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**INFLUENCE OF SOCIAL STRESS IN THE REWARDING  
EFFECTS OF MDMA AND ALCOHOL**

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**DOCTORAL THESIS**

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VNIVERSITAT  
DE VALÈNCIA

La Dra. Maria Asunción Aguilar Calpe, Profesora Titular de Psicobiología de la Unidad de Investigación Psicobiología de las Drogodependencias, del Departamento de Psicobiología de la Universitat de Valencia,

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Que la tesis doctoral presentada por Doña María Pilar García Pardo, con el título "Influence of Social Stress in the Rewarding Effects of MDMA and Alcohol" ha sido realizada bajo su dirección y que tras haberla examinado hace constar su autorización para que se realicen los tramites conducentes a su defensa.

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Fdo. Maria A. Aguilar.



*A mis padres y hermanas por confiar siempre en mí.*



*A Antonio, simplemente por TODO.*





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## ABBREVIATIONS

2,5-dimethoxy-4-bromoamphetamine (DOB)

2,5-dimethoxy-4-methylamphetamine (DOM)

3,4 methylenedioxy-methylamphetamine (MDMA)

5-hydroxyindolacetic acid (5-HIAA)

6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)

7-nitroindazole (7-NI)

Adrenocorticotropin hormone (ACTH)

Anterior cingulate cortex (ACC)

Bed nucleus of stria terminalis (BNST)

Brain-derived neurotrophic factor (BDNF)

Cannabinoid receptor 1 (CB1)

Central nervous system (SNC)

Conditioned place preference (CPP)

Conditioned stimuli (CSs)

Corticotropin-releasing factor (CRF)

Cyclooxygenase-2 (COX-2)

Deoxyribonucleic acid (DNA)

Dopamine (DA)

Dopamine regulated phosphoprotein-32 (DARPP-32)

Drug Enforcement Administration (DEA)

Elevated plus maze (EPM)

Encuesta Domiciliaria sobre Alcohol y Drogas en España (EDADES)

Encuesta Estatal sobre el Uso de Drogas en Enseñanzas Secundarias (ESTUDES)

Ethanol (EtOH)

European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)

Extracellular signal-regulated kinase (ERK)

Fixed interval (FI)

Fixed ratio (FR)

Food and Drug Administration (FDA)

Gamma-aminobutyric acid (GABA)

Government Delegation for the National Plan on Drugs (DGPNSD)

High performance liquid chromatography (HPLC)

Histone deacetylases (HDAC)

Hypothalamus-pituitary-adrenal (HPA)

Inducible NO synthase (iNOS)

Instituto Nacional de Toxicología (INT)

kappa opioid receptors (KORs)

Medial PFC (mPFC)

Memantine (MEM)

Messenger ribonucleic acid (mRNA)

Methylene-dioxy-methamphetamine (MDM)

DNA Methyltransferases (DNMT)

Nitric oxide (NO)

Nitric oxide synthase (NOS)

N-methyl-D-aspartic acid (NMDA)

Noradrenaline (NA)

Nucleus accumbens (NAcc)

Orbitofrontal cortex (OFC)

p38 mitogen-activated protein kinase (MAPK)

Positron emission tomography (PET)

Post natal day (PND)

Prefrontal cortex (PFC)

Proopiomelanocortin (POMC)

Repeated social defeat (RSD)

Self-administration (SA)

Serotonin transporters (SERT)

Serotonine (5-HT)

Shell of the NAcc (NAccsh)

Single photon emission computed tomography (SPECT)

United Nations Office on Drugs and Crime (UNODC)

Ventral Palid (VP)

Ventral tegmental area (VTA)

$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)





## RESUMEN

La adicción a las drogas es un importante problema en nuestra sociedad con graves consecuencias jurídicas, médicas y sociales para los consumidores. Se trata de un trastorno caracterizado por la pérdida de control sobre el uso de la droga, su búsqueda compulsiva y la aparición de un estado emocional negativo en ausencia de la misma. Por su categoría legal, existen dos amplios tipos de sustancias de abuso, legales e ilegales. El alcohol es la droga legal más consumida tanto por adultos como por adolescentes. Por otra parte, entre las sustancias ilegales, los datos epidemiológicos apuntan que el éxtasis o MDMA es una sustancia ampliamente consumida entre los adolescentes y adultos jóvenes sobre todo en fiestas “rave” donde los consumidores pasan horas, incluso días, haciendo uso de esta sustancia. La adolescencia constituye un periodo de alta vulnerabilidad debido a la inmadurez cerebral, siendo muy perjudicial en esta fase de la vida el consumo de drogas y la exposición a situaciones ambientales negativas. En concreto, se ha demostrado que el estrés social puede provocar el inicio, escalada y la reinstauración en el consumo de drogas en modelos animales. Por ello, el objetivo del presente trabajo ha sido estudiar el efecto del estrés social agudo y repetido sobre las propiedades reforzantes de MDMA (1.25 y 10 mg/kg) y alcohol (1.25 y 2.5 g/kg) en ratones macho adolescentes y adultos jóvenes. Para ello se ha utilizado tres paradigmas principales, la derrota social, el condicionamiento de lugar y el procedimiento de “two-bottle choice”. Para inducir estrés social los animales fueron sometidos a dos tipos de derrota social en un encuentro agonístico con un animal coespecífico agresivo. En la derrota social aguda los ratones experimentales son expuestos a un encuentro agonístico con otro ratón macho en un área neutral inmediatamente antes de la exposición a la droga (cuatro sesiones separadas 48 horas). En la derrota social repetida los ratones experimentales son expuestos a un encuentro agonístico en la jaula del oponente (modelo residente-intruso) de forma repetida e intermitente (cuatro sesiones separadas 72 horas) tres semanas antes de la exposición a la droga. Se realizó una medición de los niveles de corticosterona

en sangre tras la primera y cuarta derrota en los dos modelos de estrés social. En el condicionamiento de lugar se utiliza una caja con dos compartimentos claramente diferenciados en función del color y de la textura del suelo. Para la adquisición del condicionamiento, tras la administración de la droga el animal es confinado en uno de los compartimentos, por tanto, es un modelo de los efectos reforzantes o aversivos condicionados de las drogas. Tras la adquisición del condicionamiento se puede evaluar también su extinción y su reinstauración por la re-exposición a la droga ("priming"). En el procedimiento de "two-bottle choice" el animal tiene acceso a dos botellas, una con agua y otra con alcohol, en su propia jaula. Por tanto, es un modelo del consumo voluntario de alcohol. Asimismo también hemos evaluado el efecto del estrés social sobre otras conductas o procesos como la ansiedad (en el laberinto elevado en cruz), depresión (en el "tail-suspension test"), interacción social, aprendizaje (reconocimiento de objeto, "Hebb-Williams maze") y memoria (evitación pasiva). A su vez, hemos estudiado la base neurobiológica que subyace a los efectos reforzantes de la MDMA y su modulación por el estrés, evaluando el papel del sistema glutamatérgico y de la vía del óxido nítrico. Por otra parte, dado que el alcohol es la principal sustancia adictiva consumida a nivel mundial, y que la finalidad última del trabajo es avanzar en el conocimiento de los mecanismos neurobiológicos que subyacen a la adicción, hemos estudiado el papel de los receptores opioides  $\mu$  en la ingesta de alcohol en el paradigma de "two-bottle choice". Los ratones adultos jóvenes adquieren un condicionamiento de preferencia de lugar (CPL) tras el condicionamiento con 1.25 y 10 mg/kg de MDMA. Este efecto reforzante del MDMA es bloqueado por la exposición a derrota social aguda. Por su parte los ratones adolescentes sólo muestran CPL tras el condicionamiento con 10 mg/kg de MDMA y la derrota social aguda no modifica este efecto. Además la derrota aguda induce una menor respuesta de estrés en adolescentes que en adultos jóvenes, ya que los niveles de corticosterona sólo aumentan inmediatamente después de la derrota social en ratones adultos jóvenes. Estos resultados indican que los ratones

adultos jóvenes son más sensitivos a los efectos reforzantes del MDMA y que el estrés inducido por la derrota social en adultos jóvenes interfiere con la adquisición del CPL. Por el contrario, los ratones adolescentes y adultos jóvenes expuestos a derrota social repetida (DSR) muestran un aumento a largo plazo en su sensibilidad a los efectos reforzantes del MDMA. Tres semanas después de la exposición a DSR se observó un incremento en la duración del CPL inducido por 1.25 y 10 mg/kg de MDMA en ratones adolescentes y adultos jóvenes. Asimismo se observó un aumento de la vulnerabilidad a la reinstauración inducida por priming en ratones expuestos a DSR durante la adolescencia y condicionados tres semanas después con 1.25 mg/kg de MDMA. El incremento en los efectos reforzantes y reinstauradores del MDMA en ratones expuestos a DSR durante la adolescencia es observado a pesar de que esos ratones muestran niveles más bajos de corticosterona que los ratones adultos, indicando la elevada vulnerabilidad del cerebro adolescente a los efectos de la exposición al estrés. Con respecto al papel del sistema glutamatérgico en los efectos reforzantes condicionados del éxtasis, hemos visto que estos efectos dependen de la activación de los receptores glutamatérgicos NMDA ya que el antagonista NMDA memantina inhibe la adquisición del CPL inducido por éxtasis y bloquea la reinstauración inducida por la re-exposición a una dosis "priming" de MDMA. Asimismo, con respecto a la influencia del sistema opioide en los efectos reforzantes del alcohol, hemos observado que la administración del antagonista opioide mu GSK1521498 (0.1, 1 y 3 mg/kg) reduce el consumo de alcohol en el paradigma de "two-bottle choice" en ratas con preferencia por el alcohol, lo que sugiere la utilidad de este compuesto para el tratamiento del alcoholismo. Nuestros resultados también han demostrado que la combinación de MDMA (10 mg/kg) con la derrota social aguda induce déficits cognitivos tales como una inhibición de la memoria implícita (evitación pasiva) y un deterioro de la memoria de reconocimiento (test de reconocimiento de objeto), así como un incremento de la inmovilidad en el "tail suspension test" que es un modelo animal de depresión, y una

respuesta motora reducida al “priming” con MDMA. Con respecto al alcohol, la derrota social aguda y repetida revierte la aversión condicionada a un lugar inducida por la dosis alta de alcohol (2.5 g/kg) e incrementa el consumo voluntario de esta sustancia en el paradigma de “two-bottle choice” en ratones, lo que sugiere que el estrés social aumenta los efectos reforzantes del alcohol. Con respecto al papel de otros receptores glutamatérgicos en los efectos reforzantes del éxtasis y en la influencia que ejerce el estrés social sobre dichos efectos, hemos demostrado que los receptores NMDA, AMPA y la vía del óxido nítrico están implicadas en los efectos reforzantes condicionados del éxtasis. La administración del antagonista NMDA memantina (10 mg/kg), del antagonista AMPA CNQX (0.25, 1 y 5 mg/kg) y del inhibidor de la síntesis de óxido nítrico 7-nitroindazole (7.25 y 12.5 mg/kg) bloquean la adquisición del CPL inducido por éxtasis en ratones. Asimismo, la administración de memantine (5 mg/kg) y de 7-nitroindazole (7.25 mg/kg) revierte los efectos deteriorantes de la derrota social aguda sobre el CPL inducido por éxtasis, lo que sugiere que el sistema glutamatérgico y la vía del óxido nítrico están implicados en estos efectos del estrés social.

En conjunto estos resultados indican que la exposición al estrés social (tanto agudo como a largo plazo) incrementa los efectos reforzantes de MDMA y alcohol y produce diferentes alteraciones conductuales, por ejemplo una reducción de la conducta social, que pueden estar relacionadas con el incremento en el consumo. Los antagonistas glutamatérgicos y la inhibición de la síntesis de óxido nítrico bloquean los efectos reforzantes de la MDMA y el antagonismo de los receptores opiáceos mu disminuye la búsqueda y consumo de alcohol. El avance en el conocimiento de los sistemas de neurotransmisión implicados en los efectos reforzantes de la MDMA y el alcohol puede contribuir al desarrollo de estrategias farmacológicas para el tratamiento de la adicción a la estas drogas.

Palabras clave: MDMA, Ethanol, Derrota social, alteraciones conductuales, CPL, "two-bottle choice", ratón, rata, adolescencia

## ABSTRACT

Drug addiction is a serious problem in our society and has serious legal, medical and social consequences for consumers. This disorder is characterised by an impaired control over substance use, compulsive drug seeking, and the emergence of a negative emotional state in the absence of the drug. With regard to legality, there are two broad types of substances of abuse: legal and illegal. Alcohol is the most consumed legal substance by adults and adolescents. Among illegal substances, epidemiologic data suggest that *ecstasy*, or MDMA, is a widely used substance among adolescents and young adults, especially in "raves", where consumers spend hours, even days, using this substance. Adolescence is a period of enhanced vulnerability due to the lack of brain maturation, and consumption of drugs of abuse and exposure to different negative environmental conditions is especially harmful at this stage of life. It has been demonstrated that social stress can trigger onset, escalation and reinstatement of drug use in animal models. Therefore, the objective of this work was to study the effects of acute and repeated social stress on the rewarding properties of alcohol and MDMA using the place preference paradigm (CPP) and the two-bottle choice procedure. In addition, we set out to evaluate the effects of social stress on several behaviours or processes (anxiety, depression, social interaction, learning and memory). We have also studied the neurobiological aspects underlying the rewarding effects of MDMA and their modulation by stress, assessing along the way the role of the glutamatergic system and nitric oxide (NO) pathway. Moreover, since alcohol is the main addictive substance consumed in the world, we have studied the role of the opioid system in the rewarding effects and intake of ethanol. Overall, our results indicate that exposure to social stress (both acute and long-term) increases the rewarding effects of MDMA and ethanol and induces different behavioural alterations, such as a reduction of social interaction, which is related with an increase in drug consumption. Glutamate antagonists and inhibition of NO synthesis block the rewarding effects of MDMA and

antagonism of mu opioid receptors decreases alcohol seeking and intake. Advances in knowledge of the neurotransmitter systems implicated in the rewarding effects of MDMA and alcohol are likely to contribute to the development of pharmacological strategies for the treatment of drug addiction.

Key word: MDMA, Ethanol, Social Defeat, Behavioural alterations, CPP, two-bottle choice, mice, rat, adolescence,

# 1.- GENERAL INTRODUCTION







## 1.- GENERAL INTRODUCTION

Drug addiction can be defined as a chronic, relapsing disorder that has been characterised by a compulsion to seek and take drugs, loss of control over drug intake, and emergence of a negative emotional state (e.g., dysphoria, anxiety, and irritability) that defines a motivational withdrawal syndrome when access to the drug is prevented (Koob, 2013). In this sense, it is a chronic and recurrent illness characterised by a relapse to drug consumption even after long-term periods of abstinence (Koob, 2010; Koob & Volkow, 2010). Moreover, it is well known that environmental factors can influence the addictive disorders (Ellenbroek et al., 2005; Enoch, 2006). Exposure to stressful events can render people more prone to abusing addictive substances (Sinha, 2001; Koob & Kreek, 2007; Miczek et al., 2008) and there is a positive association between stress and increased drug intake and / or relapse to drug use (Cruz et al., 2011; Boyson et al., 2014; Burke & Miczek, 2014; Fox et al., 2015) in humans and animal models. Using the self-administration (SA) and the conditioned place preference (CPP) paradigms, it has been demonstrated that stress enhances acquisition and reinstatement of drug seeking (Rodríguez-Arias et al., 2013; Aguilar et al., 2013).

Although there are different types of stressors such as pharmacological, physical, emotional or social, the last ones (often studied in the form of social defeat, subordination stress, maternal separation and social isolation) may represent types of stressors with ecological and ethological validity, since in humans, emotional stressors

are primarily activators of stress response. In this work we induced the stress response by the exposure to different forms of social defeat, an important factor that may lead to psychopathological changes and disorders (Björkqvist, 2001; Miczek et al., 2008; Garcia-Pardo et al., 2014). In rodents, after being defeated, profound physiological and behavioral changes were observed (de Groot et al., 1999; Lumley et al., 1999; Keeney et al., 2001; Griebel et al., 2002). It has been shown several times that exposure to different procedures of social defeat increases the rewarding and reinstating effects of different types of drugs chiefly psychostimulant drugs, such as cocaine and amphetamine, in the self-administration and conditioned place preference (CPP) paradigms (Miczek et al., 2008; Neisewander et al., 2012; Aguilar et al., 2013).

Data from human and animal studies confirm that *ecstasy*, or MDMA (3,4-methylenedioxymethylamphetamine), is a drug of abuse with addictive potential (Daza-Losada et al., 2007; Davis & Rosenberg, 2014). MDMA abuse has been documented as a result of chronic consumption (Cottler et al., 2001; Leung et al., 2010), and some *ecstasy* users admit becoming concerned about their use (Degenhardt et al., 2010; Roger-Sanchez et al., 2013b). The typical recreational use of MDMA is often characterized by a pattern of repeated frequent administrations during a short period of time, also known as a binge administration (Badon et al., 2002) in night parties, raves or discos, with short-term effects after consumption such as hyperactivity, mental perspicacity and reduced fatigue (Nichols, 1986; Daza-losada et al., 2007), and long-term effects (Llorente-Berzal et al., 2013; Lopez-Rodriguez et al., 2014). Another important aspect is

that, in people, most drug use begins in adolescence, and drug use in adolescence is more likely to lead to drug abuse in the adulthood (Grant & Dawson, 1998; Lessem et al., 2006). It is known that adolescence is a highly vulnerable developmental period for the consequences of exposure to drugs of abuse (Schneider, 2008). Moreover, in relation with stress, different studies have shown that stress in adolescence increase the risk for drug abuse (Hoffmann et al., 2000; King & Chassin, 2008; McCormick, 2010). Social interactions are highly rewarding in adolescence (Carpenter-Hyland & Chandler, 2007; McCormick, 2010) and social stress can influence adolescent and adults in different ways (Schramm-Sapyta et al., 2009; Garcia-Pardo et al., 2014).

Ethanol (EtOH) is the most common legal drug consumed among adolescent people, according to data from ESTUDES (Encuesta Estatal sobre el Uso de Drogas en Enseñanzas Secundarias) 2012/2013 and recent studies demonstrated that social stress can enhance its effects (Lopez & Laber, 2014; Karkhanis et al., 2014; Norman et al., 2014; Rodriguez-Arias et al., 2014). Moreover, the combination of legal and illegal drugs is evident among consumers, and MDMA is frequently used in combination with EtOH (Barrett et al., 2006; Breen et al., 2006) and an important percentage of consumers of ecstasy (98%) admitted to taking it with alcohol (Rodriguez-Arias et al., 2011; Do Couto et al., 2011). For this reason, it is important to evaluate the effects of both drugs separately and, in future studies, the effects of their combination.

The main objective of the present thesis is to determinate the influence of social stress in the effects of illegal and legal drugs (MDMA and EtOH) using as the main paradigm CPP, although other paradigms have been used such as, second order or two-bottle choice (to study relevant

variables in the drug addiction such as motivation), elevated plus maze (EPM), tail suspension test, passive avoidance, Hebb-Williams maze, etc. Moreover, we have researched the involvement of the age variable (adolescence or adult life) and the role of the different neurotransmitter systems (such as glutamate or opioids) in the rewarding effects of MDMA and alcohol.

To sum up, after a wide theoretical framework, different experimental studies will be presented in the next pages, which will end with a general discussion about the results obtained.

## 2.- EPIDEMIOLOGY OF MDMA AND ETHANOL CONSUMPTION



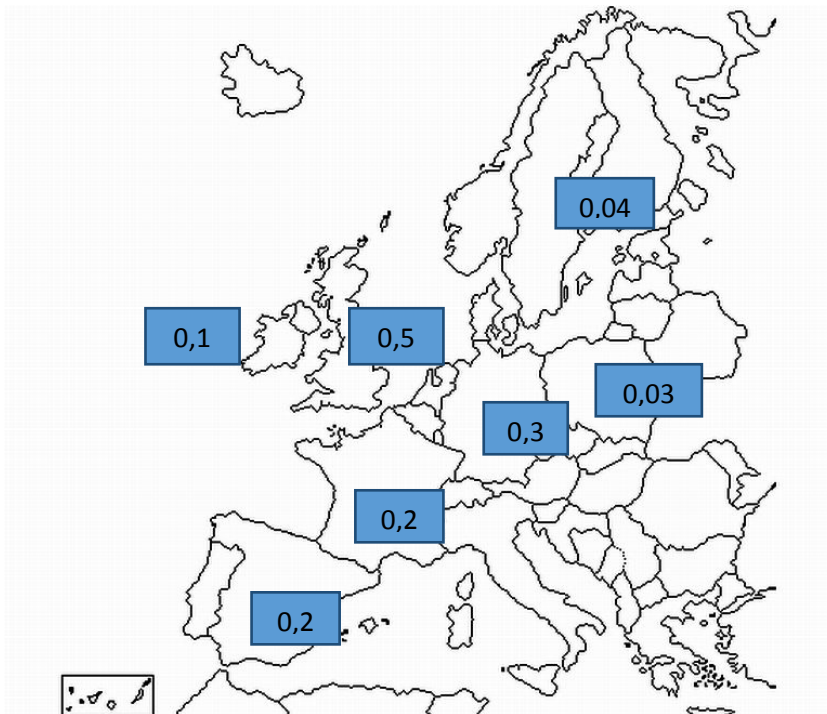
## 2.- EPIDEMIOLOGY OF MDMA AND ETHANOL CONSUMPTION.

Drug consumption continues to be an important problem in our society. The World Drug Report 2014 elaborated by the Commission on Narcotic Drugs of the United Nations Office on Drugs and Crime (UNODC) reports relevant data about MDMA consumption. *Ecstasy* and other amphetamine-type stimulants constitute the second most commonly used group of illicit substances worldwide, with 13.9 million to 54.8 million estimated users.

According to the European Report on drugs 2014 of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), *ecstasy* production seems to be concentrated in Belgium and the Netherlands and there are signs of market recovery, after the decrease in the number of laboratories in Europe in 2010-2012. This trend is also reflected in the available data on the content of MDMA tablets analysed, which declined until 2009 and then increased in the last three years of reporting. Nowadays, these data indicates that MDMA is becoming more frequent (see Figure 1). If we focus our attention on the different European countries, the dates show that in 2012 four million *ecstasy* tablets were seized in the European Union, mainly in the Netherlands (2.4 million), followed by the UK (0.5 million) and Germany (0.3 million). In addition to this, Turkey seized 3 million tablets that year.

Figure 1:

Quantities (million tablets) of ecstasy seized 2012 in countries with high values



Source: The European Monitoring Centre for Drugs and Drug Addiction 2014 (EMCDDA).

In Spain there are two regular surveys, EDADES (Encuesta Domiciliaria sobre Alcohol y Drogas en España), which polls people aged 15-64 residing in family homes; and ESTUDES (Encuesta Nacional sobre Uso de Drogas en la Enseñanza Secundaria), aimed at students aged 14-18 years who attend Secondary School; both funded and promoted by the

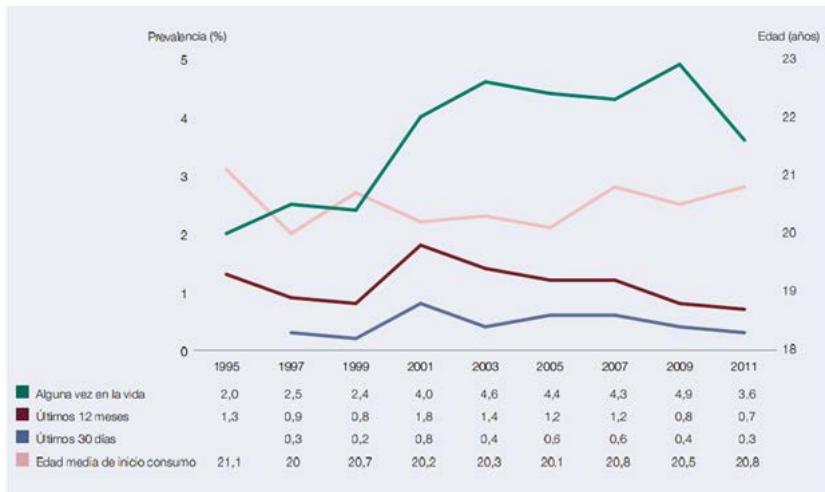


Government Delegation for the National Plan on Drugs (DGPNSD) and Ministry of Health in Spain.

According to the last version of EDADES (1999-2011) published in 2012, a general decline was seen in 2011 in *ecstasy* use prevalence for the usual three time indicators, 3.6% for consumption ever in life, 0.7% for the last 12 months and 0.3% in the last 30 days. This decline is most striking in experimental use (once in life), which is of particular significance, given that *ecstasy* is mainly consumed in this way and considering that in 2009, "Once in a lifetime" consumption rebounded and had the highest prevalence of the time series (4.9%) level. This proportion is down 1.3 percentage points in 2011 to stand at the lowest level of the last decade. However, in relation with the consumption in the last period it is possible to observe an increase again (near to consumption in 2009). Disaggregating by sex and age, it is observed that, as with other illegal substances, consumption of *ecstasy* is more common among men than women, with the highest prevalence (last 12 months) in the group of 15-24 years and 25 to 34 years of age (EDADES 1999-2011) (see figures 2 and 3, for detail). The preliminary report of EDADES 2013 report the same data for consumption in the last 12 months (0.7%).

Figure 2

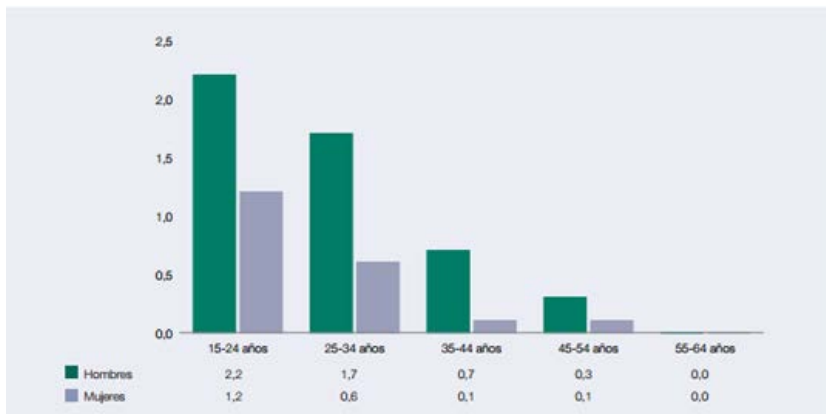
Evolution of the prevalence of ecstasy consumer and average age of first use in a population aged 15-64 (percentages). Spain 1995-



Source: OEDT. Encuesta sobre alcohol y drogas en España (EDADES)

Figure 3

Prevalence of ecstasy in the last 12 months in the population aged 15-64 according to sex and age group (percentages). Spain 2011.



Source: OEDT. Encuesta sobre alcohol y drogas en España (EDADES)

The other epidemiological report ESTUDES (2012/2013) showed that in 2012, the main illegal drugs consumed by students aged 14 to 18 years were cannabis, hypnotics, cocaine and MDMA. Prevalence of *ecstasy* use at least once in life was higher in men than women (3% versus 1,4%), representing an increase of 0.5% compared to 2010. This increase occurred mainly among men whose prevalence of consumption doubled that registered among women and the age of first consumption of MDMA was by 15, 8 years of age (see Figure 4).

Figure 4

Evolution of the prevalence of ecstasy and average age of first use among high school students of 14-18 years (percentages).

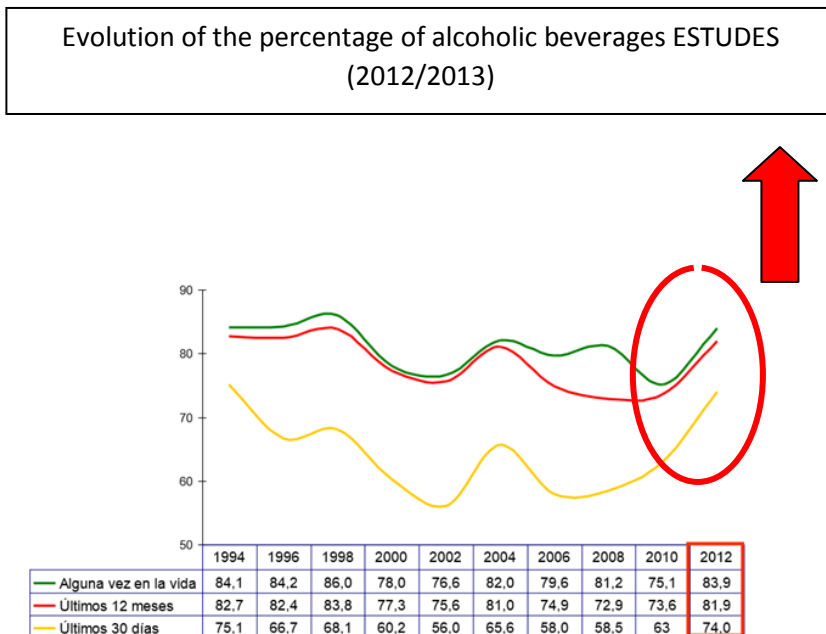


Source: ESTUDES 2012/2013

On the other hand, data about consumption of alcohol shows that it is a problematic topic, mainly among adolescents with binge drinking patterns of consumption. According to data from EDADES the extent of drinking in the Spanish society is practically universal. Thus, in 2013, 93.1% of the Spanish population between 15 and 64 years had consumed at least once in their lives, 78.3% of the population admitted to having consumed in the past year, and 64.4% had done so in the last

month. According to ESTUDES 2012-2013, alcohol is the first legal drug of abuse consumed by adolescents in Spain. In 2012, 81.9% had consumed alcohol in the past year and 74% in the last month. Moreover, the percentage of youth who consumes alcohol remains at high levels: 3 out of 10 in the last month and more than half of the 16 year olds has been drunk in the last year. Related with the binge drinking patterns the data show that this practice increases with age: 4 out of 10 adolescents (14 years) and 8 of 10 (18 years) have a binge pattern of consumption in the last year. The most important aspect in this data is that there is a tendence toward an increase in consumption of alcohol among adolescents (see figure 5).

Figure 5



Source: ESTUDES 2012/2013. Observatorio español sobre drogas. DGPNSD. MSSSI

### 3.- ABOUT MDMA....



### 3.- ABOUT MDMA

MDMA (3,4-Methylenedioxy-methamphetamine) belongs to a designer drug group made with different chemical agents. It is an illegal drug well known for its recreational use, primarily because of its empathogenic properties. For example, it increases the feeling of love, sociability, intimacy, confidence, elevated empathy, closeness to others and euphoria (Parrott, 2001; Morgan et al., 2013; Hysek et al., 2014; Davis & Rosenberg, 2014) and it is consumed mainly in places similar to dance club, discos or rave parties where people spend several hours (or even days) dancing in environments with electrical and house music (Bobes et al., 2002). Positive effects after consumption have also been demonstrated in experimental animals, MDMA inducing different changes in social behaviour such as prosocial and anti-aggressive effects (Thompson et al., 2008; Thompson et al., 2009; Machalova et al., 2012).

There are many code names and popular terms for this drug. An early street name, *ecstasy* has give rise to the initials XTC (Shulgin, 1990), but other names are also being used to indicate the same substance (Adam, Essence, lovely drug, "E" or "X") or "molly" or "mandy" when the drug is in powdered form. The acronym MDM stands for methylene-dioxy-methamphetamine, and MDMA in its full form, are a reference to the initials of the synthetic base 3, 4- methylenedioxy-methamphetamine.

#### 3.1.- History

MDMA has been among the most popular illegal psychotropic drugs since the mid 1980s (Kalant, 2001; Freudenmann et al., 2006). However, MDMA was discovered much earlier. MDMA was first synthesized and

patented by the German pharmaceutical company Merck in Darmstadt around 1912, probably unintentionally, with plans to market a compound very different from MDMA, an appetite suppressor (Freudenmann et al., 2006).

Also, in 1953, MDMA was used by American Army Chemical Centre as a potential brainwashing agent and its toxic effects were evident, although they were never published. Later, in the 1960s, the longer-acting analogue of MDMA, MDA acquired a recreational use. Its rediscovery in the late 1970s probably had little to do with the fact that it was a legal drug. But its effects were unknown for the moment. In 1978, the biologist and chemist Alexander Shulgin showed the psychoactive effects of MDMA and in 1980s MDMA began to be used as a recreational substance in rave parties among young people mainly (Pentney, 2001; Sessa, 2007).

