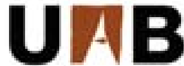

Universitat Autònoma de Barcelona

DEPARTAMENT DE FARMACOLOGIA, DE TERAPÈUTICA I DE TOXICOLOGIA
UNIVERSITAT AUTÒNOMA DE BARCELONA (UAB)

"Pharmacogenetic mechanisms (*COMT* and *hSERT*) modulating deleterious effects of MDMA and cannabis on cognitive performance in drug users"

Memòria presentada per Elisabet Cuyàs Navarro per optar al títol de doctora per la Universitat Autònoma de Barcelona. Treball realitzat sota la direcció del Dr. Rafael de la Torre Fornell i la tutoria del Dr. Magí Farré, en el Grup de Recerca Clínica en Farmacologia Humana i Neurociències, IMIM-Hospital del Mar, Parc de Recerca Biomèdica de Barcelona. Programa de Doctorat de Bioquímica i Biologia Molecular de la Universitat Autònoma de Barcelona.

Dr. Rafael de la Torre Fornell (Director de tesi)	Dr. Magí Farré Abaladejo (Tutor)	Elisabet Cuyàs Navarro (Doctoranda)

RESUM

La neurotransmissió dopaminèrgica i serotonèrgica al còrtex preforntal i a les regions del sistema límbic són un dels principals substrats cerebrals de les funcions executives (implicades en l'organització i el control) i la regulació emocinal. Tant la MDMA com el cannabis alteren la funcionalitat d'aquests sistemes de neurotransmissió. Certs polimorfismes funcionals de la catecol-O-metiltransferasa (COMT) i del transportador de serotonina (hSERT) s'han associat a diferències individuals en el funcionament cognitiu. La interacció entre ambdós gens juntament amb factors ambientals poden explicar la major o menor susceptibilitat dels consumidors a els efectes deleteris de les substàncies psicoactives en les arees.
L'objectiu és investigar la interacció de diversos polimorfismes relacionats amb els sistemes serotonergic i amb el rendiment d'una població de consumidors de MDMA i cannabis en la realització de diverses tasques neuropsicològiques (memòria i funcions executives pricipalment).

Was it all worth it, giving all my heart and soul,

Staying up all night, was it all worth it,

Living breathing rock n'roll this never ending fight,

Was it all worth it, was it all worth it,

Yes, it was a worthwhile experience,

It was worth it.

Queen, *The Miracle* (1989)

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Gràcies a tots!

18s rRNA: 18S ribosomal RNA

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: serotonin

5HT2A: serotonin receptor 2A

AACD, Ddc: aromatic amino acid decarboxylase

Actb: β-actin

BDNF: brain-derived neurotrophin

bp: base pairs

cDNA: complementary DNA

CNS: central nervous system

COMT: catechol-O-methyltransferase

CVLT: California Verbal Learning Test

CYP: cytochrome P450 isozyme

DA: dopamine

DOPA: dihydroxyphenylalanine

DRN: dorsal raphe nucleus

Gfap: glial fibrillary acidic protein

HHA: 3,4-dihydroxyamphetamine

HHMA: 3,4-dihydroxyymethamphetamine

HMMA: 3-methoxy-4-hydroxymethamphetamine

Hspa1a: heat shock 70kD protein 1A

LTD: long term depression

LTP: long term potentiation

MAOA: monoamine oxidase A

MAOB: monoamine oxidase B

MDA: 3,4-methylenedioxyamphetamine

MDMA: 3,4-methylenedioxymethamphetamine

mGluRs: glutamate metabotropic receptors

mRNA: messenger RNA

rRNA: ribosomal RNA

tRNA: transport RNA

NE: norepinephrine

NGF: nerve growth factor

NMDARs: N-methyl-D-aspartate receptors

NT-3: neurotrophin-3

NT-4: neurotrophin-4

p75NTR: p75 neurotrophin receptor

PCR: Polymerase chain reaction

PLC: phospholipase C

PLD: phospholipase D

RAVLT: Rey Auditory Verbal Test

RIN: RNA integrity number

RNS: reactive nitrogen species

ROCFT: Rey-Osterrieth Complex Figure Test

ROS: reactive oxygen species

Slc18a2, VMAT: vesicular monoamine transporter

Slc6a2, NET: norepinephrine transporter

Slc6a3, DAT: dopamine transporter

Slc6a4, 5-HTT: serotonin transporter

Snca: synuclein α

Sncg: synuclein γ

SNP: single nucleotide polymorphism

SRI: serotonin reuptake inhibitors

TH: tyrosine hydroxylase

TLDA: TaqMan® Low Density Array

TPH1: tryptophan hydroxylase 1

TPH2: tryptophan hydroxylase 2

Trk: tyrosine kinase receptor

VNTR: variation number tandem repeat

WAIS III: Wechsler Adult Intelligence Scale III

WHO: World Health Organization

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BACKGROUND

The use of MDMA has been associated to acute toxicity and long term-term neurotoxicity. In animal models, neurotoxicity involves mainly an axonopathy of the serotonergic neurotransmission system. In humans, part of these neurotoxic effects is translated into cognitive impairments, particularly in the memory and executive functions domains. In this regard, several studies were undertaken by our research group in order to shed some light into these issues.

The ENTE study (**E**cstasy **N**euro**T**oxic **E**ffects) recruited one hundred seventeen participants (37 MDMA polydrug users , 23 cannabis users, and 34 non drug users) followed during four years. Participants were evaluated in terms of immune system functionality (Pacifici et al., 2007), cognitive performance (de Sola Llopis et al., 2008a;de Sola Llopis et al., 2008b) and prevalence of psychopathology (Martín-Santos et al., 2010).

Regarding cognitive performance, at baseline, ecstasy polydrug users showed significantly poorer performance than cannabis users and non drug using controls in a measure of semantic word fluency. When ecstasy users were classified according to lifetime use of ecstasy, the more severe users (more than 100 tablets) showed additional deficits on episodic memory. After two years, ecstasy users showed persistent deficits on verbal fluency, working memory and processing speed.

With respect to psychopathology associated to ecstasy use, mood and anxiety disorders are the most prevalent psychiatric diagnoses. Dysfunction in the serotonergic system is the most widely accepted mechanism in the neurobiology of depression, and also one of the main targets of MDMA-induced neurotoxicity. In the 3-year follow-up, incident cases of depressive disorders were more primary than substance-induced and only observed in the ecstasy users group.

In the ENTE study, genomic DNA was collected and preliminarily the association of the serotonin transporter polymorphism (*5-HTTLPR*) with mood disorders was, assessed. The *5-HTTLPR* polymorphism was associated with lifetime of primary mood disorders in the ecstasy group (p= 0.018). The prevalence was significantly higher among individuals with genotype *S/S* than among those with either genotype *L/L* or *L/S*.

On the light of these preliminary results, further analyses in this population were undertaken, expanding the number of genes examined. Specifically, semantic fluency impairment in ecstasy users, one of the most robust findings in clinical studies, was examined more in depth, in terms of a better exploitation of neuropsychological results and expanding the number of gene polymorphisms evaluated (two polymorphisms in the *5-HTT* and three polymorphisms in *COMT*). Positive associations were found between semantic fluency and the polymorphisms examined.

These results on the ENTE population propelled the performance of more in depth study on the association of lifetime drug use and a comprehensive array of genes targeting the dopaminergic and the serotonergic neurotransmission systems among others, on cognitive performance in an expanded population of ecstasy users.

1. INTRODUCTION

1.1. MDMA, A BRIEF HISTORY

3, 4-methylenedioxymethamphetamine (ecstasy, MDMA) is one of the most popular illegal psychostimulants used by youth. MDMA belongs to the designer drugs group. According to their chemical structure, designer drugs can be classified into five categories: phenylethylamines, synthetic opiates, arylhexylamines, derivatives of methaqualone and others. MDMA is a phenylethylamine structurally related to amphetamine and mescaline.

Ecstasy is consumed recreationally at dance clubs and “rave” or “techno” parties. Ecstasy is usually sold in the form of tablets of different colours decorated with a wide variety of designs and logos, it can also be found as crystals. The content in “ecstasy” tablets of MDMA varies greatly from batch to batch, but regularly it has been found to contain between 80 and 150 mg of MDMA.

MDMA was first synthesized in 1912 by Anton Kollisch in the Merck laboratories as a chemical intermediate in the synthesis of hydrastinine, an astringent and clotting agent (Freudenmann et al., 2006). It was patented two years later but it never became marketed.

In 1953, the US Army Chemical Centre conducted pioneering toxicological studies in animals but results were not declassified and published until two decades later (Holsten and Schieser, 1986). In the 1960’s and 1970’s the mental health community began to explore the use of MDMA in psychotherapy because of its properties to induce feelings of euphoria, friendliness, closeness to others, and empathy after its administration. The recreational use of MDMA began in the late seventies in certain cultural groups. In the United Kingdom MDMA was classified as a controlled substance in 1977. In 1985 MDMA was included in the Schedule I of illegal substances by the U.S. Drug

Enforcement Administration due to its abuse potential and its lack of medical application. Finally, in 1986 and after some debate between authorities and psychotherapists, MDMA was considered internationally illegal by the WHO (World Health Organization) Special Committee on Drug Dependencies. In Spain, the same year of the announcement of the WHO, the use, fabrication, importation, transportation and sale of MDMA was prohibited by ministerial order (*Boletín Oficial del Estado*, BOE, June 1986). Although the prohibition, its use did not stop in Europe and North America in the 1990's. The popularity of ecstasy is due to its positive effects, which include increased energy feelings, confidence, elevated mood, euphoria and empathy (Cami et al., 2000).

1.2. THE PHARMACOLOGY OF MDMA

1.2.1. Mechanism of action

The mechanism of action of MDMA is similar to that of amphetamine, causing the release of the monoamines serotonin (5-HT), dopamine (DA) and norepinephrine (NE) into the synaptic cleft. The main difference is that while amphetamine has a more prominent effect on dopaminergic and adrenergic activities, MDMA is more active in the serotonergic system.

In the neurons containing monoamines, the neurotransmitters are stored in vesicles situated in the proximity of the membrane. In normal conditions, when a stimulus arrives, the content of the vesicles is released into the synaptic cleft in order to bind to the postsynaptic receptors and induce the signal transduction.

MDMA binds to the plasma membrane monoamine transporters and is translocated into the cytoplasm. Once inside, it stimulates the

neurotransmitter release through the transporter, reversing its normal function. Besides, MDMA is a substrate for the vesicular monoamine transporter (VMAT) and possibly enters the vesicle through this transporter and depletes the vesicular neurotransmitter storage by reversing the normal function of the transporter (Partilla et al., 2006). In addition, MDMA is a mild inhibitor of monoamine oxidase A (MAOA) activity, which might increase the extracellular levels of monoamines (Green et al., 2003).

Data obtained from animal studies, namely rats, proved that MDMA exerts an acute and rapid release of serotonin in the brain, as evidenced by *in vitro* studies using rat brain slices or synaptosomes (O'Loinsigh et al., 2001;Nichols et al., 1982;Johnson et al., 1986b;Fitzgerald and Reid, 1990), followed by a depletion of brain serotonin and its main metabolite, 5-hydroxyindoleacetic acid (5-HIAA). MDMA also inhibits the activity of tryptophan hydroxylase (TPH), the rate limiting enzyme in the synthesis of serotonin (Bonkale and Austin, 2008;Kovacs et al., 2007;Stone et al., 1989). This inhibition can last up to two weeks following a single dose of MDMA. Another neurotransmitter affected by the action of MDMA is dopamine, which is also rapidly released in brain after treatment with MDMA (Green et al., 2003). MDMA is also known to inhibit the dopamine transporter (DAT), the norepinephrine transporter (NET), and the serotonin transporter (5-HTT).

In contrast to the effects of MDMA observed in rats, studies conducted in mice have demonstrated a very different action profile, specifically a neurotoxic damage to dopaminergic terminals, reflected by a decrease in the concentrations of dopamine and its metabolites, and a decrease in densities of dopamine transporter (Granado et al., 2008;Green et al., 2003;Stone et al., 1987;O'Callaghan and Miller, 1994).

1.2.2. Pharmacokinetics and metabolism

MDMA has a chiral centre being present in two optical isomers (figure 1) that display different pharmacologic activities, metabolism and body disposition. The dextrorotatory form (S-(+)-MDMA) is the most active in the CNS and is responsible for psychostimulant and empathic effects.

Figure 1: Chemical structures of the enantiomers of MDMA.

MDMA metabolism has two main metabolic pathways: (1) O-demethylenation followed by cathechol-O-methyltransferase-catalyzed (COMT) methylation and/or glucuronide/sulphate conjugation and (2) N-dealkylation, deamination, and oxidation to the corresponding benzoic acid derivatives conjugated with glycine (de la Torre et al., 2004). Both pathways operate at the same time but at different rates, being the first one predominant in humans. Different CYP450 isozymes are involved in the different metabolic pathways. In humans O-demethylenation is catalyzed by CYP2D6, CYP1A2, and CYP2B6 to form 3,4-dihydroxymethamphetamine (HHMA) while N-dealkylation of MDMA to 3,4-methylenedioxyamphetamine (MDA) is catalyzed by CYP1A2, CYP2D6 and CYP3A4 in humans (de la Torre and Farre, 2004). The further O-demethylenation of MDA, gives rise to 3,4-dihydroxyamphetamine (HHA). Catechol type metabolites HHMA and HHA are O-methylated by COMT to 3-methoxy-4-hydroxymethamphetamine (HMMA) and 3-methoxy-4-hydroxyamphetamine (HMA) (figure 2). The elimination half-life of MDMA after a single dose is about 8 to 9 h (de la Torre et al.,

2000;Mas et al., 1999). MDMA presents a non-linear kinetics above certain doses in humans, with plasmatic concentrations of MDMA not proportional to those administered, due to the inhibition of its own metabolism (de la Torre et al., 2000;Farre et al., 2004).

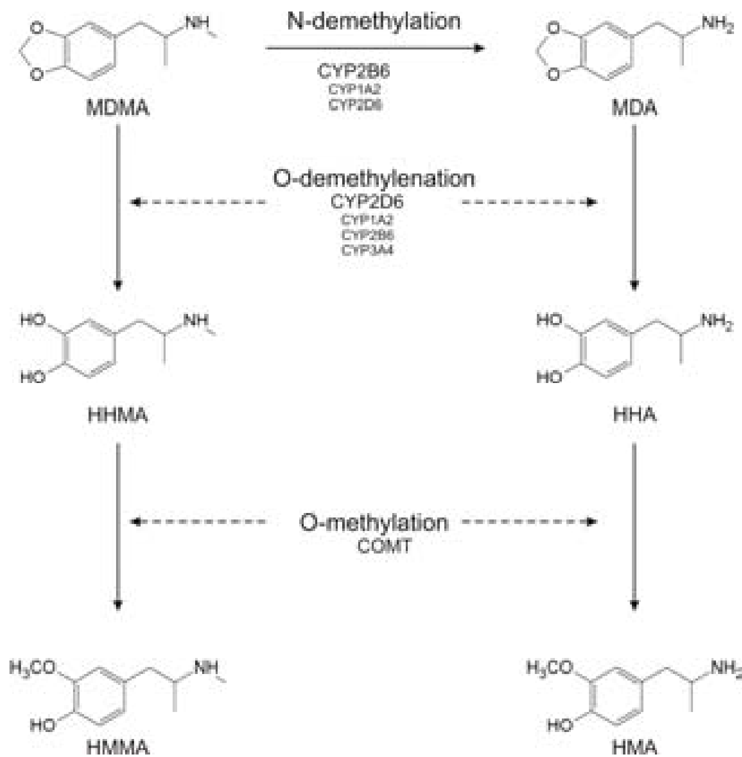


Figure 2: MDMA metabolism in humans, including the main enzymes involved.

1.2.3. Desired effects

MDMA and other designer drugs produce euphoria and psychostimulation, increased empathy and altered levels of perception. The reported desired effects are those referred to as entactogenic effects, which include tendencies to be intimate with those around them, a greater facility for communication and for interpersonal relation. Other sought effects are euphoria, the sensation of well-being and pleasure, along with the psychostimulant effects of an increased

energy, talkativeness, decrease in tiredness and appetite (de la Torre et al., 2004).

1.2.4. Acute adverse effects

The acute adverse physiological effects that occur after ingestion of ecstasy in humans include elevated blood pressure and heart rate, nausea, tremor, and hot and cold flushes, among others. Perhaps the most predominant and severe acute adverse effect is hyperthermia (with body temperatures of over 43ºC reported), which can lead to such problems as rhabdomyolysis, myoglobinuria and renal failure, liver damage and disseminated intravascular coagulopathy (Kalant, 2001). At a cardiovascular level the following physiopathological states have been observed: arterial hypertension, tachycardia, arrhythmia, myocardial ischemia (angina) and acute myocardial infarction, subarachnoideal haemorrhage, cerebral infarction and thrombosis which may arise from short-term hypertension, the possible swelling of cranial blood vessels and dehydratation.

Cephalea, trembling, muscular tension and chewing, vertigo, ataxia and dystonia can also be observed. The excess of serotonin in the central nervous system (CNS) can induce the serotonin syndrome.

At the psychological level, dysphoria, insomnia, irritability, agitation, hostility and confusion are some of the effects that may follow the ingestion of MDMA. Some references to hallucinations and certain types of paranoia are also reported (Green et al., 2003).

1.2.5. Long-term effects

There is a good body of evidence that MDMA causes long-lasting decreases in serotonin and 5-HIAA tissue levels in laboratory animals. Reduction in the activity of TPH and in the activity and density of the serotonin transporter are also observed (Colado et al., 1993;Perrine et al., 2010;Commins et al., 1987). In humans, ligand-binding imaging studies have reported lower specific binding to the 5-HT transporter in ecstasy users compared to controls (McCann et al., 2005;Kish et al., 2010;Ricaurte et al., 2000;Obrocki et al., 1999), although some authors suggest some degree of recovery after cessation of drug use (Buchert et al., 2004;Selvaraj et al., 2009;Thomasius et al., 2006). These long-term changes suggest neurotoxicity, and more specifically a neurodegeneration of the serotonergic neurotransmission system.
Long-term neuropsychological effects affecting cognitive performance and higher psychopathology prevalence resulting from recreational use of MDMA have been reported to persist long after cessation of drug use.

1.3. NEUROTOXICITY OF MDMA

1.3.1. Hypothesis and mechanisms

There is still a great controversy regarding the neurotoxic effects of MDMA. It is clear that MDMA use induces serotonin terminals damage, but it is less clear whether these effects are transient or permanent. Regardless of whether the observed changes are consequence of a process of neuroadaptation or the result of the toxic effect of MDMA, the exact mechanisms underlying this process have not yet been elucidated.

Several factors and possible mechanisms have been proposed to explain the MDMA-induced damage to serotonergic terminals and will be discussed in some detail.

Hyperthermia

A number of *in vivo* studies indicate that hyperthermia may play a major role in this process. Small changes in ambient temperature result in marked changes in the degree of serotonergic neurotoxicity in rats and also in the MDMA-induced release of DA and 5-HT (O'Shea et al., 2005;Malberg and Seiden, 1998). The administration of compounds that prevent hyperthermia has been shown to protect against the toxic effects of MDMA on serotonergic neurons, and drugs that enhance hyperthermic response therefore increase MDMA neurotoxicity.

This is of relevance for MDMA abusers, since MDMA is often taken in hot overcrowded environments which may contribute to an increase in hyperthermic response and long-term toxicity. The last observation should be combined with the fact that MDMA administration in humans induces increases in core body temperature, less relevant in controlled laboratory settings but to be taken into consideration when combined with high ambient temperature (Freedman et al., 2005).

Despite the evidences that hot environmental conditions can enhance MDMA induced neurotoxicity, hyperthermia itself is not sufficient to explain effects observed and it might interact with other known mediators of neurotoxicity as for example oxidative stress.

Oxidative stress

Animal studies have supported the involvement of oxidative stress and the formation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and lipid peroxidation products after the administration

of MDMA. Several studies reported elevated levels of markers of oxidative stress in rat brain after drug treatment. These neurotoxic effects were attenuated by free radical scavengers, antioxidants or overexpression of antioxidant enzymes in animal models (Franzese and Capasso, 2008;Shankaran et al., 2001;Jayanthi et al., 1999).

Despite there is a good agreement on the role of oxidative stress in the toxic effects of MDMA, the source of these reactive species remains controversial. It is thought that DA-derived ROS generated in 5-HT terminals either after SERT-mediated uptake of released dopamine or by the synthesis of DA from tyrosine may have a role in this process, although another plausible mechanism is the action of toxic metabolites of MDMA itself (Puerta et al., 2009).

Role of dopamine metabolism

It is well known that MDMA produces an acute and rapid release of serotonin. Dopamine is also released by the action of MDMA by both a transporter-mediated action or by the increase in postsynaptic serotonin which activates the postsynaptic 5HT2A receptors which in turn enhance DA synthesis and release.

There seems to be a close relationship between serotonin and dopamine in the long-lasting effects of MDMA. It has been suggested (Sprague et al., 1998) that dopamine may enter in the serotonergic terminals by interacting with SERT or it may also be formed within those terminals via hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and subsequently to dopamine via aromatic L-amino acid decarboxylase (AADC) (Breier et al., 2006).

Once inside the terminal, dopamine can be deaminated by MAO. MAO exists in two isoforms, MAO-A and MAO-B, being the later the predominant form in serotonergic terminals. This enzymatic process results in the production of hydrogen peroxides and other reactive

oxygen species, leading to an eventual serotonergic neurotoxicity (Alves et al., 2007;Alves et al., 2009;Hrometz et al., 2004). Consistent with this hypothesis there is the evidence that reduction of MAO-B activity results in protection against MDMA-induced toxicity to serotonergic neurons (Sprague and Nichols, 1995;Alves et al., 2009;Falk et al., 2002;Fornai et al., 2001).

Role of MDMA metabolism

MDMA metabolism may be implicated in the process of long-term serotonin depletion by the generation of free radicals through a metabolic bioactivation. Metabolism of MDMA leads to the formation of HHMA and HHA (see chapter 1.2.2.) which are very unstable reactive catechols. These species can conjugate with either sulfate and glucuronic acid, be O-methylated by COMT or autooxidize to the orthoquinones and form adducts with glutathione (GSH) (de la Torre et al., 2004;Hiramatsu et al., 1990). Several studies have revealed that thioether metabolites, accumulate in rat brain after systemic administration of MDMA (Erives et al., 2008;Jones et al., 2005). The formation of neurotoxic thioether adducts of MDMA has also been demonstrated in humans (Perfetti et al., 2009). It has been postulated that these adducts can cross the blood-brain barrier through glutathione transporters (Bai et al., 2001) and once inside the brain, can generate free radicals. Interestingly, MDMA administered intracerebrally induces neurochemical changes but not neurotoxicity, the later is only observed after the peripheral administration (Esteban et al., 2001). This observation strongly suggests a role for MDMA metabolic disposition and bioactivation. Additionally, thioether adducts discussed previously administered intracerebrally, are able to produce neurotoxicity and neurochemical changes seen after MDMA administration in the periphery (Miller et al., 1996).

These findings support the hypothesis that the bioactivation of MDMA to neurotoxic metabolites might be a relevant pathway to neurotoxicity in humans.

Serotonin transporter

The serotonin transporter is thought to play an important role in the long-term MDMA induced 5-HT depletion. This hypothesis is based in the fact that 5-HTT inhibitors such as fluoxetine and fluvoxamine prevent the 5-HT loss in rats without preventing hyperthermia (Li et al., 2010;Sanchez et al., 2001). As mentioned previously, the serotonin transporter may be involved in the transport of dopamine and/or MDMA metabolites into the serotonergic terminals which may be an important step in the formation of free radicals and their subsequent toxicity (Jones et al., 2004;Monks et al., 2004).

Tryptophan hydroxylase (TPH)

Another important factor involved in the long-term serotonin depletion induced by MDMA is tryptophan hydroxylase (TPH), the rate limiting enzyme for the synthesis of 5-HT. Some animal studies have shown that after MDMA administration there is a long term depletion of TPH activity, which starts to decline immediately after administration of the drug (Stone et al., 1989;Schmidt and Taylor, 1987). In addition, reduction of TPH-immunoreactive fibers and alterations in TPH mRNA expression have also been reported (Bonkale and Austin, 2008;Kovacs et al., 2007).

Others

Other factors have been proposed to play some role in the MDMA-induced neurotoxicity: impaired mitochondrial function (Puerta et al., 2010;Darvesh and Gudelsky, 2005), and increase in permeability of

the blood-brain barrier (Sharma and Ali, 2008;Yamamoto and Bankson, 2005), to name a few, but their relevance have not yet been elucidated.

1.3.2. Functional consequences of long-term neurotoxicity

Besides the biochemical and physiological deficits produced by the action of MDMA (previously discussed), many behavioural changes are also observed after the administration of MDMA to rats. Some studies reported subtle functional disturbances such as increased anxiety (Gurtman et al., 2002;Morley et al., 2001), decreased social behaviour (Bull et al., 2004;Clemens et al., 2007), and poor memory performance (Taffe et al., 2002;Camarasa et al., 2008) in MDMA-treated rodents and non-human primates.

Regarding all the animal studies conducted to date, one might keep in mind the difficulty to extrapolate data from animal studies to humans. It is important to point out some particular pharmacological differences among humans and the animal models, such as metabolism, doses used, route of administration and genetic polymorphisms in enzymes involved in the metabolism of MDMA (e.g. CYP2D6, COMT) or target proteins/receptors (e.g. serotonin transporter).
A further factor to consider is the fact that MDMA users are polydrug users, with cannabis, and alcohol being often substances of a concurrent use (Schifano et al., 1998).
Since MDMA induces long-lasting decrements in serotonin levels, it can be hypothesized that those functions modulated by the serotonergic systems might be affected.
In humans, several functional consequences of ecstasy use have been reported: feelings of lethargy, moodiness, irritability, insomnia,

aggressive behaviour, depression and paranoia are among the effects of ecstasy use observed on mood (Creighton et al., 1991;McCann et al., 1996;Parrott et al., 2000;Reid et al., 2007). MDMA abusers have also been reported to suffer from sleep disturbances (Parrott et al., 2000;Randall et al., 2009;Fisk and Montgomery, 2009); to display learning and memory impairments (de Sola Llopis et al., 2008a;Morgan, 1999;Quednow et al., 2006;Reneman et al., 2006;Zakzanis et al., 2007).

The most frequently reported cognitive deficit in ecstasy users is verbal memory (Schilt et al., 2008;Medina et al., 2005;Bedi and Redman, 2008). Recall deficits among MDMA users are observed, while recognition memory seems to be preserved. Life-time MDMA consumption is clearly associated with greater impairments in cognitive functions, suggesting a dose-related effect. Memory decrements in MDMA users are more clearly observable when neuropsychological tests involve a greater degree of complexity in terms of demands (Brown et al., 2010;Quednow et al., 2006). These findings suggest that high-order cognitive processes involving frontal cortex systems (e.g. attention or executive control) may be more affected by the use of MDMA.

Evidences for visual memory problems are less robust, with studies showing visuospatial memory deficits in ecstasy users (Verkes et al., 2001;Wareing et al., 2004), while others find opposite results (Medina et al., 2005).

Executive functions are also negatively affected in ecstasy users (Fisk et al., 2004;Montgomery and Fisk, 2008). Deficits in spatial working memory performance (Wareing et al., 2005) and poorer verbal fluency (Bhattachary and Powell, 2001;de Sola Llopis et al., 2008a;Heffernan et al., 2001) are described for MDMA users in comparison to control subjects although some exceptions are reported (Back-Madruga et al., 2003).

1.4. A BRIEFF APPROACH TO HUMAN COGNITION

In this chapter, basic aspects regarding the concept of learning and memory and its formation will be reviewed in order to set down the basis for a further discussion on the effects of MDMA on cognition.

Learning is defined as the process by which new information is acquired, while memory refers to the encoding, storage and retrieval of learned information.

1.4.1. Human memory categories

Human memory can be qualitatively divided in two different categories, declarative, or nondeclarative memory (figure 3). Declarative memory refers to the retrieval (and storage) of information which is available to consciousness. Some examples are the ability to remember a telephone number or some events from the past. Non declarative memory refers to skills and associations that are not available consciously (e.g. how to ride a bike).

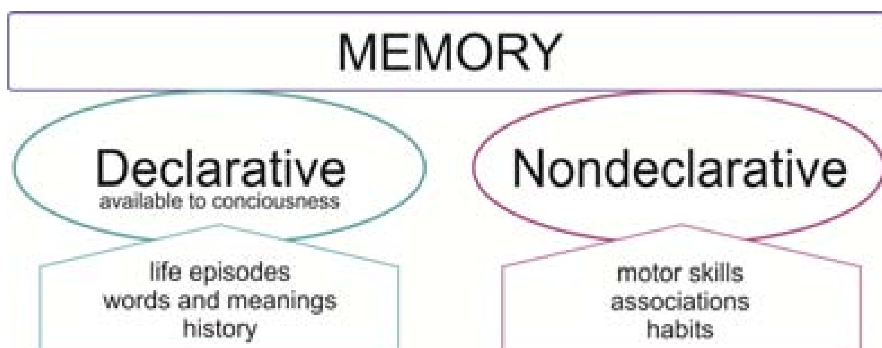


Figure 3: Major qualitative types of human memory. Adapted from (Purves, 2004a).

Human memory can also be classified in three major temporal classes (figure 4). The first class is the immediate memory, which is the ability to hold ongoing experiences in mind for seconds. Each sensory modality (verbal, visual, etc) appears to have its own register. The ability to hold information in mind for seconds or minutes once it has passed is known as working memory and represents the second group. Finally, the third temporal category is the long-term memory which is the retention of information in a more permanent form of storage for days, weeks, or years (Purves, 2004a).

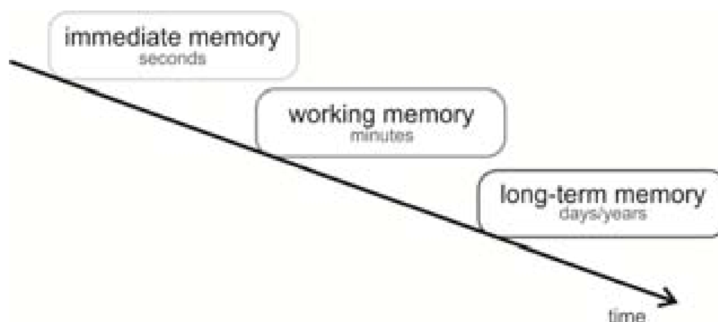


Figure 4: Temporal categories of human memory (adapted from (Purves, 2004a)).

The learning process includes several changes at molecular and cellular level that facilitates the communication among neurons. The persistence of these changes leads to memory consolidation. Neural plasticity represents the basis of higher cognitive functions such as learning and memory (Lombroso and Ogren, 2009).

Short-term or immediate memory is thought to involve only functional changes in pre-existing neuronal networks which can further undergo two processes: fade out with time (forgetting process), or be reinforced and transformed in long-term memory (memory consolidation) (Benfenati, 2007).

In order to be consolidated, the functional changes that occurred in the learning process have to be followed by gene transcription and protein

synthesis in order to promote permanent changes in the neuron as well as structural rearrangements in neuronal networks to make possible a final change in the efficiency of synaptic transmission (Benfenati, 2007).

1.4.2. Brain systems involved in learning and memory

The clinical study of the effects of different diseases or brain damages from many patients has been revealing about the brain systems responsible for the formation of memories. Medial temporal lobe structures and specifically the hippocampus are of great importance for the establishment of new declarative memories. Besides this, different lines of evidence have pointed out that this long-term storage is related to the cerebral cortex (figure 5).

Nondeclarative memories involve the basal ganglia, prefrontal cortex, amygdale, sensory association cortex, and cerebellum, but not the temporal lobe.

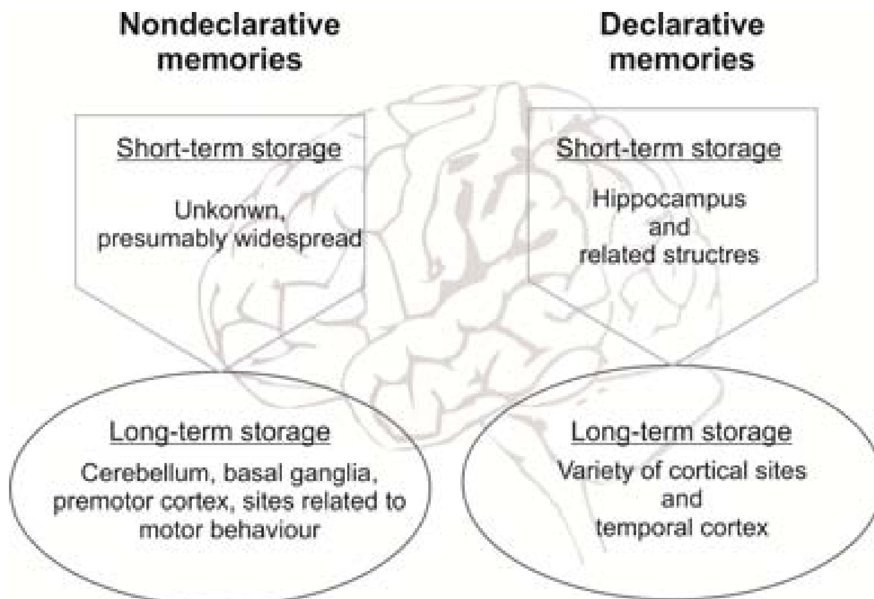


Figure 5: Schematic diagram of the memory systems of the brain.

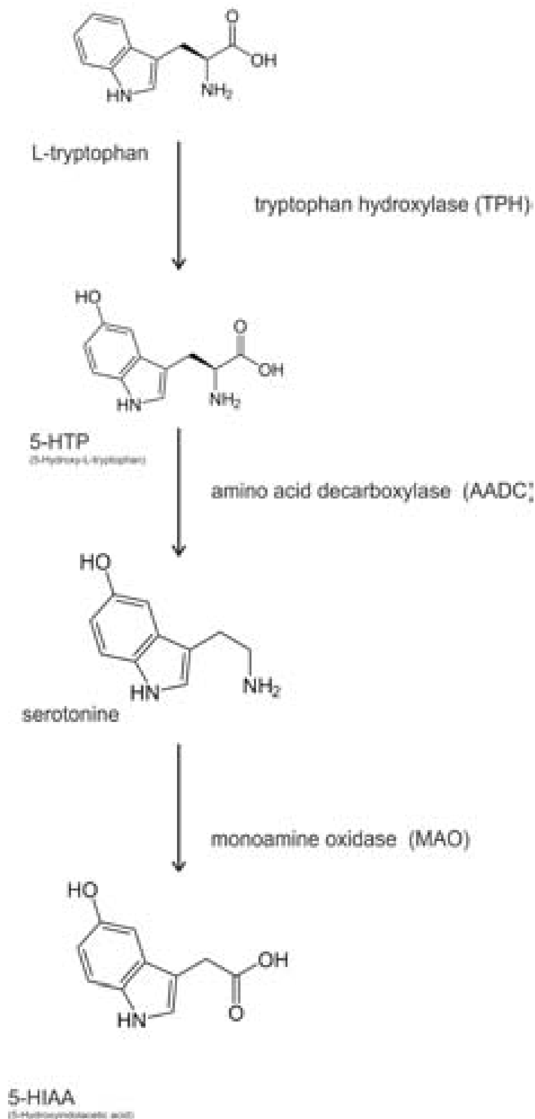
1.5. GENETIC POLYMORPHISMS OF THE SEROTONERGIC AND DOMPAMINERGIC SYSTEMS AND THEIR CONTRIBUTION TO MDMA INDUCED COGNITIVE IMPAIRMENT

1.5.1. Genetic polymorphisms

Differences in DNA sequence are found among individuals or populations. Such differences can be the result of chance or can be induced by external factors (e.g., viruses or radiation) and include sequence repeats, insertions, deletions, recombinations or single nucleotide polymorphisms (SNPs). The most abundant type of genetic variation are SNPs, which account for more than 90% of all sequence variation (Twyman, 2004). A single nucleotide polymorphism is defined as a single nucleotide variation at a specific location that is found in more than 1% of the population (Brookes, 1999). In general, they occur more frequently in the noncoding regions of genes than in the coding regions. Although SNPs in the noncoding regions of genes do not alter protein sequence, can alter regulatory regions of genes. SNPs in the coding regions can lead to alterations of protein structure and function and result in the development of disease (Kim and Misra, 2007). Apart from their importance in disease genetics studies, the study of these variations is also important for pharmacogenomic studies to understand the interindividual differences in response to drugs.

1.5.2. Genetic polymorphisms within the serotonergic and dopaminergic systems

Although there are a number of reports describing numerous genetic variants within the serotonergic and dopaminergic systems and their relationship with MDMA use, or cognitive impairments, only the most relevant polymorphisms for the objectives of this work will be discussed.



Serotonin (5-hydroxytryptamine, 5-HT) (figure 6) is synthesized and stored mainly in the enterochromaffin cells of the intestinal tract and only a small fraction of the total body serotonin is produced in the central nervous system. As mentioned previously, serotonin is synthesized by the tryptophan hydroxylase enzyme (TPH) from the essential amino acid tryptophan.

Figure 6: Serotonin biosynthesis.

Serotonin released from the gastrointestinal tract is rapidly taken by platelets via the serotonin transporter and stored in granules. In the central nervous system serotonin is stored in secretion granules into the serotonergic neurons which emanate from the cell bodies concentrated in the raphe nuclei. Once released, its action is terminated by uptake via the serotonin transporter located in the membrane of the presynaptic terminals and further metabolized by the

monoamine oxidase (MAO) enzyme. Serotonin produces its multiple effects by its interaction to serotonin receptors (for a review see (Jonnakuty and Gragnoli, 2008)). There are several families and subtypes of receptors and are found all across the human body. All the known receptors are G-protein coupled receptors that activate an intracellular cascade of second-messengers.

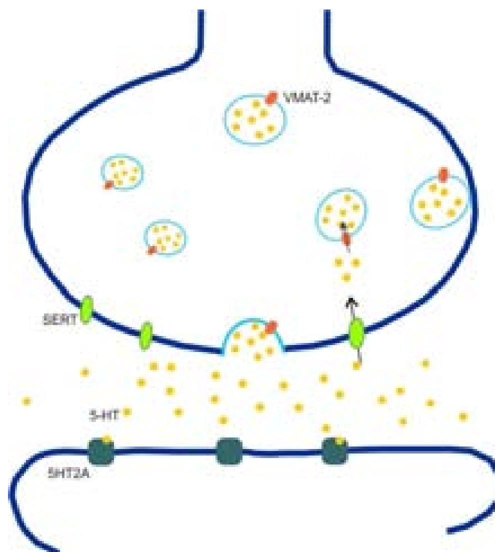


Figure 7: Representation of a serotoninergic neuron.

Serotonin has a wide range of physiological functions. It has a role in platelet aggregation and in regulation of smooth muscle in the cardiovascular and gastrointestinal system. As a neurotransmitter in

the central nervous system it is implicated in a variety of behavioural disorders such as depression, obsessive-compulsive disorder, and anxiety (Jonnakuty and Gragnoli, 2008).
The functions and the roles of the polymorphisms within the genes related to the serotonergic system will be discussed later on this chapter.

Dopamine (4-(2-aminoethyl)-benzene-1,2-diol, DA) (figure 8) is synthesised from the amino acid tyrosine. It is converted into L-DOPA by tyrosine hydroxylase (TH), which is later transformed to dopamine by the DOPA decarboxylase enzyme. Once released DA acts on dopamine receptors starting a cascade of intracellular processes leading to the transmission of the stimulus. Dopamine action in the synaptic cleft is terminated by its reuptake into the presynaptic terminals through the dopamine transporter (DAT).

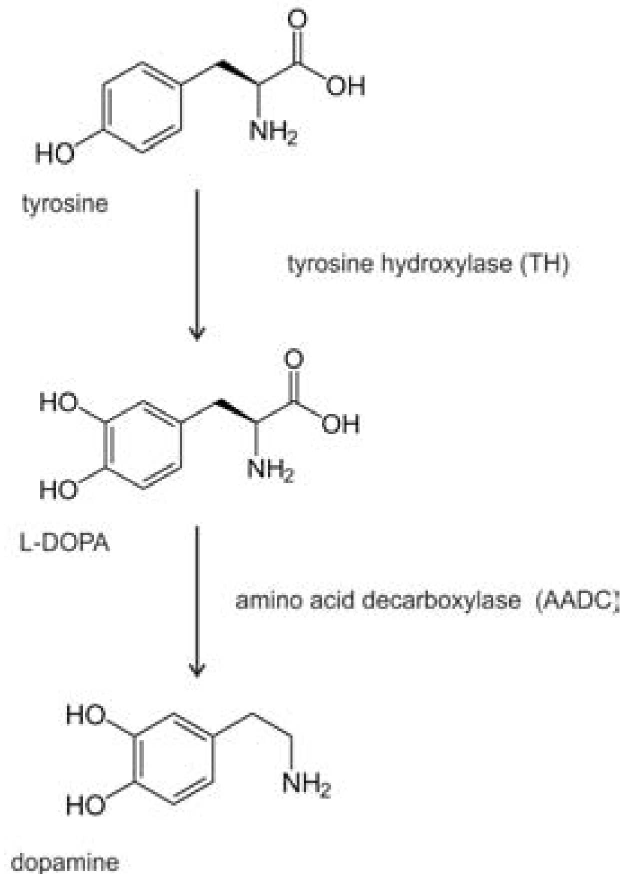


Figure 8: Dopamine biosynthesis

Once inside the cell, dopamine is further metabolized by monoamine oxidase (MAO) and catechol *O*-methyltransferase (COMT).
Dopaminergic neurons are projected from the substantia nigra and spread to several regions of the brain, the basal ganglia and the striatum among others (Purves, 2004b). Dopamine plays an important role in numerous processes such as movement, attention, learning, and some disorders (e.g., Parkinson's disease, Tourette's disorder, schizophrenia or obsessive compulsive disorder).

1.5.3. Monoamine transporters: the serotonin transporter (SERT) and its polymorphisms.

The serotonin transporter (5-HTT) is found in the brain and many peripheral tissues and is responsible of the transport of serotonin to different cells such as neurons, enterochromaffin cells, or platelets. In the brain, the serotonin transporter can be found in the perisynaptic membranes (away from the synaptic area) of neurons arising from the raphe nuclei (Torres et al., 2003).
The serotonin reuptake inhibitors (SRI) are the most frequently prescribed psychoactive drugs for the treatment of depression, obsessive-compulsive disorder, or anxiety disorders. These compounds, as well as some drugs of abuse such as MDMA or cocaine, primarily target the serotonin transporter.

The human serotonin transporter gene (*SCL6A4*) is located in the chromosome 17 (17q11) which contains 13 exons (Ramamoorthy et al., 1993) and encodes a protein of 630 amino acids. The *SCL6A4* gene comprises several domains which selectively controls the expression of the transporter in the serotonergic neurons. In humans, the transcriptional activity is modulated by a repetitive element of variant length found in the 5' flanking region. This region is termed as

the *5HTT* gene linked polymorphic region or 5-HTTLPR (for a review, (Murphy et al., 2004)). The functional polymorphism of an insertion/deletion of 43 base pairs (bp) in this promoter region give rise the long (L) or short (S) variants (Heils et al., 1996) and alters the transcriptional activity of the gene. The short variant of the polymorphism reduces the transcriptional efficiency of the *5-HTT* gene promoter, resulting in decreased *5-HTT* expression and therefore 5HT uptake activity (Lesch et al., 1996). Genotype distributions vary among different populations, but it has been reported that in European population the genotype distribution is 32% LL, 49% LS, and 19% SS (Lesch et al., 1996).

An additional functional SNP (A/G) (rs25531) within the promoter region has been recently detected in humans (Hu et al., 2006;Wendland et al., 2006). This A to G substitution generates a binding site for AP2, a nuclear factor that functions as transcriptional activator or repressor (Hu et al., 2006). In that way, the *la* variant is associated with high levels of *in vitro 5-HTT* expression, whereas *lg* is low expressing and more similar to *s* allele (Praschak-Rieder et al., 2007). The *G* allele has been also reported within the *s* allele but in a very low frequency (Kraft et al., 2005). The existence of this variant within the insertion/deletion polymorphism in the promoter region can underestimate the effect of the *5-HTTLPR* polymorphism and can explain the inconsistency of some of the results that can be found in the literature.

The *5-HTTLPR* polymorphism, and in particular the long form (*l*) has been related to better antidepressant treatment, while the *s* allele has been associated with increased risk of depression and poorer response to antidepressants (for a review (Lesch and Gutknecht, 2005)).

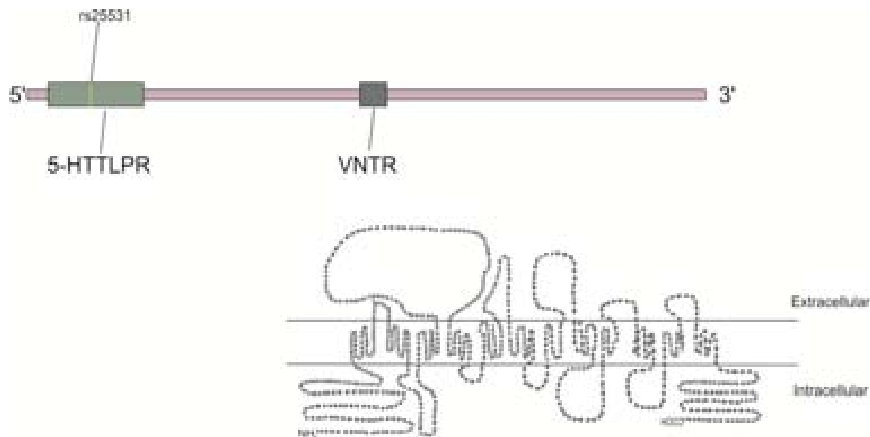


Figure 9: Human *SERT* gene representation. Protein structure of the serotonin transporter. Adapted from Murphy *et al.*,(2008).

Several studies have recently shown that the *s* allele is associated with improved cognitive functions (e.g., decision making, risk aversion, or response inhibition), and enhanced sensitivity to environmental stimuli (for review (Homberg and Lesch, 2010)).

The *5-HTTLPR* polymorphism has also been related to abnormal emotional processing and cognitive impairments in both healthy subjects and ecstasy users.

Deficits in verbal memory were observed in current and former (one year of abstinence) MDMA users, with higher lifetime use associated with greater decrements in this function (Reneman et al., 2001). Roiser et al., (Roiser et al., 2005) found that MDMA use may enhance impulsive tendencies as a function of *5-HTTLPR* genotype. MDMA users carrying the *S/S* genotype failed to reduce impulse errors in response to emotional cues. Furthermore, a later study with the same sample (Roiser et al., 2006) pointed out that ecstasy users with the *S/S* genotype were also less efficient in decision-making than controls with the same genotype.

Another study from Reneman et al., (Reneman et al., 2006) established significant impairments in memory function in heavy ecstasy users but did not find any effect of the serotonin transporter polymorphism on their cognitive performance.

Furthermore, imaging studies in humans have reported lower decreased specific binding to the 5-HT transporter in ecstasy users compared to controls (Kish et al., 2010;McCann et al., 2005;Obrocki et al., 1999;Ricaurte et al., 2000), while some others have found either an increase of SERT availability in former MDMA users or no differences compared to controls (Buchert et al., 2004;Selvaraj et al., 2009;Thomasius et al., 2006).

Another polymorphism described within the human serotonin transporter gene is a variable number tandem repeat (VNTR) within the intron 2 (*5-HTTVNTR*). This VNTR contains nine, ten, or twelve copies of a 17 bp repeat (Hranilovic et al., 2004). This polymorphism alters the transcriptional activity of the gene, with enhanced expression for the 12 repeats allele compared to the 10 repeats (Fiskerstrand et al., 1999). These effects seem to be dependent upon the individual repeat elements within the VNTR region (Lovejoy et al., 2003).

1.5.4. Serotonin receptors: serotonin receptor 2A (5HT2A)

As previously mentioned, a large number of serotonin receptors have been identified. Among them, 5-HT2A receptors are located in the medial prefrontal cortex and hippocampus of rats (Pazos et al., 1985;Xu and Pandey, 2000) and humans (Hoyer et al., 1986;Wong et al., 1987;Barnes and Sharp, 1999;Leysen, 2004) and play an important role in the serotoninergic neurotransmission in the brain.

These receptors belong to the 5-HT2 family of serotonin receptors G protein-coupled (among with the 2B and 2C subtypes).
The activation of the 5-HT2A receptor has been shown to couple G protein leading to the activation of either phospholipase C (PLC) or phospholipase D (PLD) (Parrish and Nichols, 2006) an thus increasing the inositol triphosphate (IP_3) concentrations.

In humans, the *HTR2A* gene is located in chromosome 13 (position q14-q21) (Sparkes et al., 1991) and consists of 3 exons and 2 introns (Chen et al., 1992).

A nonsynonymous polymorphism at position 1354 (C/T) occurs in the *HTR2A* gene leading to an amino acid substitution histidine (His) to tyrosine (Tyr) at codon 452 (His452Tyr) (rs6314). This amino acid change lies in the cytoplasmatic C-terminal tail of the receptor which is implicated in the G protein coupling.
As a consequence, cells containing the 452tyr variant of the receptor show reduced ability to activate phospholipases, suggesting reduced intracellular signalling capacity (Hazelwood and Sanders-Bush, 2004).
It has been also postulated that this polymorphism may also affect brain morphology with reduced grey matter concentrations in the left hippocampus for the *tyr* carriers which to some extent explained the poorer memory performances observed in these individuals(Filippini et al., 2006).

Another polymorphism within the gene is the T to C transition at position 102 (T102C, rs6313) that does not alter the amino acid composition and, therefore, has no influence on the receptor protein (Bondy et al., 1999). It has been hypothesized that this polymorphism may be associated with lower levels of gene expression and protein in healthy individuals with the *C/C* genotype compared to those with the

T/T genotype (Polesskaya and Sokolov, 2002). This polymorphism has been associated with panic disorders, schizophrenia, suicidal behaviour, and affective disorders (Maron et al., 2005;Golimbet et al., 2007;Vaquero-Lorenzo et al., 2008), although some of these results have not been replicated in other studies (Martinez-Barrondo et al., 2005;Correa et al., 2007).

Results from different studies suggest a role of this receptor in memory functioning. Specifically, De Quervain et al., (de Quervain et al., 2003) showed that individuals with the *his/tyr* genotype of the His452Tyr polymorphism performed poorer on memory recall tests than individuals with the *his/his* genotype. In the same direction, Wagner et al., (Wagner et al., 2008) found that the rare *tyr* allele of was associated with poorer delayed recall performance in the AVLT task while the immediate memory was not affected.

MDMA use has been related to reductions of serotonin receptor 2A levels in both rats and humans. Reneman et al., (Reneman et al., 2002), showed that 5HT2A receptor densities were significantly reduced in all cortical brain regions of MDMA users compared to controls, while ex-MDMA users exhibited higher receptor densities in the occipital cortex. In the same line were the results form rats studies, with decrements in the density of receptors after treatment but a time-dependent recovery was also observed after the discontinuation of MDMA administration. In addition, Kindlundh-Hoberg et al., (Kindlundh-Hogberg et al., 2006) found decrements in 5HT2A receptor mRNA and increments in 5HT2C receptor mRNA expressions in rat brain four weeks after MDMA treatment (one dose every 7 days), which would reflect neuroadaptive forces to counteract the MDMA-induced depletion of 5-HT.

1.5.5. Catechol-O-methyltransferase (COMT)

Catechol-O-methyltransferase (COMT, EC 2.1.1.6) is an enzyme involved in both, the clearance of dopamine from the synaptic cleft in the prefrontal cortex and also in the MDMA phase II metabolism in the transformation of HHMA to HMMA.

The *COMT* gene is located in the chromosome 22 at position 11 (22q11) and in humans encodes two known transcripts from two different promoters, P1 and P2 (Tenhunen et al., 1994) (figure 10). A longer mRNA from P2 promoter encodes mainly a membrane-bound COMT (MB-COMT) and a shorter mRNA from the P1 promoter encodes the soluble COMT (S-COMT). Most human tissues encode both COMT mRNA transcripts but the S-COMT is mainly found in other tissues such as liver, blood and kidney while the MB-COMT is predominantly expressed in neurons, mainly in the prefrontal cortex and at lower levels in the striatum, cerebellum, amygdala and at very low levels in the ventral tegmental area and substantia nigra (Tenhunen et al., 1994;Bertocci et al., 1991;Matsumoto et al., 2003). Despite their high sequence similarity the MB-COMT has higher affinity for dopamine (10-fold greater) than S-COMT (Lotta et al., 1995).

A functional polymorphism (rs4680) consisting in a valine (val) to methionine (met) substitution at codon 158 of the MB-COMT (codon 108 for the S-COMT variant), results in a thermolabile protein with decreased enzymatic activity (one third less activity for the *met* homozygotes compared to the *val* homozygotes) at physiologic temperatures (Mannisto and Kaakkola, 1999). Because these alleles are codominant, heterozygotes have intermediate levels of COMT activity.

A single nucleotide polymorphism (G to A) in the 3' untranslated region of the COMT gene (rs165599) has been associated with cognitive dysfunction in schizophrenia (Chan et al., 2005;Chien et al., 2009) and

bipolar disorder (Burdick et al., 2007). Little is known about the molecular function of this polymorphism but it has been proposed that this polymorphism or another nearby in linkage disequilibrium may be involved in COMT regulation (Chan et al., 2005).

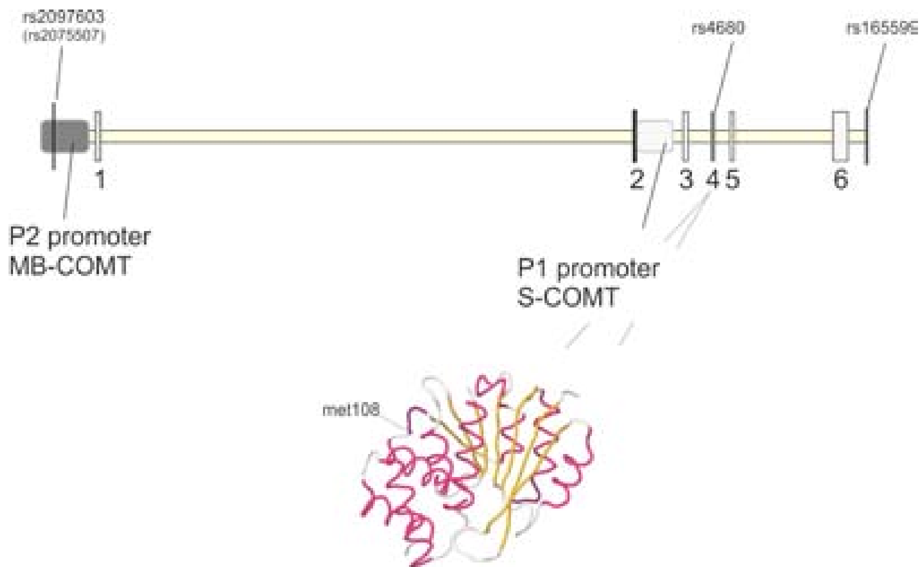


Figure 10 : Diagram of the *COMT* gene showing the locations of the SNPs genotyped in this work (adapted from Palmatier 2004). Structure of human S-COMT modified from (Rutherford et al., 2008) using Jmol software http://www.jmol.org/

Another polymorphism within a noncoding region of the COMT gene is found in the promoter P2 (A to G) (rs2097603, later on rs2075507). This SNP shows association with schizophrenia (Funke et al., 2005). Recently, this polymorphism has been suggested to interact with the *COMT* val158met variation predicting changes in the hippocampal gray matter volume (Honea et al., 2009).

Differences in COMT enzymatic activity due to genetic variations can explain to some extent the inter-individual variability in the susceptibility to MDMA-induced neurotoxicity as consequence of

MDMA metabolism and the formation of reactive species (de la Torre et al., 2004;Perfetti et al., 2009).

In addition, COMT regulated dopamine levels are critical for prefrontal-dependent cognitive functions such as working memory. Evidences suggest an inverted-U shape relationship between dopamine activity in the prefrontal cortex (PFC) and working memory performance (Goldman-Rakic et al., 2000) (figure 11). In this model, intermediate levels of dopamine appear as optimal for working memory processes.

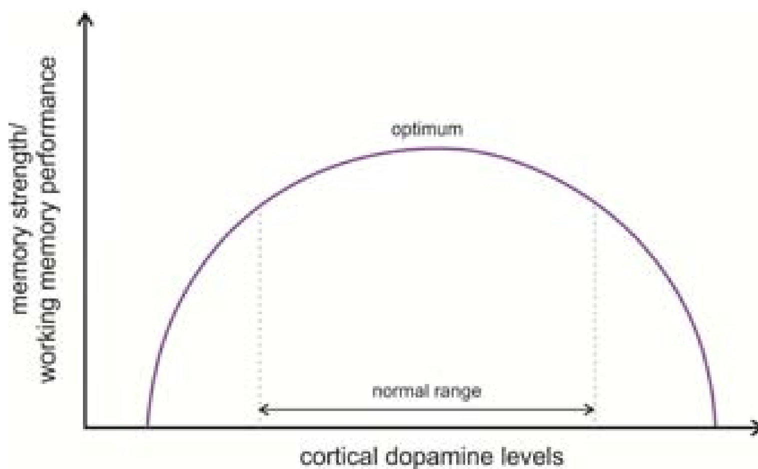


Figure 11: Inverted U-shape relationship between dopaminergic activity in the prefrontal cortex and cognitive performance. Adapted from Goldman-Rakic, 2000.

In this regard, the role of COMT and its polymorphisms in the metabolism of dopamine in brain have been extensively investigated.
Some studies have identified an association between working memory and the *COMT* val158met polymorphism (Egan et al., 2001;Goldberg et al., 2003). The *val* allele has been related to inferior working memory but higher flexibility in healthy subjects as compared to individuals with the *met* allele. In that case, a more stable prefrontal

activation was observed which facilitates working memory processes, but gives less flexibility to shift and update (Durstewitz and Seamans, 2008;Colzato et al., 2010;Schilt et al., 2009). Some studies have also pointed out a possible role (although not a main effect) of this polymorphism on verbal fluency (Alfimova et al., 2007).

As mentioned previously, the possible involvement of the COMT enzyme in the effects of MDMA on cognitive impairment can be explained either by its role in the breaking down of MDMA or its role in the metabolism of dopamine. In a recent study, Schilt et al., (Schilt et al., 2009) found an interaction between ecstasy use and the COMT genotype on verbal learning (assessed through the RAVLT test). They showed that ecstasy users with the *met* allele of the val158met polymorphism were particularly susceptible to the negative effects of the drug on verbal learning. Such observation can be explained by means of the relatively high levels of dopamine in those individuals making them more sensitive to the ecstasy effects regarding the role of dopamine on ecstasy-induced neurotoxicity.

1.5.6. Neurotrophins

Neurotrophins are a unique family of polypeptide growth factors initially identified as survival factors for some neurons but later it has been shown to play an important role in the functioning of the adult central nervous system (CNS) where they control synaptic function and plasticity, and sustain neuronal cell survival, morphology and differentiation (Poo, 2001).

Neurotrophins are synthesized as precursors (proneurotrophins) that are proteolytically cleaved to mature, biologically active neurotrophins. Four neurotrophins are expressed in mammals: nerve growth factor

(NGF), brain-derived neurotrophin (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Reichardt, 2006).

The efficacy of neurotrophins action is, besides their binding affinity to the transmembrane receptors, is also dependent on their packaging, transport, secretion and processing. Neurotrophins can be stored into granules and their exocytosis is regulated either in a “constitutive” secretion mediated by a calcium-regulated secretion or in a “regulated” activity-dependent manner (Lee et al., 2001).

Two types of receptors have been identified: the first receptor to be discovered was the low-affinity p75 neurotrophin receptor (p75NTR) (Johnson et al., 1986a) which is a member of the tumour necrosis factor superfamily. The second major class of neurotrophin receptors consists of three members of the high-affinity membrane-bound tyrosine kinase receptor (Trk). Each neurotrophin exhibit specificity in their interactions with the three members of this receptor family with BDNF activating (Ultsch et al., 1999). Trk receptor function is modulated by p75NTR on several levels (Reichardt, 2006).

The high levels of expression of BDNF in the adult central nervous system and TrkB in cortical and hippocampal structures demonstrate their critical role in the maintenance of synaptic connections, synaptic plasticity, and cognitive functions such as learning and memory (Lu, 2003).

Brain-derived neurotrophin (BDNF)

Brain-derived neurotrophin (BDNF) is the most widely distributed neurotrophin in the central nervous system, including hippocampus, neocortex, amygdala, cerebellum, and hypothalamus (Bath and Lee, 2006), key regions in the regulation of mood and behaviour. Furthermore, BDNF gives trophic support to cholinergic, dopaminergic

and 5-hydroxytryptamine containing neurons (Gratacos et al., 2007). And interaction between serotonin and BDNF has been reported, since the activation of 5-HT receptors can stimulate BDNF gene expression (Mattson et al., 2004).

As mentioned above, BDNF is secreted through two different pathways: constitutively, at low basal level, and in a regulated manner based on the level of synaptic activity. This activity-dependent secretion has been observed to be critical in the control of synaptic transmission and long-term synaptic plasticity (Lu, 2003) suggesting an important role in activity-induced long term potentiation (LTP) and long term depression (LTD).

BDNF gene is located in the chromosome 11 at position 13 (11p13). It is organized in 13 exons and due to alternative splicing it encodes two BDNF protein variants. A long form with 247 amino acids with a 5' pro-BDNF sequence which is later cleaved to form the mature protein, and a short form which is 153 amino acids long and lacks the 5' pro-BDNF region (Seidah et al., 1996).

One frequent non conservative polymorphism occurs in the human *BDNF* gene (rs6265). A single nucleotide polymorphism (SNP) at nucleotide 196 (G/A) produces an amino acid substitution (valine to methionine) at codon 66 (val66met). This polymorphism does not affect mature BDNF protein function but it has been shown to alter the intracellular trafficking and packaging of pro-BDNF and the regulated secretion of the mature protein when the *val*66 is replaced with *met* (Egan et al., 2003).

Egan et al., (Egan et al., 2003) also assessed the effect of this polymorphism in the measure of n-acetyl-aspartate (NAA), an intracellular marker of neuronal function which appears to be an indirect measure of neuronal integrity and synaptic abundance. Their results showed that *met*-carriers had lower levels of NAA compared to

val homozygotes and there was a significant linear reduction of NAA levels with increasing number of *met* alleles, suggesting a specific effect of the val66met polymorphism in the hippocampal neuronal integrity or synaptic activity.

Recently, it has been shown that the presence of the G196A mutation may block the dendritic trafficking of the BDNF mRNA by disrupting its interaction with the translin/trax complex (an RNA-binding protein complex implicated in RNA trafficking) (Chiaruttini et al., 2009). This finding can to some extent explain the phenotypic changes induced by the mutation but it is important to emphasize that the deficits in the BDNF mRNA sorting can not explain all the changes observed and that it is also plausible that this mutation can also affect the trafficking of the BDNF protein itself.

The val66met polymorphism is involved in impairments in different forms of hippocampal-dependant memory such as episodic memory (Egan et al., 2003;Hariri et al., 2003), mood disorders (Hong et al., 2003a) and personality (Sen et al., 2003). It has also been associated both positively and negatively with neuropsychiatric disorders such as Alzheimer's disease (Feher et al., 2009), Parkinson's disease (Hakansson et al., 2003;Hong et al., 2003b;Momose et al., 2002), depression (Tsai et al., 2003), and substance abuse (Liu et al., 2005) among others.

Cognitive and behavioural effects associated to the *met* allele have been shown to produce more robust effects on Caucasians than other ethnicities and it is possible that in other populations a compensatory mechanism may exist to compensate or eliminate the negative effects of the *met* change (Bath and Lee, 2006).

Using imaging techniques, bilateral reductions in hippocampal grey matter volume of healthy volunteers with *met*-BDNF were observed (Pezawas et al., 2004). In the same direction, Szeszko *et al.*, (Szeszko et al., 2005) obtained similar results but also demonstrated that *val/val*

individuals did not differ significantly from the other subjects in total intracranial volume suggesting that the effect of this polymorphism does not affect the global brain morphology.

BDNF polymorphism has been associated with impairment in some forms of learning and memory. Individuals carrying the *met* allele performed poorly than *val* homozygotes on memory tasks that rely on the hippocampus such as recalling places or events but there were no differences in less-hippocampal demanding tasks such as word learning or planning (Egan et al., 2003). At the same time, Hariri et al. (Hariri et al., 2003) using imaging techniques showed that in the execution of a simple declarative memory task, highly dependant on hippocampal formation, individuals carrying the *met* allele had lower levels of hippocampal activation compared with *val/val* subjects, in both the encoding and retrieval processes. These results are in agreement with the already known role of BDNF in activity-dependent plasticity and hippocampal long term potentiation (LTP) that underlay the formation of learning and memory.
Finally, a most recent study (Hashimoto et al., 2008) found a dose-dependent effect of the val66met polymorphism in the hippocampal activity during the encoding process but not during the retrieval process. They showed a negative correlation between the number of *met* allele and the degree of activation in the bilateral hippocampi.
In animal studies, rats treated with MDMA showed an increase in BDNF gene transcription in the frontal cortex and a decrement in the hippocampus 24h after treatment (Martinez-Turrillas et al., 2006). In this study, authors suggested that the effects observed in the hippocampus is due to a higher vulnerability of this brain region to the neurotoxic effects of MDMA and that the increments observed in the prefrontal cortex could be a compensatory mechanism to minimize the effects of the drug.

A recent study (Angelucci et al., 2010) has revealed increased BDNF concentrations in ecstasy users compared to healthy volunteers. Authors suggest two possible explanations: increased BDNF levels could be a compensatory response to MDMA neurotoxicity or might be also due to a direct effect on immune cells, which are said to be affected by MDMA use (Pacifici et al., 2002).

1.6. OTHER ENZYME/TRASNPORTER SYSTEMS CONTRIBUTING TO MDMA INDUCED COGNITIVE IMPAIRMENT

In the literature there are a number of studies involving additional enzymes, transporters, receptors and many other molecules in both the neurotoxic effects of MDMA and human cognitive performance.
The aim of this later part of the chapter is to summarize the most relevant findings related to genes that selected to fulfil the aims of this work.

1.6.1. Glutamate and its receptors

Glutamate is the main excitatory neurotransmitter within the central nervous system, and plays a crucial role in cognitive functions such as learning and memory.
It has been shown that the NR2B subunit of the NMDA receptor contributes to human memory performance by regulating aspects of synaptic plasticity (de Quervain and Papassotiropoulos, 2006). Its distribution along the human brain, with high expression levels in the frontal cortex and hippocampal pyramidal cells (Schito et al., 1997) suggests that this receptor is involved is specific cognitive processes. The activation of NMDA receptors initiates an intracellular signalling

cascade which leads to long-term potentiation (LTP) and memory consolidation.

Two main glutamate receptor families are known: the ionotropic receptors (with three subtypes, NMDA receptors, AMPA receptors, and kainite receptors), and the metabotropic receptors.
The metabotropic receptors (mGluRs) modulate postsynaptic ion channels indirectly and are coupled to different intracellular signal pathways hence producing either excitatory or inhibitory responses.
The ionotropic receptors are nonselective cation channels which always produce excitatory postsynaptic responses. These receptors are formed from the association of several protein subunits which can combine in many ways to produce different receptor isoforms (Purves, 2004b). Among them, the N-methyl-D-aspartate receptors (NMDARs) are of special interest. The NR2B subunit, encoded by the glutamate receptor, ionotropic, NMDA subunit 2B gene (*GRIN2B*) is specially involved in the stabilization of synaptic connections, long term increase/decrease of synaptic strength, necrotic/apoptotic neuronal death, and learning processes (Seripa et al., 2008;Rosenblum et al., 1996;Loftis and Janowsky, 2003).

The *GRIN2B* gene is located in chromosome 12 (12p13) (Mandich et al., 1994). A polymorphism within this subunit leads to the substitution of C to T (rs1806210) which is a silent polymorphism and has no effect on the amino acid sequence of the receptor (Thr888Thr) (Nishiguchi et al., 2000). Despite being synonymous, this SNP may have functional effects by altering the mRNA stability or translation, as the *C/T* and *T/T* genotypes might be related to increased glutamatergic neurotransmission because these genotypes are associated with early manifestations of Huntington’s disease attributable to glutamate excitotocixity (Arning et al., 2007). Increases in glutamatergic neural

transmission have been associated with reduced dopaminergic function (Seamans and Yang, 2004).

1.6.2. Monoamine oxidase (MAO) enzyme

As mentioned previously in this introduction, monoamine oxidase is involved in the termination of monoamine neurotransmitters action.
MAO enzyme is located at the outer membrane of mitochondria and is found as two isoforms, MAO-A being expressed mainly in chatecholaminergic neurons, and MAO-B predominantly in serotonergic neurons as well as astrocytes and glia (Alves et al., 2007).
The oxidative deamination of these neurotransmitters produces hydrogen peroxide which can undergo to the formation of hydroxyl radicals and oxidative stress damage can occur. This process has been proposed as a possible mechanism of MDMA-induced neurotoxicity (as discussed in chapter 1.3).
Animal studies reported the important contribution of MAO-B to MDMA-induced mitochondrial damage (Alves et al., 2007;Sprague and Nichols, 1995).

1.6.3. Enzymes involved in the synthesis of dopamine and serotonin.

Serotonin synthesis is a two-step process catalyzed by the tryptophan hydroxylase (TPH) and the aromatic amino acid decarboxylase (DDC or AADC), being the former the rate limiting enzyme (Purves, 2004b).
Tryptophan hydroxylase exists in two isoforms, TPH1 and TPH2 encoded by two different genes. Both enzymes are differentially expressed, with TPH1 found mainly in the pineal gland and gut

whereas TPH2 is selectively expressed in the brain (Patel et al., 2004;Bach-Mizrachi et al., 2006).

It has been reported that the TPH activity (Stone et al., 1989) and mRNA expression in rat brain are altered after MDMA administration (Bonkale and Austin, 2008;Kovacs et al., 2007).

The aromatic amino acid decarboxylase (DDC or AACD) is thought to play some role in the MDMA-induced toxicity to serotonergic terminals. The importance of this enzyme is twofold, due to its role in the metabolism of both serotonin and dopamine. MDMA-neurotoxicity may be in part due to oxidative damage induced by DA into serotonergic terminals. The synthesis of DA into these terminals can be explained by means of *de novo* synthesis from its precursor tyrosine by tyrosine hydroxylase (TH) and AADC endogenous in 5-HT neurons. Accordingly, Breier et al., (Breier et al., 2006) reported that systemic administration of MDMA to rats resulted in selective long-term depletions of serotonin in an AACD-dependent manner.

Tyrosine hydroxylase (TH) is the enzyme responsible of the transformation of the amino acid tyrosine into DOPA, which is further transformed into DA (see figure 8). Four different types of TH are expressed in the human brain, specifically at the substantia nigra and locus coeruleus (Nagatsu and Ichinose, 1991).

1.6.4. The vesicular monoamine transporter (SLC18A2)

The vesicular monoamine transporter (VMAT-2, *SLC18A2*) mediates the uptake of monoamines into presynaptic storage vesicles. VMAT-2 is the site of action of some pshychostimulants, such as methamphetamine and MDMA which promote the release of the intravesicular neurotransmitters ((Wimalasena, 2010) for a review).). Recent animal studies have revealed a reduction in VMAT-2 mRNA

expression in rat brain two weeks after MDMA administration but no reduction in VMAT-2 protein density was observed (Biezonski and Meyer, 2010).

1.7. GENETIC POLYMORPHISMS WITHIN THE CYP2D6 METABOLIZING ENZYME AND THEIR CONTRIBUTION TO THE PHARMACOLOGY AND THE NEUROTOXICITY OF MDMA.

As mentioned previously, in humans the CYP2D6 enzyme is supposed to be responsible for 30% of the O-demethylenation of MDMA to HHMA (Farre et al., 2004).

Interindividual differences in CYP2D6 activity can affect the MDMA pharmacology and susceptibility to toxicology. Preliminary data have shown that MDMA pharmacology differs according to *CYP2D6* genotype (de la Torre et al., 2005).

Over 70 different alleles of *CYP2D6* have been described. Allelic variants of *CYP2D6* functionally result in abolished, decreased, normal, increased or qualitatively altered catalytic activity. Among the most important variants are *CYP2D6*2*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*10*, *CYP2D6*17* and *CYP2D6*41* and gene duplications. The frequency of *CYP2D6* alleles also varies ethnically. All variant alleles are presented at the home page of the human CYP allele nomenclature committee (http://www.imm.ki.se/cypalleles/cyp2d6.htm).

Phenotypically, individuals can be divided into four categories: poor metabolisers (PM) which show a deficient enzymatic activity, intermediate metabolisers (IM), extensive metabolisers (EM) and ultra-rapid metabolisers (UM) according to increasing CYP2D6 activity. In

the Caucasian population, approximately 7 % of individuals are PM, and up to 90% are termed extensive metabolizers (EM) (Bertilsson et al., 2002).
However, it is important to point out that the non-linear pharmacokinetics of MDMA in humans is related to the inhibition of its own metabolism and independent of the genetic variability of the individuals, with PM and EM displaying a similar metabolic profile after the first dose.

A recent study has shown differences in cognitive impairment related to methamphetamine use linked to CYP2D6 genotype. Extensive metabolisers (EM) showed poorer cognitive performance than other participants, possibly due to greater neurotoxicity of the metabolic products than the parent compound (Cherner et al., 2010).

2. HYPOTHESIS AND AIMS

There are cumulating data suggesting that MDMA induces neurotoxicity in animal models and more specifically a neurodegeneration of the serotonergic neurotransmission system.

Humans

There is still debate on the question if MDMA use in humans is reliably associated with substantial neuropsychological impairment, regardless of the effects of concomitant use of other substances (e.g., cannabis, alcohol or other stimulants).

One of the main factors that may contribute to clarify MDMA-related neuropsychological findings is variation in the distribution of a number of gene polymorphisms associated with the functioning of the 5-HT system, as well as others involved in MDMA pharmacodynamics and potential neural toxicity (e.g., COMT and CYP2D6) and in neural signaling cascades involved in learning and memory processes (e.g., BDNF and glutamate genes).

In humans, it is hypothesized:

(i) that heavier MDMA use would be correlated with poorer neuropsychological performance in a dose-dependent fashion;

(ii) that heavy MDMA users would perform poorer than cannabis users and healthy individuals on neuropsychological tests of processing speed, memory and fluency (indicating robust effects of MDMA on cognition regardless of co-abuse of cannabis); and

(iii) that MDMA use would exacerbate cognitive performance decrements in individuals carrying genotypes associated with lower functionality of the serotonin, glutamate and dopamine systems (drug x gene interaction effects).

The **primary aim** of this work is to evaluate the combined effects of ecstasy use and several polymorphisms in the serotonergic and dopaminergic systems and its relationship to cognitive impairments related to the drug use in a cross-sectional study of drug users and healthy controls.

Animal model

In animal models there are still controversies concerning long-term brain damage induced by MDMA. While some authors support that MDMA at high doses induces an axonopathy of serotonergic neurons, others content this hypothesis and support the view that MDMA induces a decrease in gene expression of the serotonin transporter. This discussion is of relevance as depending on the correct hypothesis genetic polymorphisms are still (changes in gene expression) or less (axonopathy) relevant.

In animal model, it is hypothesized:

(iv) That the expression of genes related to serotoninergic system would show greater affectation than those of the dopamine neurotransmission system.

(v) That the negative effects of MDMA treatment on the serotonin transporter gene expression will be also observable in genes encoding proteins that are directly modulating the transporter and the serotonergic function.

The **second aim** of this work was to examine the MDMA-induced long-term effects on expression levels of genes related to the serotonin and dopamine biosynthesis and function in an animal model (Wistar rats) of MDMA induced neurotoxicity.

3. METHODOLOGICAL APPROACHES

To achieve the objectives of this thesis, the experimental work has been divided in two major blocks: human studies and animal model studies.

3.1. HUMAN STUDIES

In human studies, protocols required a quick genotyping of subjects. In this context it was decided to use a DNA microarray targeting most genes under study. Prior to the application of the DNA microarray, it was necessary to validate it clinically (Cuyàs et al., 2010;Fagundo et al., 2010).

Human studies include a psychiatric and neuropsychological evaluation that was carried out by a psychologist or a psychiatrist.

3.1.1. Evaluation of PHARMAchip™ DNA array for the genotyping of drug metabolizing enzymes, transporters and protein effectors.

According to the study requirements, volunteers were included in the study following a given stratification of combined genotypes for *COMT* and *5HTTLPR* genes. In order to genotype volunteers, as genotypes were inclusion criteria in the study, there was a need for a tool which allowed a rapid and effective screening for the polymorphisms of interest.

For sometime, our group had used the services of Jurilab Ltd., (Kuopio, Finland), and their DrugMEt® test to genotype the *CYP2D6* gene. Due to the discontinuation of the product, a new test had to be used. The selected test was the PHARMAchip™ DNA array, developed by Progenika Biopharma S.A. (Derio, Vizcaya, Spain). Several reasons led us to the need to assess the performance

characteristics of the new DNA array in order to ensure the results obtained.

The evaluation of the PHARMAchip™ included a single-blind comparison of genotypes obtained from 100 DNA selected samples from individuals of Spanish origin, previously analyzed with the Jurilab's Ltd DrugMEt® Test or in-house in our laboratory for those genes that were not included in this test (*COMT* and *5-HTT*).
The DNA samples selected to be re-analyzed by the PHARMAchip™ DNA array were selected for the purpose of obtaining a wide range of different genotypes to cover a maximum of common allelic variants of genes investigated.

Validation of the PHARMAchip™ DNA array

Several measures of quality were evaluated in order to determine the suitability of the new DNA array for the objectives of the study.

Accuracy

In order to assess the accuracy of PHARMAchip™, the selected samples were sent to Progenika Biopharma S.A. and were analyzed blindly.
The genotype results obtained by Progenika Biopharma S.A. were compared with those previously provided by DrugMEt®. The core set of genetic variants selected for the validation were those in common between both chips.
If the genotype call was equal in both methods for one gene, results for the whole set of individual SNPs combined/used by PHARMAchip™ software for its determination were considered correct (match). When genotype calls differed between the two methods, the putative SNPs identified as causing the discrepancy among the corresponding set of SNPs were referred to as mismatch results.

The DNA of samples with discrepant results was again sent to Progenika Biopharma S.A. and sequenced (see Materials and Methods in publication I, for further details).

Successful genotyping rate and accuracy of PHARMAchip^{TM} were calculated as the percentage of matched genotypes among the total number of evaluable genotypes and among the called genotypes, respectively.

Reproducibility and limit of detection

Reproducibility and limit of detection of the PHARMAchip^{TM} were assessed by Progenika Biopharma S.A.

Briefly, genomic DNA control samples (from a Progenika Biopharma S.A. proprietary cell line), were used. Three DNA controls were processed with PHARMAchip^{TM} in the same run over 5 consecutive days to evaluate within-day and between-day variability. The limit of detection was determined using serial dilutions (500 to 5 ng) of a DNA control (accuracy was compromised below this concentration). The limit of detection was set at the minimum DNA concentration where the signal intensity was strong enough to still give a correct genotype call.

Genotype calls in the repeated runs, and at the different DNA concentrations, were compared to the reference data obtained through sequencing. In addition, the global array fluorescence signal and that obtained in each probe set were also analyzed. The signal intensity was normalized against the background and the calculated variance on a signal level was given as a coefficient of variation for the ratio intensity/background.

3.1.2. Sample preparation

Blood samples for DNA extraction included in this study were obtained from participants in different clinical trials approved by the local Ethics Committee (CEIC-IMAS). Each volunteer gave a written informed consent before participating.

Genomic DNA was extracted from blood samples enriched with white cells (buffy coat) using FlexiGene DNA kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany).

3.1.3. Genotyping of variants not included in the DrugMEt® test

COMT val108/158met genotyping

The COMT val108/158met (rs4680) single nucleotide polymorphism (SNP) allelic variant was determined using the 5' exonuclease TaqMan® assay with ABI 7900HT Sequence Detection System (Real Time PCR) supplied by Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Primers and fluorescent probes were obtained from Applied Biosystems with TaqMan® SNP Genotyping assays (assay ID C_2255335_10). Reaction conditions were those described in the ABI PRISM 7900HT user's guide. Endpoint fluorescent signals were detected on the ABI 7900, and the data were analyzed using Sequence Detector System software, version 2.3 (Applied Biosystems).

5-HTTLPR genotyping

The 5-HTTLPR genotyping was performed using polymerase chain reaction (PCR). Each reaction mixture contained: 1x PCR Amplification buffer and 1x PCR Enhancer solution (Invitrogen, Carlsbad, CA), 1.5 mM $MgSO_4$, 300 µM dNTPs, 0.5 pmol of each primer, 0.5U of Taq DNA polymerase (Invitrogen) and 50 ng of genomic DNA as template. Primers used are described in table 1. Amplification conditions were 35 cycles of 30 s at 95ºC, 30 s at 58ºC and 1 min at 68ºC with an initial denaturation step of 3 min at 95ºC.

A 10 µl total reaction volume was used and, after PCR, the products of allelic-specific amplifications (allele L, 528 bp; allele S, 484 bp) were detected on an automatic ABI 3730XL capillary sequencer and analyzed by GeneMapper Software v3.5 (Applied Biosystems). GeneScan™-1000 [ROX]™(Applied Biosystems) was used as size standard. The analysis was performed at the Serveis Científico-Tècnics, Universitat Pompeu Fabra (UPF) premises.

Table 1: Primers and probes used

variant	Primers used
5HTTLPR	Forward: FAM-5'-GGCGTTGCCGCTCTGAATGC-3' Reverse: 5'-GAGGACTGAGCTGGACAACAACCAC-3'
5HTTVNTR	Forward: FAM-5´-GTCAGTATCACAGG-CTGCGAG-3´ Reverse: 5´-TGTTCCTAGTCTTACGCCAGT-3´
COMT P2 promoter (rs2097603)	Forward: 5'-GCCGTGTCTGGACTGTGAGT-3' Reverse: 5'-GGGTTCAGAATCACGGATGTG-3' Reporter probes for Real-Time PCR: Forward: FAM-AACAGACAGAAAAGTTTCCCCTTCCCA-3' Reverse: VIC-CAGACAGAAAAGCTTCCCCTTCCCATA-3'

3.1.4. Genotyping of additional SNPs not included in the PHARMAchip DNA array

Some other SNPs not included in the PHARMAchip DNA array were considered useful for human studies and were genotyped in-house. Different genotyping approximations were used.

Fragment analyses were performed at the Serveis Científico-Tècnics, Universitat Pompeu Fabra (UPF) premises.

For the ***5-HTT* gene, a variation number tandem repeat (VNTR)** within intron 2 (*5-HTTVNTR*) was also genotyped.
The reaction mixture contained 1 x PCR Amplification buffer and 1x PCR Enhancer solution, 1.5 mM $MgSO_4$, 300 µM dNTPs, 0.5 pmol of each primer, 0.5U of Taq DNA polymerase, and 50 ng of genomic DNA as template. Primer sequences are those detailed in table 1. The amplification conditions include an initial denaturation step of 3 min at 95ºC and 35 cycles of 30 s at 95ºC, 30 s at 60ºC and 30 s at 72ºC.
A 10 µl total reaction volume was used and, after PCR, the products of allelic specific amplifications (allele 9, 250 bp; allele 10, 267 bp; and allele 12, 300 bp) were detected on automatic ABI 3730XL capillary sequencer and analyzed by GeneMapper Software v3.5. GeneScan™-500 [ROX]™ was used as size standard.

An additional polymorphism within the **serotonin transporter promoter (5-HTTLPR (A/G) polymorphism, rs25531)** was also genotyped using restriction enzyme digestion and the subsequent fragment analysis.
The protocol used was originally described by De Luca et al., 2005, (De Luca et al., 2005) with minor modifications. Briefly, 1 µl of the *5-HTTLPR* PCR (as described in the previous section see *5-HTTLPR*

genotyping) was digested in a 10 µl reaction assay containing 1x NEBuffer 2, and 3U MspI (New England Biolabs Ipswich, MA) at 37ºC for 3 h and a final inactivation step of 20 minutes at 65ºC. The resulting fragments were detected on an automatic ABI 3730XL capillary sequencer and analyzed by GeneMapper Software v3.5. Product sizes for the digest were: long A (L_A)= 337 bp, short A (S_A)= 292 bp, long G (L_G)= 162 bp, and short G (S_g)=162 bp.

In some cases, where MspI digestion gave unclear results the samples were sequenced to assign the correct genotype. Sequencing was performed in both, the sense and antisense orientations. The excess primers and deoxynucleotides in the polymerase chain reaction (PCR) products were then degraded by adding a 2 µl of a solution of 0.8U of shrimp alkaline phosphatase (New England Biolabs), 4U of *Escherichia coli* Exonuclease I (New England Biolabs) and 0.64x shrimp alkaline phosphatase buffer. The mixture was incubated at 37℃ for 15 min, followed by deactivation for 15 mi n at 80℃. Sequencing reactions were performed with BigDye v3.1 (Applied Biosystems) in 10 µl total volume containing 1 µl template (approximately 25 ng), 3.2 pmol primer, 1 µl 5x DNA sequencing buffer (Applied Biosystems), 2 µl BigDye v3.1, and water. The reactions were cycled at 94℃ for 3 min, followed by 30 cycles at 96℃ for 10 sec, 50℃ for 5 sec, and 60℃ for 4 min. Reactions were then purified with PureLink Quick Gel extraction kit (Invitrogen) according to manufacturer's instructions. Samples were analyzed on a Prism 3730xl DNA Analyzer (Applied Biosystems). GeneScan™-500 [ROX]™ was used as size standard.

The ***COMT* 3' flanking region SNP (rs165599)**, the ***COMT* P2 promoter (rs2097603, later on rs2075507)**, and the ***BDNF* val66met (rs6265)** allelic variants were determined using an ABI 7900HT

Sequence Detection System (Real Time PCR) supplied by Applied Biosystems.

Primers and fluorescent probes for the assays were obtained from Applied Biosystems TaqMan® SNP Genotyping assays (assay ID C_2255335_10 and C_11592758_10 for rs165599 and rs6265 respectively). For the rs2097603, primers and probes used were described previously by Meyer-Lindenberg et al., 2006 (Meyer-Lindenberg et al., 2006) (see table 1). Reaction conditions were those explained in the ABI PRISM 7900HT user's guide. Endpoint fluorescent signals were detected on the ABI 7900, and the data were analyzed using Sequence Detector System software, version 2.1.

3.1.5. Recruitment of the subjects

Volunteers were recruited through an internet platform http://estudiardrogas.imim.es. In the website, participants were informed preliminarily about the scope and procedures of the study, and then asked to provide some administrative data and fill a questionnaire on status and intensity of toxic habits if they were interested in enrolling the study.

Those candidates meeting preliminarily inclusion criteria (see table 2, and the FIS-MDMA study protocol, Appendices) were contacted and interviewed. The purpose of the interview was: (i) to inform more in depth on the scope and procedures of the study, (ii) to obtain an informed consent, (iii) to confirm the questionnaire on toxic habits.

If they agreed in participating in the study, candidates provided a blood sample for genotyping and were subjected to a health and psychiatric examination.

Upon arrival to the research centre (IMIM, Hospital del Mar Research Institute), subjects were informed of the ensuing protocol and gave their written informed consent before participating in the study.

Once genotyped, individuals meeting inclusion criteria were contacted back and subjected to a neuropsychological evaluation. Previous to the evaluation, candidates were invited to provide urine and a hair sample for drug testing.

After the selection process, two hundred sixty-three participants (n=263) were recruited, of whom 60 were ecstasy polydrug users, 110 were cannabis users and 93 were non users.
In the ecstasy group, subjects were classified into heavy ecstasy users or light ecstasy users according to their lifetime consumption in order to evaluate the dose effect. The threshold was set at 100 ecstasy tablets.

Table 2: Inclusion and exclusion criteria to fulfil for all the candidates

Inclusion Criteria

1. Male or female volunteers age range 18 to 30.
2. Minimal education: secondary education or equivalent.
3. A history or results from a medical exam that demonstrated that volunteers do not present physical or psychiatric problems.
4. The ECG, general blood and urine analyses taken before the trial should be normal. Small variations within the limits of the norm were admitted, those that the investigators consider to be without clinical relevance.
5. Toxic habits:
    - Cannabis users group: Daily or almost daily cannabis consumption, with a weekly consumption of 14-28 joints (hashish + tobacco or marihuana). Minimum lifetime use of three years and at least one year of daily consumption meeting criteria of abuse and/or dependence. Consumption of less than five times of any other illicit drug, none in the last year and non meeting criteria of abuse and dependence of these drugs.
    -

- MDMA consumers group: Lifetime consumption of at least five occasions and at least one consumption in the last year, being MDMA the main psychostimulant abused. Daily or almost daily cannabis consumption, with a weekly consumption of 14-28 joints (hashish + tobacco or marihuana).
- Drug Free control group: Not meeting abuse and dependence criteria for any psychoactive drug except for nicotine. Lifetime cannabis consumption in less than 25 occasions and in five occasions for any other psychoactive drug. No consumption of any illicit drug in the last year. Less than 5 alcohol units/day for males and 2 units/day for females.
- MDMA and Cannabis groups: the consumption of two alcoholic beverages during meals, isolated episodes of acute intoxication (a maximum drunkenness once every three months) and spirits consumption during week-ends (2-3 per week) were tolerated.

6. The acceptance of trial procedures and signature of the consent form.

Exclusion Criteria

1. Previous history of brain damage with loss of consciousness (e.g. epilepsy, head injury, stroke) that may alter cognitive performance.
2. Current use or in the previous weeks of psychotropic drugs.
3. Active (last year) Axis I mental disorder following DSM-IV criteria.
4. Intelligence Quotient (IQ) <90, following the vocabulary sub-test WAIS III.
5. Subjects not able of understand study objectives and procedures.

3.1.6. Psychiatric and neuropsychological evaluation

Prior to the inclusion in the study, subjects were examined for their health status with the SF-36 questionnaire (Vilagut et al., 2005) and for psychopathology using the Spanish versions of the Psychiatric Research Interview for Substance and Mental Disorders (PRISM) (Torrens et al., 2004).

Neuropsychological evaluation (by a psychiatrist or psychologist) was organized into three main domains: (i) measures of memory and attention, (ii) measures of emotion processing, and (iii) measures of cold and hot executive functions. Tests were selected to cover the study aims, including a comprehensive assessment of key aspects of impulse control and decision-making, along with more general cognitive processes.

The neuropsychological protocol administered included the following tests: vocabulary subtest (from the WAIS-III, Spanish version, (Wechsler, 1997)); California Computerized Assessment Package CALCAP (Miller, 1990); Tower of London (Shallice, 1982); word fluency (Benton and Hamsher, 1983); semantic fluency(Benton and Hamsher, 1983); Symbol Digit Modalities Test (Smith, 1982); California Verbal Learning Test II (Delis et al., 2000); Rey complex figure test (Rey, 1941); Corsi block tapping subtest (from the WAIS-III, (Wechsler, 1997)); and letter number sequencing subtest (from the Wechsler Memory Scale III,(Wechsler, 1997)).

For the purpose of this thesis, only a subset of cognitive measures was selected and will be explained in more detail.

Memory and attention:

- Episodic memory, California Verbal Learning Test (Spanish adaptation: Test de Aprendizaje Verbal España-Complutense).
  The test consists on a list of 16 words that the participant must learn across five learning trials and then reproduce after presentation of an interference list (immediate recall) and after 20-minutes lapse (delayed recall). The dependent measures were CVLT standard indices of learning (trials 1 to 5), immediate recall, delayed recall and recognition.

- Processing Speed, Symbol Digits Modalities Test (from the Wechsler Adult Intelligence Scale –WAIS III). A brief measure of perceptual-motor speed. Participants have to rapidly convert geometric designs into Arabic numbers following a visual key. Answers must follow the correspondence shown in a visual key. The dependent measure is the total number of hits produced in 90 seconds.

- Visual Episodic Memory, Rey-Osterrieth Complex Figure Test (ROCFT): Participants have to (i) copy and (ii) reproduce (after 3- and 30-minutes delays) a complex visual figure. The dependent measures were ROCFT standard indices of immediate recall, delayed recall, and accuracy of the copy.

Executive functions:

- Verbal and Semantic Fluency, FAS and Animals. It evaluates the ability to access, to retrieve, and produce targeted information in response to a novel order (e.g., words starting with the letters F, A, and S, or words belonging to the category “animals”).

  Two types of measures were obtained from these tests:

  (1) *Total words generated*:

  Total number of correct words generated in 60 seconds. All intrusions (words not pertaining to this semantic category), perseverations (same words), and repetitions (same words with different endings) were treated as mistakes.

  (2) *Clustering and switching measures*:

  Mean Cluster Size was the main dependent variable for clustering, whereas Number of Switches was the main

dependent variable for switching. A cluster was defined as any series of two or more successively produced words belonging to the same semantic or phonemic subcategory. Cluster size was computed adding up series of words from the same subcategory starting from the second word within each cluster (i.e., a 3-word cluster has a size of 2). The Mean Cluster Size was obtained by adding the size of all the clusters and dividing it by the total number of clusters. The Number of Switches was defined and computed as the number of times the participant changed from one cluster to another within the same task. The computation of Number of Switches included single-word clusters (cluster size= 0). Intrusions, repetitions, and perseverations were excluded from the calculation of both Mean Cluster Size and Number of Switches.

3.1.7. Statistical analysis

Statistical analyses regarding human studies were performed by Dr. Klaus Langohr (statistician).

Details on the analysis performed in each case are described within the material and methods section of the different publications (included in the appendices section).

3.2. ANIMAL MODEL STUDIES

All animal handling, treatment, and biochemical measures (5-HT, 5-HIAA, and temperature) were carried out at the Departamento de Farmacología, Facultad de Farmacia de la Unversidad de Navarra (Pamplona), under the supervision of Dr. Norberto Aguirre. Procedures were approved by the local ethical committee following standards established in the Council of Europe Directive of the 24/Nov/1986, published on DOCE 18/Dec/1986. All the procedures will be described briefly.

3.2.1. Animals and treatment groups

In all cases, the doses of MDMA used refer to the hydrochloride salt. All the procedures applied in the present work were in compliance with the European Community Council Directive (86/609/EEC) and were approved by the Ethical Committee of the Universidad de Navarra.

Twelve male Wistar rats weighting 260-280 g were used for these experiments (six in each group, control and treated). Rats received three intraperitoneal injections (*i.p.*) (every 2 hours) of saline or MDMA 5 mg/kg. Seven days after treatment, rats were killed by decapitation (figure 12).

Rats were housed in constant conditions of humidity and temperature (22 ± 1ºC) with a 12-h/12-h light-dark cycle (lights on at 7:00 hours). Food and water were available *ad libitum*.

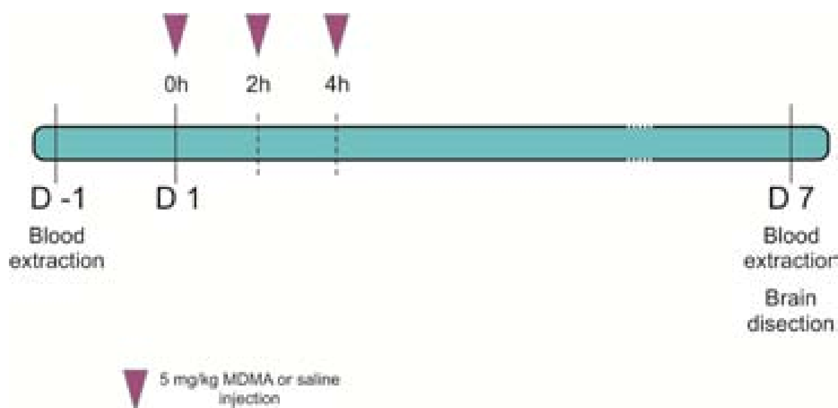


Figure 12: Scheme of the animal model study design, showing MDMA dose intervals and sample obtaining.

3.2.2. Samples

Blood

Blood samples were obtained before and after the treatment from the retro-orbital sinus with a glass capillary and immediately mixed with RNAlater® Solution (Ambion Inc., Austin TX, USA) at 4℃ overnight and then stored at -20℃ until RNA extraction.

Tissue

After decapitation, brains were rapidly removed and the different brain regions were dissected and submerged on RNAlater® Solution at 4ºC overnight and then stored at -80ºC until tissue homogenization and RNA extraction.

The brain regions selected were the hippocampus, the frontal cortex, the striatum and the dorsal raphe nucleus + substantia nigra (DRN+SN).

3.2.3. Temperature measurements

Rectal temperature of the rats was measured at an ambient temperature of 21.5±1 ℃ with a lubricated digital thermometer probe (pb 0331, Panlab, Barcelona) inserted approximately 3 cm into the rectum, the rat being lightly restrained by holding in the hand. Temperature was recorded before the first MDMA injection and thereafter every 60 min up to 8 hours. Probes were re-inserted from time to time until the temperature stabilized.

3.2.4. Biochemical measurements

Concentrations of 5-HT, 5-HIAA, in striatum, hippocampus, and cortex, were determined by high performance liquid chromatography with electrochemical detection. Briefly, samples were injected using an automatic sample injector (Waters 717 plus) onto a Spherisorb ODS-2 reverse phase C18 column (5 lm, 150 · 4.6 mm; Teknokroma, San Cugat del Vallès, Spain) connected to a DECADE amperometric detector (Antec Leyden, Zoeterwoude, The Netherlands), with a glassy carbon electrode maintained at 0.7 V with respect to an Ag/AgCl reference electrode.
The mobile phase consisted of citric acid 0.1 mol/L, Na_2HPO_4 0.1 mol/L, octanesulphonic acid 0.74 mmol/L, EDTA 1 mmol/L and methanol 16% (pH 3.4), pumped at a flow rate of 1mL/min.

3.2.5. Total RNA extraction

Total RNA refer to the whole pool of RNA molecules obtained from the extraction procedure from samples. It can contain different classes of RNA such as ribosomal RNA (rRNA), transport RNA (tRNA), messenger RNA (mRNA), etc. From all these different types, only mRNA molecules are used in gene expression analysis, as they represent the transcriptome activity of genome.

Total RNA was extracted from the different homogenized tissues or blood using the RiboPure Kit or RiboPure Blood Kit (Ambion Inc., Austin TX, USA) respectively, according to manufacturer's instructions. RNA samples were stored at -20ºC prior to its use.

3.2.6. Evaluation of total RNA integrity and purity

RNA qualities were assessed using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), and concentrations were determined using a ND-1000 spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, DE). RNA samples were stored in aliquots at -80ºC prior to use.
Gene expression analysis results may be influenced by different parameters related to RNA quality (purity and integrity) and quantity (concentration) (Khymenets et al., 2005).

1- Purity:

Total RNA purity is evaluated by the presence of protein or salts and other products of the RNA extraction procedure (e.g. ethanol). Elevated concentrations of impurities may compromise further downstream applications of the sample. The most common method for the assessment of RNA purity is based on the spectrophotometric estimation of absorbance at 260 nm (specific for nucleic acids), at 230 nm (ethanol and salts) and 280 nm (proteins). The A_{260}/A_{230} and A_{260}/A_{280} ratios are used as a representation of the relative abundance of the different impurities in the RNA sample. The accepted ranges of these ratios are within 1.6 and 2.0 for most of the commonly used applications, although in some cases, more restrictive conditions should be necessary.

2- Concentration:

Calculated on the basis of absorbance at 260 nm (specific for nucleic acids) and using the Lambert-Beer law, which predicts a linear change in absorbance versus concentration.

Some application requires high amounts or concentrations of RNA (e.g. microarrays) whereas others (e.g. TaqMan® Low Density Array) can be performed with quite diluted samples.

3- Total RNA integrity:

Typically, RNA integrity is evaluated by the estimation of the integrity of ribosomal RNA subunits bands (ratio between 28s and 18s rRNA bands) which must be around 2:1. Although useful, this ratio has been claimed to be imprecise regarding the integrity of mRNA in the samples of total RNA (Imbeaud et al., 2005;Schroeder et al., 2006).

The commercial automated capillary-electrophoresis system software used for the assessment of the RNA purity in this work (Agilent Bioanalyzer 2100) allow calculation of the RNA integrity number (RIN), which is a measure of integrity determined for the entire electrophoretic trace of the sample. This RIN is said to be independent of sample concentration, instrument and analyst, becoming a good measure of RNA integrity.

3.2.7. Reverse Transcription

The reverse transcription reaction was performed using a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems) according to manufacturer's instructions.

In these experiments, cDNA synthesis was performed using random primers to prime the reverse transcription reaction. Random primers consist of a mixture of short oligonucleotides representative of all

possible short sequences in the RNA molecules, so that, virtually all types of RNA molecules are reverse transcribed. Another frequently used method for cDNA synthesis is oligo dT primers. In that case, the primer hybridizes to the poly(A) tail of the mRNA, therefore, transcribing all mRNAs in the sample.

3.2.8. Real-Time PCR

Gene expression was assessed by quantitative TaqMan Real-Time PCR (TaqMan® Low Density Array (TLDA), Applied Biosystems). Reaction conditions were those explained in the ABI PRISM 7900HT user's guide.
β-actin and 18s rRNA were used as a reference genes. Data obtained were analysed by the Relative Quantity Manager 1.2 software (Applied Biosystems).

The TaqMan® Low Density Array is a 384-well micro fluidic card that allows the performance of 384 simultaneous real-time PCR reactions.
This is a medium-throughput array which requires lower amounts of sample and is less time-consuming than other approaches such as individual TaqMan® Gene expression assays.

Fourteen genes and two reference genes were selected for its inclusion in our study (table 3), so that 8 different samples (in triplicate) can be run in parallel in each card.

Table 3. List of selected genes.

Gene symbol	Name	Assay ID (TaqMan® Gene expression assays)	
Slc6a4	serotonin transporter	Rn00564737_m1	
Slc6a3	dopamine transporter	Rn00562224_m1	
Slc6a2	norepinephrine transporter	Rn00580207_m1	
Slc18a2	vesicular monoamine transporter	Rn00564688_m1	
Maoa	monoamine oxidase A	Rn01430955_m1	
Maob	monoamine oxidase B	Rn00566203_m1	
Ddc	aromatic amino acid decarboxylase	Rn00561113_m1	
Tph1	tryptophan hydroxylase 1	Rn01476869_m1	
Tph2	tryptophan hydroxylase 2	Rn00598017_m1	
Th	tyrosine hydroxylase	Rn00562500_m1	
Snca	synuclein α	Rn00569821_m1	
Sncg	synuclein γ	Rn00581652_m1	
Gfap	glial fibrillary acidic protein	Rn00566603_m1	
Hspa1a	heat shock 70kD protein 1A	Rn00583013_s1	
Actb	β-actin	Rn00667869_m1	Reference gene
18s rRNA	18S ribosomal RNA	-	Reference gene

Reference genes are used for normalization of data obtained in gene expression experiments. Typically, reference genes are constitutive genes transcribed at a relative constant level and that are unaffected by the experimental conditions.

3.2.9. Statistical analysis

Shapiro-Wilk test was used to test samples normality. Statistical comparisons between saline and MDMA treated groups were performed by independent samples Student's *t*-test. Differences were considered statistical significant at $p<0.05$. Data analyses were

performed using the Statistical Program for the Social Sciences (SPSS 12 for Windows).

4. RESULTS

This chapter includes the results of four different conducted to achieve the aims and objectives of this work.

Study 1 includes the results of the validation of the PHARMAchip^{TM} DNA array as part of the methodology used for the subsequent human studies conducted. Most of the results were described in an original publication (**publication I**), but some results were not included and therefore will be discussed in this work.

Results of the second study (**Study 2**) were used for an original publication (**publication II**) and examine the influence of serotonin transporter and COMT genotypes on verbal fluency in ecstasy users. The third study (**Study 3**) sought to identify the influence of a larger number of genetic polymorphisms within the serotonergic and dopaminergic systems in relation to the impact on cognitive performance in ecstasy users (**submitted publication III**).

Finally, the **Study 4** comprises the results from animal model studies, specifically the long-term effects of MDMA treatment on gene expression and serotonin metabolites in the rat brain.

Study 1

Errors and reproducibility of DNA array-based detection of allelic variants in ADME genes: PHARMAchip.

Cuyàs E, Olano-Martín E, Khymenets O, Hernández L, Jofre-Monseny L, Grandoso L, Tejedor D, Martínez A, Farré M, de la Torre R.

Pharmacogenomics. 2010 Feb;11(2):257-66

TECHNOLOGY REPORT

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Errors and reproducibility of DNA array-based detection of allelic variants in ADME genes: PHARMAchip™

Aims: Differences in adverse drug reactions can be explained by genetic variations, especially if they determine the expression of certain protein effectors and/or drug-metabolizing enzymes. Over the last decade, several tests screening for the most frequent polymorphisms in drug-metabolizing enzymes have been marketed for research and diagnostic purposes. The aim of this study was to assess the suitability of PHARMAchip™ for the genotyping of polymorphisms of genes associated with drug metabolism and response as an alternative to Jurilab Ltd's DrugMEt® Test. **Materials & methods:** In this observational study, performed using 100 previously genotyped DNA samples, we report on common genes included in the two different tests examined: the former DrugMEt test and the recently introduced PHARMAchip test. **Results & conclusion:** Although these tests are based on different methodological approaches, we have found a high concordance of results between both methods. Some of the discrepancies between tests were related to allelic variants not monitored in a particular microarray and the quality of the genomic DNA used.

KEYWORDS: CYP2D6 ▪ CYP3A5 ▪ DNA array ▪ NAT2 ▪ PHARMAchip™ ▪ pharmacogenetics

Elisabet Cuyàs[1,2], Estibaliz Olano-Martín[3], Olha Khymenets[1,4], Lorena Hernández[3], Laia Jofre-Monseny[4], Laura Grandoso[3], Diego Tejedor[3], Antonio Martínez[3], Magí Farré[1,2] & Rafael de la Torre[1,4]†

†Author for correspondence: Human Pharmacology & Clinical Neurosciences Research Group of the Neuropsychopharmacology Program, Institut Municipal d'Investigació Mèdica (IMIM-Hospital del Mar), Parc de Recerca Biomèdica de Barcelona, c/ Dr. Aiguader 88/08003 Barcelona, Spain
Tel.: +34 933 160 484
Fax: +34 933 160 467
rtorre@imim.es
[2]Universitat Autònoma de Barcelona, Barcelona, Spain
[3]Progenika Biopharma SA, Derio, Spain
[4]Universitat Pompeu Fabra, Barcelona, Spain

Differences in individual therapeutic responses to drugs and adverse drug reactions are health problems that contribute to worldwide morbidity and mortality. Several factors, such as age [1], gender [2], ethnicity [3], disease and genetics [4], contribute to these interindividual variations. Among them, the identification of pharmacogenetic biomarkers offers a promising strategy in the search for predictors of response to drugs [5,6]. Indeed, several polymorphisms have already been associated with differences in the activity of proteins involved in drug metabolism, transport and response [7].

Most therapeutically used drugs are subjected to Phase I metabolic reactions, which are mainly regulated by cytochrome P450 isoenzymes. Some of them are relevant because they metabolize either a large number of drugs (e.g., CYP2D6) or some specific drugs relevant in therapeutics (e.g., CYP2C19 and warfarin). Most of these cytochrome P450 isoenzymes account for several clinically important genetic polymorphisms, notably CYP2D6, a highly polymorphic enzyme that is, alone, responsible for more than 80 allelic variants [8]. Traditional techniques for the study of polymorphic variation in cytochrome P450 genes (especially for *CYP2D6*) are time consuming and identify a single allele per run. There is the risk, therefore, that phenotype prediction is based on a limited set of allelic variants. As a result, a large number of commercial molecular biology products have been developed to detect genetic variations of targeted cytochrome P450 isoenzymes. The most widely used is AmpliChip® CYP450 from Roche Diagnostics [9–11], which analyzes 30 allelic variants in two genes (*CYP2D6* and *CYP2C19*). The AmpliChip CYP450 Test was the first pharmacogenetic test approved by the US FDA and predicts the four CYP2D6 phenotypes and two CYP2C19 phenotypes. One of the main limitations of this test is the noninclusion of other important genes relevant in pharmacogenetics, such as *TPMT* or *VKORC*. Other tests, such as the former DrugMEt® Pharmacogenetic test from Jurilab Ltd (Kuopio, Finland), in addition to some cytochrome P450 isoenzymes (e.g., *CYP2D6*, *CYP2C9*, *CYP2C19* and *CYP2B6*), also targeted Phase II enzymes and drug-transporter genes (e.g., *NAT-2*, *TPMT* and *MDR1*, respectively), altogether genotyping for 27 SNPs from one transporter and seven different drug-metabolizing enzyme genes. DrugMEt received the European CE mark in May 2006, but nowadays the service is discontinued.

Other examples are CodeLink P450 SNP Bioarrays from Amersham Biosciences [12], designed for toxicogenetic screening and analyzing 110 SNPs, a relatively open platform but with some limitations concerning the nature of printed biochips, such as the imperfections

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existing on certain probes that are masked after printing [11], and the most recently launched by Affymetrix (CA, USA), the DMET™ Plus Premier Pack, which includes arrays, reagents and analysis software, and interrogates 1936 drug metabolism markers in 225 genes and includes all US FDA-validated genes. It contains transporter gene families, such as *MDR1*, *ABCB2* and *ABCG*, and also performs quantitative assessment of genes with whole-gene deletions, including *GSTT1*, *GSTM1*, *CYP2D6*, *CYP2A6* and *UGT2B17* [12,13].

In clinical practice, the commercialization of a given DNA microarray may be discontinued, and an alternative product must be found. Moreover, the assumption that the alternative product will produce the same results must be substantiated before releasing results with a clinical impact. The PHARMAchip™ DNA array, recently launched by Progenika Biopharma SA, has been developed for the screening of genetic variants for Phase I and Phase II drug-metabolism enzymes, drug transporters and drug protein effectors (see TABLE 1). This tool permits accurate, rapid and cost-effective screening for more than 34 genes and 85 allelic variants (73 SNPs, ten small insertion or deletions, and gene deletions and duplications [*CYP2D6*]). In contrast to the DrugMEt test previously used in our clinical studies, PHARMAchip makes possible a more accurate *CYP2D6* allele discrimination, and the examination of other genes related to not only drug metabolism but also drug response (e.g., receptors and transporters). The clinical relevance of some of the genes included in the array, particularly brain receptors and transporters, awaits further substantiation in clinical studies.

The aim of this study was to assess the suitability of PHARMAchip for the genotyping of polymorphisms of genes associated with drug metabolism and response as an alternative to Jurilab Ltd's DrugMEt Test. The evaluation of the suitability of the new tool included a single-blind comparison of genotypes obtained from 100 DNA samples selected from individuals of Spanish origin, previously analyzed with the Jurilab Ltd's DrugMEt Test.

Materials & methods

Study population

The study was performed using 100 previously genotyped DNA samples from prior clinical studies. As the DrugMEt Test interrogates a limited number of the genes included in the PHARMAchip DNA array (see TABLE 1 & BOX 1), those DNA samples were selected for the purpose of obtaining a wide range of different genotypes to cover a maximum of common allelic variants of genes investigated. Samples included in the study were analyzed initially by the DrugMEt Test at Jurilab Laboratory in Kuopio, Finland. Investigators at the Human Pharmacology and Clinical Neurosciences Research Group, IMIM-Hospital del Mar (Barcelona, Spain), selected the samples to be re-analyzed by the PHARMAchip DNA array. Blind genotyping of samples was performed at Progenika Biopharma (Derio, Spain) premises.

DNA samples

Blood samples for DNA extraction included in the current analysis were obtained from participants in different clinical trials approved by the local Ethics Committee. Each volunteer gave written informed consent before participating.

Genomic DNA was extracted from blood samples that were enriched with white cells (buffy coat) using the FlexiGene™ DNA kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Suitability of DNA multiple displacement amplification

In order to evaluate whether whole-genome amplification was compatible with PHARMAchip technology, in cases where DNA amount was a limiting factor, a comparison was carried out between the results obtained for 15 samples and their corresponding amplified material. Whole-genome amplification was performed with GenomiPhi v2 DNA Amplification kit (GE Healthcare), according to the manufacturer's instructions. Both amplified DNA and nonamplified DNA were hybridized in PHARMAchip, and the results were compared.

DNA sequencing & agarose gel analysis

In cases where genotyping results between the two compared methods were discordant, sequencing of the mutation was carried out to assign the correct genotype. Sequencing was performed in both the sense and antisense orientations with an automated sequencer (ABI PRISM® 3100 Genetic Analyzer; Applied Biosystems, CA, USA).

If the discordant result involved the presence or absence of duplication or deletion of *CYP2D6* (i.e., false positive or false negative), a modification of the methods described by Lundqvist [14] and Hersberger [15] was employed to resolve the discrepancy. The PCR product amplified was

Table 1. List of ADME genes assayed by the PHARMAchip™ versus DrugMEt® tests.

Gene	Name	Allelic variants detected by PHARMAchip	Allelic variants detected by DrugMEt
CYP1A1	Cytochrome P450 1A1	Ile462Val	
CYP1A2	Cytochrome P450 1A2	*1, *1F, *7, *11	
CYP2B6	Cytochrome P450 2B6	G516T	*1, *4, *5, *6, *7, *13, *16
CYP2C19	Cytochrome P450 2C19	*1, *2, *3, *4, *5, *7, *8, *9, *10	*1, *2, *3
CYP2C8	Cytochrome P450 2C8	*1, *2, *3, *4	
CYP2C9	Cytochrome P450 2C9	*1, *2, *3, *4, *5, *6	*1, *2, *3
CYP2D6	Cytochrome P450 2D6	*1, *2, *3, *4, *5 (deletion), *6, *7, *8, *9, *10, *11, *14A, *14B, *15, *17, *18,*19, *20, *25, *29, *31, *35, *41, duplications: *1XN, *2XN, *4XN, *10XN, *17XN, *35XN, *41XN	*1, *2, *3, *4, *5 (deletion), *6, *7, *8, *9, *10, *11, *12, *14A, *14B, *17, *41, duplications: *1XN, *2XN, *4XN, *10XN, *17XN
CYP3A4	Cytochrome P450 3A4	*1, *1B	
CYP3A5	Cytochrome P450 3A5	*1, *3, *6, *9, *10	*1, *3
GSTM1	Glutathione transferase M1	Present/absent	
GSTM3	Glutathione transferase M3	*A, *B	
GSTP1	Glutathione transferase-pi	Ile105Val	
GSTT1	Glutathione transferase-n1	Present/absent	
NAT2	*N*-acetyltransferase 2	*4, *5A, *5B, *5C, *5D, *5E, *5G, *5J, *6A, *6B, *7A, *7B, *11A, *12A, *12B, 12C, *13, *14A, *14B	*4, *5A, *5E, *6, *7, *14A, *14C, *14D
TPMT	Thiopurine *S*-methyltransferase	*1, *2, *3A, *3B, *3C, *3D	*1, *2, *3A, *3B, *3C
MDR1	Multidrug resistance 1 transporter	C3435T	C3435T
UGT1A1*	UDP-glucuronosyltransferase 1 family, polypeptide A1	*28 , *36, *37	

Underlined: common genetic variants for both PHARMAchip and DrugMEt tests.
Bold: genes recommended by the US FDA as valid genomic biomarkers [XX].
*Indicates genes included in the test but not in the array.

analyzed by electrophoresis and visualized in an agarose gel (1%; TAE buffer; 100 V; 60 min). In all PCR products, a band at 5 kb indicated successful amplification reaction (control), whereas a band at 3.5 kb indicated amplification of the duplication or deletion fragment.

■ PHARMAchip (Progenika Biopharma SA): DNA array methodology

PHARMAchip is a low-density DNA microarray based on allele-specific probes, which includes 85 variants from 34 genes that encode five categories of proteins related to drug response. These categories involve Phase I enzymes, Phase II enzymes, drug transporters, receptors and other related proteins (see Table 1).

The design, fabrication, validation and analysis of the arrays were carried out following the procedure described by Tejedor [XX] with minor modifications.

Briefly, target DNA for hybridization was prepared in seven independent multiplex PCR reactions. All genes except *CYP2D6* were amplified in multiplex PCR reactions (I–V). The gene *CYP2D6* was amplified together with a shorter deletion-specific fragment in a long-range PCR reaction (PCR-VI). Similarly, a separate long-range multiplex PCR reaction with the *CYP2D6* gene and a short duplication-specific fragment was carried out (PCR-VII) for the identification of individuals carrying multiple copies of the *CYP2D6* gene. All the multiplex PCRs reactions were performed under the same conditions (ten cycles: 94°C 10 s, 60°C 30 s, 68°C 5 min; 25 cycles: 94°C 15 s, 60°C 30 s, 68°C 5 min plus 5 s per cycle) in a Gene Amp® PCR System 9700 thermocycler (Applied Biosystems). The PCR products from each multiplex reaction were pooled and fragmented enzymatically. To improve the hybridization signal, fragments were labeled with biotinylated 11-ddUTP (New England Biolabs, MA, USA). Automatic hybridization was carried out at 42°C for 1 h in a Ventana Discovery™ station using ChipMap™ hybridization buffers and the protocol for the Microarray 9.0 Europe station (Ventana Medical Systems, AZ, USA).

Box 1. Other genes also assayed by the PHARMAchip™ test.

- ***ADRB1*** (β_1 adrenergic receptor), Arg389Gly
- ***ADRB2*** (β_2 adrenergic receptor) Arg16Gly
- ***AGTR1*** (angiotensin II receptor, type 1), A1166C
- ***BDKRB2*** (bradykinin receptor B2), C-58T
- ***DRD3*** (dopamine receptor D3), Ser9Gly
- ***GRIN2B*** (glutamate receptor, ionotropic), C2664T
- ***HTR2A*** (5-hydroxytryptamine [serotonin] receptor 2A), His452Tyr; T102C
- ***ADD1*** (adducin 1 α), Gly460Trp
- ***AGT*** (angiotensinogen), Met235Thr
- ***BCHE*** (butyrylcholinesterase), Asp70Gly; Ala539Thr
- ***COMT*** (catechol-O-methyl transferase), Val108Met
- ***DPYD*** (dihydropyrimidine dehydrogenase, IVS14+1 G>A
- ***ERCC2*** (excision repair cross-complementing rodent repair deficiency complementation group 2), Lys751Gln
- ***IL10*** (IL-10), G-1082A
- ***MTHFR*** (methylenetetrahydrofolate reductase), C677T
- ***TNFα*** (TNF-α), G-308A
- ***TYMS*** (thymidylate synthetase) deletion 3'-UTR 6 pb
- ***VKORC1*** (vitamin K epoxide reductase complex, subunit 1) G-1639A
- ***SLC6A4**** (solute carrier family 6 [neurotransmitter transporter, serotonin] member 4) promoter 44 bp deletion

Bold: genes recommended by the US FDA as valid genomic biomarkers [16].
*Indicates genes included in the test but not in the array.

Finally, DNA arrays were automatically stained with Cy3-conjugated streptavidin (Amersham Biosciences, CA, USA) in the automatic hybridization station. DNA array images were captured by an InnoScan® 700 Microarray Scanner (Innopsys, Carbonne, France). Specific software employing proprietary algorithms developed by Progenika was used to convert the intensity of the spots into the genotype of each SNP and into the presence or absence of duplications and deletions for *CYP2D6*. In addition, the software was designed to form the described combinations of SNPs in order to give the corresponding allele (in case of haplotypes), and the associated phenotype [10].

The software developed by Progenika employed clusters calculated empirically by hybridization signals from a high number of samples, so that at least ten signals were used to calculate each cluster. In the event that rare variants occurred, clones were applied.

Results were given as genotype call for samples that fell within defined clusters for each SNP. In cases of haplotypes, the software was able to assign a genotype through the combination of the set of SNPs included for each gene. If samples did not fall within the defined clusters, or failed to fulfill established quality controls (for signal intensity and coefficient of variation), the software signaled 'nonvalid'. In cases where genotypes calls for single SNPs were given, but their combination did not coincide with any included in the software, the result was given as 'unknown combination'.

In a given sample with a rare mutation that was not included in PHARMAchip, the result would be, by default, the most genetically similar.

DrugMEt pharmacogenetic test approach

The former service offered by Jurilab Ltd included three separate assays to assess the genetic variations and their corresponding alleles in eight genes. These assays were the drug-metabolism enzyme microarray, the *CYP2D6* deletion/duplication PCR assay, and *CYP2D6*41* sequencing. The drug-metabolism enzyme microarray is based on a previously described technique that uses allele-specific primer extension on chip [17]. Briefly, after DNA extraction, 40 ng of genomic DNA is amplified into two separate Multiplex PCRs, thus producing 16 PCR products in a range of 320–137 bp. The dsDNA amplification products, containing recognition sequences for T7-phage RNA polymerase, are used in the *in vitro* transcription to produce ssRNA. The ssRNA is fragmented by heat treatment, mixed with the extension mixture and applied onto the microarray. The array contains two allele-specific detection primers for each genetic variant, differing at their 3'-nucleotide in order to recognize either the wild-type, or the variant nucleotide. Depending on the nucleotide at a SNP location, the specific primer hybridizes to the ssRNA and a fluorescent label is attached to the primer. During the extension, fluorescently-labeled (Cy5) nucleotides are added to the newly built strand, resulting in the illumination of the spots. The signal intensities of the spots are recorded with a microarray scanner. The relative intensities on the specific detection primer pair are converted into genotype information with SnpRater® software.

For the *CYP2D6* deletion and/or duplication PCR assay, 40 ng of genomic DNA is amplified in a multiplex PCR, producing one, two or three amplification products, depending on the genotype, in a range of 2954–3704 bp. The amplification products are separated electrophoretically in an agarose gel. The presence of bands that are indicative of a particular genotype is translated to genotype information by visual inspection of the agarose gel image.

Finally, allele **41* is determined by sequencing the polymorphic site in the *CYP2D6* gene 2988G>A. For the analysis, 20 ng of genomic DNA is amplified in PCR, producing an amplification product from the corresponding genomic region. The amplification product is

run on analytical agarose gel to verify the specific functioning of the amplification reaction. The amplification product is sequenced with an internal primer.

PHARMAchip validation studies

Accuracy

In order to validate the accuracy of PHARMAchip, 100 samples, selected to represent a wide number of allelic variants, were sent to Progenika Biopharma SA. Samples were analyzed blindly, and the genotype results obtained by Progenika Biopharma SA were compared with those previously provided by DrugMEt. The core set of genetic variants selected for validation were those in common between both chips (see Table 1).

If the genotype call was equal in both methods for one gene, results for the whole set of individual SNPs combined/used by PHARMAchip software for its determination were considered as correct (match). When genotype calls differed between the two methods, the putative SNPs identified as causing the discrepancy among the corresponding set of SNPs were referred to as mismatch results.

The DNA of samples with discrepant results was again sent to Progenika Biopharma SA and sequenced, as described in the 'Materials and methods' section.

Finally, successful genotyping rate and accuracy of PHARMAchip were calculated as the percentage of match genotype among the total number of evaluable genotypes and among the called genotypes, respectively.

PHARMAchip reproducibility & limit of detection

Genomic DNA control samples (from a Progenika Biopharma SA proprietary cell line), were used to assess the reproducibility of the assay. Three DNA controls were processed with PHARMAchip in the same run over 5 consecutive days to evaluate within-day and between-day variability. Serial dilutions (500 to 5 ng) of a DNA control were processed in duplicate to determine the limit of detection (accuracy was compromised below this concentration). The limit of detection was set at the minimum DNA concentration where the signal intensity was strong enough to still give a correct genotype call.

Genotype calls in the repeated runs, and at the different DNA concentrations, were compared with the reference data obtained through sequencing. In addition, the global array

Table 2. Diversity of genotypes detected in the test set of samples.

Gene	Genotypes (subjects, n)									
CYP2C19	*1/*1 (67)	*1/*2 (31)								
CYP2C9	*1/*1 (67)	*1/*2 (25)	*1/*3 (3)	*2/*2 (1)	*2/*3 (2)					
CYP3A5	*1/*3 (18)	*3/*3 (79)	*3/*6 (1)							
CYP2D6	*1/*1 (9)	*1/*10 (1)	*1/*17 (1)	*1/*2 (14)	*1/*3 (2)	*1/*35 (2)	*1/*4 (10)	*1/*41 (8)	*1/*5 (1)	*1/*6 (1)
	*1/*2xN (1)	*1/*9 (1)	*1XN/*2 (1)	*1XN/*41 (1)	*2/*10 (1)	*2/*2 (2)	*2/*3 (1)	*2/*35 (3)	*2/*4 (8)	*2/*41 (6)
	*2/*9 (2)	*2XN/*2 (2)	*2XN/*4 (2)	*2XN/*41	*2XN/*5 (2)	*2XN/*6 (1)	*3/*35 (1)	*3/*4XN (1)	*4/*35 (1)	*4/*4 (1)
	*4/*41 (3)	*4/*10 (1)	*41/*41 (1)	*5/*35 (1)	*5/*41 (1)	*6/*35 (1)	*9/*10 (1)	UC (2)		
NAT2	*4/*4 (10)	*4/*5B (15)	*4/*5C (2)	*4/*6A (15)	*5A/*5B (1)	*5B/*5B (21)	*5B/*6A (21)	*6A/*12A (1)	*6A/*6A (9)	*6A/*7B (3)
TPMT	*1/*1 (88)	*1/*2 (4)	*1/*3A (4)	*3A/*3A (2)						
MDR1	C/C (32)	C/T (48)	T/T (18)							

UC: Unknown combination.

fluorescence signal and that obtained in each probe set were also analyzed. The signal intensity was normalized against the background, and the calculated variance on a signal level was given as a coefficient of variation for the ratio of intensity to background.

Results

Suitability of DNA multiple displacement amplification

One of the first evaluations carried out was of the suitability of the DNA samples obtained through multiple displacement amplification before using PHARMAchip.

A group of 15 matched-pair samples (the original DNA sample and its corresponding amplified material) covering different SNPs, deletions and duplications of CYP2D6 was included in the evaluation. All samples (original and amplified material) gave 100% concordance rates for the SNPs analyzed. The amplified samples, however, gave erroneous *CYP2D6* duplication results in two samples (**1xN/*41* vs **1/*41*, **5/*41xN* vs **5/*41*) and *CYP2D6* deletion (**1/*1* vs **1/*5*) in one sample.

Analysis of these three samples via electrophoresis and agarose visualization showed no band at 3.5 kb in the duplication primer-specific amplification, and a band at 3.5 kb in the deletion primer-specific PCR. This indicated the presence of two false positives and one false negative in the DNA-chip duplication and deletion results of the samples amplified with GenomiPhi™ (GE Healthcare). To prevent inaccurate deletion and duplication being assigned to the *CYP2D6* gene, the use of whole-genome amplified DNA should be avoided.

Genotype results of selected samples

The diversity of the core genes of the genotypes detected in the 100 samples is shown in TABLE 2. In total, over 60 different combinations were represented in the sample set, among which more than 30 different combinations and 12 allele variants, including multiplication and deletion of the whole gene, were found in the test set of samples for *CYP2D6* (TABLE 2).

Accuracy

A single-blind genotyping study with 100 samples was carried out using PHARMAchip to validate its accuracy, and the results were then compared with the previous ones obtained from the DrugMEt test. Of the 100 samples in the study, 98 fulfilled pre-established hybridization quality controls and could, therefore, be used for the comparison. In two cases, the samples, owing to poor DNA quality and quantity, could not be analyzed because the hybridization intensity was below the limit of detection.

Of the analyzed SNPs, 15 presented discrepant results, which were further analyzed by sequencing. Three samples could not be resolved because there was no DNA available to carry out sequencing.

Of the 12 remaining cases, one was a mismatch in PHARMAchip and another was a mismatch in the DrugMEt test. The ten remaining discrepancies were due to an allele detected by PHARMAchip but not by DrugMEt. Sequencing analysis of those samples confirmed results given by Progenika's DNA microarray. The alleles detected by PHARMAchip were *NAT2*12* (one case), *CYP3A5*6* (one case) and *CYP2D6*35* (eight cases).

The genotyping success rate of the PHARMAchip assay for the core set of genes analyzed was 97.9%, with the majority of failures resulting from the lack of hybridization (2.0%). Accuracy of the DNAchip was found to be 99.9% (TABLE 3).

PHARMAchip reproducibility & limit of detection

The between- and within-day variability of the PHARMAchip results was assessed by analyzing the global- and single set-probe intensity and genotyping the results of the repeats of the DNA control samples. The between- and within-day differences in global and single-probe intensity were also compared, since changes in the fluorescence intensity could eventually lead to genotype miscalls. The between-day median fluorescence intensity CVs was 37%, and the within-day intensity ranged from 9 to 24%. After normalization of the data with the background fluorescence, the percentage of variation was reduced to 24% for between-day and to 12–22% for within-day differences. No differences in genotyping calls were found between any of the repeats, giving a result reproducibility of 100%.

With quantities of up to 50 ng of DNA, the global and probe-set signals and genotype calls were not compromised. Nevertheless, an increased rate of errors and nonvalid results (not enough signal to genotype) was observed at DNA concentrations below 100 ng; therefore, the limit of detection was set at 75 ng. Saturation was not reached at 500 ng, and no higher amounts were tested.

Table 3. Quality parameters for PHARMAchip™ validation.

Parameter	Value (%)	Counts
Successful genotype	97.9	4896 out of 5000
Mismatch	0.02	1 out of 5000
No call/no hybridization	2.1	103 out of 5000
Reproducibility	100	

Discussion

Pharmacogenetics, defined as the study of variations in the DNA sequence in relation to drug response, is believed to provide a new area of knowledge that will improve therapeutic success, mainly by predicting toxicity and the lack of treatment efficacy. As a result, the development of DNA microarray technology provides an opportunity to simultaneously test hundreds of SNPs in order to achieve higher throughput and speed. Potential clinical and research applications of these tools can include the selection of subjects in clinical trials, the study of the mechanism of adverse drug reactions, and the association between specific SNPs and phenotypes or diseases.

There are several genomic biomarkers considered to be valid by the FDA in the context of Approved Drug Labels. Many of them (variants of *CYP2D6*, *CYP2C9*, *CYP2C19*, *DPD*, *NAT2*, *TPMT* and *VKORK*) are included in PHARMAchip and have a clear clinical utility [104]. For example, *CYP2D6* genotype is used to tailor treatment of hormone therapy in breast cancer. CYP2D6 poor metabolizers (i.e., patients carrying two nonfunctional alleles) have worse clinical outcomes than patients with two functional *CYP2D6* alleles when treated with tamoxifen. These patients could benefit from an alternative treatment, such as aromatase inhibitors [illegible]. Similarly, other clinical applications are warfarin dose selection after *CYP2C9* and *VKORK* genotyping [illegible], or toxicity management of capecitabine according to *DPD* mutation detection [illegible]. However, in order to increase the clinical use of other biomarkers, prospective studies and research are needed.

As a result, the clear need for tools applicable in the area of pharmacogenomics, both in the clinical setting and research, has led to the search for new genotyping techniques. In this context, the development of DNA microarray technology provides an opportunity to simultaneously test hundreds of SNPs in order to achieve higher throughput and speed. Nevertheless, it has been already foreseen that future pharmacogenomic testing will have to combine information from DNA microarrays with environmental and personal variables that influence pharmacokinetic and pharmacodynamic drug response for a proper personalized medicine, the ultimate goal of applying such methodologies [illegible].

The validation of genetic tests before use, in order to ensure that they perform according to the laboratory's needs, is a formal requirement of many accreditation standards, including ISO 15189 and ISO 13485. The 'gold standard' approach of validation is sequencing, although the comparison of results obtained using a different technology has also been routinely used [illegible]. The Secretary's Advisory Committee for Genetics, in its report on Personal Health Care Initiative (SACGHS), has incorporated a number of wise recommendations on how we should deal with concepts such as analytical validity, proficiency testing and clinical validity applied to genetic testing [103]. A practical development of these concepts would change the present approach of evaluating the present device. Validating a test by comparison with another test is not ideal. Nevertheless, there are situations where clinical genotyping was performed with a given test and, for unforeseen reasons (e.g., discontinuation of the product), a new test must be used. Therefore, the present study does not pretend to validate the PHARMAchip test with the former DrugMEt test. It is understood that manufacturers of PHARMAchip have performed their internal validations. The aim of this study was to assess the performance characteristics of PHARMAchip, developed by Progenika Biopharma SA in 2008. Hence, the following parameters were determined: the suitability of amplified DNA, the limit of detection, the reproducibility, the accuracy and the successful genotype rate (by comparison with DrugMEt, plus sequencing in discrepant cases).

Adequate amounts of high-quality genomic DNA are not always easy to obtain. For example, DNA obtained from oral swabs can sometimes lead to a poor yield, rendering them unfit for high-throughput SNP genotyping. DNA polymerase *Phi29* used for whole-genome random-primed amplification could offer a

solution to this problem. The performance of DNA amplification with GenomiPhi Gv2 was, therefore, assessed for this purpose with PHARMAchip. The occurrence of false positives with the use of GenomiPhi-amplified DNA may be explained by the generation of template-independent products (TIPs) during the process. Although every effort is made to reduce TIPs during the amplification process, up to 10 ng/µl of TIPs have been reported while using GenomiPhi Gv2 [22,23]. The presence of these products could lead to unspecific hybridization in PHARMAchip, resulting in a false-positive call in the microarray. In addition, amplification bias results from the failure of one of the two alleles to amplify and the excess amplification of one allele, giving rise to potential false negatives [24]. Multiple displacement amplification with *Phi29* DNA polymerase has yielded less biased and longer amplified products, but the successful amplification of whole-genome DNA still requires a small amount of unfragmented, high-quality DNA [24], which might not always be available. Since only one method of whole-genome DNA amplification was tested, it cannot be excluded that other procedures could be suitable. The use of nonamplified DNA is recommended to avoid the inaccurate assignment of false *CYP2D6* duplications or deletions when genotyping with PHARMAchip.

In order to assess accuracy, a single-blind comparison study was carried out. A set of 100 samples, enriched with a wide number of genetic variants (>30 different allele combinations for *CYP2D6*), were analyzed with PHARMAchip and the results compared with previously determined genotypes by DrugMEt. While the distribution of allelic variants in the sample set is not representative of their frequency of occurrence in the Caucasian population, it was selected for technical purposes and in order to conduct the evaluation of the suitability of PHARMAchip. As only those genes common in both tests were evaluated, the other genes present in PHARMAchip deserve further suitability studies.

Of the 15 detected mismatch results (out of 5000 available genotypes), ten could be explained by the presence of three alleles in PHARMAchip that were not included in DrugMEt, which were: *NAT2*12* (SNP 803A>G), *CYP3A5*6* (14690G>A), and *CYP2D6*35* (SNP 31G>A; 2850C>T; 4180G>C). It is important to point out that, according to published data (HapMap_CEU, [104]), the allelic frequency in European population for SNP 803 = G (*NAT2*12*) and 31 = A (*CYP2D6*) are 0.425 and 0.07, respectively. As a consequence, the high presence of these alleles makes the inclusion of these SNPs relevant for the correct genotyping of a European population. On the other hand, the allele *6 for *CYP3A5* is very rare in this type of population [15], but its importance is, as a consequence of its phenotype modification, due to a splicing defect associated with an absence of activity [16].

The comparison study showed a high rate of agreement between the two methods despite their technical differences: PHARMAchip is based on allele-specific oligonucleotide hybridization, while DrugMEt uses the allele-specific primer extension (ASPEX) approach.

In allele-specific oligonucleotide hybridization approximation, no enzyme is involved and allelic discrimination is dependent on the design of the probes. It is a simple assay widely used in SNP genotyping. The limiting factor for its use in large-scale genotyping originates from the inherent properties of nucleic acid hybridization reaction. In this reaction, the efficiency of hybridization and thermal stability of hybrids formed between the target nucleic acid and the probe are sequence dependent and can only be controlled by reaction conditions, such as temperature, washing stringency or salt concentrations [17].

The ASPEX technique is based on the ability of DNA polymerase to incorporate specific deoxyribonucleotides complementary to the sequence of template DNA [18]. The major advantage of the ASPEX reaction is that the discrimination is based on the correct 3′ end base pairing for primer extension rather than the differences in thermal stability between mismatched and perfectly matched hybrids [20].

Conclusion

PHARMAchip was designed to detect many alleles that are rare in a Caucasian European population and which were not found among the test set of samples for the comparison study. However, it is important to bear in mind that these alleles are more frequent in other populations, and this fact could be a clear advantage in multicultural societies, such as those of present-day North America and Europe.

The high rate of accuracy and reproducibility of PHARMAchip makes it a reliable test for its application in a clinical setting. In addition, it is an interesting tool for use in research, since it analyzes a wide number of alleles in the most relevant gene panel for pharmacogenetics.

Financial & competing interests disclosure

All clinical samples of this study were genotyped free of charge at Progenika Biopharma SA premises. Investigators from the Human Pharmacology and Clinical Neurosciences Research Group, IMIM-Hospital del Mar, did not receive any monetary compensation for the present study. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- The analytic validity of a genetic test defines its ability to accurately assign a genotype in the SNP under analysis.
- The following parameters were determined for the PHARMAchip™: suitability of amplified DNA, limit of detection, reproducibility, accuracy and successful genotype rate.
- The use of whole-genome amplified DNA is not recommended for increasing the amount of DNA, as it leads to inaccurate assignment of false *CYP2D6* duplications or deletions when genotyping with PHARMAchip.
- The high rate of accuracy and reproducibility of PHARMAchip makes it a reliable test for its application in a clinical setting.
- PHARMAchip was designed to detect many alleles that are rare in a Caucasian European population but common in other populations. This is an advantage in multicultural societies, such as those in North America and Europe.
- Only those genes common to both tests were evaluated.
- Further studies are needed to examine the other genes present in PHARMAchip.

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Study 2

The influence of 5-HTT and COMT genotypes on verbal fluency in ecstasy users.

Fagundo AB, Cuyàs E, Verdejo-Garcia A, Khymenets O, Langohr K, Martín-Santos R, Farré M, de la Torre R.

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Original Paper

The influence of *5-HTT* and *COMT* genotypes on verbal fluency in ecstasy users

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Ana B Fagundo[1,2], Elisabet Cuyàs[1,2], Antonio Verdejo-Garcia[1,3], Olha Khymenets[1], Klaus Langohr[1,4], Rocio Martín-Santos[1,5], Magí Farré[1,2] and Rafael de la Torre[1,6]

Abstract

Deficits in verbal fluency associated with ecstasy use have been well established; however, the mechanisms underlying this impairment have yet to be elucidated. In this study we investigated for the first time whether there was a disproportionate impairment in two cognitive subcomponents of verbal fluency: *clustering* (ability to generate words within the same subcategory) and *switching* (ability to change the subcategory). We also investigated a possible association between ecstasy use and verbal fluency in subjects genotyped for *5-HTT* (*5-HTTLPR* and *5-HTTVNTR*) and *COMT* (*val*[108/158]*met*, *rs165599* and *rs2097603*) polymorphisms, in order to find a potential implication of genetic factors. Ecstasy polydrug users ($n = 30$) and non-ecstasy users ($n = 41$) were evaluated in both semantic and phonemic fluency. Results showed that ecstasy users had poorer semantic (but not phonemic) fluency performance than controls. Detailed analysis of *clustering* and *switching* performance revealed that this impairment was associated with poorer *clustering* mechanisms. *Clustering* was also modulated by the *COMT rs165599* polymorphism independently of the group. A specific effect of the *5-HTTLPR* polymorphism on *switching* performance was also found, with ss carriers performing significantly worse than ls and ll carriers, suggesting a serotonin modulation of frontal-executive flexibility. Based on the impaired *clustering* and *switching* strategies observed in ecstasy users, it might be proposed that both semantic knowledge and retrieval are impaired in this population. The verbal fluency deficit in ecstasy users may be attributable to a disruption of frontal-striatal circuits directly related with the serotonin function as well as a depletion of lexical-semantic stores mediated by temporal structures.

Keywords

5-HTT polymorphism, clustering/switching scoring, *COMT* polymorphism, ecstasy (MDMA), verbal fluency

Introduction

3,4-methylenedioxymethamphetamine (MDMA or ecstasy) is one of the most widely used illegal psychostimulant drugs among young people (Landry, 2002). The word 'ecstasy' rather than MDMA has been adopted throughout the manuscript, as the latter term implies the administration of a pharmaceutically pure drug when in fact subjects are consuming street ecstasy that may or may not contain MDMA. Ecstasy tablets are consumed recreationally at dance clubs and are associated with acute effects including increased energy, euphoria and empathy (Camí et al., 2000), and also unpleasant effects such as fatigue, anhedonia, irritability, low mood, rebound midweek depression, and concentration difficulties (Morgan, 2000).

Two recent meta-analyses addressing cognitive deficits in ecstasy users have concluded that learning and memory are the abilities most significantly affected by the drug. Executive functions, while encompassing a range of relatively independent cognitive processes (Fisk and Sharp, 2004), showed medium effect size differences between drug users and controls (Kalechstein et al., 2007; Zakzanis et al., 2007). Of these processes, verbal fluency (involving access to long-term memory) is one of the most consistently affected by ecstasy use across studies (Bhattachary and Powell, 2001; De Sola et al., 2008; Fox et al., 2002; Heffernan et al., 2001; Montgomery et al., 2005; Yip and Lee, 2005). Verbal fluency deficits in ecstasy users have been observed both for phonemic (e.g. producing words beginning with F) (Fox et al., 2002;

[1]Human Pharmacology and Clinical Neurosciences Research Group-Neuropsychopharmacology Program, Institut Municipal d'Investigació Mèdica (IMIM), Barcelona, Spain.
[2]Department of Pharmacology, Therapeutics and Toxicology, Universitat Autònoma de Barcelona, Barcelona, Spain.
[3]Institute of Neuroscience, Universidad de Granada, Granada, Spain.
[4]Department of Statistics and Operations Research, Universitat Politècnica de Catalunya, Barcelona, Spain.
[5]Department of Psychiatry, Institute of Neurosciences, Hospital Clínic, IDIBAPS, CIBER-SAM, Barcelona, Spain.
[6]CEXS, Universitat Pompeu Fabra, Barcelona, Spain.

Corresponding author:
Rafael de la Torre PhD, Human Pharmacology and Clinical Neurosciences Research Group – Neuropsychopharmacology Research Program, Institut Municipal d'Investigació Mèdica, Parc de Recerca Biomèdica de Barcelona, c/ Dr. Aiguader 88 /08003 Barcelona, Spain. Email: rtorre@imim.es

Montgomery et al., 2005) and semantic modalities (e.g. producing words belonging to the category 'animals') (Heffernan et al., 2001). Furthermore, alterations in verbal fluency have been described cross-culturally in both European and Asian ecstasy-using populations (Yip and Lee, 2005). In addition, a recent longitudinal study from our laboratory demonstrated that semantic fluency was significantly impaired in ecstasy users both at baseline and after a 2-year follow-up, indicating considerable stability in this deficit (De Sola et al., 2008).

In spite of the consistent evidence of the impairment of verbal fluency as a major cognitive correlate of ecstasy use, little is known about the specific mechanisms underlying this fluency problem in ecstasy users.

Verbal fluency is a function commonly evaluated using neuropsychological tests which examine the ability to spontaneously produce words orally in a fixed time span. The most common fluency measure assessed is the total number of correct words generated. However, successful performance in this complex task requires a number of distinct processes, ranging from lexical selection and phonemic encoding to working memory and executive control. Troyer et al. (1997) suggested that optimal performance in verbal fluency tests depends on the functioning of two different, but complementary, cognitive strategies: (1) *clustering*, which reflects the ability to track words within the same semantic or phonemic subcategory, and (2) *switching*, which reflects the ability to change the subcategory when the former one is exhausted. *Clustering* relies on the integrity of semantic stores, whereas *switching* depends on flexibility skills needed to perform subcategory changes when necessary. A deeper analysis of these strategies has been useful in revealing the cognitive mechanisms underlying fluency deficits across different clinical populations, including schizophrenia (Bozikas et al., 2005; Sumiyoshi et al., 2005), Parkinson's disease (De Gaspari et al., 2006), Huntington disease (Ho et al., 2002; Troster et al., 1998), vascular dementia (Duff et al., 2004), focal brain lesions (Troyer et al., 1998), and Alzheimer's disease (Epker et al., 1999; Fagundo et al., 2008; Troster et al., 1998). In addition, these studies have contributed to the finding that *clustering* is associated with temporal lobe functioning (Troster et al., 1998; Troyer et al., 1998), while *switching* is mostly impaired among patients with left dorsolateral and superior medial frontal lobe lesions (Troyer et al., 1998) as well as in fronto-subcortical pathologies (Ho et al., 2002; Troster et al., 1998). Thus, *clustering/switching* analyses could contribute to the understanding of the brain substrates of ecstasy-induced cognitive deficits, as different studies point to temporal versus frontal-striatal dysfunction in ecstasy users (Fox et al., 2002; Quednow et al., 2006). Structural brain imaging studies, for instance, suggest that MDMA polydrug users showed multiple regions of reduced brain grey matter concentration including left prefrontal cortex (Cowan et al., 2003) and also hippocampus bilaterally (Nifosi et al., 2008). Recent studies using functional magnetic resonance imaging (fMRI) suggest differences in the activation of the hippocampus between MDMA users and healthy controls during working memory tasks and so supported the view of a hippocampal dysfunction in MDMA users (Daumann et al., 2005; Jacobsen et al., 2004; Moeller et al., 2004). However, two imaging studies did also show abnormalities in the activation of frontal and parietal regions of MDMA users during working memory tasks (Daumann et al., 2004; Moeller et al., 2004), suggesting an implication of cortical regions. All in all, the neural substrate affected by ecstasy use remains unclear. Therefore, the *clustering/switching* scoring system could shed light on the neural network underlying the cognitive alterations associated with ecstasy use.

Although it remains rather controversial, these cognitive deficits associated with ecstasy use have been linked to drug-induced alterations in the serotonergic function (McCann et al., 2008). Findings from positron emission tomography and single photon emission computed tomography imaging have consistently revealed reductions in *5-HTT* ligand binding in MDMA users and recently abstinent (roughly those abstinent for less than six months) MDMA users (Buchert et al., 2004; De Win et al. 2004; McCann et al., 1998; Reneman et al., 2001a, b; Semple et al., 1999). However, *5-HTT* levels in former MDMA users (abstinent from MDMA at least 1 year) seem to be comparable to controls (Buchert et al., 2004; Reneman et al., 2001a). The fact that long-abstinent MDMA users showed no differences in *5-HTT* ligand binding suggests that MDMA-associated decrements in *5-HTT* binding are potentially reversible. On the other hand, studies in humans have shown that some of the subjective effects of MDMA, including positive mood, extroversion, and heightened sensory perception, are blocked by selective serotonin reuptake inhibitors, supporting the involvement of *5-HTT* in the mechanism of action of MDMA (Farré et al., 2007; Liechti et al., 2000). In addition, there is evidence of *5-HTT* modulation of several aspects of cognitive function, especially those mediated by the ventromedial prefrontal cortex, such as flexibility or decision-making (Clarke et al., 2004; Riedd et al., 1999; Rogers et al., 1999). These findings have stimulated research into the cognitive domains of MDMA users, not only from a neuropsychological standpoint, but by using a genetic approach as well (Reneman et al., 2006).

Genetic polymorphisms of the *5-HTT* gene have prompted studies combining the evaluation of cognitive performance in ecstasy users with the genetic traits that may act as modulating factors of ecstasy-related cognitive impairment (Reneman et al., 2006). The human *5-HTT* gene is located on chromosome 17 (17q11.1–17q12) (Ramamoorthy et al., 1993). Two functional genetic polymorphisms for human *5-HTT* have been identified (Hranilovic et al., 2004). The first one is the insertion/deletion of 44 base pairs (bp) in the promoter region (*5-HTTLPR*), giving the long (*l*) or the short (*s*) variants. The second one is a variable number of repeats (9, 10 or 12) in tandem of 17 bp in the second intron (*5-HTTVNTR*) (Hranilovic et al., 2004). These polymorphisms alter transcriptional activity of the *5-HTT* gene and ultimately the level of available functional transporters (Fiskerstrand et al., 1999; Greenberg et al, 1999; Lesch et al., 1996). Some studies have suggested a relationship between the cognitive impairment in ecstasy users and genetic variations of the *5-HTT*, although the results are inconsistent (Reneman et al., 2006; Roiser et al., 2005, 2006). Recently, an association between MDMA use and cognitive function was studied in moderate, heavy, and ex-MDMA users as well as in non-users genotyped for *5-HTTLPR* (Reneman et al., 2006). In this study no evidence

of any effect of *5-HTTLPR* on ecstasy-related memory impairment was found. However, it has been previously reported that the *5-HTTLPR* polymorphism may play an important role in decision-making deficits in ecstasy users (Roiser et al., 2006), with a clearly attenuated response in the homozygous *ss* ecstasy users. Based on these considerations it might be hypothesized that the *s* allele is associated with ecstasy cognitive impairments, and consequently could be an important confounding variable when investigating cognitive function in ecstasy users.

The influence of *COMT* genotype polymorphisms in cognitive performance, mainly frontal-executive functions, has also been extensively investigated (Barnett et al., 2008; Egan et al., 2001; Goldberg et al., 2003). The substitution of *met* for *val* at codon 108/158 results in the transcription of a thermolabile variant of the *COMT* enzyme with approximately 40% less enzymatic activity in humans (Chen et al., 2004). The reduced activity associated with the met variant of the *COMT* gene presumably results in greater availability of dopamine in the prefrontal cortex and, thus, may be linked to some aspects of cognition in humans (Gogos et al., 1998; Meyer-Lindenberg et al., 2005). Several studies have identified an association between working memory or executive functions and *COMT* $val^{108/158}met$ genotype (Egan et al., 2001; Goldberg et al., 2003). More recent studies have also pointed out the influence of *COMT* genotypes on verbal fluency performance (Alfimova et al., 2007; Woodward et al., 2007). The $val^{108/158}met$ polymorphism appears to have a major effect on semantic verbal fluency in both healthy subjects and patients with schizophrenia (Alfimova et al., 2007), and seems to be also involved in differential verbal fluency improvement with atypical antipsychotic drugs (Woodward et al., 2007). Taking into account that deficits in executive functions and verbal fluency have been consistently described in recreational and chronic ecstasy users (Zakzanis et al., 2007), a possible effect of *COMT* polymorphisms could be expected. MDMA users, for instance, made more errors than non-users on a measure of matching familiar figures, indicating elevated impulsivity (Morgan et al., 1998). Results from a different study confirmed these findings and suggested that heavy use of MDMA may elevate behavioural impulsivity and impair decision-making cognition (Quednow et al., 2007). Other executive functions such as planning (Zakzanis and Young, 2001) also appear to be impaired with continued use of MDMA. Overall, it could be hypothesized that these cognitive deficits might be arising to some extent from changes in dopaminergic transmission in the prefrontal cortex. Nevertheless, to our knowledge there are no studies that have investigated the influence of the *COMT* polymorphism on cognitive performance in ecstasy users.

The purpose of this study was twofold: (1) to study the specific cognitive processes underlying the verbal fluency deficits in ecstasy users by examining, for the first time, whether there was a disproportionate impairment in two cognitive subcomponents of verbal fluency: *clustering* and *switching*; and (2) to investigate a possible association between ecstasy use and verbal fluency performance in participants genotyped for *5-HTT* (*5-HTTLPR* and *5-HTTVNTR*) and *COMT* ($val^{108/158}met$ *(rs4680)*, *rs165599* and *rs2097603*) polymorphisms. Considering that ecstasy use is associated with both aspects of memory – storage and retrieval (Zakzanis et al., 2007) – and with specific components of executive function modulated by dopamine and serotonin genes, we hypothesized that in ecstasy users, (1) semantic and phonemic fluency problems would be related to inefficient strategic retrieval processes, predominantly related to *switching* impairment; (2) semantic fluency deficits would be specifically related to a disorganization of the semantic store reflected in *clustering* impairment; (3) impairments in verbal fluency would be greater in *5-HTTLPR s* carriers than in *5-HTTLPR l* carriers; (4) the *5-HTTLPR s* allele would be associated predominantly with *switching* problems; and (5) *COMT* polymorphisms would modulate verbal fluency performance.

Methods

Sample

Participants were classified into three experimental groups: current ecstasy polydrug users ($n = 30$), cannabis users ($n = 18$), and non-drug users ($n = 23$). All participants were recruited in the course of an earlier neuropsychological study previously presented in detail (De Sola et al., 2008). All participants were healthy, self reporting an adequate functioning within their social and professional context, and comparable in terms of years of education and socioeconomic status. All included subjects were Caucasian. Participants were recruited through several sources including word-of-mouth, notices in neighbourhoods, advertisements in the local university and a non-governmental organization (Energy Control) specialized in providing harm reduction guidelines among drug users. A telephone interview was carried out in order to determine drug history. Telephone screening was followed by an exploration that included a detailed medical history, physical examination, urine toxicology screens, a brief neurological examination, and a structured psychiatric interview (PRISM, Torrens et al., 2004) by a psychiatrist or psychologist who approved the final subject selection. Participants were excluded if they had neurological or relevant medical diseases as well as current psychiatric disorders diagnosed by DSM-IV classification.

Depending on the group, specific exclusion criteria were also applied: for the cannabis group, a current history of regular use of other illegal psychotropic drugs apart from cannabis during last year, and past use of illegal drugs on more than five occasions; for non-users, a current history of use of illegal drugs during the past year and past use of illegal drugs on more than five occasions. Alcohol and nicotine were allowed. Regarding the ecstasy group, because it was impossible to recruit exclusive ecstasy users, it was decided to include ecstasy consumers with moderate use of other illicit drugs, ecstasy being the main psychostimulant drug abused.

Procedure

All participants were tested at the Institut Municipal d'Investigació Mèdica (IMIM-Hospital del Mar) after they had been informed of the subsequent protocol and had provided written informed consent. The study protocol was approved by the local Ethical Committee (CEIC-IMAS

99/935/I 2001/1226/I). After the testing was completed, all participants received monetary compensation for their participation. Testing consisted of obtaining the drug history, medical checking, drug screens, biochemical analyses, and psychiatric and neuropsychological assessments. Drug history was verified by hair testing and participants were screened for the presence of drugs of abuse in urine before the neuropsychological assessments. Participants were requested to refrain from drug use 72 hours prior to assessments. A 10 ml blood sample was taken and used to extract DNA. As described in more detail by De Sola et al. (2008), all participants underwent a comprehensive neuropsychological assessment session of 90 min. The neuropsychological test battery included: vocabulary subtest (from the WAIS-III, Spanish version, Wechsler, 1997); California Computerized Assessment Package CALCAP (Miller, 1990); Tower of London (Shallice, 1982); word fluency (Benton and Hamsher, 1983); semantic fluency (Benton and Hamsher, 1983); Symbol Digit Modalities Test (Smith, 1982); California Verbal Learning Test II (Delis et al., 2000); Rey complex figure test (Rey, 1941); Corsi block tapping subtest (from the WAIS-III, Wechsler, 1997); and letter number sequencing subtest (from the Wechsler Memory Scale III, Wechsler, 1997). Verbal intelligence quotient was estimated using the vocabulary subtest. For the purpose of this study, additional analyses from the semantic and phonemic fluency tests were also performed.

Verbal fluency assessment

Semantic fluency (Benton and Hamsher, 1983): for this task, participants were requested to produce in 1 min as many words as possible that belonged to a specific semantic category: *animals*.

Phonemic fluency (Benton and Hamsher, 1983): for this task, participants were requested to produce in 1 min as many words as possible beginning with a specific letter: F, A, and S.

Two types of measures were obtained from these tests: (1) total words generated – the total number of correctly generated words in 60 sec. All intrusions (words not pertaining to this semantic category), perseverations (same words), and repetitions (same words with different endings) were treated as mistakes; and (2) *clustering* and *switching* measures. Mean Cluster Size was the main dependent variable for *clustering*, whereas Number of Switches was the main dependent variable for *switching*. These variables were computed according to standard scoring rules proposed by Troyer et al. (1997) and Troyer (2000). A cluster was defined as any series of two or more successively produced words belonging to the same semantic or phonemic subcategory. On phonemic fluency, clusters were defined as groups of successively generated words that began with the same first two letters, differed only by a vowel sound, rhymed, or were homonyms. On semantic fluency, clusters were defined as groups of successively generated words that belonged to the same semantic subcategory, such as farm animals, pets, aquatic animals, African animals, and insects. The subcategories were determined a priori (Fagundo et al., 2008; for examples see also the Appendix). Cluster size was computed by adding up series of words from the same subcategory, starting from the second word within each cluster (i.e. a three-word cluster has a size of two). The Mean Cluster Size was obtained by adding the size of all the clusters and dividing it by the total number of clusters. The Number of Switches was defined and computed as the number of times the participant changed from one cluster to another within the same task. The computation of Number of Switches included single-word clusters (cluster size = 0). Intrusions, repetitions, and perseverations were also included in the calculation of both Mean Cluster Size and Number of Switches.

Genotype analysis

Genomic DNA was extracted from the peripheral blood leukocytes of all the participants using Flexi Gene DNA kit (Qiagen Iberia, S.L., Spain) according to the manufacturer's instructions.

5-HTTLPR and *5-HTTVNTR* genotyping was performed using polymerase chain reaction (PCR). Each reaction mixture contained: 1× PCR Amplification buffer and 1× PCR Enhancer solution (Invitrogen, Carlsbad, CA), 1.5 mM MgSO4, 300 μM dNTPs, 0.5 pmol of each primer, 0.5 U of Taq DNA polymerase (Invitrogen) and 50 ng of genomic DNA as template. PCRs were performed with the following pairs of primers: FAM-5′-GGCGTTGCCGCTCTGAATGC-3′ and 5′-GAGGGACTGAGCTGGACAACAACCAC-3′, and FAM-5′-GTCAGTATCACAGG-CTGCGAG-3′ and 5′-TGTTCCTAGTCTTACGCCAGT-3′ for 35 cycles at 58°C and at 60°C as annealing temperatures for *5-HTTLPR* and *5-HTTVNTR* amplification, respectively. A 10-μl total reaction volume was used and, after PCR, the products of allelic-specific amplifications (allele L, 528 bp; allele S, 484 bp for *5-HTTLPR*; and allele 9, 250 bp; allele 10, 267 bp; and allele 12, 300 bp for *5-HTTVNTR*) were detected on an automatic ABI 3730XL capillary sequencer and analysed by GeneMapper Software v3.5 (Applied Biosystems, Foster City, CA, USA).

The *COMT* promoter (*rs2097603*), *val*$^{108/158}$*met* (*rs4680*) and 3′ flanking region (*rs165599*) single nucleotide polymorphism (SNP) allelic variants were determined using the 5′ exonuclease TaqMan assay with ABI 7900HT Sequence Detection System (Real Time PCR) supplied by Applied Biosystems (Foster City, CA, USA). Primers and fluorescent probes were obtained from Applied Biosystems with TaqMan SNP Genotyping assays (assay ID C_25746809_50 and C_2255335_10 for *rs4680* and *rs165599*, respectively). Reaction conditions were those described in the ABI PRISM 7900HT user's guide. Endpoint fluorescent signals were detected on the ABI 7900, and the data were analysed using Sequence Detector System software, version 2.1 (Applied Biosystems). For the *rs2097603*, primers and probes used were described previously by Meyer-Lindenberg et al. (2006).

Statistical analysis

The associations between drug consumption group and socio-demographic variables, drug use characteristics, and genotype distributions were studied by means of either the *F*-test (quantitative variables) or the chi-square test

(qualitative variables). Since the main interest was to analyse the association between semantic and phonemic fluency and both drug consumption and *5-HTTLPR*, two-way analysis of variance (ANOVA) models with drug consumption group and *5-HTTLPR* as factors were applied. Initially, the interaction between both factors was also taken into consideration, but was subsequently abandoned, since the corresponding *p*-value exceeded 0.1 in all cases. In addition, to study the associations between the main test results (Mean Cluster Size and Number of Switches) and the four other polymorphisms *5-HTTVNTR*, *COMT val*$^{108/158}$*met*, *COMT rs165599*, and *COMT rs2097603*, the previous models were extended by including – separately – each of these polymorphisms. That is, for each of these four polymorphisms, a model was fitted including this polymorphism as well as drug consumption and *5-HTTLPR* as factors; in addition, two-way interactions between the polymorphisms and drug consumption were tested. Main effects reported for these polymorphisms in the Results section below are all from models without interactions since these were not statistically significant. Since the cannabis group and the non-drug users showed similar results with respect to verbal fluency measures and had similar genetic polymorphism distributions, they were pooled for these analyses in order to increase statistical power. In case a significant effect of a polymorphism with three genotypes was detected, the Tukey test for multiple post-hoc comparisons was applied in the framework of the corresponding ANOVA model. The genotypes 9/10 (of polymorphism *5-HTTVNTR*) and G/G (of both *COMT rs165599* and *COMT rs2097603*) were discarded for the analyses because of their low frequency among the study groups. Regarding the Mean Cluster Size in phonemic fluency, an extreme outlier, seven times larger than the mean, was excluded from the analysis. The significance level used in all models was 0.05. After the redistribution of groups, all polymorphisms tested were in Hardy-Weinberg equilibrium. We did not find evidence of linkage dysequilibrium either between *COMT rs2097603*, *val*$^{108/158}$*met*, and *rs165599* or between *5-HTTLPR* and *5-HTTVNTR*. The statistical software package R (The R Foundation for Statistical Computing) was used for all the analyses.

Results

Demographic variables, drug use characteristics and genotype distributions are presented in Table 1. In addition, among ecstasy users, consumption of other drugs was recorded (data not presented): 27 (90%) participants had used at least once cocaine; 20 (66.7%) speed; 19 (63.3%) LSD; 7 (23.3%) ketamine; 5 (16.7%) GHB, and 1 (3.3%) heroin. Among the cannabis and control group, no one had used these drugs before.

Total words generated

For semantic fluency, ecstasy users generated significantly fewer correct words than non-ecstasy users ($p < 0.01$). However, for phonemic fluency, ecstasy users and non-ecstasy users did not show significant differences in the number of words produced ($p = 0.947$).

An additional analysis of intrusions and perseverations did not show differences between ecstasy users and non-users in the number of errors for semantic fluency ($p = 0.065$), but ecstasy users committed more errors in phonemic fluency ($p < 0.001$) (see Table 2).

Clustering performance (Mean Cluster Size)

Semantic clusters: For the Mean Cluster Size a main group effect was observed: ecstasy users showed significantly smaller cluster sizes than non-ecstasy users ($p < 0.01$) (Figure 1). Furthermore, a significant effect of the *COMT rs165599* polymorphism was found ($F(1,54) = 4.73$, $p < 0.05$). Participants carrying the A/G genotype displayed a significant reduction in the number of words generated in each semantic subcategory compared with participants carrying two copies of the G allele ($p < 0.001$). No group and genotype interaction was observed ($F(1,58) = 0.24$, $p = 0.626$).

Semantic Mean Cluster Size was unaffected by *5-HTTLPR*, *5-HTTVNTR*, *COMT val*$^{108/158}$*met* and *COMT rs2097603* genotypes, with no group and genotype interactions (see Table 3).

Phonemic clusters: Results from a two-way ANOVA model showed significant main effects of group and *5-HTTLPR* genotype (Figures 1 and 2) (See Table 2), but no group and genotype interaction. Group comparisons indicated that ecstasy users generated more words within each subcategory ($p < 0.01$) (Figure 1). Genotype comparisons showed that participants with the *ls* genotype had reduced Mean Cluster Size compared with *ll* carriers ($p < 0.05$) (Figure 2).

Phonemic Mean Cluster Size was unaffected by *5-HTTVNTR*, *COMT val*$^{108/158}$*met*, *COMT rs165599* and *COMT rs2097603* genotypes, with no group and genotype interactions (see Table 3).

Switching performance (number of switches)

Semantic switches: Results from a two-way ANOVA model showed only a trend for group main effect with a slightly higher number of switches among the ecstasy users ($F(1,60) = 3.01$, $p = 0.088$; see Figure 1). However, there was a main effect of *5-HTTLPR* genotype, indicating that participants carrying the *ss* genotype had significantly lower Number of Switches compared with both *ls* ($p < 0.05$) and *ll* ($p < 0.05$) carriers (Figure 2). No group and genotype interaction was observed ($F(2,60) = 0.38$, $p = 0.687$).

Semantic Number of Switches was unaffected by *5-HTTVNTR*, *COMT val*$^{108/158}$*met*, *COMT rs165599* and *COMT rs2097603* genotypes. No group and genotype interactions were observed (see Table 3).

Phonemic switches: The phonemic *switching* score was unaffected by group ($F(1,63) = 1.79$, $p = 0.185$). However, there was a significant main effect of *5-HTTLPR* genotype ($p < 0.01$) (Figure 2). Post hoc analysis showed that participants carrying the *ss* genotype had a significantly lower

Table 1. Sociodemographic variables, drug use characteristics and genotype distributions

	Ecstasy ($n=30$)	Cannabis ($n=18$)	Control ($n=23$)	p
Age (Years)	24.2 (3.84)	22.7 (1.87)	22.4 (2.93)	0.092
Males	16 (53.3%)	6 (33.3%)	5 (21.7%)	0.057
Education (years)	14.6 (2.95)	14.0 (2.83)	15.0 (1.82)	0.504
QI- Vocabulary WAIS-III	12.0 (1.87)	12.8 (1.75)	12.8 (2.01)	0.229
Ecstasy Use				
Age of onset of use	18.1 (3.87)			
Duration of use (year)	5.5 (3.49)			
Total lifetime consumption (tablets)	200 (212)			
Cannabis use				
Age of onset of use	15.9 (2.37)	16.1 (1.54)		0.7
Duration of use (year)	7.55 (2.78)	6.19 (2.79)		0.123
Total lifetime use (joints)	4854 (4462)	2347 (3654)		0.061
Alcohol use				
Age of onset of use[†]	14.5 (2.37)	14.8 (1.17)	16.1 (1.46)	0.013
Duration of use (years)[‡]	8.97 (3.12)	6.88 (1.82)	5.70 (2.62)	<0.001
Tobacco use				
Current smokers[§]	22 (73.3%)	8 (44.4%)	6 (26.1%)	0.002
Age of onset of use	14.7 (1.84)	16.2 (3.15)	16.7 (1.63)	0.066
Duration of use (year)	7.84 (3.64)	6.12 (2.95)	4.83 (1.60)	0.106
Cigarettes per day	11.76 (6.78)	7.88 (7.40)	7.48 (5.78)	0.175
5-HTTLPR				0.213
ll	10 (33.3%)	7 (38.9%)	11 (47.8%)	
ls	11 (36.7%)	8 (44.4%)	11 (47.8%)	
ss	9 (30.0%)	3 (16.7%)	1 (4.3%)	
rs165599				0.28
A/A	12 (40.0%)	5 (27.8%)	11 (47.8%)	
A/G	16 (53.3%)	11 (61.1%)	8 (34.8%)	
G/G*	2 (6.7%)	2 (11.1%)	4 (17.4%)	
5-HTTVNTR				0.414
10/10	3 (10.0%)	5 (27.8%)	5 (21.7%)	
10/12	20 (66.7%)	9 (50.0%)	11 (47.8%)	
12/12	7 (23.3%)	3 (16.7%)	7 (30.4%)	
*9/10**	0	1 (5.6%)	0	
COMT val$^{108/158}$ met				0.172
A/A	6 (20.0%)	1 (5.6%)	8 (34.8%)	
A/G	15 (50.0%)	8 (44.4%)	9 (39.1%)	
G/G	9 (30.0%)	9 (50.0%)	6 (26.1%)	
rs2097603				0.537
A/A	12 (40.0%)	11 (61.1%)	9 (39.1%)	
A/G	14 (46.7%)	7 (38.9%)	11 (47.8%)	
G/G*	4 (13.3%)	0	3 (13.0%)	

Results are presented as mean (standard deviation) for continuous variables and absolute frequency (relative frequency) for categorical variables.
*Because of their low frequencies, this genotype was excluded from the analyses.
[†]MDMA < controls; [‡]MDMA>cannabis>controls; [§]MDMA > controls.

Number of Switches than both *ls* ($p<0.01$) and *ll* ($p<0.01$) carriers. No significant interaction was found ($F(2,64)=2.36$, $p=0.102$).

No genotype effects or group and genotype interactions were observed for *5-HTTVNTR*, *COMT val$^{108/158}$ met*, *COMT rs165599* and *COMT rs2097603* genotypes (See Table 3).

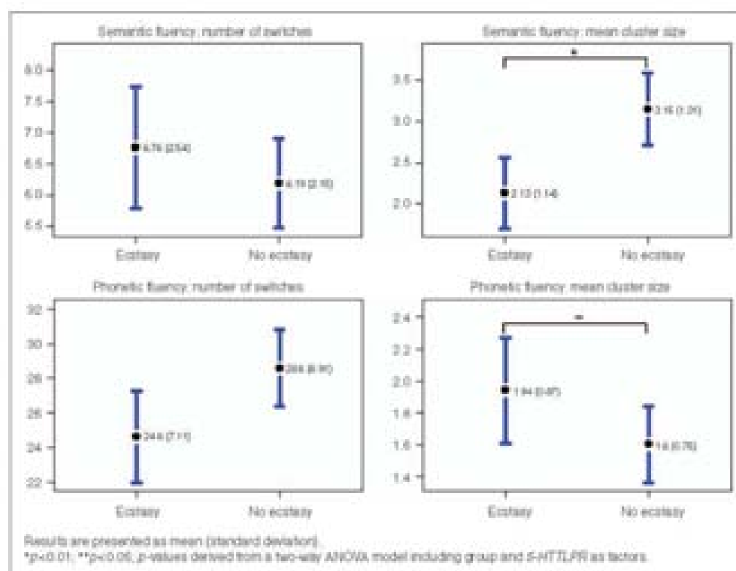
Discussion

Results showed that ecstasy users exhibited poorer semantic (but not phonemic) fluency performance than controls in traditional measures of total number of words. Detailed analyses of *clustering* and *switching* performance revealed that the poorer semantic fluency observed in this group was associated with poorer *clustering* mechanisms. *Clustering* performance was also modulated by the *COMT rs165599* polymorphism independently of the group. On the other hand, we found a specific effect of the *5-HTTLPR* polymorphism on *switching* performance across semantic and phonemic fluency modalities, with *ss* carriers performing significantly worse than *ls* and *ll* carriers. There was a trend for ecstasy users to perform worse than non-ecstasy users on *switching* measures, but *overall*

Table 2. *Clustering and switching performance within ecstasy and non-ecstasy users and 5-HTTLPR genotypes*

	Groups			*5-HTTLPR* genotypes			
	ecstasy ($n = 30$)	Non-ecstasy ($n = 41$)	ANOVA	ll ($n = 25$)	ls ($n = 29$)	ss ($n = 12$)	ANOVA
Semantic fluency							
TWG	23.33 (3.56)	26.48 (5.73)	$F(1,67) = 7.53$, $p = 0.008$	25.14 (4.78)	25.13 (5.22)	25.23 (6.08)	$F(1,67) = 0.30$, $p = 0.744$
Mean Cluster Size	2.13 (1.14)	3.15 (1.31)	$F(1,62) = 9.53$, $p = 0.003$	2.79 (1.47)	2.79 (1.30)	2.30 (1.13)	$F(2,62) = 0.14$, $p = 0.880$
Number of switches	6.76 (2.54)	6.18 (2.15)	$F(1,62) = 3.01$, $p = 0.088$	6.76 (2.47)	6.86 (2.07)	4.75 (2.01)	$F(2,62) = 5.35$, $p = 0.007$
Errors	1.03 (0.88)	0.68 (0.69)	$F(1,67) = 3.50$, $p = 0.065$				
Phonemic fluency							
TWG	40.66 (10.42)	40.67 (10.06)	$F(1,65) = 0.00$, $p = 0.947$	41.85 (11.75)	39.33 (10.37)	41.38 (5.22)	$F(2,65) = 0.46$, $p = 0.636$
Mean Cluster Size	1.94 (0.87)	1.60 (0.75)	$F(1,65) = 4.54$, $p = 0.037$	2.00 (0.81)	1.56 (0.68)	1.62 (0.79)	$F(2,65) = 3.18$, $p = 0.048$
Number of switches	24.6 (7.11)	28.6 (6.91)	$F(1,66) = 2.15$, $p = 0.148$	28.3 (7.38)	28.2 (6.47)	20.4 (5.33)	$F(2,66) = 4.85$, $p = 0.010$
Errors	3.70 (2.46)	0.80 (0.87)	$F(1,65) = 43.7$, $p = 0.000$				

Results are presented as mean (standard deviation).
p-values derived from a two-way ANOVA model including Group and *5-HTTLPR* as factors.
TWG: Total words generated.

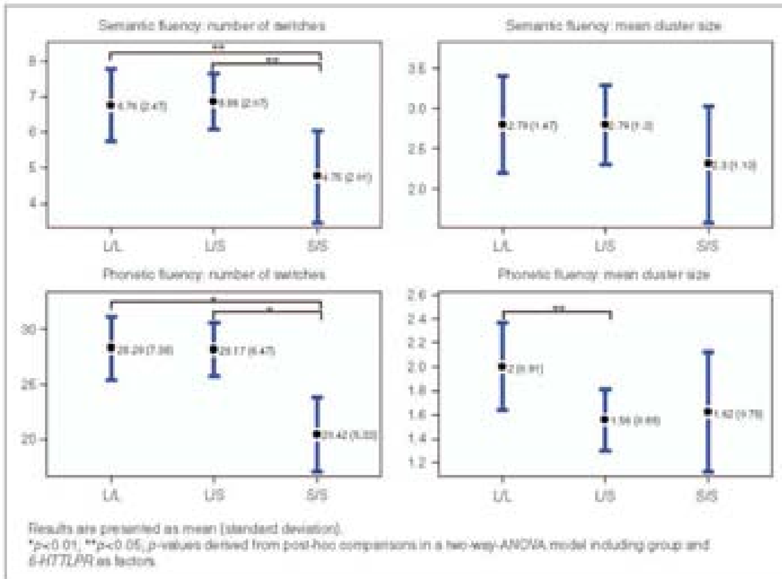


Results are presented as mean (standard deviation).
$^*p<0.01$; $^{**}p<0.05$, *p*-values derived from a two-way ANOVA model including group and *5-HTTLPR* as factors.

Figure 1. *Clustering and switching performance in the ecstasy and non-ecstasy groups.*

Table 3. *F*- and *p*-values concerning interaction and main effects of polymorphisms in 3-way ANOVA models. Values corresponding to main effects are from 3-way ANOVA models without interaction

	5-HTTLPR	*COMT val$^{158/108}$met*	*COMT rs165599*	*COMT rs2097603*
Semantic fluency				
Number of switches				
Interaction	$F(2,57) = 0.16$, $p = 0.855$	$F(2,58) = 3.00$, $p = 0.058$	$F(1,53) = 0.23$, $p = 0.635$	$F(1,54) = 1.99$, $p = 0.164$
Main effect	$F(2,59) = 0.08$, $p = 0.925$	$F(2,60) = 0.07$, $p = 0.936$	$F(1,54) = 2.02$, $p = 0.161$	$F(1,55) = 0.13$, $p = 0.725$
Mean cluster size				
Interaction	$F(2,57) = 1.73$, $p = 0.186$	$F(2,58) = 0.46$, $p = 0.636$	$F(1,53) = 1.67$, $p = 0.202$	$F(1,54) = 0.02$, $p = 0.879$
Main effect	$F(2,59) = 0.92$, $p = 0.404$	$F(2,60) = 0.24$, $p = 0.786$	$F(1,54) = 4.73$, $p = 0.034$	$F(1,55) = 0.02$, $p = 0.893$
Phonemic fluency				
Number of switches				
Interaction	$F(2,61) = 1.29$, $p = 0.283$	$F(2,62) = 0.68$, $p = 0.509$	$F(1,56) = 0.09$, $p = 0.772$	$F(1,57) = 0.16$, $p = 0.686$
Main effect	$F(2,63) = 0.79$, $p = 0.457$	$F(2,64) = 2.58$, $p = 0.084$	$F(1,57) = 2.28$, $p = 0.137$	$F(1,58) = 1.17$, $p = 0.285$
Mean cluster size				
Interaction	$F(2,60) = 0.13$, $p = 0.883$	$F(2,61) = 1.81$, $p = 0.173$	$F(1,55) = 0.88$, $p = 0.351$	$F(1,57) = 0.06$, $p = 0.802$
Main effect	$F(2,62) = 0.74$, $p = 0.483$	$F(2,63) = 0.21$, $p = 0.808$	$F(1,56) = 0.05$, $p = 0.816$	$F(1,58) = 0.90$, $p = 0.348$



Results are presented as mean (standard deviation).
*$p<0.01$, **$p<0.05$, *p*-values derived from post-hoc comparisons in a two-way-ANOVA model including group and *5-HTTLPR* as factors.

Figure 3. *Clustering and switching performance for each 5-HTTLPR genotype in both groups.*

switching performance seemed to be mostly modulated by the *5-HTTLPR* polymorphism. It is important to reiterate that the non-ecstasy user group included both drug-naive individuals and cannabis users because we had previously shown that cannabis use was unrelated to verbal fluency performance.

A major aim of this study was to disentangle the cognitive mechanisms underlying fluency deficits in ecstasy users. Our results suggest that semantic fluency deficits in ecstasy users are associated with *clustering* alterations. *Clustering* is based on the assumption that, during semantic fluency performance, individuals use a familiar search strategy based on the meaning

of the words, where the activation of the first exemplar automatically activates others semantically related words (Gleissner and Elger, 2001). It has been demonstrated that the retrieval and *clustering* of these semantically related words require an intact semantic network and memory function (Troster et al., 1998), mechanisms that are associated with temporal lobe functioning including lateral and medial temporal structures (Murphy et al., 2001; Sumiyoshi et al., 2006; Troyer et al., 1998). This notion has been highlighted by studies showing that semantic fluency *clustering* is one of the best neuropsychological markers of focal regions affecting the temporal lobe (Troyer et al. 1998). *Clustering* performance is also impaired in pathologies such as Alzheimer's disease, characterized by memory deficits and neurodegenerative damage to medial temporal structures (Fagundo et al., 2008; Pastel et al., 2004). Our results suggest, therefore, that the poorer semantic-based word fluency observed in ecstasy users (De Sola et al., 2008) can be explained in terms of a disorganization or a degradation of the semantic store, putatively associated with MDMA effects on hippocampus/temporal lobe functioning. Our findings are in agreement with recent meta-analyses concluding that memory deficits are the strongest cognitive correlate of ecstasy use (Kalechstein et al., 2007; Zakzanis et al., 2007). Our results are also consistent with the results of brain imaging studies showing ecstasy-related structural and functional alterations in the medial temporal regions that underline this cognitive function. It is of interest that neuroimaging studies in ecstasy users have found a reduced left hippocampal metabolism at resting state (Obrocki et al., 1999) and reduced left hippocampal activation during an episodic memory paradigm (Daumann et al., 2005), as well as a strong association between this pattern of predominant memory dysfunction and a reduced activation on hippocampal regions (Nifosi et al., 2008). This suggests that the functioning of the hippocampus might be affected by the neuromodulatory effects of ecstasy.

In addition, our study shows a modulatory effect of *COMT rs165599* genotype on semantic *clustering* performance irrespective of group. Results indicated that heterozygotes fail to use semantic *clustering* strategies as effectively as G/G homozygotes. These data are partially consistent with a recent report by Burdick et al. (2007), who observed an association between *COMT rs165599* genotype and a measure of episodic verbal memory (involving *clustering* strategies that support effective encoding) in bipolar disorder patients, with subjects with G genotype showing a reduced performance. In contrast with our results, they found that the homozygote G allele carriers performed significantly worse than heterozygotes. Several factors may account for this discrepancy: first, although both studies measured a similar cognitive domain, the Burdick et al. study employed a general measure of memory encoding, whereas we carried out a detailed analysis of cognitive mechanisms underlying access to semantic stores; second, these studies addressed two different clinical populations (ecstasy users versus bipolar disorder patients) whose cognitive approaches to task performing may differ considerably.

Regarding phonemic fluency, ecstasy users showed similar performance as non-users in the total number of correct words; however they performed better in the phonemic clustering. In accord with the methodology of the scoring system we have included errors in the *clustering/switching* analysis (Troyer et al., 1997). A supplementary analysis of errors was carried out for both semantic and phonemic fluency in order to provide additional information about the mechanism underlying verbal fluency deficits in ecstasy users. Of interest, the additional analysis of intrusions and perseverations showed that ecstasy users committed more errors than the non-users in phonemic fluency, which might explain the increased number of words generated in each subcategory. Intrusion errors correspond to word productions that do not agree with production criteria. Thus, in phonemic fluency tasks, intrusion errors include all words not belonging to the required phonemic field. To prevent these kinds of errors, participants must assess whether or not the item belongs to the explored semantic field. Perseveration errors are defined as a repetition of a word already produced in the sequence. To avoid this type of error, participants must use a process of output monitoring and, in particular, control the suppression of previously recalled items (Hurks et al., 2004, 2006). Processes to avoid perseveration errors are a form of executive monitoring (Rosen and Engle, 1997). In a fluency task, for instance, the number of perseveration errors has been markedly related to the working memory capacities of the participants (Rosen and Engle, 1997). Participants who had low working memory capacities made more repetitions (Rosen and Engle, 1997). Our results suggest that ecstasy users might have a reduction in the capacities needed to prevent intrusions and perseverations, and therefore ecstasy use might be associated with executive monitoring deficits.

Furthermore, in accordance with our initial hypotheses, we found a significant association between the *5-HTTLPR* genotype and *switching* performance across semantic and phonemic modalities. This is consistent with the notion that the ability to switch between elements when a cluster is exhausted relies on executive functions irrespective of the input (Troyer, 2000). The index of Number of Switches is associated with frontal lobe functioning and reflects the necessary cognitive flexibility for new subcategory search (Troyer, 2000). These assumptions are supported by several neuropsychological studies showing a decrease in the number of switches in patients with frontal lobe lesions or Parkinson's disease (Troster et al., 1998; Troyer et al., 1998) as well as by neuroimaging studies linking *switching* performance to activity in the ventrolateral prefrontal cortex, specifically the inferior frontal gyri (Hirshorn and Thompson-Schill, 2006). Furthermore, studies conducted in non-human primates have shown that selective serotonin depletion in the orbitofrontal cortex specifically impairs flexibility or *switching* abilities (Clarke et al., 2004, 2007). The *5-HTTLPR* polymorphism is functionally significant, and the *s* allele has been specifically linked to less efficient serotonin neurotransmission (Smeraldi et al., 1998; Whale et al., 2000). In accordance, our results showed that *s* carriers in both the ecstasy and control groups presented poorer *switching* performance than both *ls* and *ll* carriers across semantic and phonemic fluency tasks. Our results are therefore consistent with the proposed role of serotonin modulation in frontal-executive flexibility skills (i.e. the capacity to spontaneously shift mental set). With respect to previous studies which have shown poorer cognitive flexibility in ecstasy users

(Lamers et al., 2006; Von Geusau et al., 2004) our results suggest that these effects may be mediated more by the *5-HTTLPR* polymorphism than by ecstasy use. Our results strengthen the findings of Roiser et al. (2006), which showed that in both ecstasy users and drug-naïve controls *5-HTTLPR* modulates cognitive performance in tests of visual planning and decision-making. Nevertheless, this is the first study to report effects of the *5-HTTLPR* polymorphism on verbal fluency, specifically on switching abilities needed to adequately perform fluency tests, with a clearly attenuated response in the homozygous *ss*.

However, our study is not exempt from limitations, including the fact that the MDMA users, as a group, used more recreational drugs than control subjects, and exposure to other drugs could have played a role in the verbal fluency deficits found in the present study. Previous studies have raised the question that recent marijuana use may confound cognitive studies in MDMA users (Dafters et al., 2004; Lamers et al., 2006). Thus we included a group of cannabis users, considering that failure to match for the use of cannabis might result in its influence on MDMA-related neuropsychological impairments being overlooked.

As a result of the large number of statistical tests applied, the likelihood for a false positive result is elevated. Nonetheless, it was decided not to use any Bonferroni correction for multiple tests, since the resulting significance level would have been very conservative, most probably causing several false negative results. The sample size was relatively small for 3-way ANOVA models with a total of $2 \times 3 \times 3 = 18$ covariate patterns, including interactions. For this reason, we did not consider any three-way interactions and are aware of the fact that two-way interactions may not have been detected because of low statistical power. For the same reason, further possible predictors of *clustering* and *switching* performance were not considered in the ANOVA models. Nevertheless, we checked whether the results hold when adjusting the two-way ANOVA models for both sex and lifetime cannabis consumption. In addition, to rule out the possibility that results were confounded by IQ, years of education and age, the correlations between these variables and each of the four response variables were calculated using Spearman's rank correlation coefficient. In all cases, its absolute value was less than 0.12 and far from statistical significance. No substantial differences were found with respect to the results of the two-way ANOVA models. Errors (intrusions and perseverations) were included in the clustering/switching analysis following the original methodology of this scoring system (Troyer et al., 1997). However, a supplementary analysis of errors was carried out for both semantic and phonemic fluency in order to provide additional information about the mechanism underlying verbal fluency deficits in ecstasy users.

Finally, it is worth noting that we interpreted the inability to change from a cluster as a *switching* deficit, in accordance with previous literature on the sub-processes underlying fluency performance (Troster et al., 1998; Troyer, 2000; Troyer et al., 1998). However, it is also plausible that this inability reflects additional executive decrements, such as updating or monitoring of information deficits, which have been previously observed in MDMA users (Fisk and Montgomery, 2009; Montgomery and Fisk, 2008). In fact, there is evidence that several executive abilities simultaneously engage in response to complex tasks demands (Duncan and Owen, 2000); thus it is reasonable to consider that aspects of updating, inhibition and switching could simultaneously contribute to decreased fluency performance in MDMA users. Nonetheless, because our data indicated that the switching deficits were more closely related to the *5HTTLPR* genotype than to MDMA use (whereas MDMA use importantly affects updating mechanisms), we support the view that these deficits mainly reflect switching dysfunction, without discarding other executive components contribution.

Clinical implications

Semantic verbal fluency deficits may result in reductions of spontaneous speech among ecstasy users at a clinical level. Our results demonstrate that these semantic fluency deficits relate to potential disorganization or degradation of semantic stores. Furthermore, we showed a gene-mediated vulnerability for executive *switching* abilities that may endorse prevention strategies in individuals at risk of ecstasy use. Overall, our results may contribute to providing a better comprehension of ecstasy neurocognitive correlates. Fluency deficits seem to be linked to memory degradation and medial-temporal dysfunction in a straight line with ecstasy use, whereas frontal-executive deficits seem to be significantly modulated by serotonin genes.

Troyer et al. (1997) distinguished *switching* processes from *clustering* ones to characterize the executive and semantic contributions to verbal fluency performance. *Clustering* is a measure of the ability to access words within phonemic and semantic subcategories while *switching* is an executive measure of the ability to activate clusters and shift efficiently between them. Based on the impaired *switching* and *clustering* strategies observed in ecstasy users, it might be proposed that both semantic knowledge and retrieval are impaired in ecstasy users. Thus, the verbal fluency deficit in ecstasy users may be attributable to a disruption of frontal-striatal circuits directly related to serotonin function as well as to a depletion of lexical-semantic stores mediated by temporal structures. Exploring the nature of cognitive deficits, specifically semantic and executive-related deficits in addiction, is likely to be a far-reaching proposition. Our re-analysis of *clustering* and *switching* has highlighted the importance of choosing sensitive tools for this undertaking and highlights the importance of the assessment of cognitive domains outside episodic memory in ecstasy users.

Appendix: Examples of Semantic Subcategories

Frequently generated examples are summarized for each subcategory, although listings are not exhaustive.

General subcategories

Wild animals: African animals, Australian animals, canines (except for dog), Arctic/Far North animals, Primates, Reptiles/Amphibians.

Water animals: Fish, seafood, Arctic animals (except for polar bear), alligator, crocodile.
Farm animals: Cock, hen, chicken, duck, turkey, cow, donkey, goat, horse, mule, pig, sheep.
Birds: Farm birds, wild birds.
Pets: Dog, cat, canary, hamster, guinea pig, parrot, rabbit.

Specific subcategories

African animals: Lion, tiger, panther, leopard, cheetah, puma, hyena, jackal, lemur, cobra, elephant, giraffe, rhinoceros, zebra, hippopotamus, gazelle, antelope, monkey, chimpanzee, gorilla.
Australian animals: Ostrich, kangaroo, koala, crocodile.
Canines: Dog, wolf, fox, hyena, coyote.
Bovines: Buffalo, cow, sheep, yak.
Felines: Cat, lion, tiger, panther, cheetah, cougar, jaguar, leopard, puma.
Fish: Whale, dolphin, shark, salmon, bass, trout, prawn, lobster.
Seafood: Lobster, prawn, mussel, oyster.
Insects: Butterfly, bee, mosquito, ant, beetle, flea, fly, cockroach.
Primates: Chimpanzee, orangutan, ape, baboon, gibbon, gorilla, human, lemur, monkey.
Reptiles: Crocodile, alligator, snake, lizard, chameleon, iguana, turtle, frog, gecko, salamander, toad, tortoise.
Rodents: Mouse, hamster, chinchilla, beaver, squirrel, guinea pig, hedgehog, marmot, rat.
Arctic/Far North animals: Polar bear, penguin, reindeer, seal.

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Study 3

The influence of genetic and environmental factors among MDMA users in cognitive performance

Cuyàs E, Verdejo-García A, Fagundo AB, Rodríguez J, Cuenca AM, de Solà S, Langohr K, Peña-Casanova J, Torrens M, Martín-Santos R, Farré M, de la Torre R.

(submitted to Biological Psychiatry)

Introduction

3,4-methylenedioxymethamphetamine (ecstasy, MDMA) is one of the most popular illegal psychostimulants abused among youth. Ecstasy tablets are consumed recreationally at dance clubs and are associated with acute pleasant effects including increased energy, euphoria and empathy (Cami, Farre, Mas, Roset, Poudevida, Mas, San, and de la Torre 2000) and also unpleasant effects such as tachycardia, fatigue, anhedonia, irritability, low mood, and concentration difficulties (Morgan 2000).

There is compelling evidence that MDMA can induce long-lasting decrements in serotonin and 5-HIAA tissue levels in laboratory animals (Capela, Carmo, Remiao, Bastos, Meisel, and Carvalho 2009). A number of studies have demonstrated reductions in the activity of enzyme tryptophan hydroxylase (TPH) and in the activity and density of the serotonin transporter in MDMA-treated rats (Colado, Murray, and Green 1993;Perrine, Ghoddoussi, Michaels, Hyde, Kuhn, and Galloway 2010;Commins, Vosmer, Virus, Woolverton, Schuster, and Seiden 1987). In humans, ligand-binding imaging studies have reported decreased specific binding to the 5-HT transporter in ecstasy users compared to controls (Kish, Lerch, Furukawa, Tong, McCluskey, Wilkins, Houle, Meyer, Mundo, Wilson, Rusjan, Saint-Cyr, Guttman, Collins, Shapiro, Warsh, and Boileau 2010;McCann, Szabo, Seckin, Rosenblatt, Mathews, Ravert, Dannals, and Ricaurte 2005;Obrocki, Buchert, Vaterlein, Thomasius, Beyer, and Schiemann 1999;Ricaurte, McCann, Szabo, and Scheffel 2000). Although some studies have found significant increases of SERT availability in the midbrain and thalamus linked to protracted abstinence (Buchert, Thomasius, Wilke, Petersen, Nebeling, Obrocki, Schulze, Schmidt, and Clausen 2004;Thomasius, Zapletalova, Petersen, Buchert, Andresen, Wartberg, Nebeling, and Schmoldt 2006) there is no data about SERT recovery in the cortex and post-mortem evidence indicates that cortical

SERT protein reductions can be more robust and durable than indicated by neuroimaging studies (Kish, Fitzmaurice, Chang, Furukawa, and Tong 2010). Overall these data may suggest MDMA-induced neurotoxicity, and more specifically a neurodegeneration of the serotonergic neurotransmission system.

Despite these relatively neat findings about MDMA induced serotonin neuroadaptations, there is still debate on the question if MDMA use is reliably associated with substantial neuropsychological impairment, regardless of the effects of concomitant use of other substances (e.g., cannabis, alcohol or other stimulants). A related fundamental issue is which neuropsychological domains become more significantly impaired by the use of MDMA, and to what extent they would impact related aspects of adaptive functioning in MDMA users. The literature on this topic is characterized by considerable heterogeneity of results, which is attributable to the large amount of confounding variables inherent to research on the potential deleterious effects of MDMA use. These confounding variables include variation in patterns of MDMA consumption (e.g., purity of tablets consumed, dose-related effects, binge effects, effects of co-abuse, types of settings), potential differences in demographic background and lifestyles between users and controls, dispositional differences related to personality traits or sleep patterns, psychiatric background and status, or differences on genetic makeup (especially those related to serotonin genes) (Kish, Lerch, Furukawa, Tong, McCluskey, Wilkins, Houle, Meyer, Mundo, Wilson, Rusjan, Saint-Cyr, Guttman, Collins, Shapiro, Warsh, and Boileau 2010;Krebs, Johansen, Jerome, and Halpern 2009). In addition, neuropsychological research in MDMA users is hindered by considerable variability in the performance measures and research designs employed, making difficult to draw strong conclusions from comparison across studies (Fernandez-Serrano, Perez-Garcia, and Verdejo-Garcia 2010).

Two recent meta-analyses of neuropsychological studies in MDMA users have concluded that MDMA use is robustly associated with learning and memory impairments, yielding effect sizes ranging from 0.5 to 0.7 (Kalechstein, De La Garza, Mahoney, Fantegrossi, and Newton 2007;Zakzanis, Campbell, and Jovanovski 2007). This conclusion is substantiated by evidence from studies employing regression models (controlling for the co-abuse of other drugs) that demonstrate specific lifetime dose-related effects of MDMA use on learning and memory performance, particularly for verbal episodic memory tests (Bedi and Redman 2008a;Medina, Shear, and Corcoran 2005;Schilt, de Win, Jager, Koeter, Ramsey, Schmand, and van den 2008). Nonetheless, the size of this impact was modest (range of 6-11% of total explained variance –(Schilt, de Win, Jager, Koeter, Ramsey, Schmand 2008)) and some of the well-known potential confounding variables (e.g., psychiatric status) were not fully controlled in these studies (Krebs, Johansen, Jerome, and Halpern 2009). There is also evidence that memory decrements in MDMA users are more neatly observable when neuropsychological probes involve a greater degree of complexity in terms of encoding/organization demands (Brown, McKone, and Ward 2010;Quednow, Jessen, Kuhn, Maier, Daum, and Wagner 2006) –both studies using the California Verbal Learning Test). These findings suggest that other higher-order cognitive processes related to frontal cortex systems (e.g., attention or executive control) may be impacted by the use of MDMA and thus contribute to decreased memory performance in MDMA users.

One of the main factors that may contribute to clarify MDMA-related neuropsychological findings (once demographic and psychopathological variables are controlled) is variation in the distribution of a number of gene polymorphisms associated with the functioning of the 5-HT system. In addition, gene polymorphisms involved in MDMA pharmacodynamics and potential neural toxicity

(e.g., COMT and CYP2D6) and those supporting neural signaling cascades involved in learning and memory processes –the key cognitive processes linked to MDMA use cumulative effects (e.g., *BDNF* and glutamate genes) should also contribute to explain MDMA-induced neuropsychological deficits in humans. Some of these polymorphisms are equally interesting for MDMA-related neurocognitive effects based on their well-recognized role in modulating prefrontal cortex functioning and selective executive skills (speed, updating and performance monitoring), which have been linked both to MDMA use (de Sola, Miguelez-Pan, Pena-Casanova, Poudevida, Farre, Pacifici, Bohm, Abanades, Verdejo-Garcia, Langohr, Zuccaro, and de la Torre 2008;Fisk and Montgomery 2009) and to *COMT* and *5-HTT* genes (see reviews in Tunbridge, Harrison, and Weinberger 2006;Ullsperger 2010). This study seeks to investigate the relevance of all these genes for MDMA use-induced neuropsychological deficits.

Functional polymorphisms within the serotonin system, including serotonin transporter (*5-HTT*) and *5HT2A* receptor genes, are thought to have substantial influence on drug-related neurocognitive effects (Verdejo-Garcia, Lawrence, and Clark 2008). A functional polymorphism involving an insertion/deletion of 43 base pairs (bp) in the promoter region (5-HTTLPR) of the human serotonin transporter gene (*Slc6a4*, *5-HTT*) give rise to the long (*l*) or short (*s*) variants (Heils, Teufel, Petri, Stober, Riederer, Bengel, and Lesch 1996) which alter the transcriptional activity of the gene. The short variant of the polymorphism reduces the transcriptional efficiency of the *5-HTT* gene promoter, resulting in decreased 5-HTT expression (Lesch, Bengel, Heils, Sabol, Greenberg, Petri, Benjamin, Muller, Hamer, and Murphy 1996) and lower 5-HTT protein availability in the human brain (Heinz and Goldman 2000). This polymorphism has been associated with altered emotional processing and poorer cognitive performance in

ecstasy users; individuals carrying the s/s genotype have greater depression levels and poorer inhibitory control when performing an affective go/no-go task (Roiser, Cook, Cooper, Rubinsztein, and Sahakian 2005; Roiser, Rogers, Cook, and Sahakian 2006).

An additional functional single nucleotide variant (A/G) within the promoter (rs25531) has to be considered in order to interpret genotyping results of *5-HTTLPR* (Hu, Lipsky, Zhu, Akhtar, Taubman, Greenberg, Xu, Arnold, Richter, Kennedy, Murphy, and Goldman 2006;Wendland, Martin, Kruse, Lesch, and Murphy 2006). The *la* variant is associated with high levels of in vitro 5-HTT expression, whereas *lg* is low expressing and more similar to *s* allele (Praschak-Rieder, Kennedy, Wilson, Hussey, Boovariwala, Willeit, Ginovart, Tharmalingam, Masellis, Houle, and Meyer 2007). Reimold et al., (Reimold, Smolka, Schumann, Zimmer, Wrase, Mann, Hu, Goldman, Reischl, Solbach, Machulla, Bares, and Heinz 2007) reported an increased binding of PET ligands to 5HTT for subjects homozygous *la/la* in the putamen and midbrain. Conversely other studies found no effects of this polymorphism on 5-HTT binding in healthy volunteers (Murthy, Selvaraj, Cowen, Bhagwagar, Riedel, Peers, Kennedy, Sahakian, Laruelle, Rabiner, and Grasby 2010;Parsey, Hastings, Oquendo, Hu, Goldman, Huang, Simpson, Arcement, Huang, Ogden, Van Heertum, Arango, and Mann 2006) and postulate that lower binding is directly related to lower amounts of protein in discrete brain regions possibly associated to neurodegenerative MDMA induced effects (Kish, Lerch, Furukawa, Tong, McCluskey, Wilkins, Houle, Meyer, Mundo, Wilson, Rusjan, Saint-Cyr, Guttman, Collins, Shapiro, Warsh, and Boileau 2010;Kish, Fitzmaurice, Chang, Furukawa, and Tong 2010).

MDMA use has also been related to reductions of serotonin receptor 2A levels in rats and humans. The 5-HT2A receptors are located in the medial prefrontal cortex and hippocampus of rats (Pazos, Cortes, and

Palacios 1985;Xu and Pandey 2000) and humans (Hoyer, Pazos, Probst, and Palacios 1986;Wong, Lever, Hartig, Dannals, Villemagne, Hoffman, Wilson, Ravert, Links, Scheffel, and . 1987;Barnes and Sharp 1999;Leysen 2004) indicating that these receptors may play an important role in modulating learning and memory. Cortical 5-HT2A receptor densities in rats were decreased after MDMA treatment (Kindlundh-Hogberg, Svenningsson, and Schioth 2006). In humans, recent MDMA users display significant low cortical 5-HT2A densities while ex-MDMA users exhibited significantly higher receptor densities in the cortical areas studied (Reneman, Endert, de Bruin, Lavalaye, Feenstra, de Wolff, and Booij 2002).

A nonsynonymous polymorphism at position 1354 (C/T) occurs in the receptor gene leading to an amino acid substitution histidine (His) to tyrosine (Tyr) at codon 452 (His452Tyr) (rs6314). The rare *tyr* allele has been associated with poorer delayed but not immediate recall performance in verbal memory tasks (Wagner, Schuhmacher, Schwab, Zobel, and Maier 2008). In agreement with neuropsychological data, *tyr* carriers also have reduced hippocampal gray matter densities (Filippini, Scassellati, Boccardi, Pievani, Testa, Bocchio-Chiavetto, Frisoni, and Gennarelli 2006). Another polymorphism within the gene is the T to C transition at position 102 (T102C, rs6313) that does not alter the amino acid composition and, therefore, has no influence on the receptor protein (Bondy, Spaeth, Offenbaecher, Glatzeder, Stratz, Schwarz, de Jonge, Kruger, Engel, Farber, Pongratz, and Ackenheil 1999). Anyhow, several studies have associated this polymorphism with panic disorders, schizophrenia, suicidal behaviour, and affective disorders (Maron, Nikopensius, Koks, Altmae, Heinaste, Vabrit, Tammekivi, Hallast, Koido, Kurg, Metspalu, Vasar, Vasar, and Shlik 2005;Golimbet, Lavrushina, Kaleda, Abramova, and Lezheiko 2007; Vaquero-Lorenzo, Baca-Garcia, Diaz-Hernandez, Perez-Rodriguez, Fernandez-Navarro, Giner, Carballo, Saiz-Ruiz, Fernandez-Piqueras,

Baldomero, de Leon, and Oquendo 2008), although some of these results have not been replicated in other studies (Martinez-Barrondo, Saiz, Morales, Garcia-Portilla, Coto, Alvarez, and Bobes 2005;Correa, De Marco, Boson, Nicolato, Teixeira, Campo, and Romano-Silva 2007).

There are other genes involved in neural cascades underlying processes of synaptic plasticity (including long-term potentiation) that also play a role in the modulation of the serotonergic system. For example, genetic variation in *BDNF* influences 5-HTT availability (Henningsson, Borg, Lundberg, Bah, Lindstrom, Ryding, Jovanovic, Saijo, Inoue, Rosen, Traskman-Bendz, Farde, and Eriksson 2009). A single nucleotide polymorphism (SNP) at nucleotide 196 (G/A) (rs6265) within the *BDNF* gene, produces an amino acid substitution (valine to methionine) at codon 66 (val66met). This polymorphism does not affect mature BDNF protein function but it has been shown to alter the intracellular trafficking and packaging of pro-BDNF and the regulated secretion of the mature protein when the *val66* is replaced with *met* (Egan, Kojima, Callicott, Goldberg, Kolachana, Bertolino, Zaitsev, Gold, Goldman, Dean, Lu, and Weinberger 2003). One of the main effects observed by imaging studies in carriers of the *met* allele is a significant bilateral reduction of hippocampal and cortical volume (Pezawas, Verchinski, Mattay, Callicott, Kolachana, Straub, Egan, Meyer-Lindenberg, and Weinberger 2004;Szeszko, Lipsky, Mentschel, Robinson, Gunduz-Bruce, Sevy, Ashtari, Napolitano, Bilder, Kane, Goldman, and Malhotra 2005). *BDNF* polymorphism has also been associated with impairment in some forms of learning and memory. Individuals carrying the *met* allele perform worse than *val* homozygotes on memory tasks that rely on hippocampal functioning (Egan, Kojima, Callicott, Goldberg, Kolachana, Bertolino, Zaitsev, Gold, Goldman, Dean, Lu, and Weinberger 2003). However, recent

evidence indicates that *val* carriers may outperform *met* carrier counterparts when performing executive control tests (Beste, Baune, Domschke, Falkenstein, and Konrad 2010).

Other receptors strongly involved in the neural signalling processes underlying learning and memory processes are the N-methyl-D-aspartate receptors (NMDARs). The NR2B subunit, encoded by the glutamate receptor, ionotropic, NMDA subunit 2B gene (*GRIN2B*) is specially involved in the stabilization of synaptic connections, long term increase/decrease of synaptic strength, necrotic/apoptotic neuronal death, and learning processes (Seripa, Matera, Franceschi, Bizzarro, Paris, Cascavilla, Rinaldi, Panza, Solfrizzi, Daniele, Masullo, Dallapiccola, and Pilotto 2008;Rosenblum, Dudai, and Richter-Levin 1996;Loftis and Janowsky 2003). A polymorphism within this subunit leads to the substitution of C to T (rs1806210) which is a silent polymorphism and has no effect on the amino acid sequence of the receptor (Thr888Thr) (Nishiguchi, Shirakawa, Ono, Hashimoto, and Maeda 2000). Despite being synonymous, this SNP may have functional effects by altering the mRNA stability or translation, as the *C/T* and *T/T* genotypes might be related to increased glutamatergic neurotransmission because these genotypes are associated with early manifestations of Huntington's disease attributable to glutamate excitotocixity (Arning, Saft, Wieczorek, Andrich, Kraus, and Epplen 2007). Increases in glutamatergic neural transmission have been associated with reduced dopaminergic function (Seamans and Yang 2004).

Two additional genes will be examined based on their involvement in dopamine (COMT) and MDMA (CYP2D6 and COMT) metabolism, as well as in modulating prefrontal cortex functioning (COMT).Different isoenzymes of the COMT enzyme are involved in both the clearance of dopamine from the synaptic cleft in the prefrontal cortex and in the MDMA phase II metabolism (i.e., transformation of 3,4-

dihydroxymethamphetamine (HHMA) to 3-methoxy-4-hydroxymethamphetamine (HMMA) (de la Torre, Farre, Roset, Pizarro, Abanades, Segura, Segura, and Cami 2004)). A functional polymorphism (rs4680) consisting in a valine (val) to methionine (met) substitution at codon 158 of the MB-COMT (codon 108 for the S-COMT variant), results in a thermolabile protein with decreased enzymatic activity (one third less activity for the *met* homozygotes compared to the *val* homozygotes) at physiologic temperatures (Mannisto and Kaakkola 1999). Because these alleles are codominant, heterozygotes have intermediate levels of COMT activity. Inter-individual variability in the susceptibility to MDMA-induced neurotoxicity may in part be explained by the different enzymatic activity of these COMT variants and the reactive metabolic species formed as consequence of MDMA metabolism (de la Torre, Farre, Roset, Pizarro, Abanades, Segura, Segura, and Cami 2004;Perfetti, O'Mathuna, Pizarro, Cuyàs, Khymenets, Almeida, Pellegrini, Pichini, Lau, Monks, Farre, Pascual, Joglar, and de la Torre 2009). In addition, the *COMT* gene is involved in prefrontal cortex dopamine break-up and thus may influence the speed and quality of operations of executive control skills (Frank and Fossella 2011). The *val* allele of the *COMT* polymorphism has been associated with inferior working memory (Egan, Goldberg, Kolachana, Callicott, Mazzanti, Straub, Goldman, and Weinberger 2001;Goldberg, Egan, Gscheidle, Coppola, Weickert, Kolachana, Goldman, and Weinberger 2003) but greater cognitive flexibility in healthy subjects and psychotic patients (Colzato, Waszak, Nieuwenhuis, Posthuma, and Hommel 2010;Durstewitz and Seamans 2008).

Finally, the CYP2D6 enzyme is responsible for 30% of the conversion of MDMA to HHMA in humans (Farre, de la Torre, Mathuna, Roset, Peiro, Torrens, Ortuno, Pujadas, and Cami 2004). Interindividual differences in CYP2D6 activity may affect the MDMA pharmacology

and susceptibility to toxicity. Preliminary data have shown that MDMA pharmacology differs according to CYP2D6 genotype (de la Torre, Farre, Mathuna, Roset, Pizarro, Segura, Torrens, Ortuno, Pujadas, and Cami 2005). In agreement with this notion, a very recent study has shown that CYP2D6 involved in methamphetamine metabolic disposition modulates neuropsychological performance users (Cherner, Bousman, Everall, Barron, Letendre, Vaida, Atkinson, Heaton, and Grant 2010).

The current study seeks to clarify several open questions of the MDMA neuropsychological literature by investigating the neuropsychological performance (as a function of relevant genotype profiles) of a large sample of MDMA users recruited over 10 years in the city of Barcelona, being representative of the typical users of this substance in a homogeneous recruitment context. This sample includes considerable variability of drug use patterns, which have allowed us to carefully characterize dose-related effects of cumulative MDMA use on neuropsychological performance. Neuropsychological testing was focused on those cognitive domains that have been consistently linked to MDMA use across studies: verbal and visual memory, attention/processing speed and executive functions. Furthermore, we have investigated the effects of a number of key functional polymorphisms, previously discussed, which may importantly affect neuropsychological profiles in MDMA users. We hypothesized: (i) that heavier MDMA use would be correlated with poorer neuropsychological performance in a dose-dependent fashion; (ii) that heavy MDMA users would perform poorer than cannabis and healthy comparison individuals on neuropsychological tests of processing speed, memory and fluency (indicating robust effects of MDMA on cognition regardless of co-abuse of cannabis); and (iii) that MDMA use would exacerbate cognitive performance decrements in individuals

carrying genotypes associated with lower functionality of the serotonin, glutamate and dopamine systems (drug x gene interaction effects).

Methods

Participants

Two hundred sixty-three participants (n=263) were recruited, of whom 60 were ecstasy polydrug users, 110 were cannabis users and 93 were non users. The ecstasy users were further classified into two subgroups according to lifetime use of the substance applying a cut-off of more or less than 100 tablets (heavy vs. light users) (de Win, Jager, Booij, Reneman, Schilt, Lavini, Olabarriaga, Ramsey, Heeten, and van den 2008). All participants were healthy, self-reporting an adequate functioning within their social and professional context. Participants were recruited through several sources: `word of mouth´ notices in the local area, advertisement in the local university, and advertisement in a local NGO (Energy Control) specialized in providing harm reduction guidelines among drug users. As for the different groups, the following exclusion criteria were applied: for cannabis group, current history of regular use of other illegal psychotropic drugs with the exception of cannabis during last year, as well as past use of illegal drugs for more than 5 occasions during lifetime; and for non users current history of use of any illegal drugs during the past year, and past use of any illegal drugs in more than 5 occasions. Alcohol and nicotine use (but not abuse or dependence) was permitted. As for the ecstasy group, because it was impossible to recruit exclusive ecstasy users, it was decided to include ecstasy consumers with moderate use of other illicit drugs (not meeting abuse or dependence criteria), being ecstasy the main psychostimulant drug abused.

Test procedure

This study was approved by and conducted in accordance with the local ethics committee (CEIC-IMAS). Upon arrival to the research centre (IMIM, Hospital del Mar Research Institute), participants were informed of the ensuing protocol and gave their written informed consent before participating in the study.

All subjects were subjected to an exploration that included a detailed medical history, biochemical analyses, physical examination, urine and hair toxicology screens, a brief neurological examination, and a structured psychiatric interview (PRISM, (Torrens, Serrano, Astals, Perez-Dominguez, and Martin-Santos 2004)) by a psychiatrist or psychologist. Subjects with neurological, relevant medical disease and active psychiatric disorders (or active in the previous year of the exploration) were excluded. Abstinence period was not strictly delimitated, although all participants were requested to observe a 72 h abstinence period. Due to this fact, urine drug screens were carried out by immunoassay (CEDIA, Thermo-Fisher) in all subjects prior to neuropsychological testing in order to avoid acute effects. Drug classes screened for included: cannabis, ecstasy, cocaine and amphetamine/methamphetamine. Drug screens were performed also in hair samples by segmental analysis (last month, previous 6 months and last year) for the same drug classes in order to verify self-reported drug consumption history. This procedure allowed us to reliably classify participants into the different subgroups according to the pattern of drug use (ecstasy/cannabis vs. cannabis). All participants meeting inclusion criteria underwent a neuropsychological assessment session of 90 minutes, although here we only report analyses from a subset of these measures. After completing testing, all subjects were economically compensated for their participation.

Neuropsychological tests

The neuropsychological tests administered are briefly described below and a more thorough description of the protocol can be found in de Sola Llopis *et al.* (2008):

- Verbal Episodic Memory, California Verbal Learning Test (CVLT II). The test consists on a list of 16 words that the participant must learn across five learning trials and then reproduce after presentation of an interference list (immediate recall) and after 20-minutes lapse (delayed recall). The dependent measures were CVLT standard indices of learning (trials 1 to 5), immediate recall, delayed recall and recognition.

- Visual Episodic Memory, Rey-Osterrieth Complex Figure Test (ROCFT): Participants have to (i) copy and (ii) reproduce (after 3- and 30-minutes delays) a complex visual figure. The dependent measures were ROCF standard indices of accuracy of the copy, immediate recall, and delayed recall.

- Semantic Word Fluency, Animals. It evaluates the ability to access to, retrieve, and produce targeted information in response to words belonging to the category (e.g. “animals”). Two types of measures were obtained from these tests: (1) Total words generated: the total number of correctly generated words in 60 seconds. All intrusions (words not pertaining to this semantic category), perseverations (same words), and repetitions (same words with different endings) were treated as errors and not computed; and (2) *Clustering* and *switching* measures as explained previously in Fagundo et al. 2010 (Fagundo, Cuyàs, Verdejo-García, Khymenets, Langohr, Martín-Santos, Farre, and de la Torre 2010). A cluster was defined as any series of two or more successively produced words belonging to the same semantic or phonemic subcategory. Cluster size was computed adding up series of

words from the same subcategory starting from the second word within each cluster (i.e., a 3-word cluster has a size of 2). The Mean Cluster Size was obtained by adding the size of all the clusters and dividing it by the total number of clusters. The Number of Switches was defined and computed as the number of times the participant changed from one cluster to another within the same task. The computation of Number of Switches included single-word clusters (cluster size= 0). Intrusions, repetitions, and perseverations were excluded from the calculation of both Mean Cluster Size and Number of Switches.

- Attention/Processing Speed, Symbol Digit Modalities Test (SDMT) (from the Wechsler Adult Intelligence Scale –WAIS III): participants have to rapidly convert geometric designs into Arabic numbers following a visual key. Responses must follow the correspondence shown in a visual key. The dependent measure was the total number of hits produced in 90 seconds.

Genotyping

Genomic DNA was extracted from the peripheral blood leukocytes of all the participants using Flexi Gene DNA kit (Qiagen Iberia, S.L., Spain) according to the manufacturer's instructions.

5-HTTLPR genotyping was performed using polymerase chain reaction (PCR) as described previously in (Fagundo, Cuyàs, Verdejo-García, Khymenets, Langohr, Martín-Santos, Farre, and de la Torre 2010).

The 5HTTLPR (A/G) polymorphism (rs25531) was detected by MspI restriction enzyme digestion (De Luca, Tharmalingam, King, Strauss, Bulgin, and Kennedy 2005). Briefly, 1 µl of PCR was digested in a 10 µl reaction assay containing 1x NEBuffer 2 and 3U MspI at 37ºC for 3 h and a final inactivation step of 20 minutes at 65ºC. The resulting fragments were detected on an automatic ABI 3730XL capillary

sequencer and analyzed by GeneMapper Software v3.5 (Applied Biosystems, Foster City, CA, USA). Product sizes for the digest were: long A (L_A)= 337 bp, short A (S_A)= 292 bp, long G (L_G)= 162 bp, and short G (S_g)=162 bp.

In some cases, were MspI digestion gave unclear results the samples were sequenced to assign the correct genotype. Sequencing was performed in both, the sense and antisense orientations. The excess primers and deoxynucleotides in the polymerase chain reaction (PCR) products were then degraded by adding a 2 µl of a solution of 0.8 U of shrimp alkaline phosphatase (New England Biolabs, Ipswich, MA), 4 U of *Escherichia coli* Exonuclease I (New England Biolabs, Ipswich, MA) and 0.64x shrimp alkaline phosphatase buffer. The mixture was incubated at 37℃ for 15 min, followed by deactivat ion for 15 min at 80℃. Sequencing reactions were performed with BigD ye v3.1 (Applied Biosystems, Foster City, California) in 10 µl total volume containing 1 µl template (approximately 25 ng), 3.2 pmol primer, 1 µl 5x DNA sequencing buffer (Applied Biosystems), 2 µl BigDye v3.1 (Applied Biosystems), and water. The reactions were cycled at 94℃ for 3 min, followed by 30 cycles at 96℃ for 10 sec, 50℃ for 5 sec, and 60℃ for 4 min. Reactions were then purified with PureLink Quick Gel extraction kit (Invitrogen) according to manufacturer's instructions. Samples were analyzed on a Prism 3730xl DNA Analyzer (Applied Biosystems).

The *COMT* val108/158met (rs4680) and *BDNF* val66met (rs6265) single nucleotide polymorphism (SNP) allelic variants were determined using the 5' exonuclease TaqMan assay with ABI 7900HT Sequence Detection System (Real Time PCR) supplied by Applied Biosystems. Primers and fluorescent probes were obtained from Applied Biosystems with TaqMan SNP Genotyping assays (assay ID C_2255335_10 and C_11592758_10 for rs4680 and rs6265, respectively). Reaction conditions were those described in the ABI

PRISM 7900HT user's guide. Endpoint fluorescent signals were detected on the ABI 7900, and the data were analyzed using Sequence Detector System software, version 2.3 (Applied Biosystems).

The CYP2D6, GRIN2B C2664T (rs1806201), the 5HT2A His452Tyr (rs6314) and T102C (rs6313) genotypes were performed using the PHARMAchip^{TM} DNA array (Progenika Biopharma, Derio, Spain) (Cuyàs, Olano-Martin, Khymenets, Hernandez, Jofre-Monseny, Grandoso, Tejedor, Martinez, Farre, and de la Torre 2010). This DNA microarray allows the screening of genetic variants for phase I and phase II drug metabolism enzymes (DME), drug transporters, and drug protein effectors. It is based on the allele-specific oligonucleotide hybridization (ASO), where no enzyme is involved and the allelic discrimination is dependent on the design of the probes.

Statistical Analyses

Baseline characteristics, including drug consumption, are described by means of either mean and standard deviation (numerical variables) or absolute and relative frequencies (categorical variables). The chi-square test was applied to study the association between drug consumption (ecstasy consumption, cannabis consumption or control group) and each of the genotypes studied. In addition, it was used to check whether the Hardy-Weinberg equilibrium holds among each of the three populations under study.

At a univariate level, the correlation between the cognitive performance and both lifetime ecstasy and lifetime cannabis consumption was quantified by Pearson's correlation coefficient among those individuals consuming ecstasy and those consuming cannabis, respectively. These correlation coefficients were also computed for the subgroups defined by the *COMT* val158met genotype, on one hand, and the *5-HTTLPR* genotype, on the other.

Since the principal interest was to study the association between cognitive performance and both drug consumption and each of the genotypes of interest, ANCOVA models were fitted for all neuropsychological variables and each genotype separately. These models included drug consumption and the respective genotype as predictive variables of interest as well as gender and the WAIS-III Vocabulary index score (as a well-accepted proxy of IQ). Both latter variables were included in all regression models in order to rule out the possible confusion due to baseline differences observed among the drug consumption groups with respect to sex and IQ (because of the high correlations between education and IQ the former variable was not included in these analyses). Initially, all models did also include the two-way interaction between genotype and drug consumption. Whenever the interaction could be discarded, both factors were studied separately using the ANCOVA model excluding interaction. If a significant effect was observed of either factor, post-hoc multiple comparisons were carried out in the framework of the corresponding model using the Tukey test. If, by contrast, the interaction was significant, the effect of drug consumption was studied separately for each genotype expression and, vice versa. Again, the Tukey test for multiple comparisons was applied for these analyses in the framework of the ANCOVA models including interaction.

Statistical significance was set at 0.05 with one exception. The two-way genotype-drug consumption interaction within all ANCOVA models was only eliminated in case of $p>0.1$ in order to reduce the probability of a possible type-II error. The statistical software package R (The R Foundation for Statistical Computing), version 2.11.1, was used for all analyses. In particular, R package multcomp (Hothorn, Bretz, and Westfall 2008) was used for the multiple pairwise comparisons.

Results

Demographic variables, drug use characteristics and genotype distributions are presented in Table 1.

Concerning sociodemographic variables, the three samples showed several differences: mean ages were similar in the three groups though somewhat higher in the ecstasy group (23.2 years) than in the other two groups (21.6 and 22.8 years for cannabis and controls respectively). The proportion of individuals with a university degree or studying at university was lower in the ecstasy (68.3%) and cannabis (68.2%) groups than among controls (90.2%). On average, scores on the Vocabulary test (WAIS-III index score) were worse in the ecstasy (mean: 11.4) and cannabis groups (11.5) compared to the control group (12.6). Among each group, individuals with a university degree obtained higher WAIS-III index scores (data not shown). Regarding gender, the proportion of males was higher in the cannabis group (62.7%) with respect to the ecstasy (55%) and the control (52.7%) groups. The distribution of the employment categories among the three samples was fairly the same.

Table 1: Demographic variables, drug consumption characteristics.

	MDMA (n=60)	Cannabis (n=110)	Control (n=93)
	n (%)	n (%)	n (%)
Age[a]	23.2 (3.1)	21.6 (2.7)	22.8 (4.1)
Vocabulary WAIS-III[a]	11.4 (2.4)	11.5 (2.1)	12.6 (2)
Gender			
Male	33 (55.0)	69 (62.7)	49 (52.7)
Female	27 (45.0)	41 (37.3)	44 (47.3)
University degree[b]			
Yes	41 (68.3)	75 (68.2)	83 (90.2)
No	19 (31.7)	35 (31.8)	9 (9.8)
Employment Status			
Employed	17 (28.3)	29 (26.6)	26 (28.3)
Unemployed	13 (21.7)	24 (22.0)	13 (14.1)
Student	30 (50.0)	56 (51.4)	53 (57.6)
Smoker			
Current Smoker	46 (76.7)	70 (63.6)	17 (18.7)
Non smoker/Ex-smoker	14 (23.3)	40 (36.4)	74 (81.3)
Age at first tobacco use[a]	16.4 (3.4)	18.5 (3.1)	18.1 (3.3)
Years of tobacco consumption[a]	6.3 (3.9)	3.1 (2.6)	5.2 (4.9)
Cigarettes per day[a]	11.1 (5.9)	8.9 (6.3)	6.8 (5.3)
Age at first alcohol use[a]	14.5 (1.8)	14.8 (1.4)	15.9 (1.4)
Years of alcohol consumption[a]	8.7 (3.0)	3.1 (2.8)	6.8 (4.4)
Age at first cannabis use[a]	15.6 (2.0)	15.5 (1.6)	
Years of cannabis consumption[a]	7.7 (2.9)	6.1 (2.8)	
Age at first MDMA use[a]	18 (2.9)		
Years of MDMA consumption[a]	5.2 (3.2)		

[a]Mean (SD)
[b]Including Students

Genotype distributions

Genotype distributions for all groups are presented in table 2.

The tests of Hardy-Weinberg equilibrium among the three different groups of this study showed that genotypes in all the populations were in equilibrium with exception of the *COMT* val158met polymorphism in the cannabis group (p=0.004).

In some cases, genotypes for the different genes were combined for several reasons. For the serotonin transporter, most of the comparisons were performed with the three possible genotypes, and only when the combinations with *COMT*, *BDNF*, or the serotonin receptor polymorphisms were assessed, the three serotonin transporter genotypes were split in order to have enough individuals in each category to have enough statistical power. In those cases, individuals were classified into *L*-allele carriers or those with the *S/S*

genotype due to the hypothesis that MDMA users with the later genotype are said to show greater impairments in the cognitive functions assessed. For the combination of the two polymorphisms within the serotonin transporter (*5-HTTLPR* and rs25531), the combinations used were the same as described elsewhere. That is, high expression genotypes (*La/La*), medium (*La/Lg*, *La/S*), and low (*Lg/Lg*, *Lg/S*, *S/S*). Regarding the *COMT* val158met polymorphism, most analyses were performed using the three genotypes, but in some cases (e.g. when making comparisons with genotype combinations), it was necessary to group them into *met*-carriers or *val*-carriers in order to fulfil the groups to test different hypothesis. As for the *CYP2D6*, there were two groups, the Poor/Intermediate (if individuals carried one o more non-functional alleles), and the Ultra-rapid/Extensive (if one or more functional alleles). Finally, for the *BDNF*, *GRIN2B* C2664T, serotonin receptor His452Tyr, and the rs25531 alone, the rare genotypes (*met/met, T/T, Tyr/Tyr,* and *G/G,* respectively) were grouped with the heterozygous genotypes.

Table 2: Genotype distributions of the participants.

	MDMA heavy	MDMA light	MDMA	Cannabis	Control	
	n (%)	n (%)	n (%)	n (%)	n (%)	*p*-value
5-HTTLPR						
L/L	7 (25.0)	9 (28.1)	16 (26.7)	31 (28.2)	29 (31.2)	0.121
L/S	12 (42.9)	12 (37.5)	24 (40.0)	58 (52.7)	49 (52.7)	*0.280
S/S	9 (32.1)	11 (34.4)	20 (33.3)	21 (19.1)	15 (16.1)	
5-HTTLPR+rs25531 (n=259)						
High (La/La)	5 (17.9)	9 (29.0)	14 (23.7)	25 (23.4)	21 (22.6)	0.478
Medium (La/Lg + La/S)	13 (46.4)	12 (38.7)	25 (42.4)	55 (51.4)	52 (55.9)	*0.605
Low (Lg/Lg + Lg/S + S/S)	10 (35.7)	10 (32.3)	20 (33.9)	27 (25.2)	20 (21.5)	
rs25531 (n=259)						
A/A	-	-	53 (89.8)	93 (86.1)	77 (83.7)	0.568
G	-	-	6 (10.2)	15 (13.9)	15 (16.3)	
5HT2A receptor his452tyr (n=259)						
His/His	21 (77.8)	21 (65.6)	42 (71.2)	59 (54.1)	59 (63.4)	0.598
His/Tyr	6 (22.2)	11 (34.4)	17 (28.8)	50 (45.9)	34 (36.6)	*0.529
5HT2A receptor T102C						
T/T	-	-	9 (15.3)	25 (22.9)	17 (18.7)	
T/C	-	-	28 (47.5)	45 (41.3)	48 (52.7)	0.448
C/C	-	-	22 (37.3)	39 (35.8)	26 (28.6)	
BDNF val66met (n=262)						
val/val	22 (78.6)	20 (62.5)	42 (70.0)	59 (54.1)	59 (63.4)	0.109
met	6 (21.4)	12 (37.5)	18 (30.0)	50 (45.9)	34 (36.6)	*0.109
GRIN2B C2664T (n=259)						
C/C	19 (70.4)	19 (59.4)	38 (64.4)	57 (52.3)	54 (59.3)	0.288
T	8 (29.6)	13 (40.6)	21 (35.6)	52 (47.7)	37 (40.7)	*0.360
COMT val158met						
val/val	12 (42.9)	9 (28.1)	21 (35.0)	26 (23.6)	27 (29.0)	0.069
val/met	13 (46.4)	13 (40.6)	26 (43.3)	70 (63.6)	45 (48.4)	***0.037**
met/met	3 (10.7)	10 (31.2)	13 (21.7)	14 (12.7)	21 (22.6)	
CYP2D6						
Poor/Intermediate	-	-	9 (16.4)	14 (14.4)	13 (16.2)	0.928
Extensive/ultra-rapid	-	-	46 (83.6)	83 (85.6)	67 (83.8)	
Genotype Combinations						
5HTTLPR + COMT val158met						
L +met	-	-	24 (40.0)	68 (61.8)	57 (61.3)	
L + val/val	-	-	16 (26.7)	21 (19.1)	21 (22.6)	0.075
S/S +met	-	-	15 (25.0)	16 (14.5)	9 (9.7)	
S/S + val/val	-	-	5 (8.3)	5 (4.5)	6 (6.5)	
5HTTLPR + BDNF val66met (n=262)						
L +met	-	-	13 (21.7)	41 (37.6)	27 (29.0)	
L + val/val	-	-	27 (45.0)	47 (43.1)	51 (22.6)	**0.042**
S/S +met	-	-	5 (8.3)	9 (8.3)	7 (7.5)	
S/S + val/val	-	-	15 (25.0)	12 (11.0)	8 (8.6)	
5HTTLPR + 5HT2A his452Tyr (n=259)						
L + His/His	-	-	27 (45.8)	70 (64.2)	56 (61.5)	
L + Tyr	-	-	21 (20.3)	18 (16.5)	20 (22.0)	0.070
S/S	-	-	20 (33.9)	21 (19.3)	15 (16.5)	

* *p*-values for comparisons between ectasy heavy, ecstasy light, cannabis and control groups.

No significant differences were observed in the genotype distributions among the different groups, except for the *5-HTTLPR* and the *COMT* val158met polymorphisms. Significant differences were observed regarding the genotype distributions for the COMT val158met polymorphism (*p*=0.037) when the distinction between heavy and light ecstasy users was taken into account. There are a higher number of individuals with the *val/val* genotype in the heavy ecstasy users group (42.9%) compared to the control group (29.0%). The *val/met* genotype is also overrepresented in the cannabis group (63.6%) compared to the control group (48.4%).

Correlations between neuropsychological variables and ecstasy/cannabis lifetime consumption

Pearson's correlation coefficient revealed negative significant associations between cannabis lifetime consumption and CVLT total A1-A5 (r= -0.155, IC95%: [-0.298, -0.004]). For the ROFCT accuracy of copy, MDMA lifetime consumption showed a negative significant association (r= -0.604, IC95%: [-0.744, -0.413]), while it was significantly positive for cannabis lifetime consumption (r= 0.266, IC95%: [0.12, 0.401]). MDMA lifetime consumption showed negative significant associations with the ROFCT immediate recall (r= -0.391, IC95%: [-0.587, -0.152]), ROFCT delayed recall (r= -0.464, IC95%: [-0.642, -0.238]), and SDMT total correct (r= -0.269, IC95%: [-0.489, -0.016]).

An analysis of the results, taking into account the *COMT* val158met or the *5-HTTLPR* genotypes and the drug lifetime consumption was performed. Results showed a negative significant correlation between ROFCT immediate recall and MDMA lifetime consumption among individuals with the *val/val* genotype (r= -0.52, IC95%: [-0.587,-0.152]) and those carrying the *met* allele (r= -0.335, IC95%: [-0.588,-0.022]) (figure 1).

In the same direction are the results for the ROFCT delayed recall. Negative correlation was observed for MDMA users with the val/*val* genotype (r= -0.572, IC95%: [-0.805,-0.187]) (figure 1) and for those with the *met* allele (r= -0.418, IC95%: [-0.648,-0.118]) (figure 1). None of the other correlations analyzed reached statistical significance.

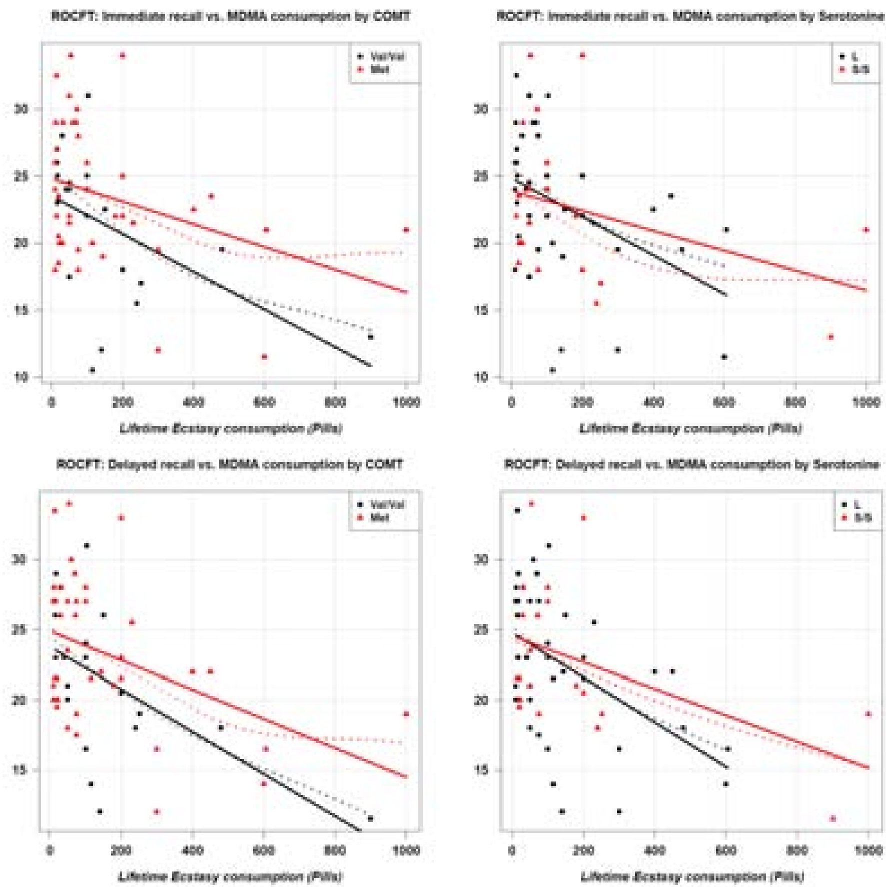

Figure 1: Ecstasy lifetime consumption and COMT val158met, 5-HTTLPR genotypes correlations for ROCT immediate and delayed recall.

Analysis of neuropsychological performance by group genotypes:

Symbol Digit Modalities Test (SDMT)

We found no significant main effect of group on performance in this test.

We found a significant Group x *5-HTTLPR* Genotype interaction (p=0.016), with heavy ecstasy users carrying the *S/S* genotype performing poorer than *S/S* controls (p=0.0152).

In addition, we found a significant Group x genotype interaction for the combination of the serotonin transporter (*5-HTTLPR*) and the *COMT* val158met genotypes (p<0.001). In the control group, individuals with the *S/S+val/val* genotype scored higher than those carrying the *L+met* combination (p=0.0219). In contrast, ecstasy users carrying the *S/S+val/val* combination performed significantly more poorly than ecstasy *L+val/val* carriers (p=0.0127).

Rey-Osterrieth Complex Figure Test (ROCFT)

Accuracy of the copy:

We found a main effect of Group and paired contrasts indicated that heavy ecstasy users had lower scores than light ecstasy users (p<0.001), cannabis users, and control group (p<0.0001, in both cases).

We found a significant Group x Genotype interaction for the COMT val158met (p=0.020). In the control group, individuals with the *met/met* genotype had lower scores than those carrying the *val/met* genotype (p=0.0312), or the *val/val* genotype (p=0.0133). However, heavy ecstasy users with the *val/val* genotype showed significantly lower scores than control individuals carrying the same genotype (p=0.016). When the *val/met* genotype was examined, heavy ecstasy users with this genotype had lower scores than light ecstasy users

(*p*<0.001), cannabis users or controls with the same genotype (*p*<0.001 for both groups).

In addition, we found a significant Group x *rs25531* (serotonin transporter) Genotype interaction (*p*=0.028). MDMA users with the *A/A* genotype had lower scores for the accuracy of copy than cannabis users (*p*=0.013), and than control individuals (*p*=0.001). Furthermore, individuals in the ecstasy group with the *G* allele, scored lower than cannabis users (*p*=0.004) with the same genotype.

When considering the combination of the *COMT* val158met and *5-HTTLPR* genotypes, we also found an effect of the Group x Genotype interaction (*p*=0.014). Individuals with the *S/S+val/val* genotype using MDMA scored poorer than those with the same genotype in the control group (*p*=0.014) or the cannabis users (*p*=0.039).

Immediate recall:

We found a main effect of Group, with heavy ecstasy users having significant lower scores than the light ecstasy users (*p*=0.009), the cannabis group (*p*<0.001), and the control group (*p*=0.002).

In addition, we found a significant main effect of the *COMT* val158met Genotype, with *met*-carriers performing significantly poorer than *val/val* individuals (*p*=0.039).

When examining the combination of the *COMT* val158met and *5-HTTLPR* genotypes, we found a Group x Genotype effect (*p*=0.015); MDMA individuals with the *S/S+val/val* genotype had significantly lower scores than those with the same genotype in the control group (*p*=0.002).

Delayed recall:

We found a main effect of Group (*p*=0.002), with heavy ecstasy users having significantly lower scores than light ecstasy users (*p*=0.022), cannabis users (*p*<0.001), and controls (*p*=0.008). We also found a main effect of *5HT2A* Genotype, indicating that individuals carrying the *His/Tyr* variant had significantly poorer performance than those with the *His/His* genotype (*p*=0.015).

The study of the interaction between the *COMT* val158met and *5-HTTLPR* genotypes showed a main effect of the Group x Genotype interaction (*p*=0.001). In the control group, individuals with the *S/S+met* genotype performed worse than those with the *L + val/val* genotype (*p*=0.036), and those with the *S/S+val/val* genotype (*p*=0.038). However, within the *L + val/val* genotype, MDMA users had significantly lower scores than controls (*p*=0.004).

California Verbal learning test (CVLT)

We found no effects of Group, Genotype or the Group x Genotype interaction on the learning (trials 1 to 5), immediate recall, and total recognition indices from this test. However, for delayed recall we found a main effect of the *GRIN2B* genotype, with individuals carrying the *T* allele recalling more words than those with the *C/C* genotype (*p*=0.023).

Semantic Word Fluency

The semantic word fluency was unaffected by group. However, there was a significant effect of the Group x *GRIN2B* genotype interaction (*p*=0.033). Post hoc analysis showed that participants with the *C/C* genotype had significantly lower scores than those with carrying the *T* allele (*p*=0.0326).

In addition, we found a significant effect of the Group x CYP2D6 phenotype interaction (*p*=0.047). In the control group,

individuals who were intermediate/poor for the CYP2D6 performed worse than those who were ultra-rapid/extensive (*p*=0.0069). In contrast, MDMA users with the ultra-rapid/extensive phenotype had significantly lower scores than those with the same phenotype in the control group (*p*=0.0146).

Number of switches:

We found a significant effect of the Group x GRIN2B genotype (*p*=0.021), indicating that both heavy ecstasy and cannabis users carrying the *C/C* genotype achieved a significantly lower number of switches than light ecstasy users (*p*=0.0057 and *p*=0.0133, respectively).

We also observed a significant effect of the Group x *5HT2A* T102C Genotype interaction, with MDMA users homozygous for the *T/T* genotype generating a significantly lower number of switches than those with the *C/C* genotype (*p*=0.023).

We additionally found a significant effect of the Group x *COMT* val158met genotype (*p*=0.026), with light ecstasy users homozygous for the *val* allele producing a higher number of switches than cannabis users with the same genotype.

Mean Cluster Size:

We observed a main effect of the *COMT* val158met genotype. Participants carrying the *met* allele displayed a significant reduction in the number of words generated in each subcategory compared with participants homozygous for the *val* allele (*p*=0.029).

Discussion

Our findings showed detrimental effects of both MDMA lifetime use and variations in candidate genes on a number of performance measures, with particular relevance of visuospatial attention and

episodic memory (taxed by the ROCFT). With respect to dose-related effects, we found that greater lifetime use of MDMA is negatively correlated with performance on visuospatial memory and visuospatial attention/speed tests. These results were further supported by group comparisons, which showed that heavy MDMA users (lifetime use>100 tablets) have significantly poorer visuospatial memory performance than light MDMA users, cannabis users and controls. Importantly, we found a number of gene x MDMA interaction effects. Results for *COMT* and *SERT* genes showed that heavy MDMA users carrying the *SERT* s/s and *COMT val/val* genotypes have poorer performance on tests of speed/attentional control (Digit Symbol) and visual attention and planning (ROCF Copy). Moreover, for ROCFT immediate recall, MDMA users carrying the *COMT val/val* and *SERT s/s* genotype (irrespective of MDMA use) perform more poorly than healthy individuals carrying the same genotype. In the case of delayed recall, MDMA users carrying the *COMT val/val* + *SERT L* genotype also perform poorer than controls carrying identical combination of genes. In addition, we found an interaction between MDMA use and both CYP2D6 extra-high metabolic activity phenotype and *GRIN2b* low glutamate activity genotype (*C/C*) associated with lower performance on verbal fluency. Finally, we also found a number of main effects of genotype (irrespective of group). The *GRIN2b* genotype is associated with verbal episodic memory, being *C/C* carriers poorer performers; the *5HT2a Tyr* allele is linked to poorer visual delayed recall; and the *COMT met* allele is associated with lower visual memory and reduced size of clusters produced during the fluency test.

The main MDMA dose-related findings and MDMA x gene interactions were found in the ROCFT visuospatial memory test. This is a complex task involving visuospatial attention and planning/organization skills during the copy, and planning and episodic memory skills during immediate and delayed recall (Shin, Park, Park,

Seol, and Kwon 2006). Copy performance is associated with dorsolateral prefrontal cortex (DLPFC) and parietal cortex functioning (Antshel, Peebles, AbdulSabur, Higgins, Roizen, Shprintzen, Fremont, Nastasi, and Kates 2008), whereas immediate and delayed recall are associated with the functioning of the DLPFC (Haroon, Watari, Thomas, Ajilore, Mintz, Elderkin-Thompson, Darwin, Kumaran, and Kumar 2009) and the hippocampus (Carlesimo, Cherubini, Caltagirone, and Spalletta 2010). The robust dose-related negative correlations with visuospatial memory are consistent with our previous results in a subsample of the MDMA users included in this study (De Sola et al., 2008). However, they are at odds with results from recent meta-analyses indicating low effect sizes for decrements on visual memory probes in MDMA users (Laws and Kokkalis 2007;Nulsen, Fox, and Hammond 2010), and with evidence from well-controlled regression-based studies showing greater effects of MDMA lifetime use on verbal rather than visual memory (Bedi and Redman 2008b;Schilt, de Win, Jager, Koeter, Ramsey, Schmand, and van den 2008;Schilt, Koeter, Smal, Gouwetor, van den, and Schmand 2010). However, a number of factors may contribute to explain this apparent discrepancy. First, the fact that the larger dose-related correlations are found with the Copy index indicates that MDMA cumulative use may have greater detrimental effects on visuospatial attention and planning skills than actual visuospatial memory; this is consistent with the finding that MDMA cumulative use is also negatively associated with Digit Symbol-indexed visuospatial attention. In addition, this dose-related association seems to be modulated by *COMT* and to lesser extent *SERT* genotypes. In fact, heavy MDMA users carrying the *COMT val/val* and *SERT s/s* genotypes performed significantly more poorly than all other groups on the ROCFT Copy index. Furthermore, the *val/val* genotype was associated with poorer performance of MDMA users, compared to controls, on both immediate and delayed

recall indices. This interpretation is in agreement with recent evidence showing that the *COMT* gene is significantly associated with visuospatial planning ability –high enzymatic functioning gene-carriers display poorer performance (Roussos, Giakoumaki, Pavlakis, and Bitsios 2008) and DLPFC and parietal activation during planning tasks (Williams-Gray, Hampshire, Robbins, Owen, and Barker 2007). Interestingly, fMRI data has revealed that continued MDMA use across 18 months is selectively associated with abnormally increased parietal activation during an executive attention test (Daumann, Jr., Fischermann, Heekeren, Thron, and Gouzoulis-Mayfrank 2004). There is also evidence of the influence of the SERT genotype on visuospatial attention/planning performance (Roiser, Muller, Clark, and Sahakian 2007). Similar to our findings, Roiser et al. found superior performance in healthy adults carrying the *s/s* genotype using a mental rotation task. However, in our sample (but not in Roiser's) heavy MDMA use modified this profile, and heavy MDMA s/s carriers –especially in combination with *COMT val/val* genotype performed significantly poorer than all the other comparison groups in the Copy index, and poorer than control individuals with similar genotypes in the Digit Symbol. Overall, we argue that heavy MDMA use and *COMT val/val* and *SERT s/s* genotypes interact to confer greater detrimental effects on visuospatial attention and planning skills, ultimately affecting visual memory performance.

In the last years there has been intense controversy about the "real" significance of the *COMT* gene for cognitive performance. Results from a recent meta-analysis indicated that the putative effects of the *COMT* gene on verbal memory, fluency, working memory or flexibility performance are negligible (Barnett, Scoriels, and Munafo 2008). However, the effects of the *COMT* gene on visuospatial attention and memory, as reported here, are rather less explored. A link between the *COMT* gene and these phenotypes arises from

neuropsychological research on individuals with microdeletion of chromosome 22q11.2. Individuals with this genetic disorder, in which the *COMT* is one of the genes in the deleted region, present quite prominent deficits in visuospatial memory tests, coupled with relative strength of verbal recall (Campbell, Azuma, Ambery, Stevens, Smith, Morris, Murphy, and Murphy 2010). In addition, neuroimaging studies keep on indicating that *COMT* polymorphisms are associated with distinct patterns of connectivity between the prefrontal cortex and the hippocampus, with *val/val* carriers having reduced coupling between both regions ((Dennis, Need, LaBar, Waters-Metenier, Cirulli, Kragel, Goldstein, and Cabeza 2010); previously (Bertolino, Rubino, Sambataro, Blasi, Latorre, Fazio, Caforio, Petruzzella, Kolachana, Hariri, Meyer-Lindenberg, Nardini, Weinberger, and Scarabino 2006)). These connections are critical for executive manipulation of memory contents (e.g., successful encoding, organization and strategic retrieval), and this skill is substantially affected by MDMA use (Quednow, Jessen, Kuhn, Maier, Daum, and Wagner 2006). Therefore, it is both biologically and neurocognitively plausible that MDMA use can further deteriorate prefrontal-hippocampal coupling in the case of *val/val* individuals, conferring them higher risk of impaired visuospatial memory deficits. This selective effect on *val/val* carriers may also contribute to explain our findings on ROCFT immediate and delayed recall. This effect should have been parallel for verbal memory. However, as compared to typical measures of verbal learning, the ROCFT requires deeper organization demands, both visually and conceptually (because figure items are much harder to categorize than verbal items). These deeper organization demands may need additional fine-tuning of DLPFC-hippocampal connectivity, and be therefore more susceptible to MDMA neuroadaptive effects. Another variable to take into account is interaction between different polymorphisms, which is rarely reported in the gene-cognition

literature. In our results, the combination of *COMT* and *SERT* genes contributed to explain variation in visuospatial attention and memory performance in MDMA users. On the other hand, visual delayed recall is more clearly linked with the *5HT2A His/Tyr* polymorphism in the whole group, and verbal delayed recall and verbal fluency are only associated with *GRIN2B* variations irrespective of MDMA use. These results are in accordance with previous literature indicating that the rare 5HT2A *Tyr* allele may be specifically associated with hippocampal integrity and memory consolidation (Filippini, Scassellati, Boccardi, Pievani, Testa, Bocchio-Chiavetto, Frisoni, and Gennarelli 2006;Wagner, Schuhmacher, Schwab, Zobel, and Maier 2008). Similarly, they are consistent with a purported role of *GRIN2B* on verbal memory performance and temporal cortex volume across development (Ludwig, Roeske, Herms, Schumacher, Warnke, Plume, Neuhoff, Bruder, Remschmidt, Schulte-Korne, Muller-Myhsok, Nothen, and Hoffmann 2010;Stein, Luppa, Brahler, Konig, and Riedel-Heller 2010).

A final worth noting finding was that of significant interaction between MDMA use and the CYP2D6 phenotype on semantic fluency performance. The CYP2D6 enzyme is one of the main products involved in MDMA metabolism (de la Torre, Farre, Roset, Pizarro, Abanades, Segura, Segura, and Cami 2004) and variations in this gene are thought to play a key role on MDMA-induced neurotoxicity due to their effects on the formation of neurotoxic thioether adducts (Perfetti, O'Mathuna, Pizarro, Cuyàs, Khymenets, Almeida, Pellegrini, Pichini, Lau, Monks, Farre, Pascual, Joglar, and de la Torre 2009).

According to our initial hypothesis, MDMA users with extremely high metabolic activity phenotypes produced a significantly lower number of words in response to a semantic prompt. These results are in agreement with recent findings about the link between higher CYP2D6 activity and impaired executive performance –including semantic

fluency in methamphetamine users (Cherner, Bousman, Everall, Barron, Letendre, Vaida, Atkinson, Heaton, and Grant 2010). However, we extend their findings by showing significant specific effects of the rare ultra-rapid/extensive phenotype on executive performance in MDMA users, a drug-using group in which greater cognitive dysfunction was expected based on specific pharmacodynamic mechanisms. Furthermore, fluency is one of the executive skills more consistently impaired in MDMA users (Fernandez-Serrano, Perez-Garcia, and Verdejo-Garcia 2010), conferring clinical significance to this gene x drug interaction effect. This finding supports the proposal that *CYP2D6* genotype polymorphisms modulate MDMA-induced neurotoxic effects and subsequent decrements in executive performance. More research including additional executive phenotypes and larger sample sizes are warranted to further substantiate these promising findings.

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Study 4

Gene expression study in an animal model of MDMA induced neurotoxicity

Gene expression experiments were carried out in order to study the long-term effects of MDMA treatment in different rat brain areas.
A second objective of this study was to investigate a possible correlation between differences in gene expression observed in brain and differences in expression levels for the same genes in blood, as the existence of this relationship between changes observable in brain and blood could be very important for studies with human volunteers as we have only access to blood cells.

Quality parameters for RNA samples

Purity and integrity

The purity of RNA samples is evaluated by the presence of protein, salts or remaining products from the extraction procedure. As described previously, the most common method for the assessment of RNA purity is based on the A_{260}/A_{230} and A_{260}/A_{280} ratios (UV ratios), which are used as a representation of the relative abundance of the different impurities in the RNA sample. Acceptable values for these ratios are within 1.6 and 2.0 for most of the applications.
RNA integrity was evaluated by two different approximations: by the typical estimation of the integrity of ribosomal RNA subunits bands (28s/18s ratio), and by the RNA integrity number (RIN), as described previously in the methodological approaches chapter.
Blood and brain samples included in this study had ratio values within the accepted range for both ratios, A_{260}/A_{230} and A_{260}/A_{280}, except for one hippocampal control sample, which had A_{260}/A_{230}= 0.9. For that sample, all qPCR reactions were examined to ensure that contamination did not affect the reaction.
Regarding integrity values, RNA samples extracted from blood had higher 28s/18s ratio values than RNA extracted from brain samples. For brain samples, all ratios values were under 1.0 (except for one cortex sample which was 1.1). RNA samples extracted from blood, had

higher 28s/18s ratios, although were under the desired value of 2.0 (except for one sample). Besides, RIN values for all the samples (extracted from both brain and blood) were considered as indicative of acceptable RNA integrity.

RNA samples were frozen at -20ºC prior to its use for reverse transcription. Random samples (six) were selected after thawing in order to check their integrity before its further use. All samples selected had similar values of integrity as those obtained before freezing, suggesting that the freeze and thaw cycle did not affect their integrity.

Therefore, we considered that all the samples were suitable for their inclusion in the gene expression assay.

Gene expression assays in rat brain.

Four different brain regions [hippocampus, striatum, cortex, and dorsal raphe nuclei + substantia nigra (DRN+SN)] were selected to carry out the gene expression studies. The DRN+SN were studied as a single brain region due to the difficulties to remove only the DRN.

Initially two reference genes (18s rRNA, and β-actin) were selected for data normalization. However, amplification of 18s rRNA started approximately at cycle 5 whereas most of the genes of interest (and also β-actin) started between cycles 20-30. Accordingly, β-actin was selected as the most suitable reference gene for all the analyses.

Dorsal Raphe Nuclei and Substantia Nigra (DRN+SN)

All genes except *Slc6a3* (dopamine transporter) were amplified.

Significant expression levels changes were observed for serotonin transporter gene (*Slc6a4*) (*p*=0.016), vesicular monoamine transporter 2 (*Scl18a2*) (*p*=0.008), and aromatic amino acid decarboxylase (*Ddc*) (*p*=0.041). In all the cases, treated animals showed decreased expression (figure 13). However, a trend to significance was observed for *Tph2* (tryptophan hydroxylase 2) (*p*=0.059), γ synuclein (*sncg*) (*p*=0.054), and *Slc6a2* (*NET*) (*p*=0.051). For the later, great dispersion was observed for the RQ values of the non treated animals, and thus, this has to be kept in mind when considering this result.

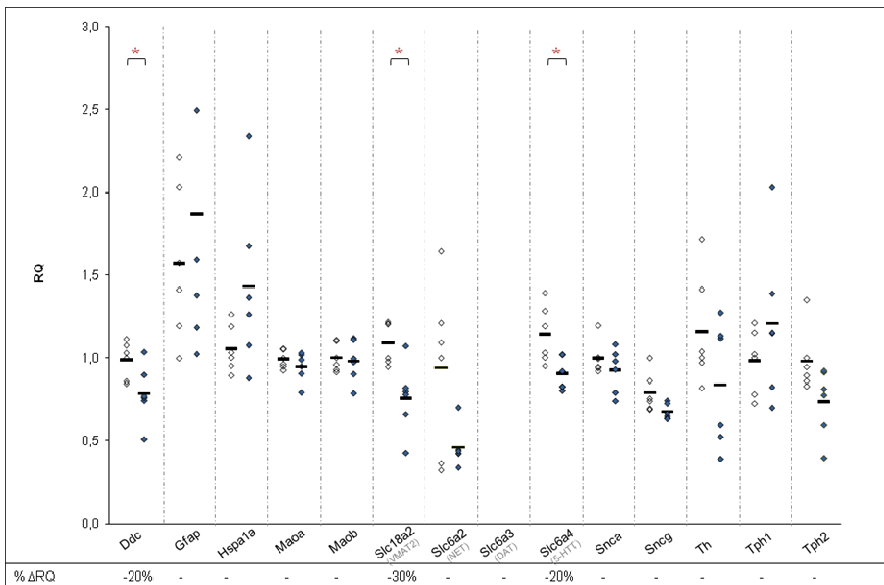

Figure 13. Relative quantification values for individual samples of the **DRN+ SN brain** area. Empty or filled diamonds represent individual RQ values for control and MDMA-treated rats individually. Lines show the mean value. Asterisks represents significant changes in gene expression (at level $p<0.05$). ΔRQ values respect control samples (in percentage) are given for samples reaching statistically significant differences.

Hippocampus

Results for relative quantification (RQ) in the hippocampus are shown in figure 14.

No amplification was observed for *Slc6a2* (*DAT*). For *Slc6a3* (*NET*) and *Tph1*, amplification started later than cycle 35, and although data

is shown in figure 2, differences observed for these genes are not considered.

When the relative quantification data were analysed, significant differences were observed for *Maob* (*p*=0.009), and *Tph2* (*p*=0.008). In both cases, levels of expression for MDMA-treated animals were increased respect to the controls (variations of around 9% and 42%, respectively).

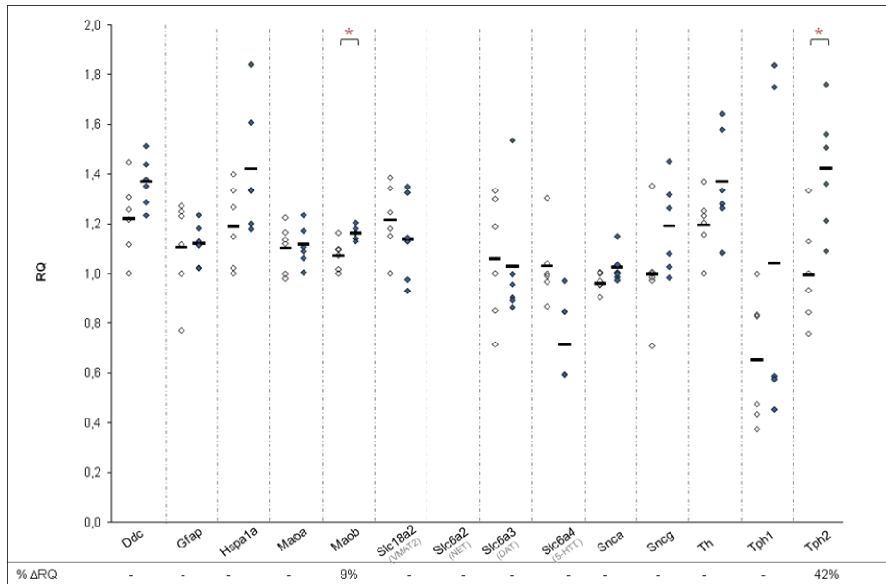

Figure 14. Relative quantification values for **hippocampal** individual samples. Empty or filled diamonds represent individual RQ values for control and MDMA-treated rats individually. Lines show the mean value. Asterisks represents significant changes in gene expression (at level $p<0.05$). ΔRQ values respect control samples (in percentage) are given for samples reaching statistically significant differences.

Cortex

Quantitative real-time PCRs results showed no amplification for *Slc6a2* (*NET*), *Slc6a3* (*DAT*) and *Tph1* genes.

Significant differences in levels of expression were observed for *Snca* (*p*=0.003) (figure 15), with treated animals displaying lower expression (variation of -20% on RQ) than control animals.

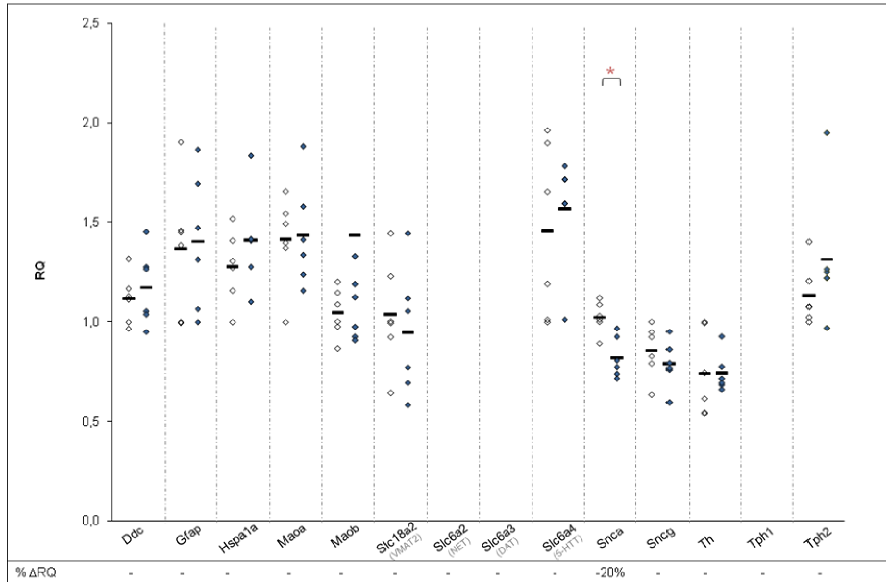

Figure 15. Relative quantification values for **cortical** individual samples. Empty or filled diamonds represent individual RQ values for control and MDMA-treated rats individually. Lines show the mean value. Asterisks represents significant changes in gene expression (at level $p<0.05$). ΔRQ values respect control samples (in percentage) are given for samples reaching statistically significant differences.

Striatum

When examining results for the striatum brain region, we observed that real-time PCRs failed to amplify *Tph1,* and *Slc6a2* (*NET*) genes.

Levels of expression were significantly different between control and treated animals only for TH (tyrosine hydroxylase) (p=0.032), with decrement in the expression (around 20%) for MDMA-treated rats (figure 16).

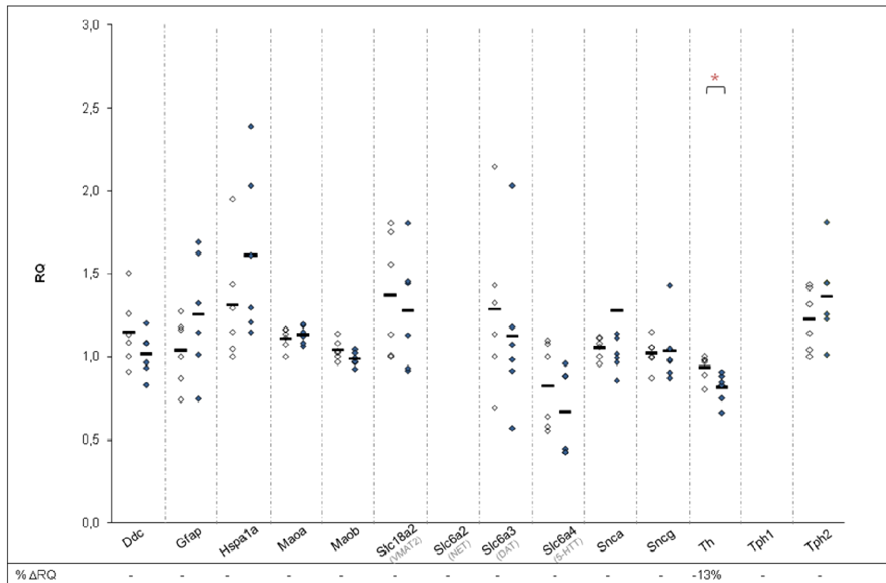

Figure 16. Relative quantification values for **striatal** individual samples. Empty or filled diamonds represent individual RQ values for control and MDMA-treated rats individually. Lines show the mean value. Asterisks represents significant changes in gene expression (at level p<0.05). ΔRQ values respect control samples (in percentage) are given for samples reaching statistically significant differences.

Gene expression assays in blood samples.

Two different types of blood samples were obtained for each animal: the day before treatment (D-1), and at the moment of the decapitation (D7).

The initial idea to study gene expression in blood samples was to carry out two different comparisons for gene expression in blood samples

a) between control and treated animals for D-1 samples, in order to ensure that gene expression prior to treatment was the same for all the animals (it would have been a good reference level of “basal” gene expression)

b) assessment of gene expression for samples obtained on day D7, analysing differences between treated and non treated animals.

In general, data from real time PCR showed weak amplification for all the samples and genes selected and most of the amplifications started at cycle 30 or later.
Comparisons performed among samples at D-1 (saline *vs.* treated groups) showed no differences in gene expression between both groups.
When performing comparisons among saline and treated groups of samples obtained at D7, no significant differences were observed for all genes analysed. The same results were obtained when the analysis was conducted comparing paired blood samples obtained at the day of decapitation and at time D-1.

Biochemical measurements

Determinations of amine concentrations in striatum, hippocampus, and cortex showed statistically significant differences in contents of 5-HT and its metabolite 5-HIAA in cortex, striatum, and hippocampus of treated animals compared to controls (table 4). Levels of dopamine and its metabolites were determined ion the striatum, significant differences in DOPAC concentrations were found (table 4).

Table 4. Amine concentrations (pg/ mg tissue) for the different brain regions (killed at 7 days).

	Striatum			Hippocampus			Cortex		
	Control	MDMA	***p*-value**	Control	MDMA	***p*-value**	Control	MDMA	***p*-value**
5-HT	452 (63)	263 (106)	**0.013**	352 (33)	118 (52)	**0.000**	304 (55)	139 (49)	**0.000**
5-HIAA	793 (146)	733 (93)	**0.004**	311 (54)	108 (58)	**0.000**	281 (70)	167 (62)	**0.015**
DA	93929 (2570)	93024 (1216)	0.454						
DOPAC	12627 (1046)	10121 (1276)	**0.004**						
HVA	413 (95)	248 (93)	0.420						

Results are presented as mean (SD)

5. DISCUSSION

Discussion of the Study 1, methodological validation of the DNA array PHARMAchip^{TM}.

In the last years, the development of DNA arrays technologies to genotype hundreds of SNPs simultaneously has marked the beginning of potential clinical and research applications of these tools. However, prior to the use of these tools, there is the need to ensure its suitability.

Study 1 sought to assess the convenience of a new DNA array for its further use in clinical studies. Two experimental approaches were possible: (i) to select a number of DNA samples and genotype them using the DNA array under evaluation (PHARMAchip^{TM}) and compare results to a sequencing of samples or (ii) to compare results of genotyping obtained with a previous commercially available DNA-array (DrugMEt®) with the new one under evaluation (PHARMAchip^{TM}). Both approaches are not incompatible although the first one is the preferred, nevertheless costs associated are quite elevated. In our case as far as a number of patients were already genotyped with the previous DNA array, it was relevant to ensure the compatibility of results obtained with both devices. Because not all the genes of interest (in particular *COMT* and *5-HTT*) in the clinical studies concerning the present PhD dissertation were included in the discontinued test, we decided to compare results obtained in our laboratory with those of the new array for these genes.

Different parameters were determined in this work: the suitability of amplified DNA, the limit of detection, the reproducibility, the accuracy and the successful genotype rate. Discussion will be focused mainly in two aspects: the relevance of DNA amplification prior to DNA-array analysis and the accuracy of results obtained by both DNA-arrays under comparison and with in-house developed genotyping assays.

The amplification of genomic DNA prior to its analysis can sometimes be useful when adequate amounts high-quality DNA are not easy to

obtain (e.g. clinical samples from children, oral swabs). The use of amplified DNA could be a solution to this problem. Although several methods for the amplification of whole genome DNA are commercially available (Park et al., 2005), only the DNA polymerase *Phi29* was tested. Results showed the occurrence of false positives with the use of GenomiPhi-amplified DNA which may be explained by the generation of template-independent products (TIPs) during the process, leading to unspecific hybridization in the array. Although the suitability of other products has not been tested, the use of non-amplified DNA is recommended to avoid inaccurate assignment of results.

The assessment of accuracy was carried out by comparing a set of samples previously genotyped by DrugMEt®, and also in some cases, the comparison of results was performed with the genotyping results obtained in-house.

For those genes common in both tests were evaluated, 15 mismatch results were detected (out of 5000 available genotypes), and therefore samples were sequenced. In ten cases, the discrepancy was explained by the presence of alleles that were not included in the DrugMEt® test, so that the assigned genotype in this case was not accurate. For the other five mismatch results, in three cases it was impossible to perform sequencing as there was not enough DNA available, and for the other two, one was a mismatch in PHARMAchip™ and the other was a mismatch for DrugMEt®.

For those genes that were not included in the DrugMEt® test (*COMT* val158met and *5-HTTLPR* variants), accuracy was assessed by comparison with the results obtained after genotyping in our laboratory (data not published). Regarding the *COMT* variant, all the samples analysed (98 samples) gave concordant results with those obtained in-house. As for the serotonin receptor genotype, three samples analysed

gave a mismatched result, so that 3 out of the 196 alleles were erroneous (1.5%). Unfortunately, further sequencing was not possible as there was no DNA available (data not published).

Overall, the comparison study showed a high rate of agreement between both methods (DrugMEt® vs. PHARMAchip^{TM}.) although there were technical differences. We consider the use of PHARMAchip^{TM} suitable for genotyping DNA samples included in our studies.

Study 2, The influence of *5-HTT* and *COMT* genotypes on verbal fluency in ecstasy users.

As described in the background chapter, the ENTE study reported verbal fluency deficits in ecstasy users. Bearing in mind these results, we decided to go one step further and investigate a possible association between ecstasy use and verbal fluency performance in participants genotyped for some polymorphisms within the serotonin transporter and the catechol-*O*-methyltransferase genes. Besides, two components of verbal fluency (clustering and switching) were also examined.

Traditionally, total number of correct words is the most common verbal fluency measure, but Troyer *et al.*, (Troyer et al., 1997) suggested that the optimal performance in verbal fluency tasks depends on two different strategies, clustering (ability to track words within the same subcategory), and switching (ability to change the subcategory). Clustering relies on the integrity of the semantic stores, whereas switching depends on flexibility.

Ecstasy users showed poorer semantic fluency performance (but not phonemic) than non-ecstasy users group (controls and cannabis users) regarding total number of correct words.

When results were analysed in more detail, the observed poorer semantic fluency of ecstasy users was associated to poorer clustering mechanisms. A modulatory effect of *COMT rs165599* polymorphism on clustering mechanism was also observed, with individuals with the *A/G* genotype performing worse than the others (irrespective of group). We also found a specific effect of the *5-HTTLPR* polymorphism on switching performance (for both, semantic and phonemic modalities), with *S/S* individuals performing significantly worse than *L/S* and *L/L* carriers (independent of group). A trend to significance regarding

switching measures was found for ecstasy users performing worse than non-ecstasy users.

A main finding of this study is that semantic fluency deficits observed in ecstasy users are mainly associated with clustering alterations rather than to switching mechanisms. It has been proposed (Gleissner and Elger, 2001), that clustering is related to a search strategy based on the meaning of the words, where the first word automatically recalls the other semantically related words. The retrieval of related words requires an intact memory function and semantic network (Troster et al., 1998). These needs are demonstrated by the evidence that clustering is impaired in pathologies such as Alzheimer's disease, characterized by memory deficits (Fagundo et al., 2008). Keeping this in mind, one can postulate that the observed effects can be explained in terms of disorganization of the semantic store. Two recent meta-analyses of neuropsychological studies in MDMA users have concluded that MDMA use is robustly associated with memory impairments (Kalechstein et al., 2007;Zakzanis et al., 2007), which suggest that hippocampal function might be more affected by the neurotoxic effects of MDMA than other regions (Gouzoulis-Mayfrank et al., 2003). This hypothesis is also supported by brain imaging studies which showed functional alterations in brain regions related to this cognitive function (medial temporal lobe/hippocampus) (for review (Verdejo-Garcia et al., 2007)).

Looking at the results, it may be surprising that ecstasy users showed similar performance in phonemic fluency in total number of words than non-users, and also performed better in phonemic mean cluster size. Once an additional analysis of the errors/perseverations was made, results revealed that ecstasy users committed more errors than non-users in phonemic fluency (which explains the high number of words generated).

One of our initial hypotheses prior to the study was the existence of an association between verbal fluency performance and some polymorphisms within the *COMT* and/or *5-HTT* genes. Our results showed a main effect of the *5-HTTLPR* polymorphism (irrespective of group) on switching performance (phonemic and semantic), with *S/S* individuals performing poorer than individuals with the *L/S* or *L/L* genotypes. As mentioned previously, switching performance depends on flexibility.

Low serotonin function has been related to impairments in cognitive flexibility ((Schmitt et al., 2006) for review), and the *5-HTTLPR* polymorphism is linked to serotonin availability (with the *S* allele associated to less efficient serotonin neurotransmission). Therefore, the effects of the serotonin transporter polymorphism on the switching ability are not surprising. According to previous studies that associated ecstasy-related cognitive impairments with the *S* allele of the *5-HTTLPR* variation) (Roiser et al., 2006), we would have expected a poorer performance on switching mechanisms for the ecstasy users group. However, our results suggest that switching is more affected by the *5-HTTLPR* polymorphism than by ecstasy use.

- Ecstasy use is related to semantic fluency impairments regarding total number of correct words. Poorer performance on clustering processes indicates a disorganization of the semantic store is linked to MDMA-toxic effects on hippocampus.

- Ecstasy users committed more errors than non-users in measures of phonemic fluency, indicating an inability to avoid perseverations.

- The *5-HTTLPR* polymorphism has a main effect on switching performance, with *S/S* individuals performing worse than those carrying the *L* allele.

Study 3, The influence of genetic and environmental factors among MDMA users in cognitive performance

Two main findings arise from this study: a strong correlation between MDMA lifetime use and detrimental effects on cognition, and also an interaction between variations in candidate genes and performance on different neuropsychological measures related to visospatial attention and episodic memory.

Greater lifetime use of MDMA is negatively correlated with performance on visuospatial memory and visuospatial attention/speed tests. Besides, such observations were further supported by group comparisons between MDMA heavy users (lifetime consumption greater than 100 pills) and MDMA light users, cannabis users, and controls, where the formers have significantly worse performance than the other groups.

These negative correlations between lifetime use and visuospatial memory are consistent with previous results of our research group in a subsample of the MDMA users included in this study (de Sola Llopis et al., 2008a), but are in disagreement with recent publications that show lower effect of MDMA use on visual memory tasks but greater effects on verbal memory (Bedi and Redman, 2008;Schilt et al., 2008;Schilt et al., 2010).

Regarding the interaction between genes and MDMA use, results showed that heavy ecstasy users carrying the *COMT val/val* and *SERT s/s* genotypes displayed poorer performance on tests of speed/attentional control (Digit Symbol) and visual attention and planning (ROCFT Copy) than individuals. Moreover, for the ROCFT immediate recall, the effect of these genotypes was irrespective of the degree of drug use, with ecstasy users performing worse than controls.

In addition, other interactions between MDMA use and genotype were found. Individuals with CYP2D6 high metabolic activity (ultra-rapid and extensive phenotypes) and *GRIN2B C/C* genotype (linked to lower glutamatergic activity) were associated with lower performance on verbal fluency. The modulation effects on speed/attention control and visual attention and planning of the *COMT val/val* observed in this study are in agreement with recent findings of the role of *COMT* gene and visuospatial planning skills (with poorer performance for *val/val* individuals) (Roussos et al., 2008). *SERT* genotype may also play a modulatory role (although to a lesser extent) on visuospatial attention/planning performance. Roiser *et al.*, (Roiser et al., 2007) found that healthy volunteers carrying the *S/S* genotype displayed higher performance in a mental rotation task. In our sample, however, this profile has been changed by MDMA use, with heavy ecstasy users performed worse than control individuals with the same genotype. Overall, we can reason that *SERT S/S* genotype combined with *COMT val/val* genotype and heavy MDMA use confer greater detrimental effects on visuospatial attention, planning skills and visual memory performance.

Moreover, significant interaction between MDMA use and the CYP2D6 phenotype on semantic fluency performance is also reported from our results. The CYP2D6 enzyme is involved in MDMA metabolism (de la Torre et al., 2004), and variations in this gene are thought to play a key role on MDMA-induced neurotoxicity (Perfetti et al., 2009). These results are in agreement with recent findings about the link between higher CYP2D6 activity and impaired executive in methamphetamine users (Cherner et al., 2010).

A number of other genotypes had effects on performance irrespective of group. These are the cases of the *GRIN2B* genotype (C/C individuals performing poorer on verbal episodic memory, CVLT delayed recall); the *5HT2A Tyr* allele which is linked to poorer visual

delayed recall (ROCFT); and the *COMT met* allele, associated with lower visual memory and reduced size of clusters produced during the fluency test. Delayed recall is clearly linked with the *5HT2A His/Tyr* polymorphism in the whole sample, and verbal delayed recall and verbal fluency are only associated with *GRIN2B* variations irrespective of MDMA use. These results are in accordance with previous studies indicating that the *Tyr* allele of the 5HT2A receptor is associated with memory consolidation (Filippini et al., 2006). Similarly, *GRIN2B* is associated to verbal memory performance (Ludwig et al., 2010).

The main effects of MDMA use and gene interactions are related to the ROFCT test, which involves visuospatial attention and planning/organization skills for the copy, and planning and episodic memory skills for the immediate and delayed recalls (Shin et al., 2006). Copy performance has been associated to the prefrontal cortex functioning, whereas the immediate and delayed recall are also associated with the prefrontal cortex and the hippocampus ((Carlesimo et al., 2010). Neuroimaging studies indicate that *COMT* polymorphisms are associated with connectivity between the prefrontal cortex and the hippocampus, with *val/val* carriers having reduced coupling between both regions (Dennis et al., 2010). Such connections are critical for executive memory contents (successful encoding and retrieval). MDMA use has been related to detrimental effects on this process (Quednow et al., 2006), so it is plausible that both MDMA use and *COMT* genotype contributes to a further decline in the prefrontal-hippocampal coupling.

- Lifetime ecstasy correlates to greater detrimental effects on visuospatial attention and planning, and speed/attention control skills.

- *COMT val/val* and *SERT S/S* genotypes are associated to poorer performance attention and planning skills on heavy MDMA users.

Study 4, Gene expression study in an animal model of MDMA induced neurotoxicity

Previous studies postulate that MDMA induces a neurodegeneration of the serotonergic neurotransmission system consistent in anatomical changes in axon terminals (axonopathy) (Green et al., 2003). However several authors administering lower MDMA doses, close to those used by humans, have not been able to demonstrate such neuroanatomical alterations (Baumann et al., 2007;Wang et al., 2005). Recent reports suggest that a lower availability of the serotonin transporter after MDMA may be related to decreased gene expression rather than to neuroanatomical alterations (Biezonski and Meyer, 2010). The aim of the study in an animal model was to examine if the MDMA induced long-term effects were related to changes on brain gene expression of several genes related to serotonin and dopamine biosynthesis and function. Doses assayed are known to induce toxicity but are lower than those assayed up to now, known to induce a high rate of mortality, and therefore close to those used in humans.
The effects of MDMA treatment on genes related to serotonergic and dopaminergic systems should be observed within brain regions involving these neurotransmission systems. Accordingly, dorsal raphe nuclei were selected as they are the largest brain region containing serotonergic cell bodies which project to other brain regions (Bonvento et al., 1991), whereas the substantia nigra is a region rich in dopaminergic neurons which projects to the striatum (Purves, 2004b). The hippocampus was chosen based on its extensive serotonergic innervations with sparse dopaminergic afferents, and its vulnerability to MDMA-induced neurotoxicity (Green et al., 2003).

The main finding was that after one week, MDMA treatment produced decrements (of about 20% in relation to control animals) in the

expression levels of the serotonin transporter gene (*Slc6a4*) in the dorsal raphe nuclei and substantia nigra region. Genes known to regulate the serotonin transporter as synucleins displayed changes in expression in the same direction. Similarly, the vesicular monoamine transporter (VMAT-2, *Slc18a2*) expression levels are also decreased. These findings are in accordance to previous published studies which reported a marked reduction of both gene expression and protein levels for the 5-HTT and only a less marked reduction in the level of expression for VMAT-2 (without alterations at the protein level) two weeks after MDMA treatment (Biezonski and Meyer, 2010). Although effects reported by these authors are significantly larger than those observed in our study, the differences observed might be attributable to the differences in MDMA doses used (4x10 mg/kg in their study compared to 3x5 mg/kg in ours). The MDMA dosage used in this study has already shown to be toxic to animals although with a lower mortality rate when compared to those reported with higher dosage regimens (Goni-Allo et al., 2008).
However, these results do not agree with those reported by Kovacs *et al.*, (Kovacs et al., 2007) who found an increment in the 5-HTT mRNA expression in the dorsal raphe nuclei seven days after the administration of a high neurotoxic MDMA dose (15 mg/kg), suggesting a potential recovery of the serotonin system. Differences in rat strains (Wistar vs. Dark Agouti) may be on the basis of different susceptibility to MDMA induced alterations in *5-HTT* gene expression.

Our results did not support this potential recovery in the seventh day after drug treatment, and results concerning decrements detected in the level of expression of the aromatic amino acid decarboxylase (*Ddc*) in this brain region further credit our findings. This enzyme is responsible for the synthesis of both dopamine and serotonin. Although we have not studied the effects of the MDMA treatment at the

level of the proteins, the decrements on the gene expression suggest that there should be lower levels of protein and therefore, lower synthesis of serotonin. Breier *et al.*, (Breier et al., 2006) showed that infusion of tyrosine in the striatum or hippocampus enhanced the raise of DA and the long-term depletion of 5-HT, but the co-administration of an inhibitor of the aromatic amino acid decarboxylase attenuated these effects in the hippocampus and returned to basal the levels of DA in the striatum, suggesting some role of this enzyme in MDMA-induced neurotoxicity.

Synucleins are proteins that primarily are localized in the presynaptic compartment of neurons. α-synuclein, and γ-synuclein regulate monoamine (DA, 5-HT, NE) transporter trafficking to or away from the cell (Wersinger et al., 2006). Moreover, α-synuclein is involved in synaptic plasticity and neurodegeneration (Mladenovic et al., 2007). γ-synuclein expression occurs mainly in limited areas such as serotonergic neurons of the raphe nuclei and recently, Wersinger *et al.*, (Wersinger and Sidhu, 2009) have shown an important role of this protein in the regulation of the serotonin transporter. Regarding their role in the correct functioning of monoamine transporters, we expected that MDMA treatment would produce some alterations in synuclein mRNA levels. Significant decreased expression levels of α-synuclein were found in the cortex of MDMA-treated rats, and also for γ-synuclein in the DRN+SN (although not reaching statistical significance).

Our results show that the MDMA treatment decreases marginally the expression levels of other genes such as *Tph2* in the DRN+SN region. Tryptophan hydroxylase is the rate limiting enzyme in the synthesis of serotonin, and several studies have stated that MDMA causes its inhibition (Stone et al., 1989), and according to these authors, the restoration of activity requires new enzyme synthesis after repeated

exposure to the drug. Our results are not in concordance with those reported by Bonkale *et al.*, (Bonkale and Austin, 2008) that found decreases in TPH immunoreactivity but marked increases in TPH2 mRNA expression in the dorsal raphe two weeks after a neurotoxic MDMA dosage regimen. Such discrepancies can be explained in terms of the different timing of the studies, as they performed their assays two weeks after treatment whereas our determinations were carried out one week after the drug administration. They may also account for the recovery and subsequent increment in mRNA expression.
However, a large increase in the expression level of the TPH2 mRNA was observed in the hippocampus of MDMA-treated rats. Without data regarding the amount of enzyme, it is reasonable, to think in a mechanism of counterbalance the decreases of serotonin related to the effects of MDMA treatment. Levels of expression in the striatum were significantly different between control and treated animals only for TH (tyrosine hydroxylase).

Slight increases in the expression level of monoamine oxidase (MAOB) are also observed in this brain region that could be related to MDMA induced inhibition of this enzyme.

Other genes, such as *Gfap* or *Hspa1a* were assessed in this study as they are biomarkers of neural damage. No alterations in gene expression were observed as reported by other authors (Wang et al., 2005).

Overall, gene expression experiments carried out in blood samples gave negative results for all comparisons performed, but it is not clear whether these negative results are due to lack of expression of selected genes in blood, or that the methodology selected for the analysis of gene expression for blood samples in these conditions is

not suitable. Another possible cause is that although there may be some correlation between changes in brain and those observed in the blood, the relatively long time span of this study may be responsible of the negative results due to the renovation of blood cells.
Further experiments should be carried out in order to establish a possible relationship between the effects of MDMA treatment in brain and peripheral tissues as blood.

- MDMA treatment procudes decrements in the expression levels of several genes in different brain regions.

- Genes related to serotonergic system function are mainly affected by MDMA treatment.

6. CONCLUDING REMARKS

Main achievements of the present work are summarized below:

Use of DNA arrays for pharmacogenetics in clinical studies

1. DNA arrays for pharmacogenetic studies require a validation with clinical samples obtained with a given DNA extraction protocol. Although the comparison study between both DNA arrays showed a high rate of agreement, it is important to check the suitability of a new tool prior to its use. It is recommendable the use of routine quality methods, as minimal changes in DNA extraction protocols may interfere in its quality, and consequently, in the results obtained from the arrays.

2. DNA arrays from different manufacturers are not interchangeables. Combinations of nucleotide changes define different alleles. Shifts from one DNA array to another, must take into account which nucleotide variations define each of the alleles included in the array.

3. Large amounts of high-quality DNA are necessary to perform the analyses. The use of whole-genome amplification methods is not advisable as they can bias the results.

The effect of MDMA lifetime consumption a genetic polymorphisms in the cognitive performance of drug users

4. Several studies have reported verbal fluency deficits in ecstasy users. These deficits are related to poorer semantic fluency performance (but not phonemic) on traditional measures of total number of words. Further analysis revealed that it is associated to clustering mechanisms, which can be explained in terms of disorganization of the semantic store.

5. A main effect of the *5-HTTLPR* polymorphism was observed on switching performance (phonemic and semantic), with individuals with the S/S genotype performing poorer than the others (L/L or L/S) genotypes.

6. There are dose-related detrimental effects of MDMA lifetime use. A greater lifetime use of MDMA is negatively correlated with performance on visuospatial memory and visuospatial attention/speed tests (ROFCT and SDMT tests). Group comparisons showed that heavy MDMA users (lifetime use>100 tablets) have significantly poorer visuospatial memory performance than light MDMA users, cannabis users and controls.

7. Gene x group interactions showed that MDMA users carrying the *SERT s/s* and *COMT val/val* genotypes have poorer performance on tests of speed/attentional control (Digit Symbol) and visual attention and planning (ROCF Copy). This suggests an interactive effect of COMT and to lesser extent SERT genotypes to confer greater detrimental effects on visuospatial attention and planning skills.

8. The CYP2D6 ultra-rapid/extensive phenotype is associated to lower verbal fluency performance. This enzyme is associated with the metabolism of MDMA and variations in its activity are associated to changes in MDMA-induced neurotoxicity.

Effects of MDMA on the expression of genes related to the derotonergic and dopaminergic neurotransmission systems.

9. Animal model studies showed that MDMA treatment produced alterations in the level of expression on genes related to the serotonergic and dopaminergic systems. These effects are different depending on the brain region assessed. Alterations observed are consistent with the widely described damage to serotonergic neurons.

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8. APPENDICES

FIS-MDMA STUDY PROTOCOL

C S B Consorci Sanitari de Barcelona

Información para los participantes en el estudio

"Mecanismos farmacogenéticos moduladores del deterioro cognitivo y emocional de consumidores de MDMA y cannabis". Código: IMIMFTCL/XXX (Versión xxx, 05-12-06).
Unitat de Farmacologia. Institut Municipal d'Investigació Mèdica. Dr Aiguader 80, Barcelona.
Investigador principal: Dr. Rafael de la Torre
Personas de contacto: Dr. M. Farré, Dr. Antonio Verdejo-García, Sra. E. Menoyo, Sra. M. Pérez

Va a ser invitado a participar en un proyecto de investigación. Antes de decidir si quiere participar es importante que entienda por qué se realiza esta investigación y cómo se va a llevar a cabo. Por favor, tómese el tiempo necesario para leer esta información cuidadosamente y para consultarla con otras personas si lo cree conveniente. No dude en preguntarnos si hay cosas que no le quedan claras, o sobre las que cree que necesita más información.

¿Cuál es el objetivo del estudio?

El consumo de cannabis, MDMA y otras drogas se ha relacionado con problemas cognitivos (por ejemplo, problemas de memoria, planificación, control de impulsos, etc.) y emocionales (por ejemplo, cambios de humor, depresión, ansiedad). Es posible que estos problemas estén relacionados con diferencias genéticas que hacen que algunas personas sean más vulnerables que otras a los efectos perjudiciales de las drogas. Algunos genes que afectan a los sistemas químicos del cerebro se expresan de manera diferente en distintas personas ("polimorfismos"), y estas variaciones se han relacionado con rendimientos diferentes en pruebas cognitivas y emocionales. En este proyecto de investigación, pretendemos investigar si estas diferencias genéticas interactúan con el consumo de drogas a la hora de producir modificaciones o alteraciones del rendimiento cognitivo y emocional de los consumidores. Específicamente, estamos interesados en la relación entre los polimorfismos de la enzima catecol metiltransferasa (COMT), relacionada con el sistema químico de la dopamina, y del transportador hSERT, relacionado con el sistema químico de la serotonina, y el rendimiento cognitivo y emocional de consumidores de MDMA y cannabis.

¿Por qué he sido seleccionado para participar en este estudio?

Ha sido seleccionado porque en función de los datos que nos proporcionó en la aplicación vía Internet del proyecto nos informó de que actualmente consume MDMA, cannabis, o ambas sustancias.

¿Qué tengo que hacer si decido participar en el estudio?

Antes de comenzar el estudio le citaremos para hacer una entrevista personal en la que podamos confirmar y ampliar los datos que nos proporcionó en la aplicación de Internet. También le haremos una entrevista específica para evaluar posibles problemas psiquiátricos. Por último, le administraremos una prueba verbal breve (aproximadamente 10 minutos) que nos permitirá estimar su cociente intelectual. Una vez iniciado el estudio tendrá que:

1. Proporcionar una muestra de sangre: para poder saber cuáles son sus características genéticas (genotipo) en relación con los genes que afectan a los sistemas químicos del cerebro relacionados con la farmacología y la toxicología de la MDMA y el cannabis, necesitaremos una muestra de su sangre. El procedimiento de extracción es indoloro, y la muestra se destinará exclusivamente a estudiar los genes de interés para la investigación como son los polimorfismos de la COMT y el hSERT. Nuestro principal interés es relacionar esos genes con su rendimiento cognitivo y emocional. Al tratarse de material genético, debemos tener su consentimiento expreso para que le podamos extraer la muestra y almacenarla hasta su análisis.

2. *Realizar una serie de pruebas de rendimiento*: para saber si las diferencias genéticas están relacionadas con el rendimiento cognitivo y emocional, hemos desarrollado una serie de pruebas, de papel y lápiz o de ordenador (parecidos a juegos), que miden sus habilidades de memoria, planificación, control de impulsos y toma de decisiones, y su capacidad para percibir y experimentar distintas emociones. La duración de estas pruebas será de aproximadamente dos horas, durante las que tendrá que procurar estar atento, concentrado, y esforzarse el máximo posible. Estas pruebas no tienen ninguna repercusión positiva o negativa sobre su salud. Las pruebas tampoco producen excesiva fatiga, aunque podrá descansar en cualquier momento durante la evaluación si lo necesita. Las instrucciones de las pruebas son sencillas, y no es necesario estar familiarizado con el uso de ordenadores para hacerlas. No obstante, tiene que saber que durante algunas pruebas escuchará algunos sonidos y verá algunas imágenes que pueden resultar desagradables. Si siente que algunos de estos sonidos o imágenes son muy desagradables, es libre de interrumpir la prueba.

¿Tengo que participar?, ¿Puedo interrumpir mi participación?

La participación en este estudio es totalmente voluntaria, es totalmente libre para decidir si participa o no en él. Una vez que decida participar, también debe saber que es libre de abandonar el estudio en cualquier momento del mismo, y sin necesidad de proporcionar ninguna explicación.

¿Que pasará con mis datos?

Los resultados de las pruebas genéticas y las pruebas de rendimiento, así como cualquier otra información recogida durante el estudio, serán utilizados única y exclusivamente para los objetivos del estudio que le hemos explicado, y serán tratados por el equipo de investigación de manera totalmente confidencial. No será identificado por su nombre en ninguno de los registros del estudio. Para todos los análisis se le asignará un número asociado a sus datos, y sólo los miembros del equipo investigador tendrán acceso a ese número.
Todos los datos recogidos sobre su participación en este estudio serán considerados como confidenciales y sólo serán utilizados por los investigadores para finalidades científicas. En caso de comunicar estos resultados a la comunidad científica, se mantendrá su personalidad en el anonimato usando un código o sus iniciales.
Según la Ley Orgánica de Protección de Datos 15/1999, el consentimiento para el tratamiento de sus datos personales y para la cesión de los mismos es revocable. Puede ejercer el derecho de acceso, rectificación y cancelación dirigiéndote al investigador, quien lo pondrá en conocimiento del promotor.

¿Que beneficios tiene el proyecto para mi?

Será compensado económicamente por su participación y esfuerzo. La compensación es proporcional a la duración de su colaboración, aumentándose si finaliza totalmente el estudio. Este estudio no pretende conseguir beneficios clínicos para el tratamiento de personas consumidoras de MDMA o cannabis, aunque confiamos que nuestros resultados contribuyan a mejorar la prevención y el tratamiento del consumo de drogas en el futuro.

SI TIENE ALGUNA DUDA SOBRE ALGÚN ASPECTO DEL ESTUDIO O LE GUSTARÍA COMENTAR ALGÚN ASPECTO DE ESTA INFORMACIÓN, POR FAVOR NO DEJE DE HACÉRSELO SABER A LOS MIEMBROS DEL EQUIPO INVESTIGADOR. PUEDE COMUNICARSE CON ELLOS (DE 8:00 A 17:00) DE FORMA PERSONAL EN EL DEPARTAMENTO DE FARMACOLOGÍA Y TOXICOLOGÍA DEL IMIM (DOCTOR AIGUADER 80, 08003 BARCELONA) O POR TELÉFONO (221-10-09 EXTENSIÓN 2226-2228-2229).

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CONSENTIMIENTO INFORMADO

En caso de que una vez leída esta información y aclaradas las dudas que pudieran haberle surgido decida participar, deberá firmar el "modelo de consentimiento por escrito" conforme marca el Real Decreto 561/1993 de 16 de Abril de 1993 (BOE nº 114 del 13 de mayo de 1993).

Este estudio fue aprobado por el Comitè Ético de Investigación Clínica del Institut Municipal d'Assistència Sanitària de Barcelona (CEIC-IMAS nº XXXX).

He recibido un ejemplar de la hoja de información, la he leído, he podido consultar mis dudas, he entendido los objetivos del estudio y lo que significa colaborar en el mismo. He entendido que mi participación es voluntaria y que puedo abandonar el mismo en cualquier momento y sin proporcionar información alguna. Estoy de acuerdo en participar.

Nombre y apellidos del participante	Firma	Fecha
Nombre y apellidos del investigador	Firma	Fecha

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Modelo de consentimiento por escrito
(Según Anexo 6, punto 2 del Real Decreto 561/1993 de 16-04-1993)

Título del ensayo: "Mecanismos farmacogenéticos moduladores del deterioro cognitivo y emocional de consumidores de MDMA y cannabis". Código IMIMFTCL/xxx (Versión xxx, xx-xx-07)

Yo, (nombre y apellidos)

____

He leído la hoja de información que se me ha entregado.
He podido hacer preguntas sobre el estudio.
He recibido suficiente información sobre el estudio.
He hablado con (nombre del investigador):

____

Comprendo que mi participación es voluntaria.
Comprendo que puedo retirarme del estudio:
1° Cuando quiera.
2° Sin tener que dar explicaciones.
3° Sin que esto repercuta en mis cuidados médicos.
Presto libre mi conformidad para participar en el estudio.

Nombre y apellidos del **participante** Fecha Firma

Nombre y apellidos del **investigador** Fecha Firma+

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Información y consentimiento para determinar el genotipo de genes relacionados con la respuesta farmacológica y toxicológica de la MDMA y el cannabis

En este estudio, se propone recoger una muestra de sangre para determinar genes que están implicados en la respuesta farmacológica y toxicológica asociados al consumo de la MDMA y el cannabis. Al tratarse de material genético, debemos tener su consentimiento expreso para que le podamos extraer la muestra y almacenarla hasta su análisis.

De hecho estos rasgos genéticos no se determinan ni se conocen para la mayoría de la población. Sólo en estudios experimentales, como en el que nos ocupa, se emplea para intentar conocer mejor la farmacología y toxicología de los fármacos. El conocimiento de los genotipos no implican hasta la fecha cambios reconocidos en la actitud ante el abuso de sustancias psicotropas. Su conocimiento tampoco parece relacionado con mayor susceptibilidad para padecer enfermedades concretas. Debe saber que:

- La sangre que Ud. nos donará sólo se utilizará para estudiar el genotipo de genes relacionados con la respuesta farmacológica y toxicológica de la MDMA y el cannabis. No se utilizará para otro propósito.
- El material genético quedará almacenado y podrá utilizarse en un futuro si los avances técnicos permiten profundizar en aspectos fármaco/toxicológicos de la MDMA y la cannabis. Usted puede solicitar en cualquier momento que su muestra sea retirada y destruida.
- Este estudio sólo tiene como finalidad la investigación. Se garantiza por tanto la confidencialidad de los datos del participante.
- En opinión de los investigadores los resultados del genotipo del participante no tienen una utilidad médica específica. Por este motivo no se cree que esta información sea relevante para la salud del participante. Si Ud. desea conocer los resultados, por favor indíquelo al final de esta página, si no lo hace nosotros no se lo comunicaremos. Ahora bien, si se sospecha que los posibles descubrimientos pueden tener relevancia para su salud, se le informará de los mismos.

Consentimiento

He leído esta información, he podido consultar mis dudas, comprendo los objetivos del estudio y lo que comporta participar en éste y consiento que se me realice la extracción de sangre para la determinación de genotipos relacionados con la respuesta farmacológica y toxicológica de la MDMA y el cannabis.

Nombre y apellidos del **participante** Fecha Firma

Deseo ser informado de los resultados de las pruebas de genotipo metabólico:

SI NO (marcar con una cruz lo deseado)

Nombre y apellidos del **participante** Fecha Firma

Nombre y apellidos del **investigador** Fecha Firma