Neurobiological Mechanisms Involved in MDMA-Seeking Behaviour and Relapse

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Neurobiological Mechanisms Involved in MDMA-Seeking Behaviour and Relapse

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A mi mama y mis hermanas,
A Karolos

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Abstract

(±) 3,4-methylenedioxymethamphetamine (MDMA), commonly known as "*ecstasy*", is currently a highly consumed drug with liability to produce addiction in some individuals. MDMA induces unique psychoactive effects that clearly distinguish it from hallucinogenic or psychostimulant drugs. MDMA mainly enhances the activity of both the serotonergic and the dopaminergic system in the mesolimbic brain reward pathways. However, the neurobiological mechanisms underlying its possible addictive properties are still not fully understood. In the present work, we have contributed to this subject by establishing that the serotonin 5-HT_{2A} receptor, in contrast to what has been observed for other drugs of abuse, is critical for MDMA-induced reinforcement. Moreover, the pharmacological blockade of this receptor can prevent cue-induced relapse. This effect is possibly mediated by its excitatory control over basal and MDMA-induced increase in midbrain dopamine, as supported by our microdialysis data. Furthermore, we have also shown that MDMA can act as an interoceptive cue to induce relapse to cocaine-seeking behaviour. Additionally, we demonstrated differential changes at the level of the dopaminergic brain reward pathway and gene expression changes in different brain areas, following self-administered MDMA in comparison to passive administration. These results underpin the impact of a learning component in the rewarding/reinforcing properties of MDMA, and provide new evidence for the serotonergic involvement in MDMA-seeking behavior and relapse.

Resumen

(±) 3,4-metilendioximetanfetamina (MDMA), popularmente conocida como "*éxtasis*", es una droga susceptible de producir adiccin en algunos individuos. Actualmente es consumida principalmente por adolescentes y jóvenes. Los particulares efectos psicoactivos inducidos por la MDMA, permiten distinguirlo de manera clara de otros psicoestimulantes o compuestos alucinógenos. Esta droga actúa principalmente activando el sistema dopaminérgico y serotonérgico en los circuitos neurales de placer. Sin embargo, los mecanismos neurobiológicos implicados en las propiedades adictivas de esta droga no han sido aún esclarecidos.

El trabajo presentado en esta Tesis Doctoral ha puesto de manifiesto algunos aspectos claves de estos procesos que eran desconocidos hasta el momento. Hemos encontrado que el receptor de serotonina 5-HT $_{2A}$ participa de forma crítica en las propiedades reforzantes de la MDMA, contrario a lo observado en el caso de otros psicoestimulantes. Además, el bloqueo farmacológico de este receptor puede prevenir la reinstauración de la búsqueda de la MDMA, desencadenada por un estímulo o clave previamente asociado a su consumo. Estos efectos pueden ser debidos al bloqueo del control excitatorio que normalmente ejercen estos receptores sobre los niveles de dopamina en estructuras mesolímbicas, como ha sido revelado en nuestros estudios de microdiálisis. Hemos demostrado también que la MDMA puede actuar como clave interoceptiva y desencadenar la recaída a la búsqueda y consumo de cocaína. Adicionalmente, nuestros estudios han mostrado que tanto la activación del sistema dopaminérgico mesolímbico, como los cambios en la expresión génica en diferentes áreas cerebrales que ocurren tras la administración de la MDMA, dependen de si el sujeto participa de manera activa en el consumo de esta droga, o si por el contrario

la recibe de forma pasiva.

En conclusión, este trabajo resalta la importancia de los procesos de aprendizaje y memoria sobre las propiedades reforzantes/recompensantes de la MDMA. Además, nuestras investigaciones aportan nuevas evidencias en relación a la participación del sistema serotonérgico en la búsqueda y recaída al consumo de esta droga.

CHAPTER 1

Introduction

1.1 Motivation and Reward

Motivation is an internal response of an individual that is the sum result of a variety of neurophysiological factors that initiate, sustain and direct behaviour. In its most primary form, it involves homeostatic processes related to hunger, thirst and temperature regulation. Homeostasis occurs thanks to coordinated and self-regulated processes, that allow an organism to achieve a dynamic equilibrium and maintain stability while adjusting to changing conditions. The result of these homeostatic processes are instances of motivation, called *drive states*. Drive states are characterized by tension and discomfort due to a physiological need followed by relief when that need is satisfied. They are related to activities that enhance survival or will ultimately support a given level of reproductive success such as eating, drinking and sexual behaviour. Thus, *rewarding* stimuli are now recognized as hedonic incentives, causing neural representations that elicit motivation and goal pursuit (Kandel, 2000). This, in turn, has resulted in the evolution of an oriented sequence of behaviours either toward specific

hedonic or rewarding *goals* or away from negative ones.

In this respect, there is a common neural substrate for the modulation of the rewarding properties of stimuli such as food, water and sex. Hence, drugs of abuse such as cocaine, heroin, nicotine and amphetamines, tap into these neural networks that apparently evolved to reinforce behaviours that are essential to survival and reproduction. Among the circuits involved, the dopaminergic mesolimbic pathway is recognized as a key component for the modulation of the rewarding properties of stimuli (Kelley and Berridge, 2002).

1.1.1 The Brain Reward Pathways

All rewarding stimuli, including drugs of abuse, activate common circuits in the mesocorticolimbic system. Most attention has been given to the medial forebrain bundle, which consists of ascending and descending fiber tracts that connect the rostral basal forebrain and midbrain structures (see Figure 1.1). Reinforcement produced by stimulation of the medial forebrain bundle appears to be caused primarily by activation of the mesocorticolimbic dopamine system (Koob and Volkow, 2010). This neural circuit is composed by cell bodies of dopaminergic neurons located in the ventral tegmental area (VTA), which project to the nucleus accumbens (NAC) as well as to the amygdala, hippocampus, and prefrontal cortex (PFC).

The NAC is strategically situated to receive important limbic information from the amygdala, PFC, and hippocampus that can be converted to motivational action through its connections with the extrapyramidal motor system (Horvitz, 2002). This structure can be subdivided by histological and connectional patterns into core and shell regions. It is within the shell region, which is closely connected to other emotion-regulating areas of the brain, where dopamine (DA) influences responses to *novel* rewarding stimuli (Pontieri et al., 1995; Ito et al., 2004). In addition to the NAC, the amygdala and PFC also play critical roles in the evaluation of rewards and the establishment of reward-associated memories. The consolidation of efficient action repertoires aimed at obtaining rewards, on the other hand,

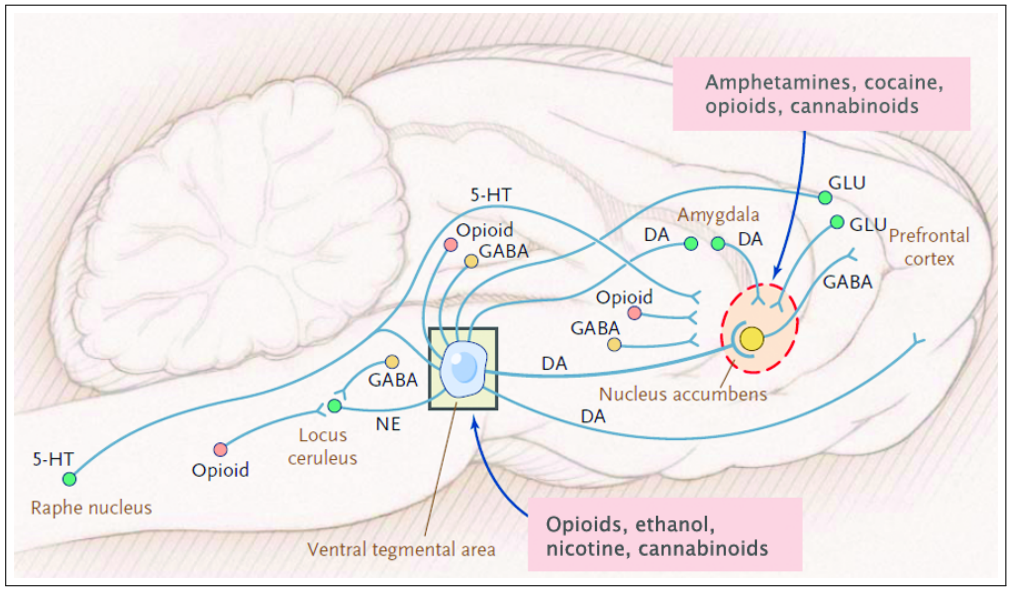

Figure 1.1: **Brain Reward Pathways.** As shown in the rat brain, mesocorticolimbic dopamine (DA) systems originating in the ventral tegmental area include projections from cell bodies of the ventral tegmental area to the nucleus accumbens, amygdala, and prefrontal cortex; glutamatergic (GLU) projections from the prefrontal cortex to the nucleus accumbens and the ventral tegmental area; and projections from the γ-aminobutyric acid (GABA) neurons of the nucleus accumbens to the prefrontal cortex. Opioid interneurons modulate the GABA-inhibitory action on the ventral tegmental area and influence the firing of norepinephrine (NE) neurons in the locus ceruleus. Serotonergic (5-HT) projections from the raphe nucleus extend to the ventral tegmental, nucleus accumbens and prefrontal cortex. This figure shows the proposed sites of action of various drugs of abuse in these circuits (Taken from Camí and Farré, 2003)

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depends on the dorsal striatum (Everitt et al., 2008).

Several neurotransmitter systems can modulate the mesolimbic dopaminergic pathway. Among the most important are glutamate in the PFC projecting to the NAC and the VTA; γ-aminobutyric acid (GABA) in the NAC which projects to the PFC; and the opioid system, which modulates the GABA-inhibitory action in the VTA. The VTA and NAC also receive projections from noradrenergic neurons in the locus ceruleus and serotonergic projections from the raphe nucleus (Camí and Farré, 2003).

Some of these areas are part of the brain's traditional memory systems.

This has led to the notion, now supported by increasing evidence, that important aspects of reward involve powerful emotional memories. Thus, the VTA-NAC pathway and the other limbic regions mentioned above similarly mediate the acute positive emotional effects of "natural rewards"[1]. (Kelley and Berridge, 2002), such as food and sex, as well as of drugs of abuse. Therefore, maladaptive neurobiological changes in these pathways are thought to underlie addiction to drugs of abuse as well as addiction to other stimuli (e.g. gambling and compulsive food taking) (Nestler and Aghajanian, 1997).

1.1.2 Role of Dopamine in Reward

All rewarding stimuli, including drugs of abuse, acutely increase DA activity in the NAC. Consequently, diverse hypotheses have been postulated from different fields in an attempt to ascertain the precise role of DA both in reward itself and in reward-related behaviours. Of the various hypotheses that have been voiced, two have most influenced the biological field, *the hedonic hypothesis* and its successor, *the learning hypothesis*.

1.1.2.1 The Hedonic Hypothesis

The hedonic hypothesis states that DA in the NAC increases as a function of the perceived *hedonic* value of a given stimulus. Is based on the fact that most pleasant rewards increase NAC DA (Wise, 1987). However, this has been shown not to be the case, since animals can exhibit positive hedonic responses after pharmacological blockade of the DAergic system (Berridge and Robinson, 1998). In addition, studies in genetically modified mice show that tyrosine hydroxylase (TH) (the rate limiting enzyme of

[1]The term *natural rewards* commonly refers to rewarding stimuli different from psychoactive drugs. However, as some drugs are natural *per se* (cannabis, opium, tobacco) and moreover, psychoactive plants and fungi are consumed by some animals in their natural environment (Samorini, 2002), I consider the term is confusing and try to avoid it in the whole document

DA synthesis) knockout (KO) mice can still display hedonic responses for sweet fluids (Cannon and Palmiter, 2003). However, animals lacking DA clearly cannot use information about rewards to motivate goal-directed behaviours, because they have a deficit in the initiation of motor responses. Therefore, they must be placed in close proximity to the goal to test preferences. The conclusion to be drawn from these experiments is not entirely clear, but it appears that, under certain circumstances, DA may not be required for hedonic responses.

Although the hedonic hypothesis was highly influential, other hypotheses are now generally more accepted. Indeed, Roy Wise, who first proposed this hypothesis, has stated that: "I no longer believe that the amount of pleasure felt is proportional to the amount of DA floating around in the brain" (Wickelgren, 1997).

1.1.2.2 The Learning Hypothesis

Conditioning is the most elementary form of associative learning. It implies the modification of synaptic efficacy through experience which finally leads to *learning*. Hence, conditioning allows organisms to adjust behaviour according to changing environmental conditions. This is the basis of adaptive behaviours, where positive outcomes are predicted from environmental stimuli that act as cues. Such behaviours enable organisms to track, locate, and secure food and necessary materials in demanding environments, with obvious survival value. Conditioning is behind both normal and maladaptive human behaviours such as addiction to drugs or other rewards (Kelley, 2004). It is present across the phylogenetic spectrum, from basic organisms such as the *Aplysia* to humans (Kandel, 2001).

Historically, conditioning has been divided into classical or Pavlovian conditioning (stimulus-outcome associations) and operant or instrumental conditioning (action-outcome associations). In the context of Pavlovian conditioning, biologically relevant outcomes such as food, water, and sexual stimuli are labeled unconditioned stimuli (US) because they are able to evoke "innate" or unconditioned responses such as salivation, approach,

and consumption (Pavlov 1927). Drugs of abuse, however, can also act as US. The Pavlovian conditioning procedure involves the pairing of a neutral sensory stimulus, termed the conditioned stimulus (CS), with a US in a temporally correlated manner called *contingency*. *Learning* occurs as the previously neutral stimulus, CS, obtains predictive value for the coming US, based on repeated pairings. Eventually, this novel cue is able to evoke a response that is often similar to that produced by the US itself. The learned response that the CS elicits is called the conditioned response (CR).

On the other hand, operant conditioning involves a voluntary response which is contingent to the presentation of a reinforcer or "outcome". For example, lever pressing (response) results in drug delivery (outcome). The *learning* of such contingencies leads to changes in the response rate. In these terms, an event that increases the probability of a response is called a *positive reinforcer*. Drugs of abuse increase the probability of lever pressing (Skinner, 1963) and are therefore both rewarding and reinforcing. It is important to note that often reinforcement and reward are used interchangeably, however not all reinforcers are rewarding. For example, in negative reinforcement, pressing a lever to avoid a foot shock. In operant conditioning, the reinforcer can be delivered in contingency with a neutral stimulus, called the *discriminative stimulus*. For example, a "cue" light above the lever presented in contingency with the delivery of the drug may, after repeated pairing, acquire reinforcing properties itself. This is relevant for drug addiction, where cues associated with drug taking often lead to relapse to drug-seeking behaviour.

Therefore, based on the premise that conditioning implies learning, this hypothesis states that DA is involved in reward learning processes. The learning hypothesis is comprised of separate but closely related hypotheses. Currently, the most influential is the reward prediction error hypothesis. The reward prediction error hypothesis originated from electrophysiological data obtained by Schultz and colleagues (Schultz, 2002; Tobler et al., 2005), and was subsequently supported by computational models (Montague et al., 2004). Dopaminergic neurons of the VTA and substantia nigra show phasic changes in spike activity that correlate with the history of reward delivery or previous reward experience. Schultz *et al*

have postulated that phasic activity changes encode the prediction error of future reward (Schultz et al., 2008). According to this hypothesis, a "burst" response of dopaminergic neurons indicates a positive prediction error ("things are better than expected") and a "pause" indicates negative prediction error ("things are worse than expected"). Activity that remains close to the baseline signals that "things are just as expected" (Montague et al., 2004). These responses are often activated in anticipation of reward by a CS that predicts a subsequent rewarding US. US activation of DA neurons obeys **prediction error models**, so that activation depends on whether the US reward is surprising or not *expected*, whereas, a fully predicted US reward may not activate the same neurons (Tobler et al., 2003).

The reward-prediction-error hypothesis has been successfully tested against a wide range of psychological theories of reward related learning and is a key component for appetitive approach behaviour related to drug use and for some current theories of addiction explained below.

1.2 Drug Addiction

The definition of addiction was presented in the Diagnostic and Statistical Manual of Mental Disorders, third edition (DSM IIIR)[2] by the American Psychiatric Association in 1987 (APA, 1987). According to this previous definition, an *addictive state* required the presence of physical *dependence* and *tolerance* as necessary components.

Tolerance, refers to the diminishing effect of a drug after repeated administration of the same dose, or to the need of a dose increase in order to produce the same effect. *Tolerance*, may develop to some of the effects of a drug but not to others; for example tolerance is frequently developed for the euphoric, analgesic and respiratory depressant effects of opiates, but not to the miosis[3]. *Pharmacokinetic tolerance*, is due to increased metabolism or clearance, while *pharmacodynamic tolerance* refers to a group of adaptations in the areas where the drug exerts its effects. It involves neural elements that respond to drugs initially, for example down or up regulation of brain neurotransmitter receptors.

On the other hand, "*physical dependence*", results from adaptations in brain circuits involved in the control of physiological functions, after long-term drug use. Physical dependence is unmasked by the withdrawal syndrome which refers to a group of somatic signs that occur upon the abrupt discontinuation of the drug intake or following an abrupt decrease in dosage. Withdrawal symptoms related to physical dependence are the inverse of the drug's acute effects because they result from homeostatic mechanisms that counteract the effect of the drug in an attempt to return the system to its normal or *homeostatic* state. Hence, the presence of physical withdrawal signs in a an individual implies that the body has adapted to the effects of the drug and needs it for its normal function. Drug dependence also

[2]In 1987, the DSM-III-R was published as a revision of DSM-III. Categories were renamed, reorganized, and significant changes in criteria were made. Six categories were deleted while others were added.

[3]pupillary constriction

produces alterations in emotional and motivational processes, which are revealed during withdrawal such as anhedonia, depression, anxiety and negative motivational states. Such symptoms may result from adaptation at the level of the brain reward circuitry, since they are the opposite of the initial effect of the drug.

The DSM IIIR definition was later modified because some addictive drugs, such as cocaine, produce psychological *withdrawal* symptoms without inducing physical dependence, and other drugs produce physical withdrawal symptoms but are not addictive (e.g. β-adrenergic antagonists, tricyclic antidepressants and antipsychotics). Thus, currently in the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV) (please see Table 1.1), addiction is defined as "Substance Dependence" in this definition *tolerance* and *withdrawal* symptoms may be present, but are not required for the diagnosis. Indeed, at least three of the criteria listed in table 1.1 are required for the diagnosis of substance dependence. In the DSM-IV, the term *dependence* is used to mean addiction, however physical dependence is just one of the process that contributes to *addiction*. Substance abuse is also considered in the DSM-IV (see Table 1.1). The motivational aspects of the individual are already seriously compromised in the case of substance abuse, and this term can only be used when the patient does not meet the criteria for substance dependence (addiction), and meet at least three of the substance abuse criteria listed in Table 1.1. For all these reasons, experts in the field have converged on a definition of addiction that uses three of the DSM-IV criteria for substance dependence and considers the motivational aspects of withdrawal more important. According to this definition drug *addiction* is a chronically relapsing disorder that is characterized by: (1) compulsion to seek and take the drug, despite adverse consequences; (2) loss of control in limiting intake, and (3) emergence of a negative emotional state (eg. dysphoria, anxiety, irritability) reflecting a motivational withdrawal syndrome when access to the drug is prevented (Koob and Volkow, 2010; Moal and Koob, 2007). It is important also to note that this pathological behaviour appears only in a small proportion (15% to 17%) of drug users (Anthony, 2010), and the development of the disease implies a transition from drug use to abuse and finally to dependence.

Table 1.1: **DSM-IV Criteria for Substance Dependence and Substance Abuse**

A maladaptive pattern of substance use leading to clinically significant impairment or distress as manifested by three (or more) of the following, occurring at any time in the same 12-month period:

Substance Dependence[1]	**Substance Abuse**[2]
1. Substance is often taken in larger amounts or over longer period than intended 2. Persistent desire or unsuccessful efforts to cut down or control substance use 3. A great deal of time is spent in activities necessary to obtain the substance (e.g., visiting multiple doctors or driving long distances), use the substance (e.g., chain smoking), or recover from its effects 4. Important social, occupational, or recreational activities given up or reduced because of substance abuse 5. Continued substance use despite knowledge of having a persistent or recurrent psychological, or physical problem that is caused or exacerbated by use of the substance 6. Tolerance, as defined by either: (a) need for markedly increase amounts of the substance to achieve intoxication or desired effect; or (b) markedly diminished effect with continued use of the same amount of a substance 7. Withdrawal, as manifested by either: (a) characteristic withdrawal syndrome for the substance; or (b) the same (or closely related) substance is taken to relieve or avoid withdrawal symptoms	1. Recurrent substance use resulting in a failure to fulfill major role obligations at work, school, or home (e.g., repeated absences or poor work performance related to substance use; substance-related absences, suspensions or expulsions from school; neglect of children or household) 2. Recurrent substance use in situations in which it is physically hazardous (e.g., driving an automobile or operating a machine when impaired by substance use) 3. Recurrent substance-related legal problems (e.g., arrests for substance-related disorderly conduct 4. Continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance (e.g., arguments with spouse about consequences of intoxication, physical fights)

1. DSM uses the term dependence to mean addiction as discussed in the text

2. According to DSM, substance abuse is diagnosed when symptoms have never met the criteria for substance dependence.

1.2.1 Theories of Drug Addiction

The main theories currently available to explain addictive processes include: 1) the opponent process theory (Solomon and Corbit, 1974); 2) the allostasis theory (Koob and Le Moal 1997); 3) the incentive sensitization theory (Berrige and Robinson 1998); and more recently. These theories are not mutually exclusive and combined can give a better understanding of drug addiction.