As it has been said before, MDMA promotes relaxation, an increase in communication with others, it facilitates a loosening of the ego and encourages elevated thoughtfulness and contemplativeness (Nichols, 1986; Morgan et al., 2013; Hysek et al., 2014). MDMA was also used, sometimes, as a therapy substance. For example, the warmth and feelings of empathy that are experienced under MDMA effects can be used to promote a positive therapy. Utilisation of MDMA as an agent to assist in couples therapy in the United States in the early 1980s was common because it allowed users to approach previously difficult intimate relationship conflicts (Greer & Tolbert 1986; Sessa et al., 2007).

However, years after it appeared, different studies showing addictive effects in MDMA consumers and different neurotoxic effects in rats,

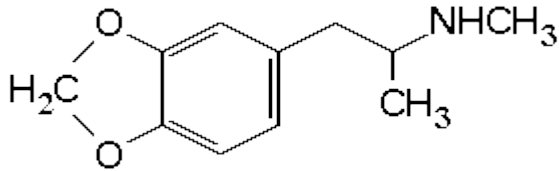


dogs or non human-primates (Mokler et al., 1987; Frith et al., 1987; Ricaurte & McCann, 1992). In fact, in 1985 the “Drug Enforcement Administration” (DEA) included MDMA in the list I of controlled substances showing that MDMA had a wide potential as an addictive substance and it did not meet the reglamentary norms required by the “Food and Drug Administration”. In this decade, several studies have shown the same perspective about the dangers of consuming MDMA and other amphetamines (Ricaurte et al., 1985; Stone et al., 1986; Schmidt, 1987; Commins et al., 1987; Battaglia et al., 1987; Ricaurte et al., 1988; O'Hearn et al., 1988; Pentney, 2001) and argues against its administration in therapy (Parrot, 2012a; Parrot, 2013a; Parrot, 2014a; Parrot, 2014b) while others maintained that *ecstasy* 's negative effects continue being a controversial topic and support the therapeutic potential of MDMA (Cole, 2014; Doblin et al, 2014).

### 3.2.- Structure

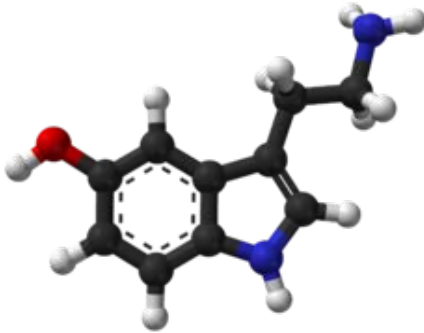
MDMA has a particular structure and although there are other drugs (mainly amphetamines) with similar structure it is clearly stands apart from other hallucinogenic amphetamines, such as DOB (2,5-dimethoxy-4-bromoamphetamine) or DOM (2,5-dimethoxy-4-methylamphetamine) (Morimoto et al., 1998) in that it is a secondary amine. That is, the basic nitrogen is substituted with an N-methyl, while hallucinogenic amphetamines are most potent as primary amines (Nichols & Oberlender, 1989). The molecular formula for MDMA is  $C_{11}H_{15}NO_2$ .

Figure 6



*Chemical structure of MDMA*

Figure 7



*Dimensional structure of MDMA*

### 3.3.- Composition

Although MDMA is a designer (not natural) drug, the precursors of this substance are vegetables that suffer different forms of adulteration to produce its addictive properties. In its pure form it is a powder, but in

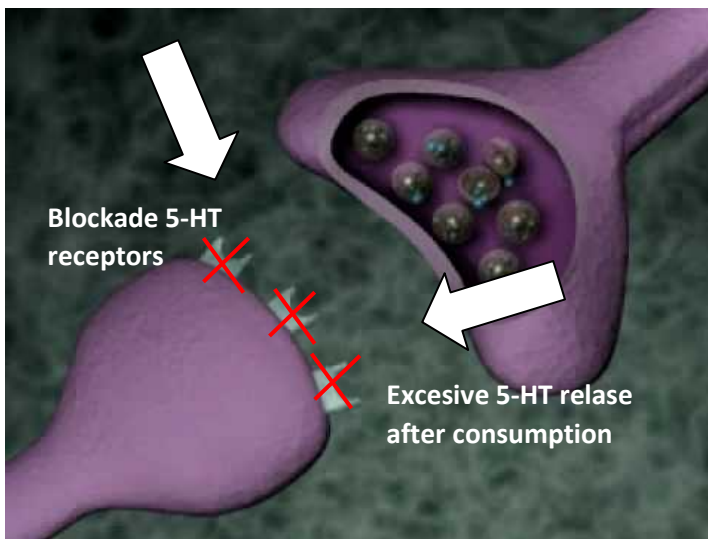
the street it is presented in tablets with different shapes, textures and colours. In Spain, according to data from the Instituto Nacional de Toxicología (INT) the amount of active ingredients present in the seized samples (tablets or capsules of approximately 300 mg) is only between 90 and 166 mg. Normally, MDMA is adulterated with other drugs or their derivatives (cannabis, benzodiazepines or methylphenidate). Sometimes, other substances with compounds very different from MDMA are considered *ecstasy*, making it a risky aspect about consumption (Sherlock et al., 1999; Baggott et al., 2000; Cole et al., 2002). Other dangerous effects of MDMA are related with the dose. Lethal doses are above 500 mg, although there have been lethal cases with lower doses. A low-middle dose is considered to be between 50-160 mg and a high dose 180-200 mg (Kalant, 2001). The price in the illegal market oscillates between 5 and 10 euros (data by EMCDDA, 2014). Therefore, it is not very expensive for adolescent and young adults to acquire and it is easy for them to buy the drug in the street.

### 3.4.- Mechanism of action of MDMA

The effects of MDMA in the central nervous system (SNC) are diverse because this substance interacts with different neurotransmission systems (Breivik et al., 2014; Yubero-Lahoz et al., 2014; Liechti, 2015). The acute effects of MDMA on brain neurotransmitter systems have been well documented. MDMA blocks the serotonin (5-HT), dopamine (DA), and noradrenaline (NA) transporters and stimulates the release of monoamines by reverse transport, mainly 5-HT (Figure 8), NA and DA (Breivik et al., 2014; Colado et al., 2004; Lizarraga et al., 2015), in

several regions of the brain mainly in the nucleus accumbens (NAcc) (Yamamoto & Spanos, 1988; Marona-Lewicka et al., 1996; White et al., 1996; Kankaanpää et al., 1998). Data shows crucial and distinct involvement of the 5-HT system in processes related with drug addiction and compulsive behaviour (Müller & Homberg, 2015). In fact, different studies have attributed its locomotor and rewarding effects to both the activation of serotonergic and dopaminergic systems (Meyer, 2013; Müller & Homberg, 2015), although other systems like endocannabinoid seem to be also associated (Valverde & Rodriguez-Arias, 2013).

Figure 8



*Mechanism of action of MDMA*

Glutamatergic system also plays an important role in the mechanism of action of MDMA. Microiontophoretic application of MDMA has been shown to inhibit glutamate evoked firing of most cells in the NAcc (White et al., 1994). Anneken & Gudelsky (2012) reported that in the hippocampus MDMA produces a delayed and sustained increase in the extracellular concentration of glutamate. The MDMA-induced increase in hippocampal glutamate release was suppressed by fluoxetine and the 5-HT<sub>2</sub> antagonist ketanserin but was still evident in the presence of tetrodotoxin (a lethal neurotoxin with rapid and potent action) and the authors concluded that 5-HT, released by MDMA, activates 5-HT<sub>2A/C</sub> receptors, thereby promoting this release of glutamate in the hippocampus.

### 3.5.- Long-term neurotoxicity and related behavioural alterations

*Ecstasy* exerts its acute effects by increasing the extracellular concentration of monoamines in the brain by reversing the functions of reuptake mechanisms and these elevations in extracellular monoamine concentrations result in serious damages for the organism, elicit significant neurobehavioral adverse effects, and they can induce even neurotoxicity and neuroinflammation (Parrott, 2002; Ricaurte et al., 2003; Cadet et al., 2007; Capela et al., 2009; Mueller et al., 2011; Torres et al., 2011; Mueller et al., 2013; Parrot, 2013b; Benningfield & Cowan, 2013; Halpin et al., 2014).

In rats, the pharmacological actions of MDMA lead to the long-term loss of 5-HT nerve terminals (Commins et al., 1987; O'Hearn et al., 1988) and biochemically the damage is reflected by a substantial decrease in the

concentration of 5-HT and its metabolite, 5-hydroxyindolacetic acid (5-HIAA) (Colado et al., 1993; Lew et al., 1996; Colado et al., 1997; Shankaran & Gudelsky, 1998; O'Shea et al., 1998; Aguirre et al., 1998; Wallace et al., 2001; Green et al., 2003; Thompson et al., 2004) and a reduction in the density of 5-HT transporter, a hallmark of 5-HT nerve terminal integrity (Colado et al., 1995; O'Shea et al., 1998; Teixeira-Gomes et al., 2014; Halpin et al., 2014).

In mice, repeated administration of *ecstasy* induces dopaminergic neurotoxicity, decreasing the functionality of DA transporters, and produces lasting impairments in recall of alternation behaviour, reducing cognitive flexibility (Viñals et al., 2013). MDMA-induced neurotoxicity can be responsible for cognitive impairment involving different areas in the brain, such as the limbic and cortical regions (Costa et al., 2014).

Similar results were found in non-human primates (Banks et al., 2008) and in baboons (Szabo et al., 2002) with reductions in brain serotonin transporters (SERT) after a treatment with a MDMA dose of 40 mg/kg during 4 days.

Studies in humans through brain imaging, using either positron emission tomography (PET) or single photon emission computed tomography (SPECT), and measuring brain SERT levels as an indicator of neuronal integrity, have suggested that MDMA abusers have damage in serotonergic neurons, with reduced levels of 5-HIAA and SERT densities (McCann et al., 1998; Semple et al., 1999; Reneman et al., 2001; McCann et al., 2005; Parrot, 2012a; Parrot, 2013b). A classical study showed that density of 5-HT<sub>2A</sub> postsynaptic receptors was lower after

recent abuse, but higher in former *ecstasy* abusers (Reneman et al., 2001), probably as consequence of the wide reduction in serotonin induced by MDMA. Imaging studies have shown lowered brain SERT binding in current MDMA users but it is uncertain for how long this abnormality might persist following abstinence (Selvaraj et al., 2009).

### 3.6.- Pharmacology of MDMA

MDMA and other amphetamines are rapidly absorbed following oral administration in the form of a pill commonly (Gouzoulis-Mayfrank & Daumann, 2009). MDMA is detectable in the blood within 30 minutes, reaches its max within 1-2 hours after consumption and has a half life of about 6–8 hours (Green et al., 2003; Dumont & Verkes, 2006), although its effects can be biochemically detected 24-48 hours after consumption (Curran, 2000). The psychoactive effects last for approximately 2–4 hours in spite of persisting blood levels and objective impairment of mental functioning lasting longer than the subjective effects in concordance with the persisting MDMA levels (Lamers et al., 2003). Approximately 40 hours are needed in order for 95% of MDMA to disappear. This is why some consumers describe psychological and physical effects days after consumption (Kalant, 2001).

In rodents or other non-human primats (Chu et al., 1996; Mueller et al., 2008), as well as in humans (de la Torre et al., 2000) the pharmacokinetic of MDMA is nonlinear and MDMA is one of the most dangerous drugs. In effect, (Schifano et al., 2010) analysed the government data on recreational stimulant deaths in the UK between 1997 and 2007 and found that over this period, there were 605

recorded deaths related to *ecstasy*/MDMA. The pharmacokinetics and metabolism of MDMA are described in more detail in other works (de la Torre et al., 2000; Green et al., 2003).

### 3.7.- Effects of MDMA

The physiological and psychological effects of MDMA are thoroughly described in several studies (Dumont & Verkes 2006; Parrott, 2013 a). There are broad data about the effects of MDMA in the organism because it acts in several parts. So, we will organize the next section in different areas. First we will describe the physiological and behavioural effects of MDMA in humans and then the same effects in experimental animals.

#### A.- HUMANS

##### ∞ Physiological effects

MDMA administration in humans evidences salient cardiovascular and neurologic sympathomimetic effects. Increased blood pressure and pulse rate and vasoconstriction are commonly observed, as also are diaforesis, mydriasis, dry mouth, jaw clenching, trismus, and bruxism (for this reason, MDMA consumers have baby pacifiers in raves parties). Fatalities can result from cardiac arrest, brain seizure, 'rhabdomyolysis' or the destruction of skeletal muscle tissue, and 'disseminated intravascular coagulation' or the failure of blood clotting which results in uncontrollable bleeding through multiple sites (Henry et al., 1992;



Hall & Henry, 2006; Parrot, 2013a; Michael White, 2014). These symptoms are known as serotonin syndrome.

Another characteristic effect of MDMA is the reduction in local cerebral blood flow and hyperthermia (Parrott, 2012 b; Kiyatkin et al., 2015), that is the major acute adverse event that can follow ingestion of MDMA by recreational users (Parrott, 2013 a). Most people report feeling hot, with pronounced sweating and feelings of dehydration (Parrott et al., 2008) and some consumers even said that they felt like their body was at 115 grades (Cohen, 1998).

These physiological effects, have sometimes been associated with the places where MDMA is consumed, such as dance clubs, discos or parties, where the ambient temperature is high and where some *ecstasy* users dance for prolonged periods with minimal breaks (Suy et al., 1999; Parrott et al., 2006). Other medical problems are related with hyperthermia including myoglobinuria, renal failure, liver damage and disseminated intravascular coagulopathy (Green et al., 2003; Docherty & Green, 2010; Halpern et al., 2011; Parrott, 2012 b). Such problems can be fatal and are identical to those seen in persons suffering from heatstroke (Kalant, 2001).

MDMA can enhance some aspects of acute and chronic stress in humans. MDMA led to an increase in the levels of cortisol (Harris et al., 2002). Gerra et al., (2003) reported that baseline cortisol was significantly lower in abstinent *ecstasy* users compared with non-user controls and that they displayed reduced cortisol responses to stress, which might indicate a neuroendocrine dysfunction induced by repeated MDMA use. Acute *ecstasy* use increases cortisol levels by 100-200% in drug-free

regular *ecstasy* users, whereas *ecstasy* consumed at dance clubs induces an 800% increase in cortisol levels, because of the combined effects of the stimulant drug and dancing. Also, in three-month abstinent users the cortisol levels were 400% higher than those in controls. Chronic users show heightened cortisol release in stressful environments accompanied by deficits in complex neurocognitive tasks. So, acute and subchronic MDMA increase cortisol levels and induce changes in the hypothalamus-pituitary-adrenal (HPA) axis (Parrot et al., 2014).

### ∞ Psychological effects

MDMA consumers report increased subjective feelings of love, euphoria, intimacy, personal closeness, elevated vigor and arousal (Liechti et al., 2000 a, b; Kuypers et al., 2007). Psychological effects of MDMA depend on 5-HT, while the euphoric effects appear to relate to DA (Liechti & Vollenweider, 2001). In fact, some consumers have entactogenic effects and positive responses such as feeling like 'floating, flying, highly sensual' and that 'everyone is your friend'.

MDMA, associated with a subjective feeling of confusion that could be related to its serotonergic-induced hallucinogenic effects, seems to have some deleterious effects on psychomotor performance (De la torre et al., 2000). Different reviews with meta-analytic studies indicated that *ecstasy* users are significantly impaired compared to non-consumer subjects on psychomotor performance (Kalechstein et al., 2007; Zakzanis et al., 2007). MDMA increases motor activity in humans and prolongs physical activity (e.g., dancing) when used in nightclubs, increasing the chances for hyperthermia (Gilpin et al., 2011).

One of the most prominent adverse effects of MDMA is related to cognitive functioning (Parrot, 2013 b; Wagner et al., 2014). Memory is an important capacity frequently reported to be affected by MDMA (Verkes et al., 2001; Verbaten, 2003; Daumann et al., 2004; Camarasa et al., 2012; Gallagher et al., 2014). Heffernan et al., (2001) reported a prospective memory damage in MDMA consumers that can persist after acute consumption because Parrott & Lasky (1998) demonstrated that 1 and 4 days after MDMA administration the users have significantly lower scores than controls (60–70% recalled fewer words) on the memory recall task.

Using Wechsler Adult Intelligence Scale memory subscales in a small group of abstinent *ecstasy*/MDMA users, Krystal et al. (1992) found mild to moderate levels of impairment in memory. In the same line, (Morgan, 1999) reported significantly poorer prose recall in abstinent *ecstasy* users, compared to non-user controls. Fox et al., (2002) administered the Cambridge Automated Neurocognitive Test Battery to abstinent *ecstasy* consumers and reported a cognitive function similar to patients with brain damage (problems in a temporal lobe). Also, with a battery of cognitive tasks Reay et al., (2006) found various deficits (including complex decision-taking) in MDMA users. Not surprisingly, long-term MDMA use impairs performance in other cognitive domains different to memory such as complex attention (McCann et al., 1999; Gouzoulis-Mayfrank et al., 2000).

Negative emotional states have been described after MDMA consumption, such as increases in self-rated apprehensiveness, depression, and other negative moods (Bedi et al., 2010; Parrott et al., 2011; Kirkpatrick et al., 2012; Taurah et al., 2014). With recreational

consumption of *ecstasy*, acute negative reactions sometimes occur. For example, acute feelings of anxiety, overstimulation, panic, and loss of personal control (Davison & Parrott, 1997; Cohen, 1998). Positive and negative mood changes often develop in the same individual, with feelings of happiness and depression, and extraversion and introversion, during the same *ecstasy*/MDMA experience (Liechti et al., 2000 a, b). In relation with aggression, Reid et al., (2007) reported that those with a higher prevalence of lifetime *ecstasy* use exhibit higher levels of aggressive and violent behaviour. Individuals with low self-control appear to be most affected by *ecstasy* use maybe because aggression is a behaviour related with 5-HT.

## B.- EXPERIMENTAL ANIMALS

### ∞ Physiological effects

In general terms, it is well known that while administration of higher doses of MDMA usually causes hyperthermia in rats and rodents, in some conditions it can also induce hypothermia, particularly following a low dose, or when animals are housed alone or at a cool ambient temperature (Docherty & Green, 2010). Both MDMA- induced hyperthermia and hypothermia result primarily from monoamine release in the brain (Docherty & Green, 2010; Shortall et al., 2013). On the other hand, changes in the cardiovascular system have been described after MDMA consumption, which causes tachycardia and arrhythmias (O’Cain et al., 2000; Badon et al., 2002). Administration of MDMA is also associated with alterations in the immune system (de Paula et al., 2008), rendering the animal treated with MDMA more

susceptible to infectious diseases (Boyle & Connor, 2010). Finally, exposure to MDMA decreases levels of gonadotrophin release hormone in the hypothalamus and serum testosterone (Dickerson et al., 2008).

### ∞ Behavioural effects

MDMA produce an increase in motor activity in rats (Balogh et al., 2004; Cassel et al., 2004; Colussi-Mas & Schenk, 2008; Roodsiri et al., 2011) and mice (Itzhak et al., 2003; Daza-Losada et al., 2008; 2009a; Ferraz-de-Paula et al., 2011), but decrease this behaviour in non-human primates (Taffe et al., 2006; Von Huben et al., 2007).

On the other hand, exposure to a wide range of drugs has been shown to produce an enduring change in behavior termed sensitization. Behavioral sensitization refers to the augmentation of the psychomotor effects of drugs following repeated intermittent exposure and represents a striking form of behavioral plasticity determined by consumption of drugs (Ball et al., 2011). MDMA produces behavioral sensitization (Ramos et al., 2004; Ball et al., 2006, 2009, 2010). For example, after 10 or 15 days of withdrawal of repeated MDMA treatment, a sensitization of locomotor activity was evident after an MDMA challenge (Ball et al., 2011; Varela et al., 2011). A recent study demonstrated that rats that had a significantly larger locomotor response to the first MDMA injection developed tolerance to the locomotor-activating effects of MDMA instead sensitization. Moreover, while rats that develop sensitization maintained relatively stable levels of MDMA SA over days and did not show cue-induced reinstatement of MDMA-seeking following extinction, rats that developed tolerance

displayed an escalation of MDMA SA and cue-induced reinstatement of MDMA seeking (Ball & Slane, 2014).

With regard to anxiety, MDMA induces mainly an anxiogenic action in animals, but this result depends on the dose, the species of animals and the method used for evaluating anxiety. Low doses (4-8 mg/kg) induce anxiogenic effects and high doses (10mg/kg-20mg/kg) induce anxiolytic effects (Ho et al., 2004; Ferraz-de-Paula et al., 2011). In addition, an anxiolytic effect has been observed after acute treatment with cocaine plus MDMA in mice (Daza-Losada et al., 2009a) or 3 weeks after this combination (Daza-Losada et al., 2008).

MDMA, though considered a prosocial drug, has been shown to have both anxiogenic and anxiolytic effects on social interaction in rodents after acute administration. In mice, the effects of MDMA on social behaviour have been studied by confronting MDMA-treated individuals with an anosmic "standard opponent" in a social encounter that takes place in a neutral area. On the other hand, in rats, different studies have reported increases in anxiety undergoing the social interaction test following several weeks of withdrawal from an MDMA dosage regimen that caused modest serotonergic neurotoxicity (Morley et al., 2001; Gurtman et al, 2002; McGregor et al., 2003). Thus, an anxiogenic profile in the social interaction test has been associated with the long-term depletion of serotonin when there is abstinence following MDMA binges. On the other hand, high impulsivity and aggression levels seen in MDMA consumers may be due to the damage induced by this drug on the serotonergic neurons.

Administration of MDMA can alter learning and memory in animal models using different species like monkeys, mice and rats (Taffe et al., 2001; Moyano et al., 2004; Daza-Losada et al., 2009a; Plaza-Zabala et al., 2010; Camarasa et al., 2012; Shariati et al., 2014). Plaza-Zabala et al. (2010) demonstrated that mice treated with MDMA showed reduced learning and recall of active avoidance task when compared with saline-treated controls. Functional deficits in learning/memory have been observed long-term after repeated administration of MDMA in experimental animals including monkeys (Taffe et al., 2001), rats (Skelton et al., 2006) and mice (Trigo et al., 2008). Daza-Losada et al. (2009a) reported that only the high doses of MDMA (20 mg/kg) induce impairments in passive avoidance test. More recently, Abad et al. (2014) showed that after binge administration of MDMA to adolescent rats a neurotoxic damage of hippocampal serotonergic terminals was observed. However, MDMA treatment under restricted conditions of learning and memory (associated with the training of a difficult water maze) increases brain-derived neurotrophic factor (BDNF) expression, stimulates synaptic plasticity and facilitates learning of a water maze task.

## 4.- ABOUT ALCOHOL....

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#### 4.-ABOUT ALCOHOL

Alcohol is the most socially accepted and most commonly used drug of abuse, and it is also the one that induces the most social and health-related problems (Nutt et al., 2010).

EtOH, the main component of alcoholic drinks, is the name for a chemical compound known as ethyl alcohol that in normal conditions is a colorless and flammable liquid with a boiling point of 78.4°C. The concentration of EtOH is variable according with the elaboration of the drink. There are two main categories that are different for their amount of alcohol: fermented beverages (such as beer or wine) with a low graduation (5-10°), and distilled beverages (for example, gin, vodka or whiskey) with a high graduation (40° - 50°).

EtOH affects the CNS, causing disinhibition, confusion, dizziness, euphoria, low glare, drowsiness, and poor motor coordination. In some cases, it increases irritability and aggressiveness. At higher concentrations, it slows movement and causes temporary loss of vision and visual hallucinations (for example, double vision is very typical in drunk people). If the dose of intoxication is very high, it can provoke coma and death.

Disinhibition and sedative effects are characteristic because EtOH is an allosteric modulator of many transmembrane receptors (Pohorecky & Brick, 1988) and it works as a CNS depressant, potentiating the action of  $\gamma$ -amino-butyric (GABA) at the GABA A receptor (Suzdak et al., 1986).

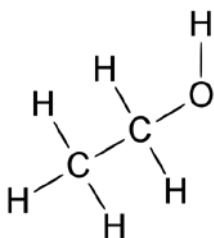
#### 4.1.- History of ethanol

Alcohol is the oldest drug of abuse used by people. For centuries, alcohol consumption has been part of our culture and society. Anciently, drinks with alcohol, have been linked with the divine by its nature and its effects and soon were associated with religious rituals. Later, the consumption of alcohol continued to extend, and nowadays, EtOH is the most important drink in our lives, even in different cultures, in spite of knowing its negative effects.

#### 4.2.- Structure of ethanol

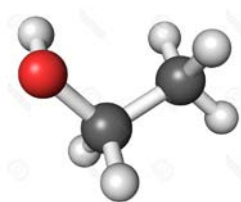
EtOH is a primary alcohol because it has the hydroxyl group connected to a primary carbon atom. EtOH has a lot of polarity because there are several links between oxygen and hydrogen neighboring molecules. Its chemical formula is  $\text{CH}_3\text{-CH}_2\text{-OH}$  ( $\text{C}_2\text{H}_6\text{O}$ ).

Figure 9



*Chemical structure of ethanol*

Figure 10



*Dimensional structure of ethanol*

#### 4.3.- Pharmacokinetics of ethanol

EtOH is consumed orally. In the body, it is distributed more easily in aqueous media in lipid and it can access the bloodstream from different organs. Once absorption of alcohol has already occurred, it is distributed in the body, and since it is a water-soluble molecule, it is distributed easily. Moreover, EtOH crosses the blood-brain barrier. In this way EtOH represents a toxic that is rapidly and completely absorbed in the intestinal tract being distributed to most tissues and organs (Szalontay, 2014). The average duration of gastric EtOH absorption process has been estimated at 1.7 minutes. In any case, this time also depends on the dose, because when it increases, the absorption time is also increased. Differences in the distribution of EtOH between individuals are due to different body fat ratio, even when the amount of the substance ingested and body weight are identical, since solubility of EtOH is different in water and in lipid media (Dasgupta, 2015). Differences in the absorption on EtOH between women and men have also been observed (Dettling et al., 2008).

#### 4.4.- Mechanism of action of ethanol

The form of actuation of EtOH in the brain is a controversial topic because alcohol has a complex mechanism of action. EtOH acts on several neurotransmitter systems such as gabaergic, glutamatergic, serotonergic, cannabinoid, opiod or dopaminergic. It is well known that EtOH is an inhibitor substance for the brain and the main neurotransmissor involved is GABA, but it is not the only one. In

addition, EtOH affects most other neurochemical and endocrine systems (Erdozain & Callado, 2014).

\*Ethanol and the GABAergic system.

Primarily,  $\gamma$ -amino-butyric (GABA) is considered the main inhibitory neurotransmitter in the CNS. The GABAergic system plays an important role in the mechanism of action of EtOH, mediating different behavioural and pharmacological effects (Erdozain & Callado, 2014). EtOH allosterically potentiates the actions of GABA, stimulating the flow of chloride through the GABAA receptors (Grobin et al., 1998; Aguayo et al., 2002). Chronic EtOH consumption and repeated EtOH withdrawal produce many adaptations of the GABAA receptor function. For instance, after chronic exposure to EtOH different behaviours (like sedative, motor incoordinating and cognitive-impairing effects) are modified due to changes in the sensitivity of GABAA receptor-mediated responses (Silvers et al., 2003; Erdozain & Callado, 2014). EtOH also enhances the GABAB induced synaptic responses being important for example, in the altered mental and motor performance after an acute EtOH intoxication (Federici et al., 2009).

\*Ethanol and glutamatergic system

Glutamate is the principal excitatory neurotransmitter in the brain and plays a crucial role in the pharmacological effects of the EtOH (Erdozain & Callado, 2014). Glutamate receptors can be divided into different groups according to the mechanism by which their activation gives rise to a postsynaptic current. There are three types of ionotropic glutamate receptors: NMDA (N-methyl-D-aspartic acid), AMPA ( $\alpha$ -amino-3-

hydroxyl-5-methyl-4-isoxazole-propionic acid), and kainate (Traynelis et al., 2010; Chandrasekar, 2013). The effect of EtOH over the glutamatergic systems lays on the modulation of the ionotropic glutamate receptors. Particularly the NMDA receptors are the most sensitive to the effects of EtOH (Dodd et al., 2000), being its depressant effects based on the antagonism of glutamate action at these receptors (Grobin et al., 1998; Wirkner et al., 1999).

EtOH inhibits the long-term potentiation phenomenon, which is important in learning and memory, probably through NMDA receptors (Givens, 1995). In the alcoholic brain, glutamatergic abnormalities have been observed, such as a lack of glia in the prefrontal cortex of alcoholics (Miguel-Hidalgo & Rajkowska, 2003; Miguel-Hidalgo et al., 2006; Smith et al., 2014). Also, blocking NMDA receptors decreases EtOH reinforcement and inhibits the expression of dependence that confirmed the role of the NMDA receptor and glutamatergic neurotransmission in EtOH reinforcement and dependence (Krystal et al., 2003). Recently, it has been demonstrated that alterations occur in the intrinsic electrical membrane properties and enhanced glutamatergic synaptic transmission in the NAcc core of rats during protracted withdrawal from chronic intermittent EtOH treatment, a model of alcohol dependence (Marty & Spigelman, 2012).

#### \*Ethanol and dopaminergic system

According to Koob & Volkow (2010), the anatomical core of the reward system are dopaminergic neurons of the ventral tegmental area (VTA) that project to the different important structures, such as, NAcc, amygdala or prefrontal cortex and other forebrain structures. Various

techniques have indicated that the mesolimbic DAergic system is activated when alcohol is administered to laboratory animals (Spanagel, 2009) and it has been suggested that the mesocorticolimbic DA system is involved both in the positive and negative reinforcing effects of EtOH (Ericson et al., 2009).

In particular, the VTA is involved in the effects of alcohol. It has been suggested that acute alcohol administration increases extracellular DA within the NAcc via changes in GABAergic feedback into the VTA (Spanagel, 2009). Low systemic doses of EtOH produce a dose-dependent increase in the firing rate of DAergic neurons, and alcohol stimulates DA transmission in the mesolimbic pathway (Gessa et al., 1985; Di Chiara & Imperato, 1988). Genetic factors are important since EtOH-induced DA release is greater in rats bred for alcohol preference (Bustamante et al., 2008), and alcohol SA produces a considerably greater relative stimulation of mesolimbic DA release in alcohol-preferring than in control rats (Katner et al., 1996; Bell et al., 2006). Moreover, alcohol cue-evoked DA release in the ventral striatum is greatest in men with a higher genetic risk for alcoholism (Oberlin et al., 2013).

The involvement of the DA system in the rewarding effects of EtOH is also described in the section 5 (page 60-61).

\*Ethanol and the endocannabinoid system

This system participates in drug reward through the release of endocannabinoids in the VTA. Accumulating evidence indicates a central role for the endocannabinoid system in the regulation of the rewarding properties of drugs of abuse including alcohol (Maldonado et al., 2006; Solinas et al., 2007; Serrano & Parsons, 2011; Erdozain & Callado, 2011; Filbey & DeWitt, 2012). Neuroadaptation to chronic EtOH involves changes in the endocannabinoid system (Vinod & Hungund, 2005). The agonism of the endocannabinoid system increases EtOH intake (Linsenhardt & Boehm, 2009) while the cannabinoid receptor 1 (CB1) antagonist rimonabant reduces the increase in DA in the NAcc induced by acute ethanol (Cheer et al., 2007), blocks the rewarding effects of alcohol and prevents reinstatement (Parolaro & Rubino, 2008). These results confirm that CB1 contributes to the motivational and reinforcing properties of EtOH, and chronic consumption of EtOH alters the levels of endocannabinoids and CB1 expression in the brain nuclei associated with addiction pathways (Pava & Woodward, 2012).

\*Ethanol and opioid system

EtOH reinforcement mechanisms also involve the endogenous opioid system. EtOH may alter opioidergic transmission at different levels and several studies suggest that mu and delta opioid receptors, as well as the enkephalins and beta-endorphins, play a major role in the effects of EtOH (Gianoulakis, 2009; Nutt, 2014). Similarly, kappa opioid receptors (KORs) are involved in the consumption, withdrawal, and escalation of EtOH (Zhou et al., 2013; Faisal et al., 2014). EtOH increases NAcc levels

of endorphins (Olive et al., 2001) and dynorphin (Marinelli et al., 2006). Moreover, chronic alcohol exposure caused changes of the dynorphin system in the brain of alcohol dependent rats and humans (Shippenberg et al., 2007; D'Addario et al., 2013) and it has been suggested that following discontinuation of alcohol administration the increased level of dynorphins may induced a negative emotional state, with craving and negative-reinforcement when alcohol becomes available (Walker & Koob, 2008a, 2008b; Wee & Koob, 2010; Nutt, 2014).

Opioid antagonists can have a potential therapeutic role in alcoholism (Walker et al., 2012; Heilig & Schank, 2014; Nutt, 2014). The administration of mu opioid antagonists decreased the release of DA in the NAcc induced by EtOH and its reinforcing effects (Gonzales & Weiss, 1998; Erdozain & Callado, 2014). Indeed, administration of mu and delta antagonists and KOR agonists also attenuated the responding for alcohol in operant paradigms (Henderson-Redmond & Czachowski, 2014). In addition, in alcohol preferring rats, KOR antagonists prevent alcohol SA (Cashman & Azar, 2014).

Naltrexone, a nonselective opiate antagonist with high affinity for the mu-opioid receptor, was approved by the FDA (Food and Drug Administration) in 1993 as a treatment for alcoholism. Naltrexone reverses alcohol-induced DA release in the NAcc in rats, and suppression of operant alcohol-reinforced behavior by naltrexone is associated with attenuation of the alcohol-induced increase in dialysate DA levels in the NAcc (Gonzales & Weiss, 1998; Henderson-Redmond & Czachowski, 2014). GSK1521498, is a novel selective mu-opioid receptor antagonist in clinical development for behavioral and drug addictive disorders (Nathan et al., 2012). GSK1521498 has been



reported to acting more selectively than naltrexone as an antagonist at the mu-opioid receptor. Under conditions of high receptor expression and endogenous receptor activation in cellular assays, GSK1521498 has inverse agonist properties, thereby reducing receptor activation in the absence of an exogenous agonist (Giuliano et al., 2012; Giuliano et al., 2013). In an experiment of the present thesis we have studied the effect of this antagonist on the alcohol consumption in rats (Giuliano et al., submitted in *Neuropsychopharmacology*).

#### 4.5.- Effects of Ethanol

##### A.- HUMANS

The effects of EtOH in humans are diverse because they are different depending on dose, gender, age, total amount ingested, the speed of consumption, body weight, the presence of food in the stomach or state of mind. Two types of effects have been observed, each with different characteristics: acute effects, caused by the massive intake of EtOH, which are proportional to the blood concentration, and chronic effects caused by excessive and repeated or continued consumption.

##### ∞ Acute physiological and behavioural effects

EtOH is toxic to most systems and tissues in the body, the CNS being the most affected system. At low concentrations (<0.5 g/l), EtOH produces a depression of inhibitory neural control mechanisms. In consequence, behaviours such as pseudoexcitation, feelings of euphoria, optimism or increased sociability are observed. If the dose of EtOH is higher (1-3

g/l), it produces a central depression and the consequence is a general slowing, ataxia, dysarthria, loss of reflexes and sleep. At very high (4-5 and >5 g/l) concentrations EtOH can induce coma or death caused by respiratory depression (Pohorecky & Brick, 1988; Sarasa-Renedo et al., 2014).

### ∞ Chronic physiological and behavioural effects

The brain is one of the major targets of the actions of EtOH, and heavy alcohol consumption produces significant alterations of the structure, physiology and function of this organ (Harper & Matsumoto, 2005). EtOH produces permanent effects in users, time after consumption, especially if consumption has been continuous over time, such as neuropsychological negative effects, changes in mood, anxiety, depression, confusion, etc. Even in severe cases, psychosis and mental disorders can appear. The Wernicke-Korsakoff syndrome is a mental disorder, caused by a nutritional deficiency or by excessive alcohol abuse, in which memory, learning and other cognitive functions are affected (Kopelman et al., 2009; Ridley et al., 2013; Brion et al., 2014). It has been observed a reduction in weight and volume of the brains of alcoholic people, correlating the degree of brain atrophy with the speed and the amount of alcohol consumed over the course of life (Sutherland et al., 2014; de la Monte & Kril, 2014).

Other physiological systems, such as the gastrointestinal, cardiovascular, immune, and sexual systems modify their activity after alcohol consumption. For example, EtOH induces digestive disorders (such as cirrhosis, hepatitis, gastritis or pancreatitis); heart disease or

hypertension, it also makes people prone to infections, decreases testosterone levels, etc. (Sarasa-Renedo et al., 2014).

Moreover, EtOH consumption is dangerous during gestation because during brain ontogeny alcohol causes irreversible alterations to the brain structure with long-term cognitive, behavioral and physical anomalies, known as fetal alcohol spectrum disorders (Alfonso-Loeches & Guerri, 2011; Moore et al., 2014).

## B.- ANIMALS

### ∞ Acute physiological and behavioural effects

Nowadays a binge-drinking pattern of consumption is typical in adolescent humans. So, a lot of animal studies focus in the acute effects of alcohol binges in adolescent rodents (Pascual et al., 2007; Do Couto et al., 2011; Rodríguez-Arias et al., 2011; Vidal-Infer et al., 2012 b; Pascual et al., 2014; Montagud-Romero et al., 2014). EtOH binges induces inflammatory mediators in the brain by activating glial cells and stimulating intracellular signalling pathways that trigger induction of cytokines, cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS) and neural cell death (Vallés et al., 2004; Blanco et al., 2005; Pascual et al., 2007). Elevated levels of COX-2 and iNOS are observed during excitotoxicity, ischaemia and neural injury (O'Banion, 1999). These changes are associated with neurobehavioural and cognitive deficits because by altering the activity of different ion channels and receptors, alcohol modulates plasticity and synaptic function in the brain (Zorumski et al., 2014).

It has been reported that the acute administration of EtOH before training leads to impairments in memory using different learning tasks, such as fear conditioning (Gulick & Gould, 2009 a, b, c), Morris water maze (Berry & Matthews, 2004), object and spatial recognition (García-Moreno & Cimadevilla, 2012), radial maze (Hoffmann & Matthews, 2001), and avoidance tasks (Gulick & Gould, 2011). Sometimes, these effects are state-dependent learning. For example, (Sanday et al., 2013) showed that the administration of 1.2 g/kg of this drug during pretraining caused memory impairment in mice, which was counteracted by the pretest administration of the same dose, revealing the participation of the state-dependency. Conversely, the administration of a higher dosis eliminated the influence of state-dependency because 2.4 g/kg EtOH led to amnestic effects irrespective of the time of administration (pretraining and/or pretest). Also in mice, Kameda et al. (2007) demonstrated that acute EtOH induced learning (1.2-3.0 g/kg) and memory deficits (0.3-3.0 g/kg) at different doses.

With regards to anxiety, EtOH (1 g/kg) produces anxiolytic-like effects in adult rats, using the light-dark box and EPM exploration tests (Pandey et al., 2008; Sakharkar et al., 2012). In the same line, Sanday et al. (2013) demonstrated that low and high doses of EtOH (1.2 and 2.4 g/kg) induced anxiolysis. The involvement of epigenetic mechanisms has also been studied, in particular, the function of histone deacetylases (HDAC) and deoxyribonucleic acid (DNA) methyltransferases (DNMT) in different areas of the brain, like the amygdala and bed nucleus of stria terminalis (BNST) of adolescent rats. The results showed that the lower dose of EtOH (1 g/kg) produced neither anxiolysis, nor inhibited the HDAC and DNMT activities in the amygdala and BNST. Anxiolysis by EtOH was

observed at 2 and 2.25 g/kg, whereas higher doses (2.5 and 3 g/kg) were found to be sedative (Sakharkar et al., 2014).

On the other hand, EtOH has well documented acute effects on motor function. Low doses of EtOH increased locomotion (1.2-1.8 g/kg) while higher doses (2.4-3.0 g/kg) decreased this behaviour (Kameda et al., 2007; Sanday et al., 2013). A recent study demonstrated that the motor and anxiolytic effects of EtOH in adolescent rats are relatively independent (0.5 g/kg exerted significant anxiolytic effects in the EPM in the absence of stimulating effects in the open field). Moreover, adolescents with a higher frequency of rearing behavior in the open field, higher percentage of open arm entries in the EPM, and lower propensity to enter the central area of the open field exhibited greater EtOH intake (Acevedo et al., 2014).

Behavioural and cognitive symptoms of EtOH are dependent on the age of the animals. Adolescent rats, compared to adults, are less sensitive to the anxiogenic (Doremus et al., 2003), hypnotic (Matthews et al., 2008) and motor impairing effects (White et al., 2002 a, b; Ramirez & Spear, 2010; Van Skike et al., 2010) of acute alcohol. Conversely, adolescent rodents are more sensitive to alcohol-induced hypothermia (Ristuccia & Spear, 2008), although in general terms EtOH induce hypothermia also in adulthood (Tanchuck-Nipper et al., 2014) an effect that is mediated by sleep rhythms (Damaggio & Gorman, 2014).

∞ Chronic physiological and behavioural effects

Chronic EtOH induces apoptosis in the rat's brain (Ikonomidou et al., 2000) and episodic alcohol intoxication or binge-type administration increases cell death in the neocortex, hippocampus and cerebellum (Pascual et al., 2007; McClain et al., 2014), and it increases oxidative stress in the brain (Collins & Neafsey, 2012). In adulthood, "alcohol-preferring" rats show reductions in spine density and terminal branching and increases in mushroom and multi-headed spines following chronic EtOH drinking and repeated deprivation (Zhou et al., 2007). EtOH exposure predominately causes reductions in medium spiny neurons branching, length, and/or spine density (McMullen et al., 1984; Rice et al., 2012; Zhou et al., 2007). Spiga et al. (2014) also recently reported reductions in thin spines within the NAcc early during alcohol withdrawal in young rats. Similar results have been showed in rabbits (Romero et al., 2013).

Moreover, animals exposed to EtOH during adolescence exhibit long-term behavioral deficits in the adulthood, such as memory impairments in the passive avoidance test, changes in social interaction behaviors and locomotor activity, and an increase of anxiety in EPM (in this late case in combination with MDMA) (Rodríguez-Arias et al., 2011). Also, animals treated with EtOH (alone and with MDMA) showed poor learning in Hebb Williams test (Vidal-Infer et al., 2012 b).



**5.- BRAIN REWARD SYSTEM AND  
REINFORCING EFFECTS OF MDMA  
AND ALCOHOL**

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## 5.- BRAIN REWARD SYSTEM AND REINFORCING EFFECTS OF MDMA AND ALCOHOL

The rewarding properties of natural stimuli and drugs of abuse are mediated by the activation of the brain reward system. The mesocorticolimbic DA system is known to play a major role in appetitive behaviors (Kelley & Berridge, 2002; Wise, 2008; Carlezon & Thomas, 2009; Dalley & Everitt, 2009) and is the main neural substrate of the rewarding effects produced by drugs of abuse (Wise, 1998; McBride et al., 1999). It is originated in the VTA and projecting to the NAcc and to several key cortical loci, including the anterior cingulate cortex (ACC), orbitofrontal cortex (OFC) and prefrontal cortex (PFC) (Cohen et al., 2002; Everitt & Robbins, 2005; Hyman et al., 2006; Feltenstein & See, 2008; Dalley & Everitt, 2009; Rodriguez-Arias et al., 2013).

Pathological disturbances of the brain DA systems are involved in a number of neurological and neuropsychiatric disorders, including Parkinson's disease, schizophrenia, attention-deficit hyperactivity disorder and drug addiction (Nieoullon, 2002; Goodman, 2008). Alcohol, psychostimulants, nicotine, opioids, and cannabinoids (Chen et al., 1990; Yoshimoto et al., 1992; Pontieri et al., 1996; Koob & Le Moal, 2001) activate the mesolimbic dopaminergic system and increase DA transmission in the NAcc and associated limbic areas (Wise, 2008). On the other hand, there is another important aspect that is necessary to highlight. Drug addiction is an illness not only characterised by a physical stimulus, because sometimes other cognitive superior process such as learning or memory are involved and they influence the circuit system described before. Responses to drugs can acquire motivational significance by being associated with environmental stimuli through

pavlovian conditioning (O'Brien et al., 1998). These drug associated conditioned stimuli (CSs) may then predict drug availability, evoke memories of drug's effects, or of withdrawal, to result in craving even long into abstinence, and, perhaps most importantly, may elicit and maintain the instrumental behaviours of drug seeking and taking (Childress et al., 1999; Garavan et al., 2000; Robbins & Everitt, 2002). Addiction can be understood in terms of pavlovian and instrumental learning and memory processes, and their subversion by the actions of addictive drugs on dopaminergic transmission within corticostriatal systems, that normally mediate learning and memory processes in the context of natural rewards (Robbins & Everitt, 1999; Everitt & Robbins, 2005; Dalley & Everitt, 2009; Everitt 2014). Other structures play a role in drug addiction. For example, PFC is important to take decisions about consumption and it is involved in behavioural inhibition, flexibility, decision-making and cognitive decisions about the negative consequences of consumption while hippocampus is important for remembering different keys associated with consumption (Cohen et al., 2002; Dalley & Everitt, 2009; Everitt, 2014). For example, using CPP it is very evident, because the experimental animals associated the environmental cues with the rewarding effects of the drug after connecting their mesolimbic area with other neuroanatomical structures such as hippocampus (see Figure 11).

In this regard, the main neurotransmitter in MDMA and EtOH reward and addiction is DA although other neurotransmitters, such as serotonin, are also involved (Müller & Homberg, 2015). MDMA increased the release of DA in NAcc that interacts with other neurotransmitters such as 5-HT or GABA (O'Shea et al., 2005; Amato et

al., 2007; Touriño et al., 2008; Reveron et al., 2010; Kehr et al., 2011). Using the CPP paradigm, it has been demonstrated that MDMA produces reinforcing effects in rats (Meyer et al., 2002; Braida et al., 2005; Diller et al., 2007; Feduccia & Duvauchelle, 2008; Catlow et al., 2010) and mice (Daza-Losada et al., 2007; 2009 b; 2011; Manzanedo et al., 2010; Rodriguez-Arias et al., 2010; Do Couto et al., 2011). Using the AA paradigm, MDMA also induces reinforcing effects in rhesus monkeys (Fantegrossi et al., 2002; Lile et al., 2005), rats (Schenk et al., 2007; 2008; 2011; Cornish et al., 2003) and mice (Trigo et al., 2006; 2007; Touriño et al., 2008; Orejarena et al., 2009; 2011; Ruiz-Medina et al., 2011).

It has been demonstrated that activation of DA D1-like and D2-like receptors contributes to the maintenance of MDMA SA. The reinforcing effects of MDMA were attenuated by pretreatment with the D1 antagonist SCH 23390 (Daniela et al., 2004) and with D2 receptor antagonist eticlopride (Brennan et al., 2009). Similarly, DA is involved in the rewarding effects of MDMA in the CPP paradigm. The mixed serotonin (5-HT<sub>2A</sub>)/DA D<sub>2</sub>) antagonist risperidone, the DA D1 antagonist SCH 23390, the DA D<sub>2</sub> antagonist haloperidol, the D2 antagonist raclopride and the DA release inhibitor CGS 10746B block acquisition of MDMA CPP (Vidal-Infer et al., 2012 a; Roger-Sanchez et al., 2013 a).

As regards alcohol, it has been repeatedly reported that this substance induces stimulant and motivational effects in animal models (Camarini et al., 2010; Brabant et al., 2014). Behavioural and biochemical studies have demonstrated that alcohol takes action in central reward areas and enhances DA neurotransmission from neurons of the VTA increasing DA

levels within the NAcc and prefrontal cortex (Di Chiara & Imperato, 1988; Boileau et al., 2003; Robinson et al., 2009; Söderpalm & Ericson, 2013; Erdozain & Callado, 2014; Mrejeru et al., 2015). Rodents self-administer EtOH directly in the VTA (Rodd et al., 2004) and the activation of DA receptors in the shell of the NAcc (NAccsh), ventral pallidum (VP) or medial PFC (mPFC) are involved in mediating the reinforcing effects of EtOH in the VTA, suggesting that the 'alcohol reward' neuro-circuitry consist in the activation of the DA projections in important brain reward areas like VTA, VP and PFC (Ding et al., 2014).

