of bupropion used in the present experiments (15 mg/kg), did increased locomotion assessed as total of entries to all arms in the EPM paradigm, and TBZ decreased total crosses between compartments in the DL box and total of entries to the EPM paradigm. Bupropion at 10 mg/kg was not able to reverse this effect in TBZ-treated animals. Thus, none of the doses used in the present experiments seem to modulate anxiety although some of them can affect locomotion in these paradigms, an effect that was not seen as total number of crosses in the T-maze. Thus, behavioral activation seems to be slightly affected by these two drugs as seen in parameters such as locomotion and exploration but also as the more active behaviors in the FST.

All these results seem coherent with the well know functions of Nacb DA which has demonstrated to have a key role in the regulation of voluntary locomotion and effort related aspects of motivated behavior (Salamone and Correa 2002, 2012). Bupropion, which acts as a DAT blocker, increases extracellular DA levels in Nacb as measured by microdialysis (Randall et al, 2014b), and also a high increase on immunoreativity of the postsynaptic marker DARPP-32 phosphorilated at different sites depending on the type of DA receptor that is activated (Randall et al, 2014b; Svenningson et al, 2004; Bateup et al, 2008). On the contrary, TBZ reduces DA levels (Nunes et al., 2013; López-Cruz et al., 2018), and produces the opposite pattern of DARPP-32 phosphorilation than bupropion (Nunes et al, 2013; Randall et al, 2014b; López-Cruz et al, 2018). In the present results, in mice we demonstrate using immunoreactivity methods that TBZ significantly increased pDARPP-32(Thr34) compared to vehicle treated mice, and this effect of TBZ was reversed by bupropion (10 mg/kg) in Nacb core, but not shell (data not shown), indicating the core subregion of Nacb is a more critical site for regulating persistent and sustained vigor in effort-choice tasks that have already been learned (Sokolowski et al., 1998; Segovia et al., 2011, 2012). Interestingly for the present results, Randall and colleagues (2012) showed an increase of pDARPP-32(Thr34) in Nacb core in high lever pressing responder rats compared with low responders in the PROG/chow task that is the most work requiring operant task. In addition, the administration of S-CE-123, an atypical and selective DAT inhibitor, which has demonstrated to reverse TBZ-induced shift on an

FR5/choice task, also produced an increase on extracellular DA levels in Nacb core (Rotolo et al, 2019).

However, it seems that in mice TBZ tends to suppress phosphorylation of DARPP-32 at threonine 75 (pDARPP-32(Thr75)) in Nacb core. Thus, using different antibodies and using two different techniques (immunoblotting and immunohistochemistry) pDARPP-32(Thr75) in TBZ treated mice seems to be reduced, although results are not significant, probably because it is difficult to see lower levels of phosphorilation than in the vehicle group. Using immunofluorescence techniques could be a more sensitive way to be able to account for the reduction, as it is suggested by the sample pictures shown in figure 5. These results seem to differ from previous studies using immunohistochemical techniques in rats, in which TBZ demonstrated an increase in both phosphorylated forms of DARPP-32 (Thr34 and Thr75) in Nacb core and shell (Nunes et al, 2013), but they seem in line with previous results in mice using western blotting in which TBZ only produced an increase on pDARPP-32(Thr34), but not on pDARPP-32(Thr75) in ventral striatum (López-Cruz et al, 2018). Moreover, as suggested by the literature and the co-localization images presented here (lower part of figure 5), it seems that in mice TBZ induced changes are mainly occurring in D2 containing neurons (Nunes et al, 2013; Svenningsson et al, 2000; Bateup et al, 2008; Bonito-Oliva et al, 2011). D2 receptors are co-localized on enkephalin-containg medium spiny neurons (MSNs), while D1 receptors are co-localized on substance-P containing MSNs (Svenningsson et al, 2000; Bateup et al, 2008). In addition, in the present studies bupropion was able to reverse the induction of pDARPP-32(Thr34) produced by TBZ, pointing to a predominant effect of both drugs in D2 receptors situated in striatal enkephalin-containing MSNs. However, further work should assess if this cells also contain D1 receptors, and also if there is co-localization with pDARPP-32(Thr34) positive cells.

In summary, our results show how bupropion increases the motivation to choose and to keep active under different type of behavioral settings (stressful or rewarding). Together with previous studies using different rodent and behavioral models (Randall et al, 2012, 2014a,b; Yohn et al,2015a, 2016a), our results also show how bupropion improves impaired behavior after DA depletion but also baseline responding, specially, in low responders (Randall et al., 2012, 2014a).These results are supported by other studies in which this DAT blocker exerts great behavioral effects at doses that augment pre- and post- synaptic markers of DA transmission (Nunes et al, 2013; Randall et al, 2014b, Yohn

et al, 2015a). Moreover, bupropion also has demonstrated to have therapeutical effects on motivational symptoms in humans (Pae et al, 2007; Papakostas et al, 2006; Argyropoulos and Nutt, 2013; Soskin et al, 2013), and the rank order of clinical effectiveness in depressed patients with psychomotor retardation, paralleled the specificity of antidepressants as dopaminomimetic agents (Rampello et al, 1991; Brown and Gershon, 1993; Treadway and Zald, 2011; Treadway and Pizzigali, 2014). These motivational symptoms are not alleviated by classical antidepressant treatments such as serotonin uptake inhibitors (Fava et al, 2014; Sthal et al, 2002), which seem more effective in patients affected by anxious depression (Rampello et al, 1995). The present results support the idea that alterations in dopaminergic transmission could contribute to the pathophysiology of motivational impairments in depression (Salamone et al, 2016b,c; Sthal et al, 2002; Treadway and Zald, 2011), and they emphasize the recent therapeutical approach that considers necessary to take into consideration the cluster of symptoms that patients with depression show to create a more effective pharmacological and individualized approach (Cuthbert and Insel, 2013).

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CHAPTER III

Fluoxetine has antidepressant effects in the forced swim test, but anergic effects in the T-maze three-choice task: potentiation of dopamine depletion induced impairments.

Abstract

Impairments in behavioral activation and effort-related motivational functions like fatigue, anergia and apathy are treatment resistant symptoms of depression. Depressed people show a decision bias towards selection of low effort alternatives. *Objectives.* In studies with male CD1 mice, we evaluated the ability of fluoxetine to modulate behavioral activation in different models. Fluoxetine was assessed using a classical depression model (forced swim test (FST)), and an effort-based choice task (running wheel (RW) T-maze task). We also studied the ability of fluoxetine to reverse the effects of the dopamine depleting agent tetrabenazine, which reduces activity in both tasks. In the FST experiment, fluoxetine increased active behaviors (swimming, climbing) while reducing passive behaviors (immobility). Fluoxetine did not reverse tetrabenazine-induced anergia, in fact exacerbating it. With the T-maze task, fluoxetine produced the same pattern of effects as tetrabenazine, reducing time running, but increasing time consuming sucrose. Fluoxetine did not reverse tetrabenazine-induced suppression of RW activity, but did suppress time consuming sucrose in tetrabenazine-treated mice. The pattern of effects produced by fluoxetine in tetrabenazine-treated mice was dissimilar from that seen after reducing food value by prefeeding or making the food bitter tasting, since in both cases, time in the RW increased. The suppressive effect of tetrabenazine+fluoxetine in the RW was consistent with the decrease in locomotion seen under anxiogenic conditions, and the reduced crossing between compartments in the T-maze. These treatments reduced pDARPP32 at both phosphorylating sites in Nacb. These results have implications for the use of fluoxetine in the treatment of depressive symptoms such as fatigue and anergia in humans.

Fluoxetine, serotonin, dopamine, depression, motivation, effort, anxiety, sedentarism.

Introduction

Motivational symptoms such as psychomotor retardation, anergia, lack of energy, lassitude, fatigue and reduced exertion of effort are common and critical in major depressive disorder (Stahl, 2002; Demyttenaere et al, 2005; Salamone et al, 2007; Treadway et al, 2011; Fava, 2014). These highly debilitating symptoms in depression are highly correlated with problems in social function, and employment (Tylee, 1999; Stahl, 2002, 2017; Hodgetts et al, 2017). They are also highly resistant to treatment, and remain as residual symptoms after remission (Stahl, 2002; Nutt et al. 2007; Fava et al. 2014). Many common antidepressants including serotonin (5-HT) transporter (SERT) inhibitors such as fluoxetine and citalopram are useful for treating mood symptoms in depression (Hieronymus et al., 2016; Rizvi et al., 2013; Rosenblau et al, 2012), but they are relatively ineffective for treating motivational dysfunctions, and, in fact, it has been suggested that SERT inhibitors can exacerbate or induce these symptoms in some patients (Nutt et al, 2007; Targum and Fava, 2011; Padala et al., 2012; Stenman and Lilja, 2013; Rothschild et al, 2014; Fava, 2014).

Because of the clinical significance of motivational dysfunctions, it is critical to develop animal models that allow researchers to study a broad range of impairments, and also to assess the ability of different drugs to reverse them. Thus, some studies have focused upon different tasks involving effort-based decision making that offer rodents the possibility of choosing between high effort instrumental actions leading to more valued reinforcers or to choose the low effort options leading to less valued reinforcers. Conditions associated with depression, including stress (Shafiei et al, 2012; Bryce and Floresco, 2019, 2016), pro-inflammatory cytokines (Nunes et al, 2014; Yohn et al, 2016c; Goldsmith et al, 2016), and dopamine (DA) receptor antagonism (Yang et al, 2019; Pardo et al, 2012; 2015; Yohn et al, 2015b; Correa et al, 2016) or DA depletion (Nunes et al, 2013; Randall et al, 2014; Pardo et al, 2015; Yohn et al, 2015a; López-Cruz et al, 2018; Rotolo et al, 2019; Carratalá-Ros et al, 2020) can affect effort-related decision making and bias animals towards the low effort options. These results obtained from animal studies are consistent with clinical data showing a reduced selection of high effort alternatives in depressed people tested on tasks of effort-based choice (Treadway et al, 2012; Chong et al, 2016). Administration of the vesicular monoamine transporter (VMAT-2) inhibitor tetrabenazine (TBZ), which blocks the monoamine storage and leads to a striatal DA depletion at low doses in primates (Pettibone et al, 1984), rats (Nunes et

al, 2013; Podurgiel et al, 2015, 2016; Yohn et al, 2016b), and mice (López-Cruz et al, 2018; Carratalá-Ros et al, 2020), has been used to induce effort-related motivational impairments in rodents (Nunes et al, 2014; Yohn et al, 2015a,b; Yohn et al, 2016a,b; López-Cruz et al, 2018; Correa et al, 2018; Rotolo et al, 2019; Carratalá-Ros et al, 2020). This drug is used in the treatment of Huntington's disease and has been reported to produce depressive symptoms including fatigue in people (Frank et al, 2009; Guay et al, 2010).

Voluntary physical activity is important for phenomena as varied as motivational performance in laboratory experiments, and workplace activity in humans. It has been observed that a lack of activity can contribute to the development of depression (Lambert 2006), and in depressed people, symptoms such as loss of interest, motivation and energy, and generalized fatigue problems interfere with participation in exercise (Knapen et al, 2015). Of course, the choice to engage in voluntary physical activity is always undertaken in relation to the possible selection of other alternatives, such as sedentary behaviors or food consumption. Voluntary running wheel was used in a recent T-maze choice task as the high effort/highly preferred option (Correa et al, 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020). This paradigm assesses the impact of drugs, homeostatic or environmental manipulations on behavioral activation and effort-related choice. This task allows the animal to freely choose between running on a running wheel (RW), consuming sucrose pellets, or sniffing a neutral non-social odor. It has been demonstrated that TBZ produced a dose dependent shift in response selection, reducing preference for the energy requiring reinforcer (wheel running), but concurrently increasing time with the second preferred reinforcer (palatable food) that required little effort to obtain (López-Cruz et al, 2018; Carratalá-Ros et al, 2020).

The ability of monoamine uptake inhibitors to reverse the effects of TBZ on effort-based choice in rodents differs depending upon their specific mechanism of action. For example, SERT inhibitors such as fluoxetine or s-citalopram failed to reverse the low-effort bias induced by TBZ in rats tested in operant paradigms in which animals had to work (lever press) to get access to more palatable food or could choose to approach and consume a less preferred but freely available chow (Yohn et al, 2016a,b,c). However, DAT inhibitors reverse TBZ effects (Randall et al, 2014; Yohn et al, 2016a,b,c). In classical animal paradigms that evaluate the therapeutic actions of antidepressant drugs such as the forced swim test (FST), TBZ suppressed active behaviors (swimming and climbing), while increasing passive ones (immobility) (Carratalá-Ros et al, 2020). Some studies have

shown that fluoxetine effectively enhances active behaviors and reduces passive ones in the FST (Jang et al, 2009; Castagné et al, 2010; Petit-Demouliere et al, 2005).

The present study explored the effect of fluoxetine administered alone or in combination with the DA depleting agent TBZ in the preference for active reinforcers in the 3-choice RW T-maze task, comparing those results with actions in classic paradigms that evaluate depressive-like behaviors such as the FST, which involves a stressful test setting. We also evaluate the effect of these drugs and their combination on anxiety paradigms (dark and light box; DL box and the elevated plus maze; EPM). Finally, we also assess the effect of behavioral manipulations that change the emotional or homeostatic value of food in this novel task. The proposed work was not presenting a global model of depression per se, or providing a general screening of antidepressant drugs, but rather focused on a behavioral component (active exertion of physical effort) that is particularly important for the motivational symptoms of depression, and potentially other disorders as well.

Materials and Methods

Animals. CD1 adult male mice (N=106) purchased from Janvier, France S.A. were 8-14 weeks old (40-50 g) at the beginning of the study. Mice were housed in groups of three or four per cage, with standard laboratory rodent chow and tap water available *ad libitum*. The colony was kept at a temperature of 22 $\pm$ 2 ºC with lights on from 08:00 to 20:00 h. All animals were under a protocol approved by the Institutional Animal Care and Use committee of 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 Behavioral Research", National Research Council 2003, USA. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Pharmacological agents. Fluoxetine (CIMYT Quimica SL, Spain) was dissolved in 0.9% saline and administered 30 minutes before testing. The range of doses of fluoxetine was based on studies involving classical mice antidepressant screening tests (Lucki et al, 2001) and effort-related behavioral tests (Yohn et al, 2016a,b). In order to conduct the reversal behavioral experiments, the VMAT-2 blocker tetrabenazine (TBZ; CIMYT Quimica SL, Spain) was used. TBZ was dissolved in a vehicle solution of 0.9% saline (80%) plus dimethylsulfoxide (DMSO 20%, final pH 5.5) and administered 120 minutes

before testing. Time elapsed after injection and the dose of 8.0 mg/kg TBZ were selected based on previous behavioral work (López-Cruz et al, 2018; Correa et al, 2018; Carratalá-Ros et al, 2020) and neurochemical studies (López-Cruz et al, 2018) demonstrating that in mice this is an optimal dose and time lead to deplete DA. DMSO (20% v/v) and saline was used as the control group. All the substances were administered intraperitoneally (IP).

Testing procedures

All behavioral procedures started two hours after the light period started. The behavioral test room was illuminated with a soft light, and external noise was attenuated.

Forced swim test (FST). This paradigm is considered to be a model of behavioral despair and is used as a test for assessing depressive-like states and for evaluating drugs with potential as antidepressants (Porsolt et al, 1977). Immobility was defined as the animal remaining motionless, making only minor movements to balance the body and keep the head above the water. In addition, we also assessed escape-related mobility such as climbing or struggling (Armario et al, 1988). Climbing is defined as any energetic and vertical movement of all four limbs against the wall of the tank. Mild 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). Naïve mice were placed in a transparent cylindrical glass tank (26 cm high and 18 cm diameter) filled with water (14 cm) and maintained at a temperature of 25ºC. Water was changed between animals. During the 6-minute test, mice were videotaped from the side, and climbing, immobility and swimming were later measured by an observer unaware of the experimental condition. After the test, mice were dried with a soft towel, put back in a box with absorbing paper under a warming light, and were monitored for 10 minutes.

Dark and light box (DL). The DL test is based on the conflict between the tendency to explore a novel environment and the avoidance of a brightly lit open area (Blumstein and Crawley 1983). The DL apparatus consisted of a polypropylene chamber divided in two compartments by a partition containing a small opening (5 cm H x 5 cm W). The light compartment (25 cmW x 25 cm H x 25 cm L) was open, painted in white and illuminated (335 lx), while the dark compartment (25 cm W x 25 cm H x 18 cm L) was painted in black and had a removable ceiling to close it (Kulesskaya and Voikar, 2014). To start the test session, mice were individually placed in the dark chamber facing one corner. Test

sessions were videotaped, and the latency of the first entry into the lit chamber, the total number of crosses and the total time spent in the lit chamber were recorded for 5 min (López-Cruz et al, 2014; Carratalá-Ros et al, 2020).

Elevated plus maze (EPM). The EPM consists of two open and two enclosed arms (65 cm L x 5 cm W) arranged in a plus configuration and intersecting in a central platform. It is made of black polypropylene and is elevated 50 cm above the floor. The open arms have a 1 cm border around their perimeter and the closed arms have a 20 cm translucent wall. This anxiety paradigm measures the avoidance that rodents show to high open spaces. Under normal conditions, mice spend more time in and make more entries into the closed arms of the maze (Lister 1987). Animals were placed in the central platform with their head pointing at one enclosed arm, and they were assessed during 5 minutes. Sessions were videotaped and a trained observer registered total time spent in the open arms, ratio of entries into the open arms compared to total arm entries, and total entries in the 4 arms as an index of locomotion. An entry into an arm was recorded when the animal crossed with all four legs the line that connected that arm with the central platform (procedure is based on López-Cruz et al, 2014).

T-maze RW-sucrose-odor choice task. The T-maze apparatus consisted of a central area that lead to 3 arms (based on López-Cruz et al, 2018). In one of them, sucrose pellets (TestDietTM, 50% sucrose, 45 mg 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. Training as well as test sessions lasted 15 minutes. Mice were trained in one session, 5 days a week. Training phase 1: to avoid neophobia to the sweet tasting pellets, animals were enclosed in that arm with the food during 5 sessions. Training phase 2: during 2 more weeks animals were exposed, one 15 min session a day to the T-maze with free access to the three stimuli. Test phase: This phase lasted between 2 or 5 more weeks depending on the experiment. For each week, there were 4 training sessions plus a testing session in which animals received drug injections or were exposed to the food manipulations. The day before the manipulation was considered as the baseline (BL). Entries into the arms of the T-maze and time spent in the reinforcer compartment of the arms were simultaneously recorded. All these measures were used based on previous studies (Correa et al, 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020). Time was selected as the main dependent measure because it allowed for the evaluation of the three different stimuli

with the same units. Time allocation is a useful measure of preference, relative reinforcement value, and response choice (Baum and Rachlin, 1969).

Immunohistochemistry for phosphorylated DA and c-AMP related phosphoprotein-32 kDaltons (pDARPP-32). After drug treatments, animals were anesthetized with CO2 and transcardially perfused with 0.9% physiological saline for 5 minutes, followed by perfusion with 3.7% formaldehyde for 5 minutes and brains were extracted. Tissue was fixed in 3.7% formaldehyde overnight and moved to 30% sucrose cryo-protectant. Then 40-um sections were cut using a cryostat. To measure the immunoreactivity to phosphorylated DA and c-AMP-regulated phosphoprotein 32kDa (pDARPP-32) nonspecific binding sites were blocked with a solution of 3% H202 for 30 minutes at room temperature following then with 1% bovine serum albumin and 0.1% Triton X-100 in PBS for 1 hour at room temperature. Phosphorilated DARPP-32 immunoreactivity was visualized with a polyclonal rabbit antibody for DARPP-32 phosphorilated at threonine 34 residue (Thr34, 1:1000; Santa Cruz Biotechnology) or polyclonal rabbit antibody for DARPP-32 phosphorilated at threonine 75 residue (Thr75, 1:500, Santa Cruz Biotechnology). These antibodies were dissolved in solutions that also contained 0.1% bovine serum albumin and 0.1 Triton X-100 in PBS for 24 h incubation on a rotating shaker at 4ºC. After the primary antibody treatment, the sections were rinsed in PBS (3 times for 5 minutes) and incubated in the secondary antibody, anti-rabbit HRP conjugate envision plus (DAKO) for 1 hour and 30 minutes on a rotating shaker at room temperature. Finally, sections were washed and rinsed for 3-5 minutes in 3,3' diaminobenzidine chromagen. The sections were then mounted to gelatin-coated slides, air dried and coverslipped using Eukitt quick-hardening (Sigma-Aldrich) as a mounting medium. The tissue was then examined by light microscopy.

Quantification of the number of cells that express immunoreactivity for pDARPP-32 (Thr34) and pDARPP-32 (Thr75) in the Nacb was performed by photographing the sections with a 20X (0.125 mm^2 / field) objective (Eclipse E600; Nikon) upright microscope equipped with a Leica DFC 450C camera (Leica Microsystems) and captured digitally using LASX software. Cells that were positively labeled for pDARPP-32 (Thr34) and pDARPP-32 (Thr75) were quantified with ImageJ software (version 1.51) and a macro written to automate particle counting within the region of interest. The size of the region of interest was 1000 x 1000 um. For each animal, cell counts were at levels that correspond to 0.70-1.70 mm anterior to bregma (Paxinos and Watson, 1997)

bilaterally from at least three sections and counts were averaged across slides and sections.

Experiments

Experiment 1. Effect of fluoxetine on depressive-like behaviors measured in the FST. Animals (N=31) received one dose of fluoxetine (vehicle, 10.0, 15.0 or 20.0 mg/kg) and 30 min after the injection were placed in the FST during 6 minutes. Mice were exposed only once to the FST since behavioral habituation develops in one session.

Experiment 2. Effect of TBZ and fluoxetine combination on depressive-like behaviors measured in the FST. Three groups of naïve mice (N=38) received a combination of TBZ (8.0 mg/kg, a dose that was shown to be effective in the FST) (Carratalá-Ros et al, 2020) plus vehicle or the highest dose of fluoxetine (20 mg/kg) or the combination of both vehicles. Fluoxetine was given 90 minutes after TBZ and 30 minutes before animals were placed in the FST.

Experiment 3. Effect of fluoxetine on anxiety parameters as measured in the DL and EPM paradigms. Mice (N=35) received one dose of fluoxetine (vehicle, 10.0, 15.0 or 20.0 mg/kg) and 30 min after injection were first place in the DL box for 5 minutes. Immediately after this test, they were placed in the EPM for 5 more minutes. Mice were exposed only once to both paradigms, since behavioral habituation develops in one session.

Experiment 4. Effect of TBZ and fluoxetine combination on anxiety parameters as measured in the DL and EPM paradigms. Three groups of naïve mice (N=32) received a combination of TBZ (8.0 mg/kg) plus vehicle or the highest dose of fluoxetine (20.0 mg/kg) or the combination of both vehicles. Fluoxetine was given 90 minutes after TBZ and 30 minutes before animals were placed in the DL box for 5 minutes, and immediately after this test, they were placed in the EPM for 5 more minutes.

Experiment 5. Effect of fluoxetine on preference for active reinforcers as measured in the 3-choice-T-maze task. After reaching a stable baseline level of performance in the T-maze, animals (N=10) received fluoxetine (vehicle, 10.0, 15.0 and 20.0 mg/kg) and, after 30 minutes, were placed in the T-maze for 15 minutes. Animals received one dose of the drug every week in a randomly varied order. The T-maze paradigm requires a

baseline performance of two weeks before tests starts, and that performance is maintained across weeks, thus allowing a repeated measures design. We have observed that fluoxetine does not produce sensitization or tolerance when administered once per week.

Experiment 6. Effect of TBZ and fluoxetine combination on preference for active reinforcers as measured in the 3-choice-T-maze task. A new group of mice (N=9) received TBZ (veh, 8.0 mg/kg) and, 90 minutes later, a dose of fluoxetine (veh, 10.0, 15.0, 20.0 mg/kg), and 30 minutes later they were placed in the T-maze during 15 minutes. Animals received one drug combination (veh-veh, TBZ-veh, TBZ-10, TBZ-15 and TBZ20 mg/kg) every week during 5 weeks in a random varied order.

Experiment 7. Manipulations that change the relative value of the sucrose pellets: Change in taste with bitter pellets. Animals (N=8) were trained as described before, and after reaching stable levels of interaction with the three different reinforcers, a drop of quinine (1.0 g/L) was added to the sweet pellets in order to make them bitter. BL behavior was assessed the day before the bitter pellets substituted the regular ones.

Experiment 8. Manipulations that change the relative value of the sucrose pellets: change in appetite by pre-feeding. After training, BL performance of mice (N=12) was recorded. Overnight animals were pre-exposed to sweet pellets *ad libitum,* and the following day, the test session started.

Experiments 9 and 10. Effect of pharmacological and food manipulations on amount of sucrose consumption, or locomotion in the T-maze. Data on pellets consumed (in milligrams) or crosses between compartments in the T-maze from experiments 5, 6, 7, and 8 were analyzed comparing each experimental condition with its respective control condition (vehicle or BL).

Experiment 11. Effect of fluoxetine alone and in combination with TBZ on DARPP-32 phosphorylation patters in Nacb as measured by immunohistochemistry. Mice (N=16) received (veh-veh, veh-fluoxetine 20 mg/kg, or TBZ 8 mg/kg-fluoxetine 20 mg/kg) at the same time leads used for the behavioral studies before perfusion. Coronal sections were analyzed by immunohistochemistry for pDARPP-32 (Thr34) and pDARPP-32 (Thr75).

Statistical Analyses. Normally distributed and homogenous data (according to Kolmogorov-Smirnov test) for the FST, DL, and EPM as well as pDARPP-32 immunoreactivity experiments employed a between groups design and data were analyzed by one-way ANOVA. Normally distributed data in the T-maze experiment followed a within groups design. Thus, when more than 2 experimental conditions were used, such as in the fluoxetine or the TBZ plus fluoxetine experiments, data were analyzed by repeated measures ANOVA. When the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used to compare each treatment with the vehicle control group (Keppel, 1991). For these comparisons, α level was kept at 0.05 because the number of comparisons was restricted to the number of treatments minus one. The effects of changing the taste of the pellets and pre-feeding animals were evaluated by Student's t-test for dependent samples. All data were expressed as mean ± SEM, and significance was set at $p<0.05$. STATISTICA 7 software was used.

Results

Experiment 1. Effect of fluoxetine on depressive-like behaviors measured in the FST. The one-way ANOVAs showed a significant effect on immobility time ($F(3,27)=5.0$, $p<0.01$), time swimming ($F(3,27)=3.41$, $p<0.05$), and time climbing ($F(3,27)=5.68$, $p<0.01$). Planned comparisons revealed that the groups that received 10.0 and 20.0 mg/kg displayed significantly less time been immobile ($p<0.05$, $p<0.01$ respectively) in comparison with the vehicle group. The two highest doses of fluoxetine (15.0 and 20.0 mg/kg) produced an increase on time spent swimming ($p<0.01$ and $p<0.05$ respectively), and only 20.0 mg/kg of fluoxetine increased time climbing in the FST ($p<0.01$) in comparison with the vehicle group (Figure 1A).

Experiment 2. Effect of TBZ and fluoxetine combination on depressive-like behaviors measured in the FST. The one-way ANOVA for time spent swimming ($F(2,35)=0.34$; $p=0.70$) was not significant. However, the ANOVAs for immobility and climbing were significant ($F(2,35)=3.76$, $p<0.05$; $F(2,35)=9.10$, $p<0.01$ respectively). Planned comparisons revealed that the group that received TBZ 8.0 mg/kg plus vehicle, and also the group that received TBZ 8.0 + fluoxetine 20.0 mg/kg displayed significantly

less climbing than the vehicle group ($p<0.01$). Finally, TBZ+ fluoxetine at 20.0 mg/kg increased time of immobility compared with the vehicle group ($p<0.01$) (Figure 1B).

Fig 1

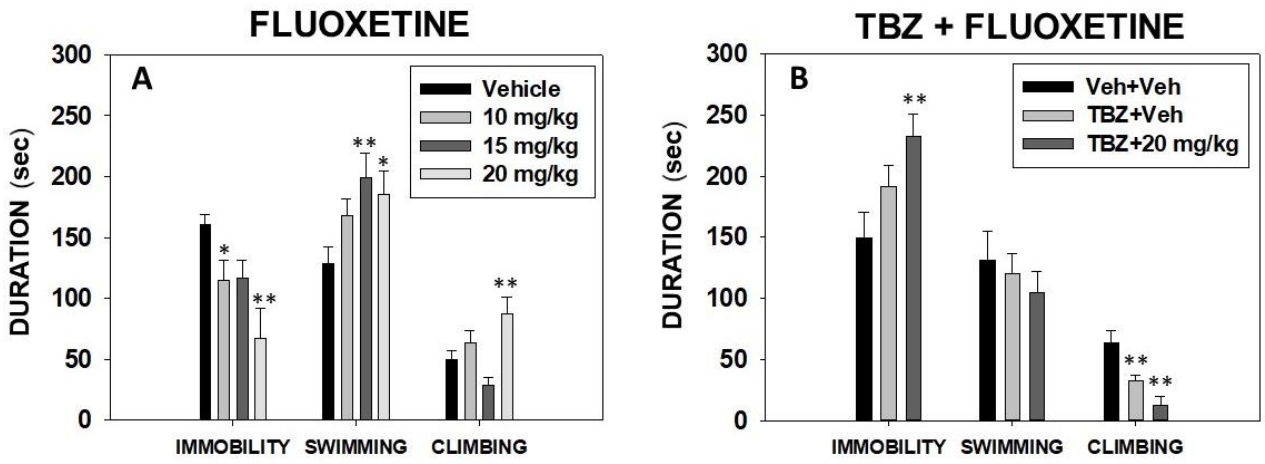

Figure 1. Effect of fluoxetine (Vehicle, 10, 15 or 20 mg/kg) (A) and TBZ plus fluoxetine combination (Vehicle+Vehicle, TBZ 8 mg/kg +Vehicle, or TBZ8 mg/kg and fluoxetine 20 mg/kg (B) on duration of immobility, swimming and climbing behavior in the FST assessed during 6 minutes. Bars represent mean ± S.E.M. of accumulated seconds. * $p<0.05$, **$p<0.01$ significantly different from the control group.

Experiment 3. Effect of fluoxetine on anxiety parameters as measured in the DL and EPM paradigms. The one-way ANOVA showed a significant effect on time spent in the illuminated arena ($F(3,31)=5.11$, $p<0.01$) of the DL box. Moreover, the one-way ANOVA also revealed a significant effect on the total number of entries into both compartments of the DL box ($F(3,31)=2.99$, $p<0.05$). Planned comparisons indicated that mice treated with fluoxetine at 15.0 and at 20.0 mg/kg spent less time in the lit chamber ($p<0.01$) in comparison with the vehicle group. Moreover, the highest dose of fluoxetine produced a decrease in total number of entries into both compartments ($p<0.01$) in comparison with the vehicle group (Figures 2A, B).

The one-way ANOVA for the effect of fluoxetine in the EPM showed a significant effect on the ratio of entries into the open arms ($F(3,31)=7.49$, $p<0.01$). Planned comparisons showed that all doses of fluoxetine reduced ratio of entries compared to vehicle ($p<0.01$). However, the one-way ANOVA did not show any significant effect on time spent in the

open arms (F(3,31)=1.91, p=0.14). The ANOVA for the total number of entries into all arms of the EPM was not significant either (F(3,31)=0.25, p=0.85) (Figures 2C-E).

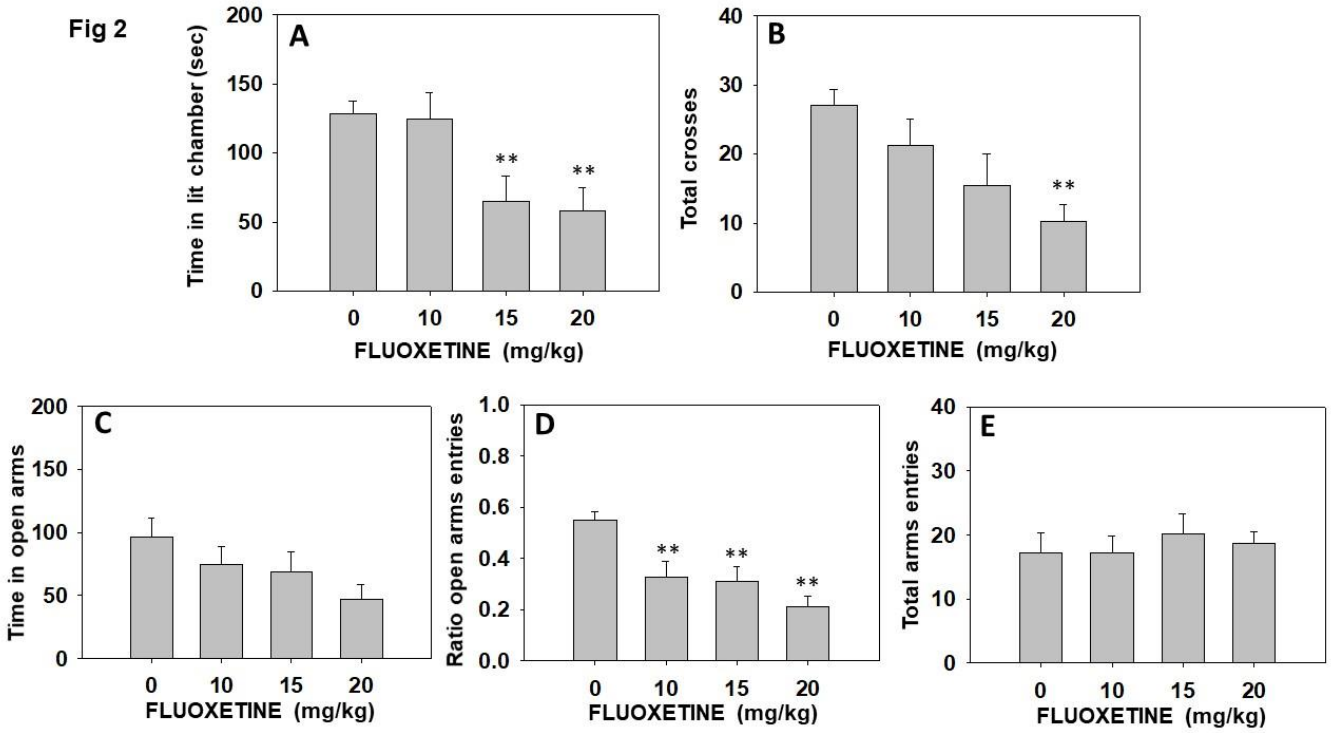

Figure 2. Effect of fluoxetine (Vehicle, 10, 15 or 20 mg/kg) on time spent in the lit chamber (A) and total of entries between the two compartments (B) of the DL box, and on time spent in the open arms (C), ratio to the open arms (D), and total of entries to the four arms (E) of the EMP during 5 minutes each test. Bars represent mean ± S.E.M. of accumulated seconds. **p<0.01 significantly different from the vehicle group.

Experiment 4. Effect of TBZ and fluoxetine combination on anxiety parameters as measured in the DL and EPM paradigms. The one-way ANOVAs did not show any significant effect of the treatment on anxiety parameters such as time spent in the lit chamber of the DL box (F(2,27)=1.23, p=0.30), time spent in the open arms (F(2,27)=2.27, p=0.80) or ratio of entries (F(2,27)=1.69, p=0.20) evaluated in the EPM paradigm (Figure 3). However, the one-way ANOVAs did show significant effects on locomotion seen in the total of entries between the two compartments of the DL box (F(2,27)=16.93, p<0.01), and total of entries into all arms of the EPM (F(2,27)=6.64, p<0.01). Planned comparisons revealed that the groups treated with 8.0 mg/kg of TBZ plus vehicle and the combination of TBZ plus fluoxetine both decreased the total number

of entries in comparison with the vehicle group ($p<0.01$) in the DL box (Figure 3B). The same effect was observed in the EPM: the groups treated with TBZ plus vehicle, and TBZ plus fluoxetine were different from the vehicle treated group ($p<0.01$) (Figure 3E).

Fig 3

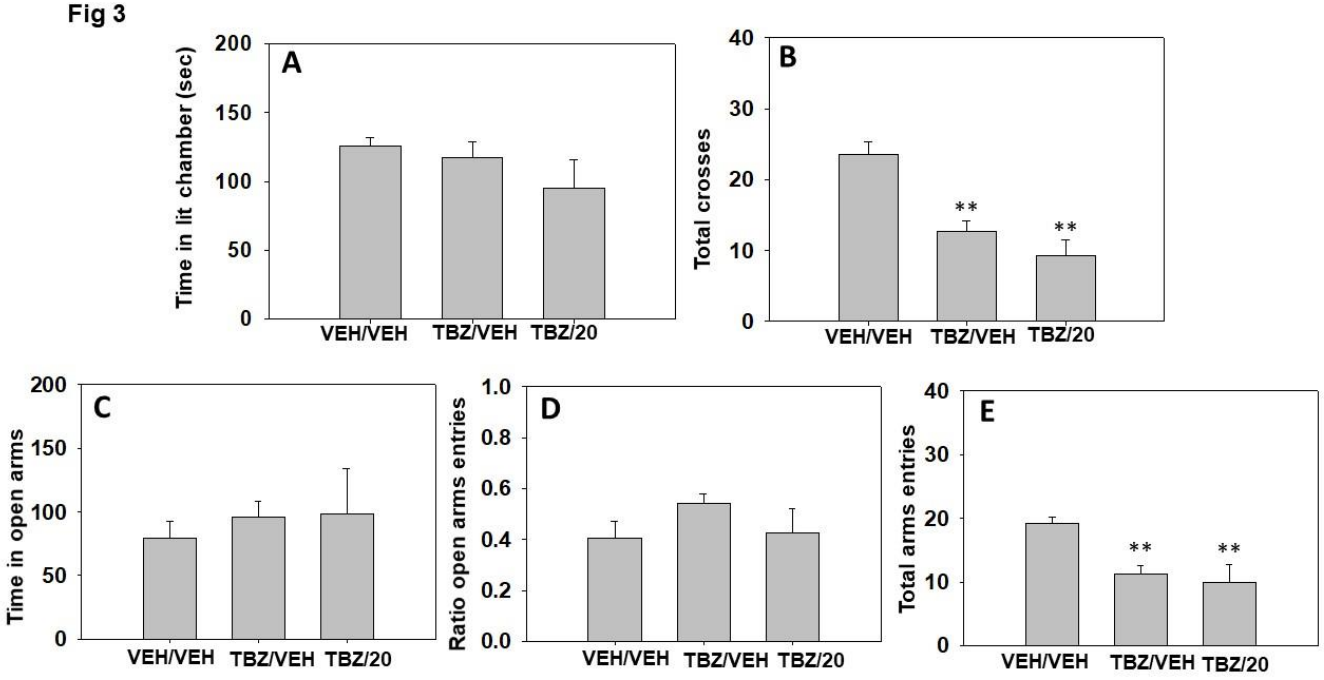

Figure 3. Effect of TBZ plus fluoxetine combination (Vehicle+Vehicle, TBZ 8 mg/kg +Vehicle, or TBZ8 mg/kg and fluoxetine 20 mg/kg) on time spent in the lit chamber (A) and total of entries between the two compartments (B) of the DL box, and on time spent in the open arms (C), ratio to the open arms (D), and total of entries to the four arms (E) of the EMP during 5 minutes each test. Bars represent mean ± S.E.M. of accumulated seconds. **p<0.01 significantly different from vehicle-vehicle group.

Experiment 5. Effect of fluoxetine on preference for active reinforcers as measured in the 3-choice-T-maze task. Repeated measures ANOVA showed fluoxetine did not produce any significant effect on time spent sniffing the neutral odor ($F(3,27)=0.90$, $p=0.45$). However, fluoxetine produced a significant effect on time spent eating ($F(3,27)=6.26$, $p<0.01$), and time spent running in the RW ($F(3,27)=15.13$, $p<0.01$). Planned comparisons revealed that fluoxetine 20 mg/kg treated-mice spent significantly more time consuming sucrose pellets ($p<0.01$), and less time running in the RW ($p<0.01$) compared to the vehicle group (Figures 4A-C). As for the other dependent variables, the repeated measures ANOVA showed that total time spent in the neutral odor compartment

was not significant (F(3,27)=1.21, p=0.32). However, fluoxetine showed a significant effect on time spent in the RW compartment (F(3,27)=9.69, p<0.01) and food compartment (F(3,27)=6.15, p<0.01). Planned comparisons revealed that when mice received the highest dose of fluoxetine (20.0 mg/kg) they spent less time in the RW compartment (p<0.01), and more time spent in the food compartment (p<0.01), in comparison with their respective vehicle conditions (Figure 4D). Finally, repeated measures ANOVAs indicated that fluoxetine did not produce significant effects on the number of entries into the food compartment (F(3,27)=0.97, p=0.42), and into neutral odor compartment (F(3,27)=1.98, p=0.14). However, the SERT inhibitor did produce a significant effect on total number of entries into the RW compartment (F(3,27)=5.43, p<0.01). Planned comparisons revealed that all doses of fluoxetine produced a decrease on total of entries to the RW compartment in comparison with the vehicle group (p<0.01) (data shown in Figure 4E).