1.2.1.1 The Opponent Process Theory

Solomon and Corbit (1974) postulated that hedonic, affective, or emotional states, once initiated, are automatically modulated by the central nervous system with mechanisms that reduce the intensity of hedonic feelings, called *"opponent-processes"*. Therefore, the manifested emotional state of an individual results from the sum of both processes (see Figure 1.2). In a first instance an "A" process (positive hedonic effect) occurs shortly after presentation of a rewarding stimulus (e.g. drug) and is directly proportional to its intensity, quality and duration. As a consequence, a subsequent "B" process (negative hedonic effects) takes place, opposite to the initial "A" process. This phenomenon is assumed to occur under normal physiological conditions. When process "A" is greater than the process "B", the subject is in a positive emotional state. In the opposite situation, when process "B" is bigger than process "A", the subject is in a negative dysphoric state. After repeated drug exposure, the individual develops tolerance to process "A" while process "B" is slow to decay, and gets larger with repeated exposure (Solomon and Corbit, 1974). This results in a dysphoric or negative emotional state when access to the drug is prevented. This theory was the

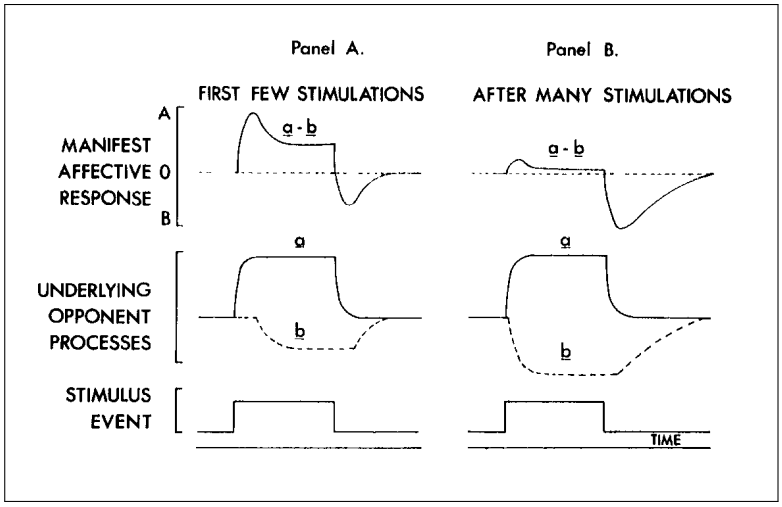

Figure 1.2: **Opponent-Process Theory, (Solomon and Corbit, 1974)** Panel A: The operation of the summing device for the first few stimulations. (The summation of the underlying opponent processes, a and b, yields the manifest affective response.) Panel B : The operation of the summing device after many repeated stimulations (Taken from, Solomon and Corbit, 1974).

basis for the development of the subsequent *allostasis* theory.

1.2.1.2 The Allostasis Theory

This theory is based in the previously explained opponent-processes theory and postulates that after chronic drug exposure, the mechanisms that are part of a "normal" or *homeostatic* limitation of reward function, fail to return within the normal range, and then produce an *allostatic* state, which is the ability of an organism to achieve chronic stability outside of the normal or homeostatic state (e.g. stress). Thus, a chronic deviation of the reward threshold is established (see Figure 1.3).

These counteradaptations in the brain reward systems give way to a spiralling distress cycle of addiction which is composed by three factors: 1) preoccupation and anticipation to take the drug (craving), 2) the compulsive consumption of large amounts of drug over a short period of time (binge), intoxication and 3) a negative emotional state during drug withdrawal (Koob and Moal, 1997). The hypothetical role of various neuroen-

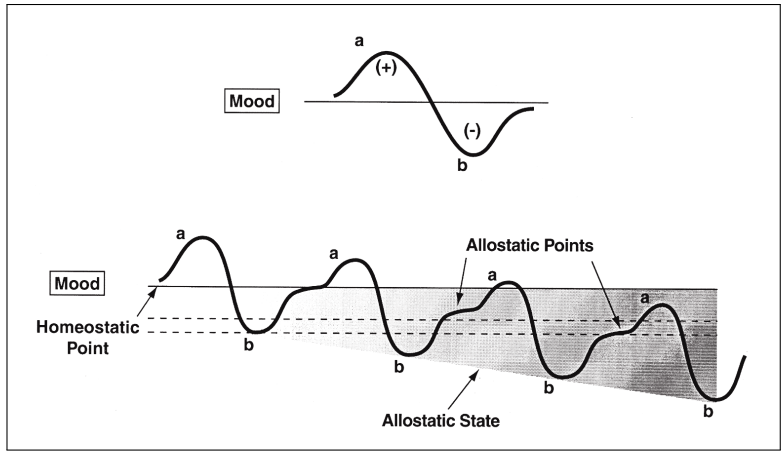

Figure 1.3: Diagram illustrating an extension of Solomon and Corbits (1974) opponent-process model of motivation, revived by the theory of allostasis of Koob and Le Moal (1997) (Taken from Koob and Moal, 1997).

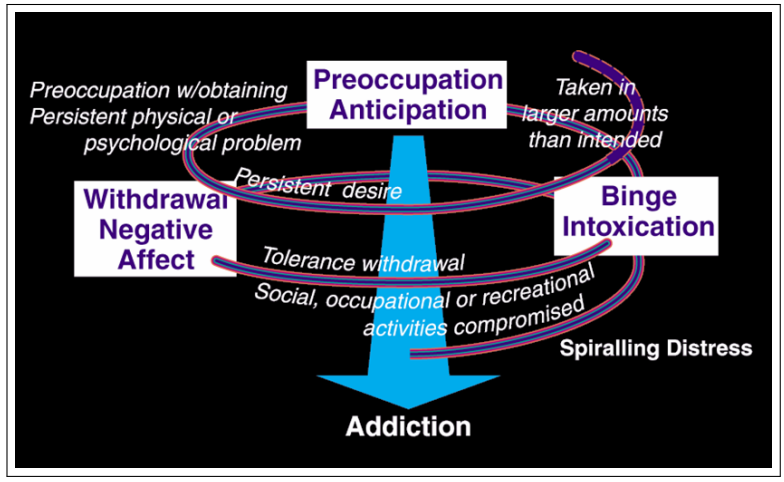

Figure 1.4: Diagram describing the spiralling cycle of addiction from the perspective of the allostasis theory (Taken and modified from Koob and Moal, 1997)

docrine systems would become important in the different components of the spiralling distress cycle of addiction. The preoccupation and anticipation of the effects of the drug is related to the neuroadaptive changes that take place after repeated drug intake and account for the negative emotional state characteristic of withdrawal. The binge-intoxication com-

ponent is reflected by activation of dopaminergic and opioid peptide systems, while the withdrawal-negative motivational state is undertaken by an over-activation of the brain stress response systems. Specifically, hyperactivation of the hypothalamo-pituitary-adrenocortical axis (HPA axis), with the consequent release of the corticotropin-releasing factor (CRF) and neuropeptide Y (Koob, 2008). These neuroadaptive process continually grow with repeated drug exposure increasing the amplitude of the spiral over time resulting in the pathological cycle of *addiction*.

These neuroadaptations have been related to prolonged and unlimited drug intake, where the goal is not the hedonic effect of the drug, but the avoidance of negative emotional state characteristic of withdrawal. Thus, the acute effect of addictive drugs seems to be responsible for the beginning of the cycle, while the counter-adaptation developed within repeated drug intake, account for the maintenance of the addictive state and the vulnerability to relapse, even after long periods of abstinence (Koob, 2008).

1.2.1.3 The Incentive Sensitization Theory

The term "sensitization" is used in pharmacology to refer to the increase in the effect of a drug upon its repeated administration. Repeated exposure to many drugs of abuse causes a progressive and lasting increase in the stimulating effects of such drugs. However sensitization may be observed for certain pharmacological effects of the drug, and not other (Robinson and Berridge, 1993).

According to this theory *reward* is a composite construct that contains an hedonic effect or "liking" component; a "learning" component that involves conditioning of drug's effects with drug-associated cues; and a "wanting" component which refers to a strong desire for the consumption of the drug and is known also as the "incentive value" of the drug. It states that after repeated exposure to the drug, a sensitization phenomenon occurs at the level of the mesolimbic dopaminergic system, specifically projections from the VTA to the NAC, which is manifested by a progressive increase in the motivation for the effects induced by the drug and the drug per se

("wanting"), this is also known as "Incentive Salience". Thus, DA is neither necessary nor sufficient to mediate changes in hedonic liking components of reward nor reward-related learning and the only reward component that is subject to DA mediated sensitization is "wanting" (Berridge, 2007).

Hence, the repeated use of addictive drugs, permanently elevates mesolimbic neural responsiveness to the drug itself as well as to drug-related cues (Berridge and Robinson, 1998). This hypersensitization produces craving, and causes compulsive drug-seeking behaviour. Through conditioning processes, the incentive value of the stimulus associated with the drug increases progressively, causing escalating compulsive drug-seeking behaviour. The continuous sensitization of this system ultimately causes addicts to relapse (Berridge and Robinson, 1998).

1.2.2 Neurocircuitry of Addiction

This section summarizes the main neuroadaptations in brain circuits related to the transition from controlled drug use to addiction (see Figure 1.5). The initial use of addictive drugs activates the brain reward pathway (see Section, 1.1.1) and trigger a DA increase in the NAC in a more prolonged and unregulated manner than "*natural stimuli*". After repeated use, the individual starts a conditioning processes (explained in Section, 1.1.2.2), that implies the modification of the synaptic efficacy in reward related brain areas, strengthening some brain circuits while weakening others. This ability of the brain to adapt and change over time, is commonly known as *plasticity*, a phenomenon that underlies all types of memory formation (Kandel, 2001). At the cellular and molecular levels, short-term plasticity (hours) involves functional changes in the effectiveness of preexisting synaptic connections, whereas long-term plasticity (days) involves morphological or structural changes in the synapses, either pruning or creating new connections (Kalivas and O'Brien, 2008; Kandel, 2000). Thus, long-term drug use induces plasticity in both, within the DA system and in DA receptive neurons (Kalivas and O'Brien, 2008), through mechanisms such as long-term potentiation (LTP) (Bonci et al., 2003).

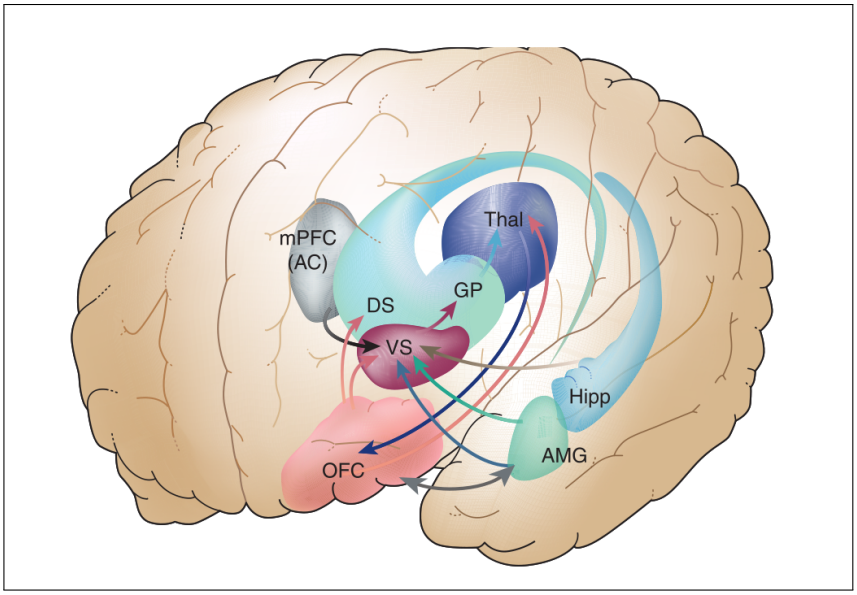

Figure 1.5: **Neurocircuitry of Addiction.** Schematic illustration of the main brain areas involved in the addiction cycle. mPFC, medial prefrontal cortex; AMG, amygdala; Thal, thalamus; VS, ventral striatum; Hipp, hippocampus; DS, dorsal striatum; GP, globus pallidus (Taken from, Everitt and Robbins, 2005).

The striatum is divided into the ventral and the dorsal part. The NAC, which is part of the ventral striatum is divided into shell and core parts, while the dorsal striatum comprises the nucleus caudate and putamen. The dorsal striatum is known to underlie motor automatic repertories, what we normally call "habits" (e.g. driving) (Balleine et al., 2007). The initial use of addictive drugs triggers DA increase in the shell part of the NAC, but with repeated voluntary drug taking, a transition to the core part of NAC has been demonstrated (Everitt et al., 2001). Hence, the NAC core has been recently postulated to function more as the dorsal than the ventral striatum (Everitt and Robbins, 2005). Over-training with cues that predict drug delivery associated with and complex drug-seeking behavioural repertoires, would be then powerfully consolidated by DA in the *dorsal striatum*, including the core part of the NAC leading to the formation of "habits". In concordance, microdialysis studies have shown that prolonged cocaine-seeking increased DA release in the dorsal striatum, but not in ventral striatum (Ito et al., 2004). Thus, after repeated drug use, certain aspects

of drug-seeking behaviour become automatic, and the ultimate goal of the behaviour, obtaining the drug, is devalued so that the behaviour is not directed to the drug, but to the action itself (Everitt and Robbins, 2005). Such automatic behaviour has been related to the characteristic compulsive behaviour present in addicts.

Furthermore, after long term drug intake, conditioned drug related cues can also induce DA increase by themselves in the NAC (Everitt et al., 2001), an effect correlated with self-reports of an intense and uncontrolled desired to take a specific drug (*craving*) (Volkow et al., 2008). The DA increase in the NAC associated with conditioned cues is not a primary response, but rather the result of feedback stimulation of *glutamatergic afferents* from the orbitofrontal cortex (OFC) and the amygdala, more specifically, the basolateral amygdala (BLA) (Everitt and Robbins, 2005). The BLA has been widely implicated in attributing "emotional values" to environmental stimuli based on comparisons from previous experience (LeDoux, 2007). In the case of drug addiction, the blockade or lesioning of this structure is able to prevent cue-induced relapse in rodents (Fuchs et al., 2005). In this sense, the activation of this network (NAC-BLA-OFC) by drug related cues can trigger *drug bingeing*, and more important, cue-induced relapse (Volkow et al., 2008).

Similarly, the context in which the drug is taken can also function as a CS. In this sense, drug-related contexts are able to trigger drug-seeking behaviour and relapse in a conditioned individual, even after long periods of abstinence (Kenney et al., 2010). This is currently one of the major problems in rehabilitation of addicted individuals in specialized centers, because most of them relapse when reinserted in their usual environment. The *hippocampal* formation, which is also a major source of glutamatergic afferents to the NAC, underlies conditioning to contextual stimuli and may therefore underlie the motivational impact of a context related to the drug in the addicted person (Atkins et al., 2008; Black et al., 2004). In animal studies, the pharmacological blockade or lesioning of this structure can effectively prevent context-induced relapse (Crombag et al., 2008).

Another important factor underling drug-seeking behaviour is the negative motivational component of *withdrawal*, characterized by dysphoria,

anxiety and/or irritability. In this respect, evidence suggests that after long periods of drug taking, an increased activation of brain stress systems (CRF, norepinephrine, dynorphin) is accompanied by a decrease of the brain anti-stress systems (such as the neuropeptide Y), in a brain macrostructure called the the extended amygdala [4]. Specifically, the increase of norepinephrine in the bed nucleus of the stria terminalis (BNST) and the corticotropin-releasing factor (CRF) in the central amygdala (CeA) are thought to contribute in the motivational state driving impulsivity to avoid unpleasant symptoms of acute withdrawal. Additionally, in an addicted person, the increase in the dynorphin-*k* opioid system, in the NAC and dorsal striatum via DA receptors stimulation (Carlezon et al., 2005), results in the release of dynorphin peptides that act as agonists at inhibitory opiate receptors in the VTA dopaminergic neurons, which finally results in a decrease of DA release in the NAC. These neurobiological mechanisms that contribute to an increase of the negative emotional state characteristic of drug withdrawal are also involved in the ability of stress to induce relapse in addicted individuals (Stewart, 2003). During abstinence, exposure to stressors activates stress response systems and induce craving, which finally leads to relapse (Goeders, 2003). Animal data have revealed that subjects exposed to an stressful stimuli/event such as a foot-shock, or movement restriction, relapse more than those that did not receive the stimulus. Furthermore, the pharmacological blockade of the systems explained above can effectively prevent stress induce relapse (for review see Stewart, 2000).

The strengthening of these limbic circuits that facilitate drug-seeking behaviour is further accompanied by an impairment of the forebrain inhibitory control. Human neuroimaging studies using positron emission tomography (PET), revealed a reduction in the activity of the OFC and the medial PFC of abstinent addicts (Volkow et al., 2002, 2004); both structures are involved in executive function (e.g. decision making). Similarly, neu-

[4]The term extended amygdala represents a macro-structure that is composed of several basal forebrain structures: the bed nucleus of the stria terminalis, the central medial amygdala, the area termed the sublenticular substantia innominata, and a transition zone that forms the medial posterior part of the nucleus accumbens (e.g. shell) . These structures have similarities in morphology, immunohistochemistry and connectivity (Alheid and Heimer, 1988; Heimer et al., 1991)

ropsychological tests have shown that chronic drug abusers show deficits in impulse control and delayed gratification (Bjork et al., 2009), which implies an impairment in goal revaluation and decision making (Hester and Garavan, 2004). Therefore, a disruption in self-control leads the addicted individual to take the drug even in the face of adverse consequences. Finally all these factors contribute to the pathological loop of compulsive drug-seeking and relapse, that characterizes the nature of addicted behaviour (Koob and Volkow, 2010).

1.3 Animal Models of Addiction

Since the neurobiological substrates involved in reward processes such as DA are conserved across species from *Drosophilae* to rodents to humans, and given that psychoactive drugs are consumed by animals in their natural environment (Samorini, 2002), it is reasonable to use animal models to study the mechanisms involved in addiction.

The ability of drugs of abuse to produce addiction can be studied using operant and non-operant animal models. Operant models are based on the concept of instrumental conditioning introduced by Skinner in 1937 (see Section, 1.1.2.2), and they measure the reinforcing properties of drugs, while non-operant models are based on Pavlovian conditioning (see Section, 1.1.2.2), and evaluate the rewarding properties of drugs of abuse. In this section, a summary of the most relevant models of each category, are described.

It is important to note that most of these models do not pretend to fully reproduce *addiction* as defined, (see Section, 1.2), but to model specific aspects of the addictive behaviour.

1.3.1 Intra-Cranial Self Stimulation

The intra-cranial self-stimulation (ICSS), evaluates the behavioural responses produced by direct electrical stimulation of brain areas that are normally activated by conventional reinforcers (e.g. food, water, sex). The animal works in an operant-conditioning task to directly receive an electric stimulation where an electrode has been implanted. The most effective electrode placement for ICSS is the lateral hypothalamus. Stimulating this area is thought to activate the medial forebrain bundle consisting of the mesolimbic DA afferents (Fish et al., 2010). The acute administration of drugs of abuse induces an hedonic state that reduces the reward thresh-

old for ICSS. Moreover, there is a correlation between the ability of drugs to decrease the ICSS threshold and their abuse potential (Esposito, 2006). After repeated administration, this threshold progressively increases, and finally, a marked increase in the reward threshold is evident during withdrawal in drug-dependent animals (Markou and Koob, 1991).

1.3.2 Conditioned Place Preference

The conditioned place preference paradigm allows to evaluate the rewarding properties of drugs of abuse using a classical or Pavlovian conditioning, where animals learn to associate a specific context with the hedonic effects of a given drug. This paradigm consists of a box with two compartments, each with different environmental characteristics (colors, texture, pattern) (see Figure, 1.6). At the beginning of the experiment, animals are allowed to freely explore both compartments. During the conditioning sessions, animals are confined to one compartment in alternate days, after the administration of either the drug or vehicle. On the test day, drug-free animals are allowed again to freely explore either environment. The time spent in the drug-paired compartment is considered an index of the rewarding value of the drug. Thus, animals that exhibit a *conditioned preference* for an environment associated with the rewarding effect of a drug, spend more time in the drug-associated compartment as compared to the vehicle-associated compartment.

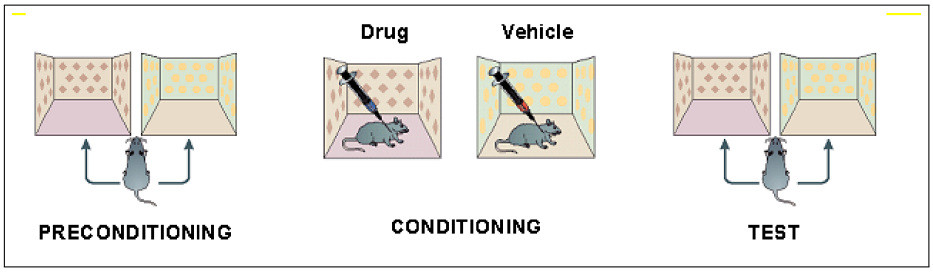

Figure 1.6: **Conditioned Place Preference Paradigm.** Schematic representation of the three phases of the conditioned place preference paradigm: preconditioning, conditioning and test.