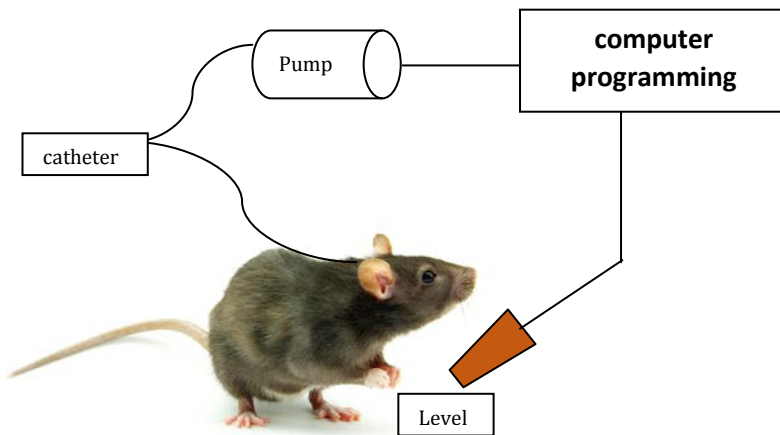
The D1 antagonist SCH 23390 administered into the core or shell of NAcc reduced context-induced reinstatement of EtOH SA (Chaudhri et al., 2009) and the development of EtOH-induced CPP (Pina & Cunningham, 2014). In addition, D2 receptor signaling in the dorsolateral striatum is also involved in EtOH SA (Corbit et al., 2014). Moreover, (Rotter et al., 2012) found that the establishment of EtOH CPP was paralleled by a decrease in frontal cortex DA D2 receptor messenger ribonucleic acid (mRNA) expression. Finally, the DA system is also involved in the rewarding effects of alcohol effects in humans (Charlet et al., 2013).

Figure 11

*Most used paradigms of drug reward*



a.- Conditioned preference place (CPP)



b.- Intravenous self-administration (SA)

# 6.- BRAIN STRESS SYSTEM AND DRUG ABUSE

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## 6.- BRAIN STRESS SYSTEM AND DRUG ABUSE

Stress is an internal and external stimulus that can produce changes in our natural organism homeostasis. Stress is a wide term and it can be used for several situations. In fact, a lot of types of stress (physical, emotional, social, pharmacological...) have been described in the literature. Biological and behavioural variables (such as cognition, motivation, emotion) are involved in the stress reaction.

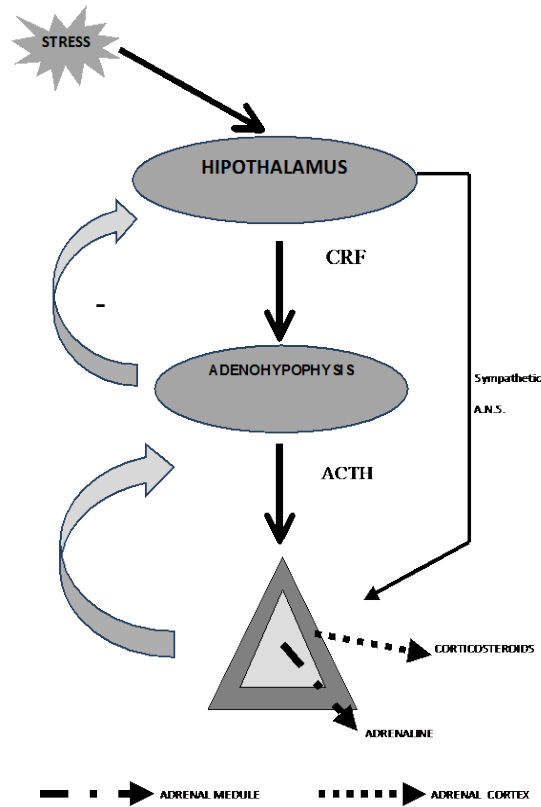
Different regulatory systems of the body are activated during stress situations, but the main structure of central control and regulation is the HPA axis. In response to stress the HPA axis begins different actions in body to go back to homeostasis, including physiological and metabolic changes (Miller & O'Callaghan, 2002), releasing hormones such as corticotropin-releasing factor (CRF), adrenocorticotropin hormone (ACTH) and glucocorticoids; in addition, the sympathetic-adrenal system releases catecholamines (adrenaline and NA) (Kupfermann, 1991). Immune system is also involved in stress response (Costa-Pinto & Palermo-Neto, 2010; Capuron & Miller, 2011; Rodriguez-Arias et al., 2013).

Under stress, the HPA axis is activated by the secretion of CRF from the hypothalamus (Turnbull & Rivier, 1997; Sarnyai et al., 2001; Goeders, 2002). CRF-containing neurons projecting from the paraventricular nucleus to the median eminence release the peptide into the adenohipophyseal portal circulation. The binding of CRF to receptors located in the anterior hypophysis results in the synthesis of proopiomelanocortin (POMC), a large precursor protein that produces several smaller peptides, including ACTH and  $\beta$ -endorphin. ACTH

diffuses through the general circulation until it reaches the adrenal glands, where it stimulates the biosynthesis and secretion of adrenocorticosteroids (e.g. cortisol in humans or corticosterone in rodents), which act at diffused body sites to assure the overall response to stress (Goeders, 2003; Kovács, 2013). Under stress, various systems are activated by the effects of cortisol (Chrousos & Gold, 1992; McEwen, 2003). The general function of the HPA axis is controlled by several negative feedback loops (Herman et al., 2012), regulated by mineralocorticoid and glucocorticoid receptors (Harris et al, 2013). Glucocorticoids act in a negative feedback mode by decreasing production and release of CRF in the hypothalamus and of POMC and its neuropeptides in the anterior pituitary (Zhou et al., 2006; Rodriguez-Arias et al, 2013).

On the other hand, in a stressful situation our organism needs to be prepared for acting (the classical flight or fight response) and ensure our survival. For this reason, the sympathetic nervous system activated the adrenal medulla by inducing adrenaline and NA release that produced an increase in heart rate, a rise in blood pressure, a shift in blood flow to the skeletal muscles, an increase in blood glucose, dilation of the pupils and stimulation of respiration (Goeders, 2003). For more information see the next figure.

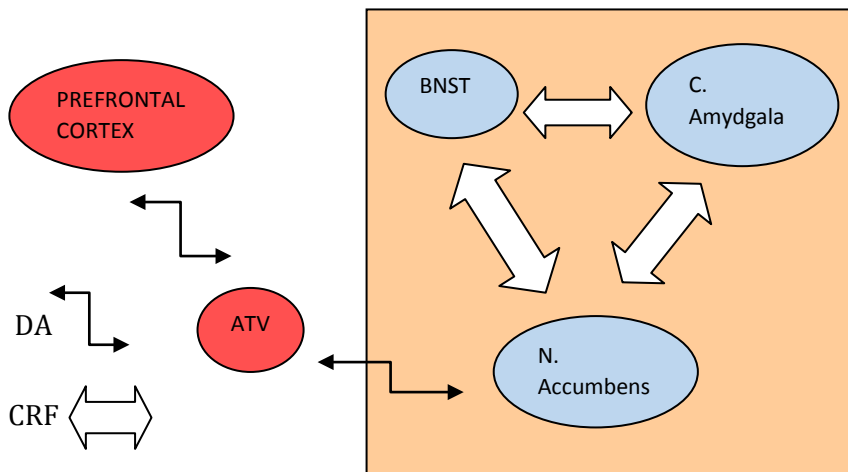


Figure 12: *Physiology of the response to stress*

The neurobiological systems involved in response to stress are close to the neurobiological system involved in drug addiction, the nexus between both systems being the circuitry of the extended amygdala (Koob, 2009; Rodriguez-Arias et al., 2013). Activation of brain stress systems seems to be a key element of the negative emotional state produced by dependence and which drives drug-seeking through negative reinforcement mechanisms (Koob, 2009). Many of the

motivational effects of drugs may involve a common neural circuitry that forms a separate entity within the basal forebrain, termed the “extended amygdala” (Alheid & Heimer, 1988). It has been demonstrated that the extended amygdala circuitry extends from the shell of the NAcc to the BNST and central nucleus of the amygdala (Alheid & Heimer, 1988; de Olmos & Heimer, 1999; Koob, 2009), where neurotransmitters such as CRF, NA, and DA interact. A detailed description of how stress exposure modifies the rewarding effects of drugs of abuse has been reported in Rodriguez-Arias et al. (2013). Moreover, we have performed an exhaustive review of the impact of social stress in addiction to psychostimulants in animal models (see first publication of the present thesis).

Figure 13

*Extended amygdala circuitry*



# 7.- ADOLESCENCE, DRUG USE AND STRESS

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## 7.- ADOLESCENCE, DRUG USE AND STRESS

As commented previously, the consumption of drugs, such as alcohol or MDMA, in adolescence is very extended (ESTUDES 2012/2013).

Adolescence refers to the gradual period of behavioral transition from childhood to adulthood but the boundaries of this period are less precisely defined (Spear 2000; Sisk & Foster, 2004). This term is closely related to puberty although the definition is not identical. Adolescence is, in general terms, the period of gradual physiological, cognitive, behavioural and psychological transitions with important changes in life (Pickles et al., 1998; Spear, 2000).

The transition from childhood to adulthood involves extensive developmental changes and reorganisation of the brain (McCormick, 2010). During puberty, neuronal maturation of the brain, which began during perinatal development, is completed such that the behavioral potential of the adult organism can be fully achieved (Schneider, 2008). In relation to these brain changes, there are numerous neurodevelopmental alterations that take place during this period, such as maturational processes in the mPFC and limbic regions, which are characterised by both progressive and regressive changes, e.g. myelination and synaptic pruning (Spear, 2000; Powell, 2006). Maturational changes are also evident during adolescence in limbic regions such as the hippocampus (Wolfer & Lipp, 1995), and gray matter reductions also take place in the striatum and other subcortical structures (Sowell et al., 2002; Rodríguez-Arias & Aguilar, 2012). For this brain reorganisation, adolescence is seen as a highly vulnerable developmental period for the consequences of exposure to drugs of abuse (Schneider, 2008).

Human adolescence is commonly considered to be from 12 to 18 years of age, although the entire second decade of life is sometimes also considered adolescence, with up to 25 years being considered late adolescence (Baumrind, 1987) or young adults. In animals, the period of adolescence is around 21-60 postnatal day (PND), which can be divided into early adolescence, between 21-34 PND, middle adolescence at 34-46 PND and young adults between 46-60 PND (Laviola et al., 2003). Precisely it is in this period when the dates about consumption of EtOH and MDMA mark an important record and there is a sizable research literature using animal models that describes age differences in the effects of drugs of abuse and the underlying mechanisms for the effects of drugs (Barron et al., 2005; Carpenter-Hyland & Chandler, 2007). Furthermore, maturation of neurotransmitter systems such as the glutamatergic, the dopaminergic and also the endogenous cannabinoid system occur during adolescence, with developmental peaks often seen concomitant with the onset of puberty (Rodriguez de Fonseca et al., 1993; Spear, 2000). Basal levels of synaptic DA are lower during this phase of development, although adolescents show a greater and faster increase in drug-induced DA release (Laviola et al., 2001; Badanich et al., 2006). In relation with it, adolescents are generally subject to a less positive impact from stimuli with moderate to low incentive value, and thus seek additional appetitive reinforcers (Spear, 2000). According to different studies (Bjork et al., 2010) it is shown that in adolescents, important centres of emotions and rewards are very active. For example, amygdala and accumbens of adolescents exhibit more activity than those of adults (Ernst et al., 2011). So, a lot of behaviours described as typical in adolescence (impulsivity, intake of drugs, disinhibition...) have a biological basis because the adolescents have immature neural

processing in the PFC and other cortical and subcortical regions involved in decision-taking, leading to a behaviour that is biased toward risk and emotional reactivity during the adolescent period (Sturman & Moghaddan, 2011; Rodriguez-Arias & Aguilar, 2012). Many typical adolescent behaviors are very related with the consumption of drugs, such as, risk taking, more autonomy, heightened responsibility, considerable peer influence, impulsivity, egocentrism, shorter periods of sleep, new interests, and increase in the number of conflicts with parents (Spear, 2000; Spear, 2011; Sturman & Moghaddan, 2011). All of these behaviours can lead to a higher incidence of risky behaviours such as a misconduct at school, drink driving, unsafe sex, antisocial behaviours and, of course, use of legal and illegal drugs (Doremus-Fitzwater et al., 2010; Eaton et al., 2012; Spear, 2011).

With regard to stress vulnerability, the adolescent nervous system is more readily shaped by environmental factors like stressful events (McCormick, 2010). In addition, the clinical literature shows that stress in adolescence increase the risk for drug abuse (King & Chassin, 2008; Hoffmann et al., 2000). The effects of stressors on brain structure and function often involve activation of the HPA axis, which results in elevations of glucocorticoid hormone concentrations and increased actions of the hormone at corticosteroid receptors distributed throughout limbic and prefrontal regions of the brain (McCormick, 2010). Elevated exposure to glucocorticoids over the course of adolescence confers sex-specific changes in behavioural responses to drugs of abuse, which may be of relevance for understanding risk factors in people (McCormick, 2010). Behavioral stress affects both the

structure and function of PFC, particularly during infancy and adolescence, though such effects are not necessarily permanent (McEwen & Morrison, 2013). Behavioural experiments in laboratory animals have revealed that adolescents are more disrupted by stressors than younger or older counterparts and that they differ behaviourally and physiologically in their response to stressors when compared to animals of other ages (Stone & Quartermain, 1997; Buwalda et al., 2011). Moreover, while adult male rats, which are repeatedly exposed to daily restraint stress, show a clear habituation in their neuroendocrine response, adolescent male rats actually exhibit a facilitation of this response (Romeo, 2010). For example, studies using physical stress (swim test) show that adolescents are more negatively affected by stressful events than are adults. Compared with adults, adolescent rats show more immobility under stressful situations in this test (Walker et al., 1995). Likewise, studies in laboratory animals have shown that adolescents sometimes exhibit a greater overall hormonal response to stress, evidenced by the increased production of corticosterone, compared with younger animals and a more prolonged increase in stress hormones relative to adults (Spear, 2000).





## 8.- METHODOLOGY



## 8.- METHODOLOGY USED IN THIS WORK.

To carry out the different experiments that compose this experimental work, different paradigms have been used with different objectives. The most important has been the conditioned preference place paradigm, which evaluates the conditioned rewarding effects of appetitive natural stimuli and drugs of abuse. The environmental cues can acquire hedonic effects when associated with positive unconditioned stimuli (Tzschentke, 2007; Aguilar et al., 2009; Manzanedo, 2001). CPP phases and procediments are described in detail in the different papers that compose this doctoral thesis (experimental study 1, 2, 3, 6, 7).

Social stress is the other basic procedure in this work. To induce social stress in the animals two different procedures have been used. First, brief or acute agonist encounters with a coespecific mouse in a neutral area have been performed with the result of defeat for the experimental animal (Do Couto et al., 2006; 2009). The second one is the exposure to repeated social defeat (RSD) using the resident/intruder model (Yap et al., 2006; Quadros & Miczek, 2009, Miczek et al., 2011). These different procedures are described in detail in different papers of the present work (experimental study 1, 2, 3, 5, 6, 7).

Also, an important aspect in the drug addiction is the free preference or motivation of the animal to look for the drug. This aspect cannot be directly evaluated in the CPP paradigm, and is especially important when a drug does not induce conditioned reward, for example, EtOH. Thus, the two-bottle choice procedure has been used in this work in different experimental studies because in this paradigm the animal chooses voluntarily whether to consume alcohol or not (Ehringer et al.,

2009; Plescia et al., 2015). A detailed explanation of this procedure can be seen in two works of the present thesis (experimental study 4 and 6).

Finally, also related to the motivation of the animal to consume the drug, second order schedules of drug reinforcement were used in this work. Under the second-order schedules, a reinforcer – usually a drug - is presented according to a schedule in which a more or less extended sequence of responses is reinforced intermittently, for example, following the completion of a fixed ratio (FR) of responses after a fixed interval (FI) has timed out. Completion of each FR is accompanied by the presentation of a stimulus that is contingent to the response, a stimulus that has been previously conditioned by association with the primary reinforcer each time it was presented during earlier training (Everitt and Robbins, 2000; Di Ciano & Everitt, 2005; Giuliano, et al., 2012; Giuliano et al., 2013). This paradigm is described in one paper of the present thesis (experimental study 4).



