Drug consumption and stressful experiences in adolescent mice: behavioural, neurotoxic and neurochemical responses

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Tesi Doctoral, Barcelona, Gener 2013

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Als meus pares, a l’Enric i al Juan

AGRAÏMENTS

Finalment ha arribat… després de 4 anys i mig d'experiències de tot tipus m'arriba l'hora de defensar la tesi doctoral. Heu sigut molts els qui heu anat aportant un granet de sorra en el camí per arribar-hi. Per això, m'agradaria escriure unes paraules per a aquells que d'alguna manera o altra m'heu ajudat en aquest projecte.

Primer de tot, agrair a la meva directora de tesi, l'Olga Valverde, per donar-me l'oportunitat de realitzar-la. Gràcies pels consells, tant a nivell científic com a nivell personal, durant aquests anys.

Gràcies a les nenes del GReNeC.... Especialment Jéssica i Neus que heu estat al meu costat des del primer dia que vaig arribar. Jéssica... què t'he de dir? saps més bé que ningú que sense tu no hauria pogut... MIL GRÀCIES!! Neus, merci per l'ajuda, de tota mena! Com a tècnic, com a persona.... quants experiments, quants cafès i quant de tot hem compartit durant aquests anys... No em vull deixar les últimes incorporacions, que encara que faci menys temps que us conec també hi heu participat. Maria, moltes gràcies per les revisions de l'anglès! Irene, Marta i Emily... com tampoc les que heu marxat... Clara, encara que estiguis lluny no m'oblido de tu. Gràcies a totes!

Albert, la primera cara que vaig veure el primer dia que vaig arribar i al final... vora dos anys de convivència! Gràcies "xavalín", va ser fantàstic pel Marina 312.

I agraïr també a tota la resta de gent del PRBB que d'alguna manera o altra heu estat amb mi aquest temps.

També vull donar les gràcies a la gent del departament de Ciències Mèdiques Bàsiques de la UdL per tota l'ajuda. Alba i Isu, en les "bidis" i els

anàlisis de "redox proteomics". Mariona i Elisa, en els estudis de metabolòmica, tant maca però tant complicada d'entendre!
Als "Amics Penyates", que últimament ens hem retrobat més i m'encanta!!! Gràcies per aquestes quedades que anem fent que són genials. Que no s'aturin. Ens veiem aviat!
Mollerussencs!!! Què faria sense vosaltres? No posaré noms perquè no acabaria mai, però bé, Colla81 "al completo" gràcies a tots i a totes. Cadascun de vosaltres teniu alguna cosa que us fa ser únics i que m'ajuden a enriquir com a persona.
A la Vero i la Carme... Nenes!!! Quantes estones compartides, quantes coses explicades, quantes sortides, quant de tot! Carme, si finalment t'embranques en fer una tesi et desitjo moltíssima sort!!! I a tu Vero, espero que tot et vagi molt bé també. Sabeu les dues que em teniu aquí pel que necessiteu.
A les de la Farmàcia... venir els estius i els caps de setmana de guàrdia és perfecte per desconnectar de ratolins i experiments... Treballar amb vosaltres és genial.
A la família, tot i que ja els dedico aquesta tesi, també voldria escriure unes línies pels de casa... Pare i mare, gràcies per ser com sou i haver-nos ajudat a nosaltres a ser com som. Us estimo molt als dos! Enric... què he de dir del meu germanet? Petit, ets únic, no canviïs mai. M'encanta poder dir que ets el meu germà! I als meus avis tampoc me'ls puc deixar que el munt d'espelmes que han arribat a encendre per nosaltres és per agraïr enormement.
I ja per acabar, i no per això menys important, al Juan. Haver-te conegut és del millor que em podia passar. Qui m'havia de dir que un dia tant bonic com és Sant Jordi coneixeria a la persona que em faria ser feliç i amb qui voldria compartir la meva vida. Gràcies perquè amb tu tot és, o sembla, més fàcil. T'estimo.

Clara Ros has been funded by a FPI fellowship (BES-2008-007915).
This work was supported by grants from Spanish Ministry of Science and Innovation (SAF2007-60249; SAF2010-15793); the Spanish Ministry of Health (PNSD 2006 and 2010); Red Temática de Investigación Cooperativa en salud (ISCIII) (RETIC-Trastornos Adictivos RTA 001/06/1001 and 12/0028/0024-FEDER) and Generalitat de Catalunya (2009SGR684).

ABSTRACT

Adolescence is a critical developmental period in which the brain emerges from an immature state to adulthood. This process of brain development is associated to greater cognitive capacity but also to altered emotional behaviour, such as anxiety and depressive symptoms; as well as increased sensation-seeking and risk taking behaviour. The proper development of brain and behaviour into adulthood can be negatively affected by external factors such as drug abuse and environmental conditions.

This work consists firstly on, studying the impact of binge ethanol, 3, 4-Methylenedioxymethamphetamine (MDMA) and its combination in adolescent mice. Secondly, study the consequences of early-life stressful experiences (social isolation) into adulthood.

Main results obtained from the first objective are that the combination of binge ethanol and MDMA induces emotional-like alterations. These alterations can be prevented by antidepressant treatment. In addition, MDMA induces memory impairments that may be associated to oxidative damage to specific proteins in the hippocampus. Neuroinflammation is also present after MDMA treatment, but not after binge ethanol treatment, in mice striatum. Metabolomic studies indicate that brain metabolism is altered after binge ethanol, MDMA or its combination. Even though these are only preliminary results, these alterations might be due to an imbalance in tryptophan metabolism.

Regarding the second objective, our findings indicate that social isolation during adolescence induces an altered response to novel and stressful situations. These alterations are probably due to altered HPA axis activity.

RESUM

L'adolescència és un període crític en el desenvolupament de l'individu en el qual el cervell va d'un estat immadur a l'edat adulta. Aquest procés va acompanyat d'una elevada capacitat cognitiva però també de freqüents alteracions de tipus emocional, com l'ansietat o els símptomes depressius, així com la cerca de sensacions de risc. Un bon desenvolupament del cervell i del comportament es pot veure negativament afectat per factors externs com són l'abús de drogues i les condicions ambientals desfavorables.

Aquest projecte consisteix en primer lloc, a estudiar l'impacte de l'alcohol en excés, la 3, 4-Metilendioximetamfetamina (MDMA) i la seva combinació en ratolins adolescents. En segon lloc, estudiar les conseqüències en l'edat adulta d'experiències estressants durant l'adolescència.

Els principals resultats obtinguts referents al primer objectiu són que la combinació d'alcohol en excés i MDMA provoca alteracions de tipus emocional. Aquestes alteracions poden ser previngudes pel tractament amb antidepressius. A més, la MDMA indueix un deteriorament de la memòria que pot estar associada amb el dany oxidatiu a proteïnes específiques de l'hipocamp. També s'ha observat una resposta neuroinflamatòria en el cos estriat dels ratolins després del tractament amb MDMA, però no després del tractament amb etanol en excés. Finalment, estudis de metabolòmica indiquen que el metabolisme cerebral es veu alterat després de l'alcohol en excés, la MDMA o la seva combinació. Tot i que només són resultats preliminars, aquestes alteracions poden ser conseqüència d'un desequilibri en el metabolisme del triptòfan.

Referent al segon objectiu, els nostres resultats indiquen que l'aïllament social durant l'adolescència indueix una resposta alterada a situacions novelles i estressants. Aquestes respostes anormals són probablement conseqüència d'alterations en l'activitat de l'eix HPA.

PROLOGUE

Adolescent drug abuse is a serious public health problem at the present time. Alcohol at a pattern of binge drinking usually combined with psychostimulants such as 3, 4- Methylenedioxymethamphetamine (MDMA) is popular among adolescents and young adults. It is concerning since adolescence is a stage of brain development with a high risk of undergoing neuropsychiatric disorders associated to emotional and cognitive alterations that can be triggered by drug abuse.

This work is mainly focused on studying the impact of the consumption of binge ethanol, MDMA or its combination in adolescent mice. In particular, emotional and cognitive alterations, neuroinflammatory response and oxidative damage to specific proteins have been determined. In addition, we have started metabolomic studies in order to know how metabolites involved in different pathways should vary as a consequence of the consumption of the abovementioned drugs of abuse.

On the other hand, it has been observed that individuals experiencing adverse environmental conditions in early-life present health affectations along their lives. For instance, social isolation is considered as a conflicting and stressful situation that may induce neurobehavioural alterations into adulthood. Therefore, a complementary objective of this work is to study the behavioural alterations induced by different home environment during the adolescent stage, particularly those related to stress.

ABBREVIATIONS

5-HT: serotonin
ACTH: adrenocorticotropic hormone
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BAC: blood alcohol concentration
cAMP: cyclic adenosine-5'-monophosphate
CNS: central nervous system
CORT: corticosterone
CRF: corticotrophin-releasing factor
CRMP2/DRP2: dihyropyrimidinase-related protein 2
CYP: cytochrome P-450
DA: dopamine
DAT: dopamine transporter
DID: drinking in the dark
EC: enriched condition
EE: environmental enrichment
GFAP: glial fibrillary acidic protein
GSH: glutathione
H_2O_2: hydrogen peroxide
HPA: hypothalamus-pituitary-adrenal
HSC: heat shock cognate
Iba-1: ionized calcium binding adaptor molecule 1
IC: isolated condition
MAO: monoamine oxidase
MDMA: 3, 4-Methylenedioxymethamphetamine
NADPH: nicotinamide adenine dinucleotide phosphate hydrogen
NE: norepinephrine
NET: norepinephrine transporter
NMDA: N-methyl-D-aspartate
O_2^-: superoxide anion
OH^-: hydroxyl radical
PCA: principal component analysis
PFC: prefrontal cortex
PLS-DA: partial least discriminate analysis
RNS: reactive nitrogen species
ROS: reactive oxygen species
SC: social condition
SERT: serotonin transporter
VTA: ventral tegmental area
WHO: World Health Organization

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INTRODUCTION

1. ADOLESCENCE

Adolescence is a critical transition period ranging from infancy into adulthood. It is characterized by the most important biological, psychological, social changes and opportunities occurring in someone's life (Dahl and Gunnar, 2009; Paus et al. 2008). Interactions between the individual and the environment are intense and may have affectations on brain development and behaviour. Adolescence is accompanied by a rapid increase in the rate of physical growth and development. Additionally, typical behavioural features of this stage are impulsivity, increased sensation-seeking and risk-taking, as well as increased social interactions. Indeed, the majority of mental health problems including anxiety, depressive symptoms or psychopathological states, appear during this stage (Young et al. 1993; Oldehinkel and Bouma, 2011). The proper brain and behaviour development from adolescence into adulthood can be affected by external factors such as drug abuse or social interactions and environmental conditions that may facilitate the development of the previously mentioned neuropsychiatric disorders.

1.1 Adolescent brain development: adolescent behaviour

Adolescence period is characterized by many dynamic cellular, molecular and anatomical modifications associated to brain development (Spear, 2000; Giedd, 2008). Brains regions such as the prefrontal cortex (PFC), hippocampus, amygdala, nucleus accumbens (NAc), ventral tegmental area (VTA) and hypothalamus undergo prominent reorganization during the adolescent stage (Crews et al. 2007). The PFC and the hippocampus are probably the two main brain areas where more changes take place during this stage. It has been observed that absolute PFC volume decreases while hippocampus increases its volume during adolescence in humans (Sowell et al. 2001; Guerri and Pascual, 2010). Even more, magnetic resonance imaging studies have demonstrated a preadolescent increase in gray matter volume

followed by a post-adolescent decrease (Giedd, 2004; Marsh et al. 2008) whereas white matter increases linearly (Sowell et al. 2004) (Figure 1). These volume changes occur probably as a result of a competitive process in which the most frequently used connections are strengthened and the most infrequently used are eliminated through environmental experiences (Blakemore and Choudhury, 2006).

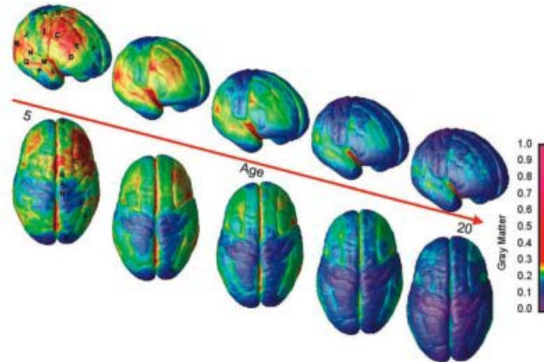

Figure 1. Magnetic resonance images indicate that gray matter decreases as the brain matures and is replaced by white matter. Red indicates more gray matter; blue, less gray matter (from Marsh et al. 2008).

It is important to mention that PFC is a brain area involved in executive functions such as cognitive flexibility, memory processes, self-regulation and evaluation of risk and reward (Crews et al. 2007). Meanwhile, hippocampus is involved in learning and memory processes (O'Keefe and Dostrovsky, 1971; Morris et al. 1982). Other abovementioned regions such as the amygdala, involved in the limbic system, are the ones implicated in the regulation of emotional behaviour (McGaugh et al. 2002; Giedd et al. 2008). Therefore, modifications in these brain areas are associated to acquisition of adult cognitive and emotional repertoires (Crews et al. 2007).

However, at a behavioural level, they are also typical hallmarks of the adolescent stage: the increased sensation-seeking, risk-taking behaviours, low levels of harm

avoidance and impulsivity (Spear, 2000; Blakemore, 2008; Eiland and Romeo, 2012). It is a time when novel experiences involving drugs, alcohol and sexual behaviours are sought, and in the majority of teens, risky behaviours are viewed as exciting and rewarding ignoring the negative consequences of this behaviour. These features are probably associated with the synaptic remodelling of limbic and frontal brain regions (Crews et al. 2007), as well as with hormonal alterations (Witt, 2007). The late development of the PFC circuits involved in judgment and inhibitory control may underlie the propensity of adolescents to impulsivity and to ignore the negative consequences of their behaviour (Guerri and Pascual, 2010). This increase in risk-taking and sensation-seeking behaviours can lead to experimentation with drugs of abuse in our modern society that may affect the proper development into adulthood (Hardin and Ernst, 2009).

Furthermore, during adolescence, social environment changes dramatically since the focus on interactions with parents shifts towards interactions with peers (Sachser et al. 2011). Peers provide positive experiences for adolescents which may help develop social skills and facilitate the transition to family independence (Spear, 2000). Additionally, there is evidence that adolescence is a highly sensitive period in which behaviour is continuously and profoundly shaped by social experiences. Thus, positive social interactions during this period of life are crucial for the correct individual development. In contrast, early-life stressful experiences may induce abnormal development into adulthood.

Therefore, drug abuse as well as early-life stressful situations may impact the correct brain development into adulthood. Consequently, the appearance of emotional disorders and cognitive alterations can be enhanced.

1.2 Emotional behaviour

Adolescence is a time of significant psychological and physiological vulnerabilities. Thus, adolescence is a period with high risk of developing neuropsychiatric disorders such as anxiety or depression (Dahl, 2004; Spear, 2000; Eiland and Romeo, 2012).

Mesolimbic regions of the forebrain in which dopamine (DA) is found such as the amygdala, show marked alterations during the adolescent stage. Given the involvement of these regions in the regulation of emotional processes, these structural modifications have been understood as to modulate the adolescent emotional behaviour (McGaugh et al. 2002; Blakemore and Choudhury, 2006; Giedd et al. 2008). A reorganization of the serotonergic system involved in mood, sleep or anxiety among other behaviours, is also occurring during adolescence. Low serotonin (5-HT) activity in adolescence has been suggested to contribute to the emotional disorders during this stage of life (Depue and Spoont, 1986). Along with the abovementioned changes in DA and 5-HT neurotransmitter systems, adolescent development is associated with changes in neuroendrocrine function (Ojeda and Terasawa, 2002; Gunnar et al. 2009). A major part of the neuroendocrine function is the *hypothalamus-pituitary-adrenal (HPA) axis* (Figure 2), that controls reactions to stress, mood, emotions, among others (Smith and Vale, 2006; Romeo and McEwen, 2006; Witt, 2007). The HPA axis is considered the major physiological stress system of the body whose activation is triggered by a wide range of psychological experiences and physiological perturbations (Armario, 2006; 2010). As its name implies, it consists of the hypothalamus, the pituitary cells and the adrenal glands located on the top of the kidneys.

Hypothalamic cells produce corticotrophin-releasing factor (CRF) hormone in response to either physical or psychological stress. The hypothalamus secretes CRF

into the blood and it binds to specific receptors on the pituitary cells, where induces the production of adrenocorticotropic hormone (ACTH). ACTH is then secreted into the systemic blood circulation and transported to its target, the adrenal glands. The adrenal glands induce the secretion of glucocorticoids, primarily cortisol in humans and corticosterone (CORT) in experimental animals (Herman et al. 2003; Romeo and McEwen, 2006). The release of cortisol/CORT initiates a series of metabolic effects aimed to alleviate the harmful effects of stress through negative feedback to both the hypothalamus and the anterior pituitary, which decreases the concentration of ACTH and cortisol/CORT in the blood once the state of stress is suppressed (Sapolsky et al. 1985; Armario et al. 1992).

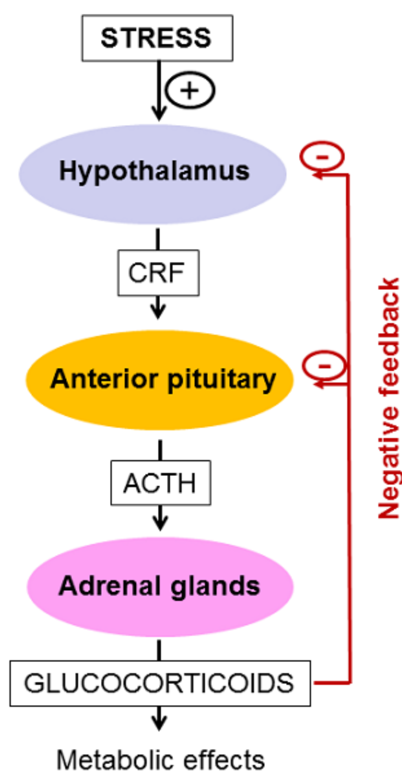

Figure 2. The HPA axis.

Apart from its own negative feedback, HPA axis actitivity is regulated by different neural systems, including the hippocampus, which exerts an inhibitory on CRF-containing neurons; and the amygdala, exerting an excitatory action (Nestler et al. 2002a).

Numerous studies have identified a link between exposure to stressful situations during adolescence and anxiety and depressive symptoms in susceptible individuals (Kendler et al. 1999). In fact, it has been observed a relationship between limbic dysfunction and HPA axis deregulation in mood disorders (Herman et al. 2005).

1.2.1 Animal models to evaluate emotional behaviour

1.2.1.1 Animal models to evaluate anxiety

There are numerous paradigms to evaluate anxiety in animal models based on different behavioural aspects (Millan, 2003; Fuchs and Flugge, 2004). These behavioural paradigms are based on the exposure of animals to a novel environment. Usually, they consist of labyrinths formed by aversive (or risky) and non-aversive (or comfortable) compartments. The animal is exposed to a conflict between the natural impulse to explore the environment and the innate tendency to avoid the unknown, especially those with some aversive characteristics (Fuchs and Flugge, 2004). The paradigms more commonly used are the following:

- The *dark-light box:* it is based on the innate aversion of rodents to brightly illuminated areas. The apparatus consists of two plastic chambers (a dark chamber and a white and illuminated chamber) connected by a small tunnel (Figure 3). The test consists in placing the animal in the dark chamber and the entrance latency into the white compartment (aversive environment), the time spent in each compartment

and number of transitions between compartments are measured. Longer time exploring the light (or white) compartment corresponds to less anxiety-like behaviour.

Figure 3. Dark-light box

- The *elevated plus maze*: it is based on the innate aversion of rodents to height. The apparatus consists of a cross shaped maze elevated 30 cm above the ground with two open arms (or aversive environment) and two closed arms (or comfortable environment) (Figure 4). The test involves placing the animal in the neutral central square and the percentage of entries in the open arms and the percentage of time spent in open arms are measured. Major entries and longer time spent in the open arms correspond to less anxiety-like behaviour.

Figure 4. Elevated plus maze

1.2.2 Animal models to evaluate depressive disorders

It is difficult to evaluate depressive disorders in animal models since depression is exclusively a human disorder (Nestler et al. 2002b). However, different paradigms have been developed to be able to identify depressive-like behaviour, and assessing antidepressant potential of different pharmacological agents, in experimental animals. One of the most currently used is the *tail suspension test* (Figure 5). In the test the animal is suspended by the tail at a certain height from the ground (Steru et al. 1985). The measuring principle is based on the energy developed by the animal trying to escape from its suspension (www.harvardapparatus.com). Thus, time of complete inactivity during this period corresponds to more despair or depressive-like behaviour. Its principle is similar to that of the forced swimming test.

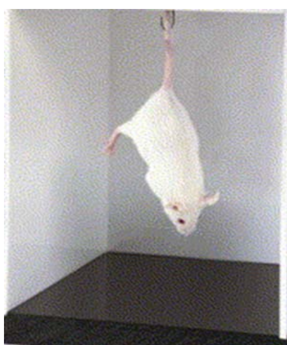

Figure 5. Tail Suspension Test

1.3 Cognition: learning and memory

As stated above, the PFC and the hippocampus are probably the two brain areas where more changes take place during the adolescent stage (Crews et al. 2007; Marsh et al. 2008). Both of them are involved in critical functions, being learning and memory processes among the most important (Morris et al. 1982; Crews et al. 2007). Learning is the process by which we acquire new information or knowledge.

Memory is the process by which this knowledge is encoded, stored and later retrieved. Memory can be classified in short-term memory, which is a temporary storage of immediately accessible information memory; and long-term memory allowing the sustainable conservation of information by encoding and storage. There are two different main categories of long-term memory, i) *non declarative or implicit memory*, it does not depend on hippocampus and includes procedural memory (involving cerebellum and striatum) and emotional or perceptive memory (involving the amygdala); and ii) *declarative or explicit memory*, which is dependent of the hippocampal formation and includes episodic and semantic memories. A particular type of explicit memory is working memory, which is dependent on the PFC (Morgado, 2005).

Learning implies synaptic plasticity, that is, structural and biochemical changes in the synapses altering the effects on postsynaptic neurons. Over the last years, a large amount of investigations have been focused on learning processes, particularly in the hippocampus, the main area involved in declarative memory (Morris et al. 1982). It has been observed that electrical stimulation of high frequency of some excitatory hippocampal circuits produces a long-term increase in the strength and effectiveness of the synapse, known as long-term potentiation. Therefore, it is suggested that there is a mechanism responsible for the synaptic changes occurring during the process of learning (Malenka and Nicoll, 1999). This process principally takes place in the hippocampal formation. Axon electric stimulation of the neuronal circuit that crosses the hippocampal formation strengthens synapses, that is, increases or produces morphological changes in dendritic spines giving rise to new or reinforcing existing synapses which may constitute the main structural basis of memory. Dendritic spines are branched protoplasmic extensions and the major site of excitatory synaptic transmission in the brain.

In the hippocampal formation, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors have a critical role in the long-term potentiation process. Activation of NMDA receptors is the critical trigger for the induction of long-term potentiation and the subsequent morphological changes associated to the process of synaptic plasticity and learning (Malenka and Nicoll, 1999). These morphological changes in the dendritic spines require the synthesis of new proteins including cytoskeleton components, kinases and receptors (Frey and Morris, 1997), essential for long-term memory.

The process of long-term potentiation has been accepted as a model of synaptic plasticity and is an essential event for memory consolidation (Prado-Alcalá and Quirarte, 2007). It is known that the hippocampus plays a crucial role in the process of memory consolidation, process information and transfer it to the cortex. Therefore, declarative memory is stored through reciprocal connections between the hippocampus and cortex (Álvarez and Squire, 1994).

It should be noted that any damage to this process could be altering the learning process as well. For instance, though the exact mechanism is not well-known, acute drug intoxication seems to produce transient alterations in these processes; and thus alter the learning process that can persist for a long time (Vik et al. 2004).

1.3.1 Animal models to evaluate declarative memory

The most obvious challenge when investigating declarative memory in experimental animals is that they cannot follow verbal instructions or “declare” their memories verbally. However, there are ways to evaluate this type of memory in laboratory animals. Two paradigms commonly used are:

- The *object recognition test*: the aim of this task is to evaluate the capacity of the animal to recognize a "familiar" object previously presented from a "novel" object. The task is conducted in a black open field. A discrimination index is calculated as the ratio between the differences in the time spent exploring either the "novel" or the "familiar" object and the total time exploring the two objects the day of the test. The hippocampus and the adjacent structure, the perirhinal cortex, are the brain areas involved in this memory task.

- The *radial arm maze:* this maze was firstly described by Olton and Samuelson (1976) to measure spatial learning and memory in rats. Particularly, it has been shown to be practicable in the application of how drugs affect memory performance (Levin, 1988). Different versions have been used over the years to adapt to the experimental conditions of each. Broadly, it consists of placing the animal in the central platform of the six or eight arm labyrinth (Figure 6). At the end of each arm there is a food site, or well, that may be baited or not with food. Animals have to learn a specific task involving spatial memory but, depending on the task, it may also involve working memory. Thus, the prefrontal cortex and the hippocampus may be involved in this task (Morris et al. 1982; Bizon et al. 2012). For that, animals are subjected to various trials a day for a determined number of days. There may be extra-maze visual cues to aid in spatial localization.

Figure 6. Eight-arm radial arm maze

2. DRUG ABUSE AND ADDICTION

As stated in the previous chapter, adolescence is a period of increased risky behaviours. Drugs such as ethanol, nicotine, psychostimulants (cocaine or amphetamines), marihuana or hallucinogens are drugs of abuse commonly consumed by adolescents (Capela et al. 2009). Individuals take drugs because of their psychotropic effects which can combine different actions such as depressant (alcohol), stimulant (cocaine, nicotine or amphetamines) or perturbation of perception (mescaline or cannabis), depending on their particular mechanism of action in the central nervous system (CNS). Despite the diversity of effects, all of them produce rewarding and positive reinforcing properties by the activation of the mesocorticolimbic dopaminergic system (Figure 7). It consists of DA neurons projecting from the VTA to the NAc and PFC resulting on a release of DA in this specific brain areas, and the associated limbic structures (Kauer and Malenka, 2007; Koob and Volkow, 2010).

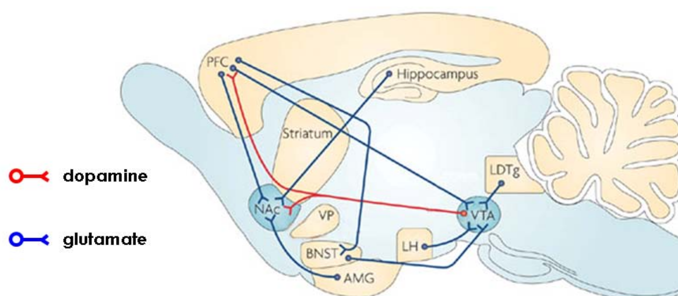

Figure 7. Simplified schematic of the circuitry of the mesocorticolimbic dopamine system in the rat brain highlighting the major inputs to the nucleus accumbens (NAc) and ventral tegmental area (VTA) (adapted from Kauer and Malenka, 2007). (**AMG**, amygdala; **BNST**, bed nucleus of the stria terminalis; **LDTg**, laterodorsal tegmental nucleus; **LH**, lateral hypothalamus; **VP**, ventral pallidum).

This system has been evolutively developed to be activated by natural rewards such as food or sex. However, drugs of abuse are able to activate it in an unusually

intense manner (from two to ten times higher than a natural reward), resulting in changes in synaptic plasticity, particularly in the dopaminergic system (Wolf, 2002). These changes induce maladaptive behaviours that may initiate the to drug addiction phenomenon (Nixon and McClain, 2010).

Repeated acute drug administration induces the development of a state known as dependence (Koob, 2009). It is postulated that the released DA after acute drug intake promotes neuroplasticity in the mesolimbic system through the activation of certain signalling pathways that results in altered gene expression. Such changes in gene expression may be associated with the transition from social drug use to drug dependence (Kalivas and O'Brien, 2008). Dependence can be manifested as altered physical symptoms (physical dependence) and negative emotional states manifested as dysphoria, anhedonia depression and anxiety (psychological dependence) occurring when drug use is discontinued or withdrawal. During this stage it may appear a process called tolerance, characterised by a decrease in the acute effects of the drug after repeated administration and thus, being necessary to increase the drug dose to produce the same response. This process is relatively usual in drug abuse but not necessarily to occur.