1.3.3 Operant Intravenous Drug Self-administration

The self-administration paradigm is a more "motivational" approach when compared to non-operant models such as the conditioned place preference, since animals have to work to obtain the drug. Therefore, drug self-administration has both reliability and predictive validity for the study of addiction. Drugs of abuse are readily self-administered by experimental animals, although, there are a few exceptions (e.g. LSD). In general, drugs that are self-administered are those that have high abuse potential.

This technique can be applied using different administration routes, including: intracerebral, intravenous or oral. In our experiments, we used intravenous drug self-administration. In this procedure, an animal is implanted with and intravenous catheter and placed in a Skinner box. The chamber is provided with active and inactive holes or levers. In general, the acquisition of an operant conditioning task is easer for mice when using holes as compared to levers. For this reason we used holes in the experiments presented in this thesis. Thus, nose-poking in the active hole activates a pump that triggers the delivery of an intravenous infusion of either the drug or saline, depending on the employed paradigm, while nose-poking in the inactive hole will have no programmed consequences. Both active and inactive responses are recorded to detect preferences for the drug-associated hole. Active responses can be paired with a discriminative stimulus such as a light or a tone.

The number and pattern of responses necessary to obtain an infusion is determined by the schedule of reinforcement imposed by the experimenter. In these experiments, we used fixed-ratio-schedules (FR) of reinforcement where the number of responses required for one infusion was fixed. Therefore, a FR1 schedule means: 1 response = 1 infusion; and a FR5 schedule means: 5 responses = 1 infusion. However, different schedules can be employed changing the ratio or the interval of reinforcement. For example, in a variable ratio schedule, the animal receives the reinforcer (e.g. drug) after

an unpredictable number of responses. The advantage of this schedule is that it produces high responding rate, real life good examples of this schedule are gambling or lottery. On the other hand, the interval between reinforcements can also be either fixed or variable. In fixed-interval schedules the response is rewarded only after a specific amount of time has elapsed (time-out period), whereas, in variable-interval schedules a response is reinforced after an unpredictable amount of time has passed. Often, different schedules of reinforcement are used to model some characteristics of addiction such as compulsive drug seeking, loss of control or impulsivity.

Among the more relevant models of addiction, we can include the work performed by Deroche-Gamonet et al. (2004). In this study, the authors show that 17 % of rats trained to self-administer cocaine met criteria for dependence, similar to those reported in the DSM-IV (see Section 1.2). This is the same percentage as human cocaine users that develop cocaine addiction. The experiments of this study where design for three of the DSM-IV criteria[5]. For the first criteria, "the subject has difficulty stopping drug use or limiting drug intake", they measured the persistence of nose-poking during a period of non-drug availability. In the second criteria "the subject has an extremely high motivation to take the drug, with activities focused on its procurement and consumption", a *progressive-ratio schedule* was employed. In this schedule, the number of responses required to receive one infusion of drug is increased progressively within the self-administration session. The main measure evaluated in this schedule is the *breaking point*. The *breaking point* refers to the maximal amount of work, (indicated by the number of responces) that the animal will perform before cessation of responding. This paradigm is considered a reliable index of the motivation of the animal to obtain the drug. Finally, the third criteria, "substance use is continued despite its harmful consequences", was assessed administering both, the drug and an electric shock when the responding ratio was completed (FR5: 5 responses = 1 drug infusion + shock). Furthermore, rats showing an addiction-like behaviour exhibited a high propensity to prime and cue-induced cocaine relapse. This protocol is consid-

[5]According to the DSM-IV at least three criteria should be met in order to diagnose drug dependence (see Table 1.1)

ered as a rat model of addiction. Another model of addiction was recently developed by Vanderschuren and Everitt (2004). This second model was based on the premise that a transition from controlled/moderate to uncontrolled/excessive drug intake requires repeated and extended exposure to the drug. Thus, previous studies using different drugs such as cocaine, alcohol and heroin had shown that the pattern of self-administration dramatically changes depending on the duration of the access to the drug (Roberts et al., 2000; Ahmed and Koob, 1998; Ahmed et al., 2000). For example, in a limited access session (e.g. 1 hour session), drug intake remains at the level of training and is stable over time. However, when extended access is allowed (e.g. 8 hours session), drug intake scaled over time to levels significantly above the training baseline. Vanderschuren and Everitt (2004) showed that rats with a limited cocaine self-administration experience were able to suppress drug seeking behaviour when an aversive stimulus (foot-shock) was presented every time they accessed to the active lever. In contrast, after 20 more self-administration sessions, including eight extended access sessions where the animals could get up to 80 cocaine infusions per session, the animals were unable to stop cocaine seeking-behaviour regardless of the aversive outcome. These experiments highlighted the compulsive and inflexible nature of an addictive behaviour that only appears after prolonged access to the drug has occurred.

However, technical difficulties prevent the use of these complex reinforcement schedules and long protocols in mice. Therefore, the experiments presented in this thesis are mainly focused on the reinforcing potency of the drug that leads to drug-seeking behaviour and relapse without trying to model DSM-IV addiction criteria. The protocols used in the self-administration experiments presented in this thesis include acquisition, extinction and relapse.

1.3.3.1 Acquisition

During the acquisition phase in our self-administration experiments, animals learn to discriminate between active and inactive holes by acquir-

ing the response-reward contingencies, as well as contingencies between neutral sensory stimuli (e.g. context, light cue). To consider that an animal has acquired a drug-seeking-behaviour, it has to meet previously established acquisition criteria, based on: 1) discrimination between active and inactive hole/lever; 2) a minimum number of drug infusions; 3) stability in the number of infusions at least during three consecutive session. Many factors may influence the acquisition and maintenance of drug-seeking behaviour, such as the dose employed, the subject vulnerability, the reinforcing potency of the drug and the pharmacological characteristics of the drug. As in humans, not all animals exposed to the drug acquire a drug-seeking behaviour. Indeed, this is generally considered as a result of individual vulnerability, which in turn is influenced, at least in part, by the subject genetic (Stewart, 2003) and epigenetic (Tsankova et al., 2007) background that interact with environmental conditions. In this sense, stressful environmental stimuli have shown to affect drug self-administration behaviour. For example, the administration of a tail-pinch or a foot-shock facilitates the acquisition of cocaine (Goeders, 2003) and amphetamine (Piazza et al., 1990; Moffett and Goeders, 2005) self-administration in rodents. Food deprivation is also commonly used to facilitate the acquisition of drug self-administration (e.g. Corrigall and Coen, 1989). Similarly, psychological stressful conditions, such as isolation, have also shown to improve acquisition (for review see, Sanchis-Segura and Spanagel, 2006).

The dose is another important factor that can modify the responding rate during acquisition. Thus, animals are able to modify their responding rate as a function of the dose employed, self-regulating their final drug intake (e.g. Trigo et al., 2006b). The dose-response curve can also give information about the vulnerability of a subject. In this respect, pharmacological studies have shown that a leftward shift of the dose-response curve indicates an increase in the animal sensitivity to the drug, whereas a rightward shift of the dose response curve denotes a reduction in this parameter (Sanchis-Segura and Spanagel, 2006). In the same line, vertical (upward or downward) shifts of the dose-response curve may indicate the vulnerability of the subject (Piazza et al., 2000). Animals that exhibit an upward shift, receive higher amount of drug and are consider more vulnerable to de-

velop addiction than those presenting a downward shift (Vanderschuren and Everitt, 2004).

The reinforcing potency of a drug modifies the rate of acquisition of the self-administration behaviour. For example, cocaine is a high responding drug and animals acquire this operant behaviour readily. Cognitive and motor skills may also interfere in the acquisition of an operant-conditioning task, and drug-self administration is not the exception. All these factor should be taken into account when establishing the acquisition criteria and analyzing the data from self-administration experiments.

1.3.3.2 Extinction

Extinction is defined as the decrease in the frequency of a response when it no longer predicts the reinforcer (drug). Extinction is not due to forgetting the original stimulus-response association. Rather, extinction is an active learning process that counteracts the expression of S-R association by the development of a new connection that effectively says "the response no longer predicts drug". This process is described as the generation and strengthening of a second, inhibitory association between the response and the stimulus, which acts in parallel with the excitatory association and directly opposes the tendency of the excitatory association to activate the response. Inhibitory associations are generally more labile, or subject to disruption, than excitatory associations, and hence, are lost with the passage of time (spontaneous recovery) or a shift of context (renewal) (Myers and Davis, 2002; Richardson et al., 2004).

In drug self-administration models, extinction sessions are identical to the training sessions except that no drug is delivered after completion of the response requirement, the CS (cue-light) may or may not be present, depending on the reinstatement paradigm. Resistance to extinction and high responding during the initial extinction sessions is often seen for drugs with high relapse potential (Shaham et al., 2003).

1.3.3.3 Reinstatement to Drug Seeking Behaviour

One of the hallmarks of pathologic drug-seeking behaviour is the high rate of relapse observed even after long periods of withdrawal (Shaham et al., 2003). There are three mayor factors that can induce relapse in humans and have also been modeled in animals: 1) the presentation of a CS associated with the administration of the drug (*cue-light* or *context*); 2) the administration of the drug itself or *primings* or 3) the presentation of a stressful stimulus (foot shock) (Stewart, 2000). Animal models of relapse have allowed to study the different neurobiological substrates implicated in each of these forms of relapse (Fuchs et al., 2005) (previously explained in Section, 1.2.2).

1.3.3.4 The Yoked Control-Operant Paradigm

As explained above, current influential theories of addiction supported by a growing body of evidence have explained addiction as a learning and memory disease (see Section, 1.1.2.2) (Hyman, 2005; Kelley, 2004; Robbins et al., 2008). This has fueled the emergence of new animal models that allow the dissociation of the direct pharmacological effects of drugs of abuse from those that result from the cognitive processes associated with self-administration in an operant conditioning task. In this sense, the yoked control-operant paradigm provides important advantages to study the conditioning processes that take place during repeated active drug intake. In this paradigm, an animal that is self-administering the drug through active responding in an operant situation, causes another subject, a yoked animal, to receive the same dose of the drug passively. The ”master“ animal (contingent) can also be connected to a third subject, which receives a saline infusion every time the master animal self-administers the drug. The yoked (non-contingent) animals do not have control over drug taking and thus, no learning takes place under these conditions. Therefore,

this paradigm is an effective methodology to dissociate the behavioural, cellular and molecular mechanisms related to active drug taking from those caused by the direct pharmacological effects of the drug (see Figure, 1.7).

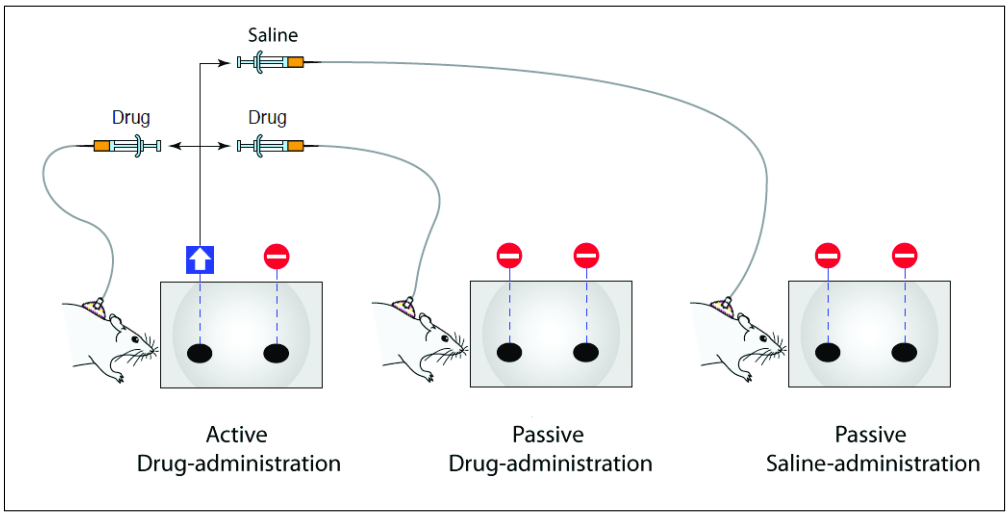

Figure 1.7: **The Yoked Control-Operant Paradigm** (Taken and modified from, Jacobs et al., 2003b).

1.3.4 Complementary Biochemical and Genetic Methods Used in Our Studies

The behavioural models described above are often complemented with biochemical and genetic methods in order to ascertain the neurobiological mechanisms underlying the behavioural responses observed. In this sense, the in vivo microdialysis and the microarray techniques are two unique approaches currently used for this purpose, which we have applied in the course of this thesis.

1.3.4.1 Microarray

Microarray is a small solid support (usually glass) onto which specific, tagged, nucleotide sequences are immobilized at a particular fixed spot. These fixed sequences, called probes, can be DNA, cDNA, or oligonucleotides. A microarray works by exploiting the ability of a given mRNA molecule to hybridize with the DNA template from which it originated. In this support, fluorescently labelled target sequences that bind to a probe sequence generate a signal. The intensity of the signal depends on the amount of target sequence that has bound a specific probe. Arrays can contain from hundreds to thousands of genes or transcripts.

There are three basic types of DNA microarrays, two are genomic and the third is "transcriptomic". The differences depend on the kind of immobilized DNA used to generate the array and the type of control and sample DNA that is used in the hybridization solution (see Figure, 1.8).

Microarray for mutation analysis: This type of arrays carry all the possible variations of one or several genes in a grid pattern. A DNA sample is extracted, multiple copies of the gene or genes of interest are generated using the polymerase chain reaction (PCR), and the sam-

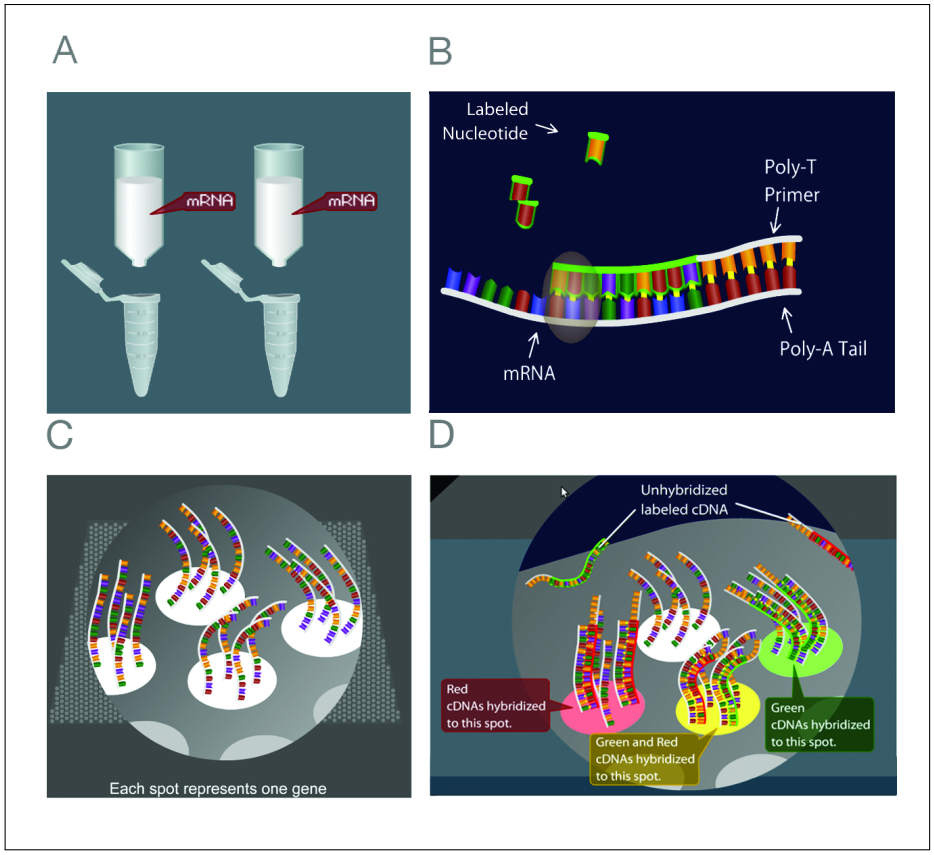

Figure 1.8: **Microarray expression analysis:** (A) mRNA extracted from experimental samples; (B) Primer adding to the adenine tail of mRNA enabling fluorescent colored labeled nucleotides to generate cDNA from mRNA samples; (C) Each spot on an array contains many copies (probes) of cDNA coding one gene; (D) Hybridizing cDNA from samples to the probes in the array. GREEN spots, represent that "only" the green-labeled cDNA (e.g. control) derived from samples is hybridized to that spot; RED, represent that "only" red-labeled cDNA (e.g. pathologic sample) is hybridized to that spot; YELLOW, represents a combination of green and red cDNA from samples are hybridized equally to the target DNA sequence. BLACK, represents areas where neither the sample groups of cDNA hybridized to the probes.

ple is then applied to the chip. The spots that light up correspond to the particular gene variants the individual has.

Comparative Genomic Hybridization: Used to identify large regions of DNA that are either missing (deletions) or present in more copies

(amplifications). Large pieces of genomic DNA are used as probes. The hybridization mixture will contain fluorescently labeled genomic DNA harvested from each of the cell populations studied.

Microarray expression analysis: We used such a microarray to perform the microarray expression analysis presented in article 3. This type of microarray, also called a ”transcriptomic” microarray is used to compare the levels of expression of specific genes under different conditions. The probes are cDNA derived from the mRNA of known genes. Biotin-labeled cRNA or cDNA fragments from experimental samples are hybridized to the probe array. These biotin molecules will act as a molecular glue for fluorescent molecules that will later be washed over the array. The hybridized probe array is scanned and the amount of light emitted is proportional to the bound target at each location on the probe array. A variation of this method is adding colored ”fluorescent protein tags” to the cDNA. Such an expression array is used to compare the expression profile of specific genes from two samples. Different tags (red and green) are used so that the samples can be distinguished in subsequent steps. The two labeled samples are then mixed and incubated with a microarray containing the immobilized DNA probes. During incubation most of the cDNA molecules from the samples hybridize to their complementary DNA strand in the microarray. Any spot that contains equivalent cDNA from both samples (”green“ and ”red“) turns yellow, while red or green spots represent genes that are expressed more in either sample. Black represents areas where neither of the samples cDNA hybridized to the target DNA.

1.3.4.2 Microdialysis

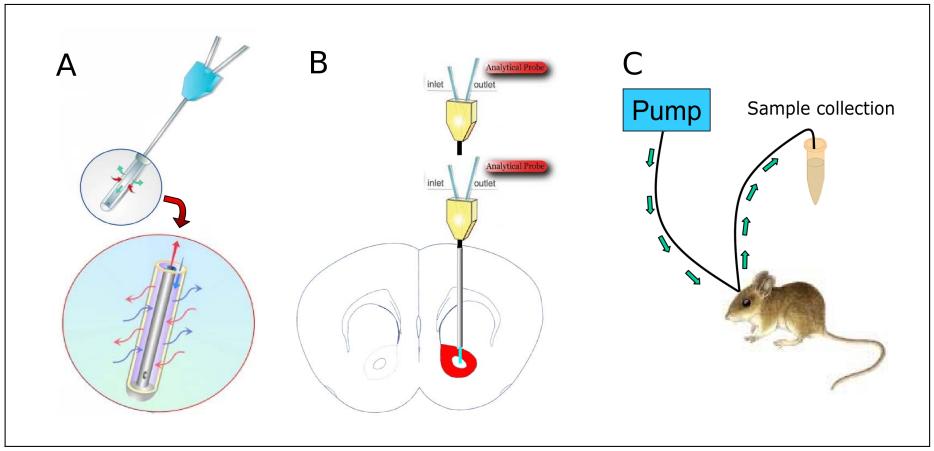

Figure 1.9: **Microdialysis** Schematic representation of the microdialysis technique (A) Semi-permeable microdialysis probe; (B) Microdialysis probe implanted in the nucleus accumbens; (C) Mice under perfusion for sample collection.

Microdialysis is a unique technique that allows to measure concentrations of a specific molecule in extracellular living tissue fluids. The microdialysis probe is designed to mimic a blood capillary. It has a semi-permeable membrane that when perfused with a physiological salt solution allows an exchange of compounds from an area of high concentration to an area of low concentration. A representative proportion of molecules from the extracellular fluid is then carried to the probe outlet for collection and analysis. With a wide range of microdialysis membranes it is possible to sample molecules in size ranging from few hundred daltons up to about 30.000 daltons making it feasible to monitor neurotransmitters, amino acids, metabolites, disease biomarkers, as well as available drug levels in target tissues. Once the samples are collected, they are analyzed using chromatographic methods including high pressure liquid chromatography (HPLC), which separates molecules based on their polarity (see Figure, 1.9).

1.4 MDMA

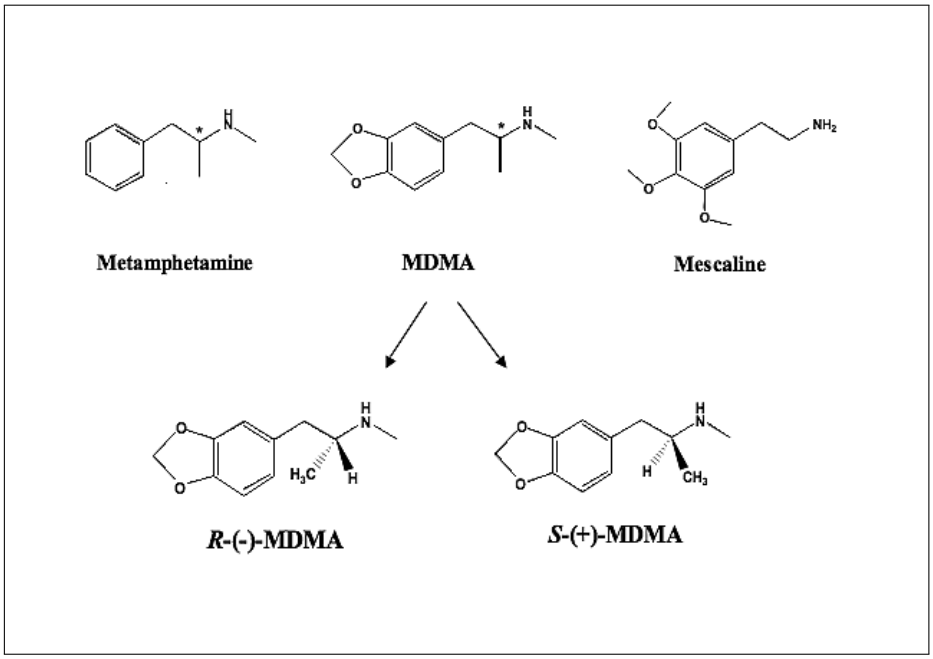

Figure 1.10: **MDMA Structure and Steroisomers** (±)3,4-methylenedioxymethamphetamine (MDMA), R(-)-MDMA and S(+)-MDMA forms. Molecular formula: C11H15NO2. **IUPAC** name:(RS)-1-(benzo[d][1,3]dioxol-5-yl)-N-methylpropan-2-amine

3,4-methylenedioxymethamphetamine (MDMA), commonly known as "**ecstasy**", is a substituted amphetamine which structure also resembles hallucinogenic compounds, such as LSD and mescaline (see Figure, 1.10). However, MDMA induces "unique' psychoactive effects that clearly distinguish it from both hallucinogenic or psychostimulant phenethylamines (Baylen and Rosenberg, 2006). MDMA contains a chiral center and, thus, exists as a pair of optical isomers, S(+)-MDMA and R(-)-MDMA, with different and sometimes opposite behavioural and pharmacological characteristics (Fantegrossi et al., 2009). The work presented was performed with

the racemic form of (±) MDMA, because it is the form normally consumed by humans.

1.4.1 History

MDMA was first synthesized by the German pharmaceutical company Merck in 1912. It was first believed that Merck created ecstasy to suppress appetite in soldiers in World War I. However, it was recently reported that MDMA was indeed synthesized by Merck, as a precursor for a haemostatic drug, and it was not pharmacologically tested in either animals or humans at that moment (Freudenmann et al., 2006). Then, in 1927 Merck started studies in animals, but whether it was ever tested in humans is not clear. By 1970 MDMA tablets were appearing in the streets of Chicago.

The first MDMA studies in humans were published in 1978 (Braun et al., 1978; Anderson et al., 1978). They reported not only chemistry, dosage, kinetics and psychotropic effects, but also noted that MDMA induced "an easily controlled altered state of consciousness with emotional and sensual overtones". Initial reports about its efficacy in psychotherapy started to appear, and by the early 1980s, MDMA was used by a number of psychotherapists in California (Shulgin, 1986).

Nichols suggested in 1986 that MDMA belongs to a *new* class of compounds, the "entactogens", because it induces feelings of "empathy", which facilitated interpersonal understanding and allowed the person to establish deeper contact with their "true self". The term entactogen was used to avoid possible negative connotations of the word "empathogen" [6], initially proposed by the American psychologist Ralph Metzner (Nichols, 1986). Finally, in 1984 MDMA's street name "ecstasy" was reported (Adam, 2006), and by 1985 to 1988, MDMA had become a Schedule I controlled substance in the United States (Freudenmann et al., 2006).

[6] Pathos ($\pi\alpha\theta o\varsigma$) means suffering in ancient Greek

1.4.2 Pharmacodynamics

MDMA has a complex mechanism of action through which it increases extracellular concentration of the main monoamines, noradrenaline (NE), serotonin (5-HT) and DA; as well as acetilcholyne (Ach) and histamine. An important aspect to take into account is that there is a complex reciprocal regulation of these monoamines, which is poorly understood and that may be central for the behavioural and neurochemical effects produced by MDMA (Gudelsky and Nash, 1996) (see Section, 1.14).

MDMA enhances extracellular 5-HT concentrations mainly through reversing the membrane 5-HT transporter (SERT). Thus, MDMA behaves as a SERT substrate, promoting carrier-mediated neurotransmitter release and inhibiting 5-HT uptake in all brain regions. Inhibition of the monoamine oxidase A and B, which are enzymes that degrade these monoamines, plus reversal of the vesicle monoamine transporter (VMAT-2) contribute to the rise in monoamine extracellular concentrations produced by MDMA. In contrast to these effects, MDMA also inhibits 5-HT firing from both dorsal and medial raphe nuclei by direct or indirect activation of somatodendritic 5-HT_{1A} autoreceptors and by rapidly inhibiting tryptophan hydroxylase, the rate limiting 5-HT synthesis enzyme. Hence, an initial 5-HT increase is followed by a decrease that lasts more than 24 h. These mechanisms are involved in the development of acute tolerance and in the adverse psychological effects after taking MDMA (Cole and Sumnall, 2003) (see Section, 1.4.5).

MDMA enhances DA levels by reversing the DA transporter (DAT), inhibiting DA re-uptake and increasing the release (Green et al., 2003). Apart from this direct action, there is also a state-dependent release of DA through activation of the mesolimbic reward pathway (see Section, 1.1.1). MDMA releases NE with greater potency than DA in *in vitro* brain homogenates (Rothman et al., 2001). The drug has strong inhibitory effects on the firing rate of NE neurons in the locus ceruleus, which may be due at least in part to serotonergic and noradrenergic mechanisms. Also, the

blockade of the NE transporter (NET), rather than transmitter release may underlie MDMA effects on NE neurons. NE release together with DA release, may be responsible for many of the amphetamine-like psychostimulant effects of MDMA (Cole and Sumnall, 2003).

MDMA elicits cortical and striatal ACh release probably through histamine H1 receptor activation. It also stimulates ACh M1 and M2 muscarinic receptors. However, the mechanism by which MDMA stimulates these receptors and the resulting behavioural effects are poorly understood. It seems that dopaminergic and serotonergic systems modulate ACh increase in the PFCx through D1 and 5-HT_4 receptors, respectively (Gudelsky and Yamamoto, 2008).

1.4.3 Pharmacokinetics and Neurotoxicity

A wealth of data suggest that MDMA-induced acute toxicity and long-term neurotoxicity depend on the metabolic disposition of MDMA. MDMA is differentially metabolized among species do to diverse enzyme kinetics and polymorphisms present, which might account for differences in its neurotoxic effects.

MDMA is mainly cleared by hepatic metabolism in different animals, primates and humans (see Figure 1.11). It is N-demethylated to form 3,4-methylenedioxyamphetamine (MDA) and O-demethylenated to form 3,4-dihydroxymethamphetamine (HHMA). HHMA is highly unstable and conjugates with sulfate and glucuronic acid. HHMA can also be rapidly oxidized to its corresponding quinone and forms adducts with glutathione and other thiol-containing compounds. HHMA is further O-methylated to form 4-hydroxy-3-methoxymethamphetamine (HMMA) (de la Torre et al., 2004). MDA, also used as recreational drug, is one of the main metabolic products of MDMA.

In humans, MDMA is mainly O-demethylenatedand, and only 5 % of oral dose is N-demethylated (de la et al., 2000). For this reason HHMA and HMMA are produced in higher rate than in other species. Human elimination half-life is 8 h and the concentration peak (t_{max}) is at 2h (Segura

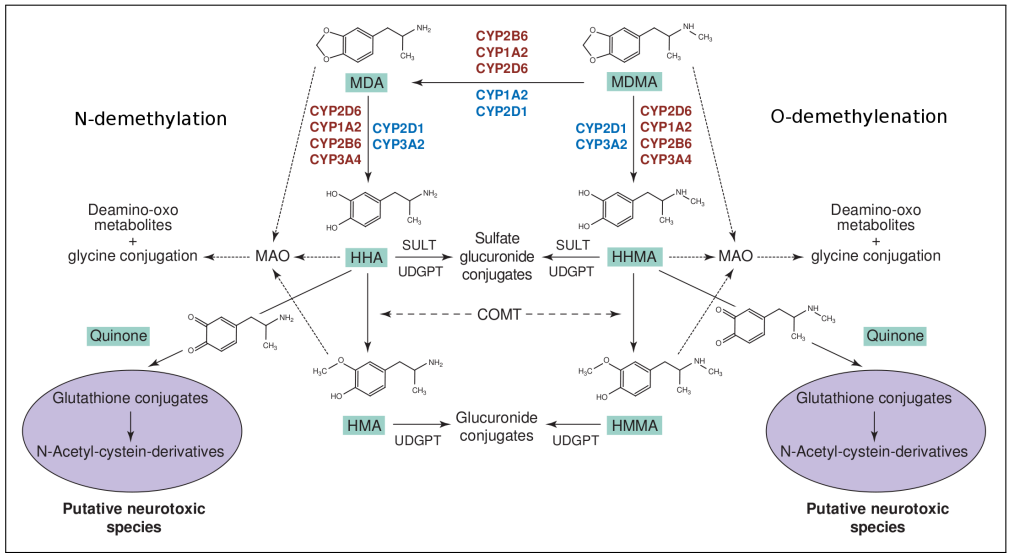

Figure 1.11: Isoenzymes of cytochrome P450 (CYP) involved in the N-demethylation and O-demethylenation metabolic reactions in rats are highlighted in blue whereas those corresponding to enzymes in humans are shown in red. The parent compound is N-demethylated to form 3,4-methylenedioxyamphetamine (MDA) and O-demethylenated to form 3,4-dihydroxymethamphetamine (HHMA). HHMA is further O-methylated to 4-hydroxy-3-methoxymethamphetamine (HMMA). In rats and mice, N-demethylation to MDA is one of the main metabolic pathways, whereas in humans and monkeys O-demethylenation to HHMA predominates. 3,4-Dihydroxyamphetamine (HHA) and HHMA are the precursors of neurotoxic species. In mice, MDMA-induced neurotoxicity is mainly DA mediated because MDMA causes the release of DA, which leads to the generation of reactive oxygen species as a result of DA oxidation. In other animal species, including humans, hepatic metabolism is a key factor involved in the production of MDMA toxicity to 5-HT-containing neurons. Abbreviations: COMT, catechol-O-methyl transferase; HMA, 3-methoxy,4-hydroxyamphetamine; MAO, monoamine oxidase; SULT, sulfotransferase; UDPGT, glucuronosyltransferase (de la Torre and Farr, 2004).

et al., 2005). MDMA pharmacokinetics is enantioselective, with (S)-MDMA eliminated faster than (R)-MDMA (Pizarro et al., 2004).

In non-human primates, MDMA is mainly O-demethylenated, as in humans, and its metabolism is also non-linear, with the main metabolic products being HHMA and HMMA (Mechan et al., 2006). In rodents (mice and rats) MDMA is mainly N-demethylated, which leads to the formation of

MDA, and 3,4-dihydroxyamphetamine (HHA). However, in mice MDMA predominates in tissues after its administration (Chu et al., 1996). Distribution half lives range from 2-26 min among species (Green et al., 2004). In rodents elimination half-life is about 1.7 h, similar to Reshus monkeys, 1.6 h. (Mechan et al., 2006; Green et al., 2004). Metabolism in animals is also enantioselective, being the clearance of (S)-MDMA faster than (R)-MDMA (Cho et al., 1990).

The cytochrome P450 isoenzyme debrisoquine 4-hydroxylase (CYP2D6) polymorphism in humans is another key point for MDMA metabolism. Approximately 5 - 9 % of Caucasians show an absence of this isoenzyme as a result of autosomal recessive inheritance of gene mutations and are classed as "poor debrisoquine metabolisers". These patients are more susceptible to hyperthermic response and serotonergic syndrome, while more resistant to neurotoxicity. By contrast, subjects who have multiple functional copies of the gene encoding CYP2D6 might exhibit a higher risk of neurotoxicity. On the other hand, concomitant use of drugs inhibiting CPY2D6 (e.g. quinidine) may increase MDMA acute toxicity (Cole and Sumnall, 2003). MDMA interacts with the polymorphic enzyme as both substrate and inhibitor. Thus, the pre-exposure of CYP2D6 to the first dose of MDMA impairs disposition of the second dose and leads to accumulation of MDMA. It has been postulated that a period of 280 h without dosing is required for the activity of CYP2D6 to return to normal because the enzyme is not inducible (de la Torre et al., 2004). In humans, this metabolite-enzyme complex underlies non-linear MDMA pharmacokinetics, as demonstrated by a clinical trial where single increasing doses of MDMA administered to volunteers (50 - 100- 125 - 150 mg) were correlated with pharmacokinetic parameters. In this study, the recovery of HMMA in urine was constant, whereas MDMA plasma recovery progressively increased, indicating constant renal clearance, and non-renal (hepatic) clearance was dose-dependent. Hence, small increases in the dose of MDMA, would then translate into a disproportionate increase in its plasmatic concentration, making the individual more prone to acute toxicity (de la Torre et al., 2000).

MDMA-induced neurotoxicity is mainly serotonergic in primates and

rats (including humans), and dopaminergic in mice (Lyvers, 2006). It is postulated that hepatic metabolism is a key factor in MDMA-induced neurotoxicity to 5-HT-containing neurons (de la Torre et al., 2005). In this respect, a decrease in the concentration of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the cerebrospinal fluid has been found in MDMA users as compared to controls (McCann et al., 1994). Similarly, using PET image techniques, a decrease in SERT was reported in MDMA exusers when compared to controls (McCann et al., 1998). However, MDMA induced neurotoxicity in humans is still controversial, and further studies are needed to clarify this important issue.

The dopaminergic neurotoxicity in mice seems to be mediated by the MDMA-induce increase in DA, which in turn is oxidated leading to the generation of reactive oxygen species that causes toxicity. In contrast, the serotonergic neurotoxicity is proposed to be mediated by hepatic metabolites in humans, non-human primates and rats (de la Torre et al., 2005) since MDMA and MDA can induce neurotoxicity only when administered systemically, but not directly into the brain (Esteban et al., 2001). Furthermore, the administration of the MDMA hepatic metabolites HHMA and HMMA into the brain also fails to reproduce neurotoxicity in rats (Johnson et al., 1992). Thus, thioether adducts with quinones that are formed from the auto-oxidation of the MDMA metabolites HHMA and HHA (Miller et al., 1996; de la Torre et al., 2005; Easton et al., 2003) are thought to induce serotonergic neurotoxicity by entering into a redox cycle and generating reactive oxygen species, which finally lead to neurotoxicity.

Thus, the neurotoxic effect of MDMA over both dopaminergic and serotonergic systems seems to be mainly mediated by oxidative stress. The administration of antioxidant compounds such as ascorbic acid (Shankaran et al., 2001) attenuate MDMA-induced neurotoxicity. However, the source of reactive oxygen species and the precise mechanisms by MDMA-induced oxidative stress, are still unknown and controversial.

1.4.4 Abuse and Addiction

The 2009 Annual Report on the State of Drugs Problem in Europe, reported ecstasy consumption to be more common among young adults (15 - 34 years). It is estimated that 7.5 million young Europeans (5.6%) have tried ecstasy, with around 2 million (1.6%) using it during the last year. Lifetime prevalence: about 9.5 million (2.8%) in European adults (see `http://www.emcdda.europa.eu/publications/annual-report/2009`).

The potential addictive properties of MDMA are currently a controversial issue. One of the reasons for this controversy, is that criteria for abuse and dependence in the DSM-IV are the same for all substances (see table 1.1), without considering that drugs are *pharmacologically diverse* and parameters such as pharmacological half life and tolerance, directly influence their *use pattern*, which in the case of is MDMA difficult to fit the addicted person behaviour within these criteria. Furthermore, under the category of specific substance disorders, MDMA is currently classified as hallucinogenic, despite its particular behavioural and pharmacological effects.

However, early studies provided some evidence of MDMA abuse and dependence (Topp et al., 1999; Yen et al., 2007; Schuster et al., 1998; Cottler et al., 2001) and found that users met dependence criteria ranging from 20 % - 64 % and for abuse from 21 % - 34 %. Symptoms of withdrawal and tolerance were also highly reported. A recent study tested DSM-IV reliability to diagnose MDMA abuse/dependence and its cross-cultural applicability. Of the total number of users included in this study, 15 % and 59 % met DSM-IV criteria for MDMA abuse and dependence, respectively (Cottler et al., 2009). This result contrasts with other data showing that the prevalence of abuse is in general higher than the one for dependence.

Reliability of the diagnoses was observed consistently across cities being “continued use despite knowledge of physical/psychological problems” and “withdrawal” the two most prevalent dependence criteria; and “physically hazardous use” was the most prevalent abuse criterion (Cot-

tler et al., 2009). Withdrawal symptoms including fatigue, loss of appetite, feelings of depression, and trouble concentrating have been reported by the National Institute on Drug Abuse (NIDA) to affect almost 60 % of MDMA users (`http://www.drugabuse.gov/pdf/rrmdma.pdf`). The authors of these studies state that there is an urgent need to create a separate substance use disorder category for MDMA (Cottler et al., 2001, 2009) due to its "unique" behavioural effects, mechanism of action and pharmacokinetic profile, that ultimately affects its use pattern.

1.4.5 Use Pattern: Relevance of Acute and Chronic Tolerance

The normal psychostimulant use pattern is to take another dose of the drug to achieve the next "high", and to avoid withdrawal symptoms, which starts the cycle of repetitive drug taking. In contrast, MDMA users stated that when tablets were taken in succession, the later capsules did not have beneficial consequences, and this effect could last up to two days (Parrott et al., 2002). This effect could be due to two different factor. First, because the subject have achieved the maximum effective dose in the dose-response curve, and second, to the pronounced acute tolerance that characterizes MDMA. Thus, the normal pattern of drug ingestion, was one "*trip*" every 1 to 4 weeks, which is very different to other psychostimulants such as cocaine. However, acute tolerance was evident, since a period between drugs is described as necessary in order to maintain the drug effectiveness. Moreover, all regular users described their first MDMA experience as "the most intense", and trips were affected by knowledge and *expectancy*, rather than any diminution in drug response (Parrott, 1998). On the other hand, chronic tolerance has also been described to affect the use pattern. Novice users generally take a single ecstasy tablet, regular users typically take 2-3 tablets, whereas the most experienced users may take 10 - 25 tablets in a single session. Reduced subjective efficacy following repeated usage is also typically described, with many users subjectively reporting the development of tolerance. Intensive self-administration or *bingeing* is often noted by experienced users. This can comprise "stacking" several tablets

together, and "boosting" successive doses over an extended period. Some experienced users snort MDMA powder nasally, whereas a small minority inject MDMA (Parrott, 2005). However, the usual pattern, is always to consume the drug on weekends and continue in a binge pattern until the early hours of Monday; it is described as an 80-h weekend, and the rest of the week is usually spent recovering (Jansen, 1999). Chronic tolerance and bingeing are statistically linked to higher rates of drug-related psychopathologic problems (Fox et al., 2002).

Acute Tolerance

Acute tolerance to MDMA is mainly due to its mechanism of action (see Section, 1.4.2). MDMA induces 80 % loss of brain 5-HT and its metabolite 5-HIAA within 4 h of a single injection (Green et al., 2003), which is followed by a direct vesicle depletion (Mlinar and Corradetti, 2003). MDMA also inhibits neuronal firing in the medial and dorsal raphe nuclei by stimulating 5-HT_{1A} autoreceptors (Sprouse et al., 1990). The 5-HT_{2A}R may also be involved in acute tolerance since Reneman et al. (2002) have shown that a high dose of MDMA decreases the density of these receptors in the cortex of rats. Moreover, this effect was positively correlated with brain 5-HT depletion. In the same study, human users showed a decrease in 5-HT_{2A}R binding sites six hours from the last exposure as revealed by brain image SPECT technique. However, 5-HT_{2A}R density recovered after thirty days of withdrawal, to a greater level than the one reported in control subjects. Hence, the 5-HT_{2A}R may be involved in both tolerance and sensitization to MDMA effects. On the other hand, the intermediate complex formed between the CYP2D6 enzyme and MDMA may also contribute to MDMA acute tolerance and thus to its use pattern. This enzyme is not inducible, and it takes several days to recover after one MDMA exposure. However, this subject is still controversial because the use pattern of MDMA would be even more spaced due to the gradual recovery of this enzyme (Heydari et al., 2004; Van et al., 2006; Yang et al., 2006). MDMA inhibits also tryptophan hydroxylase, the rate limiting enzyme for 5-HT synthesis (Schmidt

and Taylor, 1988), which results in a 5-HT *synthesis breakdown*, that finally forces users to stop taking the drug because it is no longer effective. This evidence may help to explain why the use pattern of MDMA does not seem to be clearly compulsive when compared to other psychostimulants like cocaine and amphetamine.

Chronic Tolerance

The underlying mechanisms for MDMA chronic tolerance involve mainly neuroadaptive changes in monoamine receptors, and monoamine transporters. 5-HT$_{1A}$ receptor density is significantly decreased in the brain stem region containing the dorsal raphe nucleus, after repeated (30 mg i.p. twice daily in 4 consecutive days), but not single (30 mg i.p.) MDMA treatment (Aguirre et al., 1995). As explained above, MDMA acutely inhibits neuronal 5-HT firing by stimulating 5-HT$_{1A}$ autoreceptors in the raphe nucleus (Sprouse et al., 1990). Hence, the decrease of 5-HT$_{1A}$ receptors after repeated treatment is proposed to be an adaptive response to sustained 5-HT increase in the brain. Experiments performed by Frederick et al. (1995) showed tolerance to the acute cognitive deficits produced by MDMA in an operant test battery in rhesus macaques treated with escalating doses of MDMA (0.1-20 mg/kg twice daily) during 14 days. Twenty-one months later, reduced levels of hippocampal 5-HIAA, 5-HT uptake sites and 5-HT turnover were found without altering brain 5-HT concentration levels. In concordance, monkeys pretreated with higher doses of MDMA (10 mg/kg twice a day x 4 days) also showed behavioural tolerance after drug administration, and exhibited a 50 % decrease in 5-HT in the PFC and hippocampus accompanied by a significant decrease in 5-HT receptor uptake sites in the PFC (Frederick et al., 1998). Previous studies in non-human primates self-administering MDMA for a prolonged period of time (18 months) have shown a reduction in the reinforcing effects of the R(-)-MDMA enantiomer. However, these effects were not correlated with changes in DA, 5-HT and their metabolite concentrations in any brain area studied (Fantegrossi et al., 2004a). More recently, a decrease in SERT binding was observed follow-

ing 17 days of MDMA (1 mg/kg/infusion) self-administration in rats that had been initially trained to self-administer cocaine (Schenk et al., 2007). Hence, long-term MDMA administration may lead to long-lasting deficits in 5-HT neurotransmission.

1.4.6 Acute Pharmacological Effects in Humans

MDMA acute administration in humans produces entactogenic (see Section, 1.4.1) and psychostimulant-like effects, such as *hyperactivity*, awareness and increased self-confidence accompanied by tachycardia, dry mouth and bruxism. Although users have reported slight visual effects with use of regular doses of MDMA (75 mg), hallucinogenic effects may appear only at high doses. MDMA *subjective effects* are normally pleasant and include tenderness/affection, peacefulness/calm, euphoria and decreased defensiveness (for review, see Baylen and Rosenberg, 2006). Nevertheless, some users have reported bad experiences on MDMA, characterized by confusion, paranoia, "weird or negative thoughts", such as feeling out of control, dying and feeling immobile (Parrott et al., 2007). In this sense, *expectancy* and the initial emotional state seem to be crucial for a positive outcome (Parrott et al., 2007).

An important physiological effect is hyperthermia or, more accurately, a lack of thermoregulation since it can only be observed under high environmental temperatures (Dafters, 1994; Dafters and Biello, 2003; Dafters and Lynch, 1998). *Hyperthermia* has been linked to neurotoxicity and can be found as part of the lethal serotonergic syndrome. It is a potentially life-threatening adverse drug reaction that may occur following administration of diverse drugs that enhance serotonergic activity, including MDMA. It occurs as a consequence of excess serotonergic activity at central and peripheral nervous system. The symptoms and signs may range from mild to severe and include cephalea, tachycardia, hypertension, myoclonus (intermittent tremor), mydriasis (dilated pupils), hyperreflexia and hyperthermia. In severe cases it can trigger seizures, metabolic acidosis, disseminated intravascular coagulation and renal failure with final lethal con-

sequenceses. The serotonergic syndrome triggered by MDMA has been linked to a specific CYP2D6 polymorphism related to the Caucasian phenotype (see Section, 1.4.3).

1.4.7 Acute Pharmacological Effects in Animals

A wealth of research has been undertaken in animals in an attempt to clarify MDMA effects in humans, since most of its pharmacological and behavioural effects can also be found in experimental animals. Cardiovascular effects have been reported in rodents, such as tachycardia and vasoconstriction mediated mainly through MDMA-induced sympathomimetic response (Green et al., 2003). Additionally, acute administration of MDMA induces in rodents as in humans, a hyperthermic effect under normal temperature conditions (20 - 22^{o}C) with an approximate increase of 1 - 2^{o}C (Colado et al., 1993; O'Shea et al., 1998). However, this increase depends upon environmental temperatures and strains (Green et al., 2003), and seems to be mediated by the serotonergic (Colado et al., 1993) and dopaminergic systems (Mechan et al., 2002). Among the serotonergic receptors implicated, the 5-HT$_{2A}$R, seems to play a mayor role in the hyperthermic effect of MDMA. Indeed, rodents (Herin et al., 2005; Fantegrossi et al., 2003) like humans (Liechti et al., 2000) exhibit lower MDMA-induced increase in body temperature when pre-treated with a 5-HT$_{2A}$R antagonist. Furthermore, prevention of the MDMA-induced hyperthermic response in rodents tends to provide protection against the subsequent neurotoxic loss of 5-HT (Mechan et al., 2001).

MDMA also induces hyperlocomotion in rodents. This hyperactivity seems to be modulated by the monoaminergic system through 5-HT, DA and NE receptors. Among the serotonergic receptors involved are 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$. In the case of 5-HT$_{2B}$R, MDMA-induced hyperlocomotion is abolished when mice are pre-treated with a selective 5-HT$_{2B}$R antagonist (RS127445) as in 5-HT$_{2B}$R KO mice (Doly et al., 2008). However, it has been recently published that in 5-HT$_{2B}$R KO mice, a high dose of MDMA (30 mg/kg) can induce locomotor hyperactivity (Doly et al., 2009).

On the other hand, pharmacological studies have shown that the selective 5-HT_{2A}R antagonist MDL-100907 attenuated hypermotility produced by a high dose of racemic MDMA (Fantegrossi et al., 2003; Kehne et al., 1996), while this antagonist only affected peripheral activity produced by lower doses of MDMA (Bankson and Cunningham, 2002; Herin et al., 2005). Furthermore, MDL-100907 had an opposite effect on MDMA enantiomers, potentiating S(+)-MDMA-induced hyperactivity, while decreasing it for R(-)-MDMA (Fantegrossi et al., 2003). Conversely to the 5-HT_{2A}R, 5-HT_{2C}R antagonists potentiate MDMA-induced locomotor activation (Bankson and Cunningham, 2002), whereas agonists blocked it (Ramos et al., 2005).

The dopaminergic system is also involved in MDMA-induced hyperlocomotion, stimulation of D1 and D2 receptors (Benturquia et al., 2008; Ball et al., 2003; Ramos et al., 2004, 2005). More recently, the noradrenergic system has also been implicated in the hyperlocomotion induced by MDMA, specifically through α-1-adrenergic receptors. Pretreatment with the α-1-adrenergic receptor antagonist prazosin, administered either systemicly or directly into the PFC or VTA, completely blocks the locomotor stimulant effects of (±)-MDMA in rats (Selken and Nichols, 2007). In conclusion, it is likely that all monoamines are involved in MDMA-induced hyperlocomotion through a fine coupling mechanism that is still not fully understood.

MDMA can also produce anxiety-related behaviours, which may depend on the dose administered. Evidence indicates that MDMA has anxiolytic-like properties at lower doses and anxiogenic-like at higher doses (Morley and McGregor, 2000; Lin et al., 1999), when tested in the elevated plus maze.

1.5 Mechanisms Involved in MDMA Reinforcement and Relapse

The rewarding and reinforcing properties of MDMA have been demonstrated in different species. Thus, MDMA can induce conditioned place preference in rats (Bilsky et al., 1990) and mice (Robledo et al., 2007), and ICSS studies in rats have shown that MDMA produced a dose-related lowering of the reward threshold (Hubner et al., 1988). Moreover, MDMA is reliably self-administered by non-human primates (Fantegrossi et al., 2002), rats (Fletcher et al., 2001) and more recently by mice (Trigo et al., 2006a).

The DA reward pathway is modulated by serotonergic and noradrenergic projections (see Figure 1.1), and MDMA directly increases the extracellular concentrations of 5-HT, NE and DA. Therefore, complex interactions between these neurotransmitters may participate in the rewarding/reinforcing effects of MDMA.

1.5.1 Participation of the Dopaminergic System

Using acute non-contingent administration of MDMA, various authors have shown that MDMA, like other drugs of abuse, increases DA activity in the NAC (Bankson and Yamamoto, 2004; Robledo et al., 2004, 2007). A role for DA in MDMA-induced reward has been suggested by experiments showing that the DA release inhibitor CGS 10746B prevents the acquisition of MDMA-conditioned place preference in rats (Bilsky et al., 1998). More recently, the involvement of the dopaminergic system in the reinforcing properties of MDMA has been studied using the self-administration paradigm. In this way, DA D1 receptor blockade with SCH 23390 produced a rightward shift in the MDMA self-administration dose response curve (Daniela et al., 2004). Similarly, the D2 antagonist eticlopride increased responding maintained by several doses of MDMA in rats (Brennan et al., 2009). In addition, MDMA self-administration in the NAC shell

was disrupted by the administration of D1 (SCH 23390) or D2 (raclopride) receptor antagonists in rats (Shin et al., 2008). These results suggest that both D1 and D2 receptors contribute to the maintenance of MDMA self-administration. Additionally, ambient temperature also seems to modify the effects of MDMA on NAC DA since the increase of DA in the NAC shell is greater at 30 degrees ^{o}C ambient room temperature than at 20 ^{o}C in rats (O'Shea et al., 2005). Therefore, the authors postulate that MDMA is more rewarding at higher ambient temperatures than a lower ones.

1.5.2 Participation of the Serotonergic System

The serotonergic system has been involved in the rewarding properties of MDMA. Accordingly, systemic administration of the 5-HT$_3$R antagonist MDL72222 blocked the acquisition of MDMA conditioned place preference (Bilsky and Reid, 1991). In addition, 5-HT$_{2B}$ receptors also seem to modulate the rewarding properties of MDMA since blockade or ablation of this receptor can prevent MDMA-induced hyperlocomotion and conditioned place preference (Doly et al., 2009). In concordance, a 5-HT$_{2B}$ antagonist blocked the reinstatement of MDMA conditioned place preference. This effect was possibly mediated by the modulation of these receptors over the dopaminergic system since 5-HT$_{2B}$ KO and antagonist pre-treated mice exhibit a lack of MDMA-induced DA extracellular levels in the NAC (Doly et al., 2008).

In concordance, our group recently demonstrated that SERT KO mice did not self-administer MDMA at any dose tested, although they exhibit a similar increase in NAC DA extracellular levels when compared to WT littermates (Trigo et al., 2007). This result suggests that an isolated increase of NAC DA is not sufficient to produce MDMA-induced reinforcement. Therefore, it is plausible that a 5-HT - DA interaction mediates the reinforcing properties of MDMA. In this respect, several 5-HT receptors have been involved in the modulation of mesolimbic DA activity (Alex and Pehek, 2007). More specifically, the 5-HT$_{2B}$ (Doly et al., 2008) and 5-HT$_{2A}$R (Pehek et al., 2001) receptors have been implicated in MDMA-induced increase in NAC DA levels.

Some of the characteristic pharmacological effects of MDMA that distinguish it from other psychostimulants (e.g. hallucinogenic effects at high doses) have been specifically ascribed to the 5-HT$_{2A}$R (Gonzlez-Maeso et al., 2007). In contrast to the 5-HT$_{2B}$R and other 5-HT receptors, the 5-HT$_{2A}$R has been directly involved in the physiopathology of relevant personality traits which are further related to addiction (Bubar and Cunningham, 2008) such as impulsivity (Nomura and Nomura, 2006). For these reasons, we hypothesized that, in contrast to what has been seen with other drugs of abuse, the 5-HT$_{2A}$R could be critical for MDMA-induced reinforcement as well as reinstatement of MDMA seeking behaviour.

1.5.2.1 5-HT$_{2A}$ Receptors

MDMA, like other drugs such as ergotamine, DOI, lisuride as well as all hallucinogenic compounds, except Salvinorin A, are known to activate the 5-HT$_{2A}$R. The hallucinogenic effects of these compounds have been ascribed to specific G protein-dependent signaling responses through cortical 5-HT$_{2A}$R activation (Gonzlez-Maeso et al., 2007). This receptor has also been involved in the pathophysiology of schizophrenia and is the main pharmacological target for atypical antipsychotics. Furthermore, it has been implicated in other psychopathologic conditions, such as obsessive compulsive disorder, suicide, bulimia, anorexia, borderline personality, sleep disorders and impulsivity (for review see, Serretti et al., 2007). With respect to MDMA, these receptors seem to modulate neurotoxicity (Capela et al., 2006), hyperthermia (Fantegrossi et al., 2004b; Herin et al., 2005) and cognitive impairment (Reneman et al., 2000) due to MDMA administration.

1.5.2.2 5-HT$_{2A}$R: Structure, Second Messenger Activation Pathway and Distribution in the Brain

The 5-HT$_{2A}$R is a member of the 5-HT$_2$ receptor family which also includes the 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors. It was discovered in 1979 and, by the mid-1980s, some of its pharmacological properties and second mes-

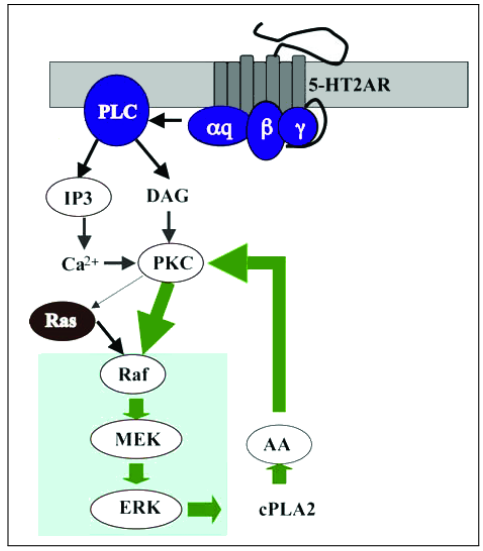

Figure 1.12: **5-HT$_{2A}$R Second Messenger Activation Pathway** 5-HT$_{2A}$R receptors activate PLC and PKC via G-protein α-q subunit (Gaq), which in turn acts on Ras and Raf. The ERK regulation consists of positive and negative feedback loops, shown in green and red thick lines, respectively. Elevation of the active form of MAPK (ERK) stimulates PLA2, which releases arachidonic acid which in turn, has a positive effect on PKC. This creates a potential positive feedback loop in the regulation of MAPK. On the other hand, the two key phosphatases in the system are PP2A and MKP. PP2A dephosphorylates both Raf and MEK. MAPK also phosphorylates MKP, which reduces ubiquitination and degradation of MKP. The increased amount of MKP results in a negative feedback. Phospholipase C (PLC), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), phospholipase A2 (PLA2), extracellular signal-regulated kinase (ERK), protein phosphatase 2A (PP2A), MAP kinase phosphatase (MKP) (Adapted, Chang et al., 2009).

senger systems involved in its responses had been described. The human *5-HT$_{2A}$* gene is located on chromosome 13q14-q21, and is conserved in human, chimpanzee, dog, cow, mouse, rat, chicken, and zebra fish.

5-HT$_{2A}$R belongs to the family of seven transmembrain domain G protein coupled receptors (see Figure 1.12). Activation of 5-HT$_{2A}$R results in depolarization due to a decrease in potassium conductance (Tanaka and North, 1993). It is positively coupled with the phospholipase C (PLC) via a G-protein, which leads to an increase of inositol phosphates (IP3) and intracellular Ca^{++} that further triggers MAPK activation via PKC. This MAPK stimulation in turn acts on the Ras and Raf pathway to produce activation

of ERK1/2 signal pathways which also stimulate PLA2 to produce arachidonic acid (Chang et al., 2009).

The CNS distribution of the 5-HT$_{2A}$R mapped with [11C]-MDL-100907, a new selective 5-HT$_{2A}$ receptor antagonist, using positron emission tomography (PET) (Ito et al., 1998) (see Figure 1.13) found high affinity binding sites mainly in cortical areas (neocortex, cortex pyriform, claustrum), nucleus caudate, NAC, olfactory tubercle, hippocampus,VTA and BLA.

Immunocytochemical, in situ hybridization and receptor autoradiography studies suggest that the 5-HT$_{2A}$Rs are postsynaptic receptors located on cortex (GABAergic) interneurons and glutamatergic cortical pyramidal neurons (mostly in layer V), cholinergic neurons in the basal forebrain and specific nuclei in the brain stem, such as the dorsal raphe nucleus (Burnet et al., 1995).

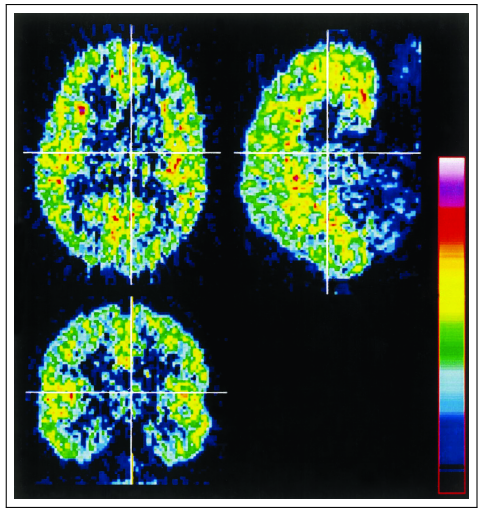

Figure 1.13: **CNS distribution of the 5-HT$_{2A}$R** Horizontal (upper) and vertical (lower) PET sections showing the distribution of radioactivity in the brain of a human volunteer following intravenous injection of a tracer dose of the 5-HT$_{2A}$ receptor ligand [11C]MDL-100907 (Taken from, Ito et al., 1998).

1.5.2.3 5-HT$_{2A}$R Modulation of Dopamine

It has been recently established that 5-HT$_{2A}$Rs are present in a large population of medial PFC and OFC (layer V) pyramidal neurons (50 - 66%), and in a smaller proportion of GABA interneurons (20%) in the same structures (Santana et al., 2004). 60 - 75% of the cortical projections to the dorsal raphe and 55% of those to the VTA express 5-HT$_{2A}$R mRNA (Vázquez-Borsetti et al., 2009). In accordance with this dual location in excitatory and inhibitory neurons, 5-HT$_{2A}$R activation in the PFC either depolarizes or hyperpolarizes pyramidal neurons depending on experimental conditions (Araneda and Andrade, 1991; Tanaka and North, 1993). The local application of a 5-HT$_{2A}$R agonist (e.g. DOI) in the medial PFC increases the firing rate of dorsal raphe 5-HTergic neurons. These, in turn, project to the medial PFC, increasing 5-HT in this structure, in a feed-back mechanism (Amargós-Bosch et al., 2004; Martín-Ruiz et al., 2001). In the same way, such stimulation with DOI has been shown to increase burst firing of DAergic neurons in the VTA, leading to an elevated mesocorticolimbic DAergic activity (Bortolozzi et al., 2005) (see Figure 1.14). Alternative indirect pathways mediating the 5-HT$_{2A}$R modulation over the mesolimbic DA system have also been described. One pathway is through the activation of pyramidal PFC neurons, which directly stimulates the NAC, and it in turn, induces dopaminergic activity in the VTA, through activation of the ventral pallidum. The other pathway described is through PFC projections to the pedunculopontine tegmental nuclei which further project to the VTA (see Figure 1.14) (Bortolozzi et al., 2005). Apart from these pathways, projections from the medial PFC to GABAergic neurons in the VTA have also been described (Carr and Sesack, 2000). These may affect DAergic neurons projecting back from the VTA to the PFC as well as GABAergic projections from the VTA to the NAC (see Figure 1.14), making the excitatory modulation of 5-HT$_{2A}$R over the mesolimbic system more complex. However, recent experimental data have revealed that 5-HT$_{2A}$R receptor stimulation by DOI, potentiates amphetamine-induced DA increase in the rat medial PFC and NAC (Kuroki et al., 2003). Hence, MDMA, through activation of the 5-HT$_{2A}$R, could increase mesolimbic DAegic transmission which may

finally modulate its rewarding/reinforcing properties.

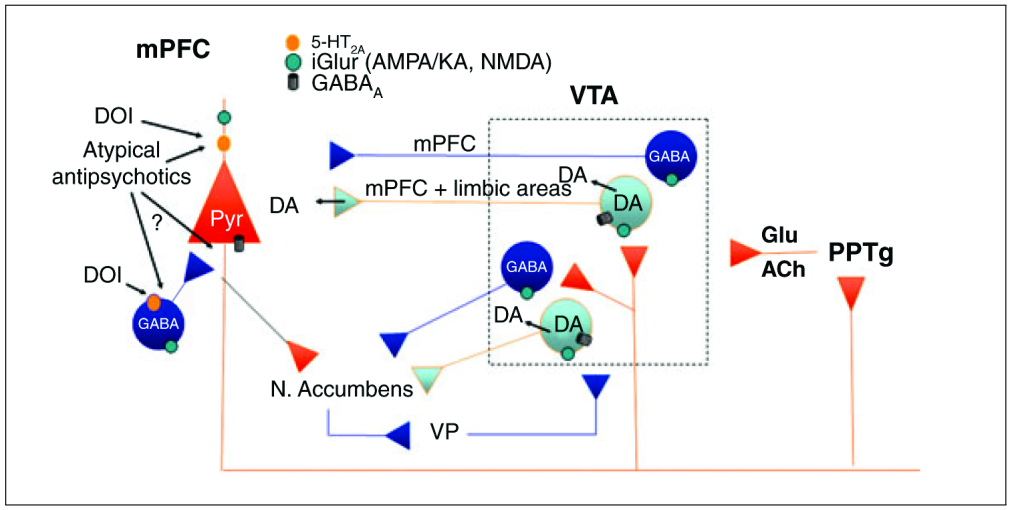

Figure 1.14: **Dopamine modulation by 5-HT$_{2A}$R** Schematic representation of the direct and indirect connectivity between the medial prefrontal cortex (mPFC) and ventral tegmental area (VTA). The dotted frame marks the VTA, with known inputs from the mPFC onto dopamine (DA) and GABAergic neurons. Inhibitory GABAergic afferents from ventral pallidum (VP) and excitatory [glutamate, acetylcholine (ACh)] afferents from the pedunculopontine tegmentum (PPTg) are not shown to synapse on DA or GABAergic neurons in the VTA. The electrical or pharmacological (e.g. DOI) activation of 5-HT$_{2A}$R in the mPFC enhance DA release in the VTA and the mPFC. The PFC facilitation of VTA DA neurons may be direct, via an excitatory pathway to VTA DA neurons projecting to the prefrontal cortex (PFC), or indirect. The latter pathways may include the above inputs from VP and PPTg. MDMA may act over 5-HT$_{2A}$R in the PFC to modulate DAergic mesolimbic transmission via these pathways (Taken and modified from, Bortolozzi et al., 2005).

1.5.2.4 5-HT$_{2A}$R and MDMA-Induced Reinforcement

Some pharmacological studies have used 5-HT$_{2A}$R blockade in an attempt to reduce psychostimulant-induced reward and reinforcement due to the known modulatory effects of these receptors on DA transmission. These studies showed that pre-treatment with the specific 5-HT$_{2A}$R antagonist, MDL-100907 had no effect on the reinforcing properties of co-

caine in rats and non-human primates (Fantegrossi et al., 2002; Filip et al., 2006; Fletcher et al., 2002a). In addition, the blockade of 5-HT_{2A}R prevents cocaine priming- (Fletcher et al., 2002a) and cue-induced relapse in rats (Nic Dhonnchadha et al., 2009).

Conversely, in the case of MDMA, recent human data report a reduction in MDMA-induced perceptual changes and emotional excitation, using the non-specific 5-$HT_{2A/C}$ antagonist ketanserin without affecting baseline positive mood (Liechti et al., 2000). In concordance, non-human primates self-administration studies showed that pre-treatment with MDL-100907 partially attenuates responding for S (+)-MDMA, and completely abolishes R (-)-MDMA self-administration (Fantegrossi et al., 2002).

1.6 Neuroadaptive Changes Following Active versus Passive Drug-Administration

Drugs of abuse can induce changes in functional and structural plasticity in neurons within the brain reward circuitry (defined in Section, 1.2.2). These changes are believed to underlie the long-lasting behavioural consequences that characterize an addictive state. Although the cellular mechanisms regulating this plasticity are not fully understood, it is well established that changes in gene expression are essential for short- and long-term plasticity. However, these changes may be influenced by whether the drug is administered actively of passively. Thus, learning processes participate in these neuroadaptive changes (Hyman, 2005), by mechanisms that include compensatory homeostatic responses due to anticipation of the previously learned drug effects (Siegel and Allan, 1998; Ramos et al., 2002). In this sense, overdose has been reported in humans when the drug is administered in the absence of the usual pre-drug cues or in a different context (Bodey et al., 2000). These neuroadaptations depend upon additional factors, such as reward predictability, and motivational aspects that can only be assessed using active drug self-administration, and thus are different from those induced by the drug itself. In this respect, animal data have demonstrated that after 21 days of abstinence from cocaine self- or yoked administration, long-term depression (LTD) in the NAC core was abolished only in cocaine self-administering rats and not in yoked animals (Martin et al., 2006). In concordance, persistent LTP, was present only in the VTA of rats that actively self-administered cocaine after 3 months of abstinence, but not in passively injected rats (Chen et al., 2008). This effect is exclusive of active drug administration since animals that self-administered food or sucrose exhibit only a transient potentiation of VTA glutamatergic signaling (Chen et al., 2008). These evidence suggest that active drug self-administration induces a persistent synaptic enhancement that is not

present after passive drug-administration or active training to obtain natural rewards. Such a persistent synaptic potentiation may contribute to the characteristic resistance to extinction and likelihood to relapse that is essential for the addictive behaviour. In this respect, a cocaine-priming injection increases extracellular levels of both DA and glutamate in the NAC during reinstatement of rats that had previously self-administered the drug. However, when yoked cocaine or saline control subjects were administered a cocaine priming, only DA levels were elevated. Therefore, glutamate increased only when animals reinstated lever pressing for cocaine, whereas DA increased regardless of behaviour (McFarland et al., 2003). Despite these previous results, only a few studies have assessed the yoked-control paradigm to reliably dissect the neuroadaptative changes involved in learning to self-administer a drug of abuse, and those produced by the direct effect of the drug.

1.6.0.5 Gene Expression Changes

There are two mayor factors that can differentially modulate the changes in gene expression induced by drugs of abuse: 1) whether the administration is acute or chronic; and 2) if the administration is active or passive (Nestler, 2000; McClung and Nestler, 2008). Regarding the first aspect, *acute* drug administration triggers cellular responses that are generally short-lived, closely tied to the molecular site of drug action and similar to the changes identified in *in vitro* models of synaptic plasticity, including the induction of such as *c-fos* and *zif268*. In this respect, an acute MDMA non-contingent administration dose-dependently increases the expression of immediate early genes in different brain areas, such as *c-fos* in the medial PFC, caudate-putamen and NAC (Stephenson et al., 1999), and *egr-1* in the PFC, striatum and hippocampal dentate gyrus (Shirayama et al., 2000). Similarly, the *Rnd3* gene involved in actin cytoskeleton modulation and cell adhesion was up-regulated in the striatum of mice after an acute non-contingent MDMA administration (Marie-Claire et al., 2007). Using microarray technology, alterations in the expression of numerous genes in-

volved in the modulation of signaling pathways, transcription regulators, or xenobiotic metabolism have been demonstrated in the frontal cortex of rats following a single MDMA (20 mg/kg, i.p.) administration (Thiriet et al., 2002).

On the other hand, chronic drug administration induces changes in different genes than those reported after acute administration. For instance, Δ-FosB accumulates in the NAC after chronic, but not after acute exposure to several drugs of abuse, including opiates, cocaine, amphetamine, alcohol, nicotine and phencyclidine, regardless if the administration is active or passive (McClung et al., 2004). Chronic passively administered MDMA increased also Δ-fosB expression in mice (Olausson et al., 2006), and induced alterations in expression of glutamate transporters, as well as glutamate receptor subunits of AMPA, NMDA and metabotropic glutamate receptors (Kindlundh-Högberg et al., 2008). In addition, differential changes in brain areas related to reward have been observed after acute or repeated passively administered MDMA in pro-dynorphin and dynorphin A gene expression (Benedetto et al., 2006). Acute MDMA markedly increased pro-dynorphin mRNA levels in the PFC and in the caudate/putamen, while it decreased pro-dynorphin gene expression in the VTA. Chronic MDMA increased pro-dynorphin mRNA in the NAC, and decreased it in the VTA (Benedetto et al., 2006). However, in the same study dynorphin A levels increased after chronic treatment in the VTA and decreased after acute treatment in the NAC, PFC and hypothalamus (Benedetto et al., 2006).

Differential patterns of gene expression have also been reported depending on the administration of the drug, active (contingent) or passive (non-contingent). In this respect, *in situ* hybridization studies reported differential gene expression changes for active versus passive cocaine repeated administration in different brain areas. Thus, c-Fos increased more in self-administering mice in the lateral and BLA after the *first* cocaine self-administration session when compared to yoked animals. In contrast, c-fos mRNA increases in the dorsal striatum independently from the administration paradigm (Kuzmin and Johansson, 1999). Furthermore, the expression of *zif268* was lower in animals that self-administered cocaine during a 16 h period, in different brain areas such as NAC, hippocam-

pus, dorsal striatum and BLA, than in those that received the drug passively (Mutschler et al., 2000). These results suggest that acute cocaine self-administration seem to up-regulate gene-expression, which has been ascribed to *sensitization* processes, whereas repeated/chronic administration leads to a down-regulation in the animals that receive the drug actively as compared to yoked groups. This later effect has been related to *anticipatory counterbalance* processes that may at least in part underlie *tolerance* (Siegel et al., 2000; Ramos et al., 2002). In agreement, NMDA receptor subunit 1 was initially increased in different brain areas including NAC, caudate-putamen and medial PFC (Crespo et al., 2002) followed by a subsequent decrease in the self-administration group when compared to yoked animals.

The use of a yoked-control operant paradigm coupled with microarray studies has shown different profiles of gene transcript alterations in the NAC shell and core following contingent versus non-contingent heroin and cocaine administration. In both cases, transcripts in the NAC shell were mainly *down-regulated* in the self-administration group, whereas expression was mostly either unchanged or *up-regulated* in the yoked group (Jacobs et al., 2004, 2005, 2002) (see Figure 1.15). This result is interesting since the general pattern of gene expression seems to change as a function of whether the subject expects the drug or not. Thus, it is plausible that anticipatory conditioned compensatory responses could precipitate long-term changes in gene expression that will later have a repercussion on reward-related behaviours, as well as, on the effects induced by subsequent exposure to the drug. Currently, no study has evaluated the learning component associated with MDMA self-administration, as a critical factor affecting gene transcription.

1.6.0.6 Changes in Dopamine

Distinct enhancement of extracellular DA levels increase in the NAC appeared using different drugs of abuse with the yoked control-operant paradigm. DA changes using this paradigm were first reported by Hemby

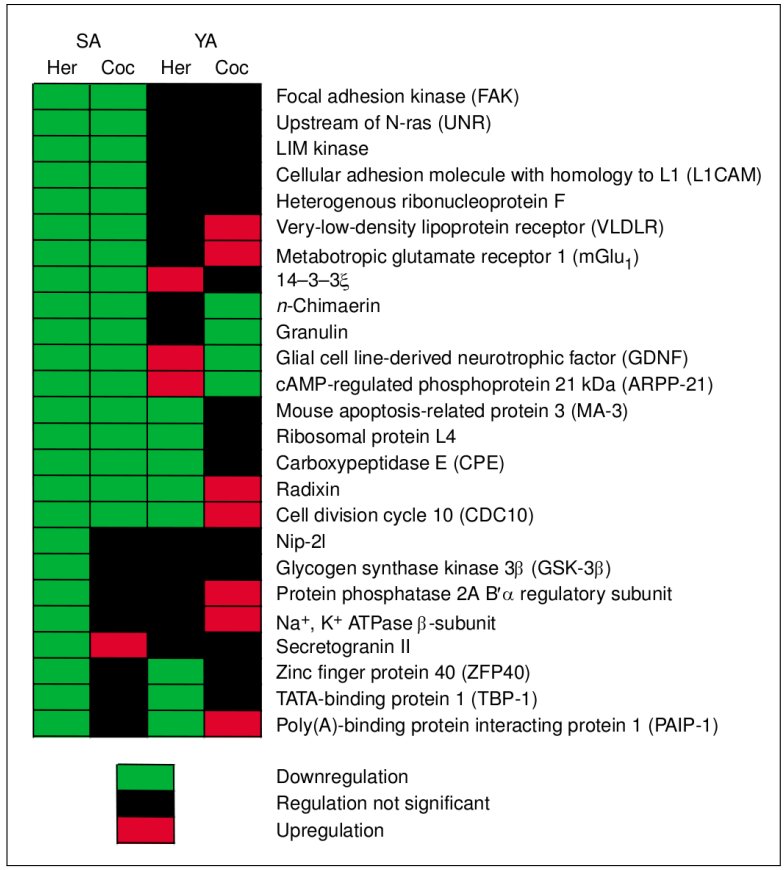

Figure 1.15: Changes in gene expression in the nucleus accumbens (NAC) shell after extinction of either active (SA) or passive (YA) administration of heroin (Her) and cocaine (Coc) (Taken from, Jacobs et al., 2003b).

et al. (1995). In this early study, passive intravenous heroin increases extracellular DA levels in the NAC, while the same dose self-administered does not modify DA levels. Later studies using similar paradigms have found that drugs of abuse can differentially affect NAC DA during acquisition, maintenance and early abstinence depending on whether the animal self-administers the drug or not (Jacobs et al., 2003a). Thus, during acquisition and maintenance, rats that self-administer cocaine exhibit a greater DA increase in the NAC shell as compared to the core during the first week of self-administration. In yoked animals, DA was also preferentially increased in the shell, but to a lesser extent than in animals self-administering

the drug. With continued exposure to the drug, the shell/core ratio of NAC DA decreased progressively and, after three weeks of self-administration, the ratio was reversed, so that DA increased more in the core than in the shell in both groups. However, in yoked animals this effect was faster than in self-administering rats. Thus, a differential biochemical sensitization at the level of the NAC core and shell has been reported depending on whether the drug is administered actively or passively (Lecca et al., 2007a). Similar results were reported by the same group using heroin in the same paradigm (Lecca et al., 2007b). These differences are in agreement with the predictive error reward function of the NA DA (see Section 1.1.2.2), and the transition of DA increase from ventral to dorsal striatum that occurs with repeated drug intake and is related to the habit learning role attributed to DA (Everitt and Robbins, 2005) (explained in Section, 1.2.2).

The effects of other drugs of abuse in similar (active *vs* passive administration) experimental designs have also been reported. Studies performed during *early abstinence* with d-amphetamine (Ranaldi et al., 1999; Di Ciano et al., 1996, 1998) have reported greater NAC DA extracellular levels in non-contingent subjects than in master rats, whereas studies performed with nicotine (Rahman et al., 2004) and cocaine (Zapata et al., 2003; Di Ciano et al., 1998) found no differences in NAC DA extracellular levels in response to a challenge of cocaine or nicotine respectively, 48 h after the last passive or active drug-administration. These differences may be due not only to distinct drug action mechanisms, but also to subtle differences in experimental conditions.

1.6.0.7 Changes in Receptor Binding Sites

Differential changes in neurotransmitter receptor binding sites have been reported using the yoked-operant paradigm. Autoradiography data show that rats self-administering methamphetamine for 5 weeks exhibit a marked decrease in DA D2 autoreceptors in the VTA and DA D1 receptors in the NAC shell 24 h after the last exposure (Stefanski et al., 1999). These decreases in the levels of DA D1 and D2 receptors did not occur in rats re-

ceiving either yoked injections of methamphetamine or saline. However, these changes did not persist in time since no changes were found in these receptors following 7- and 30-day periods of withdrawal from methamphetamine self-administration (Stefanski et al., 2002). Experiments using autoradiography techniques after cocaine self- or passive administration have shown that only passively administered cocaine produced a decrease in DA D2 receptor levels in the caudate/putamen areas, and in both the shell and core of the NAC (Stefanski et al., 2007). In contrast, when mRNA levels of D2 receptors were assessed using in situ hybridization, an increase of D2 receptor mRNA levels was found in the VTA of rats that actively self-administered cocaine and not in those that received it passively (Stefanski et al., 2007).

In the case of MDMA, a reduction of striatal DAT binding sites after passive high doses of MDMA has been reported in mice (Kindlundh-Högberg et al., 2007; Frederick et al., 1995). However, no study has assessed this question using active MDMA administration in mice. In contrast to mice, rats and monkeys present a decrease in serotonergic markers without alterations in the dopaminergic system after passive MDMA administration (Ricaurte et al., 1988; Mayerhofer et al., 2001; Frederick et al., 1998; Kindlundh-Högberg et al., 2007). A recent study has compared the effect of active versus passive MDMA administration on SERT binding sites. In this study, lower densities of SERT were found after 17 days of MDMA self-administration as compared with passively administered controls across different brain regions including the PFC, hippocampus, brain stem and striatum (Schenk et al., 2007). However, other studies in non human primates have failed to find changes in DA or 5-HT neurochemical profile after chronic self- (Fantegrossi et al., 2004c) or experimenter-administered MDMA (Frederick et al., 1995). No experiment has, so far, assessed the question of whether self- or experimenter-administered low doses of MDMA could specifically modify SERT and DAT brain binding sites in mice.

CHAPTER 2

Working Hypothesis

Given the information exposed in the previous sections, we propose that:

1. Non-contingent MDMA administration would reinstate drug-seeking behaviour in mice following extinction of MDMA and cocaine self-administration

2. MDMA administration would produce changes in the meso-accumbens dopaminergic system, and these changes can depend on whether the drug is administered actively or passively.

3. Active MDMA administration will produce gene expression changes in brain structures related to reward learning, that can be dependent on the contingency of drug administration.

4. The serotonergic system through 5-HT2*A*R will modulate the reinforcing properties of MDMA, as well as priming- and cue-induced reinstatement to MDMA-seeking behaviour.

5. 5-HT2*A*R, will participate in MDMA reinforcement and relapse to MDMA-seeking behaviour by modulating dopaminergic activity in the NAC and 5-HT and NE in the PFC.

CHAPTER 3

Objectives

1. To evaluate the efficacy of a priming injection of d-amphetamine or MDMA in reinstating cocaine-seeking behaviour in a mouse model of relapse **(Article 1)**.

2. To evaluate the effect of active and passive MDMA administration on DA extracellular levels in the NAC before and after a challenge dose of MDMA (**Article 2**).

3. To evaluate whether contingent- or yoked-administration of low doses of MDMA can produce alterations in dopamine and serotonin transporters using receptor autoradiography techniques (**Article 2**).

4. To determine gene expression changes that underlie MDMA seeking behaviour in comparison with gene expression due to the direct effect of the drug using a combined yoked-operant control paradigm with microarray techniques (**Article 3**).

5. To investigate the involvement of the 5-HT_{2A}R in the reinforcing properties of MDMA and in the cue- and priming-induced reinstatement of MDMA-seeking behaviour (**Article 4**).

6. To determine the extracellular levels of DA in the NAC, and 5-HT and NE in PFC in 5-HT_{2A}R KO mice before and after a challenge of MDMA by using microdialysis technique (**Article 4**).

CHAPTER 4

Results

4.1 Article 1, Trigo *et al* 2009

Aim: to examine whether priming injections of MDMA or d-amphetamine would reinstate previously extinguished responding for cocaine in mice

Results: acute MDMA, but not d-amphetamine or saline reinstated cocaine-seeking behaviour in mice in which cocaine selfadministration and contingent cues were previously extinguished.

Conclussions: acute MDMA can reinstate cocaine-seeking behaviour in mice.

Trigo JM, Orejarena MJ, Maldonado R, Robledo P. MDMA reinstates cocaine-seeking behaviour in mice. Eur Neuropsychopharmacol. 2009; 19 (6): 391-7.

4.2 Article 2, Orejarena *et al* 2009

Aim: to evaluate basal and 3,4-methylenedioxymethamphetamine (MDMA)-stimulated DA levels in the NAc of mice that had previously received contingent and non-contingent infusions of MDMA.

Results: Animals receiving MDMA infusions showed significantly lower basal DA levels than the yoked saline group. A reduced activation of DA was observed following MDMA in contingent mice with respect to both yoked MDMA and saline mice. No significant alterations in DA transporter or serotonin transporter were observed in the three groups of mice.

Conclussions: prolonged exposure to MDMA in mice produces changes in basal DA levels after drug withdrawal and a decreased neurochemical response at the level of the mesolimbic DA reward pathway that is, in part, related to instrumental learning during selfadministration.

Orejarena MJ, Berrendero F, Maldonado R, Robledo P. Differential changes in mesolimbic dopamine following contingent and non-contingent MDMA self-administration in mice. Psychopharmacology (Berl). 2009; 205(3): 457-66.

4.3 Article 3, Fernàndez-Castillo *et al* 2009 (submitted)

The supplementry material for this paper can be found in Appendix A, page 219

Aim: to compare the consequences of active and passive MDMA administration on gene expression in the mouse brain at transcriptomic level since all previous studies were based on passive MDMA administration.

Results: the hippocampus was the brain area with the largest changes in gene expression profiles when comparing contingent MDMA versus yoked MDMA administration, which may be related to learning self-administration behaviour. The expression of genes involved in neuroadaptative changes was also modified in the dorsal raphe nucleus. Comparing MDMA and yoked MDMA versus yoked saline mice, we also identified differential expression in genes with diverse functions, but most of the changes were observed in immunological and inflammatory genes, which pinpoints to direct effects of the drug on the brain transcriptome.

Conclussions: both hippocampus and dorsal raphe nucleus are involved in active MDMA seeking behaviour and identifies specific alterations on gene expression that support the addictive potential of this drug.

Active and Passive MDMA ('ecstasy') Intake Induces Differential Transcriptional Changes in the Mouse Brain

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Running title: MDMA-induced transcriptional changes in the mouse brain

Keywords: Addiction, MDMA, ecstasy, transcriptomics, mouse brain, gene expression

ABSTRACT

Background: 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") is a recreational drug widely used by adolescents and young adults. Although its rewarding effects are well established, there is still controversy on its addictive potential. The aim of the present study was to compare the consequences of active and passive MDMA administration on gene expression in the mouse brain at transcriptomic level since all previous studies were based on passive MDMA administration.

Methods: We used a yoked-control operant intravenous self-administration paradigm combined with microarray technology. Transcriptomic profiles of hippocampus, ventral striatum, frontal cortex and dorsal raphe nucleus were analyzed in 27 mice divided in contingent MDMA, yoked MDMA and yoked saline groups, and the observed changes were validated by quantitative RT-PCR.

Results: The hippocampus was the brain area with the largest changes in gene expression profiles when comparing contingent MDMA versus yoked MDMA administration, which may be related to learning self-administration behaviour. The expression of genes involved in neuroadaptative changes was also modified in the dorsal raphe nucleus. Comparing MDMA and yoked MDMA versus yoked saline mice, we also identified differential expression in genes with diverse functions, but most of the changes were observed in immunological and inflammatory genes, which pinpoints to direct effects of the drug on the brain transcriptome.

Conclusions: Our study suggests that both hippocampus and dorsal raphe nucleus are involved in active MDMA seeking behaviour and identifies specific alterations on gene expression that support the addictive potential of this drug.

INTRODUCTION

3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) is a drug used around the world in recreational settings by adolescents and young adults. MDMA induces euphoria and well being in humans (1), and its rewarding/reinforcing effects have been well established in animal models (2). Although the addictive potential of this substance is still a matter of debate, there is evidence showing that a high proportion of MDMA users meet the *Diagnostic and Statistical Manual of Mental Disorders* (DSM) criteria for dependence (3-5). MDMA acutely increases brain levels of dopamine (DA), serotonin (5-HT) and noradrenalin (NE) in monkeys, rats and mice by potently inhibiting neurotransmitter uptake mechanisms (6). Repeated administration of MDMA in human produces long-term neuropsychological disorders, including anxiety and mood alterations, as well as cognitive deficits (7), which may be associated with persistent neuroadaptations dependent on changes in gene expression.

Studies in laboratory animals have shown that single or repeated administration of MDMA can induce changes in gene expression similar to what has been observed following treatment with other psychostimulants such as cocaine, amphetamine or methamphetamine (8-10). Acute administration has been reported to dose-dependently increase the expression of several immediate early genes in different brain structures such as c-fos (11) and Egr-1 (12). Similarly, the Rnd3 gene involved in actin cytoskeleton modulation and cell adhesion was up-regulated in the striatum of mice after acute MDMA administration (13). Repeated treatment with MDMA increased deltaFosB expression in mice (14), and induced pronounced alterations in gene-transcript expression of glutamate transporters, as well as AMPA, NMDA and metabotropic glutamate receptor subunits in different brain regions in rats (15). In addition, changes in pro-dynorphin (PDYN) and pro-enkephalin (PENK) gene expression have been observed in several brain areas of rats treated either acutely or repeatedly with MDMA (16, 17). Using microarray technology, alterations in the expression of numerous genes involved in the modulation of

signalling pathways, transcription regulators, or xenobiotic metabolism have been demonstrated in the frontal cortex of rats following a single MDMA administration (18). Although these data provide evidence for the effects of non-contingent administration of MDMA on gene expression in the brain, there are no studies available using models of MDMA operant self-administration, which are more relevant to the human pattern of drug consumption. In this sense, the use of a yoked-control operant intravenous self-administration paradigm coupled with microarray studies have shown different profiles of gene transcript alterations in the nucleus accumbens shell and core comparing contingent versus non-contingent heroin and cocaine administration (19, 20), which suggests that the learning component associated with active drug taking is a critical factor affecting changes in gene transcription.

This study was designed to compare changes in gene expression in different brain structures including the ventral striatum, dorsal raphe nucleus, hippocampus and prefrontal cortex in mice receiving repeated contingent or non-contingent administration of MDMA in order to better understand the long-term consequences of MDMA seeking behaviour and consumption.

MATERIALS AND METHODS

Animals

Male C57Bl/6J mice weighing 20–24 g at the beginning of the experiments were initially housed five per cage in a room with controlled temperature (21 ± 1 °C) and humidity (65 ± 10%), with a reversed light/dark cycle (lights off from 08:00 to 20:00 hours), and with *ad libitum* food and water. The experiments took place during the dark phase. Behavioural tests and animal care were conducted in accordance with the standard ethical guidelines (National Institutes of Health, 1995; European Communities Directive 86/609 EEC) and approved by the local ethical committee (CEEA-PRBB).

Drugs

MDMA hydrochloride was obtained from Lipomed, A.G. (Arlesheim, Switzerland) and dissolved in sterile 0.9% physiological saline solution.

Surgery and self-administration procedure

Mice were anesthetized with an intraperitoneal (i.p.) injection of a ketamine/xylazine mixture (5:1; 0.10 ml/10 g) and then implanted with an indwelling intravenous (i.v.) silastic catheter in the right jugular vein, as previously described (21). The animals were pre-treated with ketoprofen 5 mg/kg subcutaneously (s.c.) for post-surgery analgesia. After surgery, the mice were housed individually for the remainder of the experiments. In order to avoid clots and infection, the animals were flushed through the catheter with (0.02 ml) of a solution containing heparin (30 UI/ml), cefazoline (50 mg/ml) and sodium chloride (0.09%) for 5 days. The patency of the catheters was evaluated once a week by the injection of 0.1 ml of thiopental (5 mg/ml). If prominent signs of anaesthesia were not apparent within 3 s of the infusion, the mouse and its corresponding data were removed from the experiment. Three days after surgery, the animals were randomly assigned to either contingent or yoked groups.

Contingent mice were trained to self-administer MDMA (0.125 mg/kg/infusion delivered in a volume of 23.5 µl over 2 s) in single daily 3-h sessions. Acquisition of drug self-administration was performed using a fixed ratio 1 (FR1) schedule of reinforcement such that one nose poke in the active hole resulted in one MDMA infusion, while nose poking in the inactive hole had no programmed consequences. Mice had to achieve all of the following conditions to be included in the analysis: (i) less than 20% deviation from the mean of the total number of infusions earned in three consecutive sessions (80% stability), (ii) at least 65% responses at the active hole, and (iii) a minimum of five infusions earned per session. Each contingent mouse was connected to two yoked mice; one receiving an identical dose of MDMA (yoked MDMA and the other a saline solution (yoked saline). When a contingent mouse had a failed catheter or did not meet the acquisition criteria, the corresponding yoked mice were discarded from the study. A light stimulus, located above the active hole, was paired with the delivery of the drug or saline according to the response of the contingent mouse. To avoid interference by acute transcriptional changes, animals were sacrificed eight hours after the last exposure to the self-administration boxes. The brains were quickly removed, and the following brain areas were dissected according to Franklin and Paxinos (22): frontal cortex, hippocampus, ventral striatum and dorsal raphe nucleus. Brain tissues were then frozen by immersion in 2-methylbutane surrounded by dry ice, and stored at -80 ºC for later quantification of gene expression.

RNA isolation and microarray hybridization

Twenty-seven mice (9 animals per group of contingent, non-contingent and saline) and four brain areas (hippocampus, ventral striatum, frontal cortex and dorsal raphe nucleus) were used in the expression microarray study. Three pools of three mice were performed for each experimental group (contingent MDMA, yoked MDMA and yoked saline) by minimizing the variance of the number of nose pokes in each pool. The pooled individuals were the same for all brain regions. Samples from all tissues were homogenized using the TissueRuptor system (Qiagen) and total RNA was isolated using the RNeasy

Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and integrity was evaluated using the Bioanalyzer 2100 platform (Agilent Technologies). RNA samples were stored at -80ºC until analyzed. Frontal cortex and dorsal raphe nuclei were pooled before RNA extraction, while hippocampus and ventral striatum RNA was isolated from each tissue sample separately and then pooled for the analysis. For the microarray experiment, we used the GeneChip® Mouse Expression Set 430 array (Affymetrix), which contains probes that cover over 39,000 transcripts and variants from over 34,000 well-characterized genes. A total of 36 chips were used: three pools of three individuals per condition (contingent, MDMA, yoked MDMA and yoked saline) and four brain areas. Two μg of RNA from each pool were used to hybridize arrays at the Genomics Unit of Hospital Clínic-IDIBAPS (Barcelona). Chips were scanned using a GenePix4000B scanner and raw data was obtained using the GenePix Pro 4.0 and GCOS softwares.

Quantitative RT-PCR

To confirm the most relevant results obtained in the microarray study, total RNA from the four brain regions of contingent, non-contingent and saline mice was reverse-transcribed using the High capacity cDNA Reverse Transcription Kit (Applied Biosystems). The Mouse Endogenous Control Array (Applied Biosystems) was used to select endogenous controls. Real Time-PCR experiments on 44 genes in hippocampus were perfomed using Custom Taqman Array Plates (Applied Biosystems) and the ABI Prism 7900 HT system (Applied Biosystems). Second-step Real Time-PCR experiments were performed for 15 genes using LightCycler 480 II system and the Universal Probe Library (Roche Applied Science). Gene assays were designed using the Universal ProbeLibrary Assay Design Center software (Roche Applied Science, www.roche-applied-science.com). Sequence of the primers and probes used are available upon request. Beta-actin (*Actb*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were used to normalize the relative amounts of mRNA.

Statistical and bioinformatic analyses

The self-administration data was analyzed using a three-way repeated measures ANOVA with group as a between subject factor and hole and day as within subjects factors followed by post-hoc tests for individual comparisons when appropriate. Statistical significance was set at $p < 0.05$.

For the microrray data, we used the Bioconductor software for R environment and the *affy* library (www.bioconductor.org) (23). The quality assessment of the chips was performed using the *affyPLM* library. Background correction, normalization and summarization were performed using the background, Robust Multichip Average (RMA) (24) and median-polish methods respectively. For gene filtering we discarded those probes that did not correspond to known genes and considered a threshold of log2(60) for signal filtering and IQR>25% for variability filtering. The Linear Modeling for Microarray Analysis (LIMMA) package (25) was used for class comparison, by which we compared the expression patterns of the pairs contingent-non contingent, contingent-saline, and non contingent-saline. Correction for multiple testing was achieved by adjusting the p-value with a False Discovery Rate (FDR) of 5%. Functional grouping analysis of genes with significant differential expression was performed using the DAVID Annotation Tool (david.abcc.ncifcrf.gov) (26) and was supported by literature searches.

In the quantitative RT-PCR experiments, gene expression changes for each comparison were evaluated using a U-Mann-Whitney non-parametric test, and statistical significance was set at $p < 0.05$.

RESULTS

MDMA self-administration

The average number of active and inactive nose pokes carried out by contingent mice trained to self-administer MDMA (0.125 mg/kg per infusion) as well as for yoked MDMA and yoked saline mice is shown in figure 1. Seventy percent of the contingent mice met all the acquisition criteria within a short time period (8 ± 0.76 days), and showed a mean cumulative intake of 19.7 ± 1.62 mg/kg of MDMA during the entire training period. Saline- or MDMA-yoked animals did not discriminate between holes on any of the training sessions. Eleven training sessions were performed until all contingent mice reliably acquired MDMA self-administration behaviour. An additional session was carried out in order to increase MDMA exposure. Three-way repeated measures ANOVA comparing responses in the active and inactive holes for all groups during the entire testing period revealed a significant main effect of group [$F(2,24) = 80.600$, $p < 0.001$], a significant main effect of hole [$F(1,24) = 77.770$, $p < 0.001$], a significant group x hole interaction [$F(2,24) = 77.498$, $p < 0.001$], and a significant group x day interaction [$F(20,240) = 1.969$, $p < 0.01$]. Subsequent Bonferroni post-hoc analysis revealed significant differences between contingent mice versus both yoked groups ($p < 0.001$). Discrimination between holes was significant only in the contingent MDMA group from day 1 through day 11 (Table S1).

MDMA-induced transcriptional changes

To assess possible transcriptional changes caused by active or passive MDMA administration, gene expression profiles in the four brain areas (hippocampus, frontal cortex, ventral striatum and dorsal raphe nucleus) from contingent MDMA, yoked MDMA and yoked saline mice were compared between the three possible pairs of experimental situations using microarray technology. This study design allowed to identify genes involved in the cognitive processes related to active MDMA self-

administration (those differentially expressed in the contingent MDMA–yoked MDMA comparison and in the contingent MDMA-yoked saline comparison) as well as genes modulated by the direct pharmacological effect of MDMA on the brain (those differentially expressed in both the contingent MDMA-yoked saline and yoked MDMA-yoked saline comparisons).

Contingent MDMA self administration versus yoked MDMA: drug reinforced learning

When gene expression levels were compared between contingent MDMA and yoked MDMA mice, significant differences (5% FDR) were observed only in hippocampus (n = 945) and dorsal raphe nucleus (n = 1; Figure 2A). Among them, 537 genes were also identified when hippocampus of contingent MDMA and yoked saline mice were compared and, thus, were assumed to be more consistent for further analysis (Table S2). None of these 537 genes showed a differential expression greater or lower than 2-fold (that means ±1 of Log Fold Change; Fig. 2B, Table S2). We then narrowed down the selection of genes according to functional criteria. Functional clustering was performed with DAVID and targeted at some interesting neurological functions, such as synapse, synaptosome, neurotransmitter secretion, regulation of neurotransmitter levels, nervous system development, neuron projection, axononogenesis, axon guidance, dendrite, neurite morphogenesis, neuron morphogenesis, neuron differentiation and learning and memory (Table S3). Based on this functional information, we selected 44 genes for further validation with qRT–PCR (Table 1), but no significant differences were observed for any of them.

Given these results, we followed an alternative approach for all brain structures and focused on functional candidate genes that showed a Log Fold Change of ± 1.5 in the contingent MDMA versus yoked MDMA comparison, without considering statistical significance (Table S4). Five genes were identified in the dorsal raphe nucleus: *Camk2a*, *Ddn*, *Egr3*, *Kalrn* and *Zic1* (Table 2). All of them, except for *Zic1,* were validated by qRT-PCR in this brain structure, and were shown to be significantly upregulated in contingent mice when normalized to *Actb* ($p<0.05$, Table 2) and *Gapdh* (data not

shown). Interestingly, *Ddn* and *Egr3* were also differentially expressed in the same direction in the contingent MDMA-yoked saline comparison (Table 2).

Active and passive MDMA administration versus saline: direct effect of the drug

The yoked MDMA-yoked saline comparison displayed differentially expressed genes in the four brain structures studied, ranging from 503 in hippocampus to 1340 in ventral striatum. Some of these particular genes were also identified in the contingent MDMA-yoked saline comparison, ranging from 16 in dorsal raphe nucleus to 192 in hippocampus (Fig. 2A, Tables S5-S8). Genes that were positive in the two comparisons were functionally grouped using DAVID, and similar clusters were obtained in all brain regions, the most significant ones being those involved in immune or inflammatory response, as well as response to wounding or to stress (Fig. 3). Subsequently, we selected 10 genes with a Log Fold change over 1.5 or below -1.5 for further validation: three genes involved in neuronal processes, *Sgk1* and *Sgk3* in dorsal raphe nucleus and *Slc17a7* in ventral striatum, and seven genes related to immunological functions*; Lcn2,* that was differentially expressed in all four brain regions, and *Ctla2a*, *Gbp2*, *Igtp*, *Iigp1*, *Iigp2* and *Tgtp,* that were identified both in hippocampus and frontal cortex (Table 3). qRT-PCR experiments validated the results of the microarray analysis, confirming gene overexpression caused by active and passive MDMA intake in these particular brain structures, with the exception of *Sgk3* and *Iigp2* in the dorsal raphe nucleus and hippocampus, respectively (Table 3).

DISCUSSION

In the present study, our aim was to identify alterations in brain gene expression due to neuroadaptative changes underlying the learning process associated with operant MDMA self-administration, as well as changes due to the pharmacological effect of the non contingent administration of the drug. For that purpose we have validated a new operant paradigm consisting in master mice that are trained to acquire a stable operant behaviour to self-administer a reinforcing dose of MDMA (21). Each master mouse is connected to an MDMA yoked animal that passively receives an identical dose of MDMA and to another yoked mouse that receives saline infusions. This yoked-control operant intravenous self-administration paradigm was combined with microarray technology. The results of this experimental design suggested that (i) hippocampus and dorsal raphe nucleus may play an important role in the neuroadaptative changes leading to active MDMA seeking behaviour; and (ii) MDMA modulates the expression of genes involved in neuronal function and immune response in different brain areas.

When brain expression profiles of contingent MDMA and yoked MDMA mice were compared, hippocampus, a crucial area for the control of memory and cognitive functions, was the brain structure showing the highest number of statistically significant changes in gene expression. Interestingly, these results are consistent with previous studies performed with the conditioned place preference (CPP) paradigm (27, 28), which suggest that cocaine-induced CPP depends on molecular changes that occur in hippocampus of trained rats. Additionally, in agreement with its contribution to the active self-administration of MDMA, we found that approximately half of the genes identified were also differentially expressed between contingent MDMA and yoked saline mice in the microarray data and most of them, such as *Ntrk2*, *Camk2a*, *Gsk3* and *Akt1,* play relevant roles in neuroadaptation. Nevertheless, although the majority of the differentially expressed genes were identified in hippocampus, the relationship between this brain region and learning processes related to active drug administration is less straightforward than expected since the changes observed in gene expression

were always subtle. These significant differences in the microarray experiments could not be subsequently validated by qRT-PCR in the 44 genes that were selected on the basis of their function out of 537 statistically significant hits. The lack of validation could be explained by a limited sample size that precludes detection of small differences.

In a second stage, a more stringent threshold in expression differences was considered in the microarray experiments (Log Fold Change over 1.5 or below -1.5) without considering statistical significance. This analysis identified the dorsal raphe nucleus as the most interesting brain region showing functionally relevant changes in gene expression between contingent MDMA and yoked MDMA groups. These results are supported by a recent study demonstrating that many neurons in the monkey dorsal raphe nucleus encode expected and received rewards (29), though it remains to be determined whether these signals are used for the reward-based modulation of motor behaviour or learning. Based on their function, we chose five of the differentially expressed genes in dorsal raphe nucleus for follow-up studies, and changes in *Camk2a*, *Kalrn*, *Ddn*, and *Egr3* were validated by qRT-PCR. Interestingly, although none of them had remained statistically significant under 5% FDR in the previous microarray study, all indeed passed a less restricting FDR threshold of 11%. These four genes, upregulated in the MDMA contingent group as compared to the yoked MDMA mice, are involved in neuroplasticity and neuron remodelling processes that may occur in the dorsal raphe nucleus as a consequence of learning the active MDMA self-administration behaviour. The *Camk2a* gene encodes the Ca2+/calmodulin-dependent protein kinase II alpha (CaMKIIalpha), which mediates activity-dependent synaptic plasticity. A recent study demonstrated its essential role in dendritic spine enlargement, long-term potentiation and learning (30). Dendritic spine morphogenesis is also induced by kalirin-7, an isoform encoded by the *Kalrn* gene (31). In addition, another study links these two proteins with the same signalling pathway that controls functional and structural spine plasticity (32). In this regard, the NMDA glutamate receptor activation in pyramidal neurons causes CaMKII-dependent phosphorylation of kalirin-7 leading to a rapid enlargement of existing spines. Kalirin is also

involved in neurite outgrowth through the nerve growth factor (NGF) signalling pathway (33). On the other hand, the *Ddn* gene encodes a dendritically localized mRNA that is translated to the protein dendrin, potentially involved in neuroplasticity events and modulation of post-synaptic cytoskeleton (34). In addition, *Egr3* is a member of the Egr gene family, a group of synaptic activity-inducible immediate early genes involved in neuroplasticity related to memory and learning (35-37). Interestingly, the best characterized gene of the family is *Egr1*, and its expression is increased by MDMA in rat prefrontal cortex, striatum and hippocampal dentate gyrus (12). To our knowledge, none of these genes have been so far involved in the acquisition of an operant behaviour to obtain a drug of abuse.

When comparing the contingent MDMA and yoked MDMA mice to the yoked saline mice, we also identified upregulation of genes involved in neuroadaptations and synaptic plasticity, indicating a direct consequence of exposure to MDMA. The *Sgk1* and *Sgk3* genes, which encode the serum/glucocorticoid regulated kinase 1 and 3, respectively, were identified in the dorsal raphe nucleus. Both are involved in memory consolidation (38) and regulate glutamatergic neurotransmission (39-44). In addition, Sgk1 increases neurite formation and dendrite growth (45, 46) Interestingly, *Slc17a7,* encoding the vesicular glutamate transporter 1 (Vglut1), was also upregulated in ventral striatum. Vglut1 is involved in synaptic plasticity and plays an important role in excitatory transmission (47). In this regard, a recent study described expression changes in several glutamine transporters and receptors after repeated MDMA administration (15), which is consistent with our findings. The results obtained on *Sgk1*, *Sgk3* and Slc17a7 were all validated. Although not validated, microarray data also show expression differences in other genes involved in neuroadaptations, such as the glutamate receptor *Grin1* or brain-derived neurotrophic factor (*Bdnf*), which are modulated by MDMA exposure (15, 48). Results regarding *Bdnf*, however, are contradictory as the previous study reported increased expression of the gene in rat frontal cortex after acute MDMA administration, while we observed downregulation of *Bdnf* in the same brain area.

Although we identified differential expression of genes involved in neuronal functions in the contingent MDMA/yoked MDMA versus yoked saline comparisons, most of the hits correspond to genes involved in immunological or inflammatory response, supporting new evidence for the neurotoxic effects of MDMA. Among them, we identified a strong overexpression of *Lcn2* in all the brain regions. The *Lcn2* gene encodes lipocalin2 that mediates astrocytosis under inflammatory conditions and is induced after chronic or thermal stress in brain reward regions (49-51). The results obtained after repeated MDMA acquisition in the present study, however, differ from those obtained after acute MDMA exposure in cortex (18), where mainly serotonin receptors, transcription factors, cytoskeletal and metabolic genes were differentially expressed.

In summary, the use of this yoked-control operant intravenous self-administration paradigm pointed to hippocampus and dorsal raphe nucleus as important brain regions involved in the learning behaviour associated with active MDMA self-administration. In addition, MDMA exposure induces the expression of genes related to neurotoxicity, inflammatory and immunological response in all the brain structures investigated.

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None of the authors reported any biomedical financial interests or potential conflicts of interest.

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FIGURE LEGENDS

Figure 1. Active and inactive nose-pokes in mice responding contingently for intravenous infusions of MDMA (0.125 mg/kg/infusion (A) (n = 9), in yoked MDMA mice (B) (n = 9), and in yoked saline mice (C) (n = 9). The data represent means + SEM nose pokes in 3 h sessions during the acquisition period. The asterisks denote significant differences between active and inactive nose-pokes for each training day. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (one-way ANOVA).

Figure 2. Differentially expressed genes in four brain regions in mice that self-administer MDMA (contingent MDMA), mice that receive the drug passively (yoked MDMA) and mice receiving a saline solution (yoked saline), identified thorough transcriptomic microarray analysis. (A) Venn Diagrams of the microarray data showing statistically significant genes (FDR < 5%) differentially expressed between contingent MDMA-yoked MDMA (con-non con), contingent MDMA-yoked saline (con-sal) and non contingent MDMA-yoked saline (non con-sal). (B) Volcanoplots of the contingent MDMA-non-contingent MDMA comparison showing the significance (logOdds) and the Log Fold Change. Significance threshold (FDR < 5%) is represented by a horizontal line.

Figure 3. Top ten of the most significant overrepresented biological categories that showed differential expression after exposure to MDMA (contingent MDMA and yoked MDMA mice versus yoked saline). The number of positive genes included in each category is indicated on the right side of each bar. Biological categories correspond to the following Gene Ontology (GO) terms: GO:0002376 (immune system process), GO:0006955 (immune response), GO:0006952 (defense response), GO:0006954 (inflammatory response), GO:0009611 (response to wounding), GO:0009605 (response to external stimulus), GO:0006950 (response to stress), GO:0005576 (extracellular region), GO:0005886 (plasma membrane) and GO:0048518 (positive regulation of biological process).

FIGURES

Figure 1

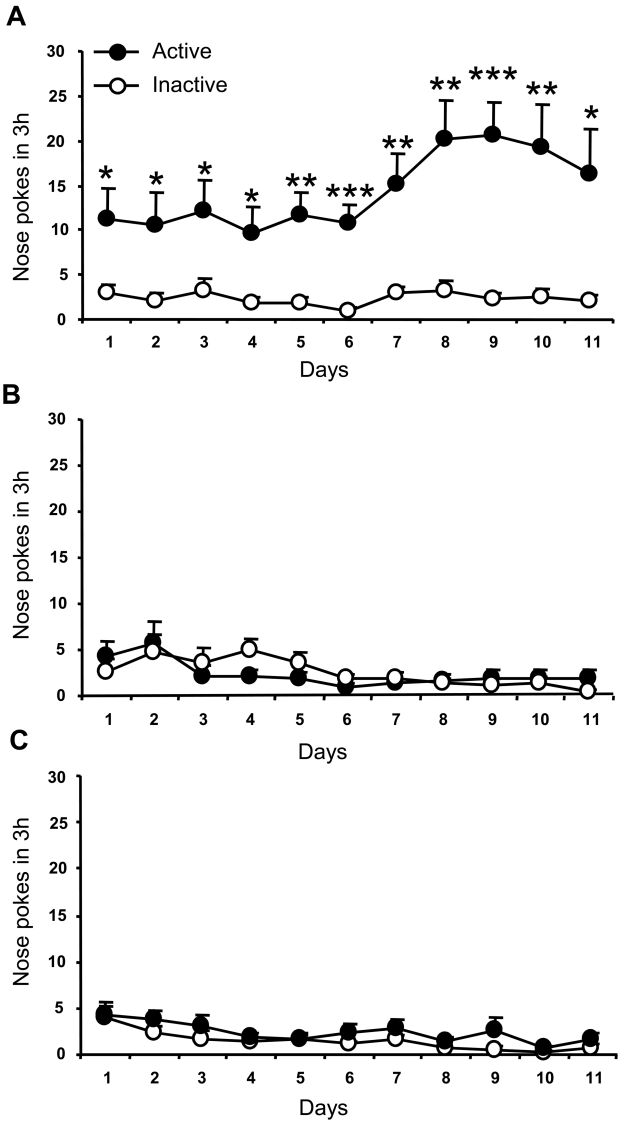

Figure 2

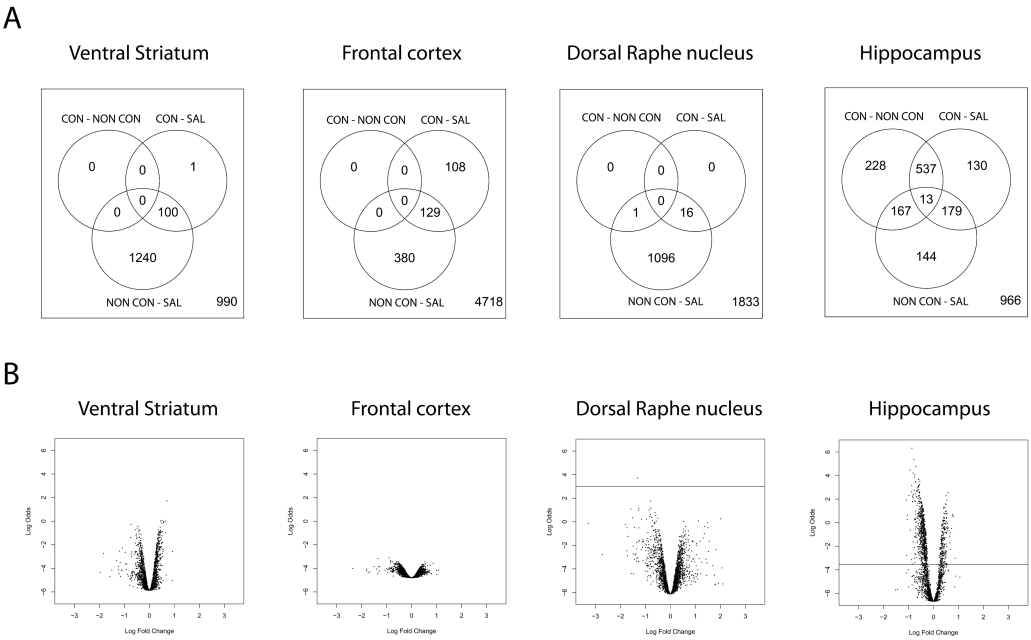
A
Ventral Striatum
Frontal cortex
Dorsal Raphe nucleus
Hippocampus
CON - NON CON
CON - SAL
NON CON - SAL
0
1
100
1240
990
108
129
380
4718
16
1096
1833
228
537
130
13
167
179
144
966
B
Log Odds
Log Fold Change

Figure 3

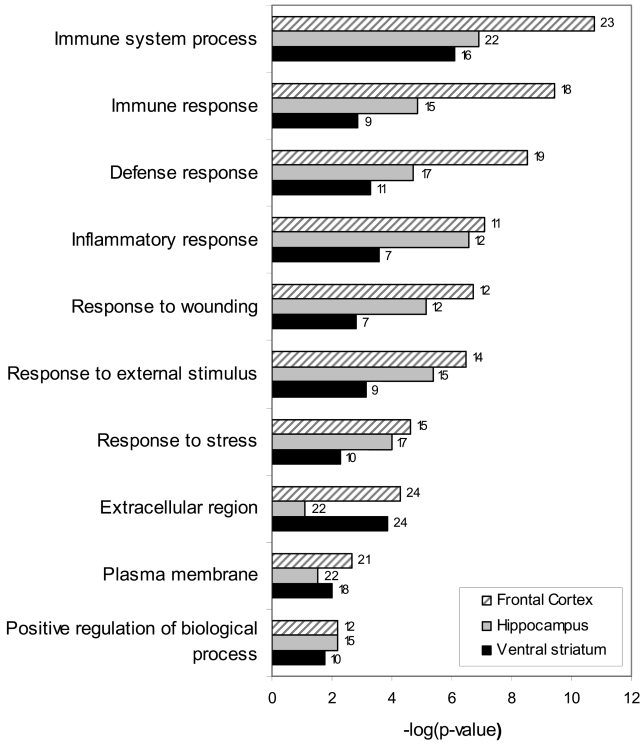
Immune system process
23
22
16
Immune response
18
15
9
Defense response
19
17
11
Inflammatory response
11
12
7
Response to wounding
12
12
7
Response to external stimulus
14
15
9
Response to stress
15
17
10
Extracellular region
24
22
24
Plasma membrane
21
22
18
Positive regulation of biological process
12
15
10
Frontal Cortex
Hippocampus
Ventral striatum
0
2
4
6
8
10
12
-log(p-value)

Table 1. Genes differentially expressed in hippocampus (5% FDR) selected for qRT-PCR validation after functional clustering.

Gene Symbol	Gene Name	Contingent-Noncontingent Fold Change	Contingent-Saline Fold Change
Downregulated			
Ablim1	actin-binding LIM protein 1	-1,25	-1,29
Adora1	adenosine A1 receptor	-1,19	-1,25
Adcyap1	adenylate cyclase activating polypeptide 1	-1,38	-1,39
Amigo1	adhesion molecule with Ig like domain 1	-1,21	-1,25
Adrbk1	adrenergic receptor kinase, beta 1	-1,38	-1,29
Acin1	apoptotic chromatin condensation inducer 1	-1,30	-1,26
Axin2	axin2	-1,26	-1,31
Bzrap1	benzodiazapine receptor associated protein	-1,40	-1,17
*Camk2a**	calcium/calmodulin-dependent protein	-1,78	-1,77
Clstn3	calsyntenin 3	-1,34	-1,35
Chrm1	cholinergic receptor, muscarinic 1, CNS	-1,30	-1,33
Chrnb2	cholinergic receptor, nicotinic, beta	-1,27	-1,29
Cplx2	complexin 2	-1,31	-1,22
Dpysl5	dihydropyrimidinase-like 5	-1,23	-1,20
Dbn1	drebrin 1	-1,35	-1,36
Evl	Ena-vasodilator stimulated phosphoprotein	-1,36	-1,31
Faim2	Fas apoptotic inhibitory molecule 2	-1,31	-1,25
*Gprin1***	G protein-regulated inducer of neurite	-1,51	-1,40
Grik5	glutamate receptor, ionotropic, kainate 5	-1,56	-1,32
*Grin1***	glutamate receptor, ionotropic, NMDA1 (zeta	-1,49	-1,33
Grin2a	glutamate receptor, ionotropic, NMDA2A	-1,30	-1,22
Grin2b	glutamate receptor, ionotropic, NMDA2B	-1,23	-1,35
Gsk3b	glycogen synthase kinase 3 beta	-1,22	-1,20
Hrh3	histamine receptor H3	-1,42	-1,25
Hap1	huntingtin-associated protein 1	-1,65	-1,32
Jund	Jun proto-oncogene related gene d	-1,33	-1,20
Junb	Jun-B oncogene	-1,44	-1,27
Madd	MAP-kinase activating death domain	-1,23	-1,34
Mt3	metallothionein 3	-1,64	-1,49
Mapk8ip	mitogen-activated protein kinase 8	-1,26	-1,26
Mapk8ip	mitogen-activated protein kinase 8	-1,44	-1,31
Nlgn2	neuroligin 2	-1,33	-1,25
Ntrk2	neurotrophic tyrosine kinase, receptor, type	-1,30	-1,22
Nos1	nitric oxide synthase 1, neuronal	-1,42	-1,48
Pde1b	phosphodiesterase 1B, Ca2+-calmodulin	-1,40	-1,21
Prkce	protein kinase C, epsilon	-1,27	-1,22
*Sort1***	sortilin 1	-1,32	-1,38
Syp	synaptophysin	-1,30	-1,30
Syt3	synaptotagmin III	-1,40	-1,27
Stxbp1	syntaxin binding protein 1	-1,27	-1,24
Akt1	thymoma viral proto-oncogene 1	-1,46	-1,30
*Vamp2***	vesicle-associated membrane protein 2	-1,52	-1,62
*Vgf**	VGF nerve growth factor inducible	-1,36	-1,47
Upregulated			
*Kcnq2***	potassium voltage-gated channel, subfamily	1,25	1,35

* Probe failure; ** Genes with differential expression following the same direction in more than one probe set. The smallest absolute fold change is shown.

Table 2. Effect of active MDMA self-administration: qRT-PCR validation of microarray data of five genes showing a Log Fold Change of ±1.5 in dorsal raphe nucleus

		Contingent-non contingent			Contingent- saline		
		Microarray		qRT-PCR	Microarray		qRT-PCR
Gene Symbol	Gene name	Fold Change	P-value (Adj P value)	Fold Change	Fold Change	P-value (Adj P value)	Fold Change
Camk2a	Calcium/calmodulin-dependent protein kinase II alpha	**2.8**	0.01 (0.11)	**2.3 ***	-	NS	**NS**
Ddn	Dendrin	**3.5**	8 e-3 (0.11)	**5.5 ***	**3.3**	0.01 (0.09)	**5.3 ***
Egr3	Early growth response 3	**4.0**	5 e-4 (0.09)	**3.0 ***	**3.3**	1.5 e-3 (0.06)	**2.4 ***
Kalrn	Kalirin, Rho GEF kinase	**2.9**	4 e-3 (0.10)	**1.5 ***	**3.2**	2.3 e-3 (0.06)	**NS**
Zic1**	Zinc finger protein of the cerebellum 1	**4.2**	0.05 (0.20)	**NS**	**3.9**	0.05 (0.18)	**NS**

*p<0.05; normalized to *Actb*
** Gene with differential expression following the same direction in two probe sets in the microarray analysis. The smallest absolute fold change is shown.
NS, not significant.

Table 3. Direct pharmacological effect of MDMA: qRT- PCR validation of microarray data of genes showing a Log Fold Change over 1.5

		Contingent-Saline			Non Contingent-saline		
		Microarray		qRT-PCR	Microarray		qRT-PCR
		Fold Change	P-value (Adj P value)	Fold Change	Fold Change	P-value (Adj P value)	Fold Change
Dorsal Raphe Nucleus							
Sgk1	Serum/glucocorticoid regulated kinase 1	**3.1**	1.3 e-5 (0.01)	**2.8** *	**4.3**	1.6 e-6 (1.4 e-3)	**4.1** *
Sgk3 **	Serum/glucocorticoid regulated kinase 3	**2.1**	0.01 (0.09)	**NS**	**4.2**	5.3 e-4 (9 e -3)	**2.8** *
Lcn2	Lipocalin 2	**26.1**	2.7 e-6 (8 e-3)	**37.7** *	**52.4**	5 e-7 (7 e-4)	**59.3** *
Ventral Striatum							
Slc17a7	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter)	**3.6**	6.9 e-4 (0.04)	**2.7** *	**3.6**	7.2 e-4 (7.4 e-3)	**2.9** *
Lcn2	Lipocalin 2	**32.8**	1.6 e-7 (3.8 e-4)	**37.7** *	**41.3**	9.5 e-8 (2.2 e-4)	**52.9** *
Frontal Cortex							
Lcn2	Lipocalin 2	**52.8**	5.5 e-8 (3 e-4)	**55.6** *	**55.7**	4.7 e-8 (2.5 e-4)	**65.9** *
Ctla2a**	Cytotoxic T lymphocyte-associated protein 2 alpha	**3.0**	8.5 e-4 (0.04)	**4.8** *	**4.9**	3.7 e-5 (8 e-3)	**7.3** *
Gbp2 **	Guanylate binding protein 2	**8.3**	2.5 e-5 (0.03)	**11.3** *	**7.7**	3.3 e-4 (0.01)	**12.9** *
Igtp	Interferon gamma Induced GTPase	**4.2**	3.7 e-4 (0.04)	**6.1** *	**4.0**	5 e-4 (0.02)	**5.3** *
Iigp1 **	Interferon inducible GTPase 1	**5.5**	2.1 e-4 (0.03)	**7.9** *	**6.4**	1 e-4 (0.01)	**9.4** *
Iigp2	Interferon inducible GTPase 2	**3.2**	1.9 e-4 (0.03)	**3.7** *	**3.0**	3 e-4 (0.02)	**3.0** *
Tgtp	T-cell specific GTPase	**5.8**	1.2 e-4 (0.03)	**7.3** *	**6.6**	6.7 e-5 (9.2 e-3)	**8.1** *
Hippocampus							
Lcn2	Lipocalin 2	**25.0**	1.7 e-6 (4 e-3)	**33.5** *	**36.4**	7 e-7 (1.6 e-3)	**49.6** *
Ctla2a**	Cytotoxic T lymphocyte-associated protein 2 alpha	**3.0**	1.5 e-3 (0.01)	**3.7** *	**5.2**	9.6 e-5 (0.01)	**8.0** *
Gbp2 **	Guanylate binding protein 2	**7.5**	4 e-3 (0.02)	**12.0** *	**6.9**	5 e-3 (0.03)	**13.4** *
Igtp	Interferon gamma Induced GTPase	**4.0**	4.8 e-3 (0.02)	**7.0** *	**4.1**	4.3 e-3(0.03)	**6.8** *
Iigp1 **	Interferon inducible GTPase 1	**3.9**	0.01 (0.04)	**5.4** *	**4.7**	6 e-3 (0.04)	**7.2** *
Iigp2	Interferon inducible GTPase 2	**2.9**	3.7 e-3 (0.02)	**NS**	**2.7**	4.9 e-3 (0.03)	**NS**
Tgtp	T-cell specific GTPase	**4.4**	1.6 e-3 (0.01)	**5.0** *	**4.7**	1.1 e-3 (0.02)	**5.5** *

* p-value < 0.05; normalized to *Actb*

4.4 Article 4, Orejarena *et al* 2010 (submitted)

The supplementry material for this paper can be found in Appendix B, page 241

Aim: to evaluated the role of 5-HT$_{2A}$R in MDMA-induced reinforcement and hyperlocomotion, and the reinstatement of MDMA seeking behaviour. Basal and MDMA-stimulated extracellular levels of DA in the nucleus accumbens (NAC) and serotonin (5-HT) and noradrenaline (NE) in the prefrontal cortex (PFC) were also assessed.

Results: basal and MDMA-stimulated extracellular levels of DA in the nucleus accumbens (NAC) and serotonin (5-HT) and noradrenaline (NE) in the prefrontal cortex (PFC) were also assessed. Self-administration of MDMA was abolished in 5-HT$_{2A}$R knockout (KO) mice compared to wild-type (WT) littermates at both doses tested (0.125 and 0.25 mg/kg/inf). Horizontal locomotion was increased by MDMA (20 mg/kg) to a higher extent in KO than in WT mice. DA outflow in the NAC was lower in KO compared to WT mice under basal conditions and after an MDMA (20 mg/kg) challenge. In WT mice, MDMA (10 mg/kg) priming did not reinstate MDMA seeking behaviour, while cue-induced reinstatement was prominent. This cue-induced reinstatement was blocked by the administration of the selective 5-HT$_{2A}$R antagonist, SR46349B (eplivanserin).

Conclussions: 5-HT$_{2A}$R are crucial for MDMA-induced reinforcement and cue-induced reinstatement of MDMA seeking behaviour. These effects are likely due to the modulation of mesolimbic dopaminergic activity.

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Involvement of 5-HT$_{2A}$ receptors in MDMA reinforcement and cue-induced reinstatement of MDMA seeking behaviour

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Research Focus: Choose one or more (maximum 5) that best describe the research focus of your paper:	Substance abuse – other substances < Clinical, Neuropsychopharmacology - behavioral < Preclinical, Neuropsychopharmacology - biochemical < Preclinical, Psychopathology – animal models < Preclinical, Genetics – pharmacogenetics/pharmacogenomics < Translational
Keywords: Enter up to 5 (minimum 3) keywords that best reflect the research reported in your paper.:	dopamine, microdialysis, food intake, locomotion, SR46349B

Involvement of 5-HT_{2A} receptors in MDMA reinforcement and cue-induced reinstatement of MDMA seeking behaviour

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Short title: 5-HT_{2A}R mediate MDMA reinforcement and cue-induced relapse

Keywords: dopamine, microdialysis, food intake, locomotion, SR46349B.

Abstract

The activation of the serotonergic system seems crucial for (±)-3,4-methylenedioxymethamphetamine (MDMA) reinforcing properties. Current evidence indicates that serotonin 5-HT_{2A} receptors (5-HT_{2A}R) modulate mesolimbic dopamine (DA) activity and several behavioural responses related to the addictive properties of psychostimulants. The present study evaluated the role of 5-HT_{2A}R in MDMA-induced reinforcement and hyperlocomotion, and the reinstatement of MDMA seeking behaviour. Basal and MDMA-stimulated extracellular levels of DA in the nucleus accumbens (NAC) and serotonin (5-HT) and noradrenaline (NE) in the prefrontal cortex (PFC) were also assessed. Self-administration of MDMA was abolished in 5-HT_{2A}R knockout (KO) mice compared to wild-type (WT) littermates at both doses tested (0.125 and 0.25 mg/kg/inf). Horizontal locomotion was increased by MDMA (20 mg/kg) to a higher extent in KO than in WT mice. DA outflow in the NAC was lower in KO compared to WT mice under basal conditions and after an MDMA (20 mg/kg) challenge. In WT mice, MDMA (10 mg/kg) priming did not reinstate MDMA seeking behaviour, while cue-induced reinstatement was prominent. This cue-induced reinstatement was blocked by the administration of the selective 5-HT_{2A}R antagonist, SR46349B (eplivanserin). Our results indicate that 5-HT_{2A}R are crucial for MDMA-induced reinforcement and cue-induced reinstatement of MDMA seeking behaviour. These effects are likely due to the modulation of mesolimbic dopaminergic activity.

Introduction

3,4-Methylenedioxymethamphetamine (MDMA), commonly known as "ecstasy", is widