UNIVERSIDAD DE JAÉN

FACULTAD DE HUMANIDADES Y CIENCIAS DE LA EDUCACIÓN
DEPARTAMENTO DE PSICOLOGÍA

TESIS DOCTORAL

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Influence of frustration on emotional self-medication in rats: A psychobiological approach.

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JAÉN, Junio, 2019

A todos los que me han ayudado en este camino,

Gracias

Acknowledgments

Dicen que una tesis se hace con sangre, sudor y lágrimas (y café, mucho café). Recorrer este duro camino sin el apoyo de familiares, amigos y personas cercanas sería imposible. Por eso, estas líneas van para ellos.

En primer lugar, me gustaría agradecer a Carmen Torres y a Nacho Morón, mis directores, que hayan confiado en mí para llevar a cabo este proyecto. Quiero agradecer vuestro entusiasmo y que hayáis estado detrás de mí para sacarlo adelante, vuestros ánimos cuando he estado más en el fondo y vuestra caña para que siguiera “pa lante”. No se puede tener unos directores de Tesis mejores que vosotros.

A mi familia, mis padres, Juan y Carmen, mis hermanos, Juanjo y Maca, mis cuñados, Edu y Patri, porque siempre habéis estado ahí, apoyándome en todo momento. A mi familia política, Cristina, Ace, Jose, Fernando, a los chiquillos, a Isa y a Pepe, a MariÁngeles y Antonio que me habéis ayudado a desconectar de este mundo loco de vez en cuando. Gracias a toda mi familia por el apoyo y el amor que me habéis dado.

Agradezco enormemente a Alex, su apoyo, su amor, sus ánimos cuando yo pensaba que no podía más. Gracias por todos los detalles que has tenido conmigo y por “aguantar el chaparrón” a mi lado, sin quejarte, y gracias a tus canciones con las letras cambiadas que me hacen reírme a carcajadas.

A mis “hermanos de armas”, mis compañeros del despacho 017. A Pablo, por sus consejos y ánimos. A Teresa, porque tu luz ilumina siempre y se nota cuando faltas del 017. A Pedro, José Andrés, David, Carmen, María Moreno, María Aranda, Rocío Linares y Marta, por estar ahí tanto para un roto como para un descosido, que con vosotros no faltan los buenos momentos. A las recién

llegadas Sonia, por su locura que anima a todos, y Loida, el antídoto de Sonia, por su ternura, amabilidad y su tranquilidad. A Gabi por esos cafés y tostadas gratinadas en la cafetería. A todos, mil gracias por vuestro apoyo.

A Pepi, Mariluz y Marijose, por ser las mejores técnicas que se pueden tener, y a Vicky, por ser amiga, ayudante y confidente. A Pedro y Lola, por los chascarrillos entre los tiempos muertos.

Quiero dar las gracias sinceramente a Juan, Ana Ibáñez, Juana, Ana Gaitán, Clara, María Jesús, Jessica, Antonio, Alba, Carmen, Laura, Noelia y Juanra, mis alumnos de TFG y prácticum, sin su enorme ayuda, no se habría podido sacar adelante el trabajo de laboratorio, además de que hubiera sido muy aburrido.

A Santos Blanco, por su paciencia en enseñarme complicadas técnicas de biología, por hacerlo sencillo para que pudiera seguir los pasos sin problema.

A Marta Sabariego y Lucas Cuenya, que me iniciaron en este loco mundo de la investigación, con vosotros di mis primeros pasos y me siento feliz por eso.

Quiero agradecer a mi amiga Ana, mi rubia linda, por tus consejos, tus risas y apoyo. Aunque no podemos estar siempre juntas, sé que estás ahí para mí, y yo para ti. Muchas gracias por hacerme ver las cosas desde un prisma distinto y darle importancia a las cosas que realmente importan.

A Pati, Esmeralda y Gabi, por acogerme en Madrid, y ofrecerme algo tan bonito y especial como vuestra amistad. Me siento muy feliz de haberos conocido y que, a pesar de las vidas ajetreadas que llevamos, siempre encontramos un hueco para disfrutar las unas de las otras. Gracias por enseñarme rinconcitos con encanto de la capital y animarme a descubrir algunas por mi cuenta. Esmeralda, gracias por abrirme las puertas de tu casa y permitirme quedarme

las visitas express. Chicas, gracias por compartir vuestro tiempo conmigo. A Ricardo Pellón, por acogerme en su laboratorio. A Antonio, por ayudarme en el laboratorio cuando me perdía por allí. A todos los que os conocí en Madrid y que me hicieron sentir en casa.

A los técnicos de los Servicios Centrales de Ayuda a la Investigación por enseñarme el manejo de los diferentes aparatos, e igualmente a la Universidad de Jaén, y al Centro de Producción y Experimentación Animal por haber facilitado los medios para desarrollar los diferentes estudios que componen este trabajo.

Also, I want to thank Dr. Mauricio Papini for welcoming me in his lab at TCU and his support throughout this period. Special thanks to Shannon Conrad and Sara Guarino, for helping me during my three months in Fort Worth, for showing me many beautiful places to visit, and for their patience when I was wrong talking to them, and for helping me to “survive.” To Mary and Linsey, thanks for your talks and coffee, you´re both amazing. Thanks to Amber and Marcos, I won´t forget that amazing show. Also, I want to thank all people I met during my stay: Andy, Randy, Lauren, Aryn, and Joanna. I won´t forget you guys!

No puedo olvidarme tampoco por su inmensa ayuda a Hugo, Clau, Cath, Romi, Analía, Daiana, Eze, Iván, Lizandro, Melissa, Cecilia, Shamy. A Ale y Gloria, que me acogieron en sus respectivas ciudades y me enseñaron cositas lindas. ¡Grupo BPPF forever!

A todos, ¡GRACIAS! THANKS!

El desarrollo de esta Tesis Doctoral ha sido financiado por el Ministerio de Economía y Competitividad del Gobierno de España mediante la concesión del Proyecto (PSI-2013-44945-P) “Mañana lo dejo: Automedicación Emocional, Cerebro y Adicción”. La vinculación de la doctoranda al contrato predoctoral se extiende desde el año 2015 hasta el 2019. La estancia de investigación realizada por la doctoranda en el año 2017 ha sido financiada por el Programa de Ayudas a la Movilidad Predoctoral para la Realización de Estancias Breves en Centros de I+D durante el año 2017, promovida por el mismo Ministerio.

De igual manera, esta Tesis también tuvo el apoyo del grupo de investigación HUM-642 (Psicología Comparada: Aprendizaje, Atención y Memoria) de la Junta de Andalucía.

Dedico además en este apartado, un especial reconocimiento al Departamento de Psicología de la Universidad de Jaén al que se adscribe el contrato predoctoral disfrutado.

Abbreviations

A/P	Anterior/Posterior axis
ACTH	Adrenocorticotropic hormone
Ad lib	Ad libitum
AMG	Amygdala
APA	American Psychological Association
BDZs	Benzodiazepines
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
CDP	Chlordiazepoxide
cE	Consummatory Extinction
Cl-	Chloride
CONT	Continuous group
CR	Continuous reinforcement
cSNC	Consummatory successive negative contrast
DA	Dopamine
DV	Dorsoventral dimension
E	Ethanol groups
ESM	Emotional self-medication
GABA	γ-aminobutyric acid
Hb	Habenula
HPA axis	Hypothalamic-pituitary-adrenal axis
i.p.	Intraperitoneal
iE	Instrumental extinction

INT	Intermittent group
iSNC	Instrumental successive negative contrast
LHb	Lateral habenula
ML	Mediolateral axis
N	Nonreinforced trials
NAc	Nucleus accumbens
NMDA	N-methyl-d-aspartate
PR	Partial reinforcement
PRCE	Partial reinforcement contrast effect
PREE	Partial reinforcement extinction effect
PTSD	Posttraumatic stress disorder
R	Reinforced trials
RHA	Roman high-avoidance rats
RLA	Roman low-avoidance rats
SM	Self-medication

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Summary

The consumption of psychoactive substances is a deeply rooted human practice since ancient times. Occasionally such practices can develop into maladaptive patterns characterized by a compulsive tendency to search and consume a substance, a loss of control for limited consumption, and the emergence of a negative emotional state when access to the drug is not possible (Koob & Volkow, 2016). Several theoretical and experimental approaches have attempted to identify the fundamental motivations leading an individual to abuse drugs. The self-medication hypothesis suggests that the type of substance chosen for consumption depends on the extent to which that substance alleviates a preexisting psychopathology or a negative state induced by aversive circumstances (Khantzian, 1985). However, the conditions that induce self-medication, the types of substances that support it, and the underlying brain areas that control the behavior are largely unknown. Recent evidence suggest that a negative emotional state of frustration induced by events involving reward loss promote the consumption of alcohol in rats.

This Dissertation systematically analyzed the role of emotional self-medication (ESM) induced by reward loss in the onset of anxiolytic consumption. First, based on prior studies (Manzo et al., 2012, 2014), I determined the appropriate experimental conditions (e.g., drugs, doses, presentation schedule) to obtain anxiolytic self-administration in male Wistar rats, using a water/drug preference test. Second, I analyzed the impact of an experience of reward loss (known to induce anxiety/frustration) to the development of self-medication with anxiolytics, including alcohol and benzodiazepines. Third, I tested the anxiolytic assumption of the emotional self-medication hypothesis, namely, that it is the

anxiolytic/anti-frustration effects of alcohol and benzodiazepines that underlie self-medication behavior. Fourth, I studied procedures that could block or ameliorate emotional self-medication, using inoculation against the effects of frustration based on exposure to partial reinforcement. Finally, I explored the role of the lateral habenula in frustration as a step toward understanding the biological basis of ESM behavior.

The studies presented in this Dissertation showed (1) that animals consumed alcohol voluntarily in the absence of frustration; (2) that emotional self-medication behavior selectively occurred during periods associated with reward devaluation, showing a reduced magnitude of the contrast effect after repeated cycles of reward downshifts (32%-4% sucrose solution); (3) that partial reinforcement training delayed the recovery from the reward devaluation effect and abolished the emotional self-medication behavior; and (4) that neurochemical lesions of the lateral habenula delayed both consummatory and instrumental extinction.

Key words: Addiction; Alcohol; Anxiolytic; Benzodiazepines; Successive negative contrast; Emotional self-medication; Extinction; Frustration; Lateral habenula; Partial reinforcement; Reward loss; Repeated reward downshifts; Wistar rats.

Chapter 1. Addiction:

Definition and clinical implications

Introduction

Drug addiction is one of the most pressing and complex current social issues, constitutes a severe mental disorder, and causes an untold amount of suffering among those with the disease and their loved ones (Ellenbroek, van der Kam, van der Elst, & Cools, 2005; Koob & Le Moal, 2006). According to the Global Burden of Disease Study (United Nations Office on Drugs and Crime, UNODC, 2016), one in 20 people 15-64 years of age consumed at least one drug in 2014. The global scene is even more complex because of the intake of multiple substances at the same time or in a successive way, a factor that increases mortality among users and induces both acute (e.g., overdose) and chronic health issues. More than 29 million people who consume illegal drugs (including opioids, cocaine, amphetamines, and cannabis) suffer drug-related diseases or premature death, resulting in the loss of 12 million life years (UNODC, 2016). Comparable data are collected in Europe, where poly-drug use is also a common practice, mainly among men. Cannabis is the most abused illegal drug, showing an upward trend in the last 10 years in almost every country. In Spain, 11% of the general population consumed cannabis in the last 12 months. Cocaine abuse is also frequent, with an average of 2% of prevalence across countries in 2015. As for opioid use, 76% of users are located in Germany, France, Italy, United Kingdom, and Spain, increasing the medical expenses derived from physical and psychiatric conditions (European Drug Report, 2017; Spanish Observatory of Drugs and Addictions, 2018).

Similar data are reported for legal drugs, including alcohol, nicotine, and medically prescribed anxiolytics. Alcohol intake is a common habit in many societies with relevant cultural and social implications (Escohotado, 2006;

Livingston, 2017). When consumed in large amounts, alcohol constitutes one of the most important contributing factor to disease, it causes death and health impairment at an early age, it triggers mental and behavioral disorders, and it increases the risk of developing cancer, cardiovascular, and infectious diseases (Kwako & Koob, 2017; Pulido et al., 2014). According to the Global Burden of Diseases (2018), alcohol is a direct cause of death and a risk factor for cancer, with the level of consumption that eliminates health risk being zero. The percentage of deaths attributable to alcohol reveals sex differences: 7.6% for men and 4% for women (European Drug Report, 2018). In Spain, alcohol is the most consumed psychoactive substance. The percentage of the general population that admits having drunk at least once in their lives was over 91% in 2017; 75.2% reported having consumed alcohol in the last 12 months; and 7.4% were daily drinkers (Spanish Observatory of Drugs and Addictions, 2017). In Spain, 10% of fatalities and 30% of deaths involving car accidents were attributable to alcohol consumption in 2011 (Pulido et al., 2014). Early consumption increases the risk of developing alcohol abuse disorders in adulthood (Prieto & Bares, 2012), especially in recent years, in which binge episodes are frequent among adolescents and young adults (Cadaveira & Corral-Varela, 2005).

Anxiolytic drugs with abuse potential, such as benzodiazepines (BZDs), are also frequently consumed in Spain, with and without medical supervision. These drugs share pharmacological properties and brain mechanisms of action with alcohol (Rosas-Gutiérrez, Simón-Arcelo, & Mercado, 2013; Tan et al., 2010). Between 2000 and 2007, the number of prescribed anxiolytics was 37% higher in our country compared with the European average (Téllez-Lapeira et al., 2017). According to the last Survey On Alcohol and Drugs in Spain (EDADES 2017-

2018), 20.8% of the general population took BZDs at least once in their lives, 11.1% during the last year, 7.5% during the last month, and 5.9% admitted to doing it daily. The average initial age of BZDs use is 47.5 years, and the proportion of women using BZDs is almost twice that of men (Spanish Observatory of Drugs and Addictions, 2018; Téllez-Lapeira et al., 2017). Overall, these data reveal that alcohol and BZD use and abuse constitute an important challenge for individual and community health.

Drug addiction, also known as substance use disorder (SUD; DSM-V, 2013), refers to a clinically significant pattern of substance use manifested by at least two of the following criteria occurring within a 12-month period:

(1) Substance is often taken in larger amounts or over a longer period than was intended.

(2) There is a persistent desire or unsuccessful efforts to cut down or control substance use.

(3) A great deal of time is spent in activities necessaries to obtain the drug, use drug, or recover from its effects.

(4) Craving, or strong desire or urge to use the substance.

(5) Recurrent substance use resulting in a failure to fulfill major role obligations at work, school, or home.

(6) Continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of substance intake.

(7) Important social, occupational, or recreational activities are given up or reduced because of substance use.

(8) Recurrent substance use in situations in which it is physically hazardous.

(9) Substance use is continued despite knowledge of having persistent or recurrent physical or psychological problems likely caused or exacerbated by the substance.

(10) Tolerance, defined as

(a) A need for markedly increased amounts of substance to achieve intoxication or desired effect.

(b) A markedly diminished effect with continued use of the same amount of substance.

(11) Withdrawal, defined as

(a) A withdrawal syndrome characteristic of the substance use.

(b) The individual consumes the substance (or a closely related substance) to alleviate or avoid negative withdrawal symptoms.

SUDs can occur in a broad range of severity, from mild to severe, depending on the number of the exhibited symptoms indicated above: mild (two to three symptoms); moderate (four to five symptoms); and severe (six or more symptoms).

A wide range of factors may predispose individuals to addiction and contribute to the passage from social-recreational drug use to compulsive use and loss of control (Koob, Arens, & LeMoal, 2014; Le Moal, 2009). This transition results from genetic (Dasgupta, 2015; Ducci & Goldman, 2008; Enoch, 2012; Le Moal, 2009; Philibin & Crabbe, 2015), age (Le Moal, 2009), personality traits (Conway, Kane, Ball, Poling, & Rounsaville, 2003), gender (Bobzean, DeNobrega, & Perrott, 2014; Fattore, Melis, Fadda, & Fratta, 2014) and

sociological factors (Crabbe, 2008; Nestler, 2000), combined with pharmacologically induced plasticity in brain circuitry (Kalivas & O´Brien, 2008).

Theories of addiction

Several different approaches have been proposed to explain the development of SUDs. As will become clear, these theories are not mutually exclusive.

One of the first and most influential theories of addiction was developed by Solomon and Corbit (1974) based on the concept of homeostasis. These authors postulated that hedonic, affective, or emotional states, once initiated, are modulated by neural mechanisms that reduce the intensity of hedonic feelings and provide a balance for the affective state (pleasure or aversion) of individuals (Volkow & Morales, 2015). The affective state of a person under the influence of drugs would result from the sum of two opposite emotional processes. The a-process usually consists of a positive hedonic response, occurs shortly after the presentation of a stimulus, and correlates closely with the intensity, quality, and duration of the reinforcer. This primary process in turn arouses a b-process (usually negative) that functions to oppose and suppress the affective or hedonic state initially generated by the onset of the a-process. The b-process drags down the strength of this a-state leading to drug tolerance. It is postulated that the b-process (the opponent process) is sluggish in onset, slow to build to its asymptote, decays slowly, and gets larger with repeated drug exposure (Koob, Caine, Parsons, Markou, & Weiss, 1997; Solomon, 1980; Volkow & Morales, 2015).

Opponent-process theory has been integrated in a neurobiological framework to explain drug addiction. Koob, Stinus, Le Moal, and Bloom (1989)

based their allostatic model of addiction on this opponent process, among other mechanisms. Allostasis is defined as a state of chronic deviation of the regulatory system from its normal homeostatic operating level caused by drug consumption (Koob & Le Moal, 2010). Drug addiction is defined as a chronically relapsing disorder characterized by: (1) compulsion to seek and take the drug; (2) loss of control in limiting intake; and (3) emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability) when access to the drug is prevented (Koob & Volkow, 2016). Drug addiction thus involves elements of both impulsivity and compulsivity that yield an addiction cycle with three stages: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (see Figure 1). These three stages interact with each other, becoming more intense and ultimately leading to the pathological state characterizing addiction. Therefore, drug addiction represents a dynamic break of homeostatic brain regulatory mechanisms that regulate the emotional state of the organism (Koob & Le Moal 1997, 2008).

Changes in the motivation for drug consumption are a key component of addiction, including sensitization and increased incentive salience. Robinson and Berridge (1993) proposed the incentive-sensitization theory, in which addictive behavior is due to progressive and persistent changes in brain cells and circuits (neuroadaptations) that normally regulate the attribution of incentive salience to stimuli. According to this theory (Robinson & Berridge, 2008), many potentially addictive drugs initially produce feelings of pleasure (euphoria), encouraging users to take drugs again. Repeated drug use sensitizes neural systems that mediate the motivational process of incentive salience (wanting), but not neural systems that mediate the pleasurable effects of drugs (liking). In other words, the

progressive increase in drug “wanting” that characterizes addiction is not accompanied by an increase in the pleasure derived from drug taking. Sensitized wanting may compel drug pursuit whether or not an addict has any withdrawal symptoms. In addition, because incentive salience is distinct from pleasure or liking processes, sensitization gives impulsive drug wanting an enduring life of its own (Robinson & Berridge, 2004). This sensitization can be extended to drug-related stimuli, even after years of abstinence.

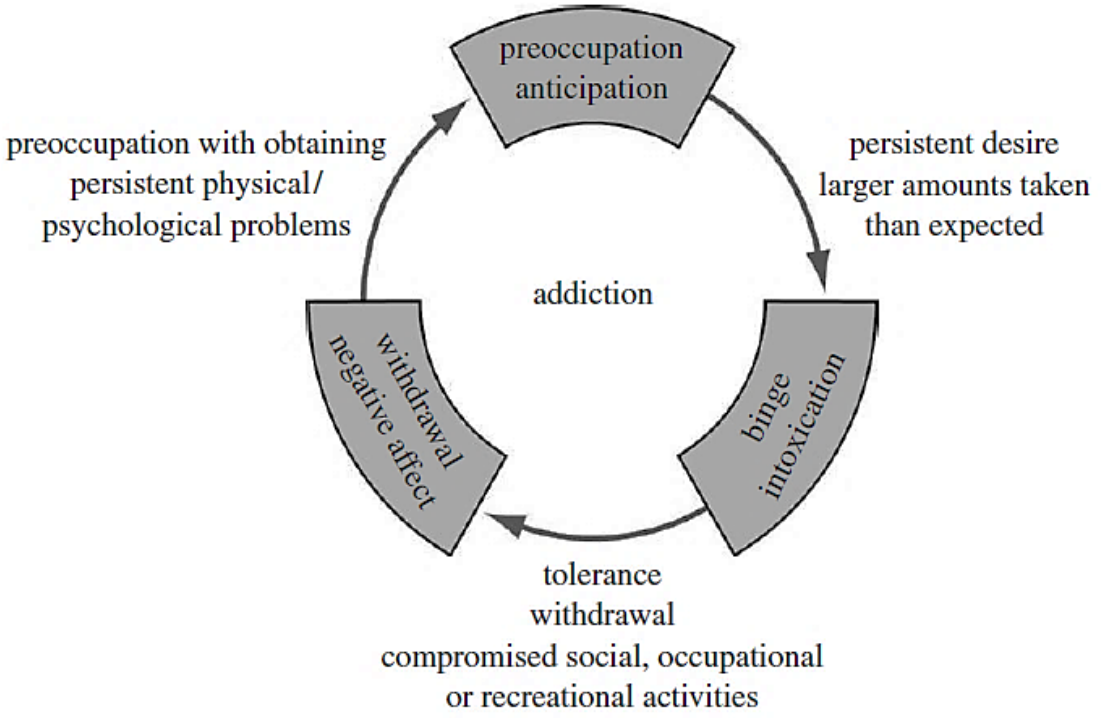

Figure 1. Diagram describing the three-stage addiction cycle with the different criteria for substance dependence incorporated from the Diagnostic and Statistical manual of mental disorders, 4th Ed (from Koob & Le Moal, 2008).

Drugs have also been shown to have habit-forming properties based on dynamic actions on the same brain reward systems that explain the control of

behavior by food reward or sexual gratification (Wise, 1996). Once established, the addictive habit becomes independent of the drug's rewarding outcome and thus difficult to extinguish despite the negative consequences derived from repeated drug intake (DiClemente, 2017).

Addictive behavior is also dependent on other forms of learning (see Vila, 2015). First, drug-related behaviors are conceptualized as instrumental responses highly dependent on the drug's reinforcing (pleasant) consequences (Skinner, 1938). In this regard, the positive reinforcement derived from drug consumption has been proposed as a key factor in the onset of drug use, even overcoming the aversive effects that some drugs induce (e.g., bitter taste, nausea, etc.). As the disorder progresses, the reinforcing properties of the drug decrease and a negative affect (withdrawal) emerges, so that drug consumption is mainly maintained on the basis of reinforcement derived from relief from the withdrawal/craving removal.

Second, certain stimuli/cues (environmental, social) have powerful effects on addicted persons, because of their association with drug effects through classical conditioning. These cues can result in conditioned tolerance, craving, and relapse, thus promoting drug-seeking behaviors (Vila, 2015). For example, Wikler (1965) found that some opioid consumers showed negative emotional responses when remembering stimuli associated with heroin consumption, even months after detoxification. Wikler called these emotional responses as "conditioned withdrawal."

Classical and operant conditioning describe how we learn from direct experience. However, humans also learn by observing others (Bandura, 1977). According to the social learning theory of addiction, addictive behavior is

mediated by cognitions or beliefs about the effects of consumption (Niaura, 2000). Such as cognitions, also referred to as expectations, are based on both personal and interpersonal experiences with the drug, as well as on the social environment and the influence of the closest models of behavior for the individual. Several factors are proposed to underlie drug consumption: the function/purpose by which the substance is consumed, the meaning the individual assigns to drug intake (e.g., cope with negative emotions; Cooper, Russell, & George, 1988), and the expected efficacy of alternative behaviors. From this perspective, drug abuse is considered a strategy to cope with existential reality, including the frustration triggered by unachievable goals (Peele, 1996).

Personality has also been considered a crucial vulnerability factor in the onset and development of an SUD. Although the notion of ‘‘addictive personality’’ has been largely rejected (Kerr, 1996; Nathan, 1988), evidence points to individual differences in several personality traits consistently associated with addiction. These traits include disinhibition, impulsivity, and novelty seeking, among others (Conway et al., 2003). In some cases, individuals can develop a psychiatric disorder and drug addiction at the same time. This co-occurrence of the two disorders is known as dual pathology (Torrens, 2008; Volkow, 2007). According to this view, the Spanish Society of Dual Pathology reports that 70% of individuals diagnosed with an SUD are also diagnosed with another psychiatric condition. Similarly, many patients primarily diagnosed with a mental disorder have a history of substance use (60% for bipolar, 30% for depression, and 80% for anxiety disorders; see Roncero et al., 2016). In fact, mental illness has been frequently reported as a risk factor for drug addiction. For example, Blume, Schmaling and Marlatt (2000) found higher opioid consumption in depressed

patients than in bipolar patients in the manic phase or schizophrenics. Similarly, individuals suffering from schizophrenia showed an improvement of their symptoms after using or being administered nicotine (Hahn et al., 2013). Generalized anxiety disorder and posttraumatic stress disorder are also associated with alcohol and cannabis abuse (Robinson, Sareen, Cox, & Bolton, 2009). This relationship between mental disorders and SUD has been frequently considered as a form of self-medication, suggesting that some mental patients use drugs to mitigate aversive emotional states and other distressing symptoms derived from their clinical conditions (Khantzian, 1985; Torres & Papini, 2016). As will be discussed below, the genesis of substance dependence could lie on the reinforcing relief from psychiatric symptoms provided by substance use (Darke, 2013).

Biological basis of addiction

In the past two decades, research has increasingly supported the view that addiction is a disease of the brain. Neuroscience research in this area not only offers new opportunities for the prevention and treatment of substance and other behavioral addictions, but may also improve our understanding of the biological processes involved in voluntary behavioral control (Volkow, Koob, & McLellan, 2016; however, see Lewis, 2017).

Current evidence shows that most drugs of abuse exert their initial reinforcing effects by activating reward circuits in the brain. Olds and Milner (1954) first reported evidence for the existence of a reward center in the brain. In their seminal paper, they described their finding that rats would continually press a lever in return for receiving a brief pulse of electrical stimulation in brain regions connected by the medial forebrain bundle. Since electrical stimulation in this

pathway was all that was needed to reinforce the lever-pressing behavior, and since the rate of reinforcement was comparable to that produced by natural rewards, the authors concluded that such stimulation was somehow rewarding by itself. Olds and Milner's experiments provided the initial evidence for a brain region that processes reward, thus bridging neurobiology of emotion and motivation with behavior (Koob, 2015).

The brain structures, neuronal pathways, and relevant neurotransmitters involved in the experience of reward and reinforcement have been further refined over the last decades. The mesolimbic pathway has been identified as the key component in reward processing. This pathway originates in dopaminergic (DA) cell bodies of the ventral tegmental area (VTA), a DA-rich nucleus located in the ventral portion of the midbrain. These DA axons primarily project in the nucleus accumbens (NAc), part of the ventral striatum, but also extend into the amygdala, bed nucleus of stria terminalis (BNST), lateral septal area, lateral hypothalamus, and prefrontal cortex (Koob, 1992). The NAc is divided into two regions: the NAc core and the NAc shell, the later being usually included in the extended amygdala. The extended amygdala includes the BNST, the central nucleus of the amygdala, the sublenticular substantia innominata, and the NAc shell, these structures sharing morphology, immunohistochemistry, and connectivity. The extended amygdala receives afferent connections from limbic cortex, hippocampus, basolateral amygdala (BLA), midbrain, and lateral hypothalamus, and send efferent connections to the lenticular nucleus of the ventral pallidum, the VTA, and the lateral hypothalamus (see Figure 2; Alheid & Heimer, 1988; Heimer & Alheid, 1991; Koob, 2008, 2009).

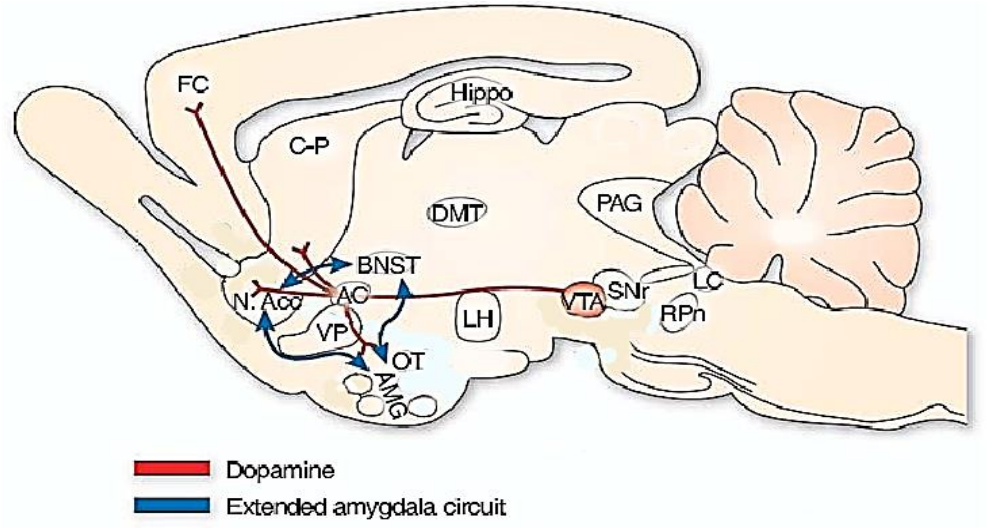

Figure 2. Sagittal section through a representative rodent brain illustrating the dopamine and extended amygdala pathways. AC: Anterior Commissure; DTM: Dorsomedial thalamus; AMG: Amygdala; BNST: Bed nucleus stria terminalis; C-P: Caudate-putamen; FC: Frontal cortex; Hippo: Hipocampus; LC: Locus coeruleus; LH: Lateral hypothalamus; N.Acc: Nucleus accumbens; PAG: Periaqueductal gray; RPn: Reticular pontine nucleus; SNr: Substantia nigra pars reticulate (Koob & Volkow, 2010).

Additional striatal regions have been widely recognized as critical for reward processing and addiction (Everitt & Robbins, 2013). The striatum can be divided into two main regions: the dorsal striatum (divided in dorsomedial and the dorsolateral subareas), and the ventral striatum or NAc. The striatum receives inputs from brainstem sources such as the VTA and the substantia nigra, as well as from the hippocampus (HC), amygdala (AMG), and raphe nuclei, among others. The outputs project mainly to brainstem nuclei and to the thalamus, which, in turn, projects back mainly to the prefrontal cortex (Lanciego, Luquin, & Obeso,

2012). Extensive evidence suggests the involvement of the striatum in the acute and chronic effects of abuse drugs, as well as in the transition from initial drug use to compulsive drug seeking and taking (Yager, García, Wunsch, & Ferguson, 2015). Thus, whereas NAc shell plays a key role in mediating the acute reinforcing effects of abuse drugs, the influence of drug-associated conditioned stimuli on drug-seeking behavior seems to depend mainly on the NAc core and on the dorsal striatum (Everitt & Robbins, 2013).

Other brain areas involved in reward processing are the anterior cingulate cortex, orbitofrontal cortex, medial prefrontal cortex, insular cortex, and AMG. Kalivas and Volkow (2005) suggested two main functions for the anterior cingulate cortex: (1) the robust motivation induced by stimuli predicting drugs of abuse, compared with stimuli predicting natural rewards, and (2) difficulties in the cognitive control of drug seeking behavior. The orbitofrontal cortex has been related to deficits in the inhibitory/executive control of drug seeking behavior, the processing of stimuli with negative emotional value, and the assignment of relative motivational value to stimuli predicting drug availability (Volkow & Baler, 2014). Abnormal functioning of this prefrontal region would thus underlie loss of control and compulsive drug taking (Volkow & Fowler, 2000). Goldstein and Volkow (2011) suggested that the medial prefrontal cortex is involved in a variety of symptoms characterizing SUDs: disrupted delayed gratification, enhanced stress reactivity, enhanced drug-stimuli seeking, and decreased motivation for stimuli other than the drug. Finally, a key function of the insular cortex is the representation of interoceptive states of the body (Craig, 2002). According to this view, Paulus and Stein (2006) found that individuals with a higher tendency toward anxiety showed increased insular activation when processing salient

stimuli. These results suggest a role of the insular cortex in the disrupting effects of interoceptive-cue processing in choice situations involving drug use and relapse (Cisler et al., 2013; Naqvi & Bechara, 2009, 2010; Volkow & Baler, 2014). For example, smokers with insular damage were able to stop smoking without experiencing craving or relapse, unlike smokers with damage in other brain regions (Naqvi, Rudrauf, Damasio, & Bechara, 2007). Similarly, inactivation of the granular insular cortex disrupted the reinstatement of nicotine self-administration after extinction in rats (Forget, Pushparaj, & Le Foll, 2010).

Prefrontal and insular cortex reciprocally connect with the AMG, a brain region specialized in establishing learned associations among events with motivational value (Everitt, Cardinal, Parkinson, & Robbins, 2003). This function has been related to changes in reward learning and memory, stress dysregulation, conditioned reward, emotional disorders, and in the transition from controlled drug use to compulsive patterns of drug seeking and taking (Egli, Koob, & Edwards, 2012; Koob & Le Moal, 2001; Volkow & Baler, 2014).

The brain evolved to respond to natural rewards such as food and sex, rather than drugs. However, humans discovered how to stimulate this system artificially with drugs (Kelley & Berridge, 2002). The hedonic value of both abuse drugs and natural rewards depends on the DA brain system described above, and on its interactions with other neurotransmitters, including γ-aminobutyric acid (GABA), glutamate, and opioids, among others (Gil-Verona, Pastor, De Paz, Barbosa, & Macías-Fernández; 2003; Kalivas & Volkow, 2005; Kelley & Berridge, 2002). Regardless of their different pharmacological effects, drugs of abuse increase the release of DA in the NAc shell, mimicking the phasic DA neuronal firing that leads to very fast DA increases signaling reward (Volkow & Morales,

2015). Several pieces of evidence support this claim. The interruption of DA activity (via anatomical lesions) drastically reduces self-administration of addictive drugs injected systemically. Similarly, DA antagonists interfere with self-administration of amphetamines, cocaine, and ethanol, among other drugs. By contrast, the administration of DA agonists increases the incentive value of otherwise neutral stimuli and induces drug-seeking reinstatement in response to drug-cues (Gil-Verona et al., 2003). According to this view, DA neurons significantly increase their activity in response to a conditioned stimulus that predicts an impending reward, therefore regulating expectancy and anticipatory responses (Hollerman & Schultz, 1998; Schultz, 2001). Additional studies also suggest a role of DA neurotransmission in associative memory processes involved in compulsive and persistent drug use (Everitt & Robbins, 2005; Kauer & Malenka, 2007). Overall, DA seems to participate in the modulation of the acute rewarding effects of abuse drugs, the formation of reward associations between neutral stimuli and the unconditioned effects of drugs, and the dysregulation of motivational processes involved in craving, drug seeking, loss of control, and over-consumption (Robinson & Berridge, 1993; Enman, Zhang, & Unterwald, 2014; Kienast & Heinz, 2006; Koob et al., 2014; Richardson & Gratton, 1996).

Finally, the neurobiological mechanisms that participate in the three stages of the addiction cycle proposed by Koob et al. (1989) have also been identified, based on both human and nonhuman animal studies. The binge/intoxication stage involves the activation of the brain reward circuit previously described, with the DA system playing a key role in the rewarding properties of nearly all drugs of abuse. The withdrawal/negative affect stage depends on both within-system neuroadaptations (e.g., decreases in DA and serotonergic transmission in the

NAc) and between-systems adaptations, in which neurochemical systems other than those involved in the positive rewarding effects of drugs of abuse are recruited or dysregulated by chronic activation of the reward system (e.g., increased activity in the hypothalamic-pituitary-adrenal axis, the extended AMG and brain-stress systems mediated by corticotropin-releasing factor, norepinephrine, and dynorphin). Finally, the preoccupation/anticipation stage has long been hypothesized to be dependent on the prefrontal cortex, BLA, ventral subiculum, dorsal striatum, and extended AMG regulating some key elements of addiction, such as craving and relapse (Koob & Volkow, 2016).

Mechanisms of action of alcohol and benzodiazepines

This section reviews the mechanisms of actions of two psychotropic depressant of the central nervous system: alcohol and BZDs. Both promote a variety of changes in several neuronal pathways, exerting a profound neurological impact that leads to behavioral alterations, including addiction.

Alcohols are organic compounds containing a hydroxyl (-OH) group attached to a carbon atom, being usually described by the general formula $C_nH_{2n+1}OH$. Ethyl alcohol or ethanol, the psychoactive constituent of alcohol, has been used recreationally for thousands of years and is one of the largest health burdens in society today. Ethanol is toxic for most body tissues, inducing changes in the cardiovascular, digestive, nervous, and muscle-skeletal systems, among others (Most, Ferguson, & Harris, 2014).

Alcohol consumption induces a number of physiological and behavioral effects in a dose-dependent manner: anxiolysis, myorelaxation, analgesia, sedation, amnesia, hypothermia, and anesthesia. These effects are partially mediated by facilitation of GABA neurotransmission in $GABA_A$ receptors (León-

Regal et al., 2018; Nestler, Hyman, & Malenka, 2009). GABA is the main inhibitory neurotransmitter in the brain and regulates the activity of many neurons. $GABA_A$ is an ionotropic receptor which contains at least five different binding sites (Figure 3). The primary binding site is for GABA. A second site binds with a class of anxiolytic drugs, the BZDs. A third binding site is for barbiturates. The fourth binding site is for various steroids, including those that produce anesthesia. Picrotoxin, a venomous substance found in a shrub, binds to the fifth site. Alcohol is coupled with one of these sites, presumably the BZD or the barbiturate binding site (Carlson & Birkett, 2017; Mendel-Matus et al., 2011; Stahl, 2013).

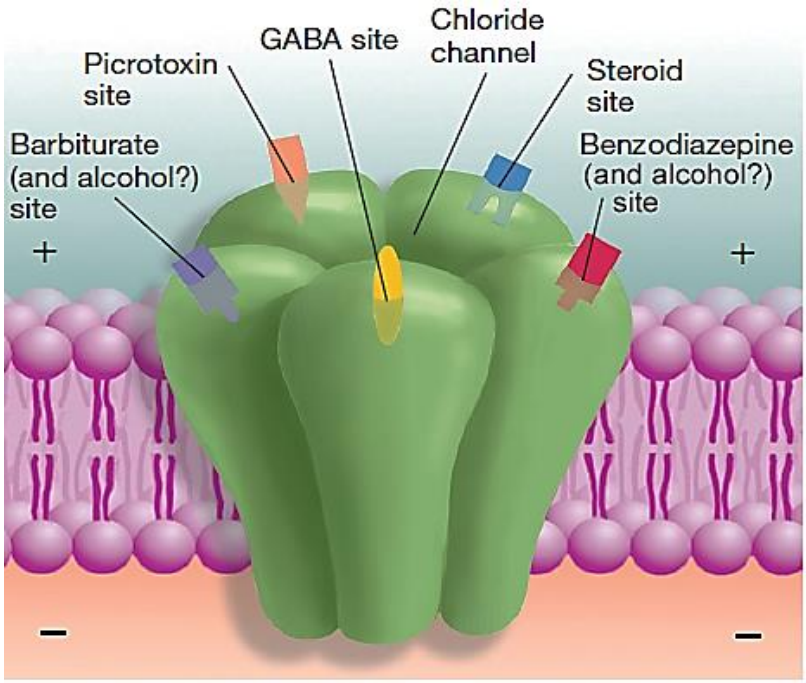

Figure 3. Graphic representation of $GABA_A$ receptor and the binding sites for GABA, steroids, picrotoxin, BZDs, and barbiturates (from Carlson & Birkett, 2017).

Alcohol facilitates the action of GABA on $GABA_A$ receptors, increasing chloride (Cl-) influx and facilitating the Cl- channel opening, which leads to inhibitory postsynaptic potentials (Carlson & Birkett, 2017; Grobin, Matthews,

Devaud, & Morrow, 1998). The facilitating action of ethanol on inhibitory GABAergic neurotransmission could explain why ethanol intoxication at high doses (>250 mg/dL) is life-threatening (León-Regal et al., 2018).

In addition, ethanol promotes DA transmission in the mesocorticolimbic pathway. This effect depends on the presence of mu (μ) opioid receptors whose activation inhibits GABAergic neurons located in the VTA. Alcohol increases endorphins release, which in turn increases DA release in the NAc via GABA inhibition. This would explain the pleasant effects that strengthen alcohol consumption (Carlson & Birkett, 2017; León-Regal et al., 2018).

Ethanol also reduces the activity of glutamate, an excitatory neurotransmitter in the central nervous system. This effect seems to be mostly mediated by N-methyl-D-aspartate (NMDA) receptors (Most et al., 2014). The NMDA receptor is an ionotropic receptor with a number of binding sites, including glutamate/NMDA, glycine, phencyclidine/ketamine and magnesium ion binding sites, among others (Carlson & Birkett, 2017; Nestler et al., 2009). The co-activation of NMDA receptors by glutamate and glycine promotes the entry of calcium ions into the neuron, which activates several intraneuronal signaling processes involved in nerve transmission and neuroplasticity (Figure 4; see Artigas, 2011).

Ethanol has a selective effect on the NMDA receptor, decreasing calcium influx and inhibiting calcium uptake (Hodge & Cox, 1998; Hoffman et al., 1990). As a consequence, the drug decreases NMDA-mediated excitatory postsynaptic potentials and inhibits NMDA-dependent long-term potentiation (Xiang, Kim, Gelernter, Park, & Zhang, 2015), thus interfering with the functions mediated by glutamate transmission, including learning and memory (Nestler et al., 2009). The

role played by glutamate in the reinforcement properties of ethanol is not clear. Stimulation of NMDA receptors exerts an inhibitory influence on reinforcement, whereas substances that block these receptors, such as phencyclidine and MK-801, are self-administered by both primates and rodents. Part of the reinforcing potential of alcohol could thus be due to its antagonistic actions on NMDA receptors (see Nestler et al., 2009).

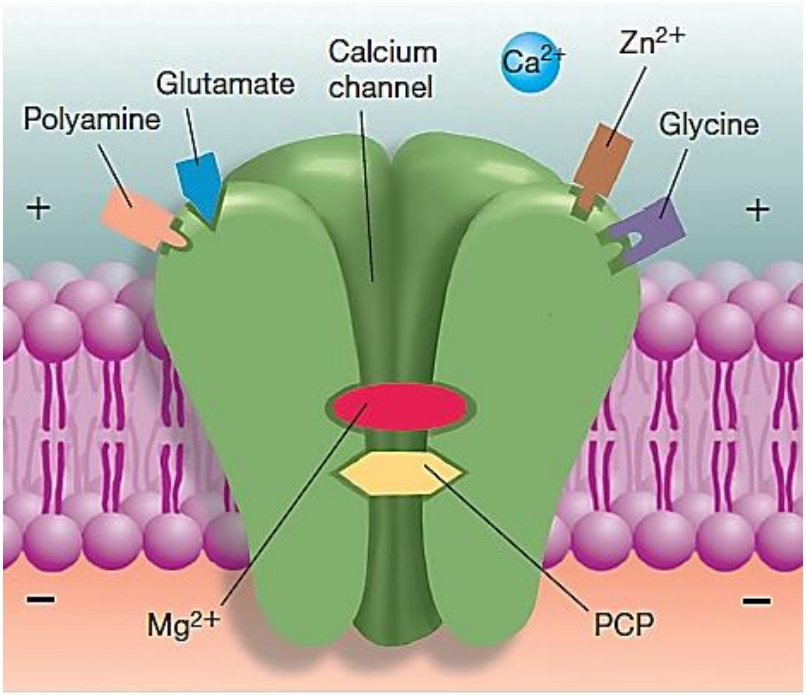

Figure 4. Graphic representation of glutamate NMDA receptor and the different binding sites (from Carlson & Birkett, 2017).

BZDs share part of their mechanism of action with alcohol. These prescription drugs are widely used to treat anxiety and insomnia, induce muscle relaxation, reduce epileptic seizures, impair memory, and are often abused after chronic use (Tan et al., 2010). BZDs are positive allosteric modulators of GABA actions in the AMG and prefrontal cortex, acting only in the presence of GABA. This effect is based on the increase in the frequency of opening of Cl- channels located in $GABA_A$ receptors (Lalive, Rudolph, Lüscher, & Tan, 2011; Stahl, 2013;

Tan, Rudolph, & Lüscher, 2011). The facilitating actions of BZDs on GABA transmission are only observed in $GABA_A$ receptors containing α-2 and/or α-3 subunits (Stahl, 2013).

BZDs have pharmacological and cellular effects in the mesolimbic DA system similar to those previously described for alcohol. Tan and colleagues (2010) demonstrated that GABAergic neurons are more strongly hyperpolarized in the presence of BZDs. BZDs reduce the inhibitory action of GABA on DA neurons, increase DA activity in the VTA, and promote DA release in the NAc—these effects underlie the reinforcing properties of BZDs (Tan et al., 2010).

In addition to the reinforcing properties of alcohol and BZDs derived from their pleasurable effects, considerable evidence points to a positive correlation between negative events and the consumption of alcohol and BZDs (Yap & Miczek, 2008). Indeed, both drugs can alleviate inner tensions that result from exposure to stressors, as was postulated by "tension-reduction theory" (Conger, 1951; Spanagel, Noori, & Heilig, 2014). Therefore, analyzing how aversive events and stress interact with the neural circuits underlying drug abuse is an important step to understand addiction and to develop therapeutic approaches (Sinha, 2008; Ungless, Argilli, & Bonci, 2010).

Self-medication hypothesis of addiction

Human beings have used stimulants, hypnotics, and other psychoactive substances since time immemorial for a variety of purposes, including pleasure, therapy, and mystical ecstasy (Escohotado, 2006; Torres & Escarabajal, 2005). Similar behaviors are observed in animals, a phenomenon studied by "zoopharmacognosy" (Huffman, 1997). Thus, animals are able to select and use specific plants with medicinal properties for the treatment and prevention of

disease in order to maintain homeostasis (Huffman, 2001; Singer, Mace, & Bernays, 2009; Villalba, Miller, Ungar, Landau, & Glendinning, 2014; Villalba & Provenza, 2007). Such behavior, known as self-medication (SM), includes leaf swallowing, ingestion, chewing, fur rubbing, etc. (Clayton & Wolfe, 1993). Janzen (1978) first showed that the intake of certain compounds obtained from plants could help animals to combat parasites. Similarly, animals consume bark and wood from some trees species as laxatives, to alleviate toothache and stomachache, and to control symptoms related with parasite infections (Huffman, 1997). Sick chimpanzees consume some plants' secondary compounds to control parasite infections and improve health (Clayton & Wolfe, 1993; Huffman, 1997, 2001, 2003; Masi et al., 2012; Villalba & Provenza, 2007). Parasitized lambs consume more tannin-containing supplements than nonparasitized lambs (Lisonbee, Villalba, Provenza, & Hall, 2009). Parasitized caterpillars improved their health when they consume a diet rich in pyrrolizidine alkaloids (Singer et al., 2009). SM can also be experimentally induced in the laboratory. Some animal models of peripheral pain showed that rats self-administered a cannabinoid agonist with analgesic properties (Gutiérrez, Crystal, Zvonok, Makriyannis, & Hohmann, 2011). Similarly, sheep exposed to a gastrointestinal disease selected a diet with the right medication (Villalba, Provenza, & Shaw, 2006).

All these examples suggest the occurrence of SM in a variety of animal species and conditions. However, many of the observations are anecdotal and equivocal (Clayton & Wolfe, 1993; Lisonbee et al., 2009), some lacking control groups to compare the biological effects of the plant (Lozano, 1998), and the mechanisms by which animals learn about the benefits of the plant are unknown (Villalba et al., 2014). Some criteria must be fulfilled to consider a behavior a SM

response: (1) the plant is not eaten regularly, despite its widespread availability; (2) the behavior must be initiated by parasitic infection, illness or discomfort; (3) the consumption of unusual food is temporary; once the animal recovers, there is a return to the regular diet (de Roode, Lefèvre, & Hunter, 2013; Fruth et al., 2014; Huffman, 2010; Lozano, 1998; Singer et al., 2009).

What does human research tell us about SM? Is SM related to addictive behavior in our species? Humans frequently use psychoactive substances for the relief of physical and emotional distress (Torres & Papini, 2016). The SM hypothesis of substance use (Khantzian, 1983) was originally focused on the frequent comorbidity observed between SUDs and mental illness. According to this theory, drug use begins as a partially successful attempt to assuage painful feelings in individuals predisposed by biological or psychological vulnerabilities (Khantzian, 1997, 2003). Thus, the SM hypothesis suggests that (1) individuals with self-regulation deficits use substances in an attempt to manage negative affective states; and (2) individuals select specific substances based on their ability to elicit the desired affective state (Darke, 2013; Gil-Rivas & McWhorter, 2013; Khantzian, 1997). Khantzian (1997) proposed three interacting factors as important to make a particular drug especially desirable to an individual: the main effect of the drug; the individual's personality; and his or her inner states of psychological suffering and disharmony.

The majority of studies aiming at testing the SM hypothesis have been conducted with clinical samples of patients diagnosed with SUDs and/or psychiatric disorders (Gil-Rivas & McWhorter, 2013), as well as with healthy subjects exposed to a variety of negative events. This research indicates that individuals exposed to chronic stress exhibit higher propensity for drug addiction

(Briand & Blendy, 2010; Yap & Miczek, 2008). Similarly, some findings support the hypothesis that SM of negative symptoms is involved in the frequent association between nicotine abuse and schizophrenia (Khantzian, 2003). There is also evidence that individuals with posttraumatic stress disorder (PTSD) are at risk of developing substance dependence after experiencing the psychotropic effects of drugs (such as alcohol) that provide relief from the negative affective states associated with trauma (Khantzian, 1997). According to this view, the rates of abuse of alcohol and other substances in war combatants who suffer from PTSD are significantly higher than those found in veterans who have not developed such disorder (Ouimette, Coolhart, Funderburk, Wade, & Brown, 2007). In the same vein, people who have been victims of physical or sexual abuse, early abandonment, natural catastrophes, death of loved ones, family conflicts, poverty, etc., show higher consumption rates of alcohol, BZDs, and illicit drugs, and more frequent relapses induced by acute stress (Duffing, Greiner, Mathias, & Dougherty, 2014; Hassanbeigi, Askari, Hassanbeigi, & Pourmovahed, 2013; Konopka, Pełka-Wysiecka, Grzywacz, & Samochowiec, 2013; Spanagel et al., 2014). SM is also common among patients suffering from anxiety disorders, social phobia, and depression (Blume et al., 2000; Menary, Kushner, Maurer, & Thuras, 2011; Robinsonet al., 2009). Therefore, the evidence of a positive correlation between emotional distress and consumption of psychoactive substances provides support for the SM hypothesis of addiction (Torres & Papini, 2016).

Despite this evidence, some clinical and experimental data cannot be explained from a SM perspective. For example, Hall and Queener (2007) measured a number of affective states and did not find any evidence of a

relationship between high levels of emotional distress and drug intake. Similarly, Castaneda (1994) assessed the expectations about the effects of drug use on psychiatric and cognitive symptoms in individuals with personality disorders. He found that patients with similar symptomatology used different drugs, experienced different effects, and developed different expectations about the consequences of drug use. In fact, the expectations of symptom aggravation or improvement varied according to the drug of choice, rather than to any particular symptom or personality trait (Castaneda, 1994). In addition, DuPont and Gold (2007) argued that individuals use drugs in nonmedical contexts to seek reward, rather than for the treatment of negative states. According to their view, confusing drug use behavior with self-treatment involves misunderstanding addictive behavior, causing unnecessary suffering, and delays and errors in the prevention and treatment of both comorbid conditions. Animal models may help us resolve this impasse by identifying factors causally related, rather than just correlated, with addictive behavior.

A number of aversive stimuli lead to increased voluntary alcohol drinking in rats, including uncontrollable foot shocks, restraint, and social stressors (Spanagel et al., 2014; Yap & Miczek, 2008). For example, Manjoch et al. (2016) found that chronic exposure to the smell of a predator increased ethanol consumption in rats. Anisman and Waller (1974) showed that the administration of inescapable foot shocks in rats increased their preference for 10% ethanol compared to a control group without shocks. This increased alcohol preference was not observed when animals could control the presentation of the aversive stimulus. Similarly, rats exposed to foot shocks exhibited slower extinction of a morphine place preference and increased tolerance to the hyperthermic effects

of morphine compared to nonshocked controls (Barsy, Mikics, Barsvári, & Haller, 2011).

Restraint stress also increases alcohol consumption under a variety of conditions. For example, Lynch et al. (1999) found increased ethanol (5%) preference in animals exposed to restraint stress in repeated cycles. Nash and Maickel (1985) showed that animals exposed to repeated immobilization increased their ethanol consumption in a choice situation.

The forced swimming test is an aversive experience that also affects alcohol intake, although null or opposite results have also been reported (see Becker, Lopez, & Doremus-Fitzwater, 2011). One study found that this experience led to increased ethanol intake (Anderson, Lopez, & Becker, 2016). Increased ethanol consumption reduced the duration of immobility (Jain, Kannamwar, & Verma, 2017).

Social stress similarly influences the voluntary intake of abuse drugs in rodents and primates, and it has been widely used based on its ecological relevance (Caldwell & Riccio, 2010; Roske, Baeger, Frenzel, & Oehme, 1994; Wolffgramm & Heyne, 1988). Wolffgramm (1990) found that animals normally housed in groups and then isolated for a period of 24 h consumed more ethanol (20%) during the isolation period than control groups (including individually housed rats, group housed rats and rats kept in contact cages). Social defeat also increases voluntary alcohol consumption in adult rats, especially under conditions of intermittent access (Blanchard, Flores, Magee, Weiss, & Blanchard, 1992; Blanchard, McKittrick, & Blanchard, 2001; Newman et al., 2018; Norman et al., 2015). In the same way, several authors found that isolation also increased

ethanol consumption (Lynch, Kushner, Rawleigh, Fiszdon, & Carroll, 1999; Nash & Maickel, 1985; Parker & Radow, 1974).

Whereas the relationship between SM and drug-taking behavior seems consistent, some results show the complexity of this relationship. The type of stressful experience (e.g., duration, intensity, quality), biological factors (e.g., strain, age, sex), and procedural variables (e.g., dose, simultaneous vs. sequential drug access, free-choice vs. operant self-administration, unlimited vs. time-restricted access) all modulate the relationship between stress/negative affect and drug use (Becker et al., 2011; Spanagel et al., 2014). Some relevant limitations must also be highlighted. First, some behavioral tasks do not report the animal's response to the stressor. Thus, there is no evidence of an aversive state before or during substance consumption and its alleviation following drug intake. Second, most studies use recreational substances such as alcohol, ignoring prescription drugs with addictive potential such as BZDs. Finally, some of these models have little ecological validity, being based on artificial stimuli and/or on responses that are not part of a rodent's behavioral repertoire (Torres & Papini, 2016). Animal models based on reward loss can overcome some of these limitations, allowing for tests of the relationship between negative states and substance use.

Reward loss and emotional self-medication

The experimental study of reward loss (including reward omission and reward devaluation) has a long tradition in the field of psychology of learning. In addition to be dependent on associative and cognitive processes, these situations may also have profound emotional and affective consequences (Huston, Souza, Komorowski, Schulz, & Topic, 2013; Papini, Fuchs, & Torres, 2015). Frustration

(also referred to as disappointment or psychological pain) is defined as a negative emotional state triggered by the violation of a reward expectancy; it occurs when the obtained incentive is of lesser value than expected on the basis of prior experience under similar conditions (Amsel, 1992a; Flaherty, 1996; Papini, 2006; Papini et al., 2015). A number of experimental preparations have been used to induce frustration in the laboratory, including successive negative contrast (SNC), appetitive extinction, and a variety of behavioral phenomena involving partial reinforcement (PR), among others (see Papini et al., 2015).

In SNC situations, a consummatory (cSNC) or an instrumental response (iSNC) is reinforced with a large reward for a number of sessions during the "preshift" phase. After this training, the magnitude or quality of the reward is surprisingly devaluated during the "postshift" phase. This unexpected reward downshift causes a behavioral decrease (Ortega, Solano, Torres, & Papini, 2017). The SNC effect thus consists of a temporary reduction in responding to a small reward in animals previously exposed to a larger reward, in comparison to the responding observed in a control group always exposed to the small reward (Figure 5; Mustaca, Bentosela, & Papini, 2000).

Appetitive extinction involves the omission, rather than devaluation, of a previously presented reward. Animals shifted from reinforcement to nonreinforcement exhibit a deterioration of the previously learned response (Figure 6; Mackintosh, 1974; Papini et al., 2015). Such response impairment has also been related with frustration (e.g., Gómez, de la Torre et al., 2009) and negative affect (Huston et al., 2013).

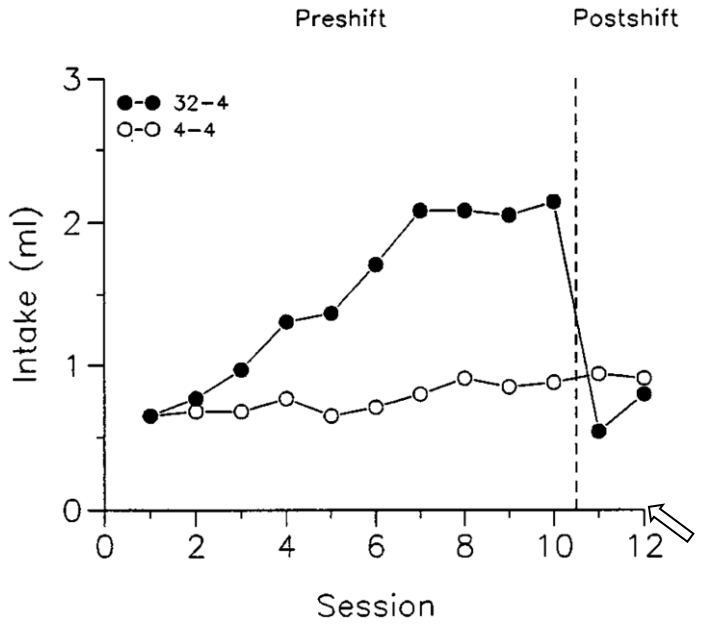

Figure 5. Example of cSNC in mice expressed in terms of daily average intake of sucrose solutions in Groups 32-4 and 4-4 during the preshift (sessions 1–10) and postshift periods (sessions 11 and 12; from Mustaca, Bentosela, & Papini, 2000).

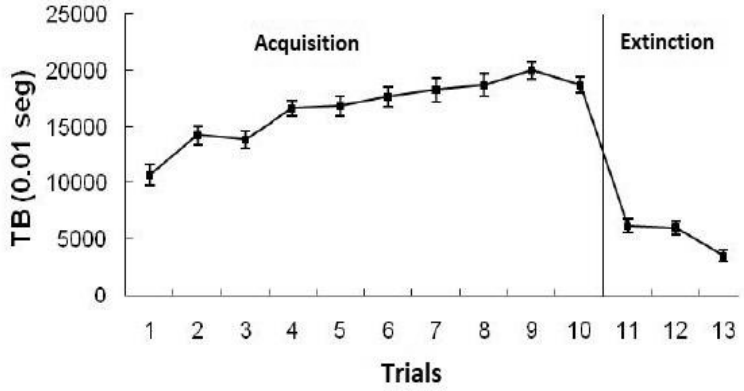

Figure 6. Example of an extinction response. Rats received 32% sucrose during acquisition trials and then nothing during extinction trials. TB: Drinking Time (from Kamenetzky, Cuenya, & Mustaca, 2011).

Finally, PR is an experimental procedure in which animals are partially reinforced in a random way after performing a consummatory or an instrumental response (reinforcement is usually delivered in 50% of the trials). This repeated/chronic frustration experience has been shown to immunize animals to the effects of subsequent reward loss (Amsel, 1992). Thus, the partial reinforcement extinction effect (PREE) consists of increased resistance to extinction after PR training compared to continuous reinforcement (CR) training (Gómez et al., 2008; Papini, 2006). Similar results are obtained when animals are partially reinforced and then exposed to reward devaluation in a SNC procedure (the partial reinforcement contrast effect, PRCE; Cuenya et al., 2012, Pellegrini et al., 2004, Figure 7).

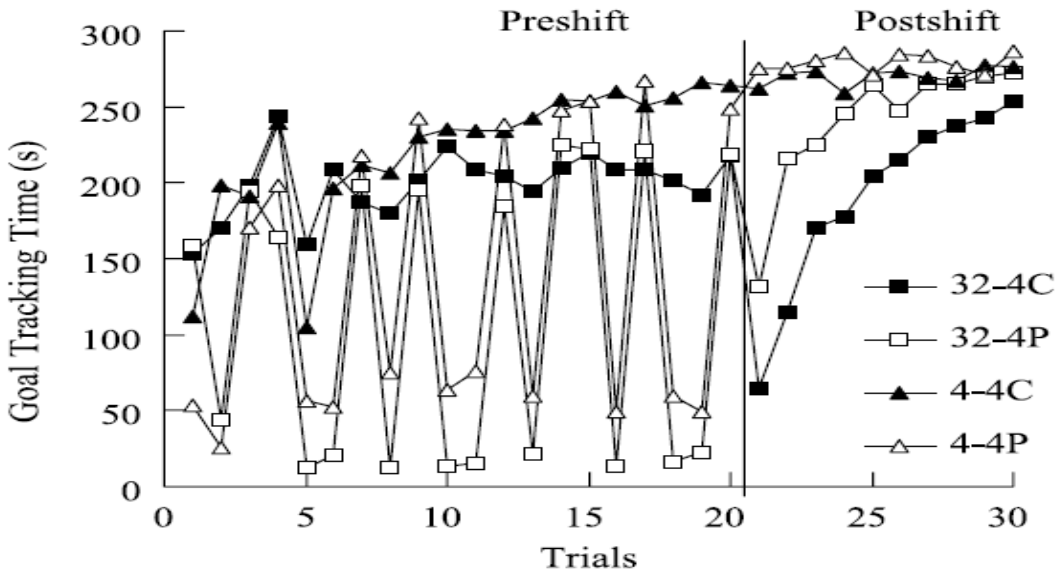

Figure 7. Example of PRCE. Mean consummatory performance, measured in terms of goal tracking time, as a function of reinforcer magnitude (32% vs. 4% sucrose) and schedule of reinforcement (P, partial reinforcement, C, continuous reinforcement) in preshift trials (from Pellegrini, Muzio, Mustaca, & Papini, 2004).

Several theories have been proposed to explain the behavioral impairment that follows reward loss. As reviewed below, most of them involve negative emotion and frustration as a critical component of the response to such events (Dantzer, 1987).

Amsel (1958, 1962, 1992a) developed a theory of frustration that includes classical and instrumental conditioning, as well as emotional and motivational variables. According to Amsel (1992a), when animals receive a smaller than expected reward, an internal state of primary frustration emerges. Primary frustration has behavioral and physiological consequences, and acts as an aversive reinforcer that can be eventually associated with environmental cues (Amsel, 1992b, 1994; Papini, 2006; Papini, Wood, Daniel, & Norris, 2006; Wood, Daniel, & Papini, 2005). The association between concurrent cues and primary frustration promotes anticipatory/secondary frustration, inducing behavioral inhibition and avoidance responses that would compete with the approach response derived from the co-existent reward expectancy. Such approach-avoidance conflict would underlie the behavioral impairment that characterizes negative contrast and extinction, among other phenomena (Amsel, 1994; Papini, 2006). Both, primary and secondary frustration, have been more recently referred to as psychological pain (Papini et al., 2006, 2015).

Gray (1982) proposed a “fear = frustration” hypothesis based on the assumption that the omission of appetitive rewards induces negative emotional responses similar to those associated with the presentation or anticipation of aversive stimuli (fear, anxiety). This hypothesis is mostly based on the observed anti-frustration effects of drugs that also alleviate anxiety, including BZDs,

barbiturates, and alcohol (Flaherty, 1996; Gray, 1987). From this perspective, signals for both punishment and nonreward would activate a brain circuit (the behavioral inhibition system) responsible for behavioral inhibition, increased arousal and vigilance, and narrowed attention (Gray, 1982, 1987; Gray & McNaughton, 2000).

Flaherty (1996) proposed a multistage theory of cSNC in which a perceptual/cognitive stage would precede the emotional reaction to reward reduction. The former would include: (a) detection that the new reward is different from the memory of the preshift reward; (b) hedonic evaluation of the new reward; and (c) search for the "missing" reward if the new reward is of lesser value (Flaherty, 1996). As a consequence of this conflict situation, a negative emotional reaction of disappointment would emerge, impending ongoing consummatory behavior.

Finally, Papini (2003) argued that situations involving reward loss promote behavioral changes based on two learning processes, and the consolidation of the corresponding memories. First, interaction with the new incentive conditions promotes the acquisition of a cognitive memory about the properties of the new reward. This was called allocentric memory because it contains information about the new reward—an external event. Second, the downshift in incentive conditions may promote, if significant enough, the acquisition of an emotional memory encoding the organism’s reaction to the loss. This was called egocentric memory since it contains information about the organism’s emotional state—an internal event (Papini et al., 2015). This egocentric mechanism is sensitive to anxiolytic treatment and to lesions in some limbic structures that regulate emotion (Papini, 2006, 2009).

As reviewed above, most of theories proposed to explain behavioral phenomena observed under reward loss conditions involve some form of negative emotion (frustration, psychological pain, anxiety, disappointment, emotional egocentric memory). Evidence for this assumption comes from behavioral, hormonal, neuroanatomical, psychogenetic, and pharmacological studies (Gómez et al., 2008; Flaherty, 1996; Papini & Dudley, 1997; Papini et al., 2015; Papini & Torres, 2017; Torres & Papini, 2017; Torres & Sabariego, 2014).

First, animals show similar responses when they are exposed to stimuli paired to either surprising nonreward or innate fear events, including escape (Daly, 1974), aggression (Azrin, Hutchinson, & Hake, 1966), potentiated startle reflex (Wagner, 1963), jumping (Adelman & Maatsch, 1956), distress vocalizations (Amsel, 1992), and odor emissions (Ludvigson, 1999), among others. Additional behavioral evidence that evokes a connection between reward loss and negative emotion is the overlap between incentive relativity and physical pain (e.g., Jiménez-García et al., 2016), leading to the conceptualization of frustration as psychological pain (Papini et al., 2015).

Exposure to incentive downshift also activates the hypothalamic-pituitary-adrenal (HPA) axis, increasing plasma levels of stress hormones, such as adrenocorticotropic hormone (ACTH) and corticosterone (Kawasaki & Iwasaki, 1997; Mitchell & Flaherty, 1998; Pecoraro, de Jong, & Dallman, 2009; Romero, Levine & Sapolsky, 1995).

Neurobiological studies indicate that emotions triggered by the presentation of aversive stimuli or the withdrawal of appetitive reinforcers depends on similar brain areas, including AMG (Henke, 1972; Glueck, Dennis, Perrotti, Torres, & Papini, 2015; Kawasaki, Glueck, Annicchiarico, & Papini, 2015;

Kawasaki, Annicchiarico, Glueck, Morón, & Papini, 2017; Liao & Chuang, 2003), hippocampus (Flaherty, Coppotelli, Tsu, & Otto, 1998), septum (Flaherty, Powell, & Hamilton, 1979), anterior cingulate (Ortega, Uhelski, Fuchs, & Papini, 2011), orbitofrontal cortex (Ortega, Glueck, Uhelski, Fuchs, & Papini, 2013; Panayi & Killcross, 2014), medial prefrontal cortex (Pecoraro & Dallman, 2005; Pecoraro, de Jong, Ginsberg, & Dallman, 2008; Glueck et al., 2015), insular cortex (Lin, Roman, & Reilly, 2009), and lateral habenula (LHb; Friedman et al., 2010, 2011), among other brain regions.

The connection between reward loss and negative emotion has been additionally supported by the use of strains of rats selectively bred on the basis of their differences in a behavioral trait known as emotional reactivity, fearfulness, or anxiety (see Torres & Sabariego, 2014). The most extensive effort to explore this topic has been conducted in the inbred Roman high- and low-avoidance (RHA-I, RLA-I) strains of rats, selectively bred for divergence (rapid vs. poor, respectively) in a two-way avoidance learning task (Driscoll, Fernández-Teruel, Corda, Giorgi, & Steimer, 2009). Roman strain differences in emotional reactivity could underlie differences found in a variety of reward loss situations, including cSNC (Gómez, Escarabajal et al., 2009), iSNC (Rosas et al., 2007; Torres et al., 2005), appetitive extinction (Gómez, de la Torre et al., 2009), the PREE (Gómez et al., 2008), and the PRCE (Cuenya et al., 2012).