The molecular basis of these processes is that within the neuroadaptations occurring during chronic drug exposure, there is a decrease in functionality of the abovementioned neurotransmitter system implicated in the acute rewarding effects of drugs of abuse, the DA system; but also 5-HT neurotransmission in the NAc. Therefore, it is necessary a higher dose of drug to induce the same neurotransmitter release and thus, the same drug response. It is believed that these systems are compromised during this stage and lead to decreased motivation for nondrug-related stimuli and increased sensitivity to the abused drug (Melis et al. 2005). Neuroadaptations occurring in systems involved in stress modulation such as the HPA axis mediated by CRF probably have a key role in the manifestation of the anxiety-like responses typical of this stage (Sarnyai, 1998; Armario, 2010; Gilpin,

2012). Up-regulation of the cyclic adenosine-5'-monophosphate (cAMP) pathway in the NAc is also a common adaptation to long-term exposure to some drugs such as ethanol, opiates and cocaine, but not to non-addictive drugs. It is suggested that up-regulation of this pathway may contribute to the negative emotional aspects of drug abuse (Koob and Nestler, 1997).

These changes in behaviour, physiology and neuroplasticity are crucial for the development from acute drug intake to addiction (Nestler, 2004; Koob and Volkow, 2010). Drug addiction impacts multiple motivational mechanisms and can be conceptualized as a disorder that progresses from impulsivity (positive reinforcement) to compulsivity (negative reinforcement). The positive reinforcement is defined as the process by which presentation of a stimulus, usually pleasant (i.e. the drug itself), increases the probability of a response. The negative reinforcement is defined as drug taking that alleviates a negative emotional state which is derived from the abovementioned deregulation of key neurochemical elements involved in reward and stress. Impulsivity often dominates at the early stages and impulsivity combined with compulsivity dominate in the later stages of addiction (Koob, 2009).

The World Health Organization (WHO) defines addiction as the "repeated use of a psychoactive substance or substances, to the extent that the user (referred to as an addict) is periodically or chronically intoxicated, shows a compulsion to take the preferred substance (or substances), has great difficulty in voluntarily ceasing or modifying substance use, and exhibits determination to obtain psychoactive substances by almost any means. Typically, tolerance is prominent and a withdrawal syndrome frequently occurs when substance use is interrupted. The life of the addict may be dominated by substance use to the virtual exclusion of all other activities and responsibilities (…) It is regarded by many as a discrete disease entity, a debilitating disorder rooted in the pharmacological effects of the drug, which is remorselessly progressive."

Regarding the vulnerability of the CNS to the effects of the drugs and the fact that the juvenile brain undergoes important structural and functional changes, adolescence drug consumption can cause irreversible abnormalities on behaviour and brain development. Thus, adolescent drug abuse can compromise future quality of life and promote addiction in adulthood.

Two drugs of abuse commonly used among adolescent and young adults are ethanol and 3, 4-Methylenedioxymethamphetamine (MDMA) (Winstock et al. 2001; Barrett et al. 2006).

2.1 Ethanol binge drinking

2.1.1 History of ethanol drinking

Ethanol (alcohol) is a substance/drug that has been consumed and abused over many years. From the earliest times to the present, alcohol has played an important role in religion and worship. Historically, it has been useful as a source of nutrients as well as for its medicinal, antiseptic and analgesic properties. Indeed, the pleasurable effects of alcohol drinking have been important in enhancing the enjoyment and quality of life. While no one knows exactly when alcohol beverages were first used, there are evidences that fermented beverages have existed since the early Neolithic period, that is 10.000 BC. Fermented beverages were used by Egyptian and China civilizations. Beer was the most commonly used alcoholic beverage by Babylonians. In the ancient Greece and Rome alcohol was also present in meals at celebrations. In the sixteenth century, alcoholic beverages (known as "spirits") were widely used for healing purposes. However, alcohol consumption was often high; therefore alcoholism was generalized among population. The nineteenth century brought a change of attitude towards alcohol and a campaign began to promote the moderate use of alcohol (Hanson, 1995).

During the last and the present century, alcohol has been the legal drug of abuse mostly accepted and consumed. In the 1940s and 1950s “binge drinking” became a usual pattern of alcohol drinking. Going on a “binge” was used to mean an extended period (days) of heavy drinking, while now it generally refers to a single drinking session leading to intoxication (Herring et al. 2008). According to the National Institute of Alcohol and Alcoholism, binge drinking is defined as a pattern of alcohol drinking that brings the blood alcohol concentration (BAC) to 0.08 g/dL or above within a 2-hours period that may or not may be associated with dependence. This pattern corresponds to consuming 70g alcohol or more (male) or 56g or more (female) in about two hours. Adolescent binge drinking would correspond to 5 or more drinks for males and 4 or more drinks for females per occasion (Wechsler et al. 1994). This pattern of binge drinking is nowadays very common among adolescents and young adults and has become a serious public health problem worldwide (Deas 2006; Labbe and Maisto, 2011).

2.1.2 Epidemiology

Alcohol is one of the first drugs of choice and binge drinking is a pattern of heavy and risky drinking that is observed all over Europe and is becoming increasingly frequent among teenagers. In fact, alcohol binge is the most commonly used drug among youth. Reports from the European School Survey Project on Alcohol and Other Drugs carried out in different European countries, indicate that young people drink more and with a clear focus on drunkenness than earlier generations (Hibell et al. 2007). Adolescents start drinking at the age of 13 – 14 years old and most important, and also alarming, is the fact that they believe that binge drinking is normal (Hernández, 2009). In Spain, a recent survey performed by Plan Nacional Sobre Drogas (Ministerio de Sanidad) in adolescents showed that 75% have consumed ethanol at some time and 36% have reported getting drunk during the previous month (ESTUDES, 2010).

2.1.3 Pharmacology of ethanol

It was long believed that ethanol was a substance "without receptor" as it was difficult to find out specific receptors. Initially, it was believed that primary targets of ethanol were membrane lipids (Guerri, 2012). The "lipid theories" postulated that alcohol action affected membrane fluidity and disordering of the bulk lipid phase of membranes. However, clear limitations have been found on this hypothesis. Nowadays it is known that ethanol has its brain specific targets, mainly the neurotransmission systems involving glutamate and γ-aminobutyric acid (GABA) (Spanagel, 2009). Acute ethanol acts as an inhibitor of the glutamate neurotransmission in different brain areas such as hippocampus, cerebellum, cerebral cortex, NAc, amygdala and VTA in a concentration-dependent manner (Lovinger et al. 1989; Hoffman, 2003). Contrary, ethanol acts as an enhancer of the GABAergic system acting presynaptically to increase GABA release in different brain regions. This effect may be through its direct union to the $GABA_A$ receptor (Mihic et al. 1997), via the activation of CRF receptors (Nie et al. 2004) or the opiate system (Foster et al. 2004). Both NMDA and $GABA_A$ receptors can be indirectly modulated by protein kinases such as protein kinase A and protein kinase C. These ethanol actions on NMDA and $GABA_A$ receptors are involved in the acute tolerance to ethanol (Spanagel, 2009; Alfonso-Loeches and Guerri, 2011).

Even though ethanol increases GABAergic neurotransmission, there is one brain region where ethanol decreases GABA release which is the VTA (Stobbs et al. 2004; Xiao et al. 2007). The DA neurons in the VTA are continuously inhibited by GABA-containing neurons (inhibitory control); accordingly, ethanol-induced decrease in GABAergic neurotransmission in this specific brain area leads to the activation of the mesolimbic pathway, inducing DA release and thus, the rewarding properties of acute ethanol exposure (Figure 8).

Additionally, ethanol has also an action on 5-HT neurotransmission system; in particular it potentiates the 5-HT_3 receptor function which regulates neurotransmission release presynaptically (Lovinger and White, 1991; McBride et al. 2004).

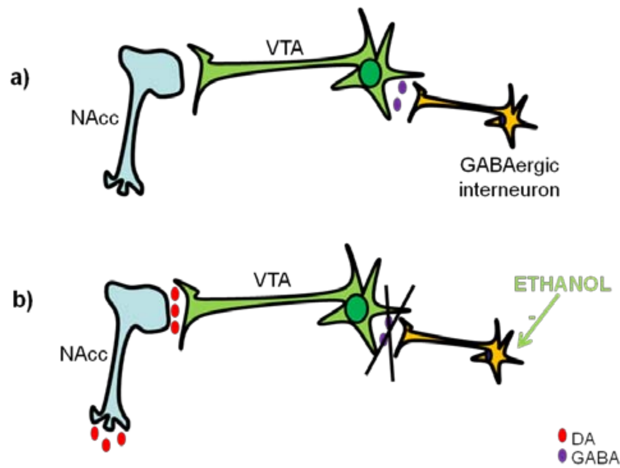

Figure 8. Main mechanism of ethanol induced reward. **(a)** Under basal conditions GABAergic interneurons are inhibiting the mesolimbic pathway and no DA is released in the NAcc. **(b)** Ethanol stimulates the release of DA in the NAcc by the inhibition of the GABAergic interneurons and subsequent deshinibition of the DA neurons in the VTA.

2.1.4 Effects on humans

2.1.4.1 Acute effects

Unlike others drugs of abuse, ethanol is a low potent substance since its effects are observed after ingestion of grams of ethanol, while for other substances such effects are observed just with milligrams (Guerri, 2012). Many of the behavioural effects of acute ethanol exposure on humans can be linked to effects on glutamatergic and GABAergic neurotransmission. These acute effects depend on the dose ingested that is, to the BAC. Thus, at a BAC of less than 50 mg/dl the effects observed are

relaxation, increase in talkativeness and impairment in some tasks requiring skill. When the BAC is higher, they begin to show signs of acute alcohol intoxication, which is the most frequent alcohol-related disorder present in adolescents. Therefore, at a BAC higher than 100 mg/dl individuals show altered perception of the environment, ataxia, hyper-reflexia, impaired judgment, lack of coordination, mood, personality and behavioural changes, prolonged reaction time or slurred speech. At a BAC higher than 200 mg/dl there are signs of amnesia, diplopia, dysarthria, hypothermia, nauseas and vomiting. Finally, at BACs higher than 400 mg/dl respiratory depression, coma and even dead can happen (Vonghia et al. 2008).

The relaxation effect induced by alcohol may have a critical role in motivating its ingestion (Ciccocioppo et al. 2006), at least in those individuals susceptible to this anxiolytic action. Alcohol consumption reduces anxiety-like behaviour, which reinforces alcohol consumption promoting future intake. Thus, alcohol consumption can be used as self-medication against anxiety symptoms. Conversely, repeated alcohol intake may induce anxiety-like behaviour in the long-term, as will be explained in the next point.

2.1.4.2 Long-term effects

Consuming huge amounts of ethanol has severe long-term adverse effects, including the increased risk of developing alcohol dependence in adulthood following binge drinking during adolescence (Gilpin et al. 2012). Although almost all body organs are affected by alcohol, the most notable effects are in the liver, pancreas, and brain (Ahmed, 1995; Crews and Nixon, 2009; Guerri and Pascual, 2010). It has been demonstrated that ethanol-induced brain damage occurs during intoxication (Crews et al. 2004). Thus, concerns have emerged since adolescents consume alcohol at a pattern of binge drinking that leads to intoxication. Indeed,

there is evidence that adolescent brain is especially vulnerable to alcohol toxicity and may induce neuropsychiatric disorders (Pagey et al. 2010), cognitive deficits and behavioural alterations (Paus et al. 2008).

Neurobehavioural and neuroimaging studies indicate that PFC and hippocampus, which are two key regions for mediating memory processes; have particular vulnerability to the deleterious effects of alcohol. For instance, smaller hippocampal volumes have been observed in adolescents who began drinking at an earlier age when compared with those who began later (Nagel et al. 2005) or PFC abnormalities such as white matter differences in adolescents with alcohol use disorders (De Bellis et al 2005). Thus, teenage drinking is associated to cognitive deficits as well as poor academic achievements (Guerri and Pascual, 2010). The alcohol-induced brain damage is associated to the frequency of heavy drinking (Sullivan and Pfefferbaum, 2005). Indeed, alterations in the PFC may also lead to compulsive behaviour that predisposes the individual to alcohol abuse, impulsivity and dependence.

On the other hand, emotional-like behaviours are also affected by chronic and acute alcohol consumption. Increased quantities and repeated alcohol ingestion are associated to symptoms of anxiety and withdrawal (Cloninger, 1987; Kliethermes, 2005). It is believed that both anxiety and withdrawal are associated, meaning that repeated withdrawal episodes enhance anxiety and compulsive behaviour that contribute to the continuation of alcohol drinking and thus, increased negative affective state or negative reinforcement that may finally lead to alcohol addiction. Furthermore, depressive disorders are also associated to heavy drinking (Swendsen et al. 1998; Torrens et al. 2005; Boden and Fergusson, 2011), which is more frequent among women than men (Epstein et al. 2007).

As explained before, adolescence is a period with frequent emotional and cognitive changes; therefore, binge alcohol drinking can exacerbate these alterations which may induce major neuropsychiatric problems in adulthood.

2.1.5 Effects on experimental animals

2.1.5.1 Acute effects

Ethanol-induced acute effects on experimental animals depend on the dosage or BAC but also on the age of the experimental animal (adolescence vs. adulthood). Adult animals are more vulnerable than adolescents to the acute effects of ethanol. As for humans, acute effects of ethanol on rodents are induced mainly by its action on the GABAergic and the glutamatergic neurotransmitter systems and include anxiolysis, sedation, hypnotic effects, motor impairments, pro-aggressive action and impaired cognitive function (Silveri and Spear, 1998; Markwiese et al. 1998; White et al. 2002; 2003).

2.1.5.2 Long-term effects

Contrary to what occurs with the acute effects, adolescent experimental animals seem to be more vulnerable to the ethanol long-term effects. Several studies have demonstrated that adolescent ethanol exposure negatively impacts immature brain, particularly on the PFC and hippocampus; leading to cognitive deficits, including learning and memory dysfunctions (Markwiese et al. 1998; Spanagel, 2009). However, these deficits seem to depend on the dose administered, meaning that a low dose of ethanol (i.e. 0.5 g/kg) did not induce memory impairments whereas a higher dose (i.e. 2.5 mg/kg) did so (Acheson et al. 2001). Others simply have found that adolescent rodents learn more slowly than adults after ethanol exposure (Chin et al. 2010). In that sense, it was suggested that ethanol-induced inhibition of

responses to NMDA receptor activation may contribute to the neural and cognitive impairments associated with intoxication (Lovinger et al. 1989). In accordance, an *in vitro* study showed that ethanol inhibits the induction of long-term potentiation more potently in adolescent than in mature hippocampal slices (Markwiese et al. 1998).

Anxiety-like behaviour has been observed during ethanol withdrawal in rats and mice (Finn et al. 2000; Sparta et al. 2007). An early study pointed that elevated levels of CRF in the central nucleus of the amygdala might be involved in this process (Rassnick et al. 1993) as well as deficits in different neurotransmitter systems such as decreased levels of GABA, DA and 5-HT in different limbic areas (Díaz et al. 2011; Weiss et al. 1996; Diana et al. 1993).

Nowadays it is known that adolescent alcohol intoxication leads to neurotoxicity and neurodegeneration in certain brain areas and cell populations such as hippocampus, PFC, cerebellum, white matter and glial cells (Crews et al. 2000; Pascual et al. 2007). Recent studies have suggested that alcohol induces brain injury and neurodegeneration through inflammatory and oxidative stress mechanisms (Montoliu et al. 1995; Blanco et al. 2005; Alfonso-Loeches et al. 2010) (see points 2.3 and 2.4). These brain changes may induce neurobehavioural alterations and cognitive deficits which can be maintained into adulthood (Pascual et al. 2007).

2.2 3, 4- Methylenedioxymethamphetamine (MDMA)

2.2.1 History of MDMA

3, 4- Methylenedioxymethamphetamine, more commonly known as MDMA or ecstasy, was firstly synthesized and patented in 1914 in Germany as a precursor for therapeutically active compounds (Steele et al. 1994). Around 1980s was used for

the first time in the clinics as an adjuvant to psychiatric treatment by Alexander Shulgin, “the stepfather of ecstasy”, and Leo Zeff (Parrott, 2007). MDMA was believed to increase patient self-esteem and facilitate therapeutic communication. It was administered orally at a dose of 75 - 175 mg which also produced acute sympathomimetic effects (Greer and Strassman, 1985). Meanwhile, the United Kingdom classified MDMA as a class A schedule 1 drug, meaning illegal to possess, sell or give away. Given that in the United States of America it became popular in the streets as a recreational drug (Pentney, 2001), the United States of America SA Drug Enforcement Administration classified MDMA as a schedule 1 drug due to its high abuse potential, lack of clinical application, lack of accepted safety for use under medical supervision and evidence that it could be neurotoxic. At the present time, the unique use of MDMA is as illicit recreational drug of abuse (Capela et al. 2009) widely consumed in raves or dance clubs by adolescents and young adults (Landry et al. 2002; Lyles and Cadet, 2003).

It is structurally related to the hallucinogenic compound mescaline and to the psychoactive amphetamine (Capela et al. 2009) (Figure 9). These similarities are responsible of its unique profile that is both, a hallucinogen and a psychostimulant. For that, Nichols (1986) proposed the term “entactogen” (psychoactive drugs with emotional and social effects) to describe the effects of MDMA.

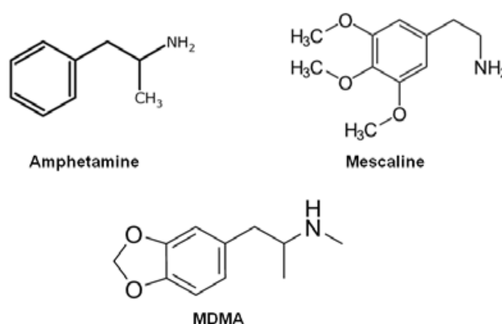

Figure 9. MDMA and related compounds mescaline and amphetamine.

2.2.2 Epidemiology

As estimated in the United Nations, the number of "MDMA-group" users ranges between 10.5 and 25.8 million people worldwide, i.e. 0.2% to 0.6% of the population between the ages of 15 and 64 (United Nations Office on Drugs and Crime, 2010). The latest data released by the European Monitoring Centre for Drugs and Drug Addiction (2011) revealed that 3.2% of Europeans have tried MDMA once but the percentage is higher (5.5%) among adolescents and young adults. The latest survey performed in Spain indicated a decrease in the MDMA use among Spanish adolescents placing such consumption at 2% (ESTUDES, 2010).

2.2.3 Pharmacology of MDMA

MDMA is a drug of abuse with a specific mechanism of action different from classical psychostimulants. In particular, MDMA induces neurotransmitter activation across several neural pathways including 5-HT, DA, norepinephrine (NE) and others (Green et al. 2003; de la Torre et al. 2004; Gudelsky and Yamamoto, 2008). In particular, MDMA binds to the plasma membrane 5-HT transporter (SERT), DA transporter (DAT) and NE transporter (NET), with higher affinity for SERT vs. DAT and less for NET. In consequence, neurotransmitter transport is inhibited and reversed, resulting in a high release of monoamines from the cytoplasm to the synaptic cleft in multiple brain regions. MDMA has a particular characteristic which is its direct monoaminergic stimulation in the NAc inducing the rewarding properties (White et al. 1994) (Figure 10). In accordance with its affinity, MDMA causes greater release of 5-HT than DA and NE (Crespi et al. 1997; Yamamoto et al. 2010).

Specifically, MDMA-induced 5-HT release occurs by two mechanisms: (1) transmitter molecules exit the cell along their concentration gradients via reversal of

normal SERT function and (2) cytoplasmic concentrations of transmitter are increased due to drug-induced disruption of vesicular storage (Wichems et al. 1995; Gudelsky and Nash, 1996; Baumann et al. 2005). In addition, it seems that MDMA is a substrate for vesicular monoamine transporter, as well. Thus, it enters the vesicles via this transporter and depletes vesicular neurotransmitter storage by reversal of transporter activity (Partilla et al. 2006).

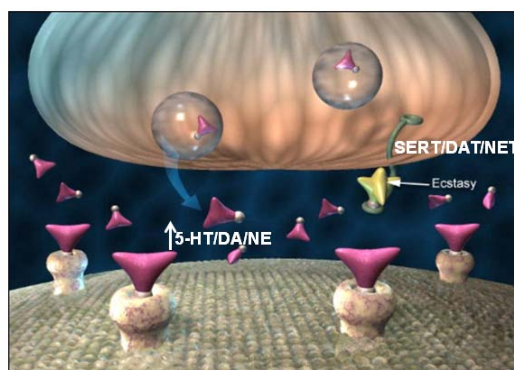

Figure 10. Mechanism of MDMA induced reward. MDMA directly stimulates monoamine release in the NAcc producing its rewarding properties.

Furthermore, other neurotransmitters have been reported to be affected by MDMA such as glutamate (Nash and Yamamoto, 1992), GABA (Bankson and Yamamoto, 2004) or acetylcholine (Fischer et al. 2000). Additionally, it has been described that MDMA has an action on 5-HT_{2A} receptor as well as on α_2-adrengergic, histamine 1 and muscarinic 1 receptor; and with less affinity on muscarinic 2, α_1-adrengergic, β-adrenergic and 5-HT_1 receptors (Battaglia et al. 1988).

2.2.4 Effects on humans

2.2.4.1 Acute effects

In humans, the most outstanding acute effects are mood-enhancement, increased energy, empathy and euphoria (Hall and Henry, 2006; Kolbrich et al. 2008). The subjective mood effects induced by MDMA are what make this substance such a popular drug of abuse among young people. In some cases, it has also been reported hallucinogenic effects (Capela et al. 2009). Alternatively, it also may produce a number of side-effects such as confusion, defensiveness, mental fatigue and anxiety as well as unhealthy medical consequences, including disruption of thermoregulation, tachycardia, hypertension, seizures and intracranial haemorrhage due to its sympathomimetic properties as psychostimulant (Green et al. 2003; Baylen and Rosenberg, 2006; Kolbrich et al. 2008).

A special focus in the MDMA-induced disruption of thermoregulation is needed as it represents a clinically relevant aspect in MDMA abusers. It has been reported that body temperature after MDMA administration can reach up to 43 ºC, which can be fatal for human organism. The environment in which MDMA is consumed is crowded conditions with high ambient temperature, physical activity and dehydration, which may contribute to an increase of the MDMA-induced hyperthermia (Capela et al. 2009). Other effects such as peripheral vasoconstriction, increased muscle tone, heat production, loss of body signals perception such as thirst or exhaustion, also contribute to the MDMA-mediated hyperthermia described in human abusers that may cause fatalities, including death (Rogers et al. 2009). In addition to the thermal increase, another relevant effect of MDMA consumption during dance clubbing is the increase in saliva cortisol up to 800% (Parrott, 2009). Cortisol is important for normal metabolic activation and for energy mobilization under conditions of high energy demand (Seyle, 1956). It is

hypothesized to have an important role in the bio-energetic stress model of MDMA, which consists in the multiple drug and non-drug influences, such as environmental and physical factors, that contribute to the psychobiological effects of MDMA (Parrott, 2006; 2009).

2.2.4.2 Long-term effects

The main MDMA-induced long-term effect on humans is neurotoxicity. There is evidence from positron emission tomography and single photon emission computed tomography scans to suggest that MDMA may reduce brain SERT densities in humans (Semple et al. 1999; Cowan et al. 2008; Kish et al. 2010). In the late 1990s the first human neuroimaging study of abstinent MDMA users was developed. Results obtained indicated reduced serotonergic markers, with dopaminergic markers unchanged, suggesting a MDMA-induced serotonergic neurotoxicity in humans (Reneman et al. 2006). Other research groups found similar results some years later, even using different techniques. In particular, Erritzoe and colleagues (2011) reported significantly lower SERT binding potential in the neocortex, pallidostriatum and amygdala correlated with lifetime MDMA usage (Erritzoe et al. 2011). Indeed, it was reported a significant reduction in hippocampal volume (den Hollander et al. 2012).

In addition, the long-term use of this psychostimulant may produce a number of neuropsychiatric disorders, including impaired impulse control, neuroticism and psychotic episodes (Morgan et al. 2002), the majority of them having a serotonergic component (Reneman et al. 2006; Kish et al. 2010). Furthermore, there are numerous studies showing memory deficits in ecstasy users. Parrott (2006) reviewed a huge amount of empirical papers revealing deficits in retrospective memory, prospective memory, complex cognitive processing, problem solving and social intelligence (Parrott, 2006), sometimes correlated to the 5-HT deficits (Green

et al. 2003; Baylen and Rosenberg, 2006). In line with these data, an Internet survey of 282 ecstasy users showed that 73% of heavy users reported memory problems, as well as anxiety (60%) and depression (65%) associated with MDMA repeated use throughout their life. It is noteworthy that the long-term effects of MDMA consumption depend on lifetime usage, intensity of use and drug co-abuse. Indeed, they seem not to be possible to reverse after prolonged abstinence, suggesting the existence of a severe neurotoxic injury (Parrott, 2006).

2.2.5 Effects on experimental animals

2.2.5.1 Acute effects

A large number of animal studies, namely on mice, rats and non-human primates, have been developed to elucidate the effects of MDMA in humans. There are many interspecies similarities and differences in terms of physiological, behavioural and toxicological effects of MDMA based on both pharmacokinetics and pharmacodynamics (de la Torre and Farré, 2004). For instance, MDMA-induced hyperthermia is observed in mice (Fantegrossi et al. 2003), rats (Brown and Kiyatkin, 2004), non-human primates (Taffe et al. 2006) and, as mentioned before, in humans (Green et al. 2003; Freedman et al. 2005). The mechanisms of MDMA-induced hyperthermia are multifactorial since a combination of serotonergic, dopaminergic and also adrenergic function is involved (Capela et al. 2009), however the exact mechanism remains unclear (see point 2.2.6).

Another remarkable acute effect observed in experimental animals is the MDMA-induced hyperactivity, which would be related to the increased physical and emotional energy reported in humans (McNamara et al. 1995; Touriño et al. 2008). 5-HT and DA components may be involved in the hyperactivity induced by MDMA (Gold and Koob, 1988; Daniela et al. 2004). However, in non-human primates,

specifically in rhesus monkeys, MDMA administered at doses similar to human recreational uses did not stimulate significant locomotor activity under normal laboratory housing conditions (Taffe et al. 2006). As described for humans, cardiovascular effects such as tachycardia, arrhythmia and enhanced vasoconstriction have been reported in rodents (Gordon et al. 1991; Fitzgerald et al. 1994; Baylen and Rosenberg, 2006).

Regarding emotional-like behaviour is reported that MDMA induces anxiogenic effects in both mice and rats (Sumnall et al. 2004; Touriño et al. 2008; Ferraz-de-Paula et al. 2011). These anxiety-related behaviours seem to be dependent on the dose, frequency of administration and the behavioural test used. Despite most studies point to the anxiogenic effect of MDMA, there are few reports describing “anxiolytic-like” effects of this particular psychostimulant. Their suggestion is that MDMA may exert a dual effect on anxiety, meaning that is capable of activating both the excitatory and inhibitory neural mechanisms that control anxiety (Lin et al. 1999; Navarro and Maldonado, 2002; Ho et al. 2004; Daza-Losada et al. 2009).

In rodents, it has also been reported alterations on the immune function associated with MDMA administration (Connor, 2004). In addition, immunological alterations are accompanied by a significant increase in plasma CORT concentration 30 minutes post-MDMA administration (Connor et al. 1998). These findings indicate that MDMA administration leads to an increase in the susceptibility to infectious diseases.

2.2.5.2 Long-term effects

As described for humans, the main long-term effect of MDMA described in experimental animals is neurotoxicity (Capela et al. 2009; Sarkar and Schmued, 2010). There are many published works showing that single or multiple doses of

MDMA to mice, rats or non-human primates result in neurotoxicity to the serotonergic or dopaminergic systems, depending on the species. MDMA induces serotonergic neurotoxicity in rats, monkeys and humans, whereas in mice produces dopaminergic neurotoxicity (Lyles and Cadet, 2003; de la Torre and Farré, 2004). Particularly, damage to 5-HT axons in primates and rats and to DA axons in mice have been observed (Lyvers, 2006); but also a broader neuronal degeneration throughout several brain areas such as the cortex, hippocampus and striatum that may lead to the MDMA-induced behavioural and cognitive alterations reported. Even though the exact mechanism of the MDMA-induced neurotoxicity remains unclear, nowadays the involvement of inflammatory processes and oxidative stress are well-known (Orio et al. 2004; 2010; Busceti et al. 2008; Alves et al. 2009) (see points 2.3 and 2.4).

MDMA-induced cognitive impairments have also been studied in experimental animals. Different models of MDMA administrations (acute, binge, chronic) on learning and memory tasks have been developed to this purpose but results are inconsistent. Reference memory impairment but not working memory alterations (Kay et al. 2011) in rats and modified active avoidance learning and recall in mice (Trigo et al. 2008) due to MDMA have been reported. However, authors did not find correlation between memory deficits and DA neurotoxicity, suggesting that other mechanisms may be involved in the MDMA-induced cognitive alterations. Contrary, some research groups have found no impairment in the passive avoidance task in MDMA-treated mice (Murnane et al. 2012) or in the radial arm maze (Hernández-Rabaza et al. 2010), who only reported memory impairment when MDMA was co-administered with other drugs of abuse, i.e. ethanol.

2.2.6 The role of hyperthermia

Several reports indicate a link between MDMA-induced hyperthermia and neurotoxicity in rats and mice (Malberg and Seiden, 1998; Green et al. 2004; Touriño et al. 2010). In rats, it has been observed that higher ambient room temperature conditions result in a hyperthermic response to MDMA, while lower ambient temperatures result in a hypothermic response (Dafters, 1994; Broening et al. 1995; Malberg and Seiden, 1998). Accordingly, significant long-term 5-HT depletion was found in MDMA-treated rats under high environment temperatures but no significant depletions were found when MDMA was administered at ambient temperature (Malberg and Seiden, 1998). A pattern of binge dosing produced increase in rat body temperature at ambient temperature and the increase was higher when administered at elevated ambient temperatures. 5-HT neurotoxicity was potentiated in the hot environment, as well (Green et al. 2004; Sánchez et al. 2004). Similarly, MDMA-induced hyperthermia may contribute to the enhancement of free radical formation in the brain (Colado et al. 1997). In mice, similar body temperature responses to MDMA treatment have been observed. However, at room temperature (20 - 22ºC) these changes seem to be more variable and depend on the mice strain used for the study (Green et al. 2003). For instance, in C57BL/6J female mice a high dose of MDMA (20 mg/kg, 4 times per day) caused an elevation of body temperature of approximately 2ºC (Miller and O'Callaghan, 1994; Johnson et al. 2002a). While in BALB/c mice a lower dose of MDMA (5 - 10 mg/kg 4 times per day) produced a hypothermic response (Johnson et al. 2002b), in CD1 mice a single dose of 5, 10 or 20 mg/kg produced an increase in body temperature (Carvalho et al. 2002; Touriño et al. 2008). Despite these contradictory results, hyperthermia is the most established response after acute MDMA administration. Even though hyperthermia can potentiate the MDMA-induced neurotoxicity, in any case is an essential factor since not all agents that prevent the neurotoxic effects do so by blocking the hyperthermic response (Malberg et al. 1996; Green et al. 2004).

There are several hypotheses for the mechanisms by which MDMA induces hyperthermia in rodents. The first hypothesis is that MDMA may interfere with heat loss mechanisms such as vasodilation of tail vessels (Mechan et al. 2002). A second possibility is that MDMA may increase metabolic rate and evaporative water loss (Gordon et al. 1991). At a molecular level, many investigations have focused on the role of 5-HT and DA on MDMA-induced hyperthermia in rats (Mechan et al. 2002). In mice, the mechanism by which MDMA produced hyperthermia seems not to be related with the acute dopaminergic effects of the drug since pre-treatment with a specific DA uptake inhibitor failed to prevent MDMA-induced hyperthermic response in mice (O'Shea et al. 2001). At the moment, the exact mechanism of the MDMA-induced hyperthermia in rats and mice remains unclear.

2.3 The role of neuroinflammation in the neurotoxicity induced by ethanol and MDMA

In the last years neuroinflammation has been associated to processes of neurodegeneration triggered by drugs of abuse, chemicals, trauma or disease (Block and Hong, 2005; Alfonso-Loeches et al. 2010; Touriño et al. 2010). Traditionally, inflammation involves the synthesis/release of proinflammatory mediators such as cytokines and chemokines. More recently, the concept of inflammation has been extended to the role of activated microglia and astroglia, known as gliosis, that is, the brain glial cells known to serve as both source and targets of proinflammatory mediators (O'Callaghan et al. 2008). Microglia is responsible for the innate immune response in the brain. Its function is to perform general maintenance and clean cellular debris (Beyer et al. 2000). Activated microglia undergoes morphologic alterations changing from resting ramified microglia into activated amoeboid microglia (Kreutzberg, 1996). On the other hand, in the normal brain astroglia plays an essential role in providing glia-neuron contact, maintaining ionic homeostasis, buffering excess neurotransmitters, secreting neurotrophic factors and serving as a

critical component of the blood-brain barrier. Although the proinflammatory function of astroglia is not as prominent as that of microglia, activated astroglia in response to brain injuries, become hypertrophic, exhibit increased glial fibrillary acidic protein (GFAP), and form glial scars which hinder axonal regeneration (Block and Hong et al. 2005).

Astrogliosis and microgliosis activate signalling pathways and promote the release of reactive oxygen species (ROS), pro-inflammatory mediators and cytokines including interleukins and tumor necrosis factor alpha. An increase in intracellular ROS occurs in microglia as a response to nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) activation that also enhances the production of neurotoxic pro-inflammatory factors (Figure 11). All these inflammatory events promote neurotoxicity and even cell death.

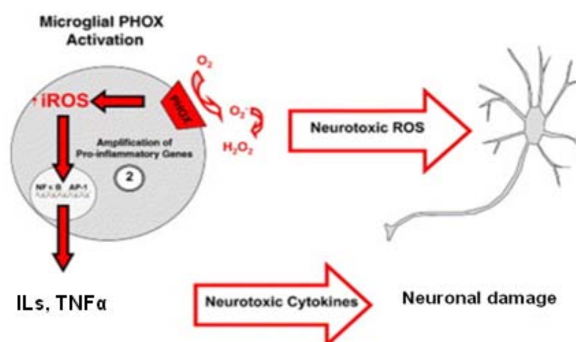

Figure 11. NADPH oxidase (PHOX) activation is the common mechanism by which microglia is toxic to neurons (modified from Block and Hong, 2005). (**ILs**, interleukins; **TNFα**, tumor necrosis factor alpha).

2.3.1 Methods of detection of microglia and astroglia

As described above, reactive astrocytes exhibit increased GFAP, which is a class-III intermediate filament. It serves as a cell specific marker that distinguishes differentiated astrocytes from other glial cells that can be determined using

antibodies against GFAP. On the other hand, microglia expression can be determined using antibodies against the ionized calcium binding adaptor molecule 1 (Iba-1) that is a 17-kDa helix-loop-helix structural domain from a family of calcium-binding proteins specifically expressed in macrophages/microglia. It is upregulated during the activation of these cells and thus, it serves as cell specific marker to determine microglia activation occurring during neuroinflammatory processes. Both can be easily detected mediating immunohistochemistry or immunofluorescence.

2.3.2 Ethanol-induced neuroinflammation

It has been observed that alcohol induces activation of glial cells such as microglia and astrocytes. It has been postulated that ethanol exerts this neuroinflammatory damage through activation of toll-like receptors, in particular toll-like receptor 4, but also through interleukin-1 receptor. Stimulation of both receptors by ethanol in glial cells induces the activation of signalling pathways that lead to production of cytokines, inflammatory mediators and subsequent neural damage (Blanco and Guerri, 2007; Guerri and Pascual, 2010) (Figure 12). Recently, it has been demonstrated that elimination of toll-like receptor 4 protects against activation of glial cells and the production of cytokines and inflammatory mediators as well as against apoptosis induced by alcohol administration (Alfonso-Loeches et al. 2010).

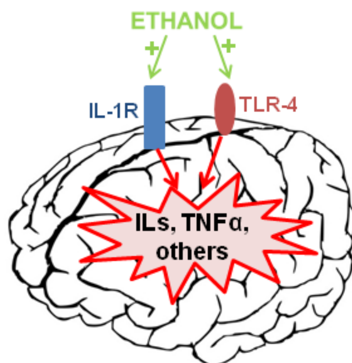

Figure 12. Mechanism of ethanol-induced brain damage by neuroinflammatory processes. (**IL-1R**, interleukin-1 receptor; **TLR-4**, toll-like receptor 4, **ILs**, interleuikins, **TNFα**, tumor necrosis factor alpha).

2.3.3 MDMA-induced neuroinflammation

Few works have reported MDMA-induced microglial activation (Thomas et al. 2004; Touriño et al. 2010; Ruiz-Medina et al. 2011). Moreover, levels of proinflammatory cytokines have been observed after toxic amphetamine treatment (Orio et al. 2004). In addition, treatment with minocycline, an inhibitor of microglial activation, prevents MDMA-induced serotonergic and dopaminergic neurotoxicity in the PFC, striatum and hippocampus (Zhang et al. 2006; Orio et al. 2010). These data indicate that a microglial inflammatory response contributes to the neurotoxic actions of MDMA in these brain regions. MDMA-induced microglial activation is usually, but not always, accompanied by astrogliosis (Miller and O'Callaghan, 1994).

2.4 The role of oxidative stress in the neurotoxicity induced by ethanol and MDMA

Metabolism of the vast majority of cells depends on oxygen. Nevertheless, all aerobic organisms are subjected to the known "oxygen paradox" since their survival depends on it during cellular respiration. At the same time, oxygen results in toxic compounds the well-known “reactive oxygen species” -ROS-. Oxidative stress takes place when the concentration of these oxidant compounds is higher than the production and/or action of the antioxidants in the cell. The term ROS encompasses the superoxide anion (O_2^-), the hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). These three chemicals are characterized by a high level of oxidation and a particular reactivity that determines its specific oxidation targets (Giorgio et al. 2007). Thus, these molecules can damage neuronal specific proteins and lipids leading to alterations in important cellular functions (Montoliu et al. 1995; Ramachandran et al. 2003). It is known that proteins are the main targets of this damage which leads to alteration of their structure causing the total or partial loss of its function (Levine et al. 1994). Inactivation of specific proteins leads to alterations in specific metabolic pathways that can induce physiological consequences (Martínez et al. 2010). Protein oxidative damage can be reversible or irreversible. Reversible modifications, such as cysteine or methionine oxidations, can be repaired while proteins that suffer irreversible modifications can either accumulate or be degraded. One of the major irreversible modifications is carbonylation, which is known to be one of the most important causes of brain protein damage and dysfunction (Berlett and Stadtman, 1997). H_2O_2 and O_2^- are involved in this process of carbonylation, which is a metal-ion-catalyzed (Fe^{2+}, Fe^{3+}) reaction, known as Fenton reaction (Figure 13).

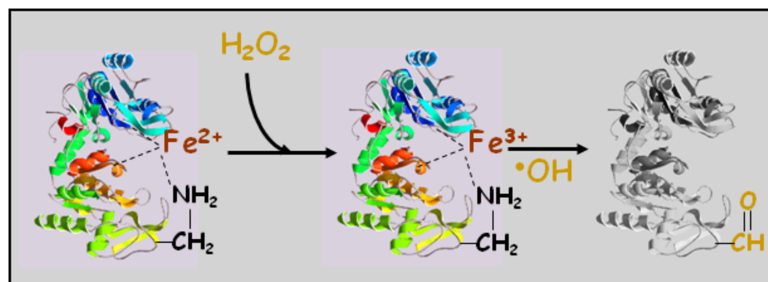

Figure 13. Fenton reaction. Protein carbonyl formation induced by oxidative stress resulting in protein dysfunction.

It is worth mentioning that the developing brain is particularly vulnerable to oxidative stress mediated damage since it has high oxygen consumption, lower anti-oxidant concentrations and high content of metals (Alfonso-Loeches and Guerri, 2011).

2.4.1 Methods of detection and quantification of protein carbonylation

Protein carbonyl formation is one of the most studied markers resulting from oxidative stress that can be easily measured by detecting carbonyl formation on amino acid side-chains (Levine et al. 1994; Levine and Stadtman, 2001; Tamarit et al. 2012). Detection can be achieved by carbonyl derivatization with several compounds containing a hydrazide group, which after forming a Schiff base result in a hydrazone that can be stabilized by reduction with cyanoborohydride. Analyses of protein carbonylation can be performed by 2-dimensional gel electrophoresis of proteins derivatized with fluorescent probes such as Bodipy-hydrazide (Tamarit et al. 2012) (Figure 14).

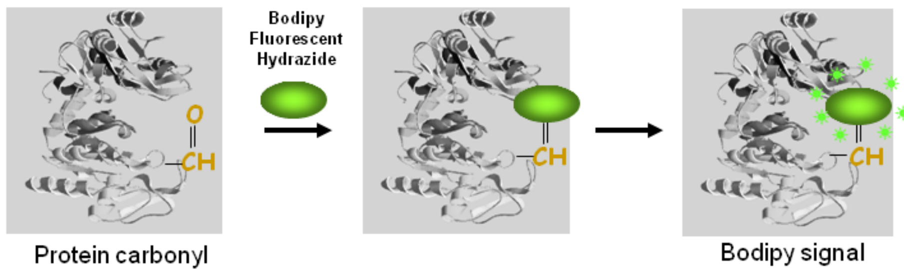

Figure 14. Protein carbonyl detection by derivatization with fluorescent hydrazides.

It is well established that both ethanol and MDMA can increase the formation of ROS by different mechanisms which will be described in the following points.

2.4.2 Ethanol-induced oxidative stress

Ethanol-induced oxidative stress can be generated by different mechanisms. Firstly, via direct activation of the multimeric enzyme complex I NADPH oxidase of the mitochondrial respiratory chain (Figure 15).

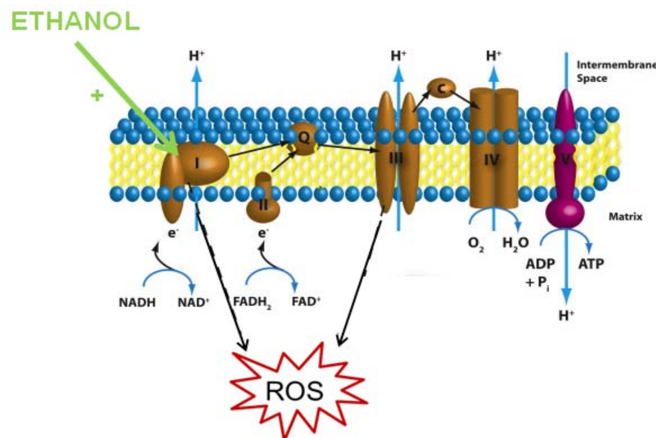

Figure 15. Ethanol-induced activation of multimeric complex I (NADPH oxidase).

A second mechanism of ethanol-induced oxidative stress is via its specific metabolism by enzymes such as cytochrome P-450 (CYP) 2E1 and alcohol

dehydrogenase to generate hydroxyethyl radicals (Ingelman-Sundberg and Johansson, 1984) and acetaldehyde, hypothesized to have toxic properties (Deitrich et al. 2006; Sarc et al. 2011). Acetaldehyde is then further oxidized to acetate by an acetaldehyde dehydrogenase (Figure 16). Indeed, alcohol may increase CYP2E1 expression and thus, the formation of ROS.

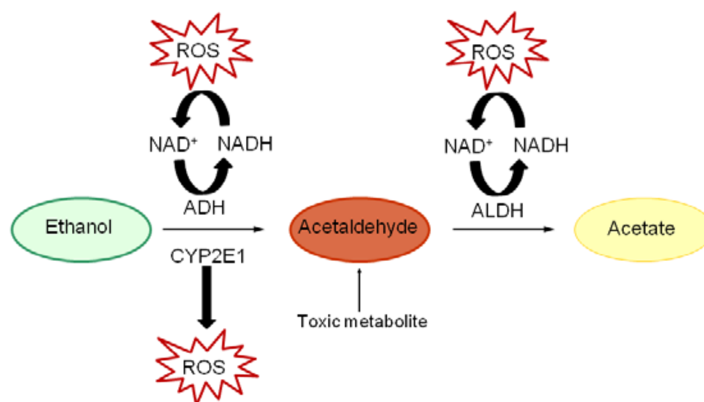

Figure 16. Ethanol metabolism by CYP2E1, alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH). Induction of oxidative stress.

Even though this specific ethanol metabolism takes place mainly in the liver, it has also been demonstrated to occur in the brain (Haorah et al. 2008).

Additionally, alcohol can reduce endogenous antioxidant levels such as glutathione and superoxide dismutase contributing to an increase in the oxidative damage (Bai et al. 2006). In accordance with the oxidative stress hypothesis, vitamin E known to have antioxidant properties has been shown to protect against ethanol-induced oxidative response (Marino et al. 2004).

Activation of microglial cells occurring after ethanol administration can also promote the release of ROS and oxidative damage, as explained before (see point 2.3).

2.4.3 MDMA-induced oxidative stress

The induction of oxidative stress by MDMA can be mediated by different mechanisms. MDMA metabolism itself leads to the production of several highly reactive metabolites, known as quinones intermediaries (Figure 17). It is hypothesized that these metabolites are responsible for the MDMA-induced neurotoxicity since several studies failed to demonstrate any neurotoxic response after direct MDMA injection into the rat brain (Esteban et al. 2001; Green et al. 2003).

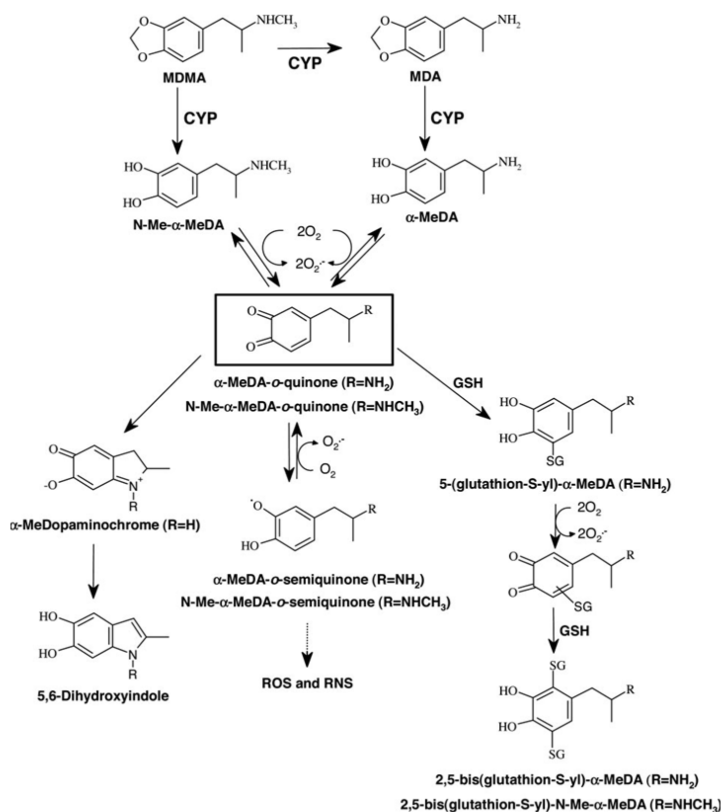

Figure 17. Toxic derivatives from MDMA metabolism, such as quinones, that can enter the redox cycling generating ROS and reactive nitrogen species (RNS). (from Capela et al. 2009).

Quinones are highly redox active molecules that can generate ROS and RNS through redox cycling (Figure 18). Additionally, reactive quinones can form adducts with glutathione (GSH) and other thiol-containing compounds that also have the ability to redox cycling, and they can be transported into the brain across the blood-brain barrier inducing brain damage (Capela et al. 2007).

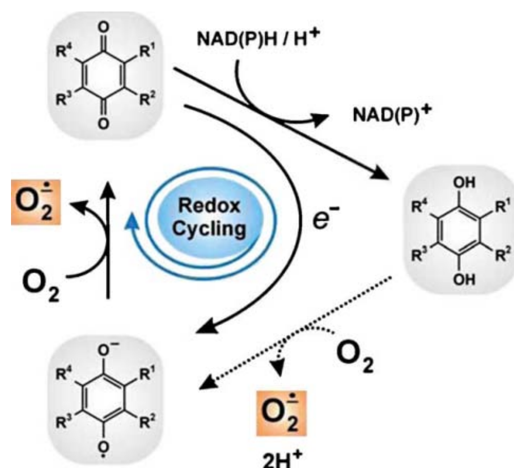

Figure 18. Redox cycling of quinones. Quinones may be reduced intracellularly to their corresponding semiquinone via quinone oxidoreductase-1, which then can react with molecular oxygen generating superoxide.

Several studies support a role for DA in the MDMA-induced neurotoxicity in rats and mice. This DA-induced neurotoxicity in mice seems to be related to the fact that DA, as a catecholamine, can be chemically oxidized. Thus, the excessive brain DA release after MDMA administration can be oxidized by enzymatic or metal catalysis in the presence of O_2^-. DA is a catecholamine easily oxidized and produces DA quinones which, in the presence of GSH, may form glutathionyl conjugates. They, in turn, induce redox cycling promoting the formation of free radicals, as explained in the previous point (Figure 18). Additionally, this event not only happens with DA since NE, another catecholamine, is also released in animal brain after MDMA administration. Oxidation of both catecholamines may promote

oxidative stress that leads to neurodegeneration. Thus, DA and NE certainly contribute to the increase of ROS and subsequent neurodegeneration after MDMA administration (Colado et al. 1997; Shankaran et al. 1999; Capela et al. 2009).

Another source of ROS is via monoamine oxidase (MAO) which is an enzyme found in the outer mitochondrial membrane that catalyses the oxidation of monoamines such as DA, 5-HT, NE and adrenaline (Hrometz et al. 2004). MAO-mediated metabolism of DA and 5-HT leads to the generation of reactive intermediates, such as 3, 4-dihydroxyphenylacetaldehyde and 5-hydroxyindole-3-acetaldehyde, respectively. Afterwards, these reactive products are converted to more stable metabolites, 3, 4-dihydroxyphenylacetic acid and hydroxyindole-3-acetic acid by an aldehyde dehydrogenase. This process induces the production of H_2O_2, as a metabolic by-product which then promotes an increase in the highly toxic OH^-, for instance, via the Fenton reaction previously described.

As stated above MDMA-induced activation of microglial cells can also induce an increase in ROS and oxidative damage (see point 2.3).

2.5 Ethanol binge drinking and MDMA combination

2.5.1 Epidemiology

A pattern of heavy ethanol binge drinking in combination with MDMA is very popular and widely abused by adolescents and young adults (Winstock et al. 2001; Barrett et al. 2006). The European Monitoring Centre for Drugs and Drug Addiction (2011) reported that the frequent alcohol users had a prevalence of ecstasy use much higher than the average population. In a recent survey 86% of students aged between 15 and 16 years who had used ecstasy in the last month, also had consumed five or more drinks on occasion, that is, a pattern of binge drinking.

In Spain, the 98.5% of students between 14 and 18 years that reported consuming MDMA in the last year was in combination with alcohol (ESTUDES, 2010). Such combination presumably enhances the subjective effects of each drug both, pharmacodynamically and pharmacokinetically (Hernández-López et al. 2002; Schifano, 2004; Oesterheld et al. 2004).

2.5.2 Previous studies

2.5.2.1 Human studies

There are few studies addressing the interaction between MDMA and ethanol in humans. Pacifici et al. (2001) focused on the effects of such combination on the immune system. Their findings were that the combination of both drugs caused highest immunosuppressive effects that either each drug alone. It was a transient effect since 24 hours later immune function showed a trend toward baseline levels. However, if repeated administrations, this transient defect into immunological homeostasis might compromise the immune response with a risk for the general health status. Hernández-López and colleagues (2002) studied the psychomotor performance and subjective effects after MDMA administration. They reported longer lasting euphoria and feelings of well-being with the combination compared to either drug alone, suggesting that potentiated effects can be perceived subjectively (Hernández-López et al. 2002). A third study by Dumont et al. (2008) evaluated the effects of single and co-administration of MDMA and ethanol on executive, memory and psychomotor functions. Their results were that co-administration of MDMA and ethanol did not exacerbate the effects of either drug alone. Impaired memory function was consistently observed after all drug conditions and moderate impairment of performance was observed. Only acute effects were evaluated in these studies. More recently, Dumont and co-workers (2010) evaluated cardiovascular function, temperature and hydration in healthy

volunteers throughout days after administration. They found that ethanol alone did not affect physiologic function, with the exception of a short lasting increase in heart rate. Contrary, MDMA potently increased heart rate and blood pressure and induced fluid retention as well as an increase in temperature. Combination of both drugs did not affect cardiovascular function compared to that of MDMA alone, but attenuated its effects on fluid retention and temperature increase. Thus, ethanol and MDMA in combination did not exacerbate physiologic effects compared to all other drug conditions, even moderated some effects of MDMA alone. The effects of MDMA plus ethanol on driving performance and laboratory tasks related to driving a placebo-controlled study were developed by Kuypers et al. (2006) and Ramaekers and Kuypers (2006). They found opposite effects between both drugs, meaning that MDMA reduced weaving in road tracking test and ethanol increased weaving and impaired brake reaction time. Moreover, MDMA seemed to ameliorate the ethanol-induced weaving increase.

2.5.2.2 Animal studies

Ethanol and MDMA interactions have been reported in animal models. Parameters such as acute responses, alterations on mood and cognition and neurotoxicity have been studied. However, results obtained are controversial and thus, further research is needed to clarify the effects of such combination. For instance, regarding body temperature, a physiological parameter widely studied when MDMA and ethanol administration is concerned; Cassel et al. (2004) reported an ethanol attenuation of the MDMA-induced hyperpyretic effect in mice while an exacerbation of the MDMA-induced hyperthermia by alcohol was described by Pontes et al. (2008). Johnson et al. (2004) described that the combination resulted in a decrease in body temperature higher than that observed after ethanol alone. Some authors have suggested a tolerance to the hyperthermic responses (Cassel et al. 2005).

At a behavioural level, ethanol administration potentiates MDMA-induced hyperlocomotion in rats (Cassel et al. 2004; Hamida et al. 2006; Riegert et al. 2008). Increased anxiety-like behaviour was observed after MDMA treatment with an enhancement of such effects when combined with a pattern of binge drinking in adolescent mice (Rodríguez-Arias et al. 2011). A few have studied possible memory deficits induced by the combination of ethanol and MDMA without consistent results. For instance, Hernández-Rabaza and colleagues (2010) only reported memory deficits in the radial arm maze when drugs were co-administered to rats. Differently, a recent study showed impaired spatial memory induced by the combination as well as either each drug of abuse separately (Vidal-Infer et al. 2012).

Neurotoxic processes of the combination of ethanol and MDMA have been studied and discrepant results have also been obtained. It is not clear whether ethanol exerts a protective or a deleterious effect on MDMA-induced neurotoxicity. According to Rodríguez-Arias et al. (2011) ethanol did not protect against MDMA-induced neurotoxicity; however, a neuroprotective role of ethanol was previously reported (Miller and O'Callaghan, 1994).

It is noteworthy, that treatment schedules, doses, times at which measurements are taken as well as specie, strain and age of rodent differ between studies. All of them are factors that may lead to the inconsistency of the reported results.

3. EARLY-LIFE SOCIAL EXPERIENCES

Early-life stressful experiences are associated to alterations in cognition, motivation and emotional behaviours (Levine, 1985; De Kloet et al. 2005). Individuals suffering an adverse environment in early-life have been shown to present health affectations along their lives (Lai and Huang, 2011). In contrast, positive social interactions may lead to a normal development of the before mentioned functions and protection against negative events in life. Home environmental conditions during childhood and adolescence are relevant for the development of a spectrum of important issues related to early-life experiences, such as neuronal development, behavioural responses, and potential future psychopathological states in humans (Penza et al. 2003; Carroll, 2003; McGowan and Szyf, 2010). For instance, loneliness during childhood and adolescence may be considered as a stressful experience while satisfying social relationships during these life periods are vital for good mental and physical health (Heinrich and Gullone, 2006).

3.1 Human evidences

Social interactions shape humans from early development through their lives and have a strong impact on many aspects of physiology and behaviour (Karelina and DeVries, 2011). Home environmental conditions during childhood as well as social interactions with peers are crucial for an appropriate growth. Social isolation during childhood is a sad, even painful emotional experience that may be considered as an early-life stressful event. Indeed, it is suggested that school environment has a major influence on adolescent mental health (Kidger et al. 2012), meaning that scholar relationships are associated with better emotional health.

It is hypothesized that the abovementioned emotional alterations are related to stress disorders. Thereupon, it seems evident the involvement of the HPA axis since

stressful situations can program the development of the HPA axis causing alterations on it (Lai and Huang, 2011). In accordance with this hypothesis, depressive and anxious symptoms in preadolescents and adolescents have been consistently linked to deregulations of the HPA axis (Oldehinkel and Bouma, 2011). It is noteworthy that the HPA axis is more sensitive during the adolescent stage; therefore, any event or environmental adversity that can be altering its function may increase the probability of emotional disorders in that individual future in life. In turn, the altered HPA axis function can cause alterations of neurochemistry and signalling pathways involved in regulating neuroplasticity and result in other neurobehavioural changes (Lai and Huang, 2011).