Fig 4

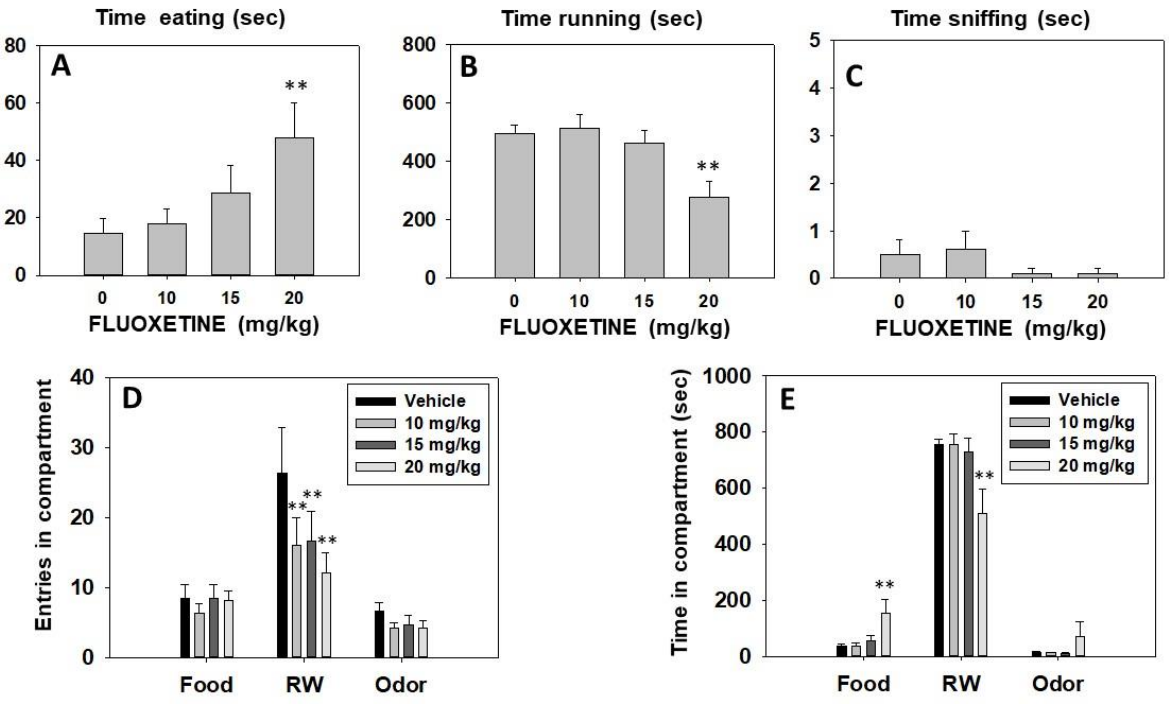

Figure 4. Effect of fluoxetine (Vehicle, 10, 15 and 20 mg/kg) on time eating (A), time running (B), time sniffing (C), entries into compartments (D), and time spent in each compartment (E) in the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. * p<0.05, **p<0.01 significantly different from vehicle.

Experiment 6. Effect of TBZ and fluoxetine combination on preference for active reinforcers as measured in the 3-choice-T-maze task. Repeated measures ANOVA showed a significant effect of treatment on time spent eating sucrose pellets ($F(4,32)=3.33$, $p<0.05$) and time spent running in the RW ($F(4,32)=3.86$, $p<0.01$), but no significant effect on time sniffing the neutral odor ($F(4,32)=0.77$, $p=0.54$). Planned comparisons showed that mice treated with TBZ 8.0 mg/kg plus vehicle spent less time running ($p<0.05$) than the vehicle-vehicle control condition. Also, fluoxetine suppressed time running in the RW in TBZ- treated mice at doses of 15.0 and 20.0 mg/kg in comparison with the vehicle-vehicle condition ($p<0.05$ and $p<0.01$ respectively). Planned comparisons for the time consuming sucrose measure revealed that mice treated with TBZ plus vehicle increased time consuming sucrose pellets ($p<0.01$) in comparison with the vehicle-vehicle group, and all doses of fluoxetine in combination with TBZ suppressed time consuming sucrose pellets (for TBZ+ 10.0 and TBZ+15.0 mg/kg $p<0.05$ and for TBZ+20 mg/kg $p<0.01$) in comparison with the TBZ plus vehicle condition (Figures 5A-C).

Repeated measures ANOVA did not show any significant effect of treatment on time spent in the RW compartment ($F(4,32)=0.77$, $p=0.54$), food compartment ($F(4,32)=1.58$, $p=0.20$), or odor compartment ($F(4,32)=1.86$, $p=0.14$) (Figure 5D, E). Finally, repeated measures ANOVAs showed a significant effect of treatment on total number of entries to the RW compartment ($F(4,32)=2.89$, $p<0.05$), total entries into the food compartment ($F(4,32)=5.46$, $p<0.01$), and total entries into the odor compartment ($F(4,32)=3.46$, $p<0.01$). Planned comparisons showed that TBZ+ fluoxetine at 15.0 and 20.0 mg/kg produced a decrease in entries into the RW compartment ($p<0.05$), and into the food compartment ($p<0.05$ and $p<0.01$ respectively) compared with their respective vehicle condition. Only the highest dose of fluoxetine (20.0 mg/kg) in combination with TBZ decreased entries to the odor compartment ($p<0.01$) in comparison with the vehicle condition.

Fig 5

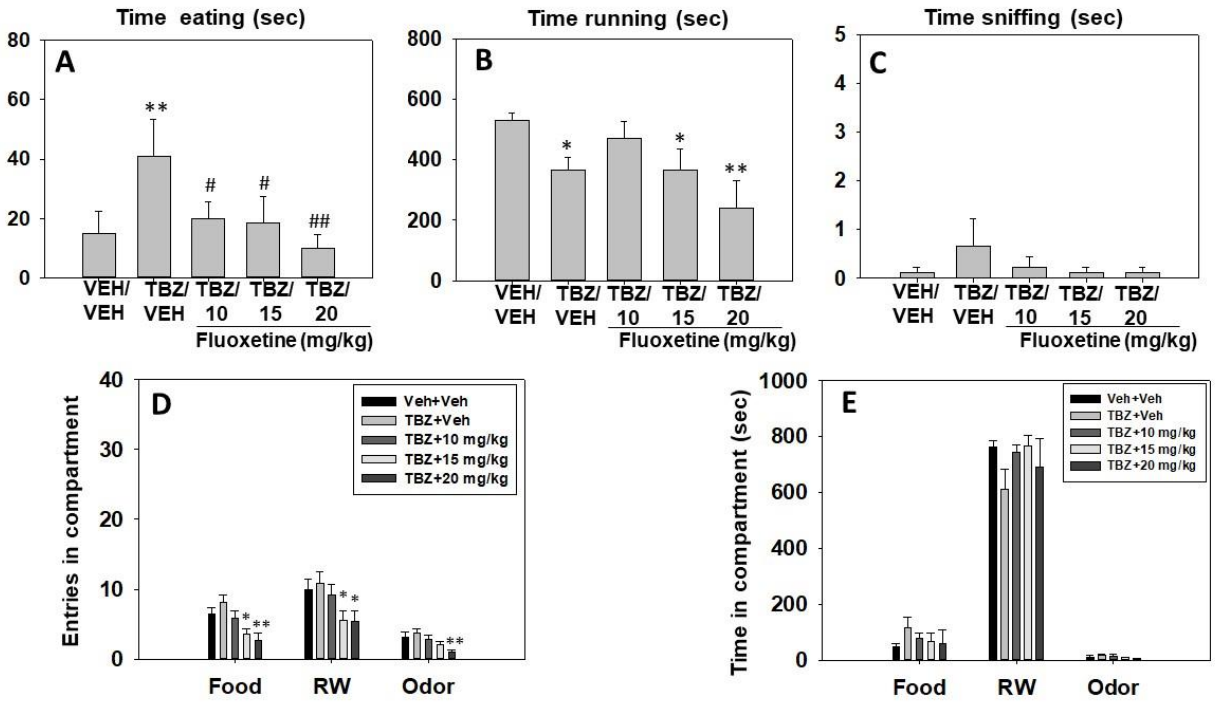

Figure 5. Effect of TBZ (Vehicle or 8 mg/kg) plus fluoxetine (Vehicle, 10, 15 and 20 mg/kg) combination on time eating (A), time running (B), time sniffing (C), entries into compartments (D), and time spent in each compartment (E) in the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. *p<0.05, **p<0.01 significantly different from vehicle-vehicle. #p<0.05, ##p<0.01 significantly different from TBZ-Vehicle.

Experiment 7. Manipulations that change the relative value of the sucrose pellets: Change in taste with bitter pellets. A series of Student`s t-tests for dependent samples for each variable showed a significant effect of bitter pellets (t(5)=4.56, p<0.01) on time running and on time spent eating compared with the control condition (t(5)=-2.53, p<0.01). However, there was no significant effect of bitter food on time sniffing the neutral odor (t=2.0, p=0.10) (Figures 6A-C). The t-tests for dependent samples comparing time in compartments between both conditions (BL vs. bitter food) did not reveal differences in time spent in RW compartment (t(5)=-1.13, p=0.30), food compartment (t(5)=1.39, p=0.22) or neutral odor compartment (t(5)=1.81, p=0.12) (Fig 6E). Finally, t-tests for dependent samples failed to demonstrate significant differences between both

conditions on any of the following variables: total entries to the food compartment (t(5)=-0.09, p=0.92), total entries to the RW compartment (t(5)=-2.23, p=0.07), and total entries to the odor compartment (t(5)=-0.58, p=0.58) (Figures 6D).

Fig 6

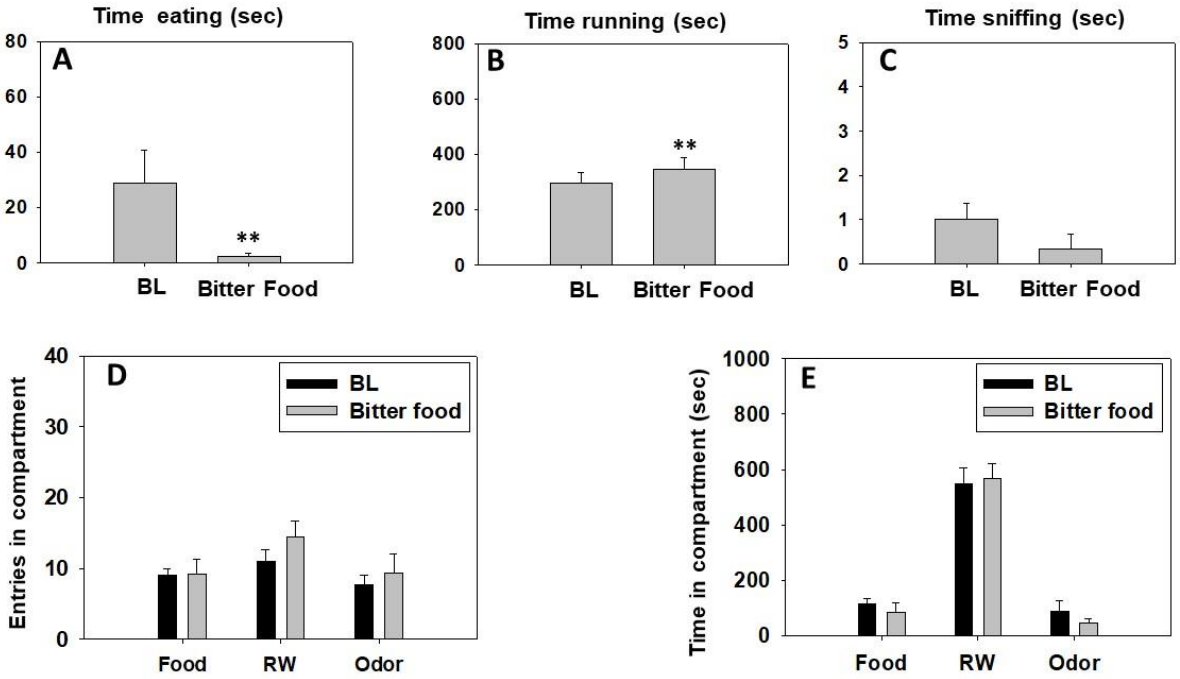

Figure 6. Effect of bittering the sucrose pellets on time eating (A), time running (B), time sniffing (C), entries into compartments (D), and time spent in each compartment (E), in the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. **p<0.01 significantly different from baseline condition.

Experiment 8. Manipulations that change the relative value of the sucrose pellets: change in appetite by pre-feeding. The Student's t-test for dependent samples showed a significant increase in time running in the RW (t(11)=-3.87, p<0.01), and a decrease in time eating sucrose pellets (t(11)=2.95, p<0.01) in the pre-feed condition compared with the control condition (Fig 7A, B). However, there was no significant effect of pre-feeding on time spent sniffing the neutral odor (t(11)=-0.82, p=0.42) (Fig 7C). Student's t-tests for dependent samples for the variable time in compartments did not show significant differences between conditions on time spent in RW compartment (t(11)=-2.06, p=0.06), and neutral odor compartment (t(11)=-0.22, p=0.82) (Fig 7E). However, Student's t-test analyses did show that there was a significantly decreased in time spent in the food compartment in the pre-feed condition compared with BL (t(11)=3.07, p<0.01). The t-

tests revealed no statistical differences in total entries into the food compartment (t(11)=1.51, p=0.15), total entries into the RW compartment (t=-1.02, p=0.32) and total entries into the odor compartment (t(11)=-1.42, p=0.18) (Fig 7D).

Fig 7

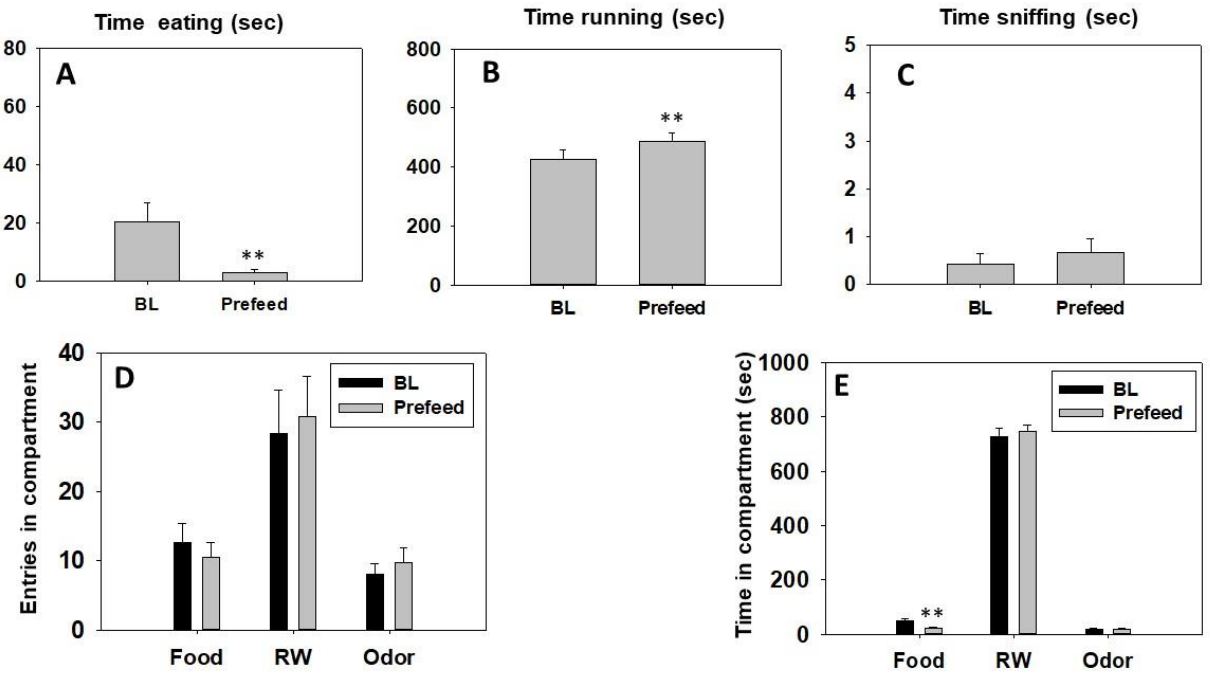

Figure 7. Effect of pre-feeding on time eating (A), time running (B), time sniffing (C), entries into compartments (D), and time spent in each compartment (E), in the T-maze task assessed during 15 minutes. Bars represent mean ± S.E.M. of accumulated seconds or number of entries. **p<0.01 significantly different from BL condition.

Experiment 9. Effect of pharmacological and food manipulations on amount of sucrose consumption in the T-maze. The Student's t-test for dependent samples showed significant differences in the amount of pellets consumed when animals received fluoxetine 20.0 mg/kg in comparison with the vehicle condition (t(9)=-3.13, p<0.01). However, the t-test comparison of vehicle-vehicle with TBZ-vehicle did not show a significant effect (t(7)=-0.67, p=0.52), and a separate t-test between vehicle-vehicle and TBZ-fluoxetine 20.0 mg/kg was not significant either (t(7)=0.88, p=0.40). There were significant differences in the amount of pellets consumed when animals were pre-feed

($t(11)=2.62$, $p<0.05$), or had access to bitter food ($t(5)=3.43$, $p<0.05$) compared to their respective controls (Table 1).

	Control condition	Experimental condition
Exp. 5. Fluoxetine	112.5 ± 40.4	306.0 ± 74.9**
Exp. 6. TBZ	129.4 ± 54.1	196.9 ± 64.7
Exp. 6. TBZ+ Fluoxetine	129.4 ± 54.1	61.9 ± 35.0
Exp. 7. Bitter food	345.0 ± 104.4	41.3 ± 28.7*
Exp. 8. Pre-feeding	240.0 ± 50.7	60.0 ± 15.0*

Table 1. Effect of tetrabenazine (TBZ) and fluoxetine alone or in combination and food manipulations on pellets intake. Mean (± SEM) of milligrams consumed during the T-maze test. *$p<0.05$, **$p<0.01$ significantly different from its respective control condition.

Experiment 10. Effect of pharmacological and food manipulations on locomotion in the T-maze. The Student's t-test for dependent samples showed significant differences in spontaneous locomotion in the T-maze when mice received fluoxetine 20.0 mg/kg in comparison with the vehicle condition ($t(9)=2.53$, $p<0.05$). However, the t-test comparison of vehicle-vehicle with TBZ-vehicle did not show a significant effect ($t(7)=-3.11$, $p=0.35$) although a separate t-test between vehicle-vehicle and TBZ-fluoxetine 20.0 mg/kg was significant ($t(7)=2.83$, $p<0.05$). Finally, there were no significant differences in spontaneous locomotion in the T-maze when animals were pre-feed ($t(11)=-0.43$, $p=0.67$) or had access to bitter pellets ($t(5)=-1.01$, $p=0.35$) (Data are shown in Table 2).

	Control condition	Experimental condition
Exp. 5. Fluoxetine	41.5 ± 8.8	24.5 ± 4*
Exp. 6. TBZ	19.4 ± 2.4	22.5 ± 2.3
Exp. 6. TBZ+ Fluoxetine	19.4 ± 2.4	9.1 ± 2.1*
Exp. 7. Bitter food	27.6 ± 2.8	33.0 ± 1.5
Exp. 8. Pre-feeding	49.0 ± 10.0	50.8 ± 9.7

Table 2. Effect of tetrabenazine (TBZ) and fluoxetine alone or in combination, and food manipulations on general locomotion. Mean (± SEM) of total entries to the three compartments of the T-maze test. **p<0.01 significantly different from its respective control condition.

Experiment 11. Effect of fluoxetine alone and in combination with TBZ on DARPP-32 phosphorylation patters in Nacb as measured by immunohistochemistry. One way ANOVAs showed an overall effect of treatment on pDARPP-32(Thr34) in Nacb ($F(2,13)=4.64$, $p<0.05$). Planned comparisons revealed that the administration of 20 mg/kg of fluoxetine decreased significantly phosphorylation of DARPP-32(Thr34) in Nacb core ($p<0.05$) in comparison with the vehicle-vehicle group. Moreover, TBZ (8 mg/kg) plus fluoxetine (20 mg/kg) was also significantly different from the vehicle-vehicle group ($p<0.01$). There was a significant effect of treatment on pDARPP-32(Thr75) in Nacb core ($F(2,12)=4.46$, $p<0.05$). Planned comparisons revealed that both the administration of 20 mg/kg of fluoxetine and the combination of 8 mg/kg TBZ plus 20 mg/kg fluoxetine significantly decreased phosphorylation of DARPP-32(Thr75) in comparison with the vehicle-vehicle group ($p<0.05$ and $p<0.01$ respectively). Data are also shown in figure 8.

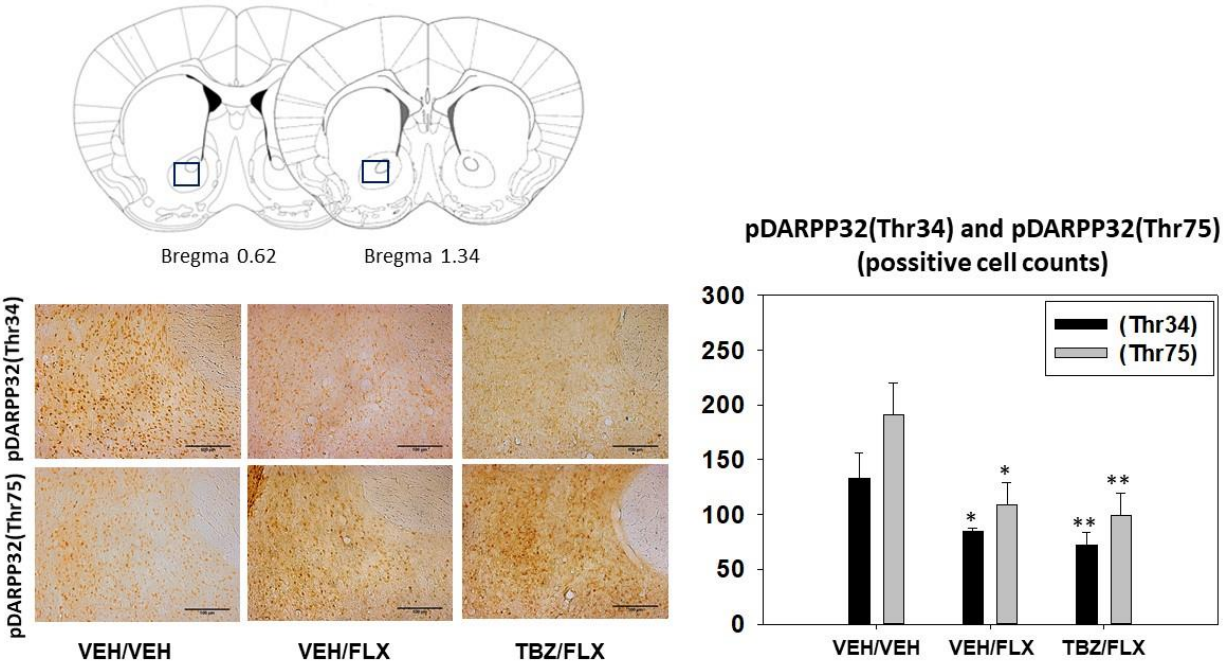

Figure 8. Effect of TBZ and fluoxetine combination (Vehicle+Vehicle, 8 mg/kg TBZ+Vehicle or 8 mg/kg TBZ+20 mg/kg fluoxetine) on number of positive cell counts for pDARPP-32(Thr34) and pDARPP-32(Thr75) immunoreactivity in Nacb core. Left: photomicrographs of individual representative brains. Right: mean ± S.E.M of number of pDARPP-32-(Thr34) and pDARPP-32-(Thr75) counts in a 100um^2 ROI. *p<0.05, **p<0.01 significantly different from Vehicle+Vehicle.

Discussion

The present experiments evaluated the ability of the 5-HT uptake inhibitor fluoxetine to produce antidepressant effects on the classical rodent paradigm, the FST, and also its potential to reverse the depression-like effects of the DA depleting agent TBZ in this paradigm (Carratalá-Ros et al, 2020). We confirmed that administration of a range of doses of fluoxetine increased significantly the time mice spent climbing and swimming, consequently reducing the time animals spent immobile in the FST (Fig 1). Previous studies have shown the same pattern; fluoxetine increases active behaviors (swimming and climbing) and decreases passive ones (immobility) in different strains of mice after acute or chronic IP administration of this SERT blocker (Costa et al, 2013; Sanmukhani et al, 2011; Lucki et al, 2001; Dulawa et al, 2004). However, when we evaluated the ability of the highest dose of fluoxetine (20.0 mg/kg) to reverse the depression-like effects produced by the DA depleting agent TBZ (8.0 mg/kg) in the FST (Carratalá-Ros et al, 2020), we observed that fluoxetine not only failed to reverse the effects of TBZ, but, in fact, it exacerbated them. Thus, animals that received the combination of TBZ and fluoxetine significantly increased time spent immobile and decreased time spent climbing, suggesting that fluoxetine is not able to alleviate motivational deficits in escaping behavior induced by DA depletion. TBZ was used in this study as a tool for altering behavioral activation and effort-related choice since this drug has been reported to induce depressive symptoms including fatigue in people (Frank et al, 2009; Chen et al, 2012) and behavioral impairments in traditional rodent depression models (Wang et al, 2010; Carratalá-Ros et al, 2020), and effort-based tasks (Nunes et al, 2013; Yohn et al, 2016a, López-Cruz et al, 2018; Rotolo et al, 2019).

The antidepressant-like effect of fluoxetine alone in the FST cannot be explained by anxiolytic actions since the same doses of fluoxetine that had an antidepressant effect in the FST, also increased anxiety-related parameters evaluated in the DL box and EPM paradigms. Thus, mice that received the two highest doses of fluoxetine (15.0 and 20.0 mg/kg) spent significantly less time in the lit chamber of the DL box, and in the open arms of the EPM paradigm (Fig 2). Some animal studies have shown that acute administration of doses of fluoxetine like the ones used in the present study (20.0 mg/kg) produce anxiogenic effects in rats (Greenwood et al, 2008), and mice (Kurt et al, 2000; Belzung et al, 2001). Moreover, it has been shown that null-SERT mutant mice usually display anxious behaviors (Holmes et al, 2003). In the present and previous experiments,

TBZ showed no anxiogenic effects at a broad range of doses (Correa et al, 2018; Carratalá-Ros et al, 2020). Interestingly, unlike fluoxetine alone, the combination of fluoxetine (20.0 mg/kg) plus TBZ (8.0 mg/kg) did not produce anxiety-related effects in any of the paradigms (Fig 3).

The FST provides some information about behaviors related to the maintenance of vigorous and persistent active responding (Gil and Armario, 1998; Slattery and Cryan, 2012) in order to escape a stressful unescapable situation. Based on this, we decided to evaluate the effects of fluoxetine in the 3-choice-T-maze-task, a rodent model that evaluates preference for vigorous physical activity vs. other sources of reinforcement that could be obtained with little effort (Correa et al., 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020). In the standard version of this paradigm, no stressor is used, and mice can freely distribute their time performing an effortful and highly preferred activity (running in a RW) or engaging in more sedentary activities (eating sucrose pellets or sniffing a fruit odor). Under basal conditions, mice spent most of their time running in the RW, some of the time eating sucrose pellets and very little time sniffing the neutral odor. In this experiment, the highest dose of fluoxetine (20.0 mg/kg) decreased significantly time mice spent running and partially shifted their behavior, increasing time consuming sucrose pellets in comparison with the vehicle condition (Fig 4). This increase in time consuming sucrose is also seen in terms of amount of food consumed; the highest dose of fluoxetine significantly increased intake of sucrose in milligrams (around 170%, table 1). This pattern of effects is surprisingly similar to the pattern previously observed for TBZ alone in this paradigm (López-Cruz et al, 2018; Carratalá-Ros et al, 2020). The administration of different doses of TBZ produced a partial shift; mice spent less time running and increased time-consuming sucrose pellets in comparison with the vehicle condition. TBZ alone also tends to increase total milligrams consumed, but the increase is milder (around 35-50%), and never reaches significance (Carratalá-Ros et al, 2020). This increase in food consumption has not been observed in rats in operant choice procedures (Yohn et al, 2016a), in which animals tend to reduce palatable food as well as chow intake. It is possible that this discrepancy is due to differences between these two species. Rats tend to consume proportionally more food and are less active than mice in T-maze paradigms (Rotolo et al., in preparation). Thus, it is possible than mice are more sensitive to the anergic effects of fluoxetine, and much less to its appetite suppressant effects. Moreover, although previous clinical studies have shown how that this SERT

blocker decreases appetite (Silverstone et al, 1992; Michelson et al, 1999), a recent metanalysis study (Serralde-Zúñiga et al, 2019) comparing different categories of anti-obesity drugs indicates that, unlike other drugs, fluoxetine treatment does not conclusively decrease weight, but there is a clear increase in the risk for drowsiness and fatigue. In addition, unlike TBZ alone, mice treated with 20.0 mg/kg of fluoxetine showed avoidance for the RW in general; they spent significantly more time in the food compartment, reducing the time spent and the total number of entries into the RW compartment. This is a pattern of effects that is similar after introducing aversive conditions in the T-maze (Carratalá-Ros et al, 2020). Thus, placing an intense light over the RW that is known to induce anxiogenic effects in the DL shifts behavior from RW to food consumption, but animals also show place avoidance for the RW compared to baseline (Carratalá-Ros et al, 2020). Those results are different from the pattern observed for TBZ, which did not change any of these parameters; mice still highly preferred to spent time in the compartment with the active reinforcer (the RW compartment) after TBZ administration, although they did not run as much (Carratalá-Ros et al, 2020).

In the next experiment, different doses of fluoxetine were unable to reverse the partial shift from RW towards food induced by TBZ (Fig 5). However, fluoxetine at all doses suppressed the TBZ-induced increase in time consuming sucrose, making the combined administration of the two drugs no different from control conditions. In previous studies in rats, fluoxetine consistently failed to reverse the lever pressing suppression induced by TBZ. But when animals were tested on fluoxetine alone, the did not shift their behavior from high effort/highly palatable food to free chow available in the operant chamber, as is typical for TBZ (Yohn et al, 2016b). In fact, in these experiments animals also showed reduced chow consumption (Yohn et al, 2016b). Thus, it seems that fluoxetine in combination with TBZ produces both anergic effects and a suppresses food consumption in rats and mice. For that reason, we wanted to evaluate the effects of conditions related to appetite and food reinforcement on T-maze choice task performance, to compare those actions with the effects of fluoxetine plus TBZ. We altered the reinforcing value of the sucrose pellets that were available in this paradigm by pre-feeding the mice with the same type of sucrose pellets the night before the test, or by adding quinine to the sucrose pellets in order to make them bitter. Both manipulations produced a significant shift, reducing almost to zero the time mice spent consuming sucrose pellets in comparison with the baseline condition, but significantly increasing time running (figures 6 and 7), which was

not observed in the pharmacological interaction experiment. Moreover, pre-feeding or making the pellets bitter led to a robust and significant reduction of milligrams consumed, which is the did not occur either with TBZ alone, and did not reach significance with TBZ plus fluoxetine (Table 1).

The disruptive effects on time running produced by fluoxetine or fluoxetine in combination with TBZ in the T-maze could be explained by general effects of locomotor activity. Thus, in the anxiety paradigms, fluoxetine (15.0 and 20.0 mg/kg), or fluoxetine (20.0 mg/kg) plus TBZ reduced locomotion, as measured by total number of entries into compartments and crosses between arms (figures 2B, 3B and 3E). Moreover, in all the experiments, the number of overall crosses between compartments in the T-maze was only significantly reduced after fluoxetine (20.0 mg/kg) or fluoxetine plus TBZ (table 2). Previous studies have reported that SERT blockade or deletion in mice reduced locomotion (Sanders et al, 2007; Holmes et al, 2002), and fluoxetine in rats that had received TBZ showed further decreased locomotor activity compared with administration of TBZ alone (Podurgiel et al, 2015).

The inability of fluoxetine to alleviate motivational impairments induced by a DA depleting agent, and to induce psychomotor deficits, could be due to actions of fluoxetine on the mesolimbic DA system (Cameron and Williams, 1995; Daw et al, 2002). Fluoxetine alone decreased DA levels in nucleus accumbens (Nacb) as measure by microdyalisis (Yohn et al, 2016a), and coadministration of TBZ and fluoxetine further decreased DA tissue levels in the rat ventrolateral neostriatum compared with TBZ alone (Podurgiel et al, 2015). Nacb receives projections from dorsal raphe nuclei 5-HT neurons, and the stimulation of this neurons decreases DA mesolimbic activity (Browne et al, 2019; Di Giovani et al, 2000; Di Matteo et al, 2008; Ichiwaka and Meltzer, 1995; Di Mascio et al, 1998). Thus, the combination of fluoxetine with TBZ, which also has been demonstrated to reduce DA tissue content and release (López-Cruz et al, 2018; Nunes et al, 2013), suggests that these impairments in behavioral activation assessed in the 3-choice-T-maze task and in the FST could be produced by an overall reduction of mesolimbic DA activity. Thus, in the present experiments, the reduction in both forms of phosphorylated DARPP32 induced by fluoxetine and also by fluoxetine combined with TBZ in Nacb core, suggests that there is a reduction in D1 and D2 dopamine receptor activation by DA as compared to the control condition.

In summary, fluoxetine, which is the SERT inhibitor most prescribed for treating depression (Hieronymus et al, 2016; Rizvi et al, 2013; Rosenblau et al, 2012), produced a antidepressant-like effect under stressful conditions in the FST, but induced an anergic effect on its own, and did not reverse the anergic effects produced by TBZ in either the FST or the 3-choice-T-maze task. Several clinical studies have reported that fluoxetine is relatively ineffective for treating psychomotor and motivational symptoms seen in depression and, in fact, it can exacerbate or induce these effects (Katz et al., 2004; Nutt et al, 2007; Padala et al, 2012; Rostchild et al, 2014). However, it should be pointed out that drugs that facilitate 5-HT can treat other aspects of depression such as mood dysfunction, rumination and cognitive arousal (Carr and Lucki, 2011; Bell et al, 2013) and, as seen in the present study as well as previous studies, fluoxetine administered alone produces an effective response in classical antidepressant tests such as the FST (Armario et al., 1988; Cryan et al, 2005a,b; Jang et al, 2009). Thus, the present studies are consistent with the idea that not all antidepressants are adequate for the treatment of psychomotor slowing and anergia symptoms commonly seen in depression. This idea of different therapeutic drugs having positive actions for some symptoms, but no effect or even negative actions on other symptoms, is consistent with the research domain criterion (RDoC) approach that highlights the importance of describing the neural circuits that mediate specific symptoms in psychopathology, and not simply the traditional diagnostic categories (Cuthbert and Insel, 2013).

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CHAPTER IV

Are there sex differences in emotional and motivational parameters in mice after dopamine depletion? Modulation by DAT and SERT blockers.

Abstract

Disorders such as depression is twice as common in women than in men, and there are sex differences in the symptomatology and treatment response to this disorder. Mesolimbic dopamine (DA) has been implicated in effort-based decision making and in behavioral activation. Impairments in behavioral activation such as anergia are often seen in people with depression and are highly resistant to treatment. The role of DA in regulating behavioral activation has been extensively studied in male rodents, but little is known about activational processes in female rodents. In the present work, we study the impact of Tetrabenazine (TBZ) in male and female mice on measures of behavioral activation such as the Forced Swim Test (FST) and in the 3-choice-T-maze task. Social Interaction (SI), anxiety in the Dark and Light (DL) box and Elevated Plus Maze (EPM) and sucrose preference were also evaluated. TBZ reduced active behaviors in the FST such as climbing in male mice and increased immobility both in male and female mice. DA depletion reduced active behaviors (running) in the T-maze task and increased time doing more sedentary activities (consuming sucrose pellets) in both sexes. There were no major differences between sexes in SI, DL and sucrose preference even after TBZ. Finally, the DAT blocker bupropion, reversed the effect of DA depletion in both sexes. However, the SERT blocker fluoxetine, was only effective in female mice. These results indicate that DA is involved in behavioral activation, and thus, pro-dopaminergic antidepressants may be able to improve impairments related to reduce behavioral activation.

Sex differences, dopamine, decision-making, serotonin, mice

Introduction

Preclinical studies using animal models have characterized some common symptoms seen in psychiatric disorders in male rodents avoiding, most of the time, performing the same experiments in female rodents (Zucker and Beery, 2010; Dalla et al, 2010a). This is also true for the study of depression, in spite of the fact that depression is twice as common in women than in men (Kessler, 2003; Seedat et al, 2009; Parker and Brotchie, 2010; Silverstein et al, 2002). Moreover, there are sex differences in the progress and the symptomatology of this disorder (Gorman, 2006; Marcus et al, 2005; Thiels et al, 2005). Clinical studies have found differences between women and men in antidepressant response. Thus, it seems that antidepressants that inhibits the serotonin transporter (SERT) such as fluoxetine or citalopram have a better outcome in women, while men are more likely to be more responsive for tricyclic antidepressants (Khan et al, 2005; Young et al, 2009; Kornstein et al, 2000).

One of the most commonly used animal models to study differences between male and female rodents is the Forced Swim Test (FST). This paradigm, evaluates the attempt of rodents to escape in a vigorous way from a stressful non-escapable situation, being in this case a deep tank filled with water (Porsolt et al., 1977). Eventually, animals will cease the vigorous attempts to escape and will passively float in the chamber (Porsolt et al., 1977). Thus, immobility is the classical parameter that is measured and antidepressants have repeatedly shown to reverse this immobility (Armario et al, 1988; Petit-Demouliere et al, 2005; Costa et al, 2013). Studying sex differences in the FST, few studies have found that female mice and rats increase the immobility time in comparison with male rodents (Kokras et al, 2012; Kokras et al, 2015), and this effect is alleviated in female rodents following the administration of SERT inhibitors. Specifically, regarding immobility, female rats and mice are more responsive to SERT inhibitors than male rodents (Kokras et al, 2015; Férnandez-Guasti et al, 2017; Dalla et al, 2010a; Jones and Lucki, 2005). Other behaviors that can be evaluated in the FST are swimming and climbing. Both behaviors are considered active behaviors that also are modified by antidepressants (Armario et al, 1988; Lucki, 1997), and are likely to be affected by DA manipulations, more than the traditional immobility measure (Gil and Armario, 1998; Costa et al, 2013). However, little is known about the differences between male and female rodent in the execution of these two behaviors. Some animal studies have found that, under basal conditions, female rodents display less climbing than male rodents,

suggesting that females are less active than males, and antidepressant drugs are able to enhance climbing behavior in female rodents (Kokras et al, 2015; Martínez-Mota et al, 2011). Other studies fail to detect sex differences on climbing and swimming in the FST (Mourlon et al, 2010; Verma et al, 2010). Moreover, there are no studies that observed the effect of pro-dopaminergic drugs on modulation of climbing in female rodents assessed in the FST.

DA modulation can be studied using effort-related decision-making tasks where rodents have to choose between exerting effort in order to obtain a higher and preferred reinforcer or minimizing their work and obtaining a lower and less preferred reinforcer (Salamone et al, 2016c, 2018; Salamone and Correa, 2012, 2002). Thus, animals with DA depletion or receptor antagonism seem to shift their behaviors from high value/high demanding options to low-effort but less valued ones (Cousins et al, 1994; Salamone et al, 2002, 2016; Mott et al, 2009; Pardo et al, 2012, 2015; Randall et al, 2012; Mai et al, 2012; Sommer et al, 2014; Yohn et al, 2015a,b). Moreover, behavioral activation and effort-based choice can be also evaluated using a novel animal paradigm: the 3-choice-T maze task (Correa et al, 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020). Voluntary wheel running takes place in mice of all strains and in both sexes (Walker and Manson, 2018). Mice have a high preference for engaging in physical activities (Routtenberg et al, 1968; Correa et al, 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020). Thus, this T-maze task allows mice to freely choose between doing an active behavior (running on a running wheel) or doing more sedentary activities (consuming sucrose pellets or sniffing a non-social odor). Tetrabenazine (TBZ) has been used in these kind of tasks in order to alter behavioral activation and decrease selection of high effort choices in rats (Nunes et al, 2013; Randall et al, 2014; Pardo et al, 2015; Yohn et al, 2015a,b, 2016a; Hosking et al, 2015; Rotolo et al, 2019) and mice (Correa et al, 2018; Carratalá-Ros et al, 2020). TBZ acts depleting monoamines by inhibiting the vesicular monoamine transporter-type 2 (VMAT-2), and at low doses this drug has its greatest effects on striatal DA in rats and mice (Pettibone et al, 1984; Nunes et al, 2013; López-Cruz et al, 2018). Thus, TBZ produces a decrease in effortful activities (such as lever pressing at high ratios in FR/choice and PROG/choice tasks) to get palatable reinforcers, but increases the consumption of free chow available without affecting preference for food (Rotolo et al, 2019; Nunes et al, 2013; Salamone et al, 2002; Cousins et al, 1996; Salamone et al, 1994b; Sink et al, 2008). TBZ also partially shifts choice behavior in adult male mice, reducing time running but

increasing time eating sucrose pellets in the T-maze task (López-Cruz et al, 2018; Carratalá-Ros et al, 2020).