Finally, an additional source of evidence is provided by pharmacological studies showing the effects of anti-anxiety drugs (BZDs, barbiturates and alcohol; Flaherty, 1996), opioid analgesics (Papini et al., 2006; Papini, 2009), and cannabinoids (Genn, Tucci, Parikh, & File, 2004; Harloe, Thorpe, & Lichtman, 2008) on the behavioral reactions to reward loss. These results have been

explained based on the attenuating actions of these drugs on frustration and psychological pain (Papini et al., 2015). In addition to their ability to diminish aversive emotional states, these drugs have been shown to exhibit abuse potential, being frequently used for recreational purposes (León-Regal et al., 2018; Rosas-Gutiérrez et al., 2013; Tan et al., 2010). According to the SM hypothesis, anxiolytic- and alcohol-related SUDs could depend on the reinforcement derived from the relief of negative emotions (Blume et al., 2000), including those induced by reward loss (Torres & Papini, 2016). However, evidence of a relationship between reward loss and anti-anxiety drug intake is scarce, given that the majority of pharmacological studies use forced administration, rather than voluntary consumption.

The effect of reward loss on voluntary consumption of alcohol was recently explored in RHA-I and RLA-I strain of rats. Manzo et al. (2014) exposed these animals to consummatory (from 22% sucrose solution to distilled water) and instrumental extinction (from 12 pellets to none in the goal box of a runway). Both tests were immediately followed by a 2-h preference test for alcohol (2%) versus water in each daily session. When animals are tested under resting environmental conditions, RHA-I rats prefer ethanol over water (Manzo et al., 2012). However, the more anxious RLA-I rats showed greater preference for and consumption of alcohol than their RHA-I counterparts after being exposed to consummatory or instrumental extinction. Interestingly, when RLA-I rats were exposed to PR during the acquisition phase of an appetitive instrumental task, partially reinforced animals showed higher resistance to extinction and lower alcohol consumption in comparison to continuously reinforced animals (Manzo, Gómez et al., 2015).

Overall, these results suggest that reward loss can induce augmented preference for substances that are known to reduce anxiety when injected in reward loss situations (Becker & Flaherty, 1982, 1983; Kamenetzky, Mustaca, & Papini, 2008). I will refer to this phenomenon as emotional self-medication (ESM). Reward loss procedures provide thus a workable approach to study (1) how animals react and adjust to emotionally arousing events by changing the consumption of substances that modulate their own emotional states, and (2) how this behavior depends on reinforcement mechanisms that could underlie the onset of anxiolytics use and misuse.

Chapter 2. Goals and hypotheses

In Chapter 1, I presented data showing the increasing consumption of drugs in our society and the probability that an occasional drug consumer would develop an SUD. I also reviewed some of the most relevant theories proposed to explain addiction from biological, behavioral, and social perspectives, and highlighted the usefulness of animal models to study the ESM hypothesis of addiction. Animal models of frustration based on the unexpected loss of rewards are useful tools to explore the influence of negative emotions on ESM behavior. However, evidence relating frustration with drug consumption is sparse. Previous data analyzed the impact of frustration on alcohol consumption using the RHA-I and RLA-I strains (Manzo et al., 2014), showing that alcohol consumption was higher in vulnerable animals exposed to anxiety-inducing situations (RLA-I) compared to less anxious animals (RHA-I). The next step involves exploring the relationship between frustration and anxiolytic consumption in genetically nonselected animals—Wistar rats. Thus, the goal of this Dissertation is to analyze the role of negative emotions induced by reward loss in the onset of anxiolytics consumption—the ESM effect. This goal was developed by addressing the following topics.

First, a series of studies established the conditions necessary to obtain significant voluntary consumption of anxiolytics (alcohol and chlordiazepoxide) in Wistar rats (Experiments 1-3). Animals were exposed to a two-bottle, free-choice preference test during 2 h, in which they had access to a bottle containing either alcohol, chlordiazepoxide (CDP, a BDZ anxiolytic), or tap water, and a second bottle containing tap water.

Second, a study assessed the relationship between frustration anxiolytic consumption. Wistar rats were exposed to a reward devaluation situation (cSNC).

Each session in the cSNC was followed by a two-bottle, free-choice preference test lasting 2 h, in which animals had access to a bottle containing either ethanol, CDP, or tap water, and a second bottle containing tap water (Experiment 4).

Third, the anxiolytic assumption underlying ESM maintains that animals consume anxiolytics because they reduce negative emotions induced by reward loss. This assumption was tested in a series of experiments. Wistar rats were first exposed to the drug/water preference test for 2 h and subsequently exposed to: (1) the elevated plus maze (EPM), a test used to assess anxiety in rodents (Experiment 5); (2) a single episode of reward loss in the cSNC task (Experiment 7); or (3) repeated reward downshifts (Experiment 8). The anxiolytic assumption was further tested in a three-task design involving reward downshift followed by a preference test with access to alcohol, and finally followed by exposure to the EPM (Experiment 6).

Fourth, animals were exposed to a chronic experience of frustration to determine whether this would increase their resistance to consume anxiolytics, thus reducing ESM behavior. Wistar rats received PR or CR training during preshift sessions in the cSNC task. Immediately after each session, they received a preference test with ethanol vs. water, or water vs. water (Experiment 9).

Finally, the role of the lateral habenula (LHb) in reward downshift was assessed using appetitive extinction. Wistar rats with either LHb or sham lesions were exposed in a counterbalanced order to consummatory and instrumental extinction tasks. In a subsequent training phase, all animals received a series of preference tests with scalating concentrations of alcohol from 2% to 24% (Experiment 10).

Based on the evidence and theoretical models reviewed in Chapter 1, the starting hypotheses and corresponding predictions were the following:

(1) Wild and laboratory animals voluntarily consume alcohol for a variety of reasons (Dudley, 2000, 2002; Manzo et al., 2012; Wiens et al., 2008). Accordingly, it was expected that Wistar rats would spontaneously consume alcohol and CDP solution when exposed to drug-water preference tests.

(2) Events involving reward devaluation or omission would induce a negative emotional state of frustration sharing features with fear, anxiety, and stress (Amsel, 1992; Flaherty, 1996; Gray, 1987; Papini, 2006). Therefore, animals exposed to events would show a behavioral impairment compared to nonfrustrated controls.

(3) Frustration induced by reward loss would lead to an increase in the voluntary consumption of anxiolytics consistent with the ESM hypothesis (Torres & Papini, 2016).

(4) Increased preference for anxiolytics (e.g., alcohol, CDP) induced by reward loss depends on the substance's ability to reduce negative affect (anxiolytic assumption; Torres & Papini, 2016). Accordingly, previous access to anxiolytics would reduce negative affect induced by exposing animals to the EPM test for anxiety or to the cSNC task.

(5) Chronic frustration induced by exposure to PR training would immunize against subsequent experience with reward loss, making organisms more resistant to frustration and reducing ESM (Cuenya et al., 2012; Manzo, Gómez et al., 2015; Pellegrini et al., 2004). Therefore, animals exposed to PR during preshift sessions in the cSNC task would show a smaller cSNC

effect and lower alcohol consumption compared to animals exposed to CR during preshift sessions.

(6) The LHb is involved in the processing of surprising/unexpected non-reward (Bianco & Wilson, 2009; Hikosaka, 2010; Mathis & Kenny, 2018; Meye, Lecca, Valentinova, & Mameli, 2013; Proulx, Hikosaka, & Malinow, 2014; Salas, Baldwin, de Biasi, & Montague, 2010), and in alcohol consumption. Based on these assumptions, LHb lesions would abolish or attenuate the behavioral consequences of reward omission (consummatory and instrumental extinction). In addition, LHb lesions would increase alcohol consumption in an alcohol vs. water preference test.

Chapter 3. Experimental studies

Study 1. Do animals consume anxiolytics voluntarily?

Introduction

Animal models of neurobehavioral disorders have often been criticized for their limited translational value (Kamenetzky & Mustaca, 2005; Spanagel, 2017). However, much of the recent progress in understanding neurobehavioral disorders, including drug addiction, depend on animal research, due to the ethical and methodological limitations of human studies (Koob, 2012; Koob et al., 2014). Some of these models reproduce the patterns and conditions under which humans start consuming drugs, allow a precise control on many relevant parameters, and shed light on the neuropathological mechanisms involved in drug abuse and dependence (García-Pardo, Roger-Sánchez, de la Rubio-Ortí, & Aguilar-Calpe, 2017; Koob et al., 2014; Spanagel, 2017; Volkow, Koob, & McLellan, 2016). This section reviews the main animal models used in neuroscience of addiction based on voluntary self-administration, since this seems to be a necessary precondition for developing addictive behavior (Spanagel, 2003).

Self-administration models can be divided into consummatory procedures, in which animals have free access to the target drug for direct consumption; and operant procedures, in which an instrumental response (e.g., lever pressing, nosepoking) is required (Kamenetzky & Mustaca, 2006; Spanagel, 2017).

In consummatory paradigms, two bottles are usually placed in the animal's home cage, one containing a solution with the drug and the other containing water. The amount of fluid consumed (drug vs. water) and a preference index are generally used as dependent variables. Drug intake can be influenced by a number of factors, including dose, number of available bottles, time of access, taste of the drug solution, experience with the experimental situation, etc.

(Meisch, 2001; Spanagel, 2003, 2017). Drug consumption tends to be high when animals have several bottles with different doses and temporally limited access to them (Spanagel & Hölter, 1999).

Animal models of alcohol abuse based on oral voluntary consumption are widely used in preclinical addiction research because these procedures have good construct and face validity (Sanchis-Segura & Spanagel, 2006; Spanagel, 2017), despite the fact that rodents do not usually consume alcohol spontaneously. Several procedures are commonly used to encourage alcohol intake and allowing animals to experience the pharmacological effects of the drug (see Goltseker, Hopf, & Barak, 2019). For example, the drug is sometimes mixed with highly palatable substances, such as sugar or saccharin and then the concentration of these substances is progressively reduced while the alcohol dose increases (Samson, Sharpe, & Denning, 1999). An alternative procedure involves giving animals ethanol as the only source of calories or fluids (Wise, 1975). Ethanol consumption is also high if animals have 24 h of free access to two bottles, one with water and the other with gradually increasing doses of alcohol (Wolffgramm & Heyne, 1995). Intermittent presentations of the ethanol solution and brief periods of access to the drug also facilitate consumption (Pinel & Huang, 1976). Finally, if the dose of alcohol progressively increases (a procedure known as acclimation), animals tend to consume large amounts of the drug, thus reducing the impact of the aversive effects of high doses (Wayner & Greenberg, 1972).

In sharp contrast with the literature on alcohol, evidence showing voluntary oral consumption of prescription anxiolytics in animals is absent, despite the high rates of nonmedical use and abuse of BZDs in humans and the similarities

between alcohol and BZDs in terms of pharmacological effects and mechanisms of action.

The main goal of this section was to identify the experimental conditions under which male Wistar rats voluntarily consume solutions of CDP and whether they prefer these solutions to water, as observed with alcohol. Three experiments were conducted with this goal in mind. In Experiment 1 the pattern of consumption/preference for an 2% alcohol solution was compared with the pattern observed by animals exposed to progressively increasing doses of CDP (0.1, 0.2, 0.4, 0.8, 1.0, 1.2, and 1.4 mg/kg). On the basis of the anxiolytic properties of both drugs (Flaherty, 1996), and on the facilitating effects of acclimation procedures on drug intake (Goltseker et al., 2019; Manzo et al., 2012; Samson et al., 1999), high levels of intake and preference for alcohol and CDP over water were predicted.

Experiment 2 increased the dose range of CDP with respect to the range used in Experiment 1; animals were progressively exposed to solutions containing 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 1.4, 1.8, 2.0, 2.2, 2.4, 3.0, 4.0, 8.0, 16.0, 24.0, 36.0, and 60.0 mg/kg. Fluid intake and CDP preference levels were compared with control animals exposed to water, with the goal of identifying which doses would induce high voluntary consumption and preference for the drug. Despite the absence of prior data on oral voluntary consumption of CDP in animals, based on the pharmacological effects of CDP and other BZDs described in the literature (Danza, Cristiani, & Tamosiunas, 2009; Uzun, Kozumplik, Jakovljević, & Sedić, 2010), we predicted an inverted U-shaped relationship between CDP dose and oral consumption; that is, low levels of drug intake and

lack of preference for low doses of CDP; higher consumption and preference for medium doses; and rejection of the highest doses.

Finally, some studies have shown that prolonged intermittent access to alcohol leads to higher intake and loss of control, relative to the moderate patterns of alcohol intake observed with continuous access procedures (Goltseker et al., 2019). Experiment 3 tested whether voluntary CDP consumption would similarly increase if the drug were presented intermittently (every other session), as opposed to continuously (every single session). Based on the evidence showing that intermittent presentation of alcohol promotes high consumption (Goltseker et al., 2019; Kimbrough, Kim, Cole, Brennan, & George, 2017; Smutek et al., 2014; Wise, 1973), we predicted higher levels of CDP consumption and preference in animals exposed to intermittent access in comparison to continuous access.

Experiment 1

Method

Subjects. The subjects were 27 male Wistar rats, experimentally naïve, purchased from Harlan Laboratories (Barcelona, Spain). Rats were housed individually in polycarbonate cages with water continuously available, in a room with constant temperature (18–22 ^{o}C) and humidity (50–60%), and lights on between 08:00 and 20:00 h. Rats were approximately 90 days old at the start of the experiment. The mean ad lib weight was 369.0 g (SEM ±4.0 g). Animals were food deprived and maintained within 82–85% of their ad lib weight. All the experiments in this dissertation followed the European Union directive guidelines for the use of animals in research (2010/63/EU) and Spanish Law (6/2013; R.D.53/2013).

Apparatus. Preference tests took place in the rat's home cage, which measured 32 x 30 x 15 cm (L x W x H; see Figure 8). Each cage had the floor covered with sawdust, and had a grid lid where the bottles used in the preference test and food were placed. The bottles containing the fluids were covered with black tape due to the photosensitivity of CDP. Scales were used to weight the bottles (Cobos, JT-300C, Barcelona, Spain), the animals (Baxtran, Model BS3, Girona, Spain), and to prepare the CDP solutions according to the dose (Cobos, Precisa 125A, Barcelona, Spain). CDP (chlordiazepoxide hydrochloride, Sigma Aldrich, Madrid, Spain) and ethanol 96% (Panreac, Castellar del Vallés, Spain) were diluted in tap water on a v/v basis. The doses of CDP were derived using the standard formula for calculating the equivalent dose from clinical studies with humans (Reagan-Shaw, Nihal, & Ahmad, 2008). The dose refers to the concentration of the drug inside the bottle containing a 12 ml of the corresponding drug solutions. Thus, the dose would be applicable to an animal that drank the entire amount in the bottle. If, for example, an animal drank 7 ml of the solution, the corresponding dose would be 0.58 mg/kg of CDP. The solution volume was selected based on previous studies in which the amount of water consumed in a period of 2 h was registered (Manzo et al., 2012).

Figure 8. Setting for preference tests.

Procedure. Animals were matched by their ad lib weight and randomly assigned to one of three groups (*n* = 9): CDP (chlordiazepoxide), E (ethanol), or W (water). Animals were first exposed to four days of habituation to the two bottles (both containing tap water) during 2 h. Starting on the fifth day, animals in Group E received access to 2% ethanol and animals in Group CDP received increasing doses of CDP as follows: 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, and 1.4 mg/kg. Each concentration of CDP was presented for two consecutive days. Each day, animals were weighed, solutions were prepared, and bottles were weighed before and after the preference test. The position of the bottles was switched daily to minimize side preference. Animals were fed at least 30 min after the end of each test.

Dependent variables. Two dependent variables were registered. First, alcohol, CDP, and water consumption were measured in ml/kg by weighting the bottles before and after the preference test, and then dividing the amount of fluid consumed by the animal´s weight. The amount of water consumed in Group W was measured by using the same procedure based on the consumption corresponding to the black bottle. The amount of water consumed by all the groups was calculated in a similar way, by weighting the water bottle (for groups CDP and E) or the transparent bottle (for Group W). Second, a preference ratio was calculated by dividing the consumption on each target bottle (CDP, E, or W) by the total consumption (CDP + W; E + W; W + W, respectively) for each session. A preference ratio above 0.5 reflects preference for CDP or ethanol over water, below 0.5 reflects preference for water over CDP or ethanol, and 0.5 implies no preference for either fluid. These dependent variables were calculated by

averaging the consumption every two days, corresponding to the days in which animals received the same dose of CDP.

Statistics. Analyses of variance were calculated for each dependent variable with an alpha value set at the 0.05 level. Pairwise LSD tests derived from the main analysis were used to interpret significant interactions. Mean values were subjected to a two-factor analysis of variance, with Drug (CDP, E, W) and Dose (0.1, 0.2, 0.4, 0.8, 1.0, 1.2, and 1.4 mg/kg for CDP group) or Session (for ethanol group) as factors. All statistical tests were calculated with the IBM SPSS package, V. 24.0.

Results and discussion

Figure 9 shows drug consumption (panel A), water consumption (panel B) and preference ratio (panel C) data corresponding to E, CDP and W groups. There was higher fluid intake and preference in Groups CDP and E than in Group W, with no apparent effect of dose in Group CDP or session in Groups E and W. A Drug by Dose/Session analysis showed no difference in water consumption among groups. With respect to drug consumption, a Dose by Session effect was obtained, $F(6, 144) = 2.79$, $p < 0.02$, suggesting a progressive increase in fluid intake across sessions and CDP doses (based on a significant linear trend, $p < 0.014$). Although the drug effect did not reach statistical significance, $F(2, 24) = 2,985$, $p < 0.07$, a pairwise LSD test derived from this analysis indicated higher fluid consumption in Group E in comparison with Group W. Finally, a Drug by Dose/Session analysis corresponding to preference ratio values showed a significant main drug effect, $F(2, 24) = 6.16$, $p < 0.007$, showing that, regardless of the dose or session, Groups E and CDP exhibited higher preference than Group W.

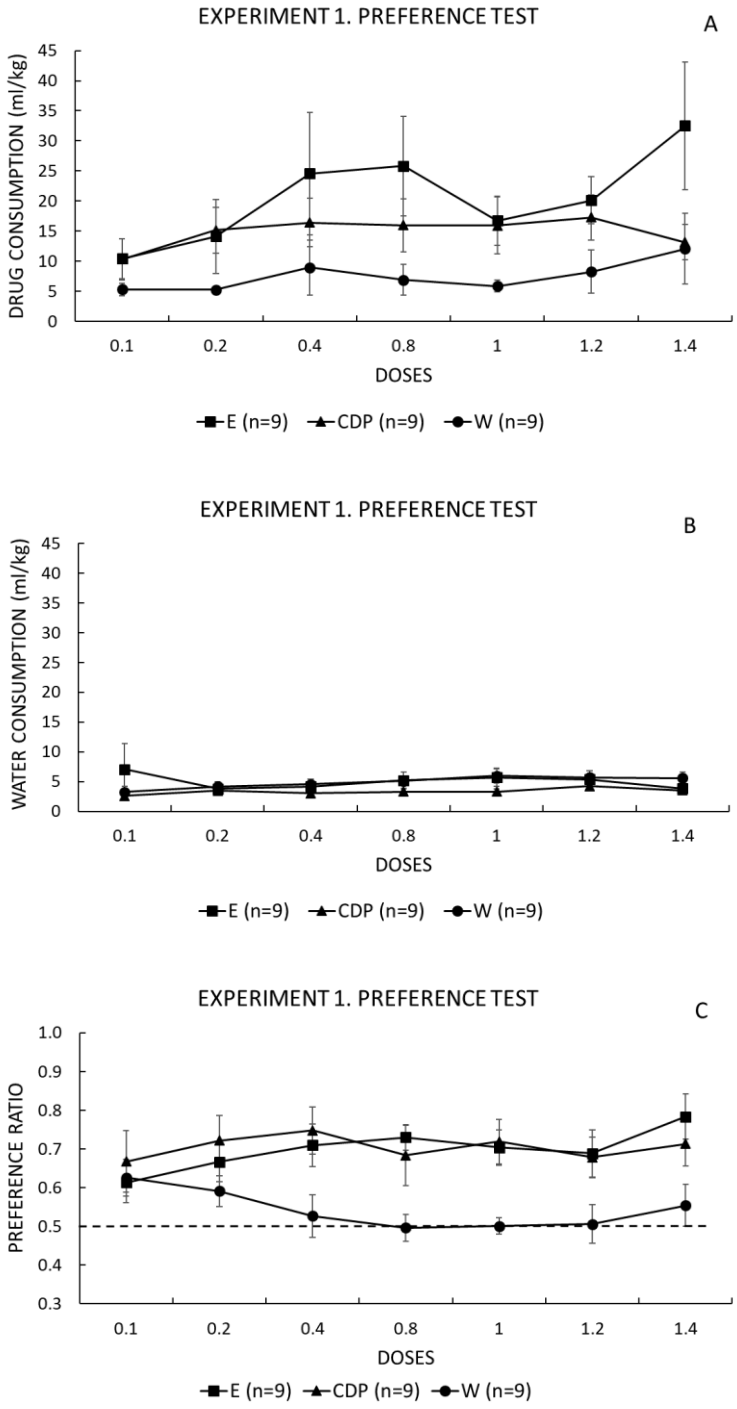

Figure 9. Drug consumption (A) and water consumption (B) (ml/kg) as a function of 2-day blocks. The CDP dose concentration avaible in each block is indicated in the x-axis. (C) Preference ratio for E, CDP, or W over water (see text for details).

The present results are in accordance with previous data showing high fluid consumption and preference for a 2% ethanol solution vs. water in rodents (Corda, Piras, Piludu, & Giorgi, 2014; Femenía & Manzanares, 2012). Present results also show that, regardless the dose, animals exposed to CDP significantly preferred the solution containing the drug instead of water, suggesting that the voluntary oral consumption of this anxiolytic had detectable and positive pharmacological effects. These results allow this drug to be used in SM experiments. However, since the boundaries of this effect, in terms of dose-dependency, were not identified in the present study, we conducted a second experiment in which the dose range of CDP was increased. We expected that low doses of the drug had no detectable effects on consumption, medium doses induced fluid intake and preference for the solution containing CDP, and high doses had aversive effects and promoted rejection, in a similar way to that observed with high alcohol concentrations presented either abruptly or gradually (Manzo et al., 2012).

Experiment 2

Method

Subjects. The subjects were 20 male Wistar rats, purchased from Charles River (Barcelona, Spain). At the start of the experiment, rats were approximately 90 days old. The mean weight was 334.4 g (SEM ±3.2). Housing and maintenance were as described in Experiment 1.

Apparatus. The apparatus used in this experiment were the same described in Experiment 1.

Procedure. Animals were matched by ad lib weight and randomly assigned to one of two groups (n = 10): CDP or W. Group CDP received an

increasing dose of CDP (0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 1.4, 1.8, 2.0, 2.2, 2.4, 3.0, 4.0, 8.0, 16.0, 24.0, 36.0, and 60.0 mg/kg). Each concentration was presented for two consecutive days. Everything else was as described in Experiment 1.

Dependent variables. The dependent variables were those described in Experiment 1: fluid consumption (in ml/kg and averaged every two days) and preference ratio.

Statistics. Analyses of variance were calculated for each dependent variable with an alpha value set at the 0.05 level. Mean values were subjected to a two-factor analysis of variance, with Drug (W, CDP) and Dose (0.1-60.0 mg/kg) as factors. All statistical tests were calculated with the IBM SPSS package, V. 24.0.

Results and discussion

Figure 10 illustrates drug consumption (panel A), water consumption (panel B), and preference ratio (panel C) data corresponding to Groups CDP and W. Drug consumption data were subjected to a Drug by Dose ANOVA, that showed significant main effects for dose, $F(17, 306) = 2.41$, $p < 0.002$, and drug, $F(1, 18) = 5.52$, $p < 0.03$, indicating changes in fluid intake in Groups CDP and W across sessions and, more interestingly, higher drug intake in CDP animals in comparison with W animals. With respect to water intake data, a Drug by Dose analysis showed significant main effects for dose, $F(17, 306) = 3.65$, $p < 0.001$, suggesting random/irrelevant differences in water consumption across sessions.

Finally, an analysis of preference ratios revealed significant main effects for dose, $F(17, 306) = 2.50$, $p < 0.001$, and drug, $F(1, 18) = 8.93$, $p < 0.008$, showing higher preference ratio values for Group CDP than for Group W.

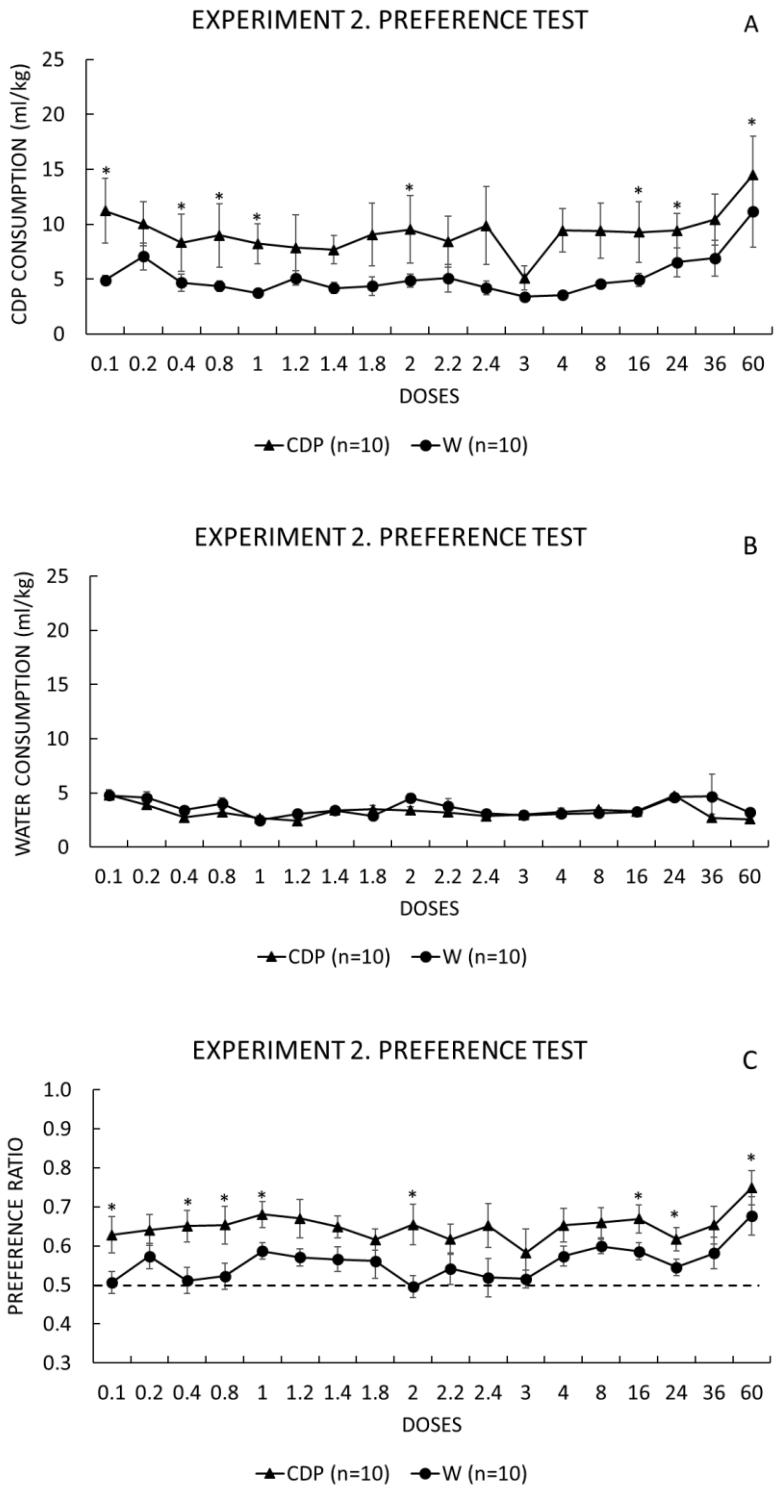

*Figure 10. (A) CDP consumption (ml/kg); (B) water consumption (ml/kg); and (C) preference ratio as a function of 2-day blocks. *: significant differences between CDP and W groups in the corresponding session.*

Overall, the present experiment showed similar results to those obtained in Experiment 1, that is, higher fluid intake and preference for CPD solutions in comparison to water regardless the dose. Although nonsignificant dose by drug interactions were obtained, asterisks in Figure 10 show a random pattern of differences in drug consumption and preference between Groups CDP and W, at low (0.1-1.0 mg/kg), medium (2 mg/kg), and high doses (16.0-60.0 mg/kg). Moreover, given that a session (dose) effect was obtained regardless the drug condition (CDP vs. W), the present data preclude any firm conclusion about the relationship between voluntary oral consumption of CDP and drug dose. Based on these results, we conducted a third experiment in which a medium, preferred dose of CDP (2 mg/kg) was presented to Wistar rats in an intermittent schedule (every other day), with the aim of increasing drug intake and preference for the drug in comparison to animals receiving the CDP solution daily.

Experiment 3

Method

Subjects. The subjects were 20 male Wistar rats, purchased from Charles River (Barcelona, Spain). Rats were approximately 90 days old at the start of the experiment. Mean ad lib weight was 334.4 g (SEM ±3.2 g). Housing and maintenance conditions were as described in Experiment 1.

Apparatus. The apparatus used in this experiment were as described in Experiment 1.

Procedure. Animals were matched by their *ad lib* weight and randomly assigned to one of two CDP groups (n = 10): intermittent (INT) or continuous (CONT). The group INT received a solution of 12 ml containing 2 mg/kg of CDP in half of sessions, whereas in the other half it was exposed to water in both

bottles. The CONT group received CDP in all sessions. Everything else was as described in Experiment 1.

Dependent variables. The dependent variables were those described in Experiment 1: fluid consumption and preference ratio.

Statistics. Analyses of variance were calculated for each dependent variable with an alpha value set at the 0.05 level. Mean values were subjected to a two-factor analysis of variance, with Drug (INT, CONT) and Session (25 sessions) as factors. INT and CONT groups were compared in terms of CDP consumption, water consumption, and preference ratio in every session, and in sessions in which CDP was available for Group INT (the 13 odd sessions). Group INT's performance was also analyzed by comparing average CDP intake on sessions in which the drug was presented (odd sessions) vs. sessions in which the drug was unavailable (even sessions). All statistical tests were calculated with the IBM SPSS package, V. 24.0.

Results and discussion

Figure 11 illustrates drug consumption (panel A), water consumption (panel B), and preference ratio (panel C) data corresponding to Groups CONT and INT. Drug consumption data were subjected to a Drug by Session analysis of variance that included all sessions. This analysis did not show significant effects for session, drug, or their interaction, Fs < 2.34, ps > 0.05. Similar results were obtained when only sessions in which INT animals received CDP were analyzed, Fs < 2.24, ps > 0.05. Finally, when the average CDP intake was compared on sessions in which the drug was available vs. unavailable in the Group INT, a marginally nonsignificant effect was obtained, $F(1, 9) = 4.27$, $p = 0.069$, indicating a slightly higher consumption in days in which the bottle

contained CDP relative to water. Nonsignificant results were also obtained when water consumption and preference ratio data were subjected to these analyses (including all sessions, only CDP available sessions, and available vs. unavailable CDP sessions in Group INT), *F*s < 1.

There was no evidence of an effect of CONT vs. INT presentation of CDP on drug intake or preference. This result contrasts with previous studies in which a higher preference for an alcohol (20%) was observed with intermittent presentations. These effect was observed regardless of whether the high dose of alcohol was presented abruptly or introduced gradually, or under forced (one bottle) or free-choice (two bottles) conditions (Wise, 1973). Momeni and Roman (2014) also found that intermittent access to 20% alcohol (three consecutive days per week for seven weeks) significantly increased alcohol intake over time. This paradigm has been useful to model binge drinking and detect individuals vulnerable to excessive alcohol intake under conditions of intermittent access (Amodeo et al., 2018; Carnicella, Ron, & Barak, 2014; Jeanblanc, Rolland, Gierski, Martinetti, & Naassila, 2018; Hwa et al., 2011).

Two results suggest that the 2 mg/kg CDP dose could be used in studies on ESM. First, there was a higher (albeit nonsignificant) consumption of CDP on days in which the bottle contained CDP compared to sessions in which animals received water in Experiment 3. Second, there was a significantly higher consumption of CDP compared to intake in animals receiving only water in Experiment 2.