3.2 Experimental animal studies

During the preadolescence and adolescence periods, which start just after weaning (postnatal day 21), rodents spent more time in social interactions and at play (Primus and Kellogg, 1989). These interactions give them the opportunity to practice adult-typical behaviour patterns which proper the correct development to adulthood (Spear, 2000). There are several procedures to study the effects of early-life social experiences on adulthood behaviour and brain development in rodents and other experimental animals. In this sense, social isolation would be considered as a stressful condition with probably negative consequences into adulthood. In rats, social isolation after weaning was found to induce a variety of behavioural and neurochemical changes such as hyperactivity, ambivalent responses to novelty, altered response to stressors, and cognitive impairments when tested as adults compared to standard conditioned rats (Robbins et al. 1996; Naert et al. 2011).

Contrary, environmental enrichment (EE) is defined as a combination of complex inanimate and social stimulation (Rosenzweig et al. 1978). It has been shown to induce benefits on rodent social interactions, behaviour and neurodevelopment in

the sense that it may facilitate sensory, cognitive and motor stimulation (van Praag et al. 2000; Nithianantharajah and Hannan, 2006). Furthermore, enrichment in the early-life can protect against stress, which can be a vulnerability factor for the development of neurodegenerative and other CNS diseases (Laviola et al. 2008).

The procedure of EE consists on housing the animals in large cages containing a variety of sensory-motor stimulus, such as tunnels, running wheels and toys (Figure 19) that are routinely changed and removed.

Figure 19. EE and the effects of enhanced sensory, cognitive and motor stimulation (adapted from Nithianantharajah and Hannan, 2006).

Despite the beneficial effects of EE reported (Cummins et al. 1973; Rosenzweig et al. 1978; Rema et al. 1999), there are several studies reporting controversial behavioural responses after housing under enriched or isolated environment. For instance, even though most studies have shown enhanced locomotor activity after rearing under isolated conditions, there are some that have found decreased activity (Hall, 1998; Schrijver et al. 2002). Regarding anxiety-like behaviour, also contrary results have been found using the same paradigm (Roy et al. 2001). Similarly, differences in exploratory behaviour in individually housed mice when compared to standard conditioned have been reported depending on the mice strain used (Võikar et al. 2005; Zhu et al. 2009).

As stated above, the HPA axis seems to have an important role in the behavioural alterations induced by different housing conditions. Hormonal responses and changes in neuroendocrine status due to environmental conditions have been studied and, likewise, controversial results have been found. Some studies have found elevated basal CORT secretions in mice kept under EE (Marashi et al. 2003). Other studies have reported no differences in CORT levels in rats exposed to different environmental conditions (Schrijver et al. 2002) and some have even found a reduction of CORT in enriched adult rats (Belz et al. 2003). More recently, no differences in basal CORT during the light period between enriched and standard housed post-weaned rats have been observed (Peña et al. 2009). On the other hand, corticosteroids levels have been found to be higher, unchanged or lower in several studies employing individually housed rodents (Bartolomucci et al. 2003). For instance, Serra and colleagues (2005) found decreased levels of plasma ACTH as well as increased sensitivity of the pituitary to CRF associated to impaired negative feedback regulation of the HPA axis in isolated rats (Serra et al. 2005). Additionally, decreased levels of plasma CORT have been shown after isolation in rats (Miachon et al. 1993; Sánchez et al. 1995). Differently, no altered levels of plasma CORT neither plasma ACTH was found by Schrijver et al. (2002).

It is worth mentioning that the influence of housing conditions on animal behaviour seems to be dependent of the particular characteristics of the home cage as well as strain, gender and age of the experimental animal used (Peña et al. 2009; Silva et al. 2011). Wahlsten and coworkers (2003) reviewed behavioural data from different laboratories suggesting different laboratory environments probably can never be made sufficiently similar to guarantee identical results on a wide range of tests (Wahlsten et al. 2003).

OBJECTIVES

As stated along this introduction drug abuse and aberrant social experiences during the adolescent stage may induce neuropsychiatric abnormalities in adulthood as well as alterations in crucial neuronal pathways. Being aware of the importance of brain development, emotional behaviour and cognition during this period of life; we decided to start a project to study the influence of drug abuse and home environment in adolescent mice. Therefore, the **general objectives** of the present doctoral thesis are:

1. To elucidate the long-term behavioural alterations and neurotoxic effects of binge ethanol, acute MDMA or its combination in adolescent mice.

2. To evaluate the impact of social isolation and enriched environment during preadolescence and adolescence stages on adulthood behaviour and HPA axis activity in mice.

Specific objectives

1. To elucidate the long-term behavioural alterations and neurotoxic effects of binge ethanol, acute MDMA or its combination in adolescent mice

- To establish a procedure of voluntary binge drinking, called drinking in the dark (DID), adapted from Rhodes et al. (2005), in combination with an acute neurotoxic dose of MDMA in adolescent CD1 mice.
- To determine body temperature before and after the abovementioned drug treatment.
- To determine long-term behavioural and cognitive alterations of binge ethanol, acute MDMA or its combination. In particular locomotor activity,

motor coordination, anxiety-like and depressive-like behaviour, and declarative or explicit memory were evaluated.

- To determine markers of neuroinflammation as astrocyte reactivity and microglia activation induced by binge ethanol, acute MDMA or its combination.
- To quantify and identify proteins becoming oxidized by binge ethanol, MDMA or its combination in mice PFC and hippocampus in order to establish a possible link with the learning and memory deficits.
- To develop metabolomic studies in the striatum, PFC and hippocampus of mice treated with binge ethanol, acute MDMA or its combination.
- To evaluate the effect of two different antidepressant drugs (imipramine and escitalopram) in order to prevent the emotional-like behaviour induced by the combination of binge ethanol and acute MDMA.

2. To evaluate the impact of social isolation and enriched environment during preadolescence and adolescence stages on adulthood behaviour and HPA axis activity in mice

- To develop an improved model of home environmental conditions in CD1 mice in which we have compared the consequences of three different home environmental conditions maintained throughout development (from pre-adolescence to adulthood): social conditions (SC), enriched conditions (EC) and isolated conditions (IC).
- To determine the behavioural consequences into adulthood of different home and social environmental conditions. In particular, determination of locomotor activity, anxiety-like and depressive-like behaviour.
- To determine HPA axis reactivity by the determination of plasma CORT before (basal level) and after a stressful situation.

METHODOLOGY

1. DRINKING IN THE DARK PLUS ACUTE MDMA IN ADOLESCENT MICE

To evaluate the impact of drug abuse on adolescent mice, the drinking in the dark (DID) procedure was used. It is a procedure of ethanol binge firstly reported by Rhodes et al. (2005). Briefly, DID procedure consists of replacing food and water bottles for ethanol or water cylinders during 2 hours. After this period, food and water bottles are replaced again. This procedure is repeated for three days and fresh fluids are provided each day. On day 4, DID procedure lasts 4 hours. Volume (ml) of water or ethanol is registered every day for each animal individually.

In our study (**Article 1 and 2**), few modifications onn the original procedure have been included. Firstly, DID procedure was repeated for two weeks in order to achieve a higher ethanol ingestion. Secondly, on day 4 of the first week animals were injected a saline i.p. injection and on day 4 of the second week, before the ethanol or water exposure, animals were injected a single dose of MDMA (20 mg/kg) or saline (i.p.). After the first recording (2 hours), animals received a second injection of MDMA (20 mg/kg) or saline (i.p.). The neurotoxic dose of MDMA was selected in accordance to previous studies performed by our team (Touriño et al. 2010; Ruiz-Medina et al. 2011). Following the 4 free access hours to fluid and immediately after recording fluid intake, ethanol and water cylinders were replaced with water bottles. Rectal temperature was monitored during experimental procedure due to its involvement in the MDMA-induced neurotoxicity (Figure 20).

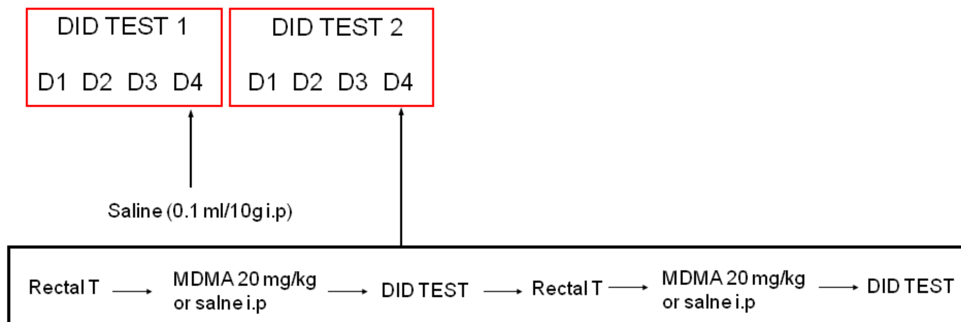

Figure 20. Experimental procedure

1.1 Behavioural paradigms

Different behavioural tests were conducted 48 hours, 72 hours and 7 days after treatment.

1.1.1 Locomotor activity

To evaluate locomotor activity, *locomotor activity boxes* were used (9 × 20 × 11 cm) (Imetronic, Lyon, France) as previously described (Soria et al. 2006). Horizontal and vertical movements were recorded (see materials and methods **Article 1**).

1.1.2 Motor coordination

Motor coordination was evaluated using an accelerating *rotarod apparatus* (five-line accelerating rotarod; LE 8200, Panlab) which provides an easy way to test the effects of drugs, brain damage, or diseases on motor coordination or fatigue resistance in rodents. Basically, the animal is placed on the roller lane of the rotarod and the timer is started. The time latency to fall (minutes and seconds) and rotation speed are automatically recorded (www.harvardapparatus.com). In our study, (see

materials and methods **Article 1**) animals were subjected to a session consisting of ten consecutive trials interleaved by 30-seconds breaks. The three last values of the session were averaged as described (Valverde et al. 2009).

1.1.3 Anxiety-like behaviour

Anxiety-like behaviour was evaluated in the dark-light box test as previously reported (Valverde et al. 2009). Mice were placed into the dark compartment and number of entries and percentage of time in the lit compartment were recorded for 5 minutes (see materials and methods **Article 1**).

1.1.4 Depressive-like or despair behaviour

Depressive-like or despair behaviour was assessed using the tail suspension test. Mice were individually suspended by adhesive tape 1 cm from the tip of the tail for a 6-minutes period as described by Steru et al. (1985) and adapted to our experimental conditions (Aso et al. 2008). The time of complete inactivity during this period was recorded (see materials and methods **Article 1**)

1.2 Evaluation of cognition

Cognition was evaluated using two different paradigms; the object recognition test and the radial arm maze test. The object recognition test was performed in a black rectangular open field. Radial arm maze was conducted in an eight-arm maze. In both tests memory consolidation was evaluated 72 hours after treatment (see materials and methods **Article 2**).

1.3 Neurotoxicity

1.3.1 Neuroinflammatory response

In order to assess the time-course neuroinflammatory response in mice striatum, presence of astrocyte reactivity and microglia was determined by immunofluorescence by using anti-GFAP and rabbit polyclonal anti-Iba1 staining, respectively (see materials and methods **Article 1**) and as previously described by our team (Touriño et al. 2010; Ruiz-Medina et al. 2011).

1.3.2 Specific protein oxidative damage analysis

Specific protein oxidation analysis was performed in the PFC and hippocampus of treated mice extracted 72 hours after treatment (see materials and methods **Article 2**). Protein carbonyls were detected using Bodipy-FL-hydrazides and analysed by 2-dimensional gel electrophoresis, as previously described (Tamarit et al. 2012).

1.4 Metabolomic studies

Metabolomic studies were performed in the striatum, PFC and hippocampus of mice 72 hours after treatment. In each treatment, 3 - 4 brain areas were analysed. For tissue processing a standard procedure was used (Wikoff et al. 2008), consisting on:

- Homogenize the tissue with a suitable buffer (see below).
- Centrifuge the extract obtained (12.000 rpm, 3 min).
- Determination of protein concentration by Bradford.
- A volume of sample containing 15 µg of protein was used.
- Protein precipitation with cold methanol. Incubate for 1 h at -20 ºC.

- Supernatant evaporation with a Speed Vac (Thermo Scientific, Rockford, United States of America).
- Resuspention of the pellet in 100 µl H_20 milli-Q.
- Filtration mediated by *Ultrafree MC filters* (UFC3LTK00, Millipore).

Metabolomic buffer	
KCl	180 mM
MOPS	1 mM
Ethylenediaminetetraacetic acid	2 mM
Dyethylenetriaminepentaacetic acid	1 mM
Butylated hydroxytoluene	1 µM

Data acquisition was performed using a LC ESI-QTOF-MS/MS 6520 instrument (Agilent Technologies). Data were collected in positive and negative electrospray mode TOF operated in full-scan mode, as previously described (Jové et al. 2011).

The Mass Hunter Qualitative Analysis (Agilent Technologies) processor was used to determine the molecular characteristics of the samples and the GeneSpring processor (Agilent Technologies) for the comparison between groups and statistical analysis. For the association of significantly different masses ($p < 0.05$), various databases were used, i.e. Suizdak, Abagyan Lab 2010, Wishart 2009, Markley, Cui 2008, German Research Center for environmental Health 2010, LIPID MAPS 2010 and KEEG pathways database 2010.

Metabolic pathway analysis was performed via MetaboAnalyst 2.0 software (www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp) to identify the affected metabolic pathways analysis and visualization (Xia et al. 2009; Xia et al. 2012).

2. SOCIAL ENVIRONMENT IN PREADOLESCENT MICE

To evaluate the effects of social environment on behaviour and neuroendocrine responses, preadolescence mice were randomly assigned to one of the three different environments: (1) SC, containing social partners only; (2) an EC, containing novel objects and social partners; and (3) an IC, without objects or social partners. SC mice were housed (5 per cage) in a Plexiglas box (25 cm wide × 25 cm long × 14 cm high). EC mice were housed (10 per cage) in a large Plexiglas box (24 cm wide × 40 cm long × 18 cm high) which was connected to another box of the same characteristics through a tunnel made of a PVC pipe. The environment contained plastic wheels and 6 - 8 novel hard plastic objects which were changed twice per week. IC mice were housed individually in small Plexiglas boxes (12.5 cm wide × 22 cm long × 12.5 cm high). All animals were maintained in the corresponding home environment for 7 weeks, that is from postnatal day 21 (preadolescence) to postnatal day 70 (adulthood) (**Article 3**).

Locomotor activity in the locomotor activity boxes, anxiety-like behaviour in the dark-light box and elevated plus maze; and despair behaviour in the tail suspension test were evaluated as described (see materials and methods **Article 3)**.

2.1 Determination of plasma CORT

Plasma CORT was determined using radioimmunoassay technique as previously described (Carrasco et al. 2008) (see materials and methods **Article 3**).

RESULTS

1. OBJECTIVE 1

1.1 Article 1

Ros-Simó C, Ruiz-Medina J and Valverde O. Behavioural and neuroinflammatory effects of the combination of binge ethanol and MDMA in mice. *Psychopharmacology.* 221: 511-525.

The *objective* of this study was to evaluate long-term behavioural and neuroinflammatory consequences of voluntary binge ethanol, acute MDMA or its combination in adolescent CD1 mice. Particularly, body temperature, locomotor activity, motor coordination, anxiety-like and despair behaviour were evaluated 48 h, 72 h, and 7 days after the treatments. Also, neuroinflammatory response to these treatments was measured in the striatum. The *main results* were that MDMA-induced hyperthermia was abolished by pre-exposition to ethanol. Ethanol plus MDMA treated mice showed lower locomotor activity. Ethanol treated mice showed motor coordination impairment and increased despair behaviour. Anxiety-like behaviour was only seen in animals that were treated with both drugs. In contrast, neuroinflammation was mostly seen in animals treated only with MDMA. *In conclusion,* binge ethanol and MDMA co-administration increases the neurobehavioural changes induced by the consumption of each one of the drugs. However, as ethanol consumption did not increase neuroinflammatory responses induced by MDMA, other mechanisms probably mediated by ethanol, are likely to account for the neurobehavioural effects.

Ros-Simó C, Ruiz-Medina J and Valverde O. Behavioural and neuroinflammatory effects of the combination of binge ethanol and MDMA in mice. Psychopharmacology. 2012; 221(3): 511-525.

1.2 Article 2

Ros-Simó C, Moscoso-Castro M, Ruiz-Medina J, Ros J and Valverde O (2013) Memory impairment and hippocampus specific protein oxidation induced by binge ethanol and 3, 4-Methylenedioxymethamphetamine in mice. *J Neurochem.* (under review).

The *objective* of this study was the detection, quantification and identification of specific proteins becoming oxidized by binge ethanol, MDMA or the combination of the two drugs in mice hippocampus and PFC 72 hours after treament. Indeed, to determine whether this oxidative damage was affecting functionality of the brain areas studied. For that, two different declarative memory tasks were performed: the object recognition test and the radial arm maze test. The *main results* revealed that proteins involved in neuronal outgrowth and stability as well as in energy metabolism, were found oxidatively damaged in hippocampus of treated animals. Oxidative damage in specific brain areas caused by MDMA was more prominent than by binge ethanol; particularly, to those proteins related to axonal and dendritic outgrowth and stability. Acute MDMA doses produced deficits in memory consolidation since memory deficits were observed 72 hours after treatment in both tasks conducted. In contrast, binge ethanol did not induce memory impairments neither in the acquisition in the radial arm maze nor in any of the tests. *In conclusion*, the observed oxidative damage to specific proteins in mice hippocampus involved in axonal and dendritic outgrowth may contribute to the MDMA-induced cognitive deficits. In contrast, the pattern of ethanol consumption tested had little effects on learning processes.

Journal of Neurochemistry

Memory impairment and hippocampus specific protein oxidation induced by binge ethanol and 3, 4-Methylenedioxymethamphetamine in mice

Journal:	*Journal of Neurochemistry*
Manuscript ID:	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Ros-Simó, Clara; Universitat Pompeu Fabra, Ciències Experimentals i de la Salut Moscoso-Castro, Maria; Universitat Pompeu Fabra, Ciències Experimentals i de la Salut Ruiz-Medina, Jéssica; Universitat Pompeu Fabra, Ciències Experimentals i de la Salut Ros, Joaquim; IRBLleida, Universitat de Lleida, Ciències Mèdiques Bàsiques Valverde, Olga; Universitat Pompeu Fabra, Ciències Experimentals i de la Salut
Area/Section:	Other
Keywords:	specific protein oxidation, binge ethanol, MDMA, hippocampus, long-term memory

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Memory impairment and hippocampus specific protein oxidation induced by binge ethanol and 3, 4-Methylenedioxymethamphetamine in mice

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Abbreviations: DID, Drinking in the Dark; MDMA, 3, 4-Methylenedioxymethamphetamine

Abstract

Binge ethanol and 3, 4-Methylenedioxymethamphetamine (MDMA) are popular recreational drugs widely abused by adolescents that may induce neurotoxic processes associated to behavioural alterations. Adolescent CD1 mice were subjected to binge ethanol using the drinking in the dark (DID) procedure, acute MDMA or its combination. Considering that both drugs of abuse cause oxidative stress in the brain, protein oxidative damage in different brain areas was analysed 72 hours after treatment using a proteomic approach. Damage to specific proteins in treated animals was significative in the hippocampus but not in the prefrontal cortex. To uncover the functions affected, the damaged hippocampus proteins were then identified revealing that they were involved in energy metabolism, neuronal function, outgrowth and stability. Damage was more prominent in MDMA treated than in ethanol treated mice. To determine whether this oxidative damage was affecting hippocampus activity, long-term hippocampal-dependent memory was evaluated using the object recognition assay and the radial arm maze; both conducted 72 hours after treatment. While acquisition in the radial arm maze was not impaired by binge ethanol, acute MDMA impaired long-term memory in both tests. Therefore, acute MDMA provokes oxidative damage to specific hippocampus proteins that may contribute to deficits in declarative memory.

Key words: binge ethanol, hippocampus, long-term memory, MDMA, specific protein oxidation

Running title: ethanol plus MDMA on memory and protein oxidation

Introduction

Adolescence is critical developmental period in which the brain emerges from an immature state to adulthood (Spear, 2000). Thus, impact of drug abuse on adolescent brain might have severe negative consequences. In addition, it is a time when novel experiences involving drugs such as ethanol or psychostimulants are sought, and in the majority of teens, risky behaviours are viewed as exciting and rewarding ignoring the negative consequences for health (Crews et al., 2007). Several reports indicate that binge ethanol and the psychostimulant 3, 4-Methylendioxymethampehtamine (MDMA or *ecstasy*) are commonly consumed among adolescence and young adults (Winstock et al., 2001; Barrett et al., 2006). It is well known that both ethanol and MDMA cause neuroinflammation and neurotoxicity in different brain areas, such as prefrontal cortex, striatum and hippocampus (Izco et al., 2006; Rodríguez-Arias et al., 2011). Thus, both types of drug abuse, alone or in combination, induce glial activation (Vallés et al., 2004; Ros-Simó et al., 2012), increase in reactive oxygen species and oxidative damage (Busceti et al., 2008; Alves et al., 2009; Rump et al., 2010), as well as proinflammatory cytokines release (Connor et al., 2001; Qin et al., 2008). Indeed, MDMA induces neuronal terminal loss (Izco et al., 2006; Touriño et al., 2010). These neurotoxic processes may lead to neurodegeneration in specific brain regions in alcohol and MDMA consumers that may alter a variety of cognitive and performance tasks, including memory and learning processes (Fadda and Rossetti, 1998; Parrott, 2001). Cognitive dysfunctions are very prominent in several neuropsychiatric disorders and often diminish patients' quality of life (Millan et al., 2012). Several investigations have studied the effects of alcohol and MDMA on memory acquisition (Able et al., 2006; Kay et al., 2011) or short-term memory (Brooks et al., 2002; García-Moreno et al., 2002). However, little is known about the effects of these drugs on memory consolidation and long-term memory, which makes it an interesting research field. Additionally, the time of exposure to the drug

and the pattern of consumption that lead to the development of neurotoxicity and cognitive alterations is still a matter of debate.

As stated above, it is well established that oxidative damage occurs in brain after ethanol and MDMA treatment in rodents and non-human primates (Busceti et al. 2008; Alves et al., 2009; Rump et al., 2010; Collins and Neafsey, 2012), but little is known about the specific oxidatively damaged proteins. This damage negatively affects protein function and can be measured by detecting carbonyl formation on amino acid side-chains (Levine and Stadtman, 2001; Tamarit et al., 2012). Therefore, we have identified carbonylated proteins induced by binge ethanol, acute MDMA (20 mg/kg x 2), and the combination of both drugs in mice hippocampus and prefrontal cortex, as they are two brain areas involved in cognitive functions (Morris et al., 1982; Bizon et al., 2012).

Indeed, we wanted to uncover whether the oxidative damage could affect activity of these specific brain areas. For that, we carried out declarative memory tasks in animals treated with binge ethanol, acute MDMA (20 mg/kg x 2), and the combination of both drugs of abuse. One of the tasks was the object recognition test, involving the hippocampus and the adjacent structure, the perirhinal cortex; both of them strongly interconnected (Wixted and Squire, 2011). The other task was the radial arm maze, which involves different aspects of spatial reference and working memory, involving the hippocampus but also the prefrontal cortex, respectively. In both tasks memory consolidation was evaluated.

Materials and Methods

Animals

All animal care and experimental procedures were conducted according to the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB). The results of all studies are reported in accordance with the ARRIVE guidelines for

reporting experiments involving animals (McGrath et al., 2010). All procedures were performed by an experimenter blind to the treatment.
A total of 120 adolescent male CD1 mice were used in this study. Mice (PND 21) were purchased from Charles River (France) and individually housed upon arrival. Animals remained on quarantine period (7 days) until the beginning of the experiment (PND 28) weighing 18-20 g. Animal rooms were controlled for temperature (22º ± 1ºC), humidity (55% ± 10%) and reversed light/dark photo-period (lights on between 20:00 h and 08:00 h). All the experiments took place during the dark phase. Food and water were available ad libitum except when water was substituted for ethanol for 2 or 4 h per day according to drinking in the dark (DID) procedure, as previously described by our team (Ros-Simó et al., 2012) with few modifications from original procedure (Rhodes et al., 2005). Briefly, DID is a procedure of voluntary binge ethanol drinking that consists of replacing food and water bottles for ethanol or water cylinders that remained in place for 2 hours. After this 2-hour period, food and water bottles were replaced again. This procedure was repeated on days 2 and 3 and fresh fluids were provided each day. On day 4, DID procedure lasted for 4 hours (DID 1). On the following week, the DID procedure was repeated again (DID 2). In addition, on day 4, subjects were injected with two injections of a neurotoxic MDMA dose (20 mg/kg, i.p) or saline (i.p.). The first injection at the beginning of the 4-hour DID procedure and the second two hours later; according to previously reported by our team (Ros-Simó et al., 2012).

Drugs

Racemic 3, 4-Methylenedioxymethamphetamine hydrochloride was purchased from Lipomed, A.G. (Arlesheim, Switzerland), dissolved in 0.9% physiological saline to obtain a dose of 20 mg/kg (2 mg/ml) expressed as the salt, and injected in a volume of 0.1 ml/10 g body weight by intraperitoneal (*i.p.*) route of administration. Ethyl alcohol was purchased from Merck Chemicals (Darmstadt, Germany) and diluted in tap water in order to obtain a 20% (v/v) ethanol solution.

Protein carbonylation analysis

To obtain crude extracts for protein oxidation analyses mice were sacrificed 72 hours after the last MDMA or saline injection and hippocampus and prefrontal cortex were immediately extracted from each subject. The tissue was solubilized with 100 µl of 50 mM Tris-HCl (pH 7) with mammalian protease inhibitors (Roche) and 2% SDS. Samples containing 250 µg of protein were treated with the fluorescent probe Bodipy-FL-hydrazide to derivatize protein carbonyls, as described (Tamarit et al., 2012) and 100 µg were applied to a 2-dimensional gel electrophoresis. Total protein staining was performed with Flamingo (Bio-Rad). Images were captured with a VersaDoc MP4000 and analysed with PDQuest software (Bio-Rad). For each spot, Bodipy signal was normalized to that of the protein signal. Spots displaying a ratio Bodipy-fluorescence/protein signal higher than 1.5 with respect to matched controls were selected for further identification. Protein identification was performed in Autoflex-Speed MALDI ToF/ToF mass spectrometer (Bruker Daltonics) by peptide-mass fingerprinting using the MASCOT Server 2.3 (www.matrixscience.com).

Hippocampus-related memory tasks

Object Recognition Test

The test was performed in a black rectangular open field (27 x 31 x 25 cm) in a moderate luminosity (100 lx). To evaluate the task, a discrimination index was calculated as the difference between the time spent exploring either the novel or familiar object divided by the total time exploring the two objects. A higher discrimination index is considered to reflect greater memory retention for the familiar object as previously reported (Maccarrone et al., 2002). Behaviour was recorded and monitored with a video camera interfaced to a computer. Experimental procedure (Figure 1A) consisted on one day of habituation to the box, without objects, for 9 min (D1 of the second week). After that, mice were exposed

to the corresponding 2 hours of DID procedure. Acquisition was performed from D2 to D4 for 9 min each day with subsequently exposure to the corresponding treatment (on D2 and D3, 2 hours of DID and, on D4, MDMA 20 mg/kg x 2 or saline and 4 hours of DID, according to the abovementioned procedure). Acquisition was performed with two identical objects (familiar object) which were maintained the three days. 72 hours later the testing phase took place, when one familiar object was replaced for a novel object.

Radial arm maze test

In a separately group of mice, radial arm maze was assessed. The radial arm maze (Panlab, Barcelona, Spain) made of black Plexiglas consisted of a central hub with eight-arms radiating from it. The maze arms were 35 cm in length, with outer arm walls 2.6 cm high, inner arm walls 15 cm high and 5.8 cm wide. The centre well of the maze was 16.7 cm in diameter and the maze was situated on a 100 cm high tripod. The food wells at the end of each arm consisted of a small piece of black Plexiglas, 1.2 cm high and 1.8 cm in diameter. The maze was situated in the middle of a dark room and surrounded by black curtains reaching from the ceiling to the base of the tripod, forming a circular enclosure 1.5 m in diameter and with moderate luminosity (100 lx). Three different extra-maze white and black visual cues (20 x 25 cm) to aid in spatial localization were affixed to the curtains. Behaviour was recorded and monitored with a video camera interfaced to a computer situated outside the curtains. Animals batched for this experiment were food restricted for requirements of the experiment (15% of its initial weight). During the first and second day of the habituation period animals were placed in the maze for 8 minutes each day (Figure 1B). The third day, one chocolate food pellet (Choco Krispis, Kellog’s) was left in the middle of each arm maze and the fourth day chocolate food pellets were moved to the wells. Both days mice were placed in the maze until the mouse collected all chocolate food pellets or 8 minutes had passed, whichever came first. The following week, coinciding with the first week of the DID procedure; training sessions were carried out for twelve days, starting on

D1 until D12, being D12 the last day of the DID procedure when acute MDMA or saline treatments were administered. During this acquisition period, animals were trained to find the chocolate pellets in three randomly selected arms. Animals were subjected to two trials of 5 minutes/day or until the mouse collected all the pellets. The day of the test (72 hours after the last day of acquisition) animals were subjected to the same procedure. Working memory errors (i.e., entries into baited arms that had already been visited during the same trial) and spatial reference memory errors (i.e., entries into non-baited arms) were recorded. The average of the values from the daily two sessions was calculated for each measure.

Statistical Analysis

Behavioural results are expressed as the mean ± SEM. Statistical analysis for treatment factor was determined by a one-way ANOVA test and subsequent Tukey post hoc test when required. Two-way ANOVA repeated measures (with treatment and day factors of variation) was used to analyse acquisition period in the radial arm maze test. In all experiments, differences were considered significant if the probability of error was less than 5%. SPSS statistical package was used.

Results

Ethanol consumption was measured every day during the DID procedure. Measures of the second week of the DID procedure are represented in Figure 2. No significant differences in ethanol consumption are found across day 1 to day 3. However, animals receiving MDMA on day 4 show less ethanol consumption than those that did not receive the psychostimulant ($p < 0.05$).

Protein oxidative damage analysis

Proteins from hippocampus and prefrontal cortex were analyzed by two-dimensional gel electrophoresis. Although protein oxidative damage in treated animals showed no significant differences in prefrontal cortex with respect to controls (data not shown), significative results were obtained in analysing hippocampus. Proteins were analysed using two pI ranges (5-8 and 7-10) to obtain a better resolution (Figure 3A). Representative gels for carbonyl detection (BODIPY signal) are shown in Figures 3B and 3C. When applying image gel analyses (described in materials and methods), ten different spots showed significatively increased carbonylation shown in amplified gel sections in Figure 4 and identified as listed in Table 1. Energy metabolism and neuronal function and stability were the cell functions mainly affected in the hippocampus.

The three treated groups exhibited oxidatively modified enzymes in energy production pathways. Two of them were glycoliytic enzymes (α-enolase and glyceralehyde-3P-dehydrogenase) and three were mitochondrial (aconitase, involved in Krebs cycle, and α and β subunits of the ATP synthase, the major ATP-producing enzyme inside the cell). Oxidation of these proteins indicates that hippocampus activity may be decreased due to the lack of ATP production.

Four additional targets were related to neuronal function. These were identified as dihyropyrimidinase-related protein 2 (DRP-2 or CRMP-2), which is a tubulin binding protein that regulates the process of axonal outgrowth and branching. Synapsin-1, carbonylated in the three treated groups, is a vesicle-specific protein implicated in neurotransmitter release to the synaptic cleft and has a role in synapse formation and maturation. The other two proteins are neurofilaments: actin, a critical element in the neuron cytoskeleton involved in cell vesicle formation and movement, cell motility, junction and signalling; and α-internexin, involved in neuronal development, axonal outgrowth and regulation of axonal stability. CRMP-2 and actin were highly carbonylated in MDMA-treated mice but not in ethanol-treated alone mice. α-internexin and synapsin-1 showed similar oxidation folds in all three treated groups compared to control (water + saline).

Finally, heat shock cognate protein 71 (HSC71) is a chaperone of the HSP-70 family, having a role in protection against apoptosis.

Hippocampus-related memory tasks

MDMA treated mice show long-term memory deficits in the object recognition test

We firstly determined the effects of the drugs of abuse studied in the object recognition test assessed 72 hours after the last MDMA or saline injection (Figure 1A). One-way ANOVA revealed effects of the treatment [$F(3, 40) = 4.51$, $p < 0.01$] (Figure 5). Post-hoc analysis showed a significant effect in MDMA treated ($p < 0.05$), and binge ethanol + MDMA treated ($p < 0.01$) when compared to water + saline group. These results indicate that long-term memory is only affected after an acute and neurotoxic MDMA dose (20 mg/kg x 2).

Radial arm maze acquisition is not impaired by binge ethanol consumption

We next examined whether binge ethanol could be affecting the acquisition process of the radial arm maze in which mice were trained for twelve days (Figure 1B). Binge ethanol consumption after daily training session in the radial arm maze did not impair memory acquisition as indicated by two-way ANOVA (treatment and day factors). Thus, statistical analysis indicated; for working memory errors (Figure 6A), no effect of the treatment [$F (1, 50) = 0.003$, n.s] but effect of the day [$F (11, 550) = 7.584$, $p < 0.001$] without interaction between day and treatment factors [$F (11, 550) = 0.491$, n.s]. Similarly, for reference memory errors (Figure 6B), no effect of the treatment was observed [$F (1, 50) = 1.374$, n.s], effect of the day [$F (11, 550) = 8.583$, $p < 0.001$] without interaction between both factors [$F (11, 550) = 0.480$, n.s]. All together these results indicate that binge ethanol did not affect acquisition of the task as they acquired in the same way as water group.

MDMA impairs reference memory but not working memory in the radial arm maze

To assess the effects of acute MDMA, alone or added to binge ethanol, on the consolidation of the previous acquired task; we tested mice in the radial arm maze 72 hours after the last MDMA or saline injection (Figure 1B). Statistical analysis showed no effects of the treatment on working memory [$F (3, 51) = 1.34$, n.s] (Figure 6C) but effects on spatial reference memory [$F (3, 51) = 4.89$, $p < 0. 01$] (Figure 6D), as indicated by one-way ANOVA. Post-hoc analysis indicated that differences in reference memory errors (Figure 5D) were observed in MDMA treated ($p < 0.05$) and binge ethanol + MDMA treated ($p < 0.05$) when compared to water + saline group. Our results indicate that MDMA treatment alone or in combination with binge ethanol impairs consolidation of spatial reference memory in mice.

Discussion

The present work shows that, under our experimental conditions, both binge ethanol and acute MDMA induced damage to specific hippocampus proteins in CD1 mice. MDMA-induced damage was more prominent than ethanol-induced, particularly to proteins involved in neuronal function and stability. Indeed, an acute neurotoxic dose of MDMA affected consolidation of declarative memory in two different paradigms. Instead, animals under a pattern of binge ethanol drinking did not exhibit significant memory deficits. The MDMA-induced memory impairments could have a relation with the specific oxidatively damaged hippocampus proteins.
Previous studies have revealed a clear involvement of oxidative stress in the neurotoxicity induced by MDMA and ethanol consumption (Alves et al., 2009; Rump et al., 2010). These studies led us to hypothesize that ethanol and MDMA could induce protein oxidation in discrete brain areas, and these changes could be associated to cognitive impairment and provide enhanced vulnerability to initiate neurodegenerative processes. Therefore, our aim in the present study was to identify oxidatively damaged proteins in different brain areas involved in learning and memory processes of treated mice. For that, we proceed to evaluate protein

oxidation in the hippocampus and prefrontal cortex of mice treated with binge ethanol and/or acute MDMA. Reactive oxygen species may react with proteins leading to oxidative modifications, which are known to be one of the most important causes of brain protein damage and dysfunction (Berlett and Stadtman, 1997). Protein carbonyl formation is one of the most studied markers resulting from oxidative stress and can be easily detected and quantified (Levine et al., 1994). In this study we have used a recent and improved procedure of carbonyl detection (Tamarit et al., 2012). Under our experimental conditions, different proteins involved in a variety of cellular functions were found oxidatively modified in hippocampus but not in prefrontal cortex (data not shown) by the consumption of binge ethanol, MDMA or its combination including α-enolase, glyceralehyde-3P-dehydrogenase, aconitate hydratase, α and β subunit of the ATP synthase, CRMP-2, actin, α-internexin, synapsin-1 and HSC 71. Carbonylation, and thus, inactivation of proteins related to energy metabolism, higher in acute MDMA than in binge ethanol treated subjects, may contribute to an energy deficiency associated with drug use. This result could explain the observation that MDMA decreases brain ATP production that results in subsequent membrane ionic deregulation, calcium entry and additional free radical formation (Darvesh and Gudelsky, 2005). In addition, carbonylation of proteins related to energy metabolism has also been found in brains affected by Huntington (Sorolla et al., 2010), Parkinson (Malkus et al., 2009) or Alzheimer (Castegna et al., 2002) neurodegenerative diseases. Furthermore, Castegna and colleagues (2002) also reported that in Alzheimer's disease brain CRMP-2 and HSC 71 were oxidatively modified hippocampus proteins. These findings suggest that the impact of binge ethanol and MDMA on brain of adolescents could lead to a neurodegeneration more typical of an elderly brain. Moreover, synapsin-1 is involved in neurotransmitters release (Evergren et al., 2007) and hippocampal neuronal development (Fornasiero et al., 2009). Finally, actin and α-internexin are neurofilaments involved in neuronal stability and axonal growth (Levavasseur et al., 1999). Considering the well-established MDMA-induced dopaminergic and serotonergic axon terminal loss (Sprague et al., 1998;

Puerta et al., 2009), it can be suggested that carbonylation of these neurofilaments would be involved in this mechanism of neurodegeneration, particularly actin, which is an important element in the growth cone cytoskeleton of the axon terminal (Fletcher and Mullins, 2010), appearing oxidized exclusively in MDMA treated mice. Altogether, our findings clearly show that free radical-mediated protein modification is a crucial event in MDMA and ethanol ingestion and support protein oxidation as an important contributor to the mechanisms underlying hippocampal neurotoxicity in MDMA and ethanol consumers. Indeed, some works have reported attenuation of ethanol and MDMA-induced neurotoxicity by free radical scavengers and antioxidants (Colado and Green, 1995; Crews et al., 2006), providing indirect evidence for the involvement of oxidative damage in the mechanism of ethanol and MDMA neurotoxicity.

To determine whether this oxidative damage caused cognitive dysfunction, we evaluated the possible impairment in declarative memory tasks induced by the exposition to binge ethanol, MDMA and its combination in mice. Our results indicated that, under our experimental conditions, acute MDMA but not binge ethanol induced memory impairments. No increased memory deficits were observed with the combination of both drugs of abuse. In accordance with this result, previous works find memory deficits with the administration of either drug alone without an enhancement of the deficits when ethanol and MDMA are administered together compared to either drug alone (Vidal-Infer et al. 2012).

Memory deficits induced by MDMA treatment were observed in the object recognition test 72 hours after treatment even after 3 training days, which was performed to ensure a good acquisition of the task. Additionally, in the radial arm maze test, animals treated with the pschostimulant performed significantly more spatial reference memory errors (Figure 6D) than the last day of acquisition, before MDMA administration (Figure 6B, day 12). Contrary, those that did not receive MDMA performed the same values of spatial reference memory errors as the last training day. Different learning and memory tasks following a MDMA treatment have been studied in rodents. For instance, non-spatial memory has been evaluated

resulting on memory impairment in MDMA treated rats (Camarasa et al., 2008). Moreover, and in agreement with our results, rats in the radial arm maze (Kay et al., 2011) or rats and mice in the Morris water maze (Camarasa et al., 2008) showed signs of spatial reference memory impairment without alterations in working memory, which depends more on prefrontal cortex. However, in all these studies animals received the MDMA treatment before acquisition period while in our procedure; animals had already acquired the task before the treatment. Thus, our results indicate that MDMA is affecting memory consolidation, that is, MDMA exhibits effects on the consolidation process of the previous learning. In line with these data, previous findings have shown that MDMA impaired acquired tasks in different behavioural paradigms in mice, such as operant-delayed alternation task involving working memory but not spatial reference memory (Viñals et al., 2012) or active avoidance performance (Trigo et al., 2008), involving emotional memory.

As stated above, binge ethanol did not provoke deficits in the consolidation of any of the tasks evaluated. Moreover, in the radial arm maze, no affectations were observed in binge ethanol treated adolescent animals on the acquisition period. There is evidence that human alcoholics show deficits in spatial memory tasks (Bowden and McCarter, 1993), and similar results have been found in animal models (Kameda et al., 2007). However, some authors do not find such damage after ethanol consumption (Popovic et al., 2004). These ethanol-induced neural changes and the potential for recovery seem to be dependent on length of ethanol exposure, volume of ethanol, degree of withdrawal signs or number of binge bouts, genetics and age (Crews and Nixon, 2009). Thus, a pattern of binge exposure during adolescence impacts the developing brain inducing neural consequences, such as brain impairments and cognitive and behavioural dysfunctions (Guerri and Pascual, 2010; Ros-Simó et al., 2012). In contrast, a pattern of alcohol dependence, more common in adulthood, liver injury due to ethanol interference in hepatic metabolism is probably the principal health problem (Ahmed, 1995). It is noteworthy that our animals have low preference (Figure 2) and as previously reported (Ros-Simó et al., 2012). Thus, there is the possibility that this amount of

ethanol ingested is not enough to produce alterations on memory consolidation, even though it can lead to long-term emotional-like behaviour alterations (Ros-Simó et al. 2012). In agreement with this hypothesis, it has been reported that low doses of ethanol (0.5 g/kg) administered 30 min before the first training session on each day for four days did not impair spatial learning in rats (Acheson et al., 2001). Indeed, it has been described that acute ethanol induces memory impairments while after repeated treatment with low doses (0.6 g/kg) a tolerance to the amnesic effects is developed (Kameda et al., 2007).

Interestingly, carbonylation of proteins related to neuronal function was higher in MDMA treated than in non-MDMA treated mice, suggesting that memory deficits could be related to affectations of the psychostimulant to these specific proteins. CRMP-2, which is highly carbonylated in MDMA treated mice (Table 1), is critically involved in axonal outgrowth and pathfinding through the transmission and modulation of extracellular signals (Fukata et al., 2002). Indeed, it has been reported to be oxidatively modified in Alzheimer's disease brain and has been related to memory loss associated with decreased interneuronal connections and to a shortened dendritic length (Coleman and Flood, 1987). Actin, only carbonylated in MDMA treated subjects, has a crucial role in the cytoskeleton network integrity (Fletcher and Mullins, 2010) and is concentrated in dendritic spines where it can produce changes in their shape that might be involved in memory function (Morgado-Bernal, 2011). Thus, considering that memory deficits are only observed in MDMA treated subjects, it could be suggested that both proteins may be involved in the MDMA-induced cognitive impairments observed. It has to be taken into account that protein carbonyl formation was also analysed in the prefrontal cortex with no significant results. Thus, it can be suggested that the impact of the MDMA-induced oxidative damage to specific proteins is distinctive for hippocampus which, moreover, is the brain area mainly involved in declarative memory (Morris et al., 1982) and where synaptic plasticity associated to the process of learning occurs (Malenka and Nicolls, 1999). Therefore, results obtained indicate that hippocampus, and the processes occurring within it, seem to be more sensitive

to the MDMA-induced neurotoxicity than other brain areas such as the prefrontal cortex. In addition, it supports the crucial role of hippocampus in declarative memory. MDMA-induced energy dysfunction and thus, low ATP production, may enhance the loss of interneuronal connections (Butterfield et al., 2006). Ethanol treated subjects that also exhibited oxidatively damaged proteins related to energy metabolism or synapsin-1, but did not display memory deficits, suggest that drug-induced oxidative stress may lead to different behavioural alterations, as previously described by our team (Ros-Simó et al., 2012).

In summary, our study shows that mainly MDMA but not binge ethanol affects consolidation of declarative memory in adolescent mice. Additionally, ethanol at this pattern of consumption does not seem to affect learning acquisition in mice. The observed oxidative damage to specific proteins in hippocampus, especially those related to axonal and dendritic outgrowth and stability, may contribute to the cognitive deficits observed. Studies of specific protein damage may provide new insights in understanding the MDMA and binge ethanol mechanisms of neurotoxicity and its behavioural consequences.

Conflict of Interest: The authors declare no conflict of interest.

Acknowledgements: Authors wish to thank to Alba Sorolla and Isabel Sánchez from "Servei de Proteòmica i Genòmica" UdL, for helping on peptide mass fingerprinting analyses.

Funding: This work was supported by grants from Spanish Ministry of Science and Innovation (SAF2010-15793 and CSD2007-00020); the Spanish Ministry of Health (PNSD 2010); Red Temática de Investigación Cooperativa en salud (ISCIII) (RETIC-Trastornos Adictivos RTA 001/06/1001-FEDER) and Generalitat de Catalunya (2009SGR684). CR-S was funded by a FPI fellowship (BES-2008-007915).

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Figure legends

Figure 1. Memory-related tasks experimental procedure. (A) Object recognition test and (B) radial arm maze. For details, see Methods and Materials.

Figure 2. Total volume of ethanol intake during the second week of the DID procedure (we show the first 2 h during the fourth day). ★ $p < 0.05$ when compared to ethanol plus saline group. Values are expressed as the mean ± SEM.

Figure 3. Protein oxidative damage analysis of mice hippocampus by 2D-PAGE (pH ranges 5-8 and 7-10). (A) Gels stained for total protein of hippocampus from control mice. (B) Representative gels (pI range 5-8) of Bodipy signal detection (carbonylated proteins) for control (water + saline) and treated samples and (C) representative gels (pI range 7-10) of Bodipy signal detection (carbonylated proteins) for control (water + saline) and treated samples. Three gels performed in duplicate were analyzed for each group (representative images are shown) (n = 3 – 4 mice per group).

Figure 4. Amplified sections of carbonylated proteins (BODIPY signal) of the identified spots shown for each experimental group. Spot numbers are described in Table 1 (n = 3 - 4 mice per group).

Figure 5. Differential effects of binge ethanol and acute MDMA on memory consolidation in the object recognition test 72 hours after the treatment (n = 10 - 11 mice per group).★ $p < 0.05$ and ★★$p < 0.01$ when compared with the water plus saline group. Values are expressed as the mean ± SEM.

Figure 6. No effects of binge ethanol on acquisition period in the radial arm maze. (A) Working memory errors and (B) reference memory errors. (n = 24 -26 mice per group). Each time point represents the mean ± SEM. Effects of acute MDMA on long-term memory in the radial arm maze test 72 hours after the treatment. (C) Working memory errors and (B) reference memory errors. (n = 12 - 15 mice per group). ★$p < 0.05$ when compared with the water plus saline group. Values are expressed as the mean ± SEM.

Table 1. Carbonylated proteins identified in hippocampus of mice submitted to different treatments (n = 3 – 4 mice per group).

Spot	Protein	Accession No*	Ox. fold MDMA**	Ox. Fold ETHANOL**	Ox. Fold ETHANOL+MDMA**
1	Heat shock cognate 71	P63018	2.44±0.38	2.19±0.29	5.52±0.89
2	Heat shock cognate 71	P63018	2.42±1.05	2.06±0.19	2.40±0.26
3	Dihyropyrimidinase related protein-2	P47942	3.95±2.05	1.69±0.51	3.35±1.27
4	α-internexin	P23565	1.55±0.39	1.54±0.22	2.32±0.49
5	ATP synthase subunit β	P10719	3.19±1.05	1.43±0.79	1.88±0.98
6	α-enolase	P04764	2.32±0.41	1.97±0.15	2.12±0.21
7	Actin	P60711	4.02±1.72	0.66±0.10	1.60±0.35
8	Aconitase	Q9ER34	1.76±0.30	1.36±0.16	1.87±0.34
9	Aconitase	Q9ER34	1.96±0.51	1.76±0.11	2.55±0.63
10	ATP synthase subunit α	P15999	1.93±0.49	1.49±0.32	1.81±0.94
11	ATP synthase subunit α	P15999	2.17±0.51	2.14±0.64	2.10±0.26
12	Synapsin-1	P09951	2.83±0.96	2.15±0.15	2.39±0.20
13	Glyceraldehyde-3-phosphate dehydrogenase	P04797	1.85±0.43	2.17±0.73	2.41±0.59
14	Glyceraldehyde-3-phosphate dehydrogenase	P04797	2.28±0.33	1.64±0.32	3.72±2.62
15	Glyceraldehyde-3-phosphate dehydrogenase	P04797	2.56±0.65	1.65±0.46	2.06±0.45

*Accession number and molecular mass according to Swissprot database.

**The oxidation fold is the mean value ± SEM of fold increase in carbonyl levels of matched pairs (treated vs control water + saline) obtained from three independent gels performed in duplicate

Figure 1

A

1st week (DID 1)
D1 D2 D3 D4
2h ethanol/water
4h ethanol/water

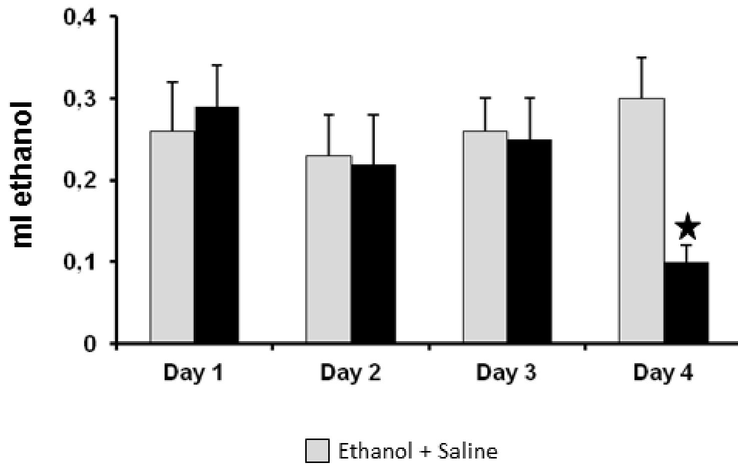
2nd week (DID 2)
D1 D2 D3 D4
72 h
Object Recognition Test
Habituation
Acquisition
+
2h ethanol/water
4h ethanol/water + MDMA/saline

B

1st week
D1 D2 D3 D4
Habituation

2nd and 3rd week (DID 1 and DID 2)
D1 ----→ D12
72 h
Radial arm maze Test
Acquisition
+
D2 to D4
2h ethanol/water
D5
4h ethanol/water
D9 to D11
2h ethanol/water
D12
4h ethanol/water +MDMA/saline

Figure 2



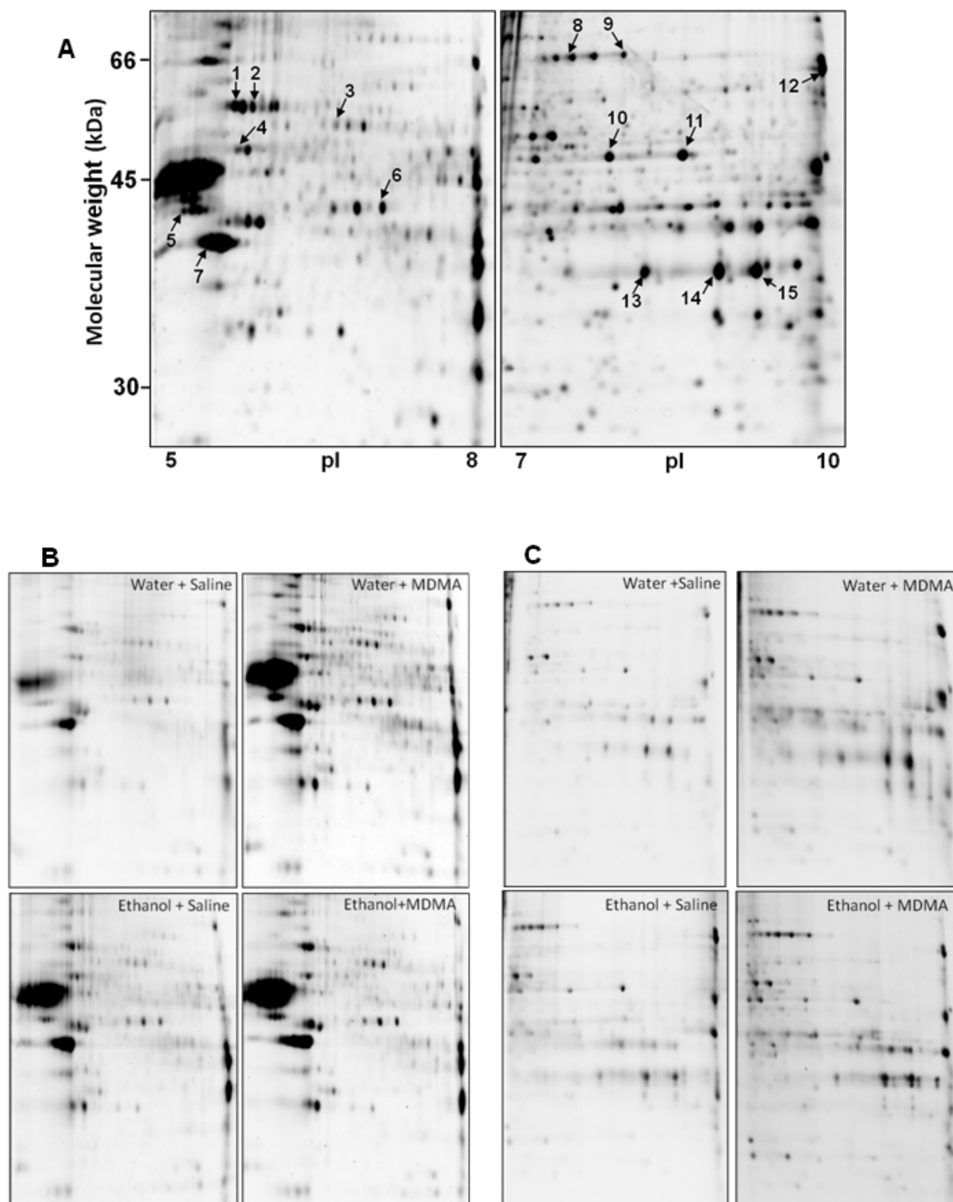


Figure 3
A
66
45
30
Molecular weight (kDa)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
5
pI
8
7
pI
10
B
Water + Saline
Water + MDMA
Ethanol + Saline
Ethanol+MDMA
C
Water +Saline
Water + MDMA
Ethanol + Saline
Ethanol + MDMA

Figure 4

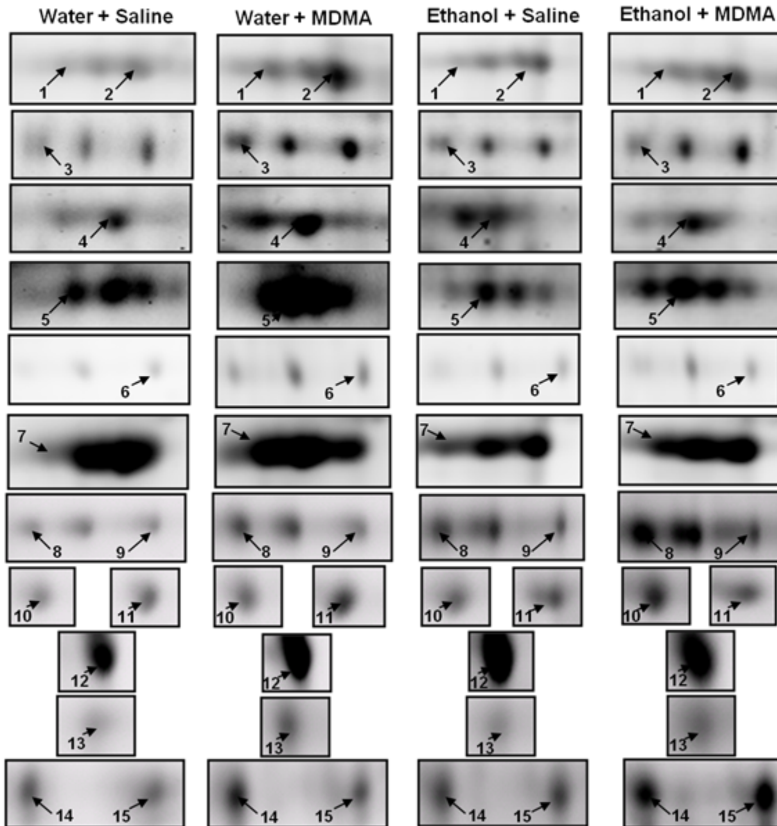


Figure 5

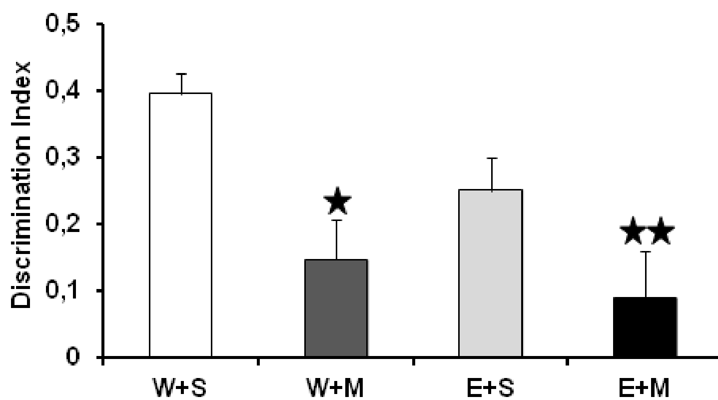
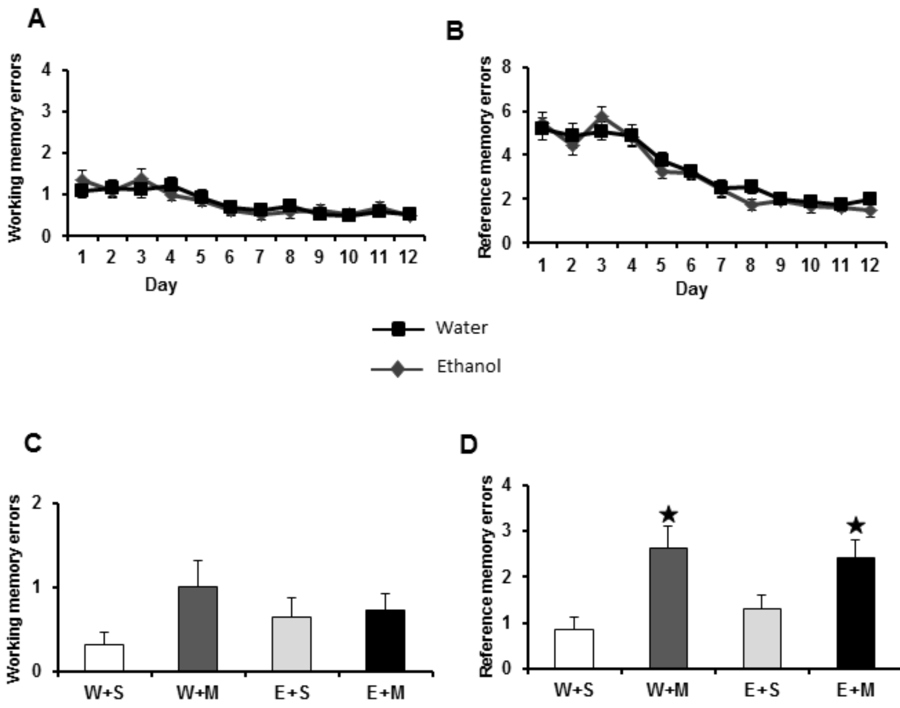


Figure 6



1.3 Metabolomic studies in mouse brains

The *objective* of this study was to evaluate whether binge ethanol, acute MDMA or its combination induce changes in the metabolomic profile in mice striatum, PFC and hippocampus. For that, untargeted metabolomic studies were performed. Our *initial results* indicate that metabolic patterns in the striatum, PFC and hippocampus are altered by binge ethanol, acute MDMA or the combination of both drugs of abuse. Indeed, to try to prevent the despair behaviour induced by the combination of binge ethanol and acute MDMA in the tail suspension test, two antidepressants (imipramine and escitalopram) were tested. Finally, we determined if these antidepressants were able to prevent changes in the metabolic profiles induced by the drugs of abuse. Antidepressant treatment with imipramine and escitalopram prevents the depressive-like behaviour observed in the ethanol + MDMA group 72 hours after the last drug administration. Metabolomic profile of mice treated with ethanol + MDMA and imipramine or escitalopram differ from that of the ethanol + MDMA. However it also differs from control group. These are initial results and further experiments will be needed. Nevertheless, *preliminary conclusions* can be summarized as follows i) ethanol, MDMA and its combination induce, in each case, changes in the metabolomic profile, ii) imipramine and escitalopram are able to prevent the depressive-like behaviour induced by the combination of binge ethanol and MDMA and iii) altered tryptophan metabolism may be involved in the depressive-like behaviour observed in the ethanol plus MDMA treated mice.

1.3.1 Metabolomic studies

The purpose of the metabolomic studies lies in the importance of knowing how metabolites involved in different pathways should vary as a consequence of changes in physiology caused by submitting animals to different conditions for instance, treatment with specific drugs. There are two types of metabolomic analyses: targeted and untargeted (Patti et al. 2012). Targeted metabolomics involve the quantification of certain metabolites of special interest in a sample. On the other hand, untargeted metabolomics consist on obtaining the general metabolic profile of each sample (Figure 21).

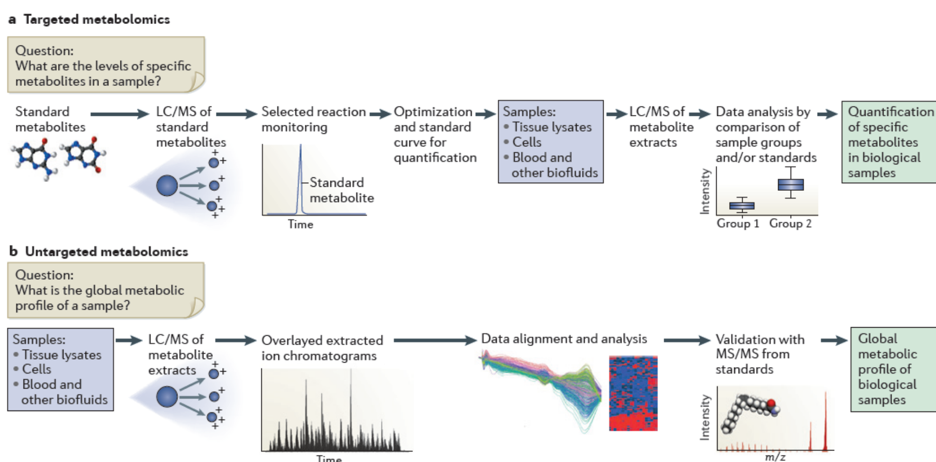


Figure 21. The targeted and untargeted workflow for LC/MS-based metabolomics (from Patti et al. 2012).

Metabolomic profiling has proved to be useful to study different diseases. It enables the study of potential biomarkers associated to a specific disease useful to identify individual risk but also to monitor its progression as well as to determine the efficacy of a pharmacological treatment. For instance, in the study of Bogdanov et al. (2008) untargeted metabolomic analyses were used to study metabolomic signatures in Parkinson disease. Metabolome obtained from plasma samples of

control subjects, non-medicated Parkinson patients and medicated Parkinson patients were analysed revealing that metabolic profile of non-medicated and medicated Parkinson patients were different. Interestingly, medicated patients showed a metabolomic profile close to that of the control subjects (Bogdanov et al. 2008).

In this study we performed studies of untargeted metabolomics to seek whether control and treated animals exhibited differences in their metabolomic profile. To analyze the obtained data, multivariate statistics analyses are performed. The objective of these analyses is to simplify complex data derived from such studies which contains thousands of variables i.e. metabolites. Thus, it reduces the variation to a two- or three-dimensional model (Trygg et al. 2007; Jové et al. 2011). There are two possible multivariate statistics analyses, "unsupervised" including the Principal Component Analysis (PCA) and "supervised" including the Partial Least Discriminate Analysis (PLS-DA). PCA analyses are used to establish whether any intrinsic clustering exists within a data set, without "a priori" knowledge of sample class and it gives raw data. Instead, PLS-DA use the class information given for training set of samples to optimize the separation according to metabolite behaviour between two or more sample classes (Nicholson et al. 2002).

Technically, a PCA analysis tries to group samples as best represented in a coordinate system. Each axis, which is each principal component, represents a group of molecules with a similar behaviour so that the first axis represents the major variance in the data set; the second axis represents the second major variance and so on. A specific PCA analysis can vary according to different study conditions and time, constituting very valuable and helpful information to explore metabolic changes associated with the progression of a treatment, an illness, etc. A PLS-DA is basically the same but clustering and reinforcing those molecules of each component to optimize the separation between the different groups studied.

Therefore, in the supervised methods, in this case PLS-DA, separation between groups is always higher than in the unsupervised or PCA methods.

1.3.2 Metabolomic patterns of mouse brains differ between drug treatments

In this work, metabolomic data are analyzed according to PCA and PLS-DA analysis of samples obtained from the different brain areas studied (striatum, PFC and hippocampus) of animals submitted to different drug treatment (control, binge ethanol, acute MDMA, binge ethanol + acute MDMA). PCA analyses for all brain areas are represented for positively ionized molecules (Figure 22) and negatively ionized molecules (Figure 23).

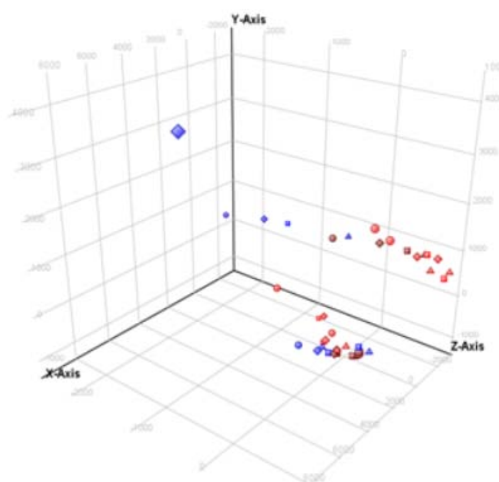


Figure 22. PCA analysis in positive ionization for all groups.
Red represents PFC; blue represents striatum and brown represents hippocampus.

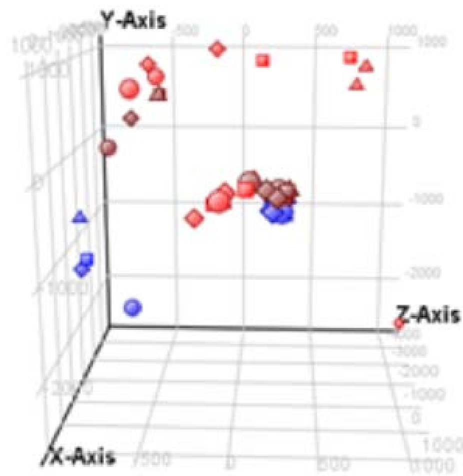
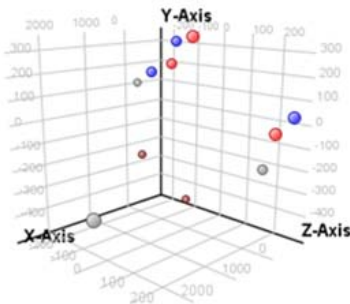


Figure 23. PCA analysis in negative ionization for all groups.
Red represents PFC; blue represents striatum and brown represents hippocampus.

Figures for each brain area are shown: striatum (Figures 24 and 25), PFC (Figure 26 and 27) and hippocampus (Figure 28 and 29).

A)



B)

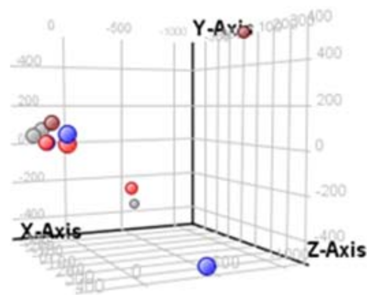


Figure 24. PCA analysis in mice striatum. A) Positive ionization and B) negative ionization. Blue balls represent water group (control); red balls, MDMA group; grey balls, ethanol group and brown balls, ethanol + MDMA group.

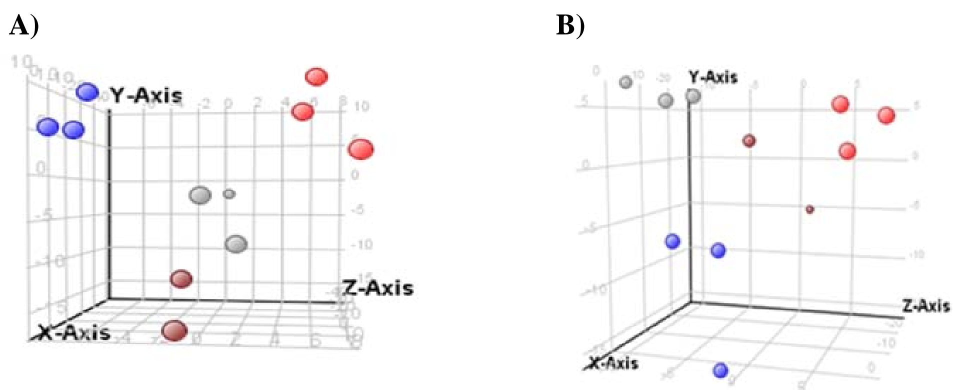


Figure 25. PLS-DA analysis in mice striatum. A) Positive ionization and B) negative ionization. Blue balls represent water group (control); red balls, MDMA group; grey balls, ethanol group and brown balls, ethanol + MDMA group.

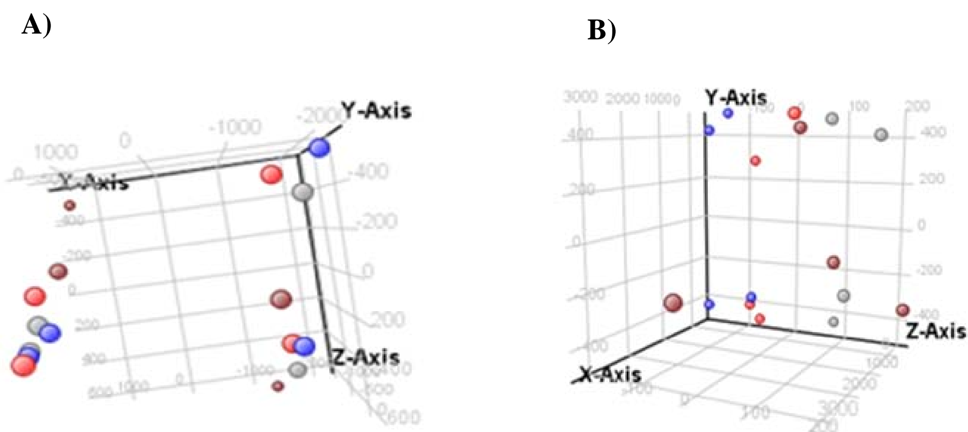


Figure 26. PCA analysis in mice PFC. A) Positive ionization and B) negative ionization. Blue balls represent water group (control); red balls, MDMA group; grey balls, ethanol group and brown balls, ethanol + MDMA group.

A) B)

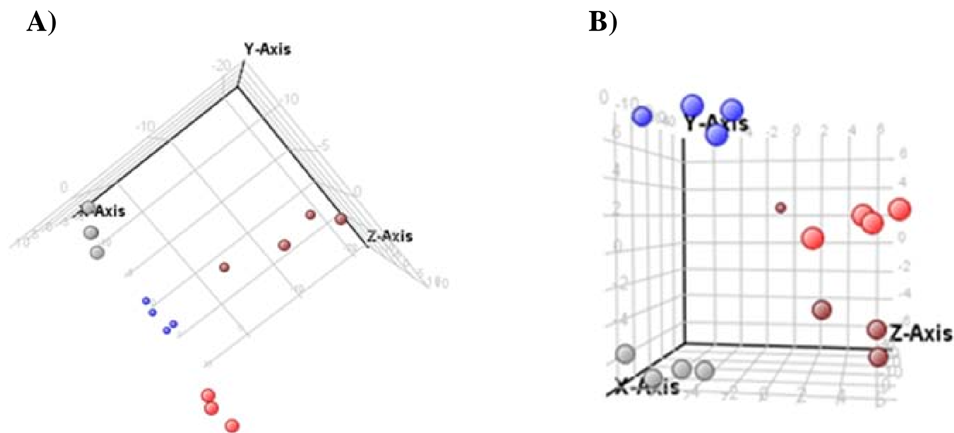


Figure 27. PLS-DA analysis in mice PFC. A) Positive ionization and B) negative ionization. Blue balls represent water group (control); red balls, MDMA group; grey balls, ethanol group and brown balls, ethanol + MDMA group.

A) B)

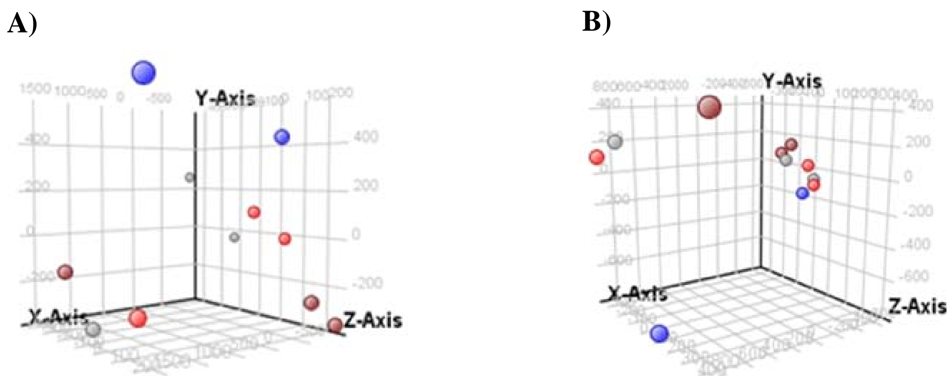


Figure 28. PCA analysis in mice hippocampus. A) Positive ionization and B) negative ionization. Blue balls represent water group (control); red balls, MDMA group; grey balls, ethanol group and brown balls, ethanol + MDMA group.

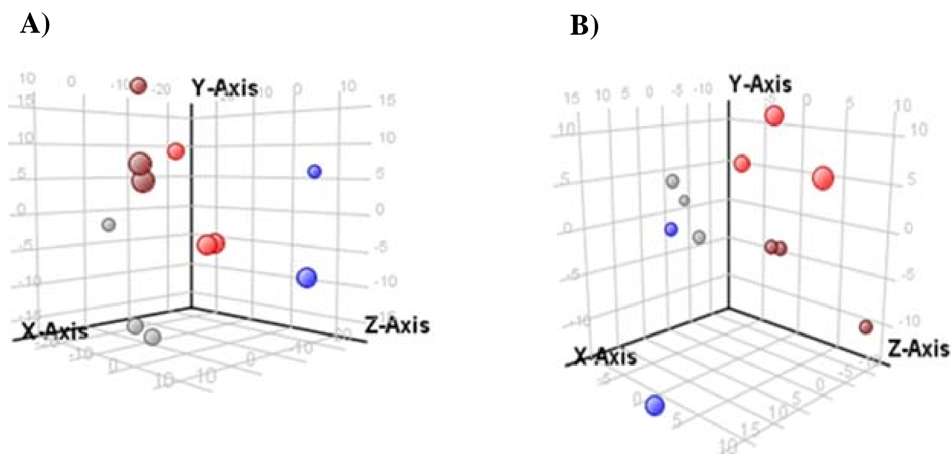


Figure 29. PLS-DA analysis in mice hippocampus. A) Positive ionization and B) negative ionization. Blue balls represent water group (control); red balls, MDMA group; grey balls, ethanol group and brown balls, ethanol + MDMA group.

In summary, these results show that i) metabolomic profile of the brain areas analyzed differ from each other and ii) each treatment shows clear-cut clustering profiles which differ from the others. To get further insight derived from these data, we used the MetaboAnalyst 2.0 software, a web-based suite for high-throughput metabolomic data analysis and interpretation firstly released in 2009 (Xia et al. 2009) but significantly improved (Xia et al. 2012), with the aim to identify the relevant pathways affected by the treatments applied to the animals. In this context, compounds identified in the previous analyses that show clear deviation from controls in the PFC and hippocampus are shown in Table 1 and Table 2. These compounds were uploaded to the MetaboAnalyst 2.0 to match with the pathways database (Figure 30 and Figure 31). Results obtained indicate that globally, amino acid metabolism is altered and thus, is the responsible for the differences in the metabolic profiles.

COMPOUND	MDMA vs. CONTROL	ETHANOL vs. COTNROL	ETHANOL+MDMA vs. CONTROL
(+)-Isomyristic acid/Myristic acid*	down	down	up
15-Keto-prostaglandin F2-alpha	down	down	up
Ribitol/L-arabitol/Deoxycitidine monophosphate (dCMP)*	down	down	up
Taurine	down	down	up

Table 1. Compounds identified in PFC showing deviation from control groups. Statistical Benjamini-Hochberg Multiple Testing Correction was used.

*compounds with the same formula and very close retention time

COMPOUND	MDMA vs. CONTROL	ETHANOL vs. COTNROL	ETHANOL+MDMA vs. CONTROL
2-Hexyldecanoic acid	down	up	down
alpha-Estradiol	up	down	down
alpha-tocopheryl acetat	down	up	up
Digitoxose	up	up	up
Phosphocreatine	up	down	down
Taurine	up	up	down
Valine	up	up	down
VitaminD3 (7-dehydrocholesterol)	down	down	up

Table 2. Compounds identified in hippocampus showing deviation from control groups. Statistical Benjamini-Hochberg Multiple Testing Correction was used.

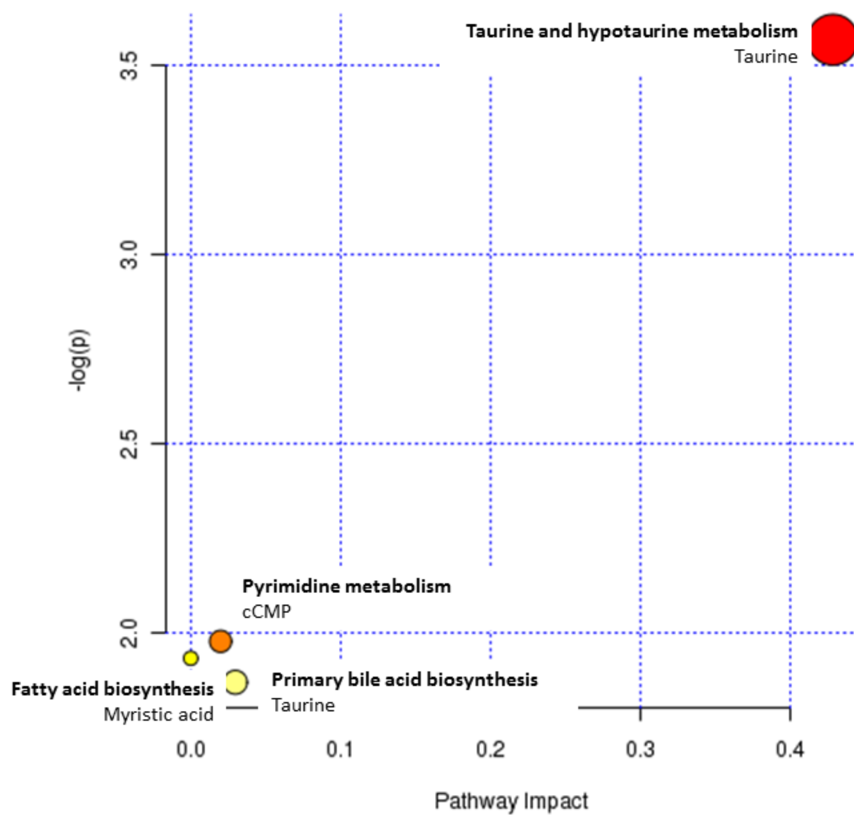


Figure 30. Pathways affected in the PFC according to the MetaboAnalyst 2.0 software.

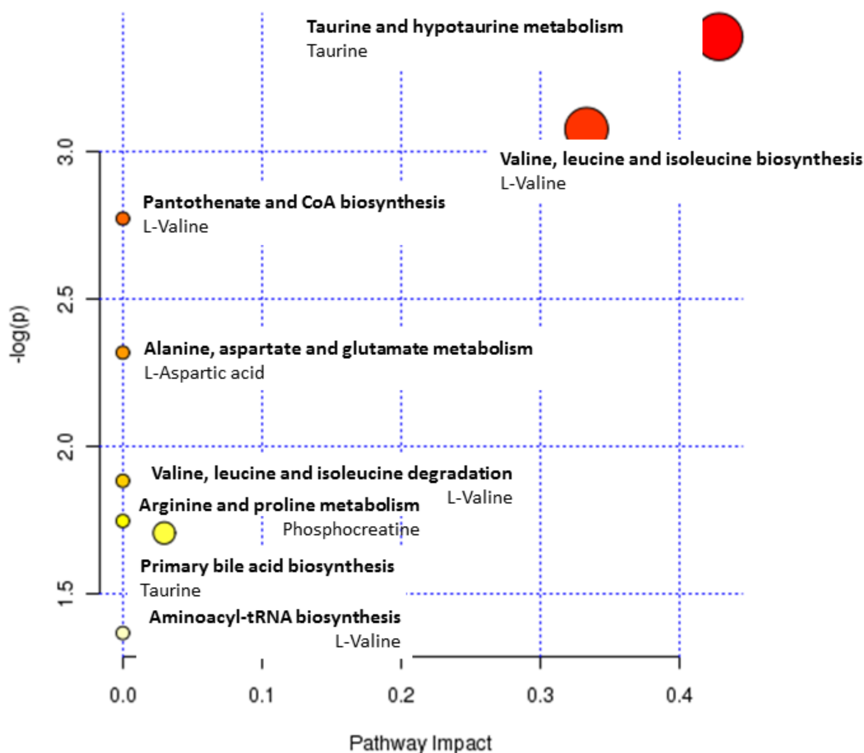


Figure 31. Pathways affected in the hippocampus according to the MetaboAnalyst 2.0 software.

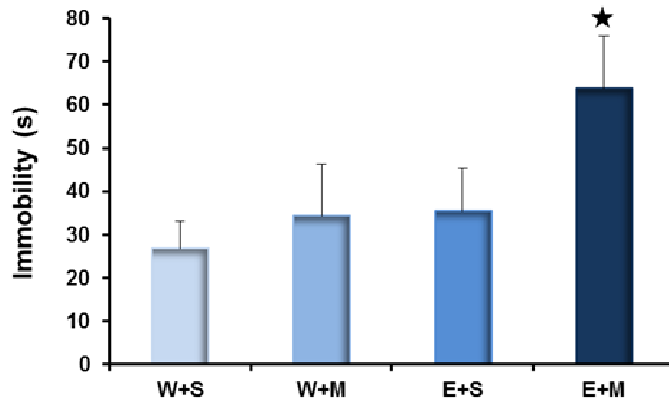
1.3.3 Imipramine and escitalopram prevent the long-term depressive-like behaviour induced by the combination of binge ethanol and MDMA

We have previously shown that the combination of binge ethanol and MDMA induced depressive-like behaviour 72 h after treatment (**Article 1**). With the aim for preventing despair behaviour, we administered imipramine (10 mg/kg), a tricyclic antidepressant and escitalopram (2, 5 mg/kg), a selective 5-HT reuptake inhibitor. Imipramine was selected since it is an effective antidepressant usually used in experimental animal studies. Escitalopram is a relatively new antidepressant and widely used in the clinic for co-morbidity syndromes of depression associated to alcohol abuse treatment at the present time (Witte et al. 2012).

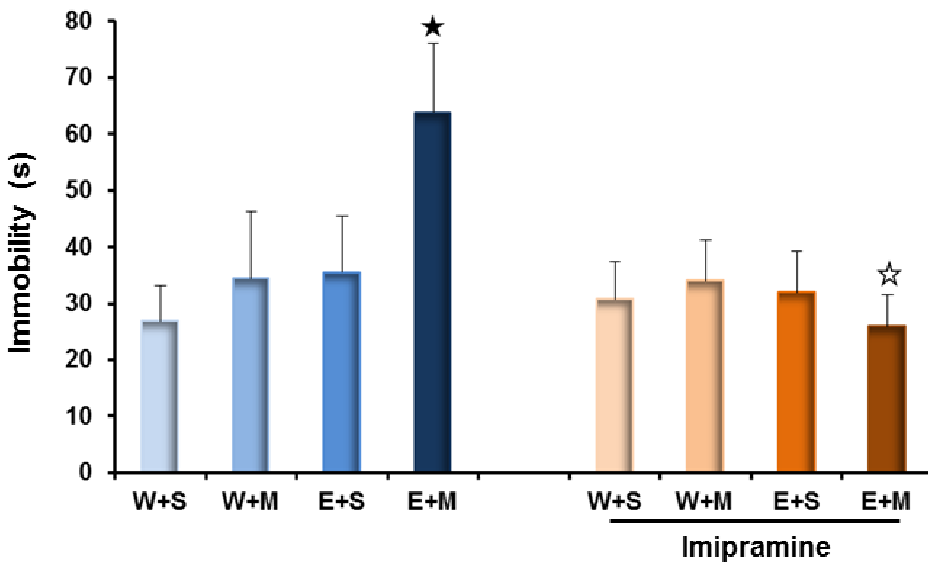
The antidepressants were administered during the second week of the DID procedure after the daily voluntary ethanol drinking period. Despair behaviour was determined evaluating the immobility time in the tail suspension test as previously described (**Article 1**).

We have demonstrated that ethanol and MDMA in combination caused depressive-like behaviour revealed as an increase in the immobility time in the tail suspension test (Figure 32A). One-way ANOVA analyses revealed a significant effect of drug administration [$F (3, 56) = 2.75$, $p < 0.05$]. As expected, Tukey post-hoc test revealed that the differences were between water + saline group and ethanol + MDMA group ($p < 0.05$). Regarding antidepressant treatment, two-way ANOVA analysis (drug treatment and antidepressant treatment factors) for imipramine treatment (Figure 32B) indicated no effect of drug abuse treatment, no effect of antidepressant treatment but interaction between both factors [$F (3, 107) = 2.5$, $p < 0.05$]. Tukey post-hoc test revealed that there were statistically significant differences between ethanol + MDMA plus imipramine and ethanol + MDMA group ($p < 0.05$). Two-way ANOVA analysis for escitalopram treatment (Figure 32C) showed no effect of the drug of abuse treatment but effect of the antidepressant treatment [$F (1, 109) = 7.5$, $p < 0.01$] with interaction between both factors [$F (3, 107) = 2.4$, $p < 0.05$]. Tukey post-hoc test revealed that there were statistical differences between ethanol + MDMA plus escitalopram when compared to ethanol + MDMA ($p < 0.01$). Therefore, we can postulate that both, imipramine and escitalopram treatment, may prevent the long-term depressive-like behaviour induced by the combination of binge ethanol and MDMA.

A)



B)



C)

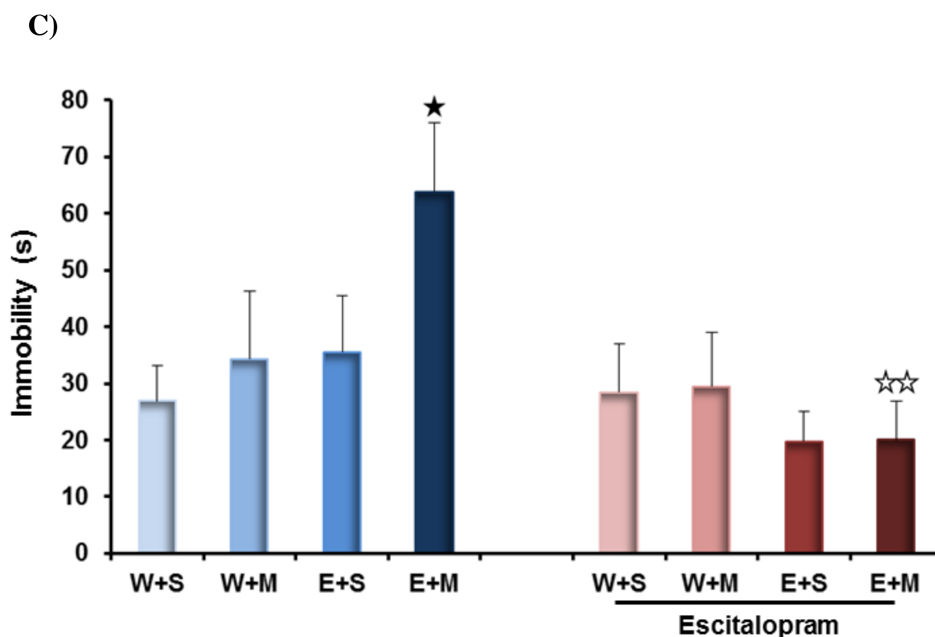


Figure 32. (A) Ethanol plus MDMA induce despair behaviour in the tail suspension test 72 hours after de last drug administration. (B) Antidepressant treatment with imipramine can prevent the depressive-like behaviour induced by binge ethanol plus acute MDMA treatment and (C) Antidepressant treatment with escitalopram can prevent the depressive-like behaviour induced by binge ethanol plus acute MDMA treatment. Data are expressed as mean ± SEM of errors performed (n = 12 - 15 mice/group).★ $p < 0.05$ when compared with water plus saline group. ☆ $p < 0.05$ and ☆☆ $p < 0.01$ when compared with ethanol plus MDMA group. (**W**, water; **S**, saline; **M**, MDMA; **E**, ethanol).

1.3.4 Imipramine and escitalopram treatment induces changes in the metabolomic profile when compared to ethanol plus MDMA treatment

Our interest is to find out a treatment that may prevent the pathological traits and analyse whether this has influence in the metabolic profile. In fact, we are trying to determine whether these antidepressants could prevent the change in the metabolic profile mentioned before. Since depressive-like behaviour was only observed in the

ethanol plus MDMA group and both antidepressants could prevent this alteration, the present metabolomic study was only performed in this specific group, with or without antidepressant treatment. Thus, analysis was performed in controls, ethanol plus MDMA, ethanol plus MDMA + imipramine and ethanol plus MDMA + escitalopram groups. A total of 4 samples were analysed for each group and each brain area studied. Analyses were performed using samples of the PFC and hippocampus since they may be involved in the depressive-like behaviour (Duman and Aghajanian, 2012). Our findings point towards that antidepressant treatment induces changes in the metabolomic profile induced by the combination ethanol and MDMA (Figures 33 to 38).

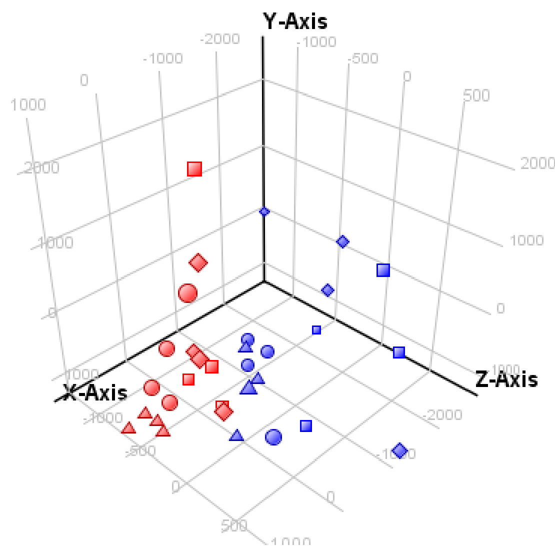


Figure 33. PCA analysis in positive ionization for all groups.
Red represents PFC and blue, hippocampus.

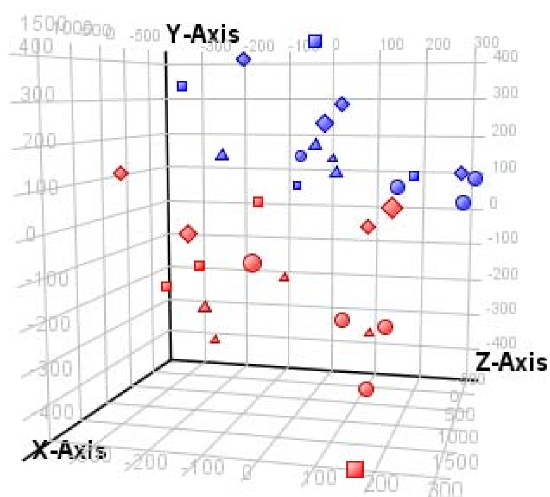


Figure 34. PCA analysis in negative ionization for all groups.
Red represents PFC and blue, hippocampus.

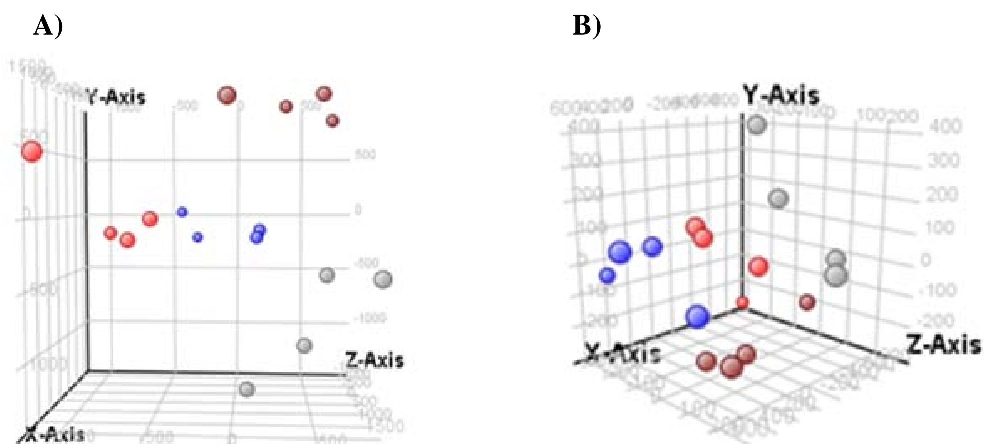


Figure 35. PCA analysis in mice PFC. A) Positive ionization and B) negative ionization. Grey balls represent control group; red balls, ethanol plus MDMA group; brown balls, ethanol plus MDMA + imipramine and blue balls, ethanol plus MDMA + escitalopram.

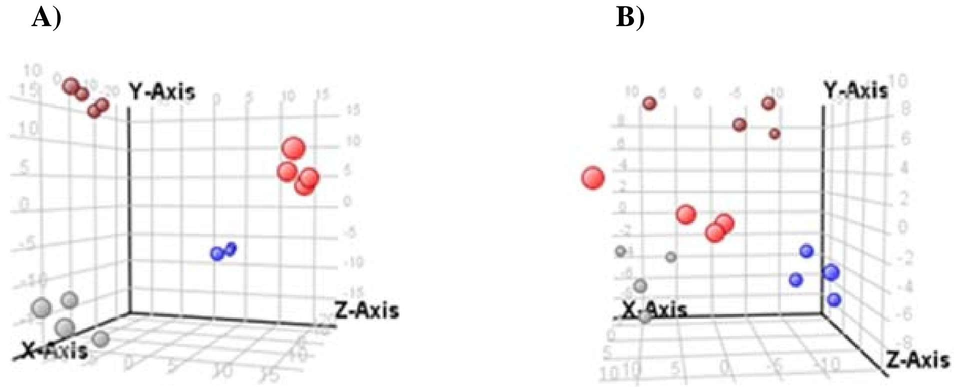


Figure 36. PLS-DA analysis in mice PFC. A) Positive ionization and B) negative ionization. Grey balls represent control group; red balls, ethanol plus MDMA group; brown balls, ethanol plus MDMA + imipramine and blue balls, ethanol plus MDMA + escitalopram.

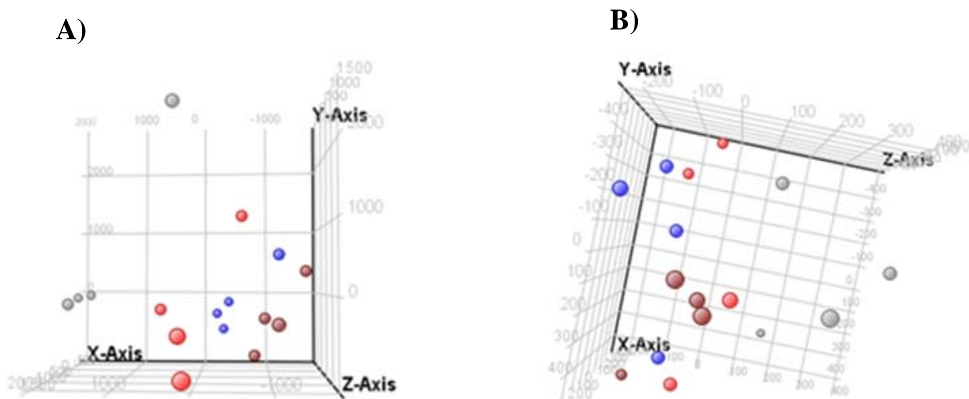


Figure 37. PCA analysis in mice hippocampus. A) Positive ionization and B) negative ionization. Grey balls represent control group; red balls, ethanol plus MDMA group; brown balls, ethanol plus MDMA + imipramine and blue balls, ethanol plus MDMA + escitalopram.

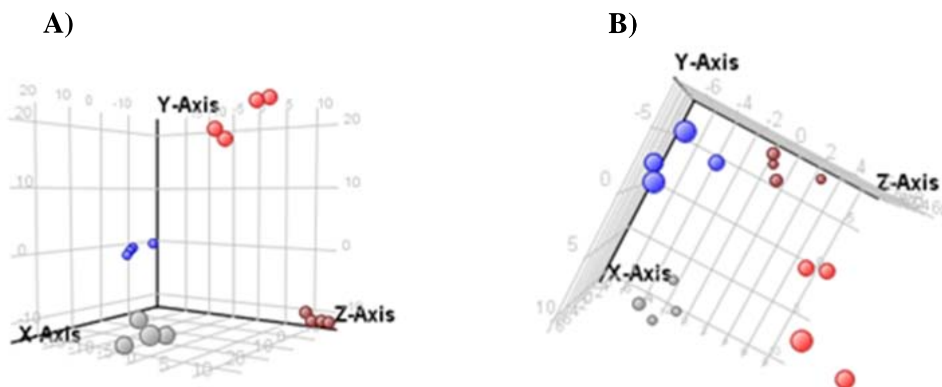


Figure 38. PLS-DA analysis in mice hippocampus. A) Positive ionization and B) negative ionization. Grey balls represent control group; red balls, ethanol plus MDMA group; brown balls, ethanol plus MDMA + imipramine and blue balls, ethanol plus MDMA + escitalopram.

These results show that i) metabolomic profile of the brain areas analyzed differ from each other and ii) antidepressant treatment changes the metabolic profile of the ethanol plus MDMA group. However, none of the antidepressants used returns the metabolic profile to the initial one (control group).

Subsequent analysis in the MetaboAnalyst 2.0 software indicated that the compounds that differ from controls (Table 3 and Table 4) were involved in different pathways (Figure 39 and Figure 40).

COMPOUND	ETHANOL+MDMA vs. CONTROL	ETHANOL+MDMA vs. E+M+Imipramine	ETHANOL+MDMA vs. E+M+Escitalopram
3-chlorotyrosine	down	down	down
Niacinamide	up	up	up
UDP-glucose	up	up	down

Table 3. Compounds identified in PFC showing deviation from control groups. Statistical Benjamini-Hochberg Multiple Testing Correction was used.

COMPOUND	ETHANOL+MDMA vs. CONTROL	ETHANOL+MDMA vs. E+M+Imipramine	ETHANOL+MDMA vs. E+M+Escitalopram
Myristic acid/ Isomyristic acid*	down	down	down
4-(diaminomethylideneamino) butanoic acid	down	up	up
7-keto-5-cholesten-3B-ol (7-ketocholesterol)	down	down	down
N-(2-hydroxyethyl)docosanamide	down	up	up
Pantothenic Acid	down	down	up
Retinaldehyde	down	down	down
UDP-N-acetyl-D-galactosamine	down	down	down

Table 4. Compounds identified in hippocampus showing deviation from control groups. Statistical Benjamini-Hochberg Multiple Testing Correction was used. *compounds with the same formula and very close retention time.

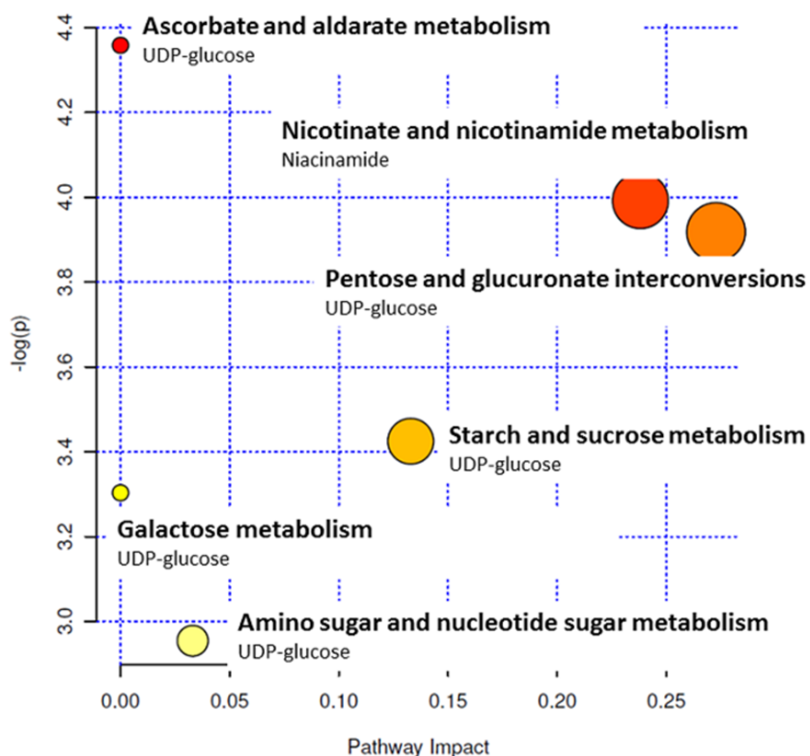


Figure 39. Pathways affected in the PFC according to the MetaboAnalyst 2.0 software.

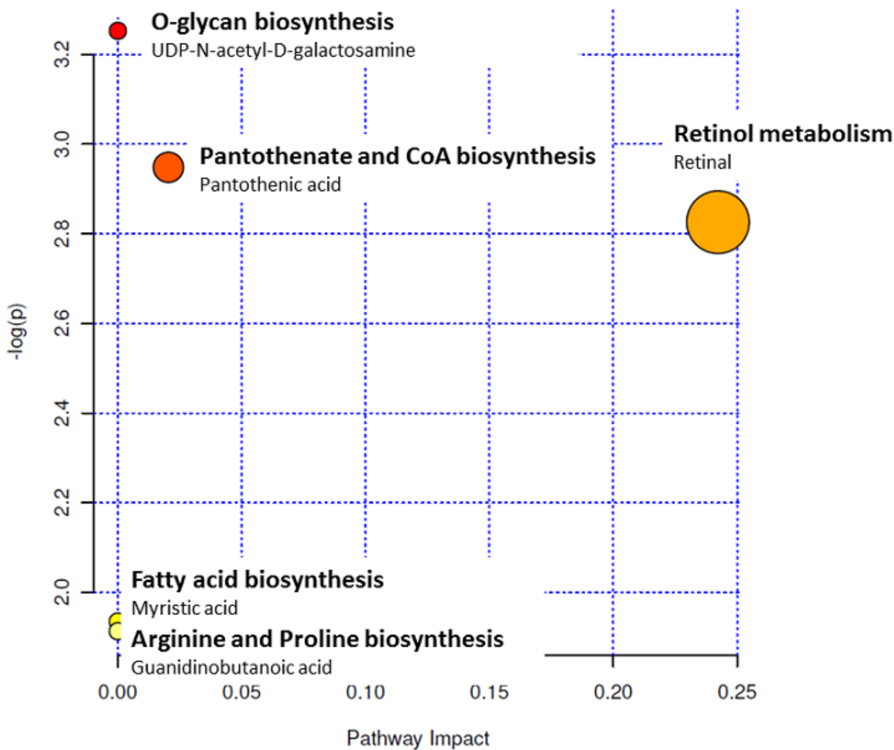


Figure 40. Pathways affected in the hippocampus according to the MetaboAnalyst 2.0 software.

Among the different pathways altered, those affecting tryptophan and 5-HT metabolism are of interest for our purposes. In that sense, amino acids such as valine, leucine or isoleucine, altered in the drug treated mice (Table 1 and 2, Figures 30 and 31) can compete with tryptophan for transport into the brain, and thus, the increase of these amino acids may be producing a decrease in 5-HT synthesis (De Myer et al. 1981; Capuron et al. 2002). Additionally, tryptophan can be metabolized to 5-HT or to kynurenines. Nicotinamide, increased in the PFC of ethanol plus MDMA treated mice (Table 3, Figure 39), is a final product of the tryptophan pathway to kynurenines (http://www.genome.jp/kegg/pathway.html). Therefore, an increase in nicotinamide can indicate increased kynurenine/tryptophan ratio and decreased 5-HT/tryptophan ratio (Kim et al. 2012).

Taking into account that escitalopram and imipramine, two 5-HT reuptake inhibitors, can prevent the depressive-like behaviour induced by binge ethanol plus MDMA, the hypothesis of the altered tryptophan metabolism is feasible. However, these are preliminary results and further analyses and experiments will be performed to provide a final conclusion to our hypothesis.

2. OBJECTIVE 2

2.1 Article 3

Ros-Simó C, Valverde O. Early-life social experiences in mice affect emotional behaviour and hypothalamic-pituitary-adrenal axis function. *Pharmacol Biochem Behav.* 102: 434-441.

The *objective* of this study was to develop an improved procedure of social isolation throughout development in CD1 mice and to elucidate its effects on behavioural parameters related to stress and neuroendocrine responses compared to enriched or social conditions. The *main results* were that CD1 mice exposed to an isolated environment exhibited higher locomotion and anxiety-like responses than animals exposed to social or enriched conditions. In addition, isolated animals showed lower basal plasma CORT than social or enriched ones but after a stressful event the elevation of plasma CORT was higher. These data suggest an enhanced response of the HPA axis to a novel and stressful situation. *In conclusion*, social interaction is an important feature to display an appropriate behavioural and neuronal development. Habituation to novel stimuli is impaired in subjects exposed to social isolation and induces increased excitability response to stressful events. Social deprivation increases the possibility of altered neuronal function and could facilitate the development of neuropsychiatric disorders in adulthood.

Ros-Simó C, Valverde O. Early-life social experiences in mice affect emotional behaviour and hypothalamic-pituitary-adrenal axis function. Pharmacol Biochem Behav. 2012; 102(3): 434-441.

DISCUSSION

Risk taking behaviours and social interactions are typical features of the adolescent stage. Of particular interest is drug consumption among human adolescents and young adults as it has become a serious public health problem in the last years (WHO, 2008; ESTUDES, 2010). In that sense, ethanol binge drinking is the most commonly used drug among youth, probably due to its legality and ease of getting. Moreover, this particular pattern of ethanol consumption has increased among Spanish adolescents between 14 and 18 years old (ESTUDES, 2010). Occasionally, ethanol binge drinking is combined with other drugs as MDMA (Winstock et al. 2001; Barrett et al. 2006). Such co-abuse usually reaches high levels of intoxication that can induce negative behavioural side effects as well as neurotoxicity and neurodegeneration.

On the other hand, early-life social and environmental experiences are essential for the growth from childhood through adolescence and into adulthood (Karelina and DeVries, 2011; Kidger et al. 2012). In that sense, social isolation or loneliness during these stages of maturation may induce emotional negative states, such as depression and/or anxiety. In contrast, social positive interactions may have beneficial effects on neurodevelopment. Growing up isolated or environmentally enriched can determine the individual's future mental health.

Furthermore, it has to be taken into account that adolescence is a critical period in which the brain undergoes important structural and physiological changes. Thus, the impact of drug abuse or early-life social stressful experiences during this critical stage of life may induce severe negative consequences that could be maintained into adulthood. In that sense, the present doctoral thesis attempts to i) uncover the long-term behavioural and neurotoxic consequences of the consumption of binge ethanol, MDMA or its combination during the adolescence period; and ii) elucidate the effects of social isolation vs. social enrichment during the preadolescent and adolescent period of life on behavioural parameters related to stress and

neuroendocrine responses into adulthood. Both studies have been conducted using adolescent CD1 mice.

Consequences of binge drinking and MDMA intake during adolescence

As stated above, binge ethanol and MDMA combination is widely abused among adolescents and young adults (Winstock et al. 2001; Barrett et al. 2006), becoming a serious public health problem. Therefore, the study of such interaction in adolescent mice would be a highly interesting research field to better understand the behavioural and neurologic consequences of such co-abuse.

Both binge ethanol and MDMA are drugs of abuse with rewarding properties in humans and experimental animals (White et al. 1994; Koob and Nestler, 1997; Volkow et al. 2011). Even though the reason why both drugs are widely taken in combination is not fully understood, it may induce longer lasting euphoria and feelings of well-being than the consumption of either drug alone (Hernández-López et al. 2002). It has also been proposed that ethanol may attenuate the negative side effects of MDMA, in particular, MDMA-induced hyperthermia (Winstock et al. 2001).

Despite the positive acute effects of these drugs of abuse, several studies have demonstrated that their consumption may cause negative consequences on brain and behaviour (Crews and Nixon, 2009; Parrott, 2006). In that sense, heavy binge drinking affects mood and cognitive performance (White, 2003; Grothues et al. 2008). Additionally, high doses of ethanol consumption can lead to neurodegenerative processes (Crews et al. 2000; Guerri and Pascual, 2010) that can be associated to the previously mentioned behavioural alterations. However, several studies have found that moderate doses of ethanol exert a neuroprotective role without inducing behavioural alterations on consumers (Collins et al. 2009; Vasanthi et al. 2012).

Meanwhile, MDMA is known to induce behavioural alterations such as hyperactivity, hyperthermia, anxiety-like behaviour and cognitive deficits in humans, non-primates and rodents (Green et al. 2001; Kuypers et al. 2006; Parrot, 2006). Its ingestion can also induce neurochemical and neuroendocrine alterations similar to those produced by exposure to acute stress, suggesting a potential role for MDMA as a "chemical stressor" (Pacifici et al. 2001; Connor, 2004). However, the main adverse consequence of its consumption is the neurotoxicity induced by this psychostimulant, which has been reported in different animal species (Green et al. 2004; Parrott, 2006; 2007; Capela et al. 2009; Sarkar and Schmued, 2010; Ruiz-Medina et al. 2011).

When ethanol and MDMA are administered together, results obtained differ between studies. While some assume an enhancement of the deleterious effects of each drug for separate (Johnson et al. 2004; Hernández-Rabaza et al. 2010; Rodríguez-Arias et al. 2011), others do not find such aggravating effects (Miller and O'Callaghan, 1994; Cassel et al. 2004; Vidal-Infer et al. 2012). It is worth mentioning that the long-term behavioural and neurotoxic alterations induced by the consumption of binge ethanol, MDMA or its combination may depend on the dose, frequency of usage, age- and sex-related differences, and genetics (Eckardt et al. 1998; Wallinga et al. 2009; Pardo-Lozano et al. 2012; Roger-Sánchez et al. 2012). Indeed, the effects will not be the same whether measured immediately after drug exposure (acute effects) or following a drug-free period (long-term effects) as is the case of our experiments.

In our studies, we used a relatively recent model of binge ethanol drinking previously described by Rhodes and co-workers (2005) called drinking in the dark, or DID, procedure with slight modifications in order to i) increase the time of ethanol exposure and ii) study the interaction between binge ethanol and MDMA in adolescent CD1 mice. CD1 mice are considered to have low preference for alcohol (Ryabinin et al. 2003; Rhodes et al. 2005). The differences in ethanol preference

between CD1 and other strains such as C57BL/6 J have been attributed to changes in ethanol metabolism and palatability (Rhodes et al. 2007). In any case, CD1 animals may reach a BAC high enough to cause behavioural effects as reported (**Article 1**). In our study, behavioural alterations were observed 72 hours and especially 7 days after drug treatment indicating that, under our experimental conditions, binge ethanol and MDMA induced long-term affectations. Contrary, 48 hours after treatment the unique alteration observed was a decreased locomotor activity in the ethanol plus MDMA group. Both ethanol and MDMA produce increases in spontaneous activity in humans and rodents (Hamida et al. 2008; Rodsiri et al. 2011). In this sense, different studies have found that ethanol potentiated the hyperlocomotion effect of MDMA in rats (Cassel et al. 2004; Hamida et al. 2006). The stimulant effects of acute ethanol are known to be mediated by the release of DA in the mesocorticolimbic system (Di Chiara and Imperato 1985; Nestby et al. 1997), whereas hyperlocomotion induced by MDMA in mice is due to both, a rapid release of DA in the mesocorticolimbic system (Touriño et al. 2008) and a release of 5-HT at the cortical level (Green et al. 2003). In these previous works such hyperactivity was observed after acute drug administration. In contrast, in our study the effect was observed 48 hours after drug administration. Thus, we suggest that the hypoactivity observed may be related to the behavioural alterations produced by the combination of both drugs rather than a specific pharmacological effect of each of the drugs.

Regarding motor coordination evaluated in the rotarod test, mice treated with ethanol showed motor skills alterations 72 hours and 7 days after treatment. Additionally, 7 days after MDMA treatment animals also performed it unsuccessfully. However, the combination of both drugs did not aggravate the motor coordination impairment observed in ethanol and MDMA groups. Ethanol administration induces long-lasting dysfunction of cerebellar neurons (Gruol et al. 1997; Wang et al. 1999), which are necessary for the integration of motor commands and sensory information to produce coordinated movements. Then,

ethanol induced damage to cerebellar neurons may cause the deficits observed in the rotarod test. MDMA produces neurotoxicity in striatal dopaminergic terminals in mice which has been shown to produce motor impairment, as previously demonstrated by our team (Touriño et al. 2010). Interestingly, previous studies reported that the highest loss of dopaminergic terminals in striatum (of about 70 %) has been observed exactly 7 days after the MDMA treatment (Colado et al. 2001, Sánchez et al. 2004). These findings agree well with the major motor alteration induced by MDMA observed in our study. An earlier study by Cassel and colleagues (2005) found that co-administration of these drugs induced a severe impairment of the sensory–motor coordination compared with ethanol or MDMA alone in rats exposed to the beam-walking test. Our results are not in agreement with this previous study since no enhanced motor coordination impairment was found in mice treated with both drugs when compared to MDMA or ethanol treated animals. The apparent discrepancy could be due to different animal species (rats vs. mice) as well as the different methodology used to assess motor coordination (rotarod apparatus vs. beam-walking test).

Emotional-like alterations were observed in mice receiving the combination of the two drugs. In that sense, anxiety-like and depressive-like behaviours were observed in binge ethanol plus MDMA treated mice 72 hours and 7 days after the last drug administration. Whereas MDMA alone did not induce mood alterations, binge ethanol alone induced despair (or depressive-like) behaviour. Anxiety and despair behaviour have been previously reported during ethanol withdrawal in experimental animals (File et al. 1993; Huang et al. 2010). It is noteworthy that in our experiments, ethanol treatment implies a voluntary ethanol drinking that lasts for two weeks. Therefore, at the doses ingested and at this specific pattern of consumption mice may have developed neuroadaptations, such as deregulation of hormonal systems (CRF) and neurotransmitters systems (5-HT and DA), that may induce such negative emotional states after ethanol withdrawal (Kalivas and O’Brien, 2008). Therefore, we suggest that the negative emotional states observed

in ethanol-treated mice might be due to ethanol withdrawal. Indeed, the acute administration of MDMA in the last day of ethanol treatment may enhance this negative emotional state. In fact, MDMA can also alter brain monoamines and hormones exacerbating the negative effects induced by ethanol withdrawal. The fact that the animals receiving both drugs are the only ones exhibiting anxiety-like behaviour together with the earlier onset of despair behaviour supports our hypothesis. Moreover, there are data in the literature demonstrating enhanced monoaminergic damage when both drugs are combined (Izco et al. 2007; Rodríguez-Arias et al. 2011; Vidal-Infer et al. 2012), which can be associated to the emotional alterations observed. In that sense, we have observed that a pre-treatment with imipramine (a tryciclic antidepressant) or escitalopram (a selective 5-HT reuptake inhibitor) can prevent the depressive-like behaviour observed in mice treated with ethanol plus MDMA, suggesting a potential role for 5-HT in the negative emotional states observed (Ros-Simó et al. data not published). Nevertheless, we cannot confirm this hypothesis since our study has not focused on the process of addiction *per se*. For that purpose, other models of ethanol administration would be necessary to be performed, i.e. ethanol self-administration.

Since the main long-term adverse consequence of adolescent drug intoxication is neurotoxicity, our objective was to get further knowledge about the neurotoxic processes induced by binge ethanol, MDMA or its combination. Body temperature was evaluated after drug administration given its close relationship with the neurotoxicity induced by the psychostimulant (O'Shea et al. 2006; Parrott, 2006). Indeed, ethanol administration could play an important role on the MDMA-induced hyperthermic response since it can modulate this response and thus, regulate the neurotoxic process (Johnson et al. 2004; Cassel et al. 2005; Izco et al. 2007). Our results showed that ethanol was able to abolish the MDMA-induced hyperthermic response (**Article 1**). The reason why ethanol induces the abolishment of the hyperthermia could be due to different aspects. For instance, causing peripheral vasodilatation, especially in the tail, where heat dissipation occurs (Green et al.

2005) or affecting the distribution, biotransformation and elimination of MDMA (Hamida et al. 2007), which may result in changes of the hyperpyretic effects of MDMA. However, the exact mechanism of this effect remains unclear. Mice receiving only ethanol did not show any difference in body temperature with respect to control group.

Regarding MDMA neurotoxicity, this may be different between species: while in humans, primates and rats it mainly causes damage to 5-HT nerve terminals, DA nerve terminals are mainly damaged in mice (de la Torre and Farré, 2004). However, the neurotoxic metabolites of MDMA that generate toxic DA quinones, ROS and RNS that lead to the increase in oxidative stress and free radical formation are common between species (Colado et al. 2001; Quinton and Yamamoto, 2006). Free radicals induce protein oxidative damage and lipid peroxidation causing neuronal damage (Jayanthi et al. 1998; Busceti et al. 2008). Simultaneously, there is an activation of microglial cells, the main immune cells in the brain, which induce the release of pro-inflammatory cytokines. Astrocytes are also activated after MDMA administration. Thus, microglial and astrocyte activation leads to an important neuroinflammation that may aggravate MDMA-induced neuronal damage (Touriño et al. 2010).

On the other hand, high amounts of ethanol intake induce oxidative stress that can be generated via its own metabolism or via the mitochondrial respiratory chain (Ingelman-Sundberg et al. 1984). As stated for MDMA, the increase in oxidative stress by high doses of ethanol may lead to protein, lipid and neuronal damage. Alcohol also induces the activation of glial cells such as microglia and astrocytes, which promote the release of ROS, nitric oxide and pro-inflammatory cytokines contributing to the ethanol-induced neurotoxicity and neurodegeneration (Vallés et al. 2004; Pascual et al. 2007; Qin et al. 2008; Zou and Crews, 2010). When MDMA and ethanol are co-administered, controversial results have been obtained regarding the aggravating or neuroprotective role of ethanol on the MDMA-induced

neurotoxicity (Miller and O'Callaghan, 1994; Johnson et al. 2004; Cassel et al. 2005; Rodríguez-Arias et al. 2011).

Our team has previously demonstrated that MDMA induced neuroinflammation in the striatum (Touriño et al. 2010; Ruiz-Medina et al. 2011). Now we have evaluated long-term neuroinflammatory responses under our experimental conditions of binge ethanol, acute MDMA or its combination. Thus, the time-course of astrocyte reactivity (anti-GFAP) and microglial expression (anti-Iba1) was determined in mice striatum by immunofluorescence (**Article 1**). Results indicated that only animals treated with MDMA alone exhibited clear astrocyte reactivity 48 hours after treatment, which was decreased after 72 hours and had disappeared 7 days after treatment. In the ethanol plus MDMA group, the astrocyte reactivity was attenuated. We suggest that the GFAP immunoreactivity response could be attributed to the observed MDMA-induced hyperthermia that may exacerbate neuroinflammation as previously demonstrated by our team (Touriño et al. 2010; Ruiz-Medina et al. 2011) and by other groups (Colado et al. 2001; Gudelsky and Yamamoto, 2003; Green et al. 2003). The attenuation of the astrocyte reactivity in ethanol plus MDMA group would confirm our hypothesis of the involvement of hyperthermia in such effect since the MDMA-induced increase in body temperature was abolished by ethanol. In addition, ethanol alone did not induce astrocyte reactivity in our experimental procedure.

Differently, microglial expression was enhanced in all MDMA treated animals with or without ethanol combination persisting more time in the MDMA alone treated group. Microglial expression was measured as a hypertrophy of microglial cells, consisting in a retraction and hypertrophy of the cell body indicating an activated state of the cells (Stoll and Jander 1999). This activation was observed 48 and 72 hours after treatment in the two MDMA-treated groups. However, one week after treatment it was only maintained in the group treated only with MDMA. Therefore, we can postulate that MDMA-induced neuroinflammatory responses can be

attenuated by its combination with ethanol (at least under the pattern of administration used in the present study). As suggested for astrocyte reactivity, this attenuation is probably associated to the abolition of MDMA-induced hyperthermia by ethanol. It is intriguing though, the absence of neuroinflammation after ethanol alone. We attribute it to the low ethanol intake by CD1 mice that, as mentioned above, are known to have low preference for alcohol (Ryabinin et al. 2003; Rhodes et al. 2005). Indeed, striatum is not a brain area highly affected by ethanol, as could be the PFC or the hippocampus (Nagel et al. 2005, De Bellis et al 2005). Thus, the lack of striatum neuroinflammation in the ethanol treated animals could be also attributed to this fact.

As described before, it is well established that oxidative damage occurs in brain after ethanol and MDMA administration in rodents and non-human primates (Busceti et al. 2008; Alves et al. 2009; Rump et al. 2010; Barbosa et al. 2012). The increased oxidative stress after drug administration can induce damage to proteins that negatively affect its function. Protein oxidative damage is known to be one of the most important causes of brain dysfunction (Berlett and Stadtman, 1997). Inactivation of specific proteins leads to alterations in specific metabolic pathways that can induce physiological consequences (Martínez et al. 2010). Although the exact proteins becoming oxidatively damaged have been studied in the livers of rats treated with MDMA (Moon et al. 2008) and mice treated with alcohol (Suh et al. 2004), they have never been detected and quantified in the brain. Therefore, we wanted to determine the specific proteins becoming oxidized after binge ethanol, MDMA or its combination in mice PFC and hippocampus (**Article 2**). Protein carbonyl formation is an irreversible protein modification and is one of the most studied markers resulting from oxidative stress that can be easily detected and quantified (Levine et al. 1994; Tamarit et al. 2012).

Despite significant results in the PFC were not obtained under our experimental conditions, different proteins involved in a variety of cellular functions were found

oxidatively damaged in the hippocampus of drug administered animals 72 hours after treatment. These proteins, involved mainly in energy production pathways, neuronal function, outgrowth, stability and repair, included α-enolase, glyceralehyde-3P-dehydrogenase, aconitate hydratase, α and β subunit of the ATP synthase, CRMP-2, actin, α-internexin, synapsin-1 and HSC 71. Even though oxidative damage was observed in all drug treated groups, animals that received MDMA exhibited higher oxidative damage than those who had not received the psychostimulant. In contrast to the results of striatum neuroinflammation, ethanol did not exert any attenuation of the oxidative damage in the hippocampus. In this case, we assume that the abolishment of the MDMA-induced hyperthermia by ethanol did not attenuate the MDMA-induced oxidative damage to specific proteins. Thus, we propose that hyperthermia may influence the neurotoxicity induced by the psychostimulant but is not the unique factor to be considered, which would be in accordance with previous published data (Malberg et al. 1996; Green et al. 2004). Furthermore, ethanol alone induced carbonylation of specific proteins related to energy metabolism or synapsin-1, which is a vesicle-specific protein implicated in neurotransmitter release to the synaptic cleft and in hippocampus neuronal development (Evergren et al. 2007).

Proteins related to energy metabolism have also become oxidized after acute MDMA contributing to an energy deficiency after drug treatment. This result could explain the observation that MDMA decreases brain ATP production that results in subsequent membrane ionic deregulations, calcium entry and additional free radical formation (Darvesh and Gudelsky, 2005). Moreover, carbonylation of proteins related to energy metabolism has also been found in brains affected by Huntington (Sorolla et al. 2010), Parkinson (Malkus et al. 2009) or Alzheimer (Castegna et al. 2002) neurodegenerative diseases. Overall, these findings strongly suggest that the impact of binge ethanol and MDMA on brains from adolescents would lead to a neurodegeneration found in elderly brains and thus, inducing an early aging.

Taking into account the results obtained in the analyses of oxidative damage to specific proteins, we hypothesized that this damage could induce impairments in the brain area affected. In order to get further into this hypothesis we evaluated whether the administration of binge ethanol, MDMA or its combination induced long-term memory deficits since hippocampus is the main brain area involved in learning and memory processes (Morris et al. 1982). For that, we studied possible deficits on declarative memory evaluated 72 hours after treatment, under drug absence. Therefore, we carried out two different paradigms. Firstly, the object recognition test involving the hippocampus and the perirhinal cortex strongly interconnected (Wixted and Squire, 2011). The other paradigm used was the radial arm maze, which involves different aspects of spatial reference and working memory, involving the hippocampus but also the PFC, respectively (Olton and Samuelson, 1976; Bizon et al. 2012). We found that under our experimental conditions, a pattern of binge ethanol drinking did not induce memory deficits in any of the tests used. Instead, the acute and neurotoxic dose of MDMA affected consolidation of declarative memory in both learning tasks conducted. The combination of both drugs of abuse did not induce an exacerbation of the cognitive deficits observed after MDMA alone.

There are few and inconsistent data in literature regarding the effects of the combination of binge ethanol and MDMA on cognition. In that sense, there are works that report memory impairments only when drugs are administered in combination (Hernández-Rabaza et al. 2010). Other studies find memory deficits with the administration of either drug alone without an enhancement of the deficits when ethanol and MDMA are administered together compared to either drug alone (Vidal-Infer et al. 2012). It is worth mentioning that discrepancies could arise from different doses and treatment schedules used. Therefore, further research in this field is necessary to elucidate the effects of such combination on memory processes in rodents. In humans, effects on cognition have been observed for each drug

separately and for the combination of them, without evidences of synergistic effects when administered together (Dumont et al. 2008).

Contradictory results have been found when each drug was studied alone. Thus, whereas several studies have described spatial memory deficits in human alcoholics (Bowden and McCarter, 1993), and animal models of alcoholism (Pascual et al. 2011), others do not have found such damage after ethanol consumption (Popovic et al. 2004). These discrepancies could be attributed to methodological differences. In fact, ethanol-induced neurologic changes and the potential for recovery seem to be dependent on length of ethanol exposure, volume of ethanol, degree of withdrawal signs or number of binge bouts, genetics and age (Crews and Nixon, 2009). In that sense, a pattern of binge ethanol exposure during adolescence impacts the developing brain inducing neurologic consequences, such as brain impairments as well as cognitive and behavioural dysfunctions (Guerri and Pascual, 2010).

In our experiments, ethanol alone did not induce long-term memory deficits 72 hours after last drug administration in any of the two cognitive paradigms used. As stated above, animals used for all these studies have low preference for alcohol since their ethanol ingestion was 1-2 g/kg/day (**Article 1**). Thus, one possibility is that the amount of ethanol ingested is not enough to produce alterations on memory when there is sufficient acquisition period (3 and 12 days in the object recognition and the radial arm maze, respectively). In agreement with this hypothesis, it has been reported that low doses of ethanol (0.5 g/kg) administered for four days 30 min before the first daily training session did not impair spatial learning in rats (Acheson et al. 2001; Novier et al. 2012). Additionally, a study by Robles and Sabriá (2008) showed that chronic ethanol consumption, at moderate levels, did not affect acquisition and performance of new associative learning and even improved some kind of learning paradigms. More recently it has been reported that adolescent intermittent ethanol treatment did not alter adult spatial learning on the Barnes maze (Vetreno and Crews, 2012). Indeed, it has been described that whereas acute

ethanol induces memory impairments, a tolerance to the amnesic effects is developed after repeated treatment with low doses (0.6 g/kg) (Kameda et al. 2007). Although numerous studies indicate memory impairment induced by ethanol (Markwiese et al. 1998; Spanagel, 2009), if administered at moderate doses these deficits are not so clearly demonstrated. Indeed, results might be different whether these memory deficits are observed during ethanol intoxication or after some days, during ethanol absence.

On the other hand, acute MDMA administration led to impairments in both, the object recognition and the radial arm maze tests. Different learning and memory tasks following MDMA administration have been studied in rodents. For instance, non-spatial memory has been evaluated resulting on memory impairment in MDMA treated rats (Camarasa et al. 2008) and mice (Nawata et al. 2010). In agreement with our results, rats in the radial arm maze (Kay et al. 2011) and rats or mice in the Morris water maze (Camarasa et al. 2008, Busceti et al. 2008) exhibited signs of spatial reference memory impairment without alterations in working memory, which depends on the PFC. However, in all these studies animals received the MDMA treatment before the acquisition period while in our procedure animals had already acquired the task before the MDMA administration. Thus, our results indicate that MDMA is affecting the memory consolidation process of the previous acquired learning. In line with these data, previous studies have shown that MDMA impaired acquired tasks in different behavioural paradigms in mice, such as operant-delayed alternation task involving working memory but not spatial reference memory (Viñals et al. 2012) or active avoidance performance (Trigo et al. 2008), involving emotional memory.

Interestingly, carbonylation of proteins related to axonal and dendritic outgrowth was higher in MDMA treated than in non-MDMA treated mice, suggesting that memory deficits could be related to affectations of the psychostimulant to these specific proteins. CRMP-2, which is highly carbonylated in MDMA treated mice, is

critically involved in axonal outgrowth and pathfinding through the transmission and modulation of extracellular signals (Fukata et al. 2002). Indeed, as well as HSC71, it has been reported to be oxidatively modified in Alzheimer's disease brain (Castegna et al. 2002) and has been related to memory loss associated with decreased interneuronal connections and to a shortened dendritic length (Coleman and Flood, 1987). Actin, only carbonylated in MDMA treated subjects, has a crucial role in the cytoskeleton network integrity (Fletcher and Mullins, 2010) and is concentrated in dendritic spines where it can produce changes in their shape that might be involved in memory function (Morgado-Bernal, 2011). α-internexin which was found carbonylated in the ethanol plus MDMA group, is also a neurofilament involved in neuronal development, axonal outgrowth and regulation of axonal stability. Thus, considering that memory deficits are observed only in MDMA treated subjects, it could be suggested that these proteins, mainly involved in axonal and dendritic stability, might have a role in the MDMA-induced cognitive impairments observed. Furthermore, considering the well-established MDMA-induced dopaminergic and serotonergic axon terminal loss (Sprague et al. 1998; Puerta et al. 2009), it can be suggested that carbonylation of proteins related to axonal stability would be involved in this mechanism of neurodegeneration. Proteins becoming oxidized in the hippocampus of ethanol treated animals may have a relation with other ethanol-induced behavioural alterations previously described (**Article 1**). Obviously, damage to the specific proteins observed in this study is not the only mechanism by which drugs of abuse induce neurotoxicity associated to behavioural and cognitive deficits. In any case, it is one step further in the understanding of the brain damage induced by drugs of abuse, in our case ethanol and MDMA.

Metabolomic approaches may be useful to identify potential biomarkers for drug-induced affectations in CNS. Therefore, we have also initiated a new line studying the metabolomic changes on brains of mice treated with binge ethanol, acute MDMA or the combination of both drugs 72 hours after last drug administration.

Our results on PCA and PLS-DA analyses indicated that metabolism of each brain area shows different traits and this metabolism is affected by the administration of drugs of abuse. We also wanted to determine the metabolites responsible for these differences. To date, only preliminary results have been obtained which indicate that taurine metabolism, pyrimidine metabolism and fatty acid biosynthesis are altered in the PFC of drug-treated animals compared to water + saline treated mice (Table 1). In the hippocampus of mice treated with binge ethanol, MDMA or its combination more metabolic pathways were altered when compared to control group (Table 2), including taurine metabolism, aminoacyl t-RNA biosynthesis, pantothenate and coenzyme A as well as metabolism of several amino acids such as valine, leucine and isoleucine biosynthesis and degradation; alanine, aspartate and glutamate metabolism; and arginine and proline metabolism. As a general comment, these results indicate that amino acid metabolism is altered 72 hours after last day of treatment, during drug absence, and accounts for the differences observed in the metabolomic profile. It has been reported that altered valine metabolism may be related to decreased 5-HT synthesis since valine competes with tryptophan for transport into the brain. Similarly to valine, other amino acids such as leucine or isoleucine are also competing with tryptophan enhancing the possibility of decreased 5-HT synthesis (De Myer et al. 1981; Capuron et al. 2002). Thus, the hypothesized decreased 5-HT synthesis could lead to the depressive-like behaviour exhibited by animals treated with ethanol plus MDMA group. Moreover, and as discussed before, imipramine and escitalopram can prevent this depressive-like behaviour (Figure 32B and 32C); which would be in agreement with our hypothesis.

Additionally, PCA and PLS-DA analysis were performed in order to determine whether the treatment with imipramine or escitalopram, which prevented the depressive-like behaviour induced by the combination of binge ethanol and MDMA, may bring the metabolomic profile of this particular group (ethanol plus MDMA) back to that of the control group. These analyses, only performed in PFC

and hippocampus for their involvement in depressive symptoms (Duman and Aghajanian, 2012), indicated that both antidepressant treatments induced a modification of the metabolomic profile. Although at the present we cannot postulate that imipramine or escitalopram are preventing the metabolomic alterations, the fact that they are preventing the depressive-like behaviour may indicate a potential protection towards the emotional negative effects of the combination of binge ethanol plus MDMA. When analysing the specific metabolites that induce these differences, and following the hypothesis of altered tryptophan metabolism, we found increased nicotinamide in PFC of ethanol plus MDMA treated mice when compared to the other groups. According to the KEGG pathways (www.genome.jp/kegg/pathway.html), nicotinamide is a product of tryptophan metabolism when this is transformed to kynurenine instead of 5-HT. Thus, if nicotinamide is increased it would mean that tryptophan metabolism is displaced to the formation of kynurenines and consequently levels of 5-HT may be decreased. In fact, there are works demonstrating a link between depression and the tryptophan breakdown and kynurenine pathway (Myint et al. 2012). Moreover, the imbalance in this tryptophan metabolism may be induced by pro-inflammatory mediators and ROS (Myint et al. 2012; Anderson et al. 2012).

It must be considered that these are only preliminary results. In order to verify our hypothesis of altered tryptophan metabolism with the subsequent decrease in 5-HT levels, more experiments will be necessary: (i) determination of other metabolites of the same pathways such as kynurenic acid or quinolinate. To that purpose, standards commercially available (or custom synthesized) would be used to unequivocally establish the retention time and mass of these specific metabolites, (ii) assessing 5-HT and tryptophan levels and (iii) determination of the expression of key enzymes in the metabolic pathway altered such as indoleamine-2, 3-dioxygenase which is the rate-limiting enzyme of tryptophan metabolism to kynurenines (Kanai et al. 2009; Kim et al. 2012).

Consequences of early-life social experiences into adulthood

The consequences of environmental and social factors during preadolescence and adolescence into adulthood have been studied for some time in humans and experimental animals (Lai and Huang, 2011; Kidger et al. 2012). Several procedures of environmental enrichment and social isolation have been developed in mice and rats (Karim and Arslan, 2000; van Praag et al. 2000; Nithianantharajah and Hannan, 2006). However, there are no consistent data from behavioural and neurochemical studies obtained from animals exposed to different environmental conditions. In that sense, while there are authors reporting reduced depressive-like responses in animals reared under enriched conditions (Brenes et al. 2008; Workman et al. 2011) or increased depressive-like behaviour in isolated animals (Ma et al. 2011), others find increased mobility in the tail suspension test after isolation associated to increased excitability (Karim and Arslan, 2000). Indeed, there are works where they simply describe no differences between groups (Silva et al. 2011). In contrast, increased anxiety-like behaviour has been mostly reported in animals housed under isolated conditions (Hellemans et al. 2004; Wei et al. 2007) and decreased anxiety in enriched animals (Benaroya-Milshtein et al. 2004; Peña et al. 2006). However, there are studies indicating increased exploration of the open arms in the elevated plus maze after social isolation (Võikar et al. 2005).

As mentioned in the introduction, the HPA axis plays an important role in the responses to stress (De Kloet et al. 1990; Armario et al. 2006). Indeed, adolescents have been demonstrated to exhibit increased HPA axis reactivity to social stressors (Gunnar et al. 2009; Stroud et al. 2009). Therefore, markers of HPA functionality determined by levels of plasma CORT or ACTH have been often evaluated when studying the effects of home environmental conditions during preadolescence and adolescence stages. Similarly, results regarding HPA response to different home environmental conditions are controversial. In this context, whereas there are studies that have found elevated plasma CORT secretion in adult mice kept under

environmental enrichment (Marashi et al. 2003), others report no differences in plasma CORT levels (Schrijver et al. 2002, Peña et al. 2009) and some even report a reduction of plasma CORT in enriched adult rats (Belz et al. 2003). Even though altered HPA axis activity is found after differently housed conditions in rodents, the exact mechanism of the HPA impaired function is not fully understood. It is important to mention that the type and strain of rodent as well as the age and sex of the animal used may be responsible for the controversial results reported.

It is worth remembering that most of the previous works have considered only two different conditions, for instance, enrichment vs. standard conditions (Moncek et al. 2004; Konkle et al. 2010) or isolation vs. standard conditions (Karim and Arslan, 2000; Bartolomucci et al. 2003). Moreover, rat is the most used experimental animal model to study environmental and social interactions effects on adulthood phenotype (Varty et al. 2000; Peña et al. 2006; Deehan et al. 2007). Therefore, the first objective of our study was to develop an improved experimental method in preadolescent CD1 mice (postnatal day 21) where the three possible environmental conditions were studied simultaneously (**Article 3**), which is methodologically more correct and may strengthen the results obtained.

Our data demonstrate mood disorders into adulthood in animals that were housed under isolation compared to enriched and social housed. Particularly, anxiety-like behaviour is observed after seven weeks of isolation in two different paradigms, the elevated plus maze and the dark-light box. Besides, isolated housed mice show increased locomotor activity. This hyperactivity is maintained through the three evaluated days suggesting that isolated animals do not habituate to the locomotor activity boxes. The increase in locomotion together with the anxiety-like behaviour observed leads us to suggest that animals housed in isolated conditions show an increased excitability and nervousness when exposed to a novel situation. Results from Varty and colleagues (2000) are in accordance with ours since they proposed that rats housed under enriched conditions have a better capability to assimilate

stimuli from their environment than rats reared under isolated conditions (Varty et al. 2000). Additionally, in the tail suspension test, isolated animals showed increased mobility as well, in accordance with previously reported by Karim and Arlsan (2000). Thus, this result would strength our hypothesis of impairment on behaviour adaptations into adulthood of mice housed in isolated condition during the pubertal stage of life.

The second objective of our study was to find an association between the altered behaviour and HPA activity. For that, levels of plasma CORT were evaluated before (basal levels) and after a stressful situation. Our results revealed lower basal levels in animals reared under isolation compared to enriched and standard housed mice. However, after a stressful event such a 5-minutes period in the dark-light box, levels of plasma CORT showed a significant increase in comparison to basal levels only in animals housed under isolation. Despite the inconsistency of the literature, our results are in agreement with those previously reported by Miachon et al. (1993), Sánchez et al. (1995) and Djordjevic et al. (2012) who also reported decreased plasma CORT levels after housing under IC. The high increase in plasma CORT after a stressful situation agrees well with our hypothesis. However, it would have been interesting to determine levels of ACTH as well. It is reported that ACTH and CORT secretion have different time-course meaning that ACTH maximum levels are observed after 5-10 minutes after the start of exposure to stressors whereas maximum CORT levels are achieved after 20-30 minutes (Armario, 2010). Moreover, adrenocortical secretion may saturate with intermediate levels of plasma ACTH, so plasma levels of CORT may not reflect ACTH release if a unique time-point is evaluated (Armario, 2006). Thus, evaluate only CORT levels can lead to erroneous interpretations (Armario, 2010).

With the results obtained we can suggest that an abnormal HPA axis response to a stressful situation seems to be responsible for the impairment in coping with novel environmental stimuli in isolated animals. In that sense, our conclusion is close to

that of Bartolomucci and colleagues (2003) who proposed that individual housing seems not to be stressful *per se*, but induces increased negative reaction to subsequent stressful events.

Overall, our findings show that drug abuse and social environment are crucial events in the development from adolescence to adulthood. Adolescents being exposed to habits of drug consumption or to negative early-life social experiences are more prone to exhibit psychopathological states such as mood disorders or poor cognitive performance in adulthood. This altered behaviour is probably associated to neurotoxic and neuroendocrine affectations that negatively impact the immature brain.

CONCLUSIONS

The final conclusions of the present doctoral thesis are the following:

1. Behaviour is affected by the combination of binge ethanol and acute MDMA. In that sense, such combination induces long-term anxiety-like and depressive-like behaviour in adolescent mice. While binge ethanol alone induces depressive-like behaviour, MDMA does not induce any affectation.

2. The MDMA-induced hyperthermic response is abolished by ethanol treatment. MDMA-induced neuroinflammation in mice striatum is attenuated by ethanol. Therefore, we postulate that ethanol modulates MDMA neuroinflammatory response by attenuating the hyperthermic effect induced by the psychostimulant.

3. Binge ethanol and acute MDMA administration induce oxidative damage to brains of adolescent mice, being a similar process to that observed in several neurodegenerative diseases.

4. MDMA alone induces deficits in declarative memory. Specific oxidative damage to hippocampal proteins related to axonal and dendritic outgrowth may contribute to these MDMA-induced memory deficits.

5. Neurotoxicity is more prominent after MDMA administration rather than binge ethanol without a significant enhancement when both drugs of abuse are co-administered.

6. Depressive-like behaviour induced by the combination of binge ethanol plus MDMA is prevented by imipramine and escitalopram treatment, indicating a role of 5-HT in this effect. Metabolomic studies suggest a possible tryptophan imbalance in drug-treated mice reinforcing the hypothesis of altered tryptophan/5-HT metabolism.

7. Early-life stressful experiences such as social isolation induce an altered response to novel situations in the adulthood. This is probably due to altered HPA axis reactivity since isolated mice show low levels of CORT at basal levels which are abnormally increased after a stressful event.

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Lexicon of alcohol and drug terms published by the World Health Organization

http://www.who.int/substance_abuse/terminology/who_lexicon/en/

MetaboAnalyst 2.0 –a comprehensive tool suite for metabolomic analysis http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp

National Institute of Alcohol and Alcoholism http://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/moderate-binge-drinking