The effects of DA depletion in effort-related decision-making tasks using male rodents are well described, but there are no studies on behavioral activation impairments in female rodents using this kind of tasks. Thus, the aim of this study is twofold: first we explored the impact of an effective dose of TBZ used previously in male mice, comparing its effect between female and male mice in two paradigms that evaluate exertion of effort, either to escape a stressful situation in the FST, or by selecting a vigorous and reinforcing activity in the effort-based decision-making task: the 3-choice T-maze task (Correa et al, 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020). We additionally assessed the impact of TBZ on paradigms that evaluate behaviors shaped by emotional factors, such as responses in anxiogenic environments (dark and light box; DL and elevated plus maze; EPM), or avoidance of novel social stimuli (evaluated in a social interaction task) and also preference and consumption of liquid sucrose that is often related to anhedonia-like behaviors. Secondly, we studied and compared in male and female mice the ability of two antidepressants with different mechanisms of action bupropion (catecholamine uptake blocker) and fluoxetine (SERT inhibitor) to reverse the effect produced by TBZ in the FST.

Materials and Methods

Animals. CD1 adult male and female mice (N=340) purchased from Janvier, France S.A. were 7-9 weeks old (25-50 g) at the beginning of the study. Mice were housed in groups of three or four per cage, with standard laboratory rodent chow and tap water available *ad libitum*. The colony was kept at a temperature of 22 $\pm$ 2 ºC with lights on from 08:00 to 20:00 h. All animals were under a protocol approved by the Institutional Animal Care and Use committee of 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 Behavioral 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; CIMYT Quimica SL, Spain) was dissolved in a vehicle solution of 0.9% saline (80%) plus dimethylsulfoxide (DMSO 20%, final pH 5.5) and administered 120 minutes before testing. Time elapsed after injection and 8 mg/kg TBZ was selected based on previous behavioral work (López-Cruz et al, 2018; Correa et al, 2018; Carratalá-Ros et al, 2020) and neurochemical studies (López-Cruz et al, 2018) demonstrating that in mice this is an optimal dose and time lead to deplete DA. Bupropion hydrochloride (BUP; Alfa Aesar, Spain) was dissolved in 0.9% saline. Bupropion was administered 30 minutes before the test started. Doses and times were selected from previous work in our laboratory (Randall et al, 2014a,b; Yohn et al, 2015a) and from studies with the forced swim test (Yamada et al, 2004; Kitamura et al, 2010). Fluoxetine (CIMYT Quimica SL, Spain) was dissolved in 0.9% saline and administered 30 minutes before testing. The range of doses of fluoxetine were based on studies involving classical mice antidepressant screening tests (Lucki et al, 2001) and effort-related behavioral tests (Yohn et al, 2016a,b) DMSO (20% v/v) and saline were used as the control group. All the substances were administered intraperitoneally (IP).

Testing procedures

All behavioral procedures started two hours after the light period started. The behavioral test room was illuminated with a soft light, and external noise was attenuated.

Forced swim test (FST). This paradigm is considered to be a model of behavioral despair and is used as a test for assessing depressive-like states and for evaluating drugs with potential as antidepressants (Porsolt et al, 1977). Immobility was defined when the animal remained motionless, making only minor movements to balance the body and keep the head above the water. In addition, we also assessed escape related mobility such as climbing or struggling (Armario et al, 1988). Climbing is defined as any energetic and vertical movement of all four limbs against the wall of the tank. Mild 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). Naïve male and female mice were placed in a transparent cylindrical glass tank (26 cm high and 18 cm diameter) filled with water (14 cm) and maintained at a temperature of 25ºC. Water was changed between animals. During the 6 minutes test, mice were videotaped from the side, and climbing, immobility and swimming were later measured by an observer

unaware of the experimental condition. After the test, mice were dried with a soft towel, put back in a box with absorbing paper under a warming light, and were monitored for 10 minutes.

Dark and light box (DL). The DL test is based on the conflict between the tendency to explore a novel environment and the avoidance of a brightly lighted open area (Blumstein and Crawley 1983). The DL apparatus consisted of a polypropylene chamber divided in two compartments by a partition containing a small opening (5 cm H x 5 cm W). The light compartment (25 cm W x 25 cm H x 25 cm L) was open, painted in white and illuminated (335 lx), while the dark compartment (25 cm W x 25 cm H x 18 cm L) was painted in black and had a removable ceiling to close it (Kulesskaya and Voikar, 2014). To start the test session, mice were individually placed in the dark chamber facing one corner. Test sessions were videotaped, and the total number of crosses and the total time spent in the lit chamber were recorded for 5 min (López-Cruz et al, 2014; Carratalá-Ros et al, 2020).

Elevated plus maze (EPM). The EPM consists of two open and two enclosed arms (65 cm L x 5 cm W) arranged in a plus configuration and intersecting in a central platform. It is made of black polypropylene and is elevated 50 cm above the floor. The open arms have a 1 cm border around their perimeter and the closed arms have a 20 cm translucent wall. This anxiety paradigm measures the avoidance that rodents show to high open spaces. Under normal conditions, mice spend more time, and make more entries into the closed arms of the maze (Lister 1987). Animals were placed in the central platform with their head pointing at one enclosed arm, and they were assessed during 5 minutes. Sessions were videotaped and a trained observer registered total time spent in the open arms, and total entries in the 4 arms as an index of locomotion. An entry into an arm was recorded when the animal crossed with all four legs the line that connected that arm with the central platform (procedure is based on López-Cruz et al, 2014).

Social preference test. Social interaction and preference was measured in a three-chambered social box. Every mouse was placed in the center of the chamber of the social interaction apparatus and they freely explored the social arena during 10 minutes in the presence of a cage conspecific on one side-compartment, and on the other side there was a small wire cage containing an object. The center compartment was empty. The placement of the conspecific or the object was counterbalanced between animals. A trained experimenter who was unaware of the experimental conditions, registered

manually time spent sniffing each target (conspecific vs object) as a measure of social preference. Time spent in each compartment and crosses between compartments was also registered. Procedures were based on López-Cruz et al, 2016.

Liquid sucrose consumption and preference. During 3 days, non-food restricted mice were place individually in standard home cages where they had *ad libitum* access to two different sucrose liquid concentrations: 10% and 5% sucrose. The amount of sucrose liquid consumed of both conditions was registered during the test day. All sessions lasted 60 minutes.

T-maze RW-sucrose-odor choice task. The T-maze apparatus consisted of a central area that leads to 3 arms. In one of them, sucrose pellets (TestDietTM, 50% sucrose, 45 mg 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). This concentration of sucrose in the pellets was selected after piloting different concentrations (i.e. 100% sucrose pellets generate avoidance), and the non-social odor used for the present studies (strawberry) was the one that generated more exploration among non-social odors in previous studies (López-Cruz et al, 2017). In general, mice were allowed to freely explore and interact with the stimulus during 15 minutes sessions, once a day, 5 days a week. Training phase 1: to avoid neophobia to the sweet tasting pellets, animals were enclosed in that arm with the food during 5 sessions (no exploration of the other arms was allowed during this phase). Training phase 2: during 2 weeks animals had free access to the three stimuli until a stable baseline was obtained. Test phase: This phase lasted during 4 more weeks. For each week, there were 4 baseline drug free sessions plus a testing session in which animals received TBZ or were tested under different behavioral manipulations. The day before the test for the behavioral manipulation was considered as the baseline (BL) for those experiments. Sessions were videotaped and a trained observer manually registered several parameters. 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 Rachlin, 1969). Entries into the arms and time spent in the arms of the T-maze were also simultaneously recorded. All these measures were taken based on previous studies (Correa et al., 2016; López-Cruz et al, 2018; Carratalá-Ros et al, 2020).

Experiments

Experiment 1. Effect of TBZ in male and female mice on behavioral activation evaluated in the FST. Naïve male (n=20) and female mice (n=20) received TBZ (vehicle or 8 mg/kg) and their behavioral output was measured in the FST.

Experiment 2. Effect of TBZ on anxiety parameters in male and female mice as measured in the DL and EPM paradigms. Male (n=26) and female (n=26) mice received TBZ (0, and 8 mg/kg) and after 120 minutes were first placed in the DL box for 5 minutes, and immediately after this test, they were placed in the EPM for 5 more minutes. Mice were exposed only once to both paradigms, since behavioral habituation develops in one session.

Experiment 3. Effect of TBZ on social motivation in male and female mice assessed in a three-chamber social preference test. Male and female mice (N=37; 19 males and 18 females) received TBZ (vehicle or 8 mg/kg) and were placed in a social preference box during 10 minutes.

Experiment 4. Effect of TBZ on preference for sucrose concentration and on fluid consumption in male and female mice. Male mice (n=25) and female mice (n=23) received vehicle or 8 mg/kg TBZ and were exposed concurrently to a bottle containing water with 5% sucrose and another bottle with 10% sucrose solution during 60 minutes.

Experiment 5. Effect of TBZ on preference for active reinforcers assessed in the 3-choice-T-maze task in male and female mice. Male (n=14) and female (n=13) mice received TBZ (vehicle and 8 mg/kg) and after 120 minutes, were placed in the T-maze for 15 minutes. Animals received one dose of the drug every week in a randomly varied order. The T-maze paradigm requires a baseline performance of two weeks before tests starts, and that performance is maintained across weeks, thus allowing a repeated measures design.

Experiment 6. Effect of TBZ and bupropion combination on behavioral activation assessed in the FST in male and female mice. Three groups of naïve male mice (n=38) and three groups of naïve female mice (n=38) received a combination of TBZ and bupropion (vehicle + vehicle, 8 mg/kg TBZ + vehicle and 8 mg/kg TBZ + 10 mg/kg BUP) and were assessed in the FST during 6 minutes.

Experiment 7. Effect of TBZ and fluoxetine combination on behavioral activation assessed in the FST in male and female mice. Male mice (n=36) and female mice (n=36) received vehicle plus vehicle, 8 mg/kg TBZ plus vehicle or 8 mg/kg TBZ plus 20 mg/kg FLX and were exposed to the FST during 6 minutes.

Statistical Analyses. Normally distributed and homogenous data (according to Kolmogorov-Smirnov test) for the FST, DL, EPM, social interaction, and sucrose liquid intake were evaluated by a two-way factorial ANOVA (sex x treatment) in which both were between factors. For the T-maze experiments data were evaluated by two-way factorial ANOVAs with a between factor (sex) and a within factor (treatment). If the sex factor was significant, two separate Students-t test for each sex comparing the effect of treatment were performed. For experiments 6 and 7, additional one-way ANOVAs for the factor treatment in each sex were performed and when the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used (Keppel, 1991). For these comparisons, α level was kept at 0.05 because the number of comparisons was restricted to the number of treatments minus one. All data were expressed as mean ± SEM, and significance was set at $p<0.05$. STATISTICA 7 software was used.

Results

Experiment 1. Effect of TBZ in male and female mice on behavioral activation evaluated in the FST. Factorial ANOVA (sex x treatment) on time spent immobile in the FST showed a significant effect of treatment ($F(1,1)=18.99$, $p<0.01$), but no effect of sex ($F(1,1)=0.75$, $p=0.38$), and no significant interaction ($F(1,36)=0.002$, $p=0.95$) (Figure 1A). The factorial ANOVA on time swimming showed an effect of treatment ($F(1,1)=15.06$, $p<0.01$), but there was no significant effect of sex ($F(1,1)=0.06$, $p=0.80$), and no interaction ($F(1,36)=0.29$, $p=0.58$; Figure 1B). Finally, the factorial ANOVA on time climbing in the FST showed an effect of sex ($F(1,1)=10.41$, $p<0.01$), and also a significant effect of treatment ($F(1,1)=7.46$, $p<0.01$), but there was no significant interaction ($F(1,36)=2.89$, $p=0.09$), (Figure 1C).

Fig 1

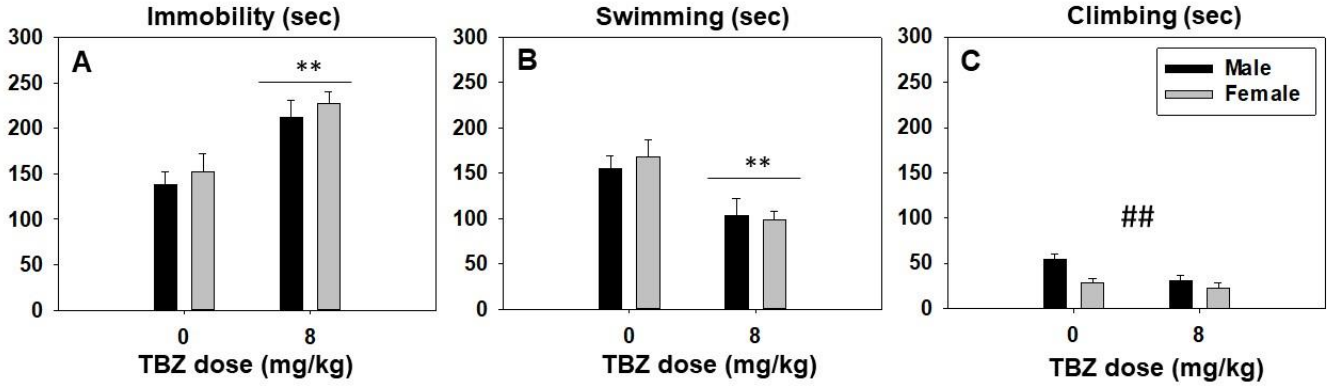

Figure 1. Effect of vehicle or TBZ (8 mg/kg) in male and female mice on measures of behavioral activation as duration of immobility (A), swimming (B) and climbing (C) in the FST assessed during 6 minutes. Bars represent mean ± S.E.M of accumulated seconds. **p<0.01 significantly different from vehicle. ##p<0.01 significant differences between male and female mice.

Experiment 2. Effect of TBZ in male and female mice on anxiety as measured in the DL and EPM paradigms. Factorial ANOVA (sex x treatment) on time spent in the lit chamber of the DL box showed no effect of sex ($F(1,1)=3.12$, $p=0.08$), no effect of treatment ($F(1,1)=0.02$, $p=0.94$), and no interaction ($F(1,30)=0.82$, $p=0.37$) (Figure 2A). However, factorial ANOVA (sex x treatment) on total number of crosses between compartments in the DL box showed an effect of treatment ($F(1,1)=13.63$, $p<0.01$), but there was no effect of sex ($F(1,1)=1.04$, $p=0.31$), and no interaction ($F(1,30)=3.49$, $p=0.07$) (Figure 2B).

Factorial ANOVA (sex x treatment) on time spent in the open arms of the EPM showed a significant effect of sex ($F(1,1)=81.40$, $p<0.01$), but no effect of treatment ($F(1,1)=0.002$, $p=0.96$), and no interaction ($F(1,31)=0.30$, $p=0.58$) (Figure 2C). Furthermore, the factorial ANOVA (sex x treatment) on total of entries in the EPM showed an effect of sex ($F(1,1)=152.72$, $p<0.01$), but no effect of treatment ($F(1,1)=1.75$, $p=0.19$) and no interaction ($F(1,31)=0.01$, $p=0.91$) (Figure 2D)

Fig 2

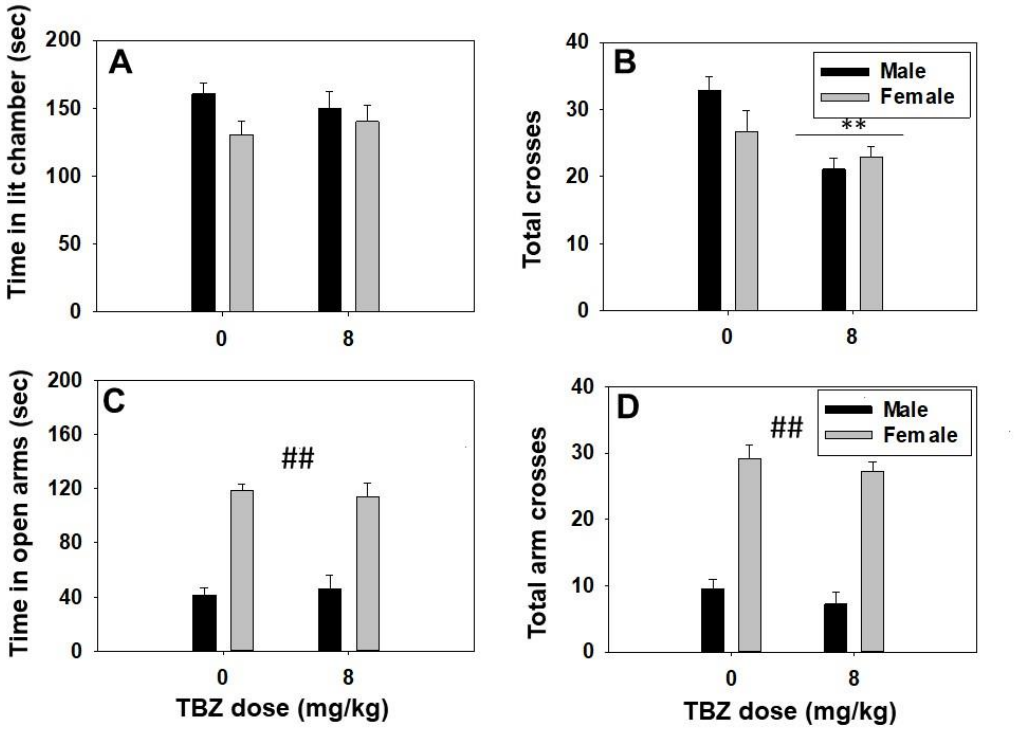

Figure 2. Effect of vehicle or TBZ (8 mg/kg) in male and female mice evaluated in the DA and in the EPM paradigms; time spent in the lit chamber (A), total of entries between the two compartments (B) of the DL box, time spent in the open arms (C), and total of entries to the four arms (D) of the EMP during two consecutive 5 minutes periods. Bars represent mean ± S.E.M. of accumulated seconds or number of crosses. **p<0.01 significantly different from vehicle, ##p<0.01 significant differences between male and female mice.

Experiment 3. Effect of TBZ on social motivation in male and female mice assessed in a three-chamber social preference test. Factorial ANOVA (sex x treatment) on time spent in the object compartment did not show an effect of sex ($F(1,1)=3.03$, $p=0.09$), or treatment ($F(1,1)=1.55$, $p=0.22$), and there was no significant interaction ($F(1,33)=1.85$, $p=0.18$). The same statistical pattern was obtained for time in the conspecific

compartment; no effect of sex (F(1,1)=1.99, p=0.16), or treatment (F(1,1)=0.15, p=0.70), and no interaction (F(1,33)=0.98, p=0.32).

Moreover, the factorial ANOVA on time interacting with the object did not show a statistically significant effect of sex (F(1,1)=3.20, p=0.08), or treatment (F(1,1)=2.45, p=0.12), and no interaction (F(1,33)=0.03, p=0.84). Finally, the factorial ANOVA on time interacting with the conspecific showed no effect of sex (F(1,1)=1.00, p=0.32), or treatment (F(1,1)=0.09, p=0.75), and no interaction (F(1,33)=1.43, p=0.24). Data are shown in table 1.

	Time sniffing				**Time in compartment**			
	Conspecific		**Object**		**Conspecific**		**Object**	
TBZ (mg/kg)	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
0.0	230.2 ± 12.5	262.0 ± 10.7	263.2 ± 11.2	215.2 ± 17.3	108.6 ± 11.8	134.6 ± 10.3	127.8 ± 10.5	102.2 ± 14.5
8.0	238.2 ± 14.1	243.7 ± 14.7	261.4 ± 15.8	255.5 ± 17.3	126.4 ± 10.1	124.1 ± 14.8	145.3 ± 14.1	124.9 ± 11.0

Table 1. Effect of TBZ (vehicle or 8 mg/kg) in male and female mice on time sniffing the conspecific, time sniffing the object, time spent in the conspecific compartment, and time spent in the object compartment in the social preference test assessed during 10 minutes. Bars represent mean ± S.E.M of accumulated seconds.

Experiment 4. Effect of TBZ on preference for sucrose concentration and on fluid consumption in male and female mice. Factorial ANOVA (sex x treatment) on the amount of 5% liquid sucrose consumed did not show an effect of sex (F(1,1)=0.56, p=0.45), or treatment (F(1,1)=0.84, p=0.36), and no interaction (F(1,44)=4.14, p=0.07). In addition, the factorial ANOVA on the amount of 10% liquid sucrose consumed did not show a significant effect of sex (F(1,1)=1.78, p=0.18), no effect of treatment

(F(1,1)=1.60, p=0.21), and no interaction either (F(1,44)=1.13, p=0.29). Data are shown in figures 3A-B.

Fig 3

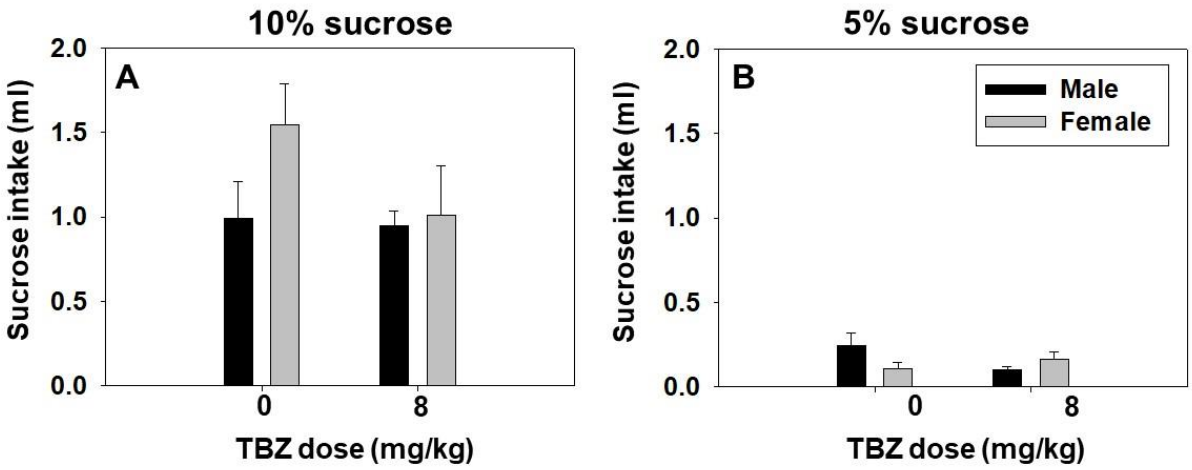

Figure 3. Effect of vehicle or TBZ (8 mg/kg) in male and female mice on the amount of 10% (A) and 5% (B) of sucrose solutions consumed during 60 minutes. Bars represent mean ± S.E.M of amount of ml consumed.

Moreover, the factorial ANOVA (sex x treatment) on the total of liquid sucrose consumed (fluid containing 10% plus fluid containing 5% sucrose) did not show an effect of sex (F(1,1)=2.21, p=0.14), or a significant effect of treatment (F(1,1)=3.29, p=0.07), and no interaction (F(1,46)=2.13, p=0.15) (Data are shown in Table 2).

Experiment 5. Effect of TBZ on preference for active reinforcers assessed in the 3-choice-T-maze task in male and female mice. Factorial ANOVA (sex x treatment) on time eating the sucrose pellets showed an effect of treatment (F(1,24)=14.74, p<0.01), but there was no effect of sex (F(1,24)=0.64, p=0.42), and no interaction (F(1,24)=0.30, p=0.58) (Figure 4A). The factorial ANOVA for time running in the RW showed a significant effect of treatment (F(1,24)=4.68, p<0.05), but there was no significant effect of sex (F(1,24)=3.41, p=0.07), and no interaction (F(1,24)=1.07, p=0.31) (Figure 4B). Finally, the ANOVA on time sniffing the neutral odor in the third arm of the T-maze did

not show an effect of sex (F(1,24)=1.32, p=0.26), or treatment (F(1,24)=1.01, p=0.32), and no significant interaction (F(1,24)=3.49, p=0.07) (Figure 4C).

Fig 4

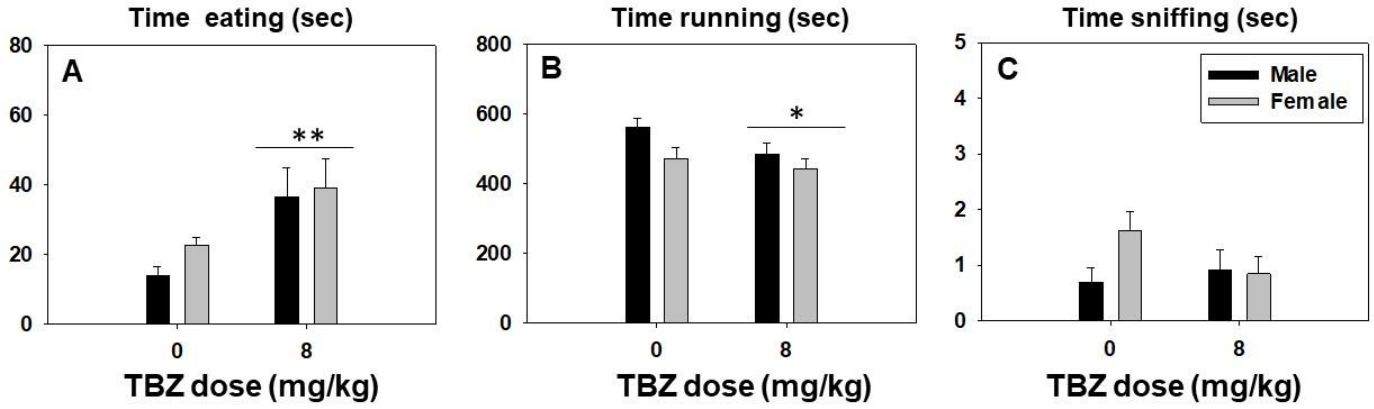

Figure 4. Effect of vehicle or TBZ (8 mg/kg) on male and female mice preferences in the 3-choice-T-maze task on time eating (A), time running (B), and time sniffing (C). Bars represent mean S.E.M of accumulated seconds. *p<0.05, **p<0.01 significantly different from vehicle.

A series of independent factorial ANOVAs (sex x treatment) on time spent in each one of the T-maze compartments showed that for the food compartment there was an effects of sex (F(1,24)=3.88, p<0.05), but not of treatment (F(1,24)=2.91, p=0.10), and no significant interaction (F(1,24)=3.89, p=0.35), (figure 5A). The factorial ANOVA on time spent in the RW compartment also showed a significant effect of sex (F(1,24)=8.70, p<0.01), but no effect of treatment (F(1,24)=1.06, p=0.31), and no interaction (F(1,24)=2.40, p=0.13) (Figure 5B). Finally, the ANOVA on time spent in the odor compartment did not show an effect of sex (F(1,24)=0.54, p=0.46), or treatment (F(1,24)=0.05, p=0.80), and no interaction (F(1,24)=1.89, p=0.18) (Figure 5C)

The independent factorial ANOVAs on total number of entries to the different compartments showed that, for the food compartment, there was a significant effect of sex (F(1,24)=27.56, p<0.01) and a significant interaction (F(1,24)=5.39, p<0.05), but there was no significant effect of treatment (F(1,24)=2.93, p=0.10). Planned comparisons

revealed that female mice that received vehicle significantly entered more times in the food compartment in comparison with male mice that received vehicle ($p<0.01$). Moreover, planned comparisons also revealed that TBZ produced different effects on male and female mice ($p<0.01$) TBZ did not produce any significant effect on total of entries to male mice in comparison with the vehicle but TBZ-treated female mice significantly reduced the total of entries to the food compartment in comparison with the vehicle group (Figure 5E).

The factorial ANOVA on entries to the RW compartment showed a statistically significant effect of sex ($F(1,24)=19.86$, $p<0.01$), but no effect of treatment ($F(1,24)=1.84$, $p=0.18$), and no interaction ($F(1,24)=1.35$, $p=0.25$) (Figure 5D). Finally, a factorial ANOVA (sex x treatment) on entries into the odor compartment showed an effect of sex ($F(1,24)=5.51$, $p<0.05$), but there was no effect of treatment ($F(1,24)=0.12$, $p=0.72$), and no interaction ($F(1,24)=0.29$, $p=0.59$) (Figure 5F)

Fig 5

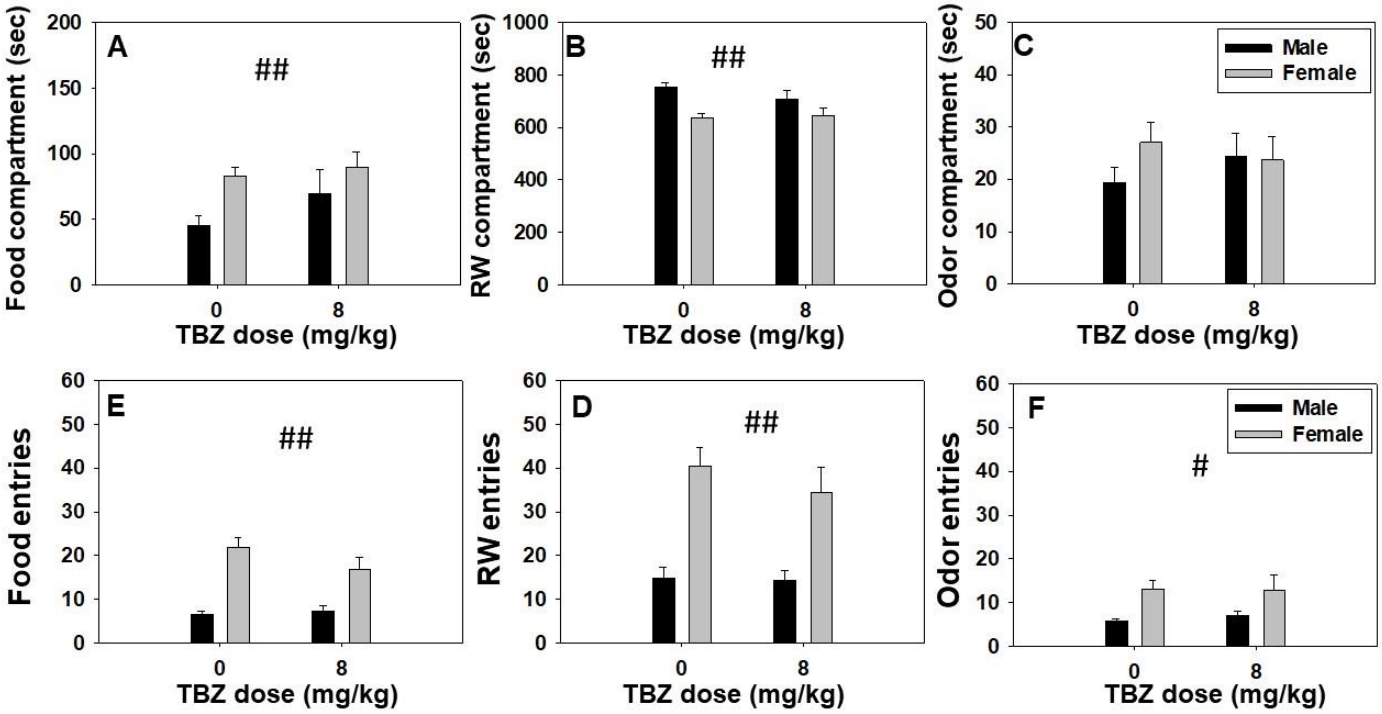

Figure 5. Effect of vehicle or TBZ (8 mg/kg) on male and female mice in the T-maze task on time spent in each compartment (A-C) and entries into compartments (D-F) assessed during 15 minutes. Bars represent mean S.E.M of accumulated seconds or number of entries. #$p<0.05$, ##$p<0.01$ significant differences between male and female mice.

Furthermore, we analyzed other dependent variables such as total food consumed and total ambulation in the T-maze (data are shown in table 2). The factorial ANOVA (sex x treatment) on the miligrams of pellets consumed did not show an effect of sex ($F(1,23)=1.84$, $p=0.18$), or of treatment ($F(1,1)=1.72$, $p=0.20$), and no interaction ($F(1,23)=0.51$, $p=0.48$). Finally, the factorial ANOVA (sex x treatment) on general locomotion in the T-maze showed an effect of sex ($F(1,24)=25.08$, $p<0.01$), but no effect of treatment ($F(1,1)=1.95$, $p=0.17$), and no interaction ($F(1,24)=3.35$, $p=0.07$).

	Vehicle		**TBZ**	
	Male	**Female**	**Male**	**Female**
Total sucrose solutions consumed (ml)	1.8 ± 0.2	1.1 ± 0.2	1.0 ± 0.3	1.0 ± 0.1
Pellet intake (mg)	100.4 ± 21.1	191.3 ± 45.4	176.5 ± 55.2	213.8 ± 41.5
General locomotion (crosses)	27.0 ± 3.4	73.6 ± 6.5##	28.5 ± 4.2	62.7 ± 9.1##

Table 2. Effect of vehicle or TBZ (8 mg/kg) on total sucrose consumed (5% sucrose fluid plus 10% sucrose fluid), on pellet intake, and on general locomotion in the T-maze task in both sexes. Mean (± SEM) of milliliters consumed during the test, or milligrams of palatable pellets consumed or crosses between compartments during the T-maze test. ## $p<0.01$ significant differences between sexes.

Experiment 6. Effect of TBZ and bupropion combination on behavioral activation assessed in the FST in male and female mice. Factorial ANOVA on immobility assessed in the FST did not show a significant effect of sex ($F(1,1)=3.07$, $p=0.08$), or sex by treatment interaction ($F(2,54)=0.44$, $p=0.64$). However, there was an effect of treatment ($F(1,2)=22.59$, $p<0.01$). Two separate one-way ANOVAs for the factor treatment in each sex demonstrated an effect on immobility time in male mice ($F(2,34)=10.71$, $p<0.01$) and also in female mice ($F(2,35)=4.20$, $p<0.05$). In males, planned comparisons revealed that TBZ-veh treated mice spent significantly more time

immobile ($p<0.01$) in comparison with the veh-veh group. Moreover, TBZ-BUP10 mg/kg was able to decrease immobility in comparison with the TBZ-veh treated male mice ($p<0.01$). In females, planned comparisons revealed the same pattern of effects than in males; TBZ-veh treated females significantly increased immobility compared to the veh-veh group ($p<0.01$), and TBZ-BUP10 mg/kg spent less time immobile than TBZ-veh treated female mice ($p<0.01$) (Figure 6A).

Factorial ANOVA (sex x treatment) on time spent swimming in the FST did not show a significant effect of sex ($F(1,1)=0.24$, $p=0.62$), and no interaction ($F(2,54)=0.73$, $p=0.48$). However, there was a significant effect of treatment ($F(1,2)=14.72$, $p<0.01$). Separate one-way ANOVAs for the factor treatment in each sex demonstrated an effect on swimming in male mice ($F(2,34)=6.94$, $p<0.01$), and also in female mice ($F(2,35)=3.43$, $p<0.05$). Planned comparisons for males revealed that TBZ-veh treated mice spent significantly less time swimming in comparison with the veh-veh group ($p<0.05$), and TBZ-BUP10 mg/kg was able to reverse the effect, increasing time male mice spent swimming in comparison with the mice TBZ-veh group ($p<0.01$). In females, planned comparisons also showed how TBZ-veh significantly decreased time swimming in comparison with the veh-veh group ($p<0.01$), and the combination of TBZ- BUP10 mg/kg significantly increased the time female mice spent swimming in comparison with the TBZ-veh treated female mice ($p<0.01$) (Figure 6B).

Finally, the factorial ANOVA on climbing showed an effect of sex ($F(1,1)=8.46$, $p<0.01$) and an effect of treatment ($F(1,2)=5.55$, $p<0.01$), but there was no significant interaction ($F(2,54)=1.10$, $p=0.33$). Separate one-way ANOVAs for the factor treatment in each sex demonstrated an effect on climbing in male mice ($F(2,34)=4.66$, $p<0.01$), but not in female mice ($F(2,35)=1.27$, $p=0.29$). Planned comparisons showed that TBZ-veh treated male mice reduced significantly climbing in comparison with the veh-veh group ($p<0.01$)

and TBZ-BUP10 mg/kg was able to reverse this effect compared with the TBZ-veh treated male mice ($p<0.05$) (Figure 6C).

Fig 6

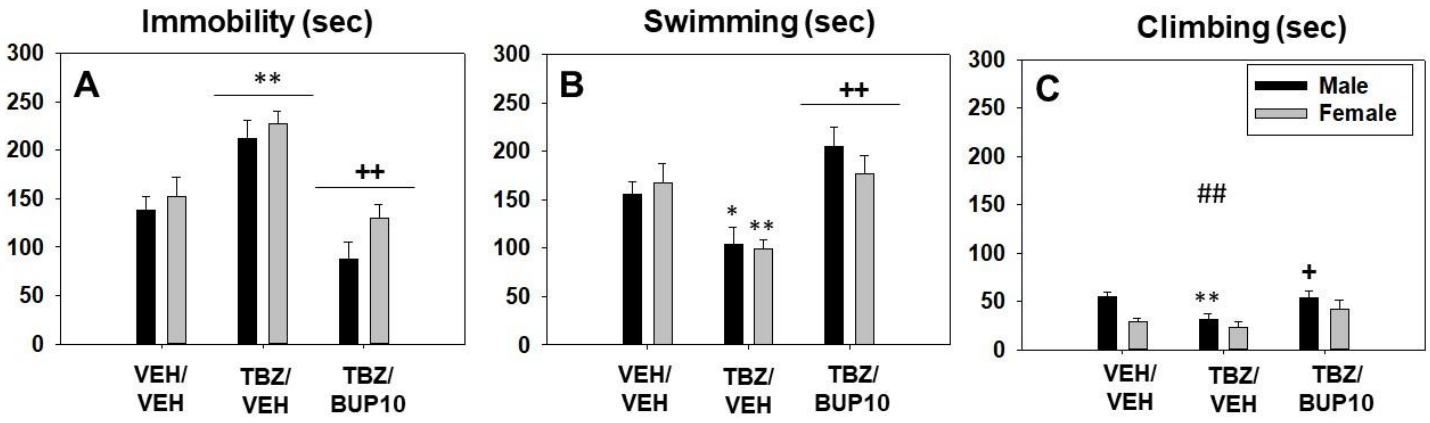

Figure 6. Effect of TBZ and bupropion combination (vehicle+vehicle, TBZ+vehicle, TBZ+10 mg/kg of bupropion) in male and female mice on duration of immobility (A), swimming (B) and climbing (C) in the FST assessed during 6 minutes. Bars represent mean ± S.E.M of accumulated seconds. *$p<0.05$, **$p<0.01$ significantly different from vehicle/vehicle. +$p<0.05$, ++$p<0.01$ significant differences between TBZ-veh and TBZ-Bupropion10 mg/kg, ##$p<0.01$ significant differences between male and female mice.

Experiment 7. Effect of TBZ and fluoxetine combination on behavioral activation assessed in the FST in male and female mice. Factorial ANOVA (sex x treatment) on immobility assessed in the FST did not show an effect of sex ($F(1,1)=0.02$, $p=0.98$) and interaction ($F(2,46)=0.83$, $p=0.44$) but there was an effect of treatment ($F(1,2)=9.90$, $p<0.01$). Separate one-way ANOVA's for the factor treatment in each sex demonstrated an effect on immobility time in male mice ($F(2,33)=4.64$, $p<0.01$) and in female mice ($F(2,33)=3.47$, $p<0.05$). Planned comparisons revealed TBZ-treated male mice and male mice that received the combination of 8 mg/kg TBZ plus 20 mg/kg fluoxetine increased significantly time spent immobile in comparison with the vehicle-vehicle group ($p<0.01$ and $p<0.05$ respectively) (Figure 7). Moreover, planned comparisons also showed TBZ-treated female mice increased the immobility time ($p<0.01$) in comparison with the

control group and 20 mg/kg fluoxetine did not produce an effect on the immobility of female mice (Figure 7A)

Factorial ANOVA (sex x treatment) on time swimming in the FST did not show an effect of sex ($F(1,1)=0.91$, $p=0.34$) and interaction ($F(2,46)=0.54$, $p=0.58$) but there was an effect of treatment ($F(1,2)=7.68$, $p<0.01$). Separate one-way ANOVA's for the factor treatment in each sex demonstrated an effect on swimming time in male mice ($F(2,33)=2.74$, $p<0.05$) and in female mice ($F(2,33)=3.49$, $p<0.05$). Planned comparisons revealed the group of male mice that received 8 mg/kg TBZ plus vehicle decreased significantly the time swimming in comparison with the vehicle-vehicle group ($p<0.05$) and fluoxetine (20 mg/kg) did not change swimming behavior. Moreover, planned comparisons also showed a significant decreased on time spent swimming of the TBZ-treated female mice ($p<0.01$) in comparison with their respective control group and 20 mg/kg fluoxetine reversed the effect produced by TBZ and female mice that received the combination of TBZ (8 mg/kg) and fluoxetine (20 mg/kg) significantly increased time swimming in comparison with the TBZ-treated female mice ($p<0.05$) (Fig 7B).

Finally, factorial ANOVA (sex x treatment) on climbing in the FST did not showed an effect of interaction ($F(2,46)=2.60$, $p=0.08$) but there was an effect of sex ($F(1,1)=7.33$, $p<0.01$) and an effect of treatment ($F(1,2)=8.28$, $p<0.01$). Separate one-way ANOVAs for the factor treatment in each sex demonstrated an effect on time climbing in male mice ($F(2,33)=4.24$, $p<0.05$) but there was any effect on climbing behavior in female mice ($F(2,33)=0.39$, $p=0.67$). Planned comparisons revealed TBZ-treated male mice significantly decreased the time spent climbing in comparison with the control group ($p<0.01$) and fluoxetine (20 mg/kg) failed to reverse the effect produced by TBZ, decreasing climbing on male mice that received the combination of both drugs ($p<0.01$) in comparison with the vehicle-vehicle group (Fig). Either TBZ (8 mg/kg) and fluoxetine (20 mg/kg) did not produce any effect on climbing behavior in female mice. Finally,

independent Students-t test demonstrated male mice spent more time climbing than female mice (t=-3.91, p<0.01) (Figure 7C)

Fig 7

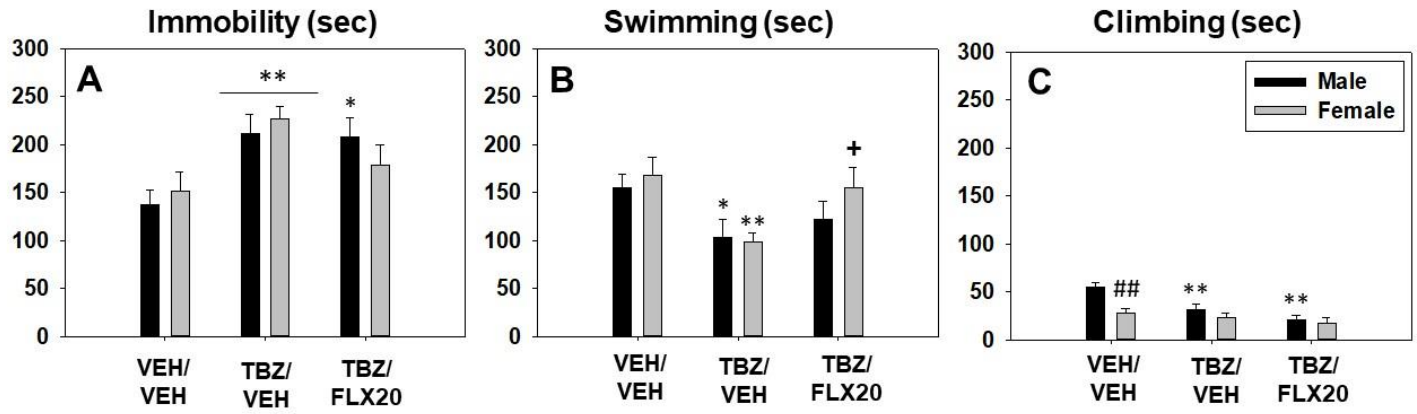

Figure 7. Effect of TBZ and fluoxetine combination (vehicle+vehicle, TBZ+vehicle, TBZ+ 20 mg/kg of fluoxetine) in male and female mice on duration of immobility (A), swimming (B) and climbing (C) in the FST assessed during 6 minutes. Bars represent mean ± S.E.M of accumulated seconds. *p<0.05, **p<0.01 significantly different from vehicle/vehicle. +p<0.05 significant differences between TBZ-veh and TBZ-Fluoxetine 20 mg/kg, ##p<0.01 significant differences between male and female mice.

Discussion

In the present work, we explored the effect of DA depletion in male and female mice on behavioral activation and preference for physical activities as sessed in the novel 3-choice-T-maze task. We also compared the results obtained in the T-maze task using other classic animal paradigms that evaluate depressive-like behaviors such as the FST, anxiety-like behaviors (DL box paradigm and EPM paradigm), social motivation evaluated in a social preference task and finally, the preference for sucrose liquid or food. Moreover, we also tried to observe if there were differences between male and female mice in the treatment response administering two antidepressants (bupropion or fluoxetine) after DA depletion and evaluating the outcome behavior in the FST.

There were no differences between male and female mice on passive behaviors (immobility) evaluated in the FST. Therefore, in control conditions, both sexes displayed almost the same time spent immobile in the tank. Moreover, male and female mice did not differ either in the time they spent swimming in the FST although it seemed that male mice were more active than female mice since male mice spent significantly more time climbing trying to escape. Previous studies have found similar results when comparing male and female rats (Hong et al, 2012; Wilkin et al, 2012) and also male and female mice (Kazavchinsky et al, 2019; Kara et al, 2017). Both sexes do not differ in time spent been immobile in the FST and display almost the same time swimming in the tank (Kokras et al, 2009; Warner et al, 2013; Kara et al, 2017). However, there are also reports showing that young and middle-aged female rats spent less time immobile, although, this sex difference disappeared at senescence (Fernández-Guasti et al, 2017). In terms of sex differences on vigorous escaping behaviors (climbing), previous studies are in accordance with the present results; female rats and mice spent less time climbing in the FST (Wegener et al, 2012; Hong et al, 2012; Masuda et al, 2001). After the administration of 8 mg/kg TBZ, a dose that has been demonstrated to decrease DA levels in mice ventral striatum (López-Cruz et al, 2018), and potentiates passive behaviors, decreasing also active behaviors in the FST (Carratalá-Ros et al, 2020), we did not observed differences between sexes due to treatment; both male and female mice decreased significantly time swimming, increasing time spent immobile in the FST. Because the baseline for climbing is very low in females, DA depletion had significant effects only in male mice.

When evaluating male and female mice on classic paradigms to assess anxiety-like behaviors, we found that, under control conditions, there were not significant differences between male and female mice on time spent in the lit chamber of the DL box. However, female mice spent significantly more time in the open arms of the EPM than male mice, indicating females are less anxious than males in this paradigm. Previous studies also have found that female rodents are less anxious than male rodents in the EPM (Voikar et al, 2001; Rodgers et al, 1993; Imhof et al, 1993). If this were the case, it would explain why female mice spent less time climbing in the FST than male mice suggesting that females could be less anxious than male mice. However, TBZ did not affect any of the anxiety parameters either in the DL box (time spent in the bright chamber) or in the EPM (time in the open arms) paradigms, and there were no sex differences. All of these results confirm the idea that drugs that modify DA transmission do not affect anxiety-related parameters. However, in terms of the activity parameter in the DL box (crosses between compartments), the TBZ administration reduced significantly locomotion equally in both sexes, consistently with previous finding in male mice (Correa et al, 2018; Carratalá-Ros et al., 2020). In the EPM the pattern of results was quite different. Under control conditions, female mice displayed more crosses than male mice, but 8 mg/kg TBZ did not affect locomotion either in male or in female mice. Higher levels of locomotion in female rats have been seen in previous studies (Fernandes et al, 1999). However, this study also found differences in anxiety in the EPM, and males were more anxious than females (Fernandes et al, 1999).

Social motivation was also assessed in male and female mice using a social preference task. This kind of task allows the study of spontaneous motivation for social contact (in this case in non-socially isolated animals) manifested by social preference or avoidance and also has been used to evaluate anxiety in rodents (File and Hyde, 1978; Guy and Gardner, 1985; López-Cruz et al, 2016). In our experiment, male and female mice had to choose between interacting with a same sex-conspecific or spent time investigating an object. Male and female mice did not differ on direct exploration of any of the stimulus or time spent in any of the compartments, and TBZ administration did not change behavior. There are only a few studies about the social interaction processes in female rodents (Perkins et al, 2016; Veenema et al, 2012), and in those few studies it has been shown that there are no differences between sexes in social exploration neither in young

nor in adult rats, although there were aging-related declines in social behavior in females (Perkins et al, 2016).

In addition, the preference for different concentrations of liquid sucrose was also evaluated in male and female mice. Both sexes clearly preferred the higher concentrated solution (10% versus 5%) and TBZ did not change this preference and did not reduced the total amount of fluid consumed. Previous studies also have shown no baseline difference between female and male rodents on liquid sucrose intake and preference, although female rats tend to drink more under conditions that generate pain (O Henderson et al, 2017; Dalla et al, 2008). Sweet taste can act as a powerful natural reward (Levine et al, 2003; Yamamoto, 2003). Moreover, preferences for higher concentrations seem to invigorate behavior to work harder for those solutions, when a lower, but freely available, sucrose concentration is concurrently available (Pardo et al, 2015; SanMiguel et al, 2018). A previous study in hour laboratory using male rats, evaluated the impact of TBZ on preference and total drinking of two sucrose solutions freely and concurrently available (Pardo et al, 2015). In this study TBZ did not produce any change on concentration preference or the total volume consumed. However, it did shift preferences when rats had to work in order to obtain the highly concentrated sucrose (Pardo et al, 2012). Thus, from present and previous work it seems that administration of TBZ, which produces DA depletion in ventral striatum (López-Cruz et al, 2018; Nunes et al, 2013; Pettibone et al, 1984), does not affect aspects of sucrose motivation such as intake, preference and hedonic reactivity when animals can freely choose without doing an effort. However, the intake of liquid sucrose can change under effortful conditions indicating that DA regulates behavioral activation, leaving intact the directional aspect of motivation (approaching to a palatable reward where effort is not required) (Pardo et al, 2015).

Behavioral activation can be studied using operant procedures and maze procedures. Most of the animal studies that use operant tasks are performed in male rodents, although there is some literature on performance of female rodents in operant tasks. Thus, it has been observed that female mice lever-press less in a progressive ratio operant task (Lloyd et al, 2018), and the same pattern is observed in female rats using fixed ratios (van Haaren et al, 1990). However, so far there are no published studies assessing female performance in effort-based decision-making tasks. The present results in the 3-choice-T-maze task demonstrate that, under control conditions, there are not differences on behavioral performance and preference for the reinforcers between male and female mice. Therefore,

both male and female mice spent most of the time running in the RW, then eating the sucrose pellets and finally, sniffing the non-social odor. Thus, the level of preference for active reinforcers seems to be identical in both sexes. However, sex differences are revealed in terms of time spent in the different compartments (figures 5 A-C); female seem to spent significantly more time than males in the food compartment provably indicating higher place preference associated to this compartment or it is also possible than females eat more slowly than males. In parallel, female mice reduced time spent in the RW compartment, but there were no differences in time spent in the odor compartment. Interestingly, the total number of entries into each compartment is higher in female mice, and total number of crosses between compartments was also higher, indicating that female mice were more active than male mice in exploring the T-maze in general, consistently with the results in the EPM. Previous studies have already shown that female rodents are more active than male rodents as demonstrated by the use of RW (Hyde and Jerussi, 1983; Slob et al, 1981; Basso and Morrell, 2017) and it seems female rodents are more likely to engage in different activities not loosing focus from the main task (van Haaren et al, 1990).

In terms of the effects of DA depletion on female preferences in the T-maze suggest that there are no differences compared to male preferences. Previous studies have demonstrated that the administration of a wide range of doses of TBZ in male mice tested in this 3-choice-T-maze task partially shift behavior from the RW to the sucrose pellets with no change in the fruit odor sniffing (López-Cruz et al, 2018; Carratalá-Ros et al, 2020). The present study corroborates those results in males, and show that TBZ significantly reduced time running in the RW and increased time eating palatable pellets. Moreover, there were no differences between sexes. In addition, TBZ did not affect either male or female mice on measures of place preference assessed as time spent in each compartment, and on exploration evaluated as total of entries to each compartment. These data indicate that male and female mice did not avoid being in close proximity to the reinforcers and showed normal exploration of the T-maze, although TBZ-treated female mice displayed more entries to each compartment and showed more general locomotion than TBZ-treated male mice.

Our second objective in the present work was to characterize, the potential “therapeutic” effect of different categories of antidepressants that differ in their mechanism of action, by assessing their capability of revering the behavioral impairments induced by the DA

depleting agent. Therefore, we evaluated the response of male and female mice in the FST after TBZ or TBZ plus bupropion, a catecholamine reuptake inhibitor that acts on the DA transporter (DAT) and elevates DA release in Nacb (Randall et al, 2014). Bupropion has demonstrated to treat effectively some symptoms of depression in humans (Feighner et al, 1986; Kiev et al, 1994; Weihs et al, 2000; Pae et al 2007; Papakostas et al, 2006; Cooper et al, 2014), and also to increase active behaviors in animal models that evaluate depressive-like behaviors (Carratalá-Ros et al.; Yamada et al, 2004; Kitamura et al, 2010; Yuen et al, 2017). However, little is known about the effect of bupropion on the behavioral output of female rodents. In our experiment, the administration of 8 mg/kg TBZ produced the same effect that we described previously: male and female mice decreased swimming behavior and increased immobility, but there were differences in climbing between male and female and TBZ only affected climbing in male mice that had a higher baseline. The administration of 10 mg/kg of bupropion, a dose that is effective at increasing active behaviors in male mice assessed in the FST (see chapter 2), alleviated the behavioral activation impairment induced by TBZ increasing time spent swimming and decreasing immobility in both sexes. However, bupropion only was able to increase climbing behavior in male mice.

Secondly, a different group of male and female mice received the serotonin transport inhibitor fluoxetine. In humans, this antidepressant is useful to treat emotional symptoms present in depression (Papakostas et al, 2008; Hieronymus et al, 2016; Rizvi et al, 2013; Rosenblau et al, 2012), but seems ineffective for treating motivational dysfunctions, and in fact, it can exacerbate or induce these symptoms such as fatigue in some patients (Nutt et al, 2007; Targum and Fava, 2011; Padala et al, 2012; Stenman and Lilja, 2013; Rothschild et al, 2014; Fava, 2014). In animal models, the administration of fluoxetine alone increases active behaviors (swimming and climbing) and decreases immobility in male rodents (Jang et al, 2009; Castagné et al, 2010; Petit-Demouliere et al, 2005; see previous chapter), and also in female rodents (Fernández-Guasti et al, 2017; Jones and Lucki, 2005). Experiments done previously in our lab have demonstrated that the combination of TBZ (8 mg/kg) and fluoxetine (20 mg/kg) exacerbates the behavioral activation impairment induced by the DA depleting agent in male mice (see previous chapter). In the present experiment, TBZ alone induced behavioral activation impairments in both male and female mice, decreasing active behaviors and increasing immobility, and the administration of fluoxetine (20 mg/kg) had sex dependent effects.

Thus, the combination of 8 mg/kg TBZ and 20 mg/kg fluoxetine did not reverse the effect of TBZ in immobility and in climbing. In females, this SERT blocker reversed the effect produced by TBZ in swimming and immobility. However, because of the low baseline in climbing in females neither TBZ nor TBZ plus fluoxetine had an impact on female climbing. Some studies have indicated that fluoxetine is more effective in female than in male rodents since lower doses of this SERT blocker are required in females than in males in order to improve behavior in the FST (Jones and Lucki, 2005; Fernández-Guasti et al, 2017). Thus, present and previous data support observation in the clinical field suggesting that women have a better outcome after fluoxetine treatment than men, at least for some depressive symptoms (Khan et al, 2005; Young et al, 2009; Kornstein et al, 2000).

In summary, this study provides information about the behavioral performance of female mice under basal conditions, and also under DA depletion in different animal paradigms, comparing them with the behavior of male mice. Moreover, the present study indicates that DA is involved in behavioral activation, and thus, suggesting that pro-dopaminergic drugs may be able to improve depressive symptoms related to reduce behavioral activation in humans. Moreover, the differential pharmacological response between male and female mice shown in this study, support the differential efficacy of distinct monoamine uptake inhibitors in the treatment on motivational symptoms in both sexes. These ideas are consistent with the research domain criterion (RDoC) approach that highlights the importance of describing the neural circuits that mediate specific symptoms in psychopathology, and not simply the traditional diagnostic categories (Cuthbert and Insel, 2013). Furthermore, it highlights the fact that relatively limited basic research has been devoted to developing animal models and consequently describing drug treatments which are sensitive to sex differences. These results suggest caution should be exercised in interpreting the results from female rodents in tests validated on males.

		Sex differences
FST	**Immobility**	=
	Swimming	=
	Climbing	Female -
DL	**Time in light**	=
	Crosses	=
EPM	**Time open arm**	Female +
	Total crosses	Female +
Social Interaction	**Time sniffing conspecific**	=
	Time conspecific compart.	=
	Total crosses	=
Sucrose Preference	**Preference**	=
	Total sucrose intake	=
Choice T-maze task	**Interaction with stimulus**	=
	Time in food compartment	Female +
	Time in RW compartment	Female -
	Time in odor compartment	=
	Total food intake	=
	Exploration of compartments	Female +

Table 3. Summary of male and female differences in spontaneous behaviors. = indicates no significant difference, + indicates more behavior, - indicates less behavior.

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GENERAL CONCLUSIONS

The present thesis characterizes behaviorally and pharmacologically a new animal model for the evaluation of preference for reinforcers that require high levels of behavioral activation: the 3-choice-T-maze task **(Chapter 1)**

The use of this paradigm allowed to understand the role of mesolimbic DA in the regulation of behavioral activation and the voluntary exertion of effort in motivation, a component that is often affected in psychiatric disorders such as depression.

It has been observed that physical activities have intrinsic motivational properties, and the choice to engage in voluntary physical activity is undertaken, most of the times, in relation and competing with other alternatives that are more sedentary (i.e. drugs, foods, social stimuli, etc.). The results from **Chapter 1** show how using the 3-choice-T-maze task, which allows mice to freely engage in running on a RW or doing sedentary activities such as consuming sucrose pellets or sniffing a non-social odor, we can stablish a hierarchy of preferences. In this case on the top, and significantly different from the other two, is interacting with a running wheel that allows a high degree of voluntary physical exercise. Moreover, the administration of a wide range of doses of the vesicular monoamine transport type 2 blocker; tetrabenazine (TBZ) which depletes DA, reduced the time engaged in the RW, although no dose suppressed the preference for the RW as the first option. Furthermore, at the highest doses, TBZ produced a reorganization of behavior, compensating for the loss of interaction with the vigorous reinforcer, by increasing time engaged in consuming non-habitual and highly palatable food, that has been used in other rodent paradigms as the most preferred reinforcer (in choice-operant procedures for example). This shift suggests that the animal is not generally anhedonic, since it increases food consumption, and is not fundamentally impaired motorically, since still spends more time running. The third stimuli generated minimal attraction since it was a fruit odor that was never associated to food or any other more powerful reinforcer. This third option only increased in preference when the fruit odor was substituted by a social odor from the same or different sex conspecific. Thus, increasing the salience and potential value of the olfactory stimuli, increased the time dedicated to explore it, specially, when it was an odor from a female.

Furthermore, ample behavioral characterization of the conditions that modulate preferences for the RW versus other less preferred reinforcers were performed in order to discard potential confounding factors such as force, aversion, loss or increase in appetite, etc., in the interpretation of the TBZ results. Thus, a series of experiments (in **Chapters 1 and 3**) were design to modify the motivational value of the reinforcers available in the T-maze task. We started by changing factors that affect the RW value, such as increasing RW resistance to turn and, consequently, increasing the force required to run. This manipulation reduced the time running but did not increase food consumption. Also, making the compartment of the RW aversive by placing an anxiogenic light over the RW produced a partial shift towards food, but also increase avoidance of the RW chamber, something that was not produced by DA depletion. In the increase force experiment animals reduced time spent running, but they stay in proximity of the RW, although taking more pauses. Increases or decreases in food appetite by satiation or deprivation of food, or making the food aversive by adding a bitter flavor, did not produce the same pattern than the pharmacological manipulations that increase or decrease DA.

This new animal model for the study of anergia after DA depletion with TBZ, was systematically compared with a classical animal model of anergia induced, in this case, by learned helplessness in an aversive context; the forced swim test (FST). TBZ produced a very similar pattern of results in both paradigms for the study of anergia; the FST and the 3-choice-T-maze task. There are important differences between both paradigms. For instance, the FST uses an acute and unescapable stressor (a tank with water) to generate baseline anergia; the animal rapidly learns that it can not escape and minimizes effort staying immobile or mildly swimming to keep afloat. This is used as a model of depressive behavior. DA depletion potentiates that baseline inactivity. However, in the case of the 3-choice-T-maze task among the 3 different reinforcers, all of them appetitive, the spontaneous behavior of any mice is to select the one that allows voluntary vigorous exercise, the RW. Thus, DA depletion is required in order to generate anergia. This anergia leads to a partial reorganization of preferences. While the FST tries to model a pathological state, the T-maze is intended for the study of factors that modulate normal behavior.

Because an important part of depressed patients also have anxiety, we evaluated if DA depletion induces an anxiogenic or anixiolitic effects. However, TBZ was not effective at modulating anxiety-related parameters (**Chapters 1-5**) or social interaction, neither in

male nor in female mice (**Chapter 5**). TBZ effects in most of those rodent paradigms (DL, EPM and SI) produced a reduction in exploration of the environment, but no change in social preference or in avoidance to aversive stimuli (intense light or open elevated spaces).

In addition, we compared the impact of two of the most common antidepressant drugs used in the clinical practice: fluoxetine and bupropion, for their effects in both models of anergia. Both of these drugs are monoamine uptake inhibitors, but they seem to differ substantially in their improvement of motivational disfunctions such as fatigue seen in some people with depression. Thus, bupropion, a cathecolamine uptake blocker which main action is to block DAT, thus, increasing striatal DA **(Chapter 2),** and fluoxetine, a SERT inhibitor **(Chapter 3),** were evaluated on their own or in combination with TBZ on these animal models of anergia and on anxiety. We also assess their impact on postsynaptic DA receptor function by analyzing phosphorylation of DARPP-32 in Nacb core, a central brain nuclei for the regulation of the activational component in motivated behavior **(Chapters 2 and 3)**.

In **Chapter 2**, bupropion on its own, increased time engaged in running in the T-maze task and also increased time climbing in the FST with no effect on anxiety. Bupropion also was able to reverse the effects of DA depletion in both animal models suggesting a "therapeutic" effect on anergia. Moreover, TBZ increased phosphorylation of DARPP-32 at Thr34, and bupropion was able to decrease TBZ induced pDARPP-32(Thr34) immunoreactivity, suggesting that the actions of these two drugs on behavior could be mediated by their actions on Nacb DA receptor containing neurons; the medium spiny neurons (MSN). From previous studies in rats (Nunes et al., 2013) we know that the effect of TBZ increasing pDARPP-32(Thr34) occurs in D2 receptor containing neurons in Nacb core, neurons that also release enkephalin. In those studies pDARPP-32(Thr75) was also increased after TBZ administration to rats, but that increase occurred in D1 containing neurons that release Substance P. In our studies with mice, TBZ did not produce a significant effect on DARPP-32 phosphorylated at Thr75 on Nacb core. However, it is worth mentioning that TBZ produced a clear tendency to reduce pDARPP-32(Thr75) immunoreactivity that did not reach statistical significance, even after using a different antibody and a different technique (western blotting). The tendency to suppress pDARPP-32(Thr75) compared to vehicle was observed better using immunofluorescent techniques. Thus, in further studies we should use this procedure to try to assess if this tendency is

stable, although, it is possible that to observe a reduction of a marker that at baseline levels is already quite low, is difficult technically. Interestingly, we found that there was a partial co-localization of pDARPP-32(Thr75) immunoreactivity in enkephalin-containing neurons located in Nacb core, suggesting that this effect of TBZ is also occurring on D2 receptor containing neurons.

The SERT inhibitor fluoxetine, was evaluated in **Chapter 3**. As with bupropion, we evaluated its effects alone or in combination with the most effective dose of TBZ on behavioral activation and on anxiety parameters. We confirmed that fluoxetine was effective at inducing behavioral activation in the FST, as it had been previously demonstrated. The highest doses of fluoxetine increased swimming and climbing. However, in the 3-choice-T-maze task fluoxetine produced anergia-like pattern of behaviors, very similar in fact to what was characteristic of TBZ: reduction in running and increasing time consuming sucrose pellets. Moreover, fluoxetine administered alone produced an anxiogenic effect. Furthermore, fluoxetine failed to reverse the behavioral impairment produced by TBZ in the FST, and it was not able to reverse TBZ-induced suppression of time running in the RW. However, fluoxetine in TBZ treated animals suppressed consumption of sucrose pellets. Thus, fluoxetine given in combination with TBZ reduced time interacting with both reinforcers; RW and food, and there was no compensation towards the neutral odor either. The reduction of food consumption produced by fluoxetine did not resemble the effect produced by changing the value of the food. It is possible that the interaction TBZ-fluoxetine produces an impairment in locomotion since crosses between compartments is reduced, not only in the EPM, but also in the T-maze. It has been previously demonstrated that DA release in Nacb core, a central area for the regulation of spontaneous locomotion, is reduced by fluoxetine alone (Yohn et al., 2016). Consistent with this finding, in chapter 3 we demonstrate that fluoxetine on its own and also the combination of TBZ-fluoxetine significantly reduced pDARPP-32(Thr34) and also pDARPP-32(Thr75) in Nacb core, suggesting an overall decrease of DA transmission in this area.

The effects of different antidepressants on the FST and on effort-based decision making operant paradigms are well known in basic studies that use male rodents. However, there is little evidence on the effect of these drugs in females, despite of the fact that in humans, epidemiological data show that depression is twice as common in women than in men, and that the pharmacological response to treatment also differs between sexes. Thus,

Chapter 4 compare male and female mice on their response to these antidepressants, and on their performance in these different behavioral paradigms that assess anergia and anxiety. The most effective dose of TBZ from chapter 1 was used in male and female mice in order to assess its impact on behavioral activation in the T-maze task and the FST. Anxiety parameters, social motivation and consumption and preference for sucrose solutions were also evaluated in male and female mice after the administration of TBZ. First of all, the results on spontaneous behavior in most of all these paradigms do not show significant differences between sexes. The most important exception been climbing in the FST, and locomotion during the exploration of the T-maze. TBZ produced a similar pattern of behavior in both sexes in the T-maze task inducing behavioral activation impairments; decreasing running on a wheel but increasing sucrose consumption. In the FST, TBZ decreased swimming in both sexes but only affected to climbing behavior in male mice, since baseline climbing was already low in females. DA depletion did not affect social interaction and sucrose preference neither in male nor in female mice, and anxiety in both sexes was not affected by TBZ, although female seem less anxious in the EPM compared to male.

Bupropion and fluoxetine were also used in both sexes in order to reverse TBZ effects in the FST. Bupropion was able to reverse the effects of DA depletion in both, male and female mice. However, fluoxetine failed to reverse TBZ-induced behavioral impairments in male in all three parameters; immobility, swimming and climbing. TBZ treated females improved after receiving fluoxetine in immobility and in swimming. However, climbing in females was neither impaired nor improved by TBZ or TBZ plus fluoxetine or TBZ plus bupropion, indicating that this is not an important behavior in the study of FST in females.

In conclusion, a translational outcome of the present dissertation is that not all antidepressants are adequate for the treatment of psychomotor slowing and anergia symptoms commonly seen in depression. Moreover, sex differences are key in order to approach the pharmacological response to these different symptoms. This idea of different therapeutic drugs having positive actions for some symptoms, but no effect or even negative actions on other symptoms, is consistent with the research domain criterion (RDoC) approach that highlights the importance of describing the neural circuits that mediate specific symptoms in psychopathology, and not simply the traditional diagnostic categories.

APPENDIX I

Effects of fluoxetine on the T-maze running wheel choice task in female and male rats: lack of modulation by 5HT-2C receptor antagonists.

Introduction

The development of novel animal models in the study of effort-related aspects of motivation are necessary in order to continue gaining new insights into this complex aspect of behavior. Effort-based choice tasks such as the FR5/chow feeding choice and PROG/chow feeding choice tasks use food as the reinforcer for both high and low effort options. Complications using these types of methods can develop when pharmacological agents known to produce appetite suppressing effects are used to alter task performance. Running on a running wheel (RW) has been shown to be an intrinsically reinforcing activity in rats (Kagan and Berkun 1954, Collier and Hirsch 1977, Pierce et al. 1986, Iversen 1993), subject to multiple types of reinforcement schedules. Recent mouse studies have reported on the use of a T-maze running wheel task choice task in mice. When given the option to run on a RW or consume sucrose, mice preferred the RW (Correa et al. 2016). With all of this in mind, a RW/chow feeding choice task for rats was developed that allowed animals to choose between consumption of food (lab chow) vs. engaging in voluntary physical activity in the RW. This new procedure with two different motivating factors can be used to tease apart the changes in behavior that can become muddied with a single motivating factor. The present studies examined the effects of the 5-HT uptake inhibitor and antidepressant fluoxetine (FLX) and the ability of the 5HT-2c receptor antagonist SB in reverse the effects produced by FLX. In addition, the blockade of the SERT transporte leads to the stimulation of the different 5-HT receptors thus, we suggest that using a 5HT-2c receptor antagonist would reverse the effects produced by FLX in the T-maze running wheel task.

Materials and Methods.

Subjects. Male (N=6) and female (N=8) Sprague Dawley rats (Envigo) were used. Rats were food restricted to their 90% free-feeding weight and allowed modest growth. Water was available ad libitum.

Pharmacological agents. Doses of fluoxetine (FLX, Tocris) (vehicle, 5, 10, and 15 mg/kg IP) were given 90 minutes prior to testing. Drug testing was completed utilizing a repeated measures design, with rats receiving one drug treatment per week, in a randomly varied order. The dose of SB (Tocris) (1.0 mg/kg, IP) was given 25 min before the test.

Running Wheel/Chow Feeding Choice Task. Behavioral sessions occurred in a Plexiglas T-maze apparatus consisting of a start arm (29 x 21 x 21 cm) and two test arms (99 x 32 x 59 cm). Within the maze, a clear box (47.6 x 25.9 x 20.9 cm) with a hole (10.3 cm diameter) cut in the front was placed in either the right or left arm. This box housed the RW (Starr Life Sciences Corp.) (33.02 cm diameter) apparatus with an attached counter to record RW rotations during sessions. A wall (32 x 59 cm) with an entry hole was placed in front of the box to keep the rats from moving around the sides of box during the task. The opposite arm contained a ceramic bowl (9 cm diameter) (PetCo) containing standard lab chow.

Training took a total of 6 weeks. Rats were first only granted access to the RW by being placed directly into the box with the entry hole blocked for two weeks. The following two weeks, the side containing the bowl was blocked off and rats were placed in the middle of the maze facing the wall. This required the rat to learn to use the entry hole of the RW box. The final two weeks of training, the maze was open for the rat to explore both arms with an empty ceramic bowl placed opposite to the RW. On completion of the training period, weighed amounts of chow became concurrently available. The start arm was never utilized during this task and each rat began the session in the middle of the maze facing the wall. Each session lasted 30 minutes. At the end of each session, each rat was immediately removed from the maze, the number of completed RW rotations was recorded and the amount of chow consumed during the task was determined by weighing the remaining chow (including spillage). Drug testing began once RW activity and chow consumption reached stable baseline levels.

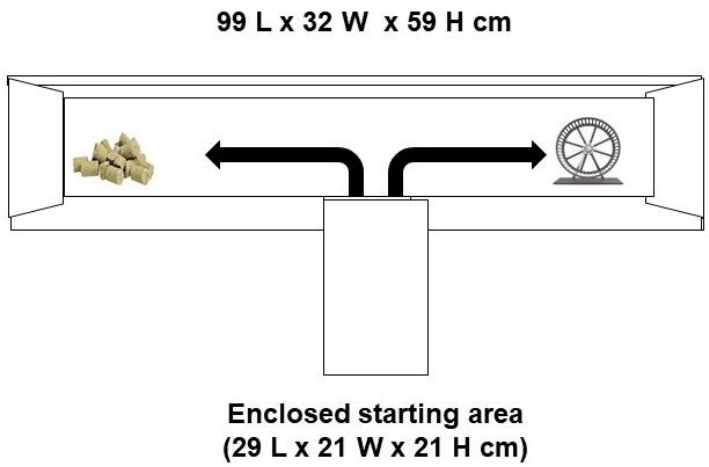

Figure 1. Schematic of RW/chow feeding choice task

Results

Experiment 1. Effect of fluoxetine on preference between RW or chow in the T-maze choice task. Factorial ANOVA (sex x treatment) on preference for the RW showed overall effects of sex ($F(1,12)=15.00$, $p<0.01$). and of fluoxetine ($F(3,36)=34.24$, $p<0.01$), and also a significant interaction ($F(3,36)=18.67$, $p<0.01$). Planned comparisons revealed that in male rats, when compared with the vehicle group the two highest doses of fluoxetine (10 and 15 mg/kg) decreased significantly RW rotations ($p<0.01$). In female rats all doses of fluoxetine (5, 10 and 15 mg/kg) significantly reduced the RW rotations in comparison with the vehicle group ($p<0.01$ for all doses). Moreover, male and female where only different when they received the vehicle treatment ($p<0.01$) but there were not differences between sexes at any other dose of fluoxetine.

The factorial ANOVA (sex x treatment) on chow intake showed significant effects of sex ($F(1,12)=197.32$, $p<0.01$), and fluoxetine ($F(3,36)=34.29$, $p<0.01$), and also a significant interaction ($F(3,36)=18.67$, $p<0.01$). Planned comparisons revealed that all doses of fluoxetine (5, 10 and 15 mg/kg) reduced significantly chow consumption in the male rats compared with the vehicle treatment ($p<0.05$ for 5 mg/kg, and $p<0.01$ for 10 and 15 mg/kg of fluoxetine). Fluoxetine-treated female rats significantly decreased the amount of chow consumed in comparison with the vehicle at all doses of fluoxetine tested ($p<0.01$). Moreover, vehicle treated females consumed significantly less chow than the male group ($p<0.01$), and that difference was also significant between both sexes when treated with 5 and 10 mg/kg ($p<0.01$).

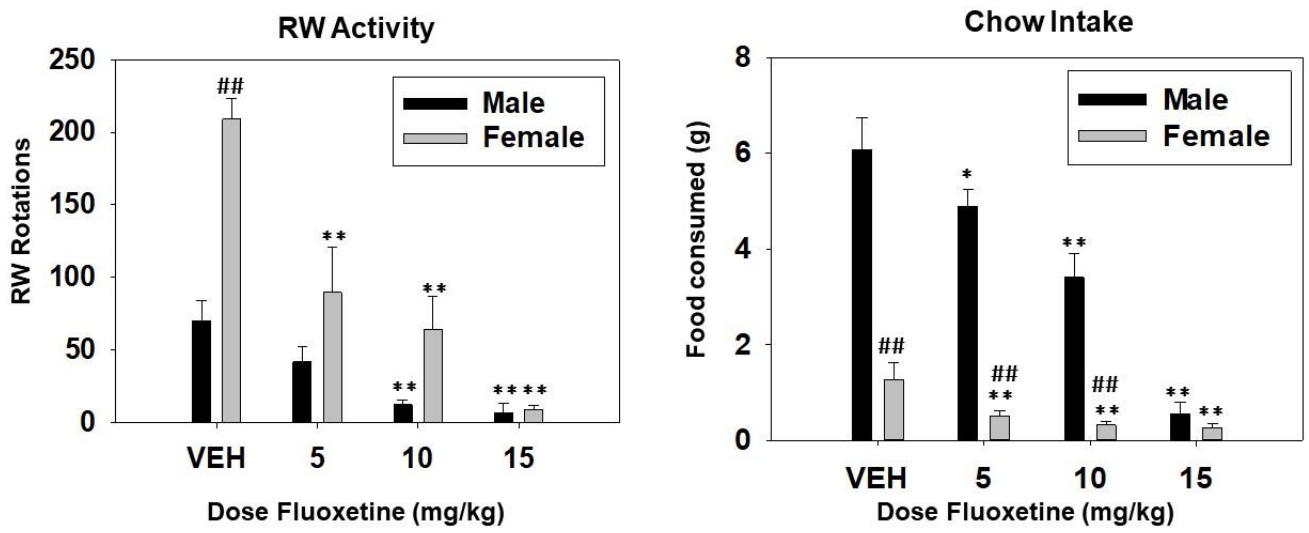

Figure 2. Comparison of RW activity measured by the number of rotations completed by the RW and the total of chow intake between male and female rats. An overall significant difference was seen in RW activity and chow intake (#p<0.01) between the sexes. An overall significant effect of FLX was seen in males (**p<0.01 at 10 and 15 mg/kg) and females (**p <0.01 at all doses) on RW activity and chow intake.

Experiment 2. Impact of the 5HT-2C antagonist on the effects of fluoxetine in the T-maze choice task. Factorial ANOVA (sex x treatment) on preference for the RW showed overall effects of sex ($F(1,12)=29.19$, $p<0.01$), treatment ($F(2,24)=58.95$, $p<0.01$), and a significant interaction ($F(2,24)=15.43$, $p<0.01$). Planned comparisons revealed that fluoxetine-treated male rats and also male rats that received the combination of fluoxetine plus 1.5 mg/kg SB1 reduced significantly RW rotations in comparison with their respective vehicle control ($p<0.01$). The same pattern was true for female rats; female rats that received fluoxetine (12.5 mg/kg), and the combination of fluoxetine plus SB1 also significantly reduced the RW rotations in comparison with the vehicle treatment ($p<0.01$). Male and female groups were different only under vehicle condition ($p<0.01$).

Factorial ANOVA (sex x treatment) on chow consumption showed significant effects of sex ($F(1,12)=22.13$, $p<0.01$), treatment ($F(2,24)=26.12$ $p<0.01$), and also a significant interaction ($F(2,24)=13.93$, $p<0.01$). Planned comparisons revealed that male rats that received 12.5 mg/kg of fluoxetine and the combination of fluoxetine plus 1.5 mg/kg SB1 reduced significantly chow consumption in comparison with vehicle ($p<0.01$). However, these treatments did not produce significant differences in female rats. Differences between male and female were only significant when receiving vehicle ($p<0.01$).

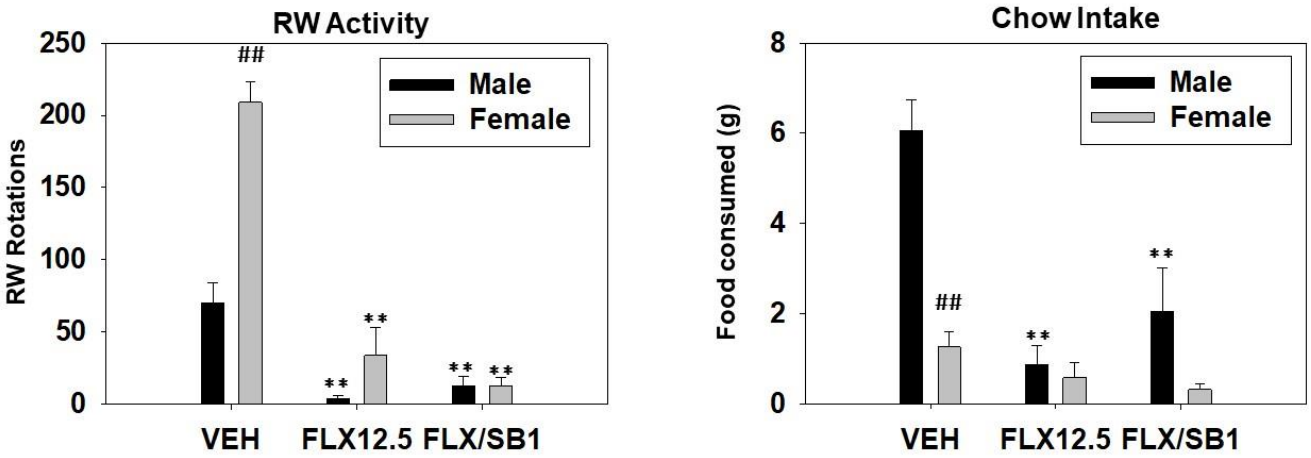

Figure 3. RW activity and chow intake (grams) between male and female rats. An overall significant difference was seen in RW activity and chow intake (##$p<0.01$) between sexes. An overall significant effect of FLX and the combination of FLX and SB was seen in males (**$p<0.01$) and females (**$p<0.01$) in RW activity. There was an overall effect of FLX and the combination of FLX and SB on the chow intake in male rodent (**$p<0.01$) but not in female rats.

Discussion

There was a significant overall effect of fluoxetine dose, a significant difference between males and females, and a significant sex x dose interaction. Whereas females tended to show more RW activity and less chow consumption the males showed the opposite. FLX administration induced an overall reduction in running wheel activity at all 3 doses administered. This is consistent with recent studies in mice (Carratalá-Ros et al. submitted). FLX administration induced a reduction in chow intake in both males and females (2 highest doses in male and all 3 doses in females). It is important to note that even with the significant difference between behavior, FLX administration had the same general effect across both sexes. Thus, these studies demonstrate that FLX decreased selection of RW activity in rats, consistent with previous mouse studies. Moreover, the 5-HT2c antagonist was not able to reverse the effect produced by FLX both in male and female rats and in fact, exacerbated the effect of the 5-HT uptake inhibitor in the RW activity. The same effect was seen in the total of chow intake in male rats but female rats did not change the total of chow consumption after the administration of FLX and the combination of FLX and SB in comparison with the control group.

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