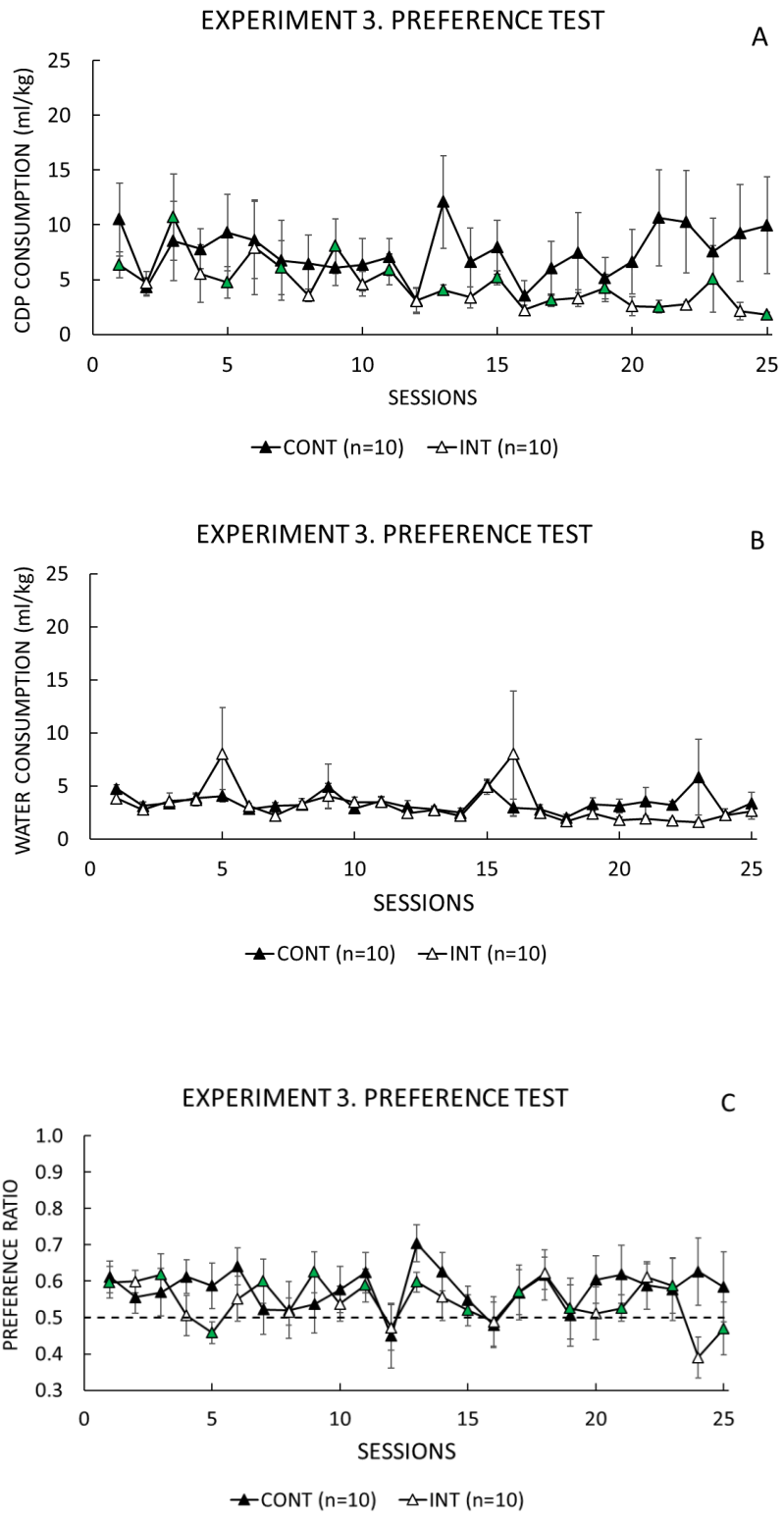

Figure 11. (A) CDP consumption (ml/kg), (B) water consumption (ml/kg), and (C) preference ratio as a function of 2-day blocks. Green triangles: CDP presented in Group INT.

Study 1: General discussion

The experimental series presented in Study 1 was conducted to identify the conditions under which animals voluntarily consume CDP (an anxiolytic prescription drug with addictive potential), and whether they prefer this drug to water. Rats were exposed to solutions containing different doses of CDP, 2% ethanol (used as positive control), or water (Experiments 1-2). The results showed significantly higher levels of CDP consumption than water regardless of the CDP dose. These results indicated that CDP had detectable and positive (probably reinforcing) pharmacological effects. The attempt at increasing CDP intake by implementing an intermittent presentation schedule, analogous to those successfully used with alcohol, were not effective with CDP (Experiment 3).

Several aspects of these results merit additional comments. First, unlike ethanol, CDP does not have caloric content, so its preference cannot be attributed to a nutritional factor. It is possible that the CDP solution has a taste or flavor distinguishable from and preferred over water, leading animals to prefer it to water regardless the dose. However, this should lead to progressively higher CDP consumption as the dosis increases, which was not observed.

Second, the poor results obtained in Experiment 3 could be due to the low levels of consumption registered in the intermittent and continuous conditions. Since CDP (2 mg/kg) was diluted in 12 ml of tap water, if an animal consumed, for example, 2 ml of the solution, the actual ingested dose of CDP was 0.33 mg. This dose was probably too low to achieve detectable pharmacological effects according to the standard formula for calculating the equivalent dose from clinical studies with humans (Reagan-Shaw et al., 2008).

In summary, these data show, for the first time, that animals voluntarily drink and prefer CDP solutions to water under free-choice conditions. Further experiments will have to be conducted to determine whether CDP can be applied to studies of ESM.

Study 2. Does frustration increase anxiolytic consumption?

Experiment 4

See Appendix A

Manzo, L., Donaire, R., Sabariego, M., Papini, M. R., & Torres, C. (2015). Anti-anxiety self-medication in rats: Oral consumption of chlordiazepoxide and ethanol after reward devaluation. *Behavioural Brain Research*, *278*, 90-97.

Study 3. Why do animals self-medicate?

Introduction

The results obtained in the previous subsection indicate that reward devaluation induces ESM: Animals exposed to an unexpected 32-4% sucrose downshift increased their consumption and preference for ethanol and CDP (but not water), compared to animals exposed to 4-4% sucrose controls. These data are consistent with the hypothesis that reward downshift induced negative affect (Amsel, 1992; Flaherty, 1996; Papini, 2003; Papini et al., 2006), and that animals consumed both ethanol and CDP based on their anxiolytic/alleviating actions on frustration. Moreover, these results fulfill the requirements proposed by Torres and Papini (2016) to conceptualize a behavior as an ESM response: (1) the induction task triggered negative affect (e.g., frustration), as inferred from the transient impairment of consummatory behavior; (2) the increased consumption of alcohol and CDP was restricted to periods of behavioral inhibition induced by reward devaluation; and (3) increased consumption was selective for anxiolytic substances that decreased negative affect, as opposed to water. Despite these encouraging results, an additional assumption underlying the ESM hypothesis has not yet been tested, namely, that the consumed substance actually reduced negative affect triggered by the induction task. This so-called “anxiolytic assumption” states that increased intake and preference for anxiolytics following the induction task is dependent on a reinforcing effect derived from their ability to reduce negative affect.

This section systematically tested the anxiolytic assumption of the ESM hypothesis. Experiments 5-6 explored whether previous free access to anxiolytic drugs influenced exploratory behavior in the elevated plus-maze (EPM) test. This test consists of a cross-shaped maze with two arms protected by high walls

(closed arms) and two unprotected arms without walls (open arms). Based on the natural conflict between the drive to explore new environments and the tendency to avoid potentially unsafe areas (Cárdenas & Navarro, 2002; Kumar, Bhat, & Kumar, 2013; Ramos, 2008), the EPM has been used to detect anxiolytic effects, indicated by increased open arms exploration, in pharmacological studies (Boerngen-Lacerda & Souza-Formigoni, 2000; Escarabajal, Torres, & Flaherty, 2003; Pellow, Chopin, File, & Briley, 1985; Rodgers, Davis, & Shore, 2002). Several studies found that ethanol (administered intragastrically or orally) significantly increased the number of entries and the time spent in the open arms (Acevedo, Nizhnikov, Molina, Marcos, & Pautassi, 2014; Brolese, Lunardi, Lopes, & Gonçalves, 2017; Colombo et al. 1995; Paré, Paré & Kluczynski, 1999). Similarly, free access to ethanol for voluntary consumption increased open-arm exploration (Pohorecky, 2008). Based on this evidence, the EPM seems potentially suitable to detect anxiolytic effects derived from ethanol and BZD consumption. Experiment 5 involved giving animals free access to ethanol, CDP, or water (respectively), followed by EPM testing on sessions 11-12. Based on previous studies (Colombo et al., 1995; Päivärinta & Korpi, 1993; Pohorecky, 2008), higher open-arm exploration in animals with previous access to ethanol or CDP was expected in comparison to animals receiving water.

Experiment 6 explored whether the impact of previous voluntary anxiolytic consumption on anxiety behaviors in the EPM would increase by exposing animals to reward downshift before the preference test. It was predicted that increased ethanol consumption induced by reward devaluation would reduce anxiety as assessed in the EPM test in terms of open-arm exploration.

Experiments 7-8 further explored the anxiolytic assumption of the ESM hypothesis by exposing animals to a cSNC task after free access to alcohol, CDP or water in a previous preference test. cSNC is a prime candidate to assess whether previous exposure to anxiolytics for free consumption ameliorates such aversive emotional state. Moreover, there is evidence that cSNC is reduced by presession systemic administration of ethanol and other GABAergic anxiolytics, including CDP (Becker & Flaherty, 1982, 1983; Kamenetzky, Mustaca, & Papini, 2008). These findings are consistent with the interpretation that an increase in preference for and consumption of ethanol and CDP after reward loss reflects ESM (Torres & Papini, 2016). In Experiment 7, animals were exposed to a single reward downshift experience (from 32% to 4% sucrose) after free access to 2% ethanol, 2 mg/kg of CDP, or water. Then, the usual order of the tasks was reversed, with the preference test administered *before* the cSNC induction task every day. According to the anxiolytic assumption of the ESM hypothesis and to the sensitivity of the cSNC to the effects of anxiolytic drugs (Flaherty, 1996), it was predicted that ethanol and CDP intake would reduce the cSNC effect, in terms of either size or recovery time.

Experiment 8 used the same arrangement of tasks described in the previous experiment, but it sought evidence for the anxiolytic assumption using repeated reward downshifts in the cSNC situation. The mechanisms underlying the cSNC effect are known to change as a function of experience with the downshift conditions. For example, systemic ethanol and benzodiazepine administration have no effect on the size of the effect when administered before the first downshift event, but they reduce the effect when administered before the second downshift event (Becker & Flaherty, 1982; Flaherty, Grigson, & Lind,

1990). If the first downshift session is lengthened from the usual 5 min to 20 min, the effects of CDP are detected during the second 5 min period (6-10 min) of the session, suggesting that a minimum of 5 min of experience with the new reward conditions is needed before the anxiolytic effect is detectable (Flaherty, Grigson, & Rowan, 1986). A similar change in the effectiveness of ethanol and CDP occurs when animals are exposed to repeated reward downshifts. For example, after three trials of access to 32% sucrose, animals receiving alternating sessions with 4% and 32% sucrose received either ethanol (1 g/kg) or saline before each 4% sucrose session. Under these conditions, a significant cSNC effect emerged after two downshift sessions in saline animals, but it failed to emerge in ethanol-treated animals (Kamenetzky et al., 2008). Similarly, CDP reduced the cSNC effect during the first session after repeated cycles of reward downshift (Flaherty, Clarke, & Coppotelli, 1996). Based on this evidence, i predicted that the attenuating effect of ethanol on consummatory behavior inhibition would become evident only after a few 32-4 downshift cycles.

Experiment 5

Method

Subjects. The subjects were 30 male Wistar rats, experimentally naïve, purchased from Charles River (Barcelona, Spain). At the start of the experiment, rats were approximately 90 days old. The mean weight was 403.3 g (SEM ±3.4 g). Other conditions were as described in Experiment 1.

Apparatus. The preference test was conducted in the home cage, as described in Experiment 1. The EPM (Figure 12) consisted of two open arms and two closed arms, each measuring 49.5 x 10 cm (L x W), with black polycarbonate floors. The open arms were bounded by 1 cm high ledges on the sides, with no

ledges at the end of the arms. The closed arms had 39.5-cm high transparent polycarbonate walls. The maze was elevated 50.5 cm above the ground. The rats were carried to the experimental room in a transport cage 32 x 30 x 15 (L x W x H). Animals were video recorded (web cam Logitech model C200, Spain), and the corresponding dependent variables were then processed with JWatcher 1.0 by two independent observers (http://www.jwatcher.ucla.edu). Time variables were measured in seconds with a manual chronometer.

Figure 12. Elevated plus maze used in these experiments.

Procedure. Animals were matched by weight and randomly assigned to one of three groups (*n* = 10): E, CDP, or W. The preference test was conducted as in Experiment 1. Animals in Group CDP received increasing doses of CDP as follow: 0.1, 0.2, 0.4, 0.8, and 1.0 mg/kg. Each dose was administered in two sessions; the highes dose was administered in all sessions after session 9. Each

rat was trained separately. The onset of training sessions was delayed 15 min to avoid overlaps with the EPM session. Rats were exposed to the EPM on sessions 11-12 after the 2-h preference test. Animals were placed in the central square, facing one of the open arms, and they were allowed to freely explore the apparatus for 5 min.

Dependent variables. For the preference test, two dependent variables were registered: fluid consumption (E, CDP, or W) and preference ratio (see Experiment 1).

Several measures were recorded in the EPM: (1) Rearing (animal stands on his hind legs) time (s) and rearing frequency in the open and closed arms. (2) Head dipping (animal drops its head under a closed or open arm) time (s) and frequency. (3) Distal portion time (s) and frequency in the open arms. (4) Grooming (animal licks all reachable parts of its body) time (s) and frequency. (5) Open- and closed-arm entries and time (s). (6) Total arms entries (closed-arm entries plus open-arm entries) and total arms time (s; closed-arm time plus open-arm time).

Statistics. Analyses of variance were calculated for each dependent variable with an alpha value set at the 0.05 level, and with pairwise LSD tests derived from the main analysis. For dependent variables registered in the preference test, mean values were subjected to a two-factor analysis of variance, with Drug (E, CDP, W) and Session (1-15) as factors. For dependent variables registered in the EPM, an analysis of variance was conducted with Drug (E, CDP, W) and Session (11-12) as factors. All statistical tests were calculated with the IBM SPSS package, V. 24.0.

Results and discussion

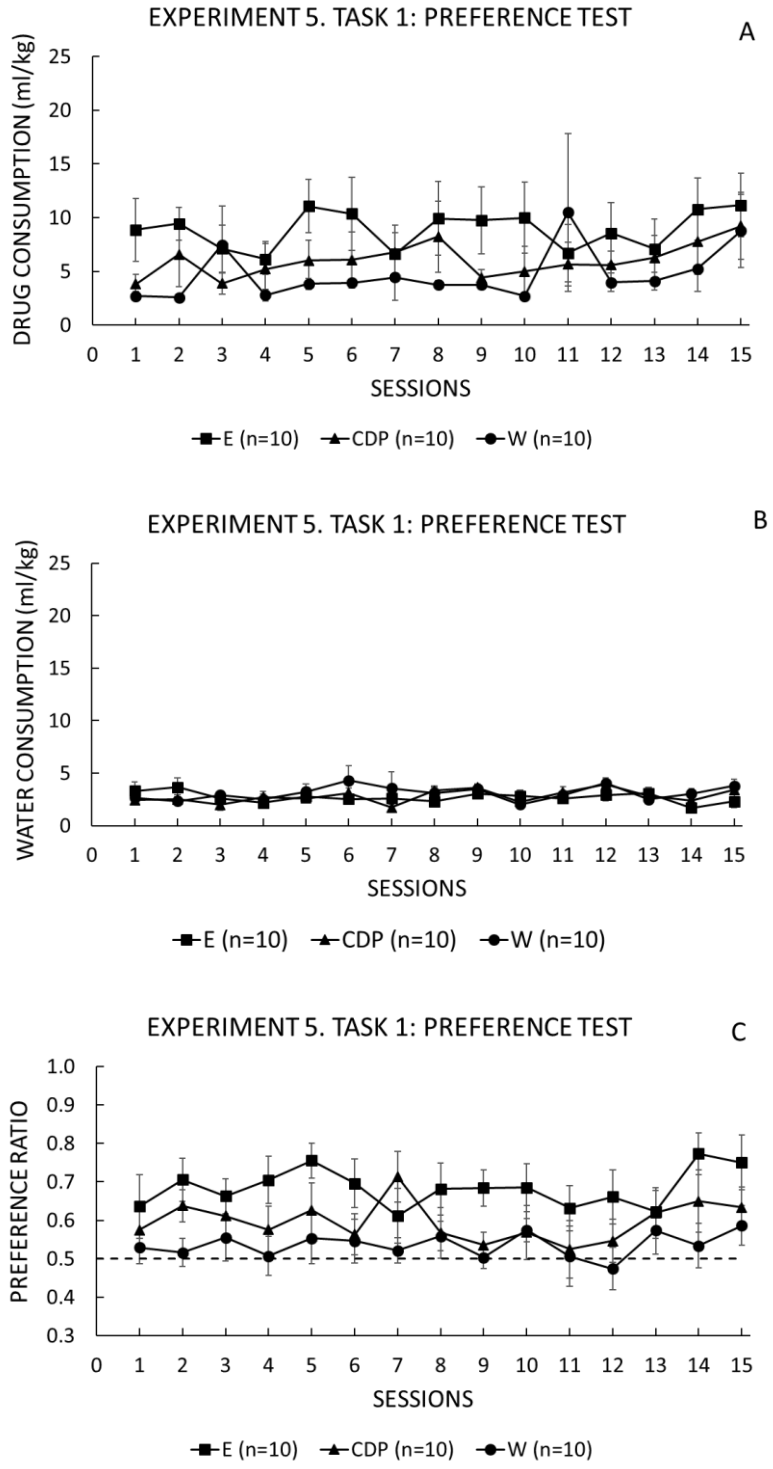

Figure 13. (A, B) Mean (±SEM) consumption of alcohol, CDP, and water in the preference test (ml/kg). (C): Mean (±SEM) preference ratio for Groups E, CDP, and W.

Preference test. Figure 13 illustrates drug consumption (panel A), water consumption (panel B), and preference ratio (panel C). Group E showed higher consumption and preference across sessions compared to Groups CDP and W. Analyses of the drug and water consumption revealed nonsignificant main effects of Session, Fs(14, 378) < 1.11, p > 0.05, and Drug, Fs < 1. The Session by Drug interactions were nonsignificant, Fs < 1. With respect to the preference ratio data, there was a significant main effect of Drug, F(2, 27) = 7.15, p < 0.003. Further pairwise tests derived from the main analysis revealed higher preference ratio for Group E than in Groups CDP and W, respectively.

EPM. Table 1 and Figure 14 represent the performance of Groups E, CDP, and W on sessions 11 and 12 in the EPM. Drug by Session analyses showed significant main effects for session in open-arm rearing frequency and time, open- and closed-arm head dipping frequency, open distal portion entries and time, entries in the open arms, and time spent in the closed arms, Fs(1, 27) > 4.82, ps < 0.04. These results indicated a general decrease in open-arm exploration and an increase in closed-arm exploration across sessions. Importantly, there was a significant main effect of drug in closed-arm entries, F(1, 27) = 4.48, p < 0.03, indicating more closed-arms entrie in Group E compared to Groups CDP and W (Figure 14). Therefore, access to ethanol significantly increased closed-arm entries in comparison with groups exposed to CDP and water, without affecting exploratory behavior registered in the open arms.

Table 1. *Group performance in the EPM (Means, ±SEMs) on sessions 11 and 12. *: p < 0.05.*

			DAY 11			DAY 12		
ARMS		VARIABLES	E	CDP	W	E	CDP	W
OPEN	FREQUENCY	**Rearing** *	1.9 ±1.00	1.7 ±0.56	1.9 ±0.64	0.8 ±0.51	0.4 ±0.22	0.6 ±0.22
		Head Dipping *	4.5 ±1.54	4.6 ±1.20	4.90 ±1.25	2 ±0.93	2.0 ±0.70	3.7 ±1.01
		Distal Portion *	3.1 ±0.87	4.6 ±0.97	5.7 ±0.97	1.9 ±0.67	2.6 ±0.91	3.2 ±0.9
		Entries *	5.3 ±0.97	6.2 ±0.92	7.8 ±0.74	3.4 ±0.99	3.9 ±1.21	5.8 ±1.07
	TIME (sec)	**Rearing** *	1.9 ±1.23	2.1 ±0.79	1.7 ±0.52	1.2 ±0.75	0.4 ±0.24	0.8 ±0.33
		Head Dipping	8.6 ±3.38	9.2 ±2.98	7.9 ±2.08	4.2 ±2.21	3.3 ±1.19	10.7 ±2.56
		Distal Portion *	28.3 ±7.79	25.7 ±5.38	43.3 ±10.10	17.7 ±7.33	13.5 ±4.39	25.4 ±6.88
		Entries	136.0 ±21.53	110.7 ±14.53	103.9 ±16.70	64.6 ±24.43	57.4 ±15.17	96.3 ±18.32
CLOSED	FREQUENCY	Rearing	11.8 ±1.72	9.0 ±0.68	9.6 ±1.35	12.3 ±1.6	11.6 ±1.46	11.4 ±1.44
		Head Dipping *	4.4 ±0.96	6.9 ±0.99	5.2 ±0.80	4.4 ±1.07	3.4 ±0.64	3.1 ±1.03
		Entries *	7.2 ±0.84	5.4 ±0.45	5.5 ±0.79	8.0 ±0.71	6.1 ±0.64	7.0 ±0.67
	TIME (sec)	Rearing	25.1 ±3.36	17.2 ±2.28	20.2 ±2.90	26.5 ±3.86	33.3 ±5.73	28.9 ±3.94
		Head Dipping	11.6 ±3.07	15.3 ±2.26	11.0 ±2.43	17.0 ±5.01	11.6 ±2.99	12.5 ±4.46
		Entries *	102.7 ±22.36	124.0 ±18.18	143.3 ±17.32	167.9 ±19.23	172.7 ±15.18	142.9 ±17.37
OPEN + CLOSED ARMS	FREQUENCY	Grooming	0.7 ±0.42	0.3 ±0.15	0 ±0	0.5 ±0.5	0.3 ±0.21	0.2 ±0.13
		Total Entries	12.5 ±0.56	11.6 ±0.97	13.3 ±0.79	11.4 ±0.79	10.0 ±1.37	12.8 ±1.06
	TIME (sec)	Grooming	4.6 ±3.55	2.0 ±1.39	0 ±0	1.9 ±1.95	3.2 ±2.45	0.6 ±0.49
		Total Entries	238.7 ±9.63	234.7 ±13.18	247.2 ±5.0	232.5 ±10.38	230.2 ±10.52	239.1 ±9.77

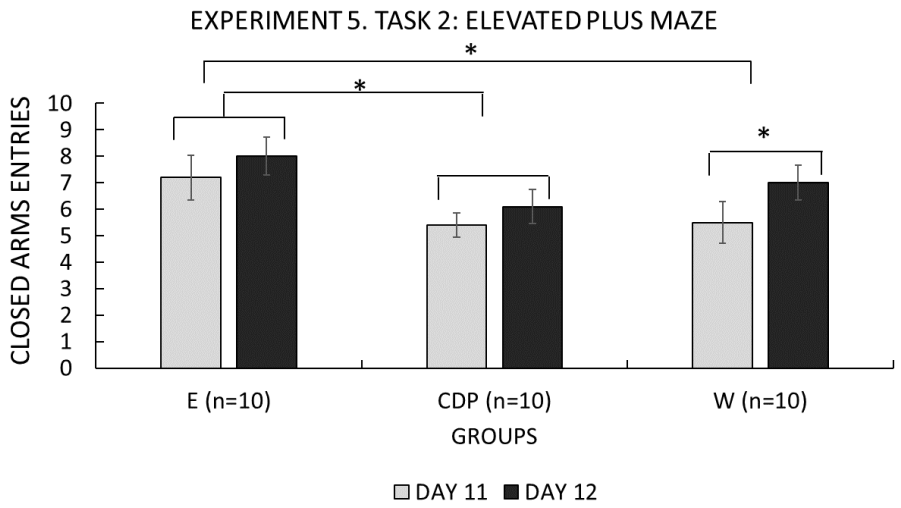

Figure 14. Mean (±SEM) frequency of closed-arm entries during sessions 11 and 12 of the EPM testing for Groups E, CDP, and W.

The lack of effect of CDP on EPM performance could rely on the low preference/consumption levels registered during the preference test, an explanation that will have to be explored in future experiments. There are precedents for increased closed-arm entries in rats receiving alcohol (3.25 g/kg administered intragastrically; Acevedo et al., 2014). Since closed-arm entries have been traditionally considered as a measure of locomotor activity in the EPM (Cárdenas & Navarro, 2002), it can be concluded that alcohol consumption had a disinhibitory motor effect, rather an anxiolytic action in the present study. This falls short of providing conclusive evidence supporting the anxiolytic assumption of the ESM hypothesis. A possible explanation for these results is that the low levels of ethanol intake prevented an anxiolytic effect from being observed. In accordance with this explanation, alcohol has been shown to have a biphasic effect on motor activity and anxiety behaviors that depends on dose, among other factors (Karlsson & Roman, 2016). To explore this possibility, alcohol intake was

increased in Experiment 6 by exposing animals to reward downshift before the ethanol preference and EPM tests.

Experiment 6

See Appendix B

Donaire, R., Conrad, S. E., Thompson, J. B., Papini, M. R., & Torres, C. (2018). Augmented voluntary consumption of ethanol induced by reward downshift increases locomotor activity of male Wistar rats in the elevated plus maze. *Behavioural Processes*, *150*, 59-65.

Experiment 7

Method

Subjects. The subjects were 48 male Wistar rats, experimentally naïve, purchased from Harlan Laboratories (Barcelona, Spain). At the start of the experiment, rats were approximately 90 days old. The mean weight was 398.4 g (SEM ±2.8 g). Housing and maintenance were as described in Experiment 1.

Apparatus. The preference test took place in the rat's home cage, as described in Experiment 1 (see Figure 8). Induction task (cSNC) training involved 8 Plexiglas boxes (Figure 15). Everything else was as described in Experiment 4. All the bottles were painted in black and adequately labelled according to the solution (E, CDP, or W).

Figure 15. Consummatory successive negative contrast boxes.

Procedure. Rats were matched for weight and randomly assigned to one of six groups (*n* = 8): E/32, E/4, CDP/32, CDP/4, W/32, and W/4. Animals were first exposed to the two-bottle preference test, as described in Experiment 1, one containing the drug (2% ethanol for groups E/32 and E/4; 2 mg/kg CDP for groups CDP/32 and CDP/4) and the other one containing tap water. Both bottles

contained water for Groups W/32 and W/4. Immediately after that, animals were exposed to the cSNC test (see Experiment 4).

Dependent variables. In the preference test, the dependent variables were those described in Experiment 1: fluid consumption in ml/kg (alcohol, CDP, or water) and preference ratio. For cSNC, the amount of sucrose solution consumed (ml/kg) was used as described in Experiment 4.

Statistics. Analyses of variance were calculated for each dependent variable with an alpha value set at the 0.05 level and with pairwise LSD tests derived from the main analysis. Mean values were subjected to a three-factor analysis of variance, with Drug (CDP, E, W), Sucrose (32, 4), and Session as factors (3 × 2 × 10 for preshift; 3 × 2 × 5 for postshift). All statistical tests were conducted with the IBM SPSS package, V. 24.0.

Results and discussion

Preference test. Figure 16a and 16b shows the values corresponding to drug and water consumption for Groups E/32, E/4 (panels A, D), CDP/32, CDP/4 (panels B, E), and W/32, W/4 (panels C, F). Animals exposed to ethanol seemed to drink more of the bottle containing the drug solution than animals exposed to CDP or water, regardless the contrast condition (32% or 4%).

A Drug by Sucrose by Session analysis revealed a main drug effect, $F(2, 42) = 23.80$, $p < 0.001$, and a drug by session interaction in the preshift phase, $F(18, 378) = 1.89$, $p < 0.02$. The analysis of this interaction indicated greater fluid intake in Group E than in Groups W and CDP in all preshift sessions. Similar effects were obtained in postshift sessions, where only a main drug effect was obtained, $F(2, 42) = 4.80$, $p < 0.02$, indicating that Groups E showed higher fluid consumption than Groups W and CDP across sessions.

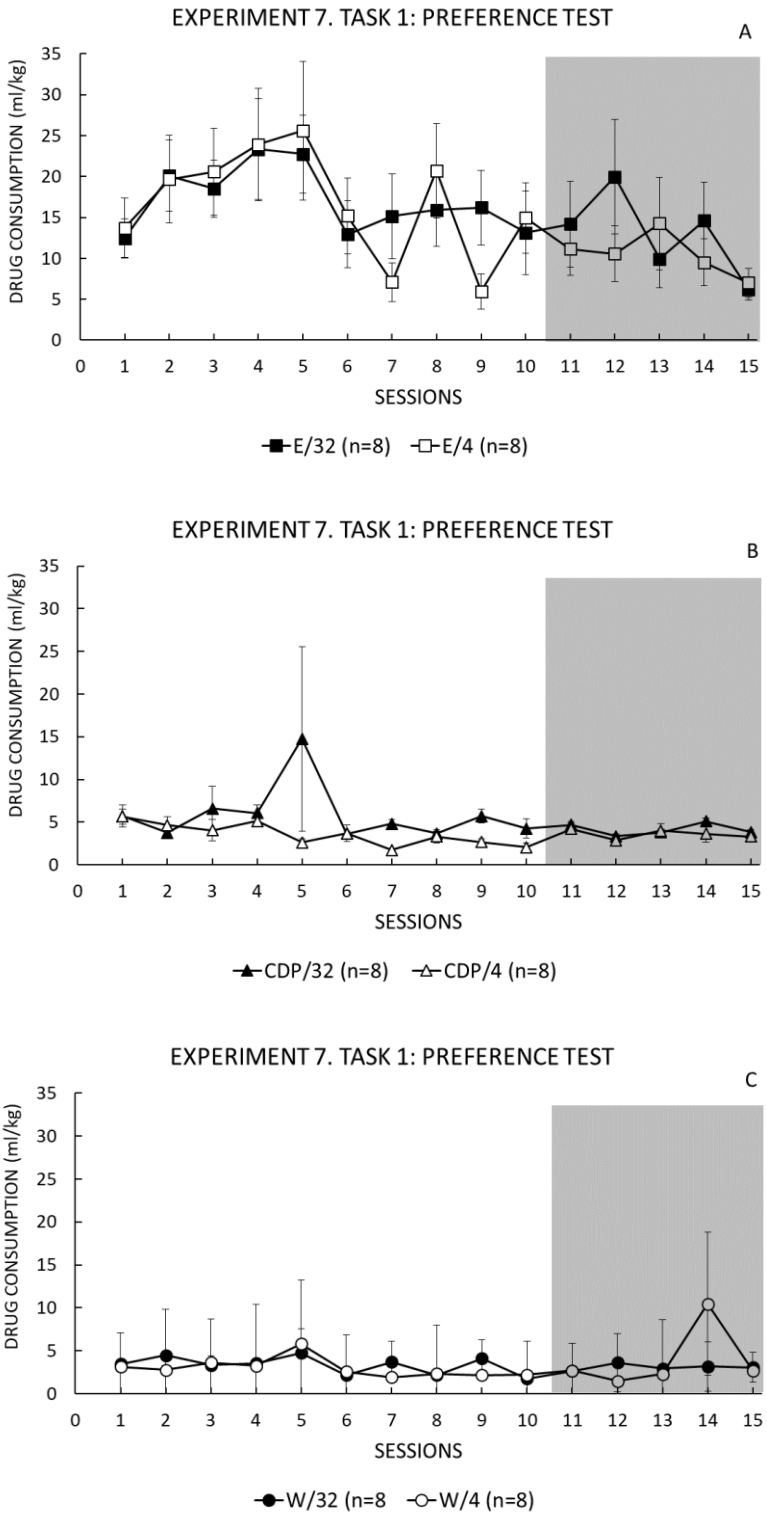

Figure 16a. Fluid consumption (ml/kg) for groups exposed to alcohol (A), CDP (B), or water (C). The gray area marks postshift sessions in the subsequent cSNC task.

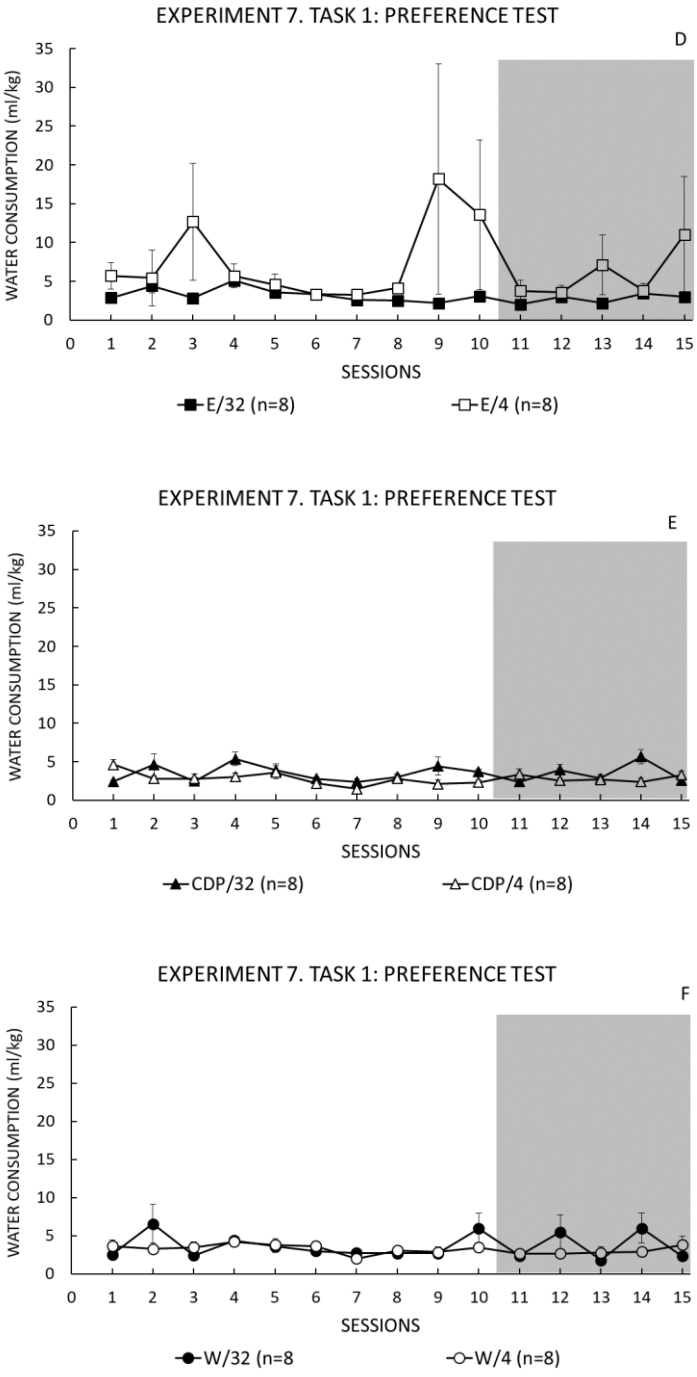

Figure 16b. Water consumption (ml/kg) for groups exposed to alcohol (D), CDP (E), and water (F). The gray area marks the postshift sessions in the subsequent cSNC task.