## 9.- JUSTIFICATION OF EXPERIMENTS

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## 9.- JUSTIFICATION OF EXPERIMENTS

Taking into consideration that drug addiction is an important problem in our society, that MDMA and EtOH are abuse substances with an extended consumption between adolescent and young adults and that stress seems to be an important factor to initiation, maintenance and relapse/reinstatement of the drug addiction, the experiments presented in this doctoral thesis have the general objective to determine how stress exposure can modify the rewarding effects of these drugs and to enhance the knowledge of the neurobiological substrates of these effects.

For this reason, the first step in this work was to do a wide review of all the information available until the moment about the impact of social stress in animal models of drug addiction. The theoretical framework (paper 1: Review) was essential to design the experimental work.

Later, we studied the influence of acute social stress on the acquisition of the CPP induced by MDMA and if there were differences between early adolescent and young adult mice (paper 2: experimental study 1).

Then, we determined the role of the glutamatergic NMDA receptors in the acquisition and reinstatement of the rewarding effects of MDMA (paper 3: experimental study 2, in press) as a previous step to evaluate the role of this neurotransmitter system in the influence that acute social defeat has on the rewarding effects of MDMA.

Following, we studied the influence of repeated social stress on the acquisition of the CPP induced by MDMA and if there were differences between early adolescent and young adult mice (paper 4, experimental study 3, submitted).

In paper 5, we report the work made in the Department of Psychology of the University of Cambridge during a 4-months period of a stay in the prestigious laboratory directed by the professor BJ Everitt. During this time, we studied the effects of opioid system in the alcohol addiction. Then, in relation with our topic, we studied the effects of social stress in the effects of EtOH (see experimental study 6).

In the following study we want to evaluate if the combination of social stress and MDMA use has synergic negative behavioural and cognitive effects because no other paper has studied this relation, even though both drugs induced impairing effects separately. For this reason, after treatment with a high dose of MDMA and exposure to acute social stress, the animals were evaluated in memory, motor activity and depression-like behaviour (experimental study 5, paper in preparation).

As mentioned above, to study how the exposure to acute and repeated social stress affects the motivational effects of ethanol in the CPP paradigm and the voluntary consumption of this drug in the two-bottle choice procedure we design the next study (experimental study 6, paper in preparation).

Finally, to extend the knowledge about the implication of glutamatergic system in the rewarding effects of MDMA, we studied the effects of NMDA and AMPA antagonists, as well as a nitric oxide synthase (NOS) inhibitor, in the CPP induced by *ecstasy*. In addition, we studied the effects of these drugs in mice exposed to acute social defeat before place conditioning with *ecstasy* (experimental study 7, paper in preparation).



The final objective of the present work is to increase our knowledge of the different biological, psychological and environmental factors that contribute to rewarding effects of MDMA and EtOH in mice and translate this knowledge to the prevention and treatment of drug addiction in humans.

10.- PUBLISHED  
PAPERS



## 10.- PUBLISHED PAPERS

10.1.- **REVIEW:** Impact of social stress in addiction to psychostimulants: what we know from animal models. Aguilar, M. A, **García-Pardo, M. P.**, Montagud-Romero, S., Miñarro, J., & Do Couto, B. R. *Current Pharmaceutical Design.*, 2013;19(40):7009-25. Doi: 10.2174/138161281940131209124708

10.2.- **EXPERIMENTAL STUDY 1:** Effects of acute social stress on the conditioned place preference induced by MDMA in adolescent and adult mice. **García-Pardo, M. P.**, Rodríguez-Arias, M., Maldonado, C., Manzanedo, C., Miñarro, J., & Aguilar, M. A. *Behavioural Pharmacology*, 2014 Sep;25(5-6):532-46. Doi: 10.1097/FBP.0000000000000065.

10.3.- **EXPERIMENTAL STUDY 2:** Involvement of NMDA glutamate receptors in the acquisition and reinstatement of the conditioned place preference induced by MDMA. **García-Pardo, M.P.**, Escobar-Valero, C., Rodríguez-Arias, M., Miñarro, J., & Aguilar, M.A. *Behavioural Pharmacology*, 2015, in press.

**11.- SUBMITTED  
PAPERS**



## 11. SUBMITTED PAPERS

11.1.- **EXPERIMENTAL STUDY 3:** Effects of repeated social stress on the conditioned place preference induced by MDMA in adolescent and adult mice. **García-Pardo, M. P.**, Blanco-Gandía, M. C., Valiente-Lluch, M., Rodríguez-Arias, M., Miñarro, J. & Aguilar, M. A. *Submitted to Progress in NeuroPsychopharmacology and Biological Psychiatry (under review)*

11.2.- **EXPERIMENTAL STUDY 4:** A novel preclinical model of alcohol seeking and drinking in alcohol-preferring rats: Selective effects of the novel  $\mu$ -opioid receptor antagonist GSK1521498. Giuliano, C., Goodlett, C. R., Economidou, D., **García-Pardo, M. P.**, Belin, D., Robbins, T. W., Bullmore, E. T., & Everitt, B. J. *Submitted to NeuroPsychopharmacology (under second review)*

**With regard to this study it is important to note that I have only performed the procedure of the two-bottle choice and the experiments to evaluate the effects of naltrexone and CGS1521498 in ethanol consumption in this paradigm (Figure 4). I have also initiated the second order procedure.**

## 12.- PAPERS IN PREPARATION



## 12.- PAPERS IN PREPARATION

12.1.- **EXPERIMENTAL STUDY 5:** Cognitive and behavioural effects induced by the combination of social stress and MDMA administration in mice. **García-Pardo, M. P.**, Rodríguez-Arias, M., Miñarro, J., & Aguilar, M. A.

12.2.- **EXPERIMENTAL STUDY 6:** Effects of social stress on the consumption of ethanol in two-bottle choice procedure and on acquisition of ethanol-induced place conditioning in adult mice. **García-Pardo, M. P.**, Rodríguez-Arias, M., Miñarro, J., & Aguilar MA. (in preparation b)

12.3.- **EXPERIMENTAL STUDY 7:** Implication of NMDA and AMPA glutamatergic receptors and nitric oxide (NO) pathway in the rewarding effects of “ecstasy” in the conditioned preference place in young adult mice and its modulation by social defeat stress. **García-Pardo, M. P.**, Rodríguez-Arias, M., Miñarro, J., & Aguilar ,M. A. (in preparation c)

# 13.- RESULTS AND LINE OF ARGUMENT

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### 13.- RESULTS AND LINE OF ARGUMENT

In developed societies, drug consumption is widely extended between adolescent and young subjects (ESTUDES, 2012; EDADES, 2013; EMCDDA, 2014; UNODC, 2014). Alcohol is the legal drug most commonly consumed by adolescents and adults in Europe while MDMA is an illegal drug frequently consumed between adolescents and young adults at weekend parties and discos. Most people used these drugs in a recreational way, but several numbers of individuals showed important problems or damages associated with drug consumption and developed dependence and addiction disorders. Although alcohol and MDMA use is very extended, only some people suffer a transition from the voluntary consumption of these drugs to dependence and addiction, characterized by loss of control over the use of the substance and the enhanced vulnerability to relapse after periods of abstinence (Everitt & Robbins, 2013). Biological as well as individual and environmental variables contribute to the transition to drug addiction, like genetics (Demers et al., 2014), alterations in DA and glutamatergic systems (Van den Oever et al., 2012; Pierce & Wolf, 2013; Quintero, 2013; van Huijstee & Mansvelder, 2015), age at the moment of drug exposure (Schramm-Sapyta et al., 2009; Doremus-Fitzwater et al., 2010; Gulley & Juraska, 2013), personality traits such as impulsivity or novelty-seeking phenotype (Jupp & Dalley, 2014; Everitt, 2014), social environment (Neisewander et al., 2012) and stress exposure (Sinha et al., 2011; Rodríguez-Arias et al., 2013), between others. Thus, it is essential to know the neurobiological substrate of the rewarding effects of drugs of

abuse and determine which variables are involved in the individual vulnerability to drug addiction. With this objective, in the present thesis we have studied, using different animal models, the influence of some variables, such as age, social stress exposure and pharmacological manipulations, on the rewarding effects of MDMA (commonly known as *ecstasy*) and alcohol (EtOH).

The results obtained in the different experiments performed have increased the knowledge of neurobiological bases of the rewarding effects of MDMA and alcohol. The original findings of our work are: 1. age is an essential factor for the acquisition of the rewarding effects of MDMA in the CPP paradigm; MDMA induces CPP at lower doses in young adults than in adolescent mice. 2. social stress induces acute and long-term effects on the CPP induced by MDMA; social defeat immediately before conditioning decreased CPP, while RSD three weeks before conditioning increased the duration of CPP and the vulnerability to reinstatement; 3. social stress, and its combination with MDMA, induced behavioural alterations that suggested an increased vulnerability to develop anxiety, depression, social avoidance and cognitive impairment; 4. acute and repeated social stress blocked the conditioned place aversion induced by alcohol and increased its intake in the two-bottle choice 5. N-metil-D-aspartato (NMDA) and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors and nitric oxide (NO) pathway are involved in the conditioned rewarding effects of MDMA and modulates the influence of acute social defeat on these effects; 6. alcohol intake in the two-bottle choice paradigm is reduced by the mu opioid antagonist GSK1521498. We hope

that an effective translation of this new knowledge in the next years may contribute to the advance in the prevention and treatment of drug addiction disorders.

Age is an important factor in determining the rewarding effects of drugs of abuse (Schramm-Sapyta et al., 2009; Doremus-Fitzwater et al., 2010; Gulley & Juraska, 2013). Although MDMA is consumed by adolescents or young adults in the context of weekend parties, no previous studies had evaluated the influence of age in the rewarding effects of MDMA in animal models. With this purpose we used mice of two different ages: adolescent and young adult mice. In two different studies of the present thesis we have observed that young adult mice are more sensitive to the rewarding effects of MDMA than adolescent mice in the CPP paradigm. A dose of 1.25 mg/kg of MDMA that is ineffective to induce CPP in adolescent mice (Daza-Losada et al., 2009b; Manzanedo et al., 2010; Rodríguez-Arias et al., 2010; Roger-Sánchez et al., 2013a; García-Pardo et al., 2014) induces such effect in adult mice (García-Pardo et al., 2014; Mateos-Garcia et al., 2015; García-Pardo et al., submitted). Moreover, after conditioning with 10 mg/kg of MDMA adult mice showed reinstatement of CPP with lower doses (2.5 mg/kg) than younger mice (García-Pardo et al., 2014; García-Pardo et al., submitted). These results are in accordance with those of epidemiologic studies reporting that MDMA consumption increases with age in subjects between 14 and 18 years of age (ESTUDES, 2012) and that young adults are the main consumers of MDMA (EDADES, 2011, 2013).

As the maturation of control brain systems in the PFC through adolescence is contextually dependent, recurrent adverse episodes of stress might shape the adolescent brain and trigger long-term maladaptive responses increasing addiction risk (Bernheim et al., 2013). It has been demonstrated that adverse life experiences may render individuals more prone to abuse addictive substances and more vulnerable to relapse into drug-seeking after periods of detoxification (Caprioli et al., 2007; Miczek et al., 2008; Le Moal, 2009; Sinha et al., 2011), being stress a risk factor for the initiation, maintenance and escalation of drug consumption and for relapse (Sinha, 2008; Koob, 2010; Sinha et al., 2011; Logrip et al., 2011, 2012; Aguilar et al., 2013; Rodríguez-Arias et al., 2013). As emotional stressors are primary activators of a stress response in humans, social defeat in an agonistic encounter is considered a stressor of ecological and ethological validity in rodents (Tornatzky & Miczek, 1993) that closely mimics real life situations in a human context (Neisewander et al., 2012). The main objective of the present thesis has been to study the influence of social defeat stress in the effects of MDMA and EtOH. With this purpose, we used two different procedures to induce social defeat in mice allowing the evaluation of both the acute and long-term effects of social defeat on the motivational properties of these drugs.

With regard to MDMA, after performing an exhaustive review of all the studies published about the effects of social stress on the rewarding effects of psychostimulants (Aguilar et al., 2013), we find different studies demonstrating that stress exposure, in particular social defeat, increased cocaine and amphetamine SA (Miczek et al., 2008; Quadros &

Miczek, 2009; Miczek et al., 2011; Cruz et al., 2011; Boyson et al., 2011; 2014; Yap et al., 2014; Han et al., 2015) and the rewarding effect of these drugs in the CPP paradigm (McLaughlin et al., 2006; Burke et al., 2011; Hymel et al., 2014); however, the influence of stress on the effects of MDMA had not been previously evaluated. Moreover, the majority of studies about the effects of social defeat on drug vulnerability have been performed only in adult mice. However, it is essential to test the effects of social defeat stress in younger animals, since adolescence is a highly vulnerable developmental period and adolescent rodents are more vulnerable to stressors than younger or older counterparts (Stone & Quartermain, 1997; Vázquez, 1998; Buwalda et al., 2011). Thus, the use of adolescent and young adult mice to test the effects of social defeat in the rewarding properties of MDMA is another essential contribution of the present work. In several studies of the present thesis we observed that both acute and RSD modify MDMA-induced CPP in adolescent and young adult mice, although the effects observed are different in function of the type of social defeat at which the mice are exposed. Acute social defeat experienced immediately before each conditioning session with MDMA reduces the rewarding effects of this drug in young adult mice, without affect CPP in adolescent mice (García-Pardo et al., 2014). We believe that this lack of MDMA CPP observed in young adult mice after exposure to acute social defeat is mainly related with specific features of this procedure of social stress. Firstly, as mice experienced social defeat immediately before each conditioning session with MDMA, the adverse experience of social defeat could reduce the interest in future social interaction and the typical prosocial and euphoric properties of MDMA. Secondly, social defeat is suffered in the context of an agonistic encounter in a neutral area, which could be less stressful than to be

defeated in the cage of a resident. Thirdly, only the short-term effects of social defeat stress are evaluated (48 hours after the last stress exposure). These results pointed out the importance of the temporal schedule of social defeat and testing, in agreement with previous studies with alcohol. While social defeat immediately before access to EtOH SA decreased intake, when there is a delay between social defeat and SA access an increase in EtOH intake is observed (see Neisewander et al., 2012 for review). It has also been reported that social stress induces a general decrease of ongoing behaviour that might interfere with the expression of CPP (Meerlo et al., 1996) and/or the negative experience of defeat may interfere with the positive effects of MDMA impairing the acquisition of CPP. This reduction in the sensitivity of mice to the rewarding properties of MDMA could lead to an increase in the consumption of this drug, thus facilitating the development of drug abuse and dependence.

Conversely, RSD induces a long-term increase in the duration of CPP in both adolescent and young adult mice and an enhancement in the vulnerability to the reinstatement of CPP in adolescent mice (García-Pardo et al., submitted). These results observed after RSD are in agreement with previous studies in which an increase in the rewarding effects of psychostimulants is generally observed after the absence (10 days or more) of RSD stress in a resident-intruder paradigm (Miczek et al., 2008; Quadros & Miczek, 2009; Miczek et al., 2011; Cruz et al., 2011; Boyson et al., 2011; 2014; Yap et al., 2014; Han et al., 2015). Similarly, in a recent study we demonstrated that mice exposed to RSD during adolescence showed an increase in EtOH consumption and motivation

to drink in adulthood (Rodríguez-Arias et al., 2014). In the present study, RSD induces a more potent long-term increase in the rewarding properties of MDMA in adolescent than in young adult mice, increasing the duration of CPP and the priming-induced reinstatement with a very low dose (0.625 mg/kg). It is important to note that the greater effect of RSD in adolescents is observed in spite of social defeat is a lower stressful stimuli for these mice (as measured by corticosterone levels after social defeat that are lower in adolescent than in young adult mice). This clearly indicates the higher vulnerability of adolescent brain to the impact of social stress.

Besides the modification of the rewarding effects of MDMA, we observed that young adult mice exposed to different kinds of social defeat showed several short- and long-term behavioural alterations. In particular, four episodes of social defeat on alternating days impaired memory in the passive avoidance task 5 days after the last episode of social defeat (García-Pardo et al., in preparation a), while exposure to RSD increased anxiety in the elevated plus maze, induced social avoidance and delayed learning of the Hebb Williams maze at least three weeks after the last episode of social defeat (García-Pardo et al., submitted). Even more significant is the fact that the combination of social defeat with MDMA administration induces profound behavioural alterations such as an increase in the time spent in immobility in the tail suspension test (indicative of a depressive-like state), an impairment of memory in the object recognition task and a reduced motor response to priming with MDMA. These results suggested that the MDMA use associated to conditions of social stress might enhance the vulnerability to develop neuropsychiatric disorders (García-Pardo et al., in preparation a).

The results obtained in the present thesis also demonstrated that the effects of social defeat are different in function of the drug of abuse tested. While acute and RSD induces opposite effects on MDMA reward, both procedures induces the same effects on the rewarding effects of other drugs of abuse. In other PhD thesis in course of our laboratory, the effects of the same procedures of acute and RSD on the rewarding properties of cocaine in the CPP paradigm are being evaluated (Montagud-Romero, PhD thesis in preparation). Both acute and RSD induce an increase in the rewarding and reinstating effects of sub-threshold doses of cocaine and in the duration of the CPP induced by effective doses of this drug (Montagud-Romero et al., under review; Rodríguez-Arias et al., submitted). In a similar way, in other studies of the present thesis we have observed the same effects of acute and RSD on the motivational effects of alcohol in the CPP paradigm and on intake of this substance in the two-bottle choice procedure (García-Pardo et al., in preparation b). Although previous studies have evaluated the effects of social defeat on the rewarding properties of alcohol (see Neisewander et al. 2012, for a review) it is impossible to obtain a clear conclusion of how social defeat modifies such effects of alcohol due to the high disparity in the methodology used in the different studies. The most important contribution of our work is the evaluation of the effects of social stress using two kinds of social defeat and two different models for the evaluation of the motivational effects of alcohol, performing the experiments in mice of the same strain and age. Our results clearly demonstrated that social stress exposure induces an acute and long-



term increase in the vulnerability to alcohol consumption, in agreement with previous studies of our laboratory (Rodríguez-Arias et al., 2014).

As commented above, repeated drug consumption induces different brain changes, which initially involve alterations in the DA system but subsequently imply the recruitment of other neurotransmitters that modulate DA mesolimbic activity, such as the opioid (Nutt, 2014) or glutamate systems (Van den Oever et al., 2012; Pierce & Wolf, 2013; Quintero, 2013; van Huijstee & Mansvelder, 2015). Other contribution of the present thesis is the evaluation of pharmacological manipulations that can reduce the rewarding effects of MDMA in the CPP paradigm or alcohol intake in the two-bottle choice procedure. These kind of experiments allow us to increase the knowledge of the neurobiological basis of the rewarding effects of these drugs and to investigate new targets for the pharmacological treatment of vulnerable subjects that develop drug dependence and addiction. We have demonstrated for the first time that the glutamate NMDA receptors are involved in the rewarding effects of *ecstasy* in the CPP paradigm; the blockade of these receptors with memantine interferes with the acquisition of *ecstasy*-induced CPP and with priming-induced reinstatement of this CPP (García-Pardo et al., in press). Although no previous studies have evaluated the role of glutamate on the rewarding effects of MDMA, the results of this study are in agreement with those demonstrating the involvement of glutamate on the rewarding effects of other drugs of abuse (Olive et al., 2012) and they are consistent with the involvement of glutamatergic system in drug addiction (Pomierny-Chamiolo et al., 2014; Tomek et al., 2013). In addition, the idea that glutamate-mediated plasticity may underlie to the long-term vulnerability to relapse of drug

consumption observed in dependent subjects even after long-term periods of abstinence (van Huijstee & Mansvelder, 2015) is supported by the fact that memantine blocks priming-induced reinstatement after extinction of MDMA CPP. The study of the role of other glutamate receptors and the related NO pathway in the rewarding effects of MDMA and in the modulation that social defeat stress induce in such effects have been the subject of other study of this thesis. The results obtained supported that AMPA receptors as well as the NO pathway are involved in the rewarding effects of *ecstasy* since mice treated with the AMPA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or the NOS inhibitor 7-nitrohindazole (7-NI) before conditioning with a low dose of *ecstasy* did not acquire CPP (García-Pardo et al., in preparation c). In addition, we have evaluated the involvement of NMDA and AMPA glutamate receptors and NO pathway in the effects of acute social stress on the CPP induced by *ecstasy*. Our results suggested that glutamate and NO signalling are involved in the reduction of the MDMA CPP induced by acute social defeat since this effect is selectively reversed by low doses of memantine (MEM) and 7-NI. These results are also in agreement with the idea that glutamate system is involved in the behavioural and physiological changes induced by social defeat stress (Krugers et al., 1993; Belozertseva & Beshpalov, 1998; Yap et al., 2005; Covington et al., 2008; Popoli et al., 2011).

With regard to alcohol, it is important to note that in spite of the current pharmacological therapies, nowadays there does not exist an effective treatment for all people with alcohol related disorders. We have demonstrated for the first time that the mu-opioid receptor antagonist GSK1521498 markedly reduced both alcohol seeking and voluntary

alcohol consumption, effects that were greater and longer lasting than those of naltrexone, other compound with the same profile clinically used for the treatment of alcohol dependence. The neurobiological relevance of this study is that we have confirmed the role of opioid system in the alcohol dependence, and have pointed out that the intervention on this system, for example that based in the antagonism of mu-opioid receptors, can help in the development of new pharmacotherapies for the alcoholism.

The main objective of the present thesis was to study the influence of social stress on the rewarding properties of MDMA and alcohol. The conclusion is that exposure to social stress induces a clear modification in the rewarding effects of both drugs which lead to the enhancement in the vulnerability to drug addiction. There are some differences in function of age (adolescent or young adult mice), type of social defeat (short- or long-term effects) and drug (MDMA or alcohol). In the case of MDMA, age and type of social defeat are essential factors determining the effects observed. MDMA induces higher rewarding effects in young adults than in adolescent mice; similarly, social defeat induces a greater corticosterone response in young adults than in adolescent mice (García-Pardo et al., 2014; García-Pardo et al., submitted). Acute social defeat did not induce short-term effects in adolescents but clearly reduces the CPP in young adult mice (García-Pardo et al., 2014). Conversely, RSD induces a long-term effect in both adolescent and young adult mice, increasing the duration of CPP; in this case, even RSD in adolescents has more consequences, enhanced the vulnerability to reinstatement of CPP after extinction (García-Pardo et al., submitted).

With regard to alcohol, both acute and RSD induces the same effect in the two different paradigms that we used to evaluate the effects of alcohol (CPP and two-bottle choice). Social defeat reversed the conditioned place aversion induced by EtOH and increased the intake of this substance. These effects suggested that social stress might enhance the rewarding effects of alcohol and so the vulnerability to develop addiction (García-Pardo et al., in preparation b). In addition, we also demonstrated that social defeat induced long-term behavioural alterations (García-Pardo et al., submitted) and that the combination of social defeat and MDMA is a very dangerous condition that increase the vulnerability to develop neuropsychiatric disorders (García-Pardo et al., in preparation a). In future studies we would like to evaluate the effects of combined administration of EtOH with social defeat as well as the consequences of co-administration of both drugs.

Moreover, the present thesis completes a line of research of our laboratory devoted to discover the neurochemical substrates of the rewarding effects of MDMA. In previous studies we have demonstrated the influence of dopaminergic and serotonergic systems (Vidal-Infer et al., 2012 a; Roger-Sanchez et al., 2013a, 2013b, 2013c; Aguilar et al., 2015). The original contribution of this thesis is the demonstration of the involvement of the glutamatergic system (NMDA and AMPA receptors) and the NO pathway in the rewarding effects of MDMA. We reported for the first time, that the NMDA antagonist memantine inhibited acquisition and blocked priming-induced reinstatement of MDMA CPP (García-Pardo et al., in press). Similarly, we observed that the AMPA antagonist CNQX and the NOS inhibitor 7-NI also inhibited

MDMA CPP (García-Pardo et al., in preparation c). Besides, our results also suggest that these glutamatergic/NO drugs can reverse the impairing effect of acute social defeat on MDMA CPP. Although these results are promising, they must be completed using the RSD procedure in both adolescent and young adult mice. Lastly, the study performed in the University of Cambridge demonstrates that the blockade of mu-opioid receptors with GSK1521498 reduces EtOH intake in the two-bottle choice paradigm (Giuliano et al., submitted). In future studies we would like to evaluate if this drug also reverses the increase in EtOH intake induced by acute and RSD in this paradigm.

The research of the neurobiological consequences of social stress exposure is a priority line of our laboratory. As commented above, the study of the effects of social stress on the rewarding effects of cocaine, MDMA and alcohol have derived into two different PhD theses. We have also demonstrated that RSD during adolescence induces a long-term impairment of brain blood barrier and modifies cocaine CPP and SA (Rodríguez-Arias et al., submitted) and induced changes in the expression of DA receptors. We are now studying the modulation of the effects of social defeat using different pharmacological approaches, such as D1 and D2 DA antagonists, CRF1 and CRF2 antagonists, etc. New promising approaches are being developed in other laboratories to reverse the effects of social stress such as the disruption of reconsolidation (Hymel et al., 2014), and the inhibition of p38 mitogen-activated protein kinase (MAPK), a protein of the cascade of molecular and cellular events activated by stress (Bruchas et al., 2011). Thus, we would like to investigate how these approaches modify the effects of

social defeat on the rewarding effects of drugs. Similarly, the manipulation of oxytocin, an anti-stress neurohormone involved in drug addiction (Lukas et al., 2011; Sarnyai & Kovács, 2014), might be a new strategy to reduce the effects of different kinds of social defeat. Future studies must also to incorporate another paradigms to evaluate the effects of social stress on the rewarding effects of MDMA and alcohol. For example, studies with the SA paradigm would allow to confirm the results obtained with the CPP, i.e. whether adult mice exposed to acute social defeat need to consume higher quantities of MDMA to feel the same rewarding effect that non-stressed animals. Further studies are also necessary to explore if stressed animals have changes in the motivation to get the drug, using for example, the progressive ratio or the second order paradigm. On the other hand, as behavioural traits such as impulsivity or high novelty seeking are significant variables modulating the development of drug addiction (Jupp & Dalley, 2014; Everitt, 2014), it will be interesting to study if the effects of social stress are modulated by these traits and/or if stressed animals became more or less impulsive to get the drug.

While in this work only corticosterone levels in stressed and non-stressed animals have been measured, in future studies we will evaluate changes in the activity of different neurotransmitter systems involved in the rewarding effects of MDMA and alcohol in mice exposed to different kinds of social stress, using different neurochemical techniques such as high performance liquid chromatography (HPLC), western-blot or microdialysis. It has been reported increases in tyrosine hydroxylase (Rodríguez-Arias et al., 2014) and DA signalling in the

mesocorticolimbic pathway (Razzoli et al., 2011) three weeks after RSD. In addition, RSD potentiates DA release after d-amphetamine challenge (Han et al., 2015) and increased extracellular signal-regulated kinases (ERK) phosphorylation in the VTA and the pharmacological inhibition of this effect attenuated the induction of sensitization and escalated cocaine taking induced by RSD (Yap et al., 2014). RSD also increase the expression of the DA cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein-32 (DARPP-32), but not of D1 or D2 receptors, in different brain areas (Jin et al., 2015). Moreover, CRF-DA interactions in the VTA can play a role in the escalation of cocaine intake induced by RSD, that is prevented by CRF1 and CRF2 antagonists (Boyson et al., 2014); enhanced glucocorticoid levels after RSD might influence mesolimbic dopaminergic activity regulating the firing rate of DA neurons via glucocorticoid receptors on medium spiny neurons of the NAcc that also expressed D1 receptors (Spanagel et al., 2014). In addition, different procedures of social defeat induces variations of serotonin levels in brain structures involved in the reward (Jacobson-Pick et al., 2013), a downregulation of the serotonergic gene expression in the Raphe nuclei two weeks after RSD (Boyarskikh et al., 2013), and up-regulates expression of SERT in rat dorsal raphe (Zhang et al., 2012) and of 5-HT(1B) in rostral NAcc shell (Furay et al., 2011). Finally, four 5-min aggressive encounters reduced the mRNA levels of the autoreceptor 5-HT1A in the dorsal raphe leading to hyperactivity of 5-HT neurons (Cooper et al., 2009). Some of these changes might alter reward mechanisms and be involved in the RSD-induced increase in vulnerability to drugs of abuse. Moreover, the knowledge of such changes might contribute to the identification of new drug targets for the treatment of drug addiction.

We consider that the present thesis offers a complete picture of the short- and long-term effects of social defeat on the motivational properties of MDMA and alcohol, making an important contribution to the knowledge on the effects of stress on drug addiction and behaviour. In addition, we have demonstrated the involvement of the glutamatergic system and NO pathway in the rewarding effects of MDMA and have found new pharmacological manipulations that reversed these effects of MDMA (such as NMDA and AMPA receptor antagonists or NOS inhibitor) and EtOH intake (such as the mu-opioid receptor antagonist GSK1521498). Our current objective is to evaluate whether these treatments can also reverse the enhancement of vulnerability to MDMA and alcohol induced by exposure to social stress. Although preliminary, the experiments performed with this objective are promising. In addition, different lines of research are opened from the results obtained in the present thesis. We expect in future years a translation of our basic research to develop better prevention and treatment strategies reducing the negative influence of stress on addictive disorders.





# 14.- CONCLUSIONS



#### 14.- CONCLUSIONS

- \* Young adult mice acquired CPP after conditioning with 1.25 and 10 mg/kg of MDMA. Mice did not show CPP after exposure to acute social defeat before each conditioning session with MDMA.
- \* Adolescent mice showed CPP only after conditioning with 10 mg/kg of MDMA and acute social defeat did not modify the effect of MDMA.
- \* Social defeat induced a lower stress response in adolescent than in young adult mice since corticosterone levels are enhanced immediately after social defeat only in young adult mice.
- \* These results suggested that young adult mice are more sensitive than adolescents to the rewarding effects of MDMA and that the stress induced by social defeat in young adult mice interferes with the acquisition of CPP.
- \* Adolescent and young adult mice exposed to repeated social defeat (RSD) showed a long-term enhancement in their sensitivity to the rewarding effects of MDMA.
- \* An increase in the duration of the CPP induced by 1.25 and 10 mg/kg of MDMA was observed three weeks after exposure to RSD in adolescent and young adult mice.
- \* An enhancement of the vulnerability to priming-induced reinstatement was observed in mice exposed to RSD during adolescence and conditioned with 1.25 mg/kg of MDMA.

- \* The increase in the rewarding and reinstating effects of MDMA in mice exposed to RSD during adolescent is observed despite these mice showed lower levels of corticosterone than young adult mice, indicating the high vulnerability of adolescent brain to stress exposure.
- \* The conditioned rewarding effects of *ecstasy* in mice depend on the activation of NMDA glutamate receptors. The NMDA antagonist memantine inhibited acquisition of *ecstasy* CPP and blocked priming-induced reinstatement.
- \* Administration of the mu-opioid receptor antagonist GSK1521498 (0,1, 1, 3 mg/kg) reduced the consumption of alcohol in the two-bottle choice procedure in alcohol-preferring rats, suggesting the usefulness of this compound for the treatment of alcoholism.
- \* The combination of MDMA (10 mg/kg) with acute social defeat induced cognitive deficits (implicit and recognition memory), depression-like behaviour in the tail suspension test and weak motor response to MDMA priming.
- \* Acute and RSD reversed the conditioned place aversion induced by alcohol (2.5 g/kg) and increased the voluntary consumption of this substance in the two-bottle choice procedure in mice, suggesting that social stress enhances the rewarding effects of alcohol.
- \* Besides glutamate NMDA receptors, AMPA receptors and NO pathway are involved in the conditioned rewarding effects of *ecstasy*. Memantine (10 mg/kg), the AMPA antagonist CNQX (0.25, 1 and 5 mg/kg) and the NOS inhibitor 7-NI (7.25 and 12.5 mg/kg) blocked the acquisition of the CPP induced by *ecstasy* in mice.

\* Memantine (5 mg/kg) and 7-NI (7.25 mg/kg) reversed the impairing effects of acute social defeat on *ecstasy* CPP, suggesting that glutamate and NO pathway are involved in these effects of social stress.

# 15.- REFERENCES



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