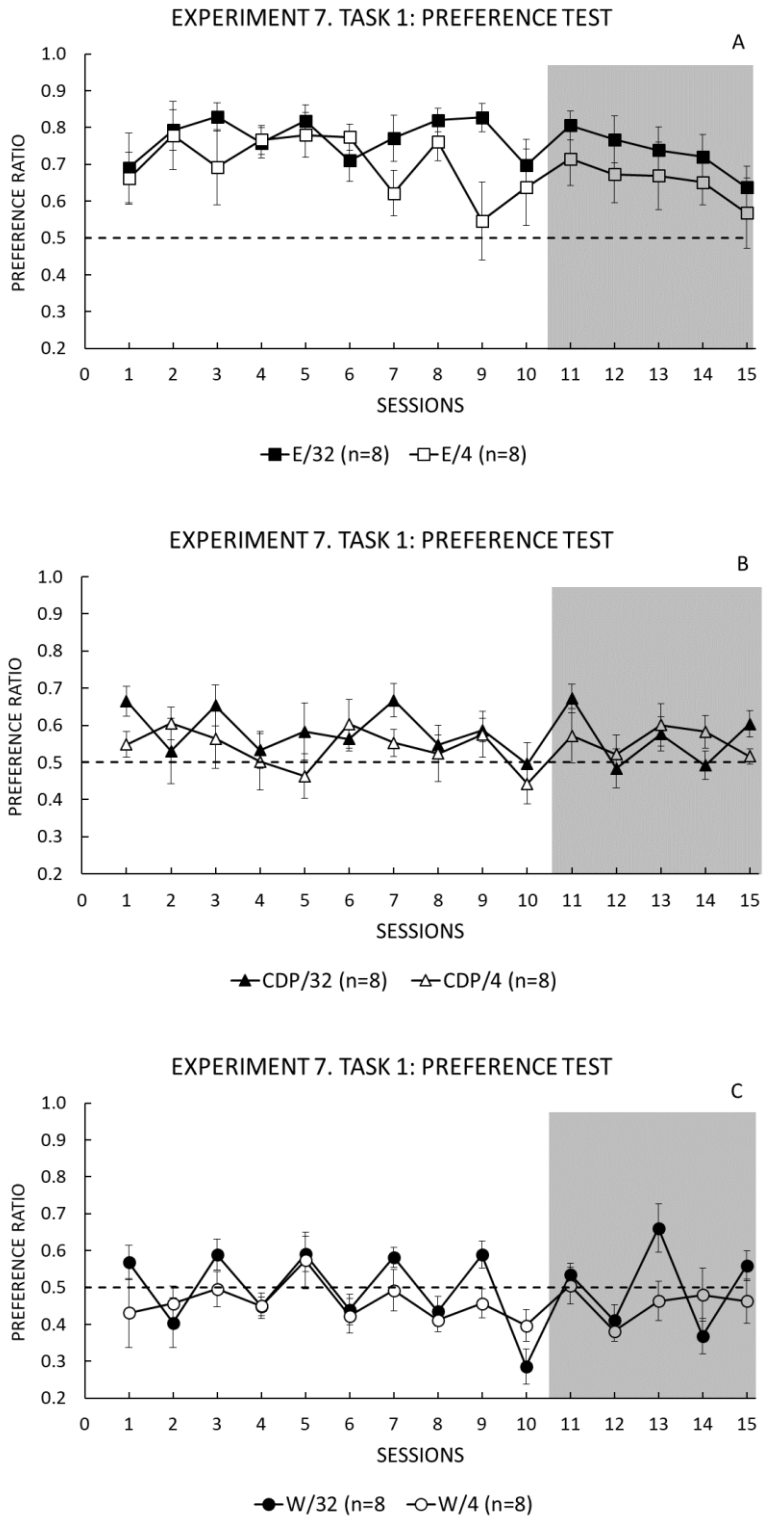

Figure 17. Means (± SEM) preference ratio values for groups exposed to alcohol (A), CDP (B), and water (C). The gray area marks the postshift sessions in the subsequent cSNC task.

A Drug by Sucrose by Session analysis conducted with the consumption data obtained from the water bottles revealed a marginally significant effect of drug on preshift phase, $F(2, 42) = 3.17$, $p > 0.05$, indicating that Group E drank more water than Group CDP. On postshift phase, a marginally significant drug by sucrose interaction was obtained, $F(2, 42) = 3.19$, $p > 0.05$. The source of this interaction was a significant difference between Groups 32 vs. 4 only in the E condition, $F(1, 42) = 6.63$, $p < 0.02$, as well as from significant E vs. W and E vs. CDP differences only in the 4% condition.

Figure 17 shows preference ratio values for Groups E/32, E/4 (panel A), CDP/32, CDP/4 (panel B), and W/32, W/4 (panel C). A Drug by Sucrose by Session analysis showed a main effect of session, $F(9, 378) = 3.36$, $p < 0.001$, sucrose, $F(1, 42) = 4.42$, $p < 0.05$, and drug, $F(2, 42) = 42.64$, $p < 0.001$ in preshift sessions, as well as significant Session by Sucrose, $F(9, 378) = 2,07$, $p < 0.04$, and Session by Drug interactions, $F(18, 378) = 1.82$, $p < 0.03$. The analysis of the Session by Sucrose interaction indicated that Groups 32 showed higher preference ratio values than Groups 4 on session 7, $F(1, 42) = 8.07$, $p < 0.007$, and session 9, $F(1, 42) = 8.79$, $p < 0.005$, whereas on session 3, differences were marginally significant, $F(1, 42) = 4.03$, $p > 0.05$. With respect to the Drug by Session interaction, the analysis revealed significant E vs. W differences in all preshift sessions, and E vs. CDP differences on sessions 2, 3, 4, 5, 6, and 10. By contrast, Group CDP showed higher preference ratio compared to Group W on sessions 6 and 8, whereas differences were marginally significant on session 2, $p > 0.05$.

On postshift sessions, a Drug by Sucrose by Session analysis showed significant main effects of session, $F(4, 168) = 4.81$, $p < 0.001$, and drug, $F(2, 42)$

= 16.50, $p < 0.001$, as well as a significant session by drug interaction, $F(8, 168) = 2.03$, $p < 0.05$. The analysis of this interaction indicated that Group E showed higher preference ratio values compared to Group W on sessions 1-4, and to Group CDP on sessions 1, 2, and 4. By contrast, differences between Groups CDP and W were marginally significant on sessions 2 and 4, ps > 0.05.

Induction task (cSNC task). Figure 18 shows the values corresponding to sucrose consumption registered in the cSNC task for Groups E (A), CDP (B), and W (C). As expected, the 32% to 4% sucrose devaluation reduced fluid consumption in Group 32 compared to Group 4, an observation that was supported by statistical analyses. On preshift sessions, a Drug by Sucrose by Session analysis revealed a significant main effect for session, $F(9, 378) = 29.45$, $p < 0.001$, as well as a drug by sucrose significant interaction, $F(2, 42) = 3.54$, $p < 0.04$. The analysis of the interaction revealed statistically significant differences between Groups E vs. W receiving 4% sucrose (E > W). In addition, as opposed to Group CDP and E, in which no differences were found between Groups 32 and 4, Group W/32 showed higher fluid consumption than Group W/4, $F(1, 42) = 6.36$, $p < 0.02$. On postshift sessions, significant main effects of session, $F(4, 168) = 10.81$, $p < 0.001$, sucrose, $F(1, 42) = 16.69$, $p < 0.001$, and their interaction, $F(4, 168) = 3.48$, $p < 0.009$, were obtained. Further analysis of this interaction indicated that, regardless the drug condition, Groups 32 exhibited lower fluid consumption than Groups 4 on sessions 1, 2, and 3, lowest $F(1, 42) = 6.22$, $p < 0.02$.

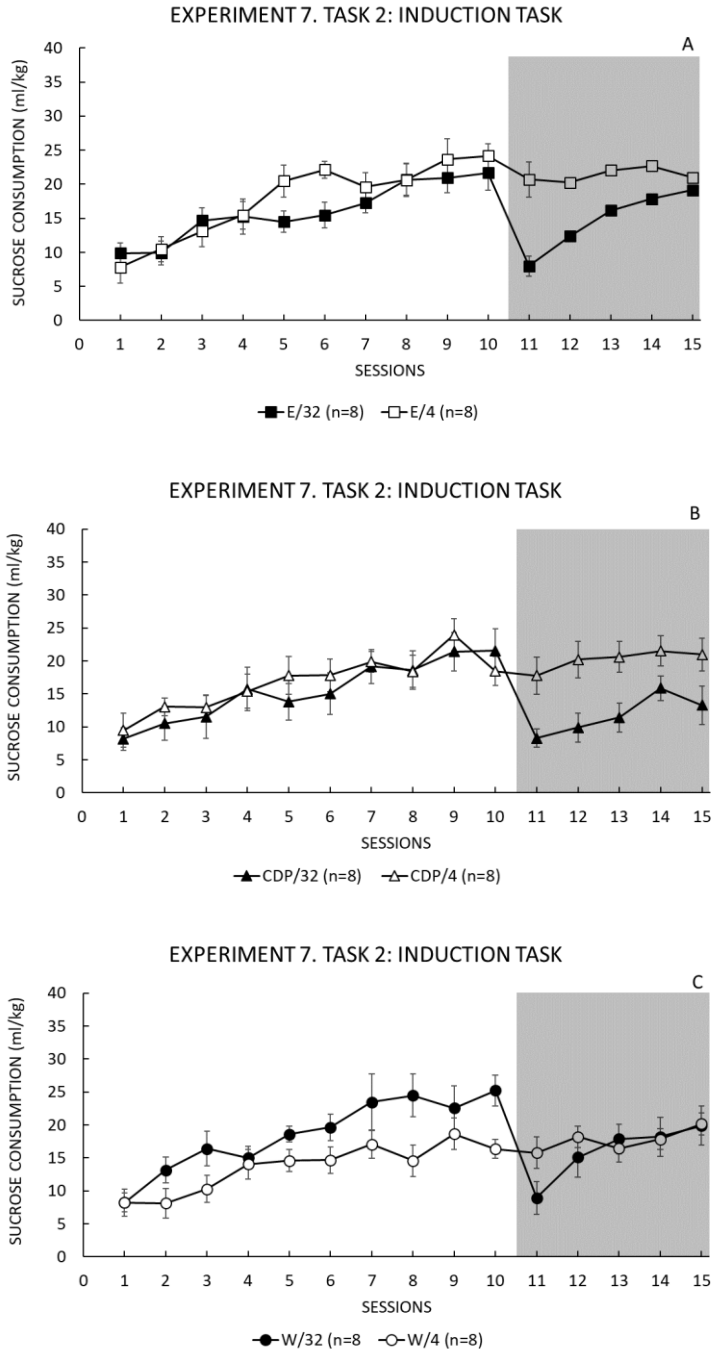

Figure 18. Means (±SEMs) of sucrose consumption during preshift (1–10) and postshift (11–15) sessions in the cSNC task, for Groups E (A), CDP (B), and W (C). The gray area marks postshift sessions.

Therefore, previous access to anxiolytics for free consumption did not affect the cSNC effect: regardless the drug condition, downshifted animals

exhibited lower fluid consumption in comparison to unshifted controls on postshift sessions 1, 2, and 3. This is the transient cSNC effect widely described in the literature (Flaherty, 1996). This provides no support for the anxiolytic assumption of the ESM hypothesis.

Experiment 8 used the same arrangement of tasks used in the present experiment, but it sought evidence for the anxiolytic assumption using repeated reward downshifts in the cSNC situation. It thus tested whether anxiolytic consumption attenuates the impact of reward loss when animals have repeated experience with both events in sequence: the preference test and the induction/frustrating task. Repeated experience may allow the organism to learn about the attenuating consequences of ethanol consumption on negative affect derived from reward devaluation, thus reducing the size or duration of the cSNC effect.

Experiment 8

Method

Subjects. The subjects were 32 male Wistar rats, experimentally naïve, purchased from Charles River (Barcelona, Spain). At the start of the experiment, rats were approximately 90 days old. The mean weight was 335.7 g (SEM ±1.5 g). Housing and maintenance were as described in Experiment 1.

Apparatus. The apparatus used in this experiment were the same described in Experiment 4 for the cSNC task and in Experiment 1 for the preference test.

Procedure. Rats were matched for weight and randomly assigned to one of four groups (n = 8): E/32, E/4, W/32, and W/4. During 18 days, each preference session (involving a choice between 2% ethanol and water; see Experiment 1)

was immediately followed by training in the cSNC situation (see Experiment 4). Animals labeled E/4 and W/4 received a 4% sucrose solution throughout the sessions (8 in preshift; 10 in postshift). Groups labelled E/32 and W/32 had access to 32% sucrose during the preshift sessions, but 32% and 4% sucrose alternated during postshift sessions (4% sucrose on odd-numbered sessions; 32% sucrose on even-numbered sessions). Everything else was as described in Experiment 1 for preference testing and in Experiment 4 for cSNC.

Dependent variables. In the preference test, fluid consumption and the preference ratio were calculated as described in Experiment 1. In the cSNC task, the dependent variable was the sucrose intake in ml/kg (see Experiment 4).

Statistics. Analyses of variance were calculated for each dependent variable with an alpha value set at the 0.05 level, and with LSD pairwise tests derived from the main analysis. Mean values registered in the preference test and the cSNC task were subjected to a three-factor analysis of variance, with Drug (E, W), Sucrose (32%, 4%), and Session as factors (8 in preshift; 10 in postshift). In addition, sessions in which groups W/32 and E/32 experienced reward downshift (that is, 4% sucrose) were compared with their respective E/4 and W/4 control groups. All statistical tests were calculated with the IBM SPSS package, V. 24.0.

Results and discussion

Preference test. Drug consumption, water consumption, and preference ratio data were plotted separately for Groups E/32 and E/4, and for Groups W/32 and W/4 (Figure 19a and 19b, panels A to F). In preshift sessions, a significant drug effect was obtained for drug consumption and preference ratio, Fs(1, 28) > 7.01, ps < 0.02, with higher values in Groups E compared to Groups W.

Significant effects of session, $F(7, 196) = 3.77$, $p < 0.001$, session by sucrose, $F(7, 196) = 2.37$, $p < 0.03$, and session by drug, $F(7, 196) = 2,18$, $p < 0.04$, were obtained relative to water consumption. Pairwise analysis derived from these interactions revealed significant differences between Groups 32 vs. 4 on session 2 (32 < 4), as well as significant differences between Groups E vs. W on session 8 (E > W). Analyses of postshift sessions yielded similar results, with main drug effects for drug consumption and preference ratio (E > W), Fs(1, 28) = 5.79, ps < 0.03, and a main session effect for water consumption, $F(9, 252) = 3.56$, $p < 0.001$. Finally, when sessions in which Groups W/32 and E/32 experienced reward downshifts were compared with their respective W/4 and E/4 controls, a significant drug effect was obtained for preference ratio values (E > W), $F(1, 28) = 5.82$, $p < 0.03$, and a main session effect appeared for water consumption, $F(4, 112) = 2.79$, $p < 0.03$.

Induction task (repeated reward downshifts). Figure 20 shows sucrose consumption in Groups E/32 and E/4 (A), and Groups W/32 and W/4 (B). On preshift sessions, a Session by Sucrose by Drug analysis only yielded a significant main effect for session, $F(7, 196) = 24.25$, $p < 0.001$, indicating a progressive increase in sucrose intake across sessions. Several analyses were computed on postshift data. First, odd-numbered sessions in which Groups W/32 and E/32 experienced reward downshift were compared with their respective E/4 and W/4 controls. There were significant effects of Session, $F(4, 112) = 21.39$, $p < 0.001$, Sucrose, $F(1, 28) = 30.03$, $p < 0.001$, and Session by Sucrose, $F(4, 112) = 2.82$, $p < 0.03$. Although pairwise LSD tests derived from this interaction revealed significant differences between Groups 32 vs. 4 in all sessions, Fs(1, 28) > 7.39, ps < 0.02, further analyses with drug condition collapsed indicated

that Group E/32 showed significantly lower sucrose consumption on sessions 9 , 11, and 13, *F*s(1, 28) > 4.77, *p*s < 0.04, but not on sessions 15 and 17, Fs < 1. By contrast, Groups W/32 and W/4 differed in each of the downshifted sessions, *F*s(1, 28) > 5.50, *p*s < 0.03.

These results provide partial support for the anxiolytic assumption of the ESM hypothesis. The size of the cSNC effect decreased across successive downshifts in animals previously receiving access to ethanol, but not in animals receiving access to water. However, there was no difference between the two alternating groups, W/32 vs. E/32, in downshifted sessions; ideally, access to ethanol should have also caused higher sucrose consumption in E/32 than in W/32. Thus, this effect must be considered with caution.

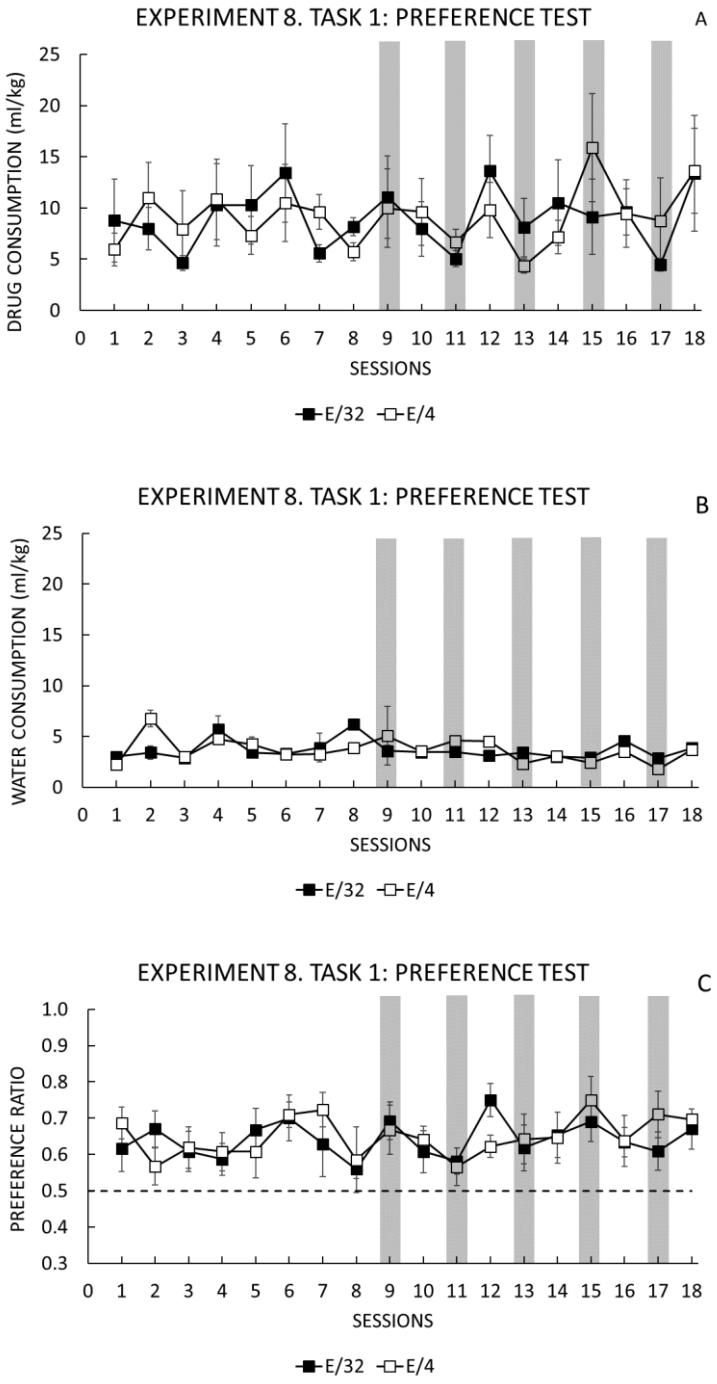

Figure 19a. Drug and water consumption (ml/kg), and preference ratio for Group E (panels A, B, C). The gray areas mark downshift sessions in the cSNC task.

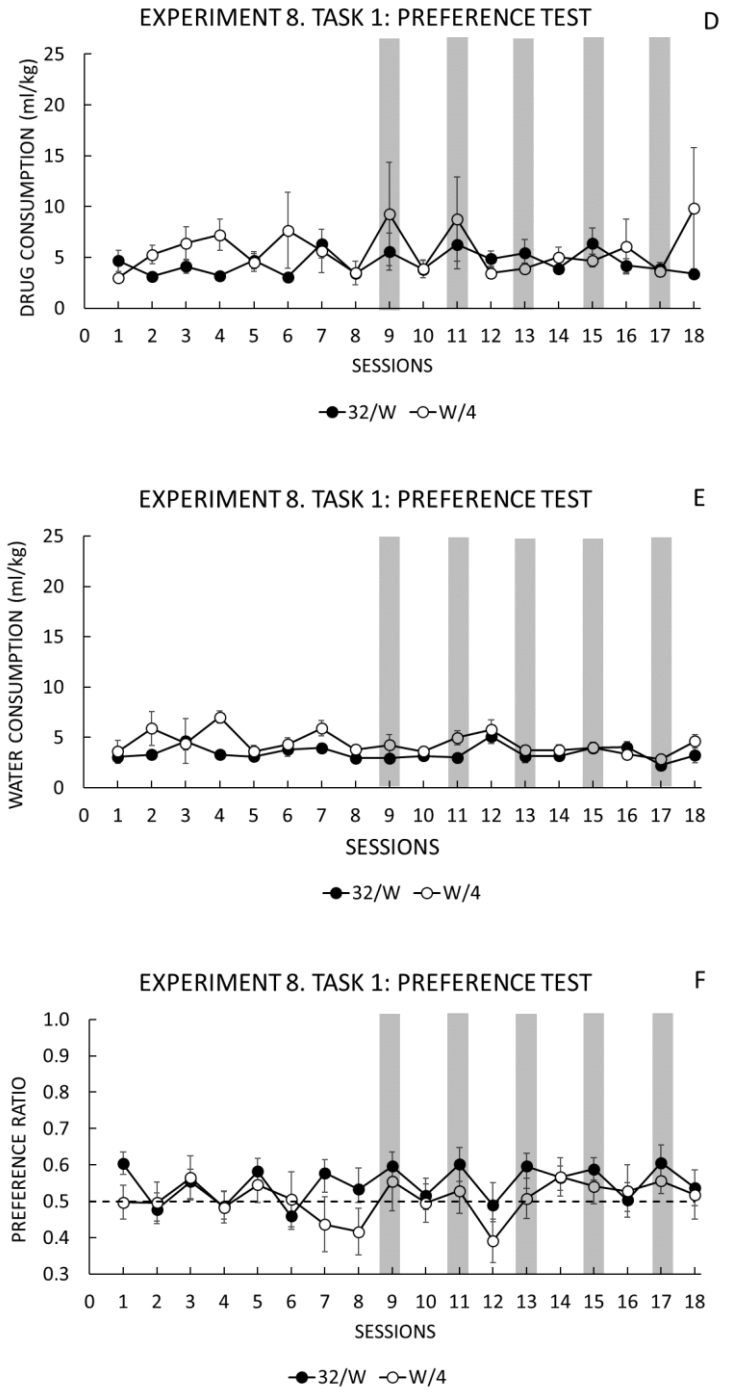

Figure 19b. Drug and water consumption (ml/kg), and preference ratio for Group W (panels D, E, F). The gray areas mark downshifted sessions in the cSNC task

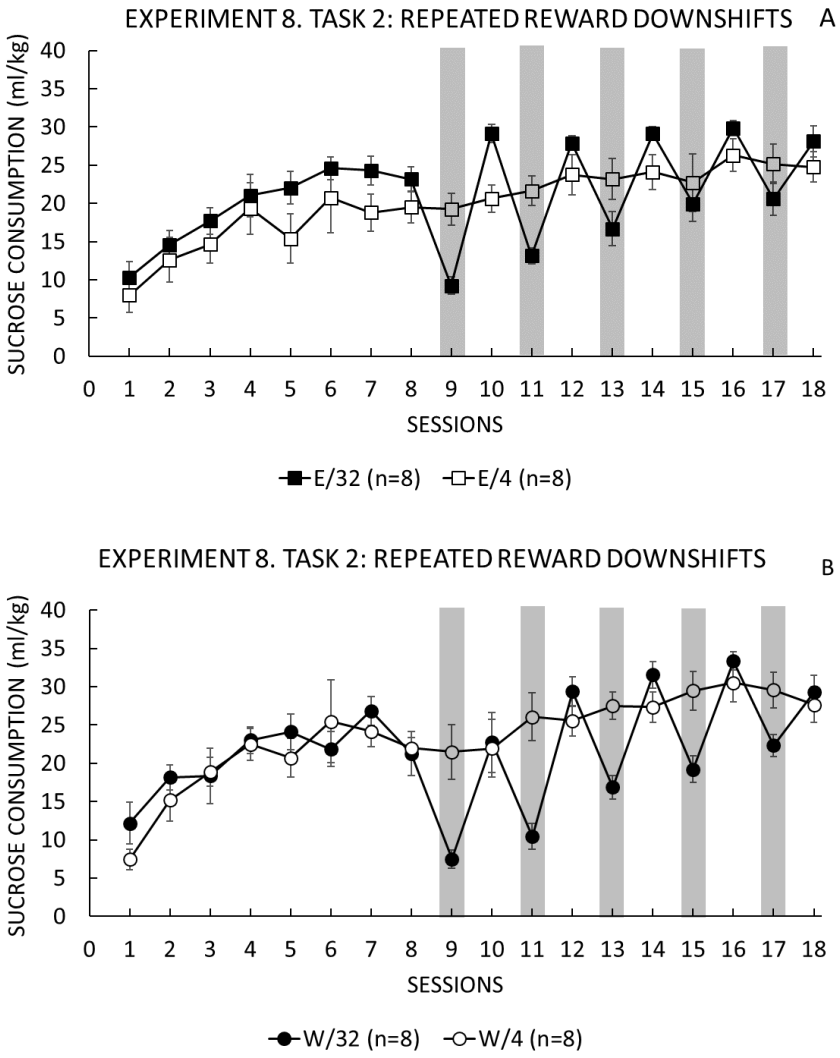

Figure 20. Means (±SEMs) of sucrose consumption during preshift (1–8) and postshift (9–18) sessions in the cSNC tasks. Groups differed in terms of the prior preference test: ethanol (E, panel A), or water (W, panel B). The gray bars signal the devaluation session in each cycle from 32% to 4% sessions in postshift phase.

Study 3: General discussion

A series of experiments designed to test the anxiolytic assumption underlying the ESM hypothesis produced limited results. The ESM hypothesis suggests that the induction of negative affect increases the preference for and

consumption of substances that reduce such emotional states (Torres & Papini, 2016). This hypothesis is based on several assumptions, including the so-called anxiolytic assumption, that states that the anxiolytics consumed in the preference test actually reduce the negative affect (frustration) induced by reward loss, thus reinforcing and maintaining drug intake behavior (Manzo et al., 2014; Manzo, Donaire et al., 2015; Manzo, Gómez et al., 2015). The approach chosen in the present experiments to test this assumption was to reverse the usual task sequence used in ESM experiments. Animals were first exposed to a preference test for anxiolytics, followed by an induction task involving negative emotion (EPM in Experiments 5-6; reward downshift in Experiments 7-8). Contrary to predictions, there was no evidence that previous free access to anxiolytic drugs (ethanol, CDP) reduced anxiety behaviors in the EPM test (Experiments 5-6), although ethanol significantly increased locomotor activity in both studies. Therefore, oral 2% ethanol had a nonspecific/disinhibitory effect on motor behavior (Karlsson & Roman, 2016). The lack of effect of ethanol and CDP on anxiety behaviors is in accordance with studies involving voluntary ethanol consumption (Wscieklica et al., 2016), but it contrasts with the consistent anxiolytic action obtained in studies based on forced administration of both drugs (Cárdenas & Navarro, 2002; Escarabajal, Torres, & Flaherty, 2003; Grahn et al., 2018; Komaki et al., 2014).

Experiment 6 replicated the increase in ethanol consumption that characterizes ESM induced by reward loss (Manzo et al., 2014; Manzo, Donaire et al., 2015; Manzo, Gómez et al., 2015), an effect that was not replicated with CDP. The increase in ethanol intake was accompanied by increased activity in the EPM on session 12. However, no effects on EPM performance were observed

on session 15, when behavioral evidence suggests that the negative affect induced by the reward downshift and the consequent increase in ethanol consumption had dissipated. Since closed-arm and total-arm entries were not reduced on session 15 relative to sessions 11–12 in Group W, the present results cannot be interpreted in terms of locomotor habituation, sensitization of fear/anxiety, or learned avoidance among other factors related to repeated testing (see Carobrez & Bertoglio, 2005; Escarabajal et al., 2003). Therefore, the present results suggest that consumption of ethanol under the same conditions used in previous ESM studies (i.e., 2% alcohol concentration, 2-h preference test; Manzo et al., 2014; Manzo, Donaire et al., 2015; Manzo, Gómez et al., 2015) affected general activity in the EPM (for further discussion see Donaire et al., 2018).

Experiment 7 showed no evidence that previous free access to ethanol or CDP significantly affected the behavioral suppression induced by reward devaluation during the subsequent induction task. The most parsimonious interpretation of this result is that there was some ineffective training parameter that, once identified and corrected, might yield evidence for (or against) the anxiolytic assumption of the ESM hypothesis. For example, the amount of CDP and water consumed by CDP groups was similar, suggesting that animals had difficulty discriminating these fluids. The absence of effect of cSNC on CDP consumption could thus be explained in terms of low levels of fluid intake in CDP animals. However, animals exposed to ethanol clearly preferred and consumed more ethanol than water immediately before they were tested in the cSNC task, but this made no difference with respect to CDP. Alternatively, it is possible that consuming low or moderate amounts of anxiolytics in the absence of negative affect has little or no impact on subsequent emotional states. However, the results

obtained in Experiments 5-6 suggest that ethanol affected performance in the EPM test regardless of whether the consumption occurred in the presence of negative affect. Whether positive results could be obtained by increasing the dose of CDP or ethanol used in the preference test or by inducing negative affect before the preference test will have to be determined in future experiments.

The lack of conclusive effects could also lie on the anxiety tests chosen in the present experiments. Pharmacological (Flaherty et al., 1990; Pellow & File, 1986), molecular (Glueck et al., 2015; Hale, Bouwknecht, Spiga, Shekhar, & Lowry, 2006), and neurobiological studies (Kawasaki et al., 2015) have validated the EPM and cSNC tasks as animal models of anxiety. However, it is unclear whether these two situations activate the same type of anxiety. In fact, performance in the EPM is typically described in terms of unconditioned anxiety, whereas the cSNC involves conditioned anxiety, as the effect depends on the violation of a learned expectancy (Flaherty, Greenwood, Martin, & Leszczuk, 1998). Furthermore, the nature of the anxiety involved in EPM seems to change with experience with the apparatus (File, 1995), a fact that could explain the differential effects of ethanol observed on sessions 11-12 compared to session 15. Therefore, the present results provided no support for the assertion that previous free access to ethanol or CDP would reduce negative affect as registered in the EPM or cSNC tasks.

Finally, Experiment 8 was based on previous evidence showing that repeated experience with reward devaluation does not affect cSNC, and that the anxiolytic properties of CDP and ethanol increase as a function of repeated downshift events. In Flaherty et al.'s (1996) study, animals received eight cycles of repeated reward reduction and CDP administration. Animals exhibited

evidence of cSNC throughout all the downshifts, but the anti-contrast properties of CDP during the first downshift session were observed only in later downshift events. In the same vein, Flaherty, Becker, and Checke (1983) reported that negative contrast occurred if the animals were alternatively shifted between 32% and 4% sucrose, regardless of whether they were exposed to a single or a double alternation schedule. In agreement with these results, previous free access to ethanol abolished the cSNC effect after repeated cycles in Experiment 8. These data suggest that drug intake can affect subsequent negative affect only if the animal has repeated experience with both the preference test and the induction task. As stated above, repeated experience may allow the organism to learn about the attenuating consequences of ethanol consumption on negative affect. However, since the performance of downshifted animals with prior access to ethanol did not differ from those with prior access to water, the present results were only partially consistent with the anxiolytic assumption of the ESM hypothesis.

In summary, the present experimental series provided encouraging, but not definitive evidence that voluntary oral access to anxiolytics reduce negative affect triggered by reward loss. Their value lies in suggesting experimental conditions that might be more appropriate to test the anxiolytic assumption underlying the ESM hypothesis of drug intake.

Extending protocols in terms of both the induction tasks and the substances available in the preference test are needed to determine the generality of ESM effects. In this respect, other forms of reward loss not involving consummatory behavior (e.g., extinction behavior in a runway), situations inducing emotional activation (e.g., changes in restraint stress conditions), and

sources of chronic physical pain (e.g., tail flick test) are alternatives worth exploring in connection with the induction of preference for and consumption of substances that modulate negative affect. Similarly, substances known to modulate reward loss when administered systemically (e.g., BZDs, opioids, and cannabinoids; Flaherty, 1996; Genn et al., 2004; Papini, 2009) are candidates for inclusion in the preference test. Interestingly, substances known to have anxiogenic effects could be used to test a prediction derived from the ESM hypothesis that their consumption should diminish selectively during periods of loss-induced anxiety. Caffeine provides a good example of a widely used anxiogenic substance with detectable emotional effects in rodent behavior (e.g., Hughes & Hancock, 2017).

Validating the main assumption underlying animal models of ESM will provide insights into the factors contributing to the emergence of addictive behavior. The ESM hypothesis suggests that coping with episodes of reward loss may be one of the forces driving the early development of substance abuse. Given the individual and social importance of drug addiction, the ESM hypothesis may ultimately provide insights into the prevention of such conditions.

Study 4. How can ESM be reduced?

Experiment 9

Experiments 4-6 indicated that reward devaluation induces voluntary consumption of anxiolytics in animals. This behavior is conceptualized as a form of ESM, provided that: (1) it was limited to transient periods of negative affect (as it matches with the also transient behavioral inhibition registered in the induction task) and (2) it was selective for drugs with anti-anxiety properties (based on the lack of effects observed in water intake). These results are in accordance with previous studies showing increased ethanol intake in high-anxiety RLA-I rats vs. low-anxiety RHA-I rats exposed to both consummatory and instrumental reward omission tasks (Manzo et al., 2014). ESM induced by reward loss constitutes thus a consistent behavioral phenomenon that can be extended to Wistar rats, to reward downshift paradigms, and to BZDs consumption. This effect seems to be dependent on the reinforced properties derived from the reduction of negative affect (Experiment 8), and can be useful to explore how this mechanism underlies the onset, progression, and/or relapse into a SUD.

The next step in this Dissertation was to explore experimental conditions that can reduce or abolish ESM. This approach will help identify behavioral interventions with potential protective effects against drug use induced by stressful daily events of reward loss in humans. Experiment 9 addressed this question by exposing animals to PR training involving a quasi-random alternation of reinforced (R) and nonreinforced (N) trials. PR experience has been repeatedly shown to attenuate the disruptive effects of subsequent reward loss, being considered as an effective treatment for developing resilience to loss-induced negative affect (Glueck, Torres, & Papini, 2018; Papini et al., 2006). Several PR-related phenomena have been developed in the laboratory. The PREE, for

example, consists of greater resistance to extinction after acquisition under PR than under CR (Amsel, 1992; Jenkins & Stanley, 1950). Similar effects have been reported in reward devaluation situations: training animals under PR preshift conditions significantly reduce the size of the SNC effect in comparison with CR. This result is referred to as the PRCE and has been shown in both instrumental and consummatory tasks (Mikulka, Lehr, & Pavlik, 1967; Pellegrini et al., 2004). Both the PREE and the PRCE seem to depend on the negative emotional effect derived from the uncertainty characterizing PR training (Amsel, 1992; Gray, 1987; Leslie, Shaw, McCabe, Reynolds, & Dawson, 2004). According to this view, when a response is nonrewarded in the presence of a reward expectancy (as occurring in animals receiving PR in N trials), an aversive internal state of primary frustration is induced. The pairing of initially neutral contextual stimuli with this emotional reaction would enable these stimuli to trigger an expectancy of frustration, called secondary or anticipatory frustration. Secondary frustration would interfere with the performance of the previously learned response of approach to the goal. However, the occurrence of an R trial in presence of secondary frustration would increase tolerance to frustration through a counterconditioning process, based on the pairing between stimuli with opposite hedonic value (Rick, Donaire, Papini, Torres, & Pellón, 2018). Therefore, counterconditioning may be the mechanism underlying response persistence during extinction after chronically uncertain conditions (see Papini, 2006).

Several sources of evidence suggest that the impact of PR on behavior is dependent on negative emotional processes. First, both the PREE and the PRCE are abolished or attenuated by the administration of anxiolytic drugs, including BZDs, barbiturates, and ethanol (Feldon, Guillamon, Gray, de Wit, &

McNaughton, 1979; Gray & Smith, 1969; Gray & Dudderidge, 1971; Ison & Pennes, 1969; McNaughton, 1984; Pellegrini et al., 2004). According to the frustration hypothesis of PR, both the PREE and the PRCE failed to occur because anxiolytic drugs prevented the animals from learning to tolerate frustration during PR training, as the response of frustration was reduced by the drug (Leslie et al, 2004). Second, frustrative nonreward derived from PR activates the HPA axis, increasing plasma levels of ACTH and corticosterone compared to CR (Earley & Leonard, 1979). Paradoxically, the PREE was abolished by injecting ACTH daily during acquisition trials (Gray, Mayes, & Wilson, 1971). Third, lesions of brain regions involved in emotion regulation (including the septo-hippocampal system, the dorsal noradrenergic bundle, and the orbitofrontal cortex, among others) eliminate the PREE in a variety of experimental situations (Feldon, Rawlins, & Gray, 1985; Gray, Quintão, & Araujo-Silva, 1972; Henke, 1977; Owen, Boarder, Gray, & Fillenz, 1982; Ortega, Glueck, Uhelski, Fuchs, & Papini, 2013; Rawlins, Feldon, & Gray, 1980; Swanson & Isaacson, 1967; Lobaugh, Bootin, & Amsel, 1985). Fourth, performance differences in response to PR vs. CR have been reported in Long-Evans rats selectively bred on the basis of differences in recovery rates from cSNC (fast vs. slow): no evidence of increased behavioral persistence after PR was found in rats with fast recovery rates, as opposed to slow recovery and control (random) animals (Ortega, Norris, López-Seal, Ramos, & Papini, 2014). In the same vein, only the more anxious RLA-I strain (as opposed to their RHA-I counterparts) showed the PREE (Gómez et al., 2008; Manzo, Gómez et al., 2015) and the PRCE (Cuenya et al., 2012). Most importantly, RLA-I animals exposed to PR during the acquisition phase of an instrumental appetitive task displayed lower ethanol consumption after reward

omission (extinction) sessions compared with CR animals (Manzo, Gómez et al., 2015). These results suggest that repeated frustration (PR) can reduce ESM in rats genetically selected for high levels of anxiety.

In the present experiment, we analyzed whether the reducing effect of PR on ESM extends to Wistar rats and to consummatory reward devaluation situations. Animals received 32% sucrose under PR or CR conditions, and were then downshifted to 4% sucrose. The effect of this induction task on ethanol intake was tested in a subsequent preference test. For two groups, one bottle contained tap water and the other 2% ethanol. For two other groups, both bottles contained water. Based on the evidence reviewed above, I predicted increased resistance to reward downshift and lower ethanol consumption in rats exposed to PR vs. CR, whereas PR and CR groups receiving water in the preference test would not exhibit any change in fluid intake.

Method

Subjects. The subjects were 32 male Wistar rats, experimentally naïve, purchased from Charles River Laboratories (Barcelona, Spain). At the start of the experiment, rats were approximately 90 days old. The mean weight was 356.5 g (SEM ±2.1 g). Housing and maintenance were as described in Experiment 1.

Apparatus. The consummatory task was conducted in the boxes described in Experiment 4. The preference test was as described in Experiment 1.

Procedure. Rats were matched by weight and randomly assigned to one of four groups (n = 8): 32C/E, 32P/E, 32C/W, and 32P/W. Groups 32C/E and 32C/W received daily access to 32% sucrose in each of the 20 preshift trials. Groups 32P/E and 32P/W received access to 32% sucrose solution on half of the

trials (R trials); on the remaining trials, these rats had access to distilled water (N trials). Groups were counterbalanced across days, although PR groups were always trained before the CR groups in N trials to minimize sucrose residue. The sequence of R and N trials was as follows: R N R R N N R N R N N R N R R N R N N R (see Pellegrini et al., 2004). During 10 postshift sessions, all the rats were downshifted and received 4% sucrose.

After each session in the consummatory task, animals received a two-bottle preference test as described in Experiment 1. One bottle contained 2% ethanol and the other contained tap water (Groups 32C/E and 32P/E); or both bottles contained water (Groups 32C/W and 32P/W).

Dependent variables. In the consummatory task, the dependent variable was the amount of sucrose solution consumed (ml/kg; see Experiment 4). In the preference test, fluid consumption and the preference ratio were calculated as described in Experiment 1.

Statistics. Analyses of variance were calculated for each dependent variable with an alpha value set at the 0.05 level, and with pairwise LSD tests derived from the main analysis. The mean values corresponding to the dependent variables registered in the reward devaluation task and the preference tests were subjected to a three-factor analysis of variance, with Drug (E, W), Reinforcement (PR, CR), and Session as factors (2 × 2 × 20 in preshift phase; 2 × 2 × 10 in postshift phase). Separate analysis for R and N trials were also conducted. All statistical tests were computed with the IBM SPSS package, V. 24.0.

Results

Induction task (reward downshift). Figure 21 plots sucrose consumption for CR and PR groups receiving ethanol (A) or water (B). There was a clear

influence of PR training on preshift sucrose consumption and also an apparently slower recovery from reward devaluation in Groups PR relative to CR (postshift sessions). These observations were confirmed by statistical analyses. On preshift sessions, a Session by Drug by Reinforcement analysis revealed main session, $F(19, 532) = 47.29$, $p < 0.001$, and reinforcement effects, $F(1, 28) = 73.53$ $p < 0.001$, and also a significant session by reinforcement interaction, $F(19, 532) = 43.13$, $p < 0.001$. Further analysis of this interaction showed significant differences between Groups CR and PR on N sessions (sessions 2, 5, 6, 8, 10, 11, 13, 16, 18, and 19; Fs$(1, 28) > 28.73$, $p < 0.005$). On postshift sessions, a Session by Drug by Reinforcement analysis yielded significant effects for session, $F(9, 252) = 25.89$, $p < 0.001$, and reinforcement by session, $F(9, 252) = 2.60$, $p < 0.007$. This interaction resulted from significant differences between Groups CR and PR on postshift session 3; the effect was marginally significant on session 1, $p > 0.05$, showing higher sucrose intake in Group CR than in Group PR.

Preference test. Figure 22a and figure 22b (panels A to F) represents the results obtained in the preference test for 32C/E, 32P/E, 32C/W, and 32P/W groups, including drug consumption, water consumption and preference ratio. The inspection of this figure suggests a sharp increase in ethanol consumption and preference ratio in most of the downshift sessions in the CR group exposed to ethanol, as opposed to partially reinforced E group, and to groups receiving only W (32C/W and 32P/W).

A Drug by Reinforcement by Session analysis conducted with data collected in preshift sessions showed: (1) a significant session effect for drug consumption values, $F(19, 532) = 2.40$, $p < 0.001$; (2) a significant drug effect for preference ratio values, $F(1, 26) = 7.65$, $p < 0.01$; and (3) significant effects for

session, $F(19, 532) = 2.79$, $p < 0.001$, drug, $F(1, 28) = 4.18$, $p < 0.05$, and session by reinforcement, $F(19, 532) = 1.61$, $p < 0.05$, with respect to the dependent variable water consumption (higher water consumption in W vs. E, and in CR vs. PR on sessions 8 and 18).

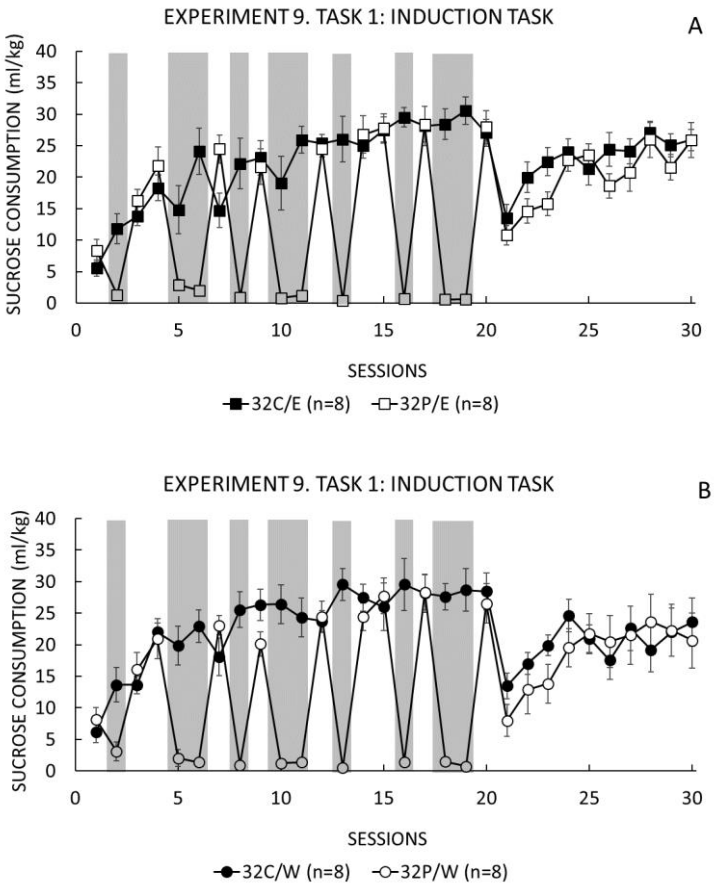

Figure 21. Means (±SEMs) of sucrose consumption during preshift (1–20) and postshift (21–30) sessions of the cSNC task. Groups differed in terms of the preference test: ethanol (E, panel A), or water (W, panel B). The gray area marks N sessions in groups receiving PR training in preshift sessions.

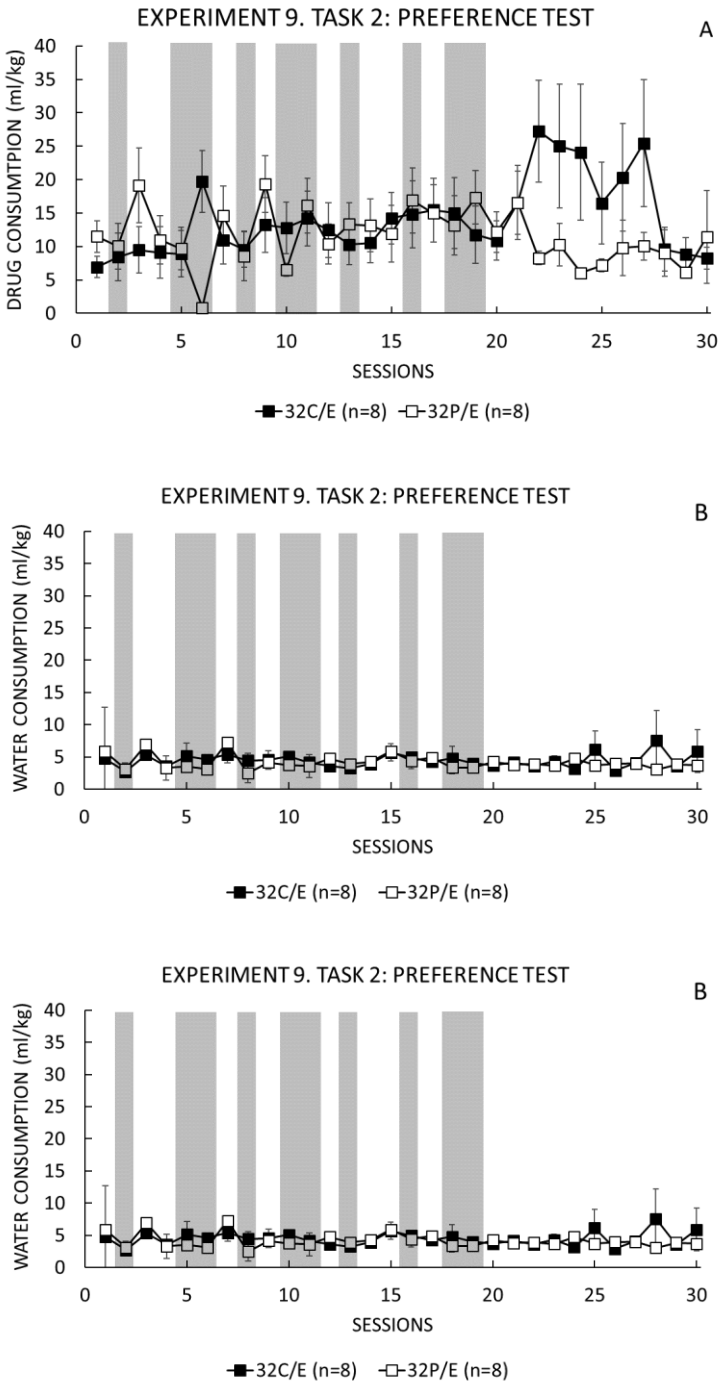

Figure 22a. Alcohol and water consumption (ml/kg), and preference ratio for Groups E (panels A, B, C). The gray areas mark N sessions for Groups PR in preshift sessions of the cSNC task.

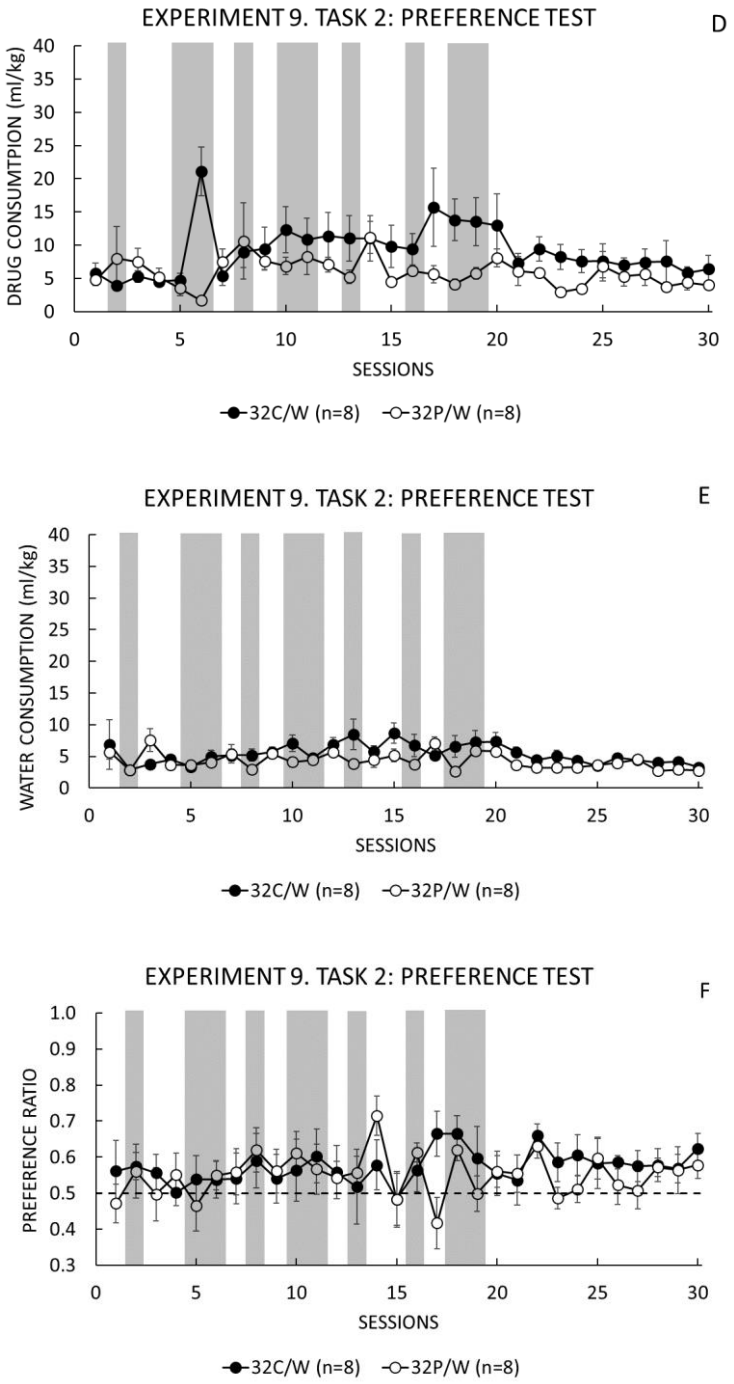

Figure 22b. Fluid consumption (ml/kg) and preference ratio for Groups W (panels D, E, F). The gray areas mark N sessions for Groups PR in preshift sessions of the cSNC task.

On postshift sessions, similar drug consumption analyses yielded significant effects for session, $F(9, 252) = 2.33$, $p < 0.02$, drug, $F(1, 28) = 6.54$, p

< 0.02, and session by reinforcement, $F(9, 252) = 2.33$, $p < 0.02$. Although the triple interaction, drug by reinforcement by session, was marginally significant, $p > 0.07$, a comparison between Groups E and W across sessions (with the reinforcement condition collapsed) showed higher fluid intake in Group 32C/E than in Group 32C/W on postshift sessions 2, 3, 4, 6, and 7, Fs (1, 28) > 4.12, $p < 0.05$, but no differences between Groups 32P/E and 32P/W. In addition, the comparison between Groups PR and CR across sessions (with the drug condition collapsed) yielded significant differences between CR vs. PR exposed to ethanol on sessions 2, 3, 4, and 7 (32C/E > 32P/E), Fs(1, 28) > 4.41, ps < 0.05, but not between CR vs. PR exposed to water. Similar results were obtained when preference ratio values were analyzed. A Drug by Reinforcement by Session analysis showed significant effects for drug, $F(1, 26) = 5.74$, $p < 0.03$, and reinforcement by session, $F(9, 234) = 2.17$, $p < 0.03$, revealing higher preference in E vs. W, and higher preference in CR vs. PR on session 4, $F(1, 26) = 5.14$, $p < 0.04$. The triple interaction did not reach statistical significance, although a comparison between PR and CR across sessions (with the drug condition collapsed) yielded significant differences between groups exposed to ethanol on postshift session 4 (32C/E > 32P/E), $F(1, 26) = 5.51$, $p < 0.04$). Finally, no significant effects were obtained when water consumption data were analyzed.

Study 4: Discussion

The present experiment was designed to determine whether PR training could attenuate the impact of reward devaluation on ESM in Wistar rats. Reduced response to reward downshift and lower alcohol consumption were expected to occur in PR animals compared to CR animals. Contrary to our prediction, PR training slightly delayed recovery from reward devaluation, as shown by

significantly less sucrose intake in PR vs. CR on postshift session 3. However, in agreement with a prediction based on the ESM hypothesis, CR training induced higher ethanol intake on postshift sessions 2, 3, 4 and 7 than PR training. This shows the attenuating effects of PR on ESM induced by reward devaluation. In agreement with a previous study (Manzo, Gómez et al., 2015), the present results suggest that reward uncertainty derived from PR training can be an effective strategy to reduce ethanol intake induced by reward loss.

The PREE has been considered as a paradoxical form of learning, and several explanations in terms of emotional processes have been proposed. According to Amsel (1992), reward inconsistency (including PR) increases resistance to extinction because the association between secondary frustration and reward that characterizes PR procedures facilitates counterconditioning. Counterconditioning occurs during pairings between stimuli of opposite hedonic value, as first reported by Pavlov (1927). Pavlov paired electric shocks (aversive) with food delivery (appetitive) and dogs eventually salivated to the delivery of a shock. Similarly, since anticipatory frustration promotes avoidance and anticipatory reward promotes approach, counterconditioning would facilitate the development of approach to stimuli that evoke anticipatory frustration, thus generating persistence under conditions of PR (Papini, 2003). The fact that treatment with anxiolytics during acquisition disrupts the PREE and the PRCE supports this theoretical explanation, as the drugs would impair counterconditioning by reducing the intensity of the frustration present in N sessions (Gray & Smith, 1969; Iwahara, Nagamura, & Iwasaki, 1967; Pellegrini et al., 2004).

In the present experiment, only weak differences between PR and CR groups were found in the reward downshift task and they went in the opposite direction to that expected (i.e., slower recovery from reward devaluation in PR than in CR animals). Although speculative, anxiolytic effects derived from ethanol consumption after N trials could have reduced counterconditioning in Group 32P/E, thus abolishing the PRCE in a similar way to that obtained by presession forced administration of CDP (Pellegrini et al., 2004). However, since the results were obtained regardless of the drug condition in the preference test (ethanol or water), this explanation should be taken with caution.

There are precedents of reversed PREE during consummatory extinction after being offered sucrose solutions as reinforcer in rats (Mustaca, Freidin, & Papini, 2002). A reversed PRCE was also found in a recent study in which animals receiving PR vs. CR in a Pavlovian taste conditioning task were subsequently transferred to a cSNC task, both tests involving licking response. Animals exposed to PR in the taste conditioning task showed increased consummatory suppression after reward downshift in comparison with continuously reinforced animals (Experiments 6-7; Glueck et al., 2018). Whether or not the reversed PREE obtained in the present study is a replicable finding and/or reveal an anxiolytic effect of ethanol will have to be determined in future experiments.

The fact that ethanol consumption and preference were higher in CR animals than in PR animals can be explained in terms of ESM (Torres & Papini, 2016). As discussed elsewhere (Manzo, Gómez et al., 2015), reward devaluation after CR would have led to high levels of frustration, thus promoting increased ethanol intake over water based in its anxiolytic properties. PR training would

have reduced frustration during the reward devaluation task, thus reducing of impact of reward downshift on ethanol consumption. The preventive effect of PR on ESM could have important clinical implications: Increasing resilience to frustration via PR could be used as a potential behavioral intervention to protect against drug use triggered by negative experiences of reward loss.

Study 5. What brain areas regulate ESM?

Experiment 10

See Appendix C

Donaire, R., Morón, I., Blanco, S., Villatoro, A., Gámiz, F., Papini, M. R., & Torres, C. (2019). Lateral habenula lesions disrupt appetitive extinction, but do not affect voluntary alcohol consumption. *Neuroscience Letters, 703*, 184-190.

Chapter 4. General discussion

General discussion

The consumption of legal drugs constitutes a widely accepted practice in occidental societies. Alcohol is frequently consumed on weekends, on special events, and on major festivities. According to the Spanish Observatory of Drugs and Addictions (2017), 91% or population drank at least once in their lives, 7.4% are daily drinkers, and the drug was involved in 30% of deaths attributable to motor vehicle accidents. Alcohol consumption is often associated with multiple myths and false beliefs about its benefits. For example, most of people think that taking a small amount of alcohol per day (e.g., a glass of wine) has a protective effect and prevents cardiovascular disease. This is a controversial issue discussed by both researchers and doctors with defenders and critics. However, it is widely accepted that the excessive consumption of alcohol leads to an SUD with multiple negative consequences for both individuals and society (Global Burden of Disease, 2018).

Understanding addiction constitutes a great challenge for both basic and clinical neuroscientists. Some consider drug addiction as neurobehavioral disorder originated in the brain (e.g., Volkow & Morales, 2015), while others do not (see Becoña, 2018; Hall, Carter, & Forlini, 2015). For example, Lewis (2017) argues that the brain disease model of addiction is flawed because brain changes in addiction are similar to those generally observed when recurrent, highly motivated goal seeking results in the development of strong habits. According to this view, addictions are bad habits than can be broken based on people's autonomous choices and in spite of the "negative" influence of genes, environments, and/or brain changes related to addiction (Fenton & Wiers, 2017).

Most of studies focus on drug consumption when the pattern is abusive and a withdrawal syndrome emerged (Belin-Rauscent, Fouyssac, Bonci & Belin, 2016; Kamenetzky & Mustaca, 2006). By contrast, there is a paucity of information about onset of drug use. In the same way as positive events (e.g., leisure, socialization, etc.) are associated with alcohol consumption (Díez-Hernández, 2003), the occurrence of negative life events also correlates with alcohol abuse (Enman et al., 2014). Imagine a person who had a bad day and consumed alcohol to relieve the discomfort and feel better; once the discomfort vanishes, that person does not need to keep drinking. This example underlines the basic assumption of the ESM hypothesis of drug abuse (Khantzian, 1985). According to this hypothesis, humans view drug use as appealing because of its ability to relieve psychological suffering (Khantzian, 2003, 2013). Recent scientific approaches include events of reward loss as significant sources of psychological pain or frustration that would underlie the onset of drug taking (Torres & Papini, 2016). This Dissertation systematically analyzed the role of reward loss on anxiolytic consumption in Wistar rats. Animals were exposed to induction tasks involving reward devaluation or omission, followed by free access to anxiolytics for voluntary consumption. Ten experimental studies were conducted and the main results were the following.

First, the experimental conditions necessary to obtain significant consumption of and preference for alcohol and CDP vs. water were analyzed (Experiments 1-3). According to previous studies (Manzo et al., 2012), animals consumed more alcohol (2%) than water under baseline conditions, that is, in the absence of negative affect induced by reward loss (Experiment 1). Inconsistencies were found with respect to CDP intake; although animals

preferred CDP over water, the pattern of fluid consumption did not depend on the drug dose (Experiments 1-2) or the presentation mode (Experiment 3).

Second, the impact of reward loss on anxiolytic consumption was analyzed by exposing animals to a sucrose devaluation task (cSNC) followed by a preference test involving free access to alcohol vs. water, CDP vs. water, or water vs. water. Unexpected reward downshift (from 32% to 4% sucrose) induced a transient increase in alcohol and CDP consumption and preference, as opposed to the lack of effect obtained in animals receiving only water or exposed to unshifted reward conditions (4% sucrose). These results were consistent with previous studies (Manzo et al., 2014) and showed for the first time that frustration associated with incentive loss promote BZD (CDP) voluntary intake in nonhuman animals, thus providing evidence consistent with the ESM hypothesis (Manzo, Donaire et al., 2015).

The anti-anxiety/frustration effects of alcohol and CDP were assumed to underlie the ESM behavior (Torres & Papini, 2016). This assumption was systematically analyzed in an experimental series in which the order of the tasks (induction and preference) was reversed. Experiments 5 to 8 showed that prior free access to anxiolytics (1) significantly increased locomotor activity in the EPM test for anxiety (regardless whether or not animals were under a frustration state); (2) did not affect subsequent cSNC when animals experienced a single event of reward downshift; and (3) reduced the impact of reward devaluation after repeated downshift trials. These results partially confirmed the anxiolytic assumption derived from the ESM hypothesis (Donaire et al., 2018).

Experiment 9 explored whether developing resilience to frustration via partial reinforcement training would reduce ESM induced by reward devaluation.

Animals received PR vs. CR during preshift sessions of a reward devaluation task involving 32% sucrose, followed by a preference test in which animals received alcohol vs. water (or water vs. water for control groups). Unlike CR animals, animals that received PR did not increase alcohol consumption after experiencing reward devaluation (4%). These PR vs. CR differences were not obtained in animals receiving only water in the subsequent preference test. In agreement with a previous study (Manzo, Gómez et al., 2015), the present results suggest that PR training could be useful to prevent ESM induced by reward loss.

Finally, the exploration of the biological basis of ESM was initiated by studying the role of the LHb on appetitive extinction and alcohol consumption. The LHb has been implicated in the processing of reward omissions (Matsumoto & Hikosaka, 2007) and alcohol intake (Friedman et al., 2010, 2011). In the present experiment, LHb lesions increased resistance to consummatory and instrumental extinction, but did not affect alcohol consumption, regardless of the concentration (2-24%) or the duration of the preference test (2 h vs. 24 h).

The present results suggest that animal models based on the unexpected loss of rewards can be useful to systematically analyze the psychobiological basis of ESM. Such studies promise to shed light on the role of negative affect in the onset of anxiolytic use.

Despite the promise of the present results, some inconsistences and negative results will have to be addressed in future experiments. First, although animals showed an increased preference for alcohol vs. water after experiencing reward devaluation, low levels of fluid intake were registered in most of studies. It is thus uncertain whether or not pharmacologically relevant blood alcohol concentrations were reached in the present experiments. This issue will be

explored in the future by collecting blood samples and analyzing alcohol concentration in the ESM task. Similarly, neither a stable dose-dependent consumption nor an influence of the drug mode of presentation (continuous vs. intermittent) was obtained with respect to CDP. Increased CDP consumption and preference induced by reward devaluation was also inconsistent (unpublished data not shown here). Therefore, future experiments will have to systematically analyze the source of these inconsistent results. Such experiments should include, for example, a better dose adjustment (according to interspecies dose conversion protocols; Nair & Jacob, 2016), an analysis of the palatability and pharmacokinetic properties of the drug (Greenblat, Shader, MacLeod, & Sellers, 1978), and a systematic study of additional experimental procedures aimed at increasing CDP intake (e.g., unlimited 24-h availability, forced choice, and their combination with intermittent access; Wise, 1973). Whether the presence of negative affect is a necessary condition for ESM with CDP also needs to be addressed in the future.

Unlike the inconsistences previously described, reward devaluation repeatedly increased alcohol consumption in the present studies (Experiments 4, 6, and 9), thus indicating that ESM is a replicable phenomenon whose boundaries will have to be further analyzed (including parametric studies that manipulate alcohol dose, interval between induction and preference tasks, pre-postshift reward disparity, preference test duration, etc.).

However, that the anxiolytic effects of alcohol and CDP underlie ESM behavior was only partially supported in the present Dissertation. Of all the experiments conducted to clarify this problem (Experiments 5-8), only one (Experiment 8) suggested that previous access to alcohol mitigated the emotional

impact of subsequent aversive events (repeated reward downshifts). As indicated above, extending protocols in terms of both the induction task, the substances available in the preference test, and/or its duration will allow a further analyses of the mechanisms responsible for ESM behavior (Donaire et al., 2018).

One of the most relevant hypotheses included in this Dissertation was that a reduction in the intensity of the reward downshift experience should attenuate ESM. Experiment 9 tested this hypothesis by training animals under PR vs. CR conditions and giving them subsequent free access to alcohol. Contrary to our prediction, PR did not reduce the impact of reward devaluation in the consummatory induction task, although it reduced alcohol intake in the preference test. This pattern of results showed a dissociation between the induction task and the preference test that will have to be analyzed in future experiments. In any case, the protective effect of PR on alcohol intake suggests that increasing tolerance to frustration could be used as a potential behavioral intervention against drug use triggered by negative affect derived from reward loss. Including experimental manipulations aimed at increasing the benefits of PR (e.g. administering vehicle after N trials) or identifying alternative interventions to also promote resilience (e.g., physical activity) will open up new avenues for future research on ESM prevention (Darlington et al., 2016; Ruiz-Juan & Ruiz-Risueño, 2011; Smith & Lynch, 2012).

Finally, the involvement of the LHb in reward omission situations (Experiment 10) was a preliminary study that will be extended in the future. Experiments in which animals have access to anxiolytics immediately after experiencing reward devaluation or omission will establish the role of the LHb in alcohol intake induced by negative affect. Similarly, the temporal inactivation of

the LHb or its disconnection from other brain structures will allow us to further explore its specific role in ESM situations (including the processing of the absolute value of the reward, the comparison between different reward magnitudes, the modulation of the emotional reaction to incentive loss, and/or the regulation of drug intake). As previously discussed, there has been a lack of attention to the role of reward loss in the onset of drug use and abuse, despite the fact that (1) many addictive drugs modulate the impact of reward loss and (2) several brain areas regulate both reward loss and drug abuse, including prefrontal cortex, AMG, insular cortex, striatum, NAc, and LHb, among others. The present Dissertation therefore suggests avenues for cross-pollination between lines of research that have proceeded relatively independently (Ortega et al., 2017).

Due to ethical restrictions derived from human research, animal models are widely used in behavioral neuroscience experiments with the aim of contributing to the understanding of both normal behavior and discrete symptoms of pathological conditions (Keehn, 2018). Animal models allow a precise control of the environmental variables that determine behavior, its objective measurement, the exploration of its biological basis, and the assessment of its responsivity to pharmacological and psychological treatments (Escorihuela & Fernández-Teruel, 1998; Mehta & Gosling, 2006). This Dissertation builds on the use of animal models of reward devaluation and omission to systematically analyze how negative emotions triggered by incentive loss impact the voluntary consumption of anxiolytic drugs. Animal models of reward loss have several advantages over other animal models of fear/anxiety: (1) tasks do not involve the presentation of aversive stimuli, but the unexpected removal of appetitive stimuli,

thus simulating a frequent, yet experimentally neglected source of emotional distress in humans (Papini et al., 2015); (2) they enable a objective record of behavioral indices of emotional stress (Torres & Papini, 2016); (3) they allow the identification of vulnerability (e.g., genetics) and protective factors (e.g., partial reinforcement) that determine individual differences in emotional reactivity to loss (Torres & Sabariego, 2014). Ultimately, the use of animal models of reward loss in research on addiction will pose new questions on the role of ESM in the onset of anxiolytic use, thus promoting progress in neuroscientific research and clinical interventions.

Chapter 5. Conclusions

The main conclusions from this Dissertation can be summarized as follows:

(1) Animals consume and prefer anxiolytics (alcohol, CDP) over water under baseline two-bottle free–choice conditions.

(2) Frustration triggered by reward devaluation (cSNC) increases alcohol (2%) and CDP (1 mg/kg) voluntary consumption and preference. This ESM behavior is not observed in unshifted animals or in animals receiving only water.

(3) Prior free access to anxiolytics (alcohol, CDP) increases locomotor activity as assessed in the EPM test for anxiety, regardless whether or not animals are exposed to reward devaluation before the preference test.

(4) Prior free access to anxiolytics (alcohol, CDP) does not affect a subsequent cSNC effect when animals experience a single event of reward downshift, but alcohol reduced the contrast effect after repeated reward downshift trials.

(5) Animals exposed to partial reinforcement during the preshift phase of a reward devaluation task do not increase alcohol consumption after reward downshift, as opposed to animals receiving continuous reinforcement.

(6) LHb lesions increase resistance to consummatory and instrumental extinction (involving reward omission), but do not affect alcohol consumption, regardless the concentration or duration of the test.

(7) Voluntary anxiolytics consumption induced by reward loss can be useful to explore how ESM underlies the onset of drug intake.

Chapter 6. Resumen extendido

Resumen

El consumo de sustancias psicoactivas constituye una práctica muy común en el ser humano desde la antigüedad. En algunas ocasiones, este consumo puede derivar en un patrón conductual caracterizado por una tendencia compulsiva a buscar y consumir dicha sustancia, una pérdida de control del consumo limitado y la aparición de un estado emocional negativo cuando la droga no está disponible (Koob y Volkow, 2016). Varios enfoques teóricos y experimentales han intentado identificar las motivaciones fundamentales que llevan a un individuo a consumir sustancias psicoactivas. La hipótesis de la automedicación sostiene que el tipo de sustancia elegida para el consumo depende de la medida en que esa sustancia alivia una psicopatología preexistente o un estado negativo inducido por circunstancias aversivas (Khantzian, 1985). Sin embargo, no se conocen las condiciones que inducen la automedicación, los tipos de sustancias la sustentan y las áreas del cerebro responsables de esta conducta. Nuestro grupo de investigación ha acumulado evidencia empírica que sugiere que los estados emocionales negativos inducidos por situaciones de pérdida de recompensa promueven en ratas el consumo de alcohol, aportando evidencia en favor de la hipótesis de la automedicación emocional (Torres y Papini, 2016).

Los cinco estudios presentados en esta Tesis Doctoral suponen la ampliación de esta línea de investigación, y su objetivo fundamental fue analizar sistemáticamente el papel de la automedicación emocional inducida por la pérdida de recompensa en el inicio del consumo de ansiolíticos (alcohol y BZD) en ratas Wistar. En primer lugar, se analizaron las condiciones experimentales necesarias para obtener un consumo voluntario significativo de ansiolíticos

(alcohol y CDP) en los animales. En segundo lugar, se exploró si la exposición a una situación de pérdida de recompensa (cSNC) aumenta el consumo de alcohol y CDP. En tercer lugar, se estudió si el efecto ansiolítico del alcohol y el CDP es el responsable de la conducta de automedicación, para lo cual los animales tuvieron acceso previo a una de estas sustancias y después fueron expuestos a distintas situaciones inductoras de afecto negativo (laberinto elevado, devaluación de recompensa). En cuarto lugar, se analizó si una experiencia crónica con frustración (reforzamiento parcial) reduce el impacto posterior de la devaluación de la recompensa y anula la conducta de automedicación. Finalmente, se analizó el papel de la habenula lateral en las respuestas inducidas por omisión de recompensa (extinción consumatoria e instrumental) y en el consumo voluntario de alcohol presentado en dosis crecientes y en diferentes tiempos de acceso a la droga.

Los principales resultados obtenidos en los cinco estudios presentados en esta Tesis mostraron que los animales consumen alcohol (2%) voluntariamente en ausencia de frustración, replicando estudios previos (Manzo et al., 2012). Sin embargo, se hallaron inconsistencias en relación con el consumo voluntario de CDP (Experimentos 1-3). La reducción súbita en la magnitud de una recompensa (una solución de azúcar del 32% al 4%) produjo un aumento selectivo y transitorio en el consumo y la preferencia por alcohol (2%) y CDP (1 mg/kg), en contraste con los resultados obtenidos en el grupo devaluado que tuvo acceso a agua en el test de preferencia y en los grupos control no devaluados (4%; Experimento 4). Por otro lado, el consumo voluntario de ansiolíticos (inducido o no por una experiencia previa de pérdida de recompensa) aumentó la actividad locomotora general registrada en el laberinto elevado en cruz (Experimentos 5-

6) y redujo la magnitud del efecto de contraste cuando se realizó con ciclos repetidos (32-4% de sacarosa; Experimento 8), pero no cuando los animales fueron expuestos a una experiencia única en devaluación (Experimento 7). Asimismo, el entrenamiento en reforzamiento parcial retrasó ligeramente la recuperación del efecto de devaluación de la recompensa y abolió la conducta de automedicación emocional (Experimento 9). Por último, la lesión neuroquímica de la habénula lateral produjo un retraso en la extinción de dos conductas apetitivas (consumatoria e instrumental), si bien dicha lesión no afectó al consumo ni a la preferencia por alcohol, con independecia de la dosis presentada (2-24%) o del tiempo de acceso a la misma (2 h vs. 24 h).

En resumen, la presente Tesis Doctoral constituye un avance en la investigación dirigida a analizar de qué modo las emociones negativas vinculadas con la pérdida de incentivos influyen en el inicio del consumo voluntario de ansiolíticos, pudiendo así constituir una puerta de entrada a la adicción.

Palabras clave: Adicción, Alcohol, Ansiolíticos, Benzodiacepinas, Contraste Sucesivo Negativo, Automedicación Emocional, Extinción, Frustración, Habénula Lateral, Pérdida De Recompensa, Ratas Wistar, Reforzamiento Parcial

Introducción

El consumo de sustancias psicoactivas constituye una de las prácticas más arraigadas en el ser humano desde tiempos remotos. Las motivaciones que lo promueven y los propósitos que se persiguen con esta conducta son variados: el placer, la evasión, la diversión, la disminución del dolor, el tratamiento de la enfermedad, la introspección, el éxtasis místico, etc. (Escohotado, 2006; Torres y Escarabajal, 2005). Sin embargo, esta conducta puede terminar derivando en un patrón comportamental desadaptativo caracterizado por una tendencia compulsiva a buscar y consumir la sustancia, por la pérdida de control en relación con su consumo limitado, y por la aparición de un estado emocional negativo (disforia, irritabilidad, ansiedad) cuando el acceso a la sustancia no está disponible (Koob y Volkow, 2016).

Para diagnosticar un trastorno por uso de sustancias se proponen once criterios, entre los que se incluyen: un consumo en mayores cantidades o durante más tiempo de lo que inicialmente se pretendía; consumo recurrente y/o continuado de sustancias en situaciones de riesgo; *craving* o ansia por consumir la sustancia; tolerancia; abstinencia, etc. (DSM-V, 2013). Entre las sustancias cuyo consumo puede derivar en un trastorno por uso de sustancias destacan el alcohol y los ansiolíticos, estos últimos frecuentemente consumidos para controlar el estrés, la ansiedad y los trastornos de sueño. De acuerdo con los datos estadísticos del Observatorio Español sobre Drogas (2017), un 91,2% de la población general manifiesta haber consumido alcohol alguna vez en su vida, un 77,6 % durante los últimos 12 meses, y un 9,3% lo consumen diariamente. Con respecto al uso de ansiolíticos, un 18,7% de la población ha tomado benzodiacepinas al menos una vez en su vida, el 12% de la población las ha

consumido en los últimos 12 meses, y un 6% de la población admite hacerlo diariamente. El impacto socioeconómico del elevado consumo de alcohol a nivel mundial viene reflejado en los datos recientemente publicados en la revista The Lancet por el *Global Burden of Diseases* (2018). Según este estudio, más de 2,5 billones de personas en todo el mundo consumen alcohol, siendo esta droga el factor causal directo de 2,8 millones de muertes en el año 2016. El estudio revela, además, que no existe dosis alguna de alcohol que tenga un efecto protector para la salud, una evidencia que contradice la creencia popular relativa al consumo de dosis bajas o moderadas de esta droga con potencial de abuso.

En la actualidad se realizan muchos esfuerzos para identificar las causas y las consecuencias de la adicción. La mayoría de la investigación neurocientífica sostiene que el principal factor implicado en el inicio del consumo de sustancias es el refuerzo positivo derivado del mismo, asociado a su vez con el placer y la euforia que todas las drogas de abuso producen al ser consumidas de forma aguda. Estos efectos se relacionan directamente con su capacidad para activar un circuito cerebral conocido como circuito de la recompensa (Everitt y Robbins, 2013; Volkow, Koob y McLelland, 2016). Aunque las drogas de abuso difieren en su perfil farmacológico, todas ellas comparten la capacidad de aumentar los niveles de dopamina en dicho circuito (Koob et al., 2014; Tan et al., 2011). Por su parte, las teorías adaptativas de la drogadicción proponen que las experiencias ambientales y la reactividad individual a las mismas constituyen un determinante crítico para iniciar el consumo de drogas de abuso y para desarrollar (o no) una patología adictiva (Conway et al., 2003). Otra teoría que intenta dar explicación a la adicción a sustancias, de corte psicodinámico, es la hipótesis de la automedicación (Khantzian, 1985, 2013). Esta hipótesis afirma

que los individuos tienden a abusar de sustancias por la capacidad de las mismas para aliviar un trastorno emocional preexistente o atenuar un estado negativo transitorio inducido por estímulos ambientales aversivos, en un intento de tolerar y comprender mejor los propios sentimientos y regular el afecto negativo (Blume et al., 2000; Gil-Rivas y McWhorter, 2013; Khantzian, 1997). Esta conducta ha sido conceptualizada más recientemente como de automedicación emocional (ESM por su sigla en inglés, "*emotional self-medication"*; Torres y Papini, 2016).

La mayoría de los estudios dirigidos a comprobar la hipótesis de la ESM se han llevado a cabo con muestras clínicas de pacientes diagnosticados con trastorno por abuso de sustancias y/o trastornos psiquiátricos, como depresión, ansiedad o esquizofrenia (Castaneda, Galanter y Franco, 1989; Gil-Rivas y McWhorter, 2013; Khantzian, 2003). También se ha estudiado en sujetos sanos expuestos a experiencias negativas, mostrando que éstos pueden desarrollar adicción cuando son expuestos a estrés crónico (Briand y Blendy, 2009; Konopka et al., 2013; Yap y Miczek, 2008). Por último, los individuos también pueden consumir drogas para eliminar la disforia e irritabilidad que aparece asociada al síndrome de abstinencia (Koob, 2014). En relación con el consumo de alcohol se ha comprobado, por ejemplo, que las tasas de abuso de esta sustancia en veteranos de guerra que sufren un trastorno por estrés postraumático son superiores a las encontradas en los veteranos que no han desarrollado dicho trastorno. Además, las experiencias de estrés agudo provocan recaídas en el consumo con más frecuencia en el primer grupo de sujetos que en el segundo (Enman et al., 2014; Hien et al., 2010; Mantsch et al., 2014; Ouimette et al., 2007). Del mismo modo, las personas que han sido víctimas de abuso físico o

sexual, abandono temprano, catástrofes naturales, que han sufrido la muerte de un ser querido, conflictos familiares, pobreza, etc. consumen más alcohol que la población no expuesta a este tipo de eventos traumáticos (Gordon, 2002; Konopka et al., 2013; Hassanbeigi et al., 2013).

La conducta de automedicación también parece observarse en animales no humanos, dado que éstos son capaces de seleccionar y usar plantas y sustancias específicas para aliviar su malestar físico y mantener la homeostasis (Huffman, 2001; Singer et al., 2009; Villalba et al., 2014; Villalba y Provenza, 2007). Por ejemplo, los chimpancés enfermos consumen plantas para eliminar infecciones parasitarias y mejorar su salud (Clayton y Wolfe, 1993; Huffman, 1997, 2001, 2003; Masi et al., 2012; Villalba y Provenza, 2007). En la misma dirección, la infección con parásitos en ovejas puede ser reducida por estos animales seleccionando dietas que contienen la medicación apropiada (taninos; Lisonbee et al., 2009). Además, los roedores son capaces de autoadministrarse infusiones de un agonista cannabinoide u opioide con propiedades analgésicas tras ser sometidos a dolor físico mediante el ligamiento del nervio ciático (Ewan y Martin, 2013; Gutiérrez et al., 2011). En relación con la ESM, se han tratado de identificar, en condiciones experimentales, qué tipo de experiencias emocionales negativas inducen el consumo de sustancias ansiolíticas. La mayoría de los estudios revisados se basan en la exposición del animal a descargas eléctricas inescapables (Anisman y Waller, 1974; Barsy, Mikics, Barsvári, y Haller, 2011; Volpicelli, Tiven, y Kimmel, 1982), estrés social (prueba intruso-residente, aislamiento social, exposición al olor de un depredador; Caldwell y Riccio, 2010; Manjoch et al., 2016; Nash y Maickel, 1985; Roske et al., 1994), inmovilización (Lynch et al., 1999; Nash y Maickel, 1985), natación forzada (Füllgrabe,

Vengeliene, & Spanagel, 2007; Siegmund, Vengeliene, Singer, y Spanagel, 2005), etc. Si bien todas estas situaciones inducen un aumento significativo en el consumo de alcohol, los estudios no siempre arrojan resultados consistentes. Tales inconsistencias dependen, entre otros factores, del tipo de experiencia estresante utilizada (duración, intensidad o cualidad), de cómo se lleva a cabo el test de consumo (mediante procedimientos operantes o de libre elección agua-droga), de cuál es el tiempo y modo de acceso a la droga (ilimitado vs. restringido), de la dosis presentada, y de diversos factores biológicos (edad, sexo, cepa, etc.; Becker et al., 2011; Spanagel et al., 2014). A pesar de su utilidad, los modelos animales comentados presentan ciertas limitaciones que merecen destacarse (Torres y Papini, 2016). En primer lugar, en todos ellos se trabaja con sustancias de uso recreativo como el alcohol, sin tener en cuenta los ansiolíticos de prescripción médica, que no han sido estudiados hasta el momento en condiciones experimentales inductoras de ESM. En segundo lugar, algunas de las pruebas de estrés utilizadas no permiten analizar de forma objetiva cómo responde el animal al estresor y cómo dicha respuesta se relaciona con el consumo de la droga (por ejemplo, los procedimientos de inmovilización forzada o de restricción física). En tercer lugar, muchas de estas pruebas tienen una reducida validez ecológica, dificultando la generalización de los resultados a lo que podría acontecer en escenarios naturales y en seres humanos. En este contexto cobran especial relevancia los modelos animales de pérdida de recompensa o frustración, los cuales pueden superar gran parte de estas limitaciones, como se comenta a continuación.

La pérdida inesperada de fuentes de reforzamiento significativas constituye una de las experiencias más estresantes en seres humanos

(Cochrane y Robertson, 1973; Scully, Tosi, y Banning, 2000), siendo una fuente de dolor psicológico y de sufrimiento en nuestra vida diaria (Papini, 2006; Papini et al., 2015). En el laboratorio animal, estas experiencias se inducen exponiendo a los sujetos a la devaluación u omisión en la cantidad o en la calidad de un reforzador, en presencia de señales previamente asociadas con un reforzador de mayor magnitud (Amsel, 1992; Papini, 2006). Por ejemplo, el contraste sucesivo negativo (SNC, por su sigla en inglés, "*successive negative contrast*") consiste en una reducción o deterioro transitorio en la respuesta que aparece ante bajos niveles de recompensa (fase de postcambio) en animales que previamente han sido expuestos a valores de recompensa superiores (fase de precambio), en comparación con la ejecución de un grupo que siempre recibe bajos niveles de recompensa (Flaherty, 1996; Papini y Torres, 2017). Otro fenómeno de pérdida relevante es la extinción: los animales reciben una recompensa tras la ejecución de una respuesta (fase de adquisición) que después es omitida (fase de extinción), lo que conduce al deterioro o disminución de dicha respuesta (Mackintosh, 1974; Torres y Papini, 2017). Se sabe, asimismo, que experiencias repetidas de omisión de recompensa pueden inmunizar al animal a situaciones futuras de pérdida, promoviendo la resistencia a la frustración. Así, el efecto del reforzamiento parcial en la extinción es el fenómeno que se obtiene al reforzar una determinada respuesta sólo en algunos ensayos (50%) durante una primera fase de adquisición, tras la cual la recompensa se suprime (fase de extinción). El fenómeno puede observarse mediante la comparación de la ejecución de este grupo con la de otro que recibe la recompensa continuamente durante la fase de adquisición. El resultado obtenido es una mayor resistencia a la extinción en el grupo de reforzamiento

parcial en comparación con el grupo de reforzamiento continuo (Amsel, 1992; Gómez et al., 2008). Resultados similares se obtienen cuando, tras un entrenamiento en reforzamiento parcial, el animal es sometido a la devaluación de la misma, un fenómeno conocido como el efecto del reforzamiento parcial en el contraste (Cuenya et al., 2012; Pellegrini et al., 2004).

Las respuestas registradas en este tipo de situaciones han sido analizadas y caracterizadas de forma muy precisa, evidenciando su vinculación con los circuitos cerebrales del miedo, la ansiedad, el dolor físico y el estrés (Ortega et al., 2017; Papini et al., 2015; Torres y Sabariego, 2014). Sin embargo, las pruebas que ponen en relación la frustración con el consumo voluntario de sustancias de abuso son prácticamente inexistente en la actualidad. Sí es sabido que tanto el alcohol como las benzodiacepinas, inyectados sistémicamente, atenúan el SNC, afectan a la extinción, y eliminan el efecto de reforzamiento parcial sobre la extinción y sobre el contraste (Flaherty, 1996; Gray, 1987; Pellegrini et al., 2004).

Recientemente, nuestro grupo de investigación ha estudiado la relación entre frustración y consumo de drogas con efectos ansiolíticos. Para ello, se utilizaron animales genéticamente seleccionados sobre la base de sus diferencias extremas en la capacidad para aprender la tarea de evitación activa en dos sentidos (las ratas Romanas consanguíneas de Alta -RHA-I- y Baja - RLA-I- Evitación). Estas diferencias se relacionan, a su vez, con divergencias en su reactividad emocional y sensibilidad a la frustración, que es baja en la cepa RHA-I y alta en la RLA-I (Steimer y Driscoll, 2003; Torres y Sabariego, 2014). Utilizando estas cepas se ha analizado el impacto de numerosas experiencias de pérdida sobre el consumo voluntario de alcohol. Los resultados demostraron

que el efecto de una experiencia en omisión de recompensa (extinción) sobre el consumo voluntario de etanol (2%) en ratas RHA-I vs. RLA-I depende de las características genéticas de los animales, dado que sólo la cepa RLA-I (más reactiva a la frustración que la RHA-I: Cuenya et al., 2012; Donaire, Sabariego, Gómez, Fernández-Teruel y Torres, 2013; Gómez et al., 2008; Gómez, de la Torre et al., 2009; Gómez, Escarabajal et al., 2009; Rosas et al., 2007; Sabariego et al., 2013; Torres et al., 2005) aumentó su consumo de alcohol después de dicha experiencia (Manzo et al., 2014). Estos resultados sugieren que las experiencias de pérdida de incentivos pueden constituir factores precipitantes en el consumo de sustancias ansiolíticas en individuos genética y temperamentalmente vulnerables, poniendo de manifiesto la relevancia que la conducta de ESM podría tener en el inicio del consumo de sustancias con potencial de abuso. Por otro lado, cuando estos animales fueron expuestos a un programa de refuerzo parcial, las ratas RLA-I mostraron una mayor resistencia a la extinción y un menor consumo de alcohol en comparación con los animales reforzados continuamente. Estos resultados sugieren que la exposición a la incertidumbre propia del entrenamiento en refuerzo parcial es capaz de reducir la conducta de automedicación en ratas genéticamente seleccionadas por su alto nivel de ansiedad (Manzo, Gómez et al., 2015).

En resumen, los datos presentados anteriormente sugieren que los modelos animales de pérdida de recompensa proporcionan una aproximación viable para estudiar cómo reaccionan los animales y cómo se adaptan ante un evento que puede activar emociones negativas, induciendo un aumento en la preferencia por sustancias que son conocidas por reducir la ansiedad cuando son inyectadas en situaciones de pérdida de recompensa (Becker y Flaherty,

1982, 1983; Kamenetzky, Mustaca y Papini, 2008). Esta conducta de consumo de sustancias ansiolíticas parece estar bajo el control tanto de factores genéticos como ambientales, como se puede ver en los estudios con las ratas Romanas (Manzo, Gómez et al., 2015; Torres y Papini, 2016). Sin embargo, la influencia de experiencias estresantes asociadas a una pérdida de recompensa no se ha puesto a prueba en individuos que no sean genética y temperamentalmente vulnerables y no se han explorado las condiciones en que este consumo de ansiolíticos tiene lugar. Por lo tanto, la presente Tesis Doctoral tiene como objetivo ampliar esta línea de investigación y analizar sistemáticamente el papel de la ESM inducida por la pérdida de recompensa en el inicio del consumo de ansiolíticos, proporcionando una posible explicación de cómo este comportamiento de automedicación podría subyacer al inicio del uso y abuso de ansiolíticos.

Los objetivos específicos, hipótesis y predicciones se describirán dentro de cada uno de las secciones dedicadas a los estudios que componen esta Tesis Doctoral.

Estudios experimentales

En esta sección se presenta un breve resumen del trabajo experimental realizado en esta Tesis Doctoral. En primer lugar, se expondrá un resumen general de la metodología utilizada. A continuación, se describirán los estudios realizados para dar respuesta a cada uno de los objetivos planteados, indicando las particularidades de la metodología utilizada en cada uno de ellos y los resultados obtenidos.

Metodología general

Sujetos. Los sujetos utilizados fueron ratas Wistar macho de tres meses

de edad, los cuales fueron privados de comida para mantenerlos entre el 82% y el 85% de su peso ad lib. Los animales se asignaron a los grupos en función de su peso corporal, y fueron alojados de manera individual, con agua disponible constantemente. Las condiciones de alojamiento y mantenimiento de los sujetos se ajustaron en todo momento a la normativa vigente europea (2010/63/EU) y española (6/2013; R.D.53/2013), contando con el visto bueno del Comité de Ética en Experimentación Animal de la Universidad de Jaén.

Aparatos y procedimientos. Se utilizaron las pruebas conductuales que se describen brevemente a continuación.

Tarea de inducción. Para inducir un estado emocional negativo de frustración se utilizaron los aparatos y procedimientos siguientes:

(1) ***Contraste Sucesivo Negativo consumatorio*** *(cSNC;* véase Figura 15): Para esta prueba se utilizaron 6 cajas de policarbonato de 30 cm x 15 cm x 30 cm. La pared frontal tenía un agujero en el que se introducía una bureta graduada de 50 ml. La solución de sacarosa fue preparada mezclando azúcar y agua destilada. Para la solución al 32% (o 4%) se mezclaron 32 gramos de azúcar (o 4 gramos) con 68 gramos (o 96 gramos) de agua destilada. Se utilizó un agitador magnético para disolver el azúcar. Los animales recibieron una única sesión diaria, con una duración de 5 min desde el primer lametón, medida con un cronómetro manual. El grupo expuesto a la devaluación de la recompensa recibió la solución de sacarosa al 32% en la fase de precambio y 4% en la fase de postcambio. El grupo control recibió la solución al 4% a lo largo del experimento. La variable dependiente (VD) registrada en esta tarea fue la cantidad de solución consumida en ml/kg.

(2) ***Extinción consumatoria*** (véase Figura 15): Para esta tarea, se utilizaron los mismos aparatos y el mismo procedimiento que para el cSNC, con la excepción de que se utilizó una solución de sacarosa al 32% en una primera fase de adquisición, y agua destilada en la fase de extinción. La VD registrada en esta tarea fue la cantidad de solución consumida en ml/kg.

(3) ***Extinción instrumental*** (véase Figura 24)*:* En esta tarea se utilizó un laberinto recto de plexiglás de 0,7 mm de grosor, cuyas dimensiones eran de 12 cm x 12 cm x 245 cm. La parte superior era de plexiglás transparente, y las paredes y el suelo de color negro. El laberinto constaba de tres secciones: compartimento de salida, recorrido, y compartimento de meta. La longitud del compartimento de salida y de meta era de 20 cm, y la sección del recorrido medía 205 cm. Los compartimentos del laberinto estaban delimitados por puertas de guillotina, también de plexiglás negras de 0,3 milímetros de grosor, que se levantaban para que la rata recorriera el laberinto y se bajaban cuando el animal entraba en el compartimento meta con las cuatro patas (con un tiempo máximo de 40 seg, medido con un cronómetro manual). La sesión experimental constaba de 6 ensayos diarios, con un intervalo entre ensayos de 10 min. El entrenamiento consistió en tres fases: habituación (con una duración de tres días), adquisición (10 sesiones en las que los animales recibían 12 pellets de comida) y extinción (5 sesiones, en las que los animales no recibían ningún pellet). La VD registrada en esta tarea fue la latencia de respuesta (Gómez et al., 2008; Gómez, de la Torre et al., 2009).

(4) Prueba de preferencia (véase Figura 8). Se llevó a cabo en la jaula-hogar

de los animales (32 cm x 15 cm x 30 cm), que tenía el suelo cubierto de serrín. Se utilizó un procedimiento de libre elección con dos botellas de 150 ml –una con agua y la otra con la sustancia ansiolítica- (o agua en ambas en el caso del grupo control; véase Manzo et al., 2014). La prueba duró dos horas. Las VD registradas en esta tarea fueron el consumo de la droga (etanol, CDP o agua) y de agua (ambas en ml/kg) y la razón de preferencia (consumo de la botella que contenía el ansiolítico/consumo total de fluidos—suma del consumo de ambas botellas).

(5) Laberinto elevado en cruz (EPM por su sigla en inglés "*elevated plus maze*", véase Figura 12): Este aparato dispone de dos brazos abiertos (49,5 cm x 10 cm con un borde de 1 cm excepto en su parte distal) y dos brazos cerrados (49,5 cm x 10 cm) que constan de paredes transparentes de 39,5 cm de altura, construido con plexiglás negro (suelo). Este aparato se encuentra elevado a 50 cm del suelo. Los animales fueron expuestos al test durante 5 minutos, colocándolos en la zona central donde confluyen los cuatro brazos y permitiéndoles el acceso libre a los mismos durante dicho periodo. La conducta de los sujetos fue grabada para su análisis posterior, mediante el programa informático JWatcher (http://www.jwatcher.ucla.edu). Las VD registradas fueron (1) número de entradas y tiempo de estancia en los brazos abiertos y cerrados; (2) número de entradas y tiempo de estancia en el extremo del brazo abierto; (3) tiempo y frecuencia de *rearing* (definida como el alzamiento sobre las dos patas traseras) tanto en los brazos abiertos como en los cerrados; (4) tiempo y frecuencia de *head-dipping* protegido (definido como asomar la cabeza por debajo del brazo abierto, bien desde el brazo cerrado o el

cuadrado central de unión de los cuatro brazos) y (5) tiempo y frecuencia de *head-dipping* no protegido (definido como asomar la cabeza por debajo del brazo desde el brazo abierto).

Fármacos. Se utilizaron como sustancias de acción ansiolítica el alcohol (2% v/v en todos los experimentos, salvo el Experimento 10, que usó un aumento gradual de la dosis desde 2% hasta 24%) y el CDP. El rango de dosis en los tres primeros experimentos varió entre 1 y 60 mg/kg. En el resto de experimentos, la dosis a utilizar para el consumo oral fue de 1 ó 2 mg/kg (véase Reagan-Shaw et al., 2008). El Experimento 10 tiene una variación en el test de preferencia, añadiendo una sesión de 24 horas de duración al final de las sesiones. Como sustancia neurotóxica en este experimento se usó ácido quinolínico (un agonista de los receptores NMDA de glutamato).

Estudio 1: ¿Consumen los animales ansiolíticos voluntariamente?

Esta sección tuvo como objetivo principal establecer las condiciones experimentales ideales (fármaco, dosis, patrón de presentación) para obtener un consumo voluntario de ansiolíticos en ratas Wistar (alcohol y CDP), usando para ello la prueba de preferencia droga vs. agua (Experimentos 1-3). En el Experimento1, los animales se dividieron en tres grupos expuestos durante dos horas en su jaula hogar a alcohol, CDP o agua en una de las botellas, y a agua en la otra (Grupos E, CDP y W, respectivamente). En el Experimento 2, los animales tuvieron acceso a una solución de CDP (Grupos CDP y W), con dosis crecientes de CDP desde 0.1 mg/kg hasta 60 mg/kg. Dichas dosis fueron presentadas cada dos días. En el Experimento 3 los animales se dividieron en dos grupos, uno con acceso a CDP (2 mg/kg) de manera continua (Grupo CONT) y otro grupo con un patrón de presentación intermitente (INT), en el que se

presentaba la solución de CDP en los días impares del experimento. Aunque no hay estudios con este patrón de consumo de CDP, sí se ha comprobado que este patrón de presentación aumenta el consumo de alcohol en animales (véase Carnicella et al., 2014; Momeni y Roman, 2014; Wise, 1975). La hipótesis de partida, basada en la revisión de la literatura, buscaba la confimación de que los animales consumen voluntariamente sustancias psicoactivas de acción ansiolítica como el alcohol o el CDP, por lo que se esperaba encontrar un mayor consumo y preferencia por ambas sustancias en comparación con el consumo y la preferencia por agua.

Los resultados obtenidos en el Experimento 1 mostraron un alto consumo y una alta preferencia por la dosis de 2% de alcohol frente a agua. Además, se observó que, independientemente de la dosis, los animales que recibieron CDP prefirieron significativamente más la solución que contenía este ansiolítico en comparación con agua, lo que sugiere que esta sustancia tiene efectos farmacológicos detectables positivos cuando se ingiere de forma oral en condiciones de consumo voluntario.

Los resultados obtenidos en el segundo experimento mostraron datos similares a los obtenidos en el Experimento 1. De nuevo, los animales presentaron un consumo alto por la solución de CDP, en comparación con el agua, independientemente de la dosis. Aunque la interacción Dosis x Droga no fue estadísticamente significativa, un análisis de la misma reveló que los animales mostraron un consumo significativamente más alto que el grupo control cuando fueron expuestos a las dosis de 0,1; 0,4; 0,8; 1,0; 2,0; 16,0; 24,0 y 60,0 mg/kg, pero no a las dosis de 0,2; 1,2; 1,4; 1,8; 2,2; 2,4; 3,0; 4,0; 8,0 y 36,0 mg/kg.

Con respecto a los resultados encontrados en el tercer experimento (patrón de presentación intermitente), no se obtuvo evidencia de que este tipo de exposición favoreciera el consumo y la preferencia por CDP en relación con la presentación continua, lo que contrasta con estudios previos realizados con alcohol (Momeni y Roman, 2014; Wise, 1975).

Estudio 2: ¿La frustración aumenta el consumo de ansiolíticos?

La finalidad de este segundo estudio (Experimento 4) fue analizar si la frustración (inducida por la devaluación en el valor de una recompensa esperada) aumenta el consumo voluntario de ansiolíticos. Para ello, los animales fueron expuestos a una situación de cSNC, y a continuación tuvieron acceso a una prueba de preferencia por alcohol, CDP o agua (Manzo, Donaire, Sabariego, Papini, & Torres, 2015; *Behavioural Brain Research*, *278*, 90-97). Se estableció como hipótesis de trabajo que las experiencias de devaluación u omisión de la recompensa inducen un estado emocional de frustración, con características similares al miedo, la ansiedad y el estrés (Amsel, 1992; Flaherty, 1996; Gray, 1987; Papini, 2006). Dado que estos estados emocionales favorecen el consumo de sustancias de abuso, como el alcohol (Becker et al., 2011), se esperaba que los animales expuestos a la reducción en la concentración de sacarosa (del 32% al 4%) mostraran un aumento selectivo en el consumo de sustancias con propiedades ansiolíticas (alcohol, CDP), pero no en el consumo de agua. Los resultados fueron en la dirección prevista, dado que la devaluación del reforzador produjo un aumento selectivo y transitorio en el consumo y la preferencia por estas sustancias, un hallazgo que no apareció en el grupo devaluado expuesto solo a agua, ni tampoco en los grupos controles no devaluados.

Los resultados obtenidos en este estudio corroboran los datos obtenidos

en estudios previos en relación con el alcohol (Manzo et al., 2014), y además sugieren que el efecto de contraste induce una emoción negativa (Amsel, 1992a; Flaherty, 1996; Gray, 1987; Papini, 2003; Papini et al., 2006). Con respecto al CDP, la emoción negativa inducida por la pérdida de recompensa condujo a un aumento en el consumo de dicho ansiolítico, siendo la primera vez que se demuestra que, ante situaciones de malestar emocional debido a la pérdida de reforzamiento, el consumo de ansiolíticos aumenta. Estos resultados confirman igualmente que la automedicación ocurre de manera selectiva durante la fase en la que la recompensa se devalúa (fase de postcambio).

Estudio 3: ¿Por qué se automedican los animales?

Tras obtener evidencia de que los animales se automedican cuando sufren malestar emocional derivado de una experiencia de devaluación de recompensa (Experimento 4), el siguiente objetivo de la presente Tesis Doctoral fue analizar si el efecto ansiolítico del alcohol y el CDP era el responsable de esta conducta. Para ello se realizaron cuatro experimentos. Dos de ellos implicaron cambiar el orden de las tareas, exponiendo a los animales a la prueba de preferencia primero, seguida de un test de ansiedad (EPM; Experimento 5), a un único episodio de devaluación de la recompensa (cSNC, Experimento 7), o a repetidas devaluaciones de la recompensa (Experimento 8). Un experimento adicional (Experimento 6) analizó el supuesto ansiolítico de la hipótesis de la ESM exponiendo a los animales a la devaluación de la recompensa (32%-4%), seguida de la prueba de preferencia por alcohol, y de la exposición posterior al EPM en las sesiones 11, 12 y 15 de la tarea de inducción (correspondientes a las sesiones 1, 2 y 5 de la fase de postcambio; Donaire, Conrad, Thompson, Papini y Torres, 2018; *Behavioural Processes,* 150, 59-65). Sobre la base del

efecto ansiolítico que tienen el alcohol y el CDP, se esperaba encontrar que la exposición a estas drogas durante la prueba de preferencia se manifestara en una mayor exploración de los brazos abiertos del laberinto elevado (como índice de ansiólisis; Experimento 5), y en una atenuación del efecto de cSNC (Experimentos 7 y 8). Asimismo, la reducción de la concentración de sacarosa del 32% al 4% realizada en el Experimento 6 debería aumentar el consumo y la preferencia por alcohol en la prueba de consumo posterior, un aumento que debería reflejarse en un efecto ansiolítico detectable en el EPM (sesiones 11 y 12).

Los resultados obtenidos pueden resumirse del modo siguiente. En primer lugar, el mayor consumo de alcohol en relación con CDP y agua registrado en el Experimento 5 produjo un aumento significativo en el número de entradas al brazo cerrado del laberinto elevado, una medida de actividad locomotora que parece sugerir que el alcohol, a la dosis consumida en este estudio, produjo un efecto desinhibitorio inespecífico, más que ansiolítico.

En segundo lugar, los resultados obtenidos en el Experimento 6 mostraron que hubo un aumento en el consumo de etanol después de las sesiones de devaluación de la recompensa (ESM). Este aumento se acompañó de un incremento en el número de entradas al brazo cerrado y en el número total de entradas registrados en el laberinto elevado en la sesión 12, en contraste con la ausencia de efecto observada en las sesiones 11 y 15 (Donaire et al., 2018).

En tercer lugar, los resultados obtenidos en el Experimento 7 mostraron que el acceso previo a alcohol para su consumo voluntario no afectó al efecto de cSNC posterior, que fue similar al observado en grupos devaluados y controles expuestos a agua en la prueba de preferencia.

Por último, los resultados obtenidos en el Experimento 8 mostraron que los animales con acceso previo a alcohol en la prueba de preferencia mostraron una atenuación del efecto de cSNC a partir del tercer ciclo de devaluación, mientras que los animales con acceso sólo a agua mantuvieron este efecto durante todos los ciclos de devaluación utilizados (cinco).

Estudio 4: ¿Cómo se puede reducir la automedicación emocional?

El objetivo de esta sección fue identificar manipulaciones experimentales efectivas para atenuar o eliminar la conducta de ESM inducida por la devaluación del incentivo. Sobre la base de estudios previos (Manzo, Gómez et al., 2015), se analizó si una experiencia crónica de frustración (derivada de un entrenamiento en reforzamiento parcial) podría aumentar la resistencia conductual en una situación de frustración posterior (cSNC) y reducir la conducta de automedicación. Con este objetivo en mente, dos grupos de animales fueron expuestos a reforzamiento parcial en la fase de precambio, uno de ellos con acceso a alcohol y otro a agua en el test de preferencia; otros dos grupos fueron expuestos a reforzamiento continuo, con las mismas condiciones en el test de preferencia (Experimento 9). Se esperaba encontrar que los animales expuestos en la fase de precambio a una solución de sacarosa al 32% bajo condiciones de reforzamiento parcial (es decir, recibiéndola en el 50% de los ensayos de forma pseudoaleatoria) mostraran un efecto de cSNC atenuado en comparación con animales expuestos a reforzamiento continuo. Esta atenuación provocada por el reforzamiento parcial debería verse acompañada, además, de un menor consumo de alcohol en la prueba de preferencia posterior.

Contrario a lo esperado (Pellegrini et al., 2004), el entrenamiento en reforzamiento parcial retrasó ligeramente la recuperación del cSNC. No

obstante, el reforzamiento parcial anuló la conducta de automedicación emocional que sí fue observada en el grupo entrenado en la fase de precambio en condiciones de reforzamiento continuo, que aumentó su consumo de alcohol cuando la recompensa fue devaluada.

Estudio 5: ¿Qué regiones cerebrales regulan la automedicación emocional?

El último estudio que se presenta en esta Tesis Doctoral tuvo como objetivo fundamental iniciar la exploración de las estructuras cerebrales implicadas en la conducta de automedicación emocional, centrando nuestra atención en la habénula lateral (LHb, por su sigla en inglés "*lateral habenula*"). Se trata de una región diencefálica conectada con regiones cerebrales especializadas en el procesamiento de la recompensa, la regulación de la conducta emocional y el consumo de sustancias de abuso, entre otras funciones (Hikosaka, 2010; Lecourtier y Kelly, 2007; Pobbe y Zangrossi, 2008). A pesar de que numerosas evidencias muestran su implicación en situaciones que suponen la omisión inesperada de recompensas, la pérdida y el fallo (Hikosaka et al., 2008; Vadovičová, 2014), lo cierto es que no se ha analizado hasta el momento su papel específico en tareas de inducción de frustración como las utilizadas en esta Tesis Doctoral. Asimismo, su función reguladora de la conducta adictiva se limita en gran medida a estudios de autoadministración en paradigmas relacionados con conducta operante, así como a situaciones de consumo voluntario con dosis altas (aversivas) de la droga en estudio (Haack et al., 2014; Lecca et al., 2014; Li et al., 2016). Por todo ello y como aproximación preliminar, en el presente estudio (Donaire, Morón, Blanco, Villatoro, Gámiz, Papini y Torres, 2019; *Neuroscience Letters, 703*, 184-190) se analizó la implicación de la LHb

en las conductas generadas tanto en tareas de inducción de frustración (extinción consumatoria e instrumental), como en pruebas de preferencia por alcohol presentado a diferentes concentraciones (2%, 4%, 6%, 8%, 10%, 12%, 16% y 24%), con dos modalidades de tiempo de acceso a la droga (2 h, 24 h). La razón de separar ambos tipos de tareas (inducción de frustración y test de preferencia) fue averiguar si la lesión podría afectar a la conducta de consumo de alcohol con independencia del estado afectivo del animal, un resultado que, de obtenerse, podría dificultar la interpretación de estudios futuros de ESM. Se esperaba encontrar que la lesión de esta estructura afectara a la capacidad de los animales para procesar o reaccionar a la omisión de la recompensa (sacarosa al 32% en la prueba consumatoria; 12 pellets en la tarea instrumental), por lo que éstos deberían mostrar una mayor resistencia a la extinción consumatoria e instrumental, respectivamente, en comparación con grupos controles falsos lesionados, los cuales recibieron todo el proceso de cirugía a excepción de la sustancia neurotóxica (ácido quinolínico), siendo inyectados con el vehículo. Por otro lado, los animales lesionados deberían mostrar un consumo más elevado de dosis altas de alcohol en comparación con los controles en la prueba de preferencia, mientras que el consumo de dosis bajas (por ejemplo, de la dosis al 2% de alcohol empleada en los experimentos anteriores) no debería verse afectada.

La lesión de la LHb se llevó a cabo utilizando técnicas y procedimientos de cirugía estereotáxica convencionales. La lesión irreversible se realizó mediante la administración intracerebral de ácido quinolínico en dosis de 0,12 M (Sigma-Aldrich, España), disuelto en 0,175 microlitros de tampón fosfato salino al 0,1 Molar (PBS) e infundido durante 1 minuto y 40 segundos. Se trata de un

agonista selectivo de los receptores NMDA de glutamato que produce la lesión localizada de las neuronas ubicadas en la región de interés, sin afectar a las fibras de paso (Beal, Swartz, Finn, Mazurek y Kowall, 1991). Se realizaron 4 infusiones, dos en cada hemisferio, siguiendo las coordenadas siguientes: AP: -3,1/-3,6; ML: ±0,7; DV: -4,7/-5, partiendo del punto Bregma (Paxinos y Watson, 2013). El tratamiento de los cerebros con procedimientos histoquímicos de marcado de astrocitos con la proteína ácida fibrilar glial (GFAP) permitió obtener evidencia de lesión en algunos animales asignados al grupo lesionado (*n* = 6), cuya conducta se comparó con la de ratas no lesionadas (*n* = 4).

Los resultados indicaron que los animales lesionados mostraron una mayor resistencia a la extinción consumatoria e instrumental en comparación con los sujetos controles. Las diferencias aparecieron al comparar ambos grupos utilizando una medida de ejecución relativa: el promedio de ejecución correspondiente a las primeras 3 sesiones de extinción (sesiones 11-13 de la fase de extinción) dividido por la ejecución registrada en la última sesión de adquisición (sesión 10 de adquisición). Por el contrario, la lesión de esta región cerebral no afectó a la conducta de consumo de alcohol durante la prueba de preferencia, con independencia de la dosis empleada y del tiempo de acceso a la misma. Estos resultados ponen de manifiesto la participación de esta región cerebral en la regulación de conductas inducidas por la omisión de una recompensa esperada, pero no en la conducta de consumo de alcohol bajo condiciones emocionales basales, es decir, en ausencia de frustración. Estos hallazgos suponen la ampliación de nuestro conocimiento sobre las bases biológicas de las conductas inducidas por la pérdida de incentivos, y permitirán en un futuro analizar si la ESM inducida por frustración depende o no de la

integridad de esta región cerebral.

Discusión general

El consumo de drogas legales constituye hoy en día una práctica socialmente aceptada en las sociedades occidentales. Se consume alcohol frecuentemente en fines de semana, en acontecimientos importantes o con ocasión de fiestas señaladas. De acuerdo a los datos presentados por el Observatorio Español sobre Drogas (2017), un 91,2% de la población española ha consumido esta sustancia alguna vez en su vida, un 7,4% son consumidores diarios, y es la causa de un 30% de los accidentes mortales en carretera. El consumo de alcohol está asociado a múltiples mitos y falsas creencias sobre sus posibles beneficios. Por ejemplo, existe la creencia popular de que tomar una pequeña cantidad de alcohol (como una copa de vino) tiene un efecto protector sobre el organismo, previniendo enfermedades cardiovasculares; se trata de un asunto discutido tanto por investigadores como por médicos, teniendo entre sus filas a detractores y a defensores. Sin embargo, el consumo desmedido de alcohol y otras sustancias psicoactivas puede llegar a desembocar en un trastorno por abuso de sustancias (Global Burden of Disease, 2018).

La comprensión de la adicción sigue siendo un gran reto para los neurocientíficos tanto para los básicos como los clínicos. Una parte de ellos considera la adicción como un trastorno neuroconductual originado en el cerebro (por ejemplo, Volkow y Morales, 2015), mientras que otros investigadores no lo consideran así (ver Becoña, 2018; Hall et al., 2015). En esta dirección, Lewis (2017) considera que el modelo de adicción como enfermedad cerebral es erróneo, dado que considera que los cambios producidos en el cerebro en la

adicción son similares a aquellos normalmente observados en el desarrollo de otros hábitos (Fenton y Wiers, 2017).

La mayoría de estudios relacionados con los trastornos por abuso de sustancias se han centrado en estudiar el consumo de drogas cuando éste es abusivo o cuando se ha instaurado un síndrome de abstinencia (Belin-Rauscent et al., 2016; Kamenetzky y Mustaca, 2006). Sin embargo, hay poca información sobre cómo o por qué se inicia el consumo de drogas. De igual manera que los acontecimientos positivos (por ejemplo, ocio, socialización, etc.) están asociados al consumo de alcohol (Díez-Hernández, 2003), la ocurrencia de eventos vitales negativos está también asociada con un consumo problemático de esta droga (Enman et al., 2014). Por ejemplo, una persona que ha tenido un mal día puede consumir alcohol para aliviar el malestar que siente y sentirse mejor. Una vez que el malestar se desvanece, esta persona no necesitaría seguir tomando ninguna sustancia más. Este ejemplo podría servir de base para enmarcar la hipótesis de la automedicación propuesta por Khantzian en 1985. Esta hipótesis planteó que los seres humanos ven las drogas de abuso atractivas porque alivian el sufrimiento psicológico. Aproximaciones más recientes establecen entre las fuentes de dolor psicológico las experiencias de pérdida inesperada de incentivos; por ejemplo, divorcio, la muerte de un ser querido, despido laboral, exclusión social, etc. (Papini et al., 2006; Torres y Papini, 2016). La presente Tesis Doctoral surge con el objetivo de abordar esta problemática desde un punto de vista experimental con modelos animales. Así, su objetivo general fue utilizar pruebas del laboratorio basadas en la devaluación de recompensa para analizar de qué modo estas experiencias influyen en el consumo voluntario de

ansiolíticos. Para ello se realizaron 10 estudios experimentales que permitieron obtener los resultados de que se comentan brevemente a continuación.

En primer lugar, se establecieron las condiciones experimentales idóneas para conseguir un consumo voluntario de alcohol y CDP en ratas Wistar macho (Experimentos 1-3). Los resultados obtenidos indicaron que los animales consumen de manera voluntaria alcohol (2%) en ausencia de frustración, replicando estudios previos (Manzo et al., 2012). Sin embargo, se encontraron inconsistencias en el consumo voluntario de CDP. En este caso, y aunque los animales prefirieron consumir la solución de CDP, el patrón de consumo no dependió de la dosis de la droga (Experimentos 1-2) ni del modo de presentación (Experimento 3).

En segundo lugar, se realizó un estudio dirigido a estudiar la influencia de la frustración (inducida por la devaluación en el valor de una recompensa esperada; cSNC) en el consumo voluntario de ansiolíticos (Experimento 4). Los resultados mostraron que la devaluación del reforzador (sacarosa) produjo un aumento selectivo y transitorio en el consumo y la preferencia por alcohol y CDP, un hallazgo que no apareció en el grupo devaluado expuesto sólo a agua, ni tampoco en los grupos controles que recibieron la baja magnitud del reforzador a lo largo del experimento. De este modo, se confirmó que la conducta de automedicación aparece de manera selectiva durante la fase en la que la recompensa se devalúa, estando así vinculada con el afecto negativo derivado de dicha experiencia (Manzo, Donaire et al., 2015).

En tercer lugar, se estudió si el efecto ansiolítico que poseen el alcohol y el CDP podría ser el responsable del aumento en el consumo de ansiolíticos obtenido en el estudio anterior, poniendo a prueba así el supuesto ansiolítico de

la hipótesis de la ESM (Experimentos 5-8). Este supuesto fue estudiado en una serie de experimentos en la que el orden de las tareas (inducción y test de preferencia) fue invertido. Los resultados obtenidos mostraron que el acceso previo a ansiolíticos aumentó la actividad locomotora general registrada en el EPM (con independencia de que los animales estuvieran bajo un estado de frustración o no) y redujo la magnitud del efecto de cSNC cuando éste se realizó con transiciones repetidas (32%-4%), pero no cuando se llevó a cabo con una única experiencia en devaluación (Donaire et al., 2018).

En cuarto lugar, se estudió cómo podría atenuarse o eliminarse la conducta de ESM (Experimento 9). Se utilizó para ello reforzamiento parcial durante la fase previa a la devaluación del reforzador. Dado que éste reduce el impacto de una experiencia posterior de devaluación de la recompensa (Pellegrini et al., 2004), este entrenamiento debería reducir o anular el consumo de alcohol inducido por la misma (Manzo, Gómez et al., 2015). Los resultados obtenidos indicaron que el entrenamiento en reforzamiento parcial retrasó ligeramente la recuperación del efecto de la devaluación y anuló la conducta de ESM, en comparación con el entrenamiento en condiciones de reforzamiento continuo, que sí indujo la conducta de ESM cuando la recompensa fue devaluada.

Por último, se inició la exploración de las bases biológicas de la conducta de ESM, centrando nuestra atención en el papel de la LHb en la extinción consumatoria e instrumental y en el consumo voluntario de alcohol. La lesión neuroquímica de esta estructura cerebral produjo un retraso en la extinción de ambas conductas apetitivas, tal y como se esperaba encontrar dada la implicación de esta región cerebral en el procesamiento de ausencia de

recompensa (Matsumoto e Hikosaka, 2007). Sin embargo, en contra de lo esperado (Haack et al., 2014), no se apreció efecto alguno en el consumo ni la preferencia por el alcohol presentado a diferentes dosis y tiempos de acceso al consumo.

De lo hallado en los experimentos que componen esta Tesis Doctoral puede concluirse que los animales (ratas Wistar) consumen de forma voluntaria sustancias ansiolíticas (alcohol y CDP) y las prefieren frente a agua, tanto en condiciones basales como en situaciones en las que la frustración está presente (por ejemplo, en condiciones de cSNC), en cuyo caso se observa un aumento significativo en el consumo. Aunque los datos referentes al consumo de alcohol son consistentes y replican estudios previos (Manzo et al., 2012; Manzo et al., 2014), los datos referentes al consumo de CDP generan inconsistencias que deberán ser abordadas en estudios futuros. En cualquier caso, puede concluirse que la ESM inducida por pérdida es un fenómeno conductual consistente y replicable, útil como modelo animal para el estudio del inicio del consumo de sustancias ansiolíticas.

Por otro lado, parece que el acceso previo a una sustancia ansiolítica no es capaz de reducir la frustración en una tarea inductora posterior (cSNC) cuando el animal tiene una única experiencia en devaluación, pero sí parece atenuarla cuando dicha experiencia se repite varias veces. Además, dicho acceso no es capaz de generar un efecto ansiolítico, aunque sí desinhibitorio, en el EPM. Estos hallazgos obligan a continuar en un futuro con el análisis experimental del supuesto ansiolítico de la ESM, aumentando la dosis de la droga utilizada y/o empleando otras pruebas conductuales de ansiedad.

Uno de los hallazgos más relevantes obtenidos en la presente Tesis es la posibilidad de atenuar la ESM entrenando al animal en condiciones capaces de hacerlo más resistente a la pérdida. Así, la anulación del consumo de alcohol en sujetos expuestos a reforzamiento parcial ofrece vías de estudio futuras para la prevención de la conducta de consumo en seres humanos.

Finalmente, la implicación de la LHb en las conductas derivadas de la omisión de una recompensa (extinción), así como la ausencia de efecto de la lesión en el consumo de la dosis de alcohol que utilizamos en nuestros experimentos de ESM (2%) permitirá en un futuro analizar de manera más precisa su papel en el consumo voluntario de ansiolíticos inducido por pérdida. En este sentido, inactivar de manera temporal esta región cerebral antes, durante o después de la experiencia inductora del consumo será una aproximación experimental valiosa que nos permitirá responder preguntas relevantes acerca de nuestra línea de investigación. Asimismo, realizar estudios de desconexión de la LHb de otras regiones con funciones cognitivas, motivacionales y emocionales conocidas mejorará nuestro conocimiento del circuito cerebral de la pérdida y su vinculación con la conducta de consumo de sustancias (Ortega et al., 2017). En este sentido, el estudio neuroanatómico de determinadas estructuras cerebrales abre una nueva puerta para explorar las bases biológicas de la ESM. Es importante señalar que el circuito de la recompensa comparte algunas regiones cerebrales que regulan el efecto de SNC, como por ejemplo la ACC, BLA, HC, IC, mPFC y NAc (ver Ortega et al., 2017). Sin embargo, actualmente no se conoce cómo interactúan estos dos circuitos, una interacción que podría ser la base que sustenta la conducta de ESM. Una posible región candidata para servir de punto de interacción para ambos circuitos sería la LHb

que, como se vio en el estudio 5 (Experimento 10) de esta Tesis Doctoral, parece afectar a la respuesta de los animales ante una situación de omisión de recompensa. Se sabe que la LHb está conectada con estructuras que regulan la información relacionada con recompensas inesperadas, con su omisión y con la presentación de estímulos aversivos (VTA; Morales y Margolis, 2017), así como con estructuras relacionadas con la toma de decisiones, la flexibilidad en la conducta y la persistencia de ésta (mPFC, ACC, respectivamente; Kolling et al., 2016; Shin, Rauch y Pitman, 2006). También conecta con áreas involucradas en la regulación de las conductas de miedo y ansiedad (AMG y BNST; Janak y Tye, 2015; Stamatakis et al., 2014). Asimismo, la LHb está relacionada con el NAc, que procesa información emocional y motivacional, al igual que regula el efecto que producen algunas drogas (Salgado y Kaplitt, 2015) y, a través de esta estructura, la LHb conecta con el HC, que está involucrado en la memoria implícita y es activado al mismo tiempo que la AMG en episodios de memoria emocional (Shin et al., 2006). Debido a las conexiones con estas estructuras cerebrales, la LHb podría influir en ambos circuitos, tanto en el circuito de recompensa cerebral como en el circuito del contraste. Aun así, se necesitan más estudios para comprender su función en relación con la ESM inducida por devaluación de la recompensa.

Dadas las limitaciones éticas propias de la investigación en seres humanos, el uso de modelos animales ha resultado sumamente valioso e imprescindible para el progreso en todos los ámbitos de la ciencia. En nuestro campo de estudio, los modelos animales permiten imitar tanto procesos psicológicos o conductuales normales como conductas psicopatológicas que ocurren en seres humanos; también permiten manipular los mecanismos

biológicos que intervienen en tales procesos, así como evaluar los efectos de tratamientos diversos, además de ayudar al desarrollo teórico, al diagnóstico y a la prevención (Escorihuela y Fernández-Teruel, 1998; Keehn, 2018). En efecto, en condiciones experimentales adecuadas es posible controlar muchos factores que afectan a la conducta, permitiendo la realización de descripciones objetivas de la misma, el control preciso de las condiciones que promueven su aparición y el estudio de sus bases biológicas; todo ello explica la amplia aceptación que tienen los modelos animales en el ámbito de estudio de la conducta (Metha y Gosling, 2006). La presente Tesis Doctoral se ha basado en este planteamiento experimental, utilizando los modelos animales de omisión y devaluación de la recompensa para abordar el estudio científico de algunas de las experiencias que pueden llevar a un individuo a consumir sustancias de abuso. Estos modelos presentan dos ventajas principalmente. Por un lado, posibilitan estudiar un estado emocional negativo sin emplear estímulos capaces de inducir daño físico, superando de este modo los problemas éticos con los que se encuentran otras pruebas basadas en la presentación de estímulos potencialmente dañinos para el sujeto experimental. Por otro lado, nos permiten medir la conducta emocional antes y después de la exposición a la prueba de preferencia basada en consumo oral; de este modo se pueden estudiar las “condiciones afectivas” que pueden iniciar, mantener o prevenir el consumo de ansiolíticos en seres humanos. Todo ello aporta una validez ecológica al paradigma experimental utilizado que merece destacarse.

La presente Tesis constituye, además, una continuación en la investigación sobre las bases psicobiológicas de la pérdida, a la vez que abre nuevas vías de estudio al analizar cómo esta experiencia, con gran impacto

emocional, aumenta el riesgo de comenzar a consumir ansiolíticos. Cada estudio presentado ya ha delineado algunas de las restricciones, deficiencias y direcciones futuras que surgen del trabajo descrito, ayudándonos a comprender mejor el fenómeno en estudio. Así, por ejemplo, los experimentos presentados en el estudio 1 aportan datos inconsistentes en cuanto al consumo voluntario de CDP sin inducción previa de frustración, dado que no se halló un efecto de la dosis ni del modo de presentación del fármaco (intermitente vs. continuo; Experimento 3). Estos hallazgos, junto con los resultados obtenidos en el estudio 2, sugieren que los animales necesitan estar en un estado afectivo negativo (en nuestro caso la frustración) para aprender que el consumo de una determinada sustancia reduce dicho estado. También parece necesario que ambas experiencias (inducción de frustración y acceso libre a la droga) se repitan varias veces, sobre todo si la prueba de preferencia se realiza antes que la de inducción (Experimento 8). Se sabe, además, que efecto de ESM es más difícil de obtener cuando los animales son expuestos a una tarea instrumental (laberinto recto), debido al alto consumo de la droga que a veces se observa durante la fase previa a la devaluación (Jiménez-García, 2015; véase, no obstante, Manzo et al., 2014; Manzo, Gómez et al., 2015). Asimismo, el intervalo temporal entre la tarea de inducción y la prueba de preferencia debe durar como máximo 15 de minutos para que se produzca la ESM (Papini, 2016). En su conjunto, estos resultados nos permiten establecer algunas de las condiciones experimentales necesarias para que se produzca el efecto de ESM, al mismo tiempo que abren vías futuras de investigación como las que se comentan a continuación.

En primer lugar, dado que hemos encontrado ciertas inconsistencias en relación con el consumo espontáneo de CDP, podría usarse otro fármaco similar

como, por ejemplo, el loracepam. Este fármaco es de acción rápida y posee una vida media intermedia (su efecto dura entre 8 y 24 horas), no crea metabolitos activos y además es igualmente consumida de forma oral por los seres humanos (De la Fuente, 1981; Stahl, 2013). También podría aumentarse la dosis de alcohol (por ejemplo, hasta un 6%), promoviendo un consumo mayor de ambas sustancias mediante su presentación intermitente (Carnicella et al., 2014; Wise, 1973).

En segundo lugar, datos no publicados sugieren que los animales consumen gran parte de la solución de alcohol durante los primeros 30 minutos de la prueba de preferencia. Una posibilidad futura sería reducir el tiempo de acceso a los ansiolíticos a este intervalo para que sea más fácil detectar sus propiedades ansiolíticas en pruebas de ansiedad aplicadas posteriormente. Ello posibilitaría analizar con más garantías el supuesto ansiolítico de la hipótesis de la ESM.

En tercer lugar, dada la mayor incidencia de trastornos de ansiedad y de consumo de ansiolíticos en mujeres en relación con los hombres (da Silva, Ramos y Takahashi, 2004; Observatorio Español sobre Drogas, 2017; Van der Heyden et al., 2009), en futuros estudios podría compararse la ejecución de machos y hembras en nuestro paradigma experimental para determinar si hay diferencias de sexo en la reactividad a la frustración (como indican estudios previos; Donaire et al., 2013), en el consumo de ansiolíticos inducido por la misma, y/o en ambos.

En cuarto lugar, la posibilidad de influir en la conducta de ESM mediante “intervenciones conductuales” como el reforzamiento parcial ofrece también vías de investigación futuras muy interesantes. Por ejemplo, se podría comprobar si

efecto del reforzamiento parcial en el consumo de ansiolíticos aumenta cuando éstos no se encuentran disponibles en los ensayos no reforzados del citado entrenamiento. En la misma dirección “preventiva” de la ESM, estudios epidemiológicos revelan que los individuos que practican actividad física aeróbica tienen menos probabilidades de usar y abusar de drogas ilícitas, incluido el alcohol (Ruiz-Juan y Ruiz-Risueño, 2011; Smith y Lynch, 2012). Asimismo, estudios con animales indican que los animales que tienen acceso a una rueda de actividad tienen un consumo reducido de alcohol (Darlington et al., 2016). Estos autores también plantearon que la rueda de actividad podría inducir un cambio en la sensibilidad al alcohol, afectando a su consumo, y propusieron una serie de genes candidatos a mediar la relación entre el consumo de alcohol y la conducta en la rueda de actividad. Estos datos proporcionarían una base para analizar si la ESM inducida por pérdida se reduce dando opción a los animales a realizar voluntariamente actividad física (por ejemplo, suministrándoles una rueda de actividad durante el test de preferencia).En definitiva, los resultados obtenidos en la presente Tesis Doctoral ayudan a comprender un fenómeno tan complejo y relevante como es la conducta de consumo de sustancias, una conducta que, cuando se vuelve patológica, es causa de un gran sufrimiento para la persona que la padece y las que están en su entorno. Más que dar respuestas definitivas, el contenido de estas páginas abre nuevos interrogantes, algo imprescindible para el avance de la investigación neurocientífica y sus posibles aplicaciones prácticas.

Conclusiones

Las principales conclusiones derivadas de esta Tesis Doctoral pueden resumirse del modo siguiente:

(1) Los animales consumen voluntariamente y prefieren ansiolíticos (alcohol, CDP) frente a agua cuando estas sustancias se presentan en condiciones de elección libre con dos botellas.

(2) La frustración generada ante situaciones de pérdida de recompensa (cSNC) aumenta el consumo voluntario y la preferencia por alcohol (2%) y CDP (1 mg/kg). Esta conducta de automedicación no se observa en animales no devaluados ni tampoco en animales que reciben sólo agua durante la prueba de preferencia.

(3) El acceso previo a ansiolíticos (alcohol, CDP) aumenta la actividad locomotora evaluada en el EPM, con independencia de si los animales son expuestos o no a devaluación de la recompensa antes del test de preferencia.

(4) El acceso previo a ansiolíticos (alcohol, CDP) no afecta al impacto de una experiencia de pérdida cuando los animales son expuestos a un único evento de devaluación de la recompensa.

(5) El alcohol reduce el efecto de la devaluación de la recompensa cuando el animal tiene experiencias repetidas con dicha devaluación.

(6) Los animales expuestos a refuerzo parcial en la fase de precambio de una prueba de devaluación de la recompensa no aumentan el consumo de alcohol en la fase de postcambio.

(7) La lesión de la LHb aumenta la resistencia a la extinción consumatoria e instrumental pero no al consumo de alcohol, con independencia de la dosis presentada o del tiempo de acceso a la droga.

(8) El consumo voluntario de ansiolíticos inducido por la pérdida de recompensa puede ser una herramienta valiosa para estudiar si la ESM

constituye una puerta de entrada al consumo de ansiolíticos.

Chapter 7. References

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Appendix A

Experiment 4

Manzo, L., Donaire, R., Sabariego, M., Papini, M. R., & Torres, C. (2015). Antianxiety self-medication in rats: Oral consumption of chlordiazepoxide and ethanol after reward devaluation. *Behavioural Brain Research*, *278*, 90-97. https://doi.org/10.1016/j.bbr.2014.09.017

Abstract

Rats increased preference for ethanol after sessions of appetitive extinction, but not after acquisition (reinforced) sessions (Manzo et al., 2014). Drinking was not influenced by appetitive extinction in control groups with postsession access to water, rather than ethanol. Because ethanol has anxiolytic properties in tasks involving reward loss, these results were interpreted as anti-anxiety self-medication. The present experiment tested the potential for self-medication with the prescription anxiolytic chlordiazepoxide, a benzodiazepine with an addictive profile used in the treatment of anxiety disorders. To test this hypothesis, Wistar rats exposed to a 32-to-4% sucrose devaluation received a two-bottle, 2-h preference test immediately after consummatory training. One bottle contained 1 mg/kg of chlordiazepoxide, 2% ethanol, or water for different groups (the second bottle contained water for all groups). Three additional groups received the same postsession preference tests, but were exposed to 4% sucrose during consummatory training. Rats showed suppression of consummatory behavior after reward devaluation relative to unshifted controls. This effect was accompanied by a selective increase in preference for chlordiazepoxide and ethanol. Downshifted animals with access to water or unshifted controls with access to the anxiolytics failed to exhibit postsession changes in preference. Similar results were observed in terms of absolute consumption and consumption relative to body weight. This study shows for the first time that a prescription anxiolytic supports enhanced voluntary consumption during periods of emotional distress

triggered by reward loss. Such anti-anxiety self-medication provides insights into the early stages of addictive behavior.

Appendix B

Experiment 6

Donaire, R., Conrad, S. E., Thompson, J. B., Papini, M. R., & Torres, C. (2018). Augmented voluntary consumption of ethanol induced by reward downshift increases locomotor activity of male Wistar rats in the elevated plus maze. *Behavioural Processes*, *150*, 59-65. https://doi.org/10.1016/j.beproc.2018.02.013

Abstract

Rats exposed to unexpected reward loss increase voluntary oral consumption of ethanol. Such consumption has been assumed to attenuate loss-induced negative affect (called emotional self-medication). To test this assumption, food-deprived male Wistar rats were exposed to 10 sessions of access to 32% sucrose followed by 5 sessions of access to 4% sucrose (reward downshift). A two-bottle preference test was initiated immediately after each consummatory session to assess ethanol intake. The experimental group received access to 2% ethanol and water, whereas the control group received access to two water bottles. On sessions 11, 12, and 15, immediately after the preference test, animals were tested in the elevated plus maze (EPM) for signs of anxiety. Sucrose consumption was reduced after the 32-to-4% sucrose downshift on sessions 11 and 12, but behavior recovered by session 15. Consummatory suppression was followed by increased ethanol intake in the preference test after sessions 11 and 12, but intake was reduced to preshift levels by session 15; no changes were observed in water controls. Finally, general activity (closed-arm entries and total arm entries) in the EPM increased in the ethanol group on session 12, but not on session 15, relative to water controls. The increase in ethanol consumption induced by reward downshift had measurable effects on activity as assessed in the EPM. These results show that voluntary oral 2% ethanol consumption after reward downshift can affect subsequent

behavior, but fall short of providing unambiguous evidence that such ethanol consumption reduces negative affect.

Appendix C

Experiment 10

Donaire, R., Morón, I., Blanco, S., Villatoro, A., Gámiz, F., Papini, M. R., & Torres, C. (2019). Lateral habenula lesions disrupt appetitive extinction, but do not affect voluntary alcohol consumption. *Neuroscience Letters, 703*, 184-190. https://doi.org/10.1016/j.neulet.2019.03.044

Abstract

This study analyzed the effects of LHb lesions on appetitive extinction and alcohol consumption. Eighteen male Wistar rats received neurochemical lesions of the LHb (quinolinic acid) and 12 received a vehicle infusion (PBS). In a runway instrumental task, rats received acquisition (12 pellets/trial, 6 trials/session, 10 sessions) and extinction training (5 sessions). In a consummatory task, rats had daily access to 32% sucrose (5 min, 10 sessions) followed by access to water (5 sessions). Then, animals received 2 h preference tests with escalating alcohol concentrations (2%–24%), followed by two 24 h preference tests with 24% alcohol. Relative to Shams, LHb lesions delayed extinction, as indicated by lower response latencies (instrumental task) and higher fluid consumption (consummatory task). LHb lesions did not affect alcohol consumption regardless of alcohol concentration or test duration. The LHb modulates appetitive extinction and needs to be considered as part of the brain circuit underlying reward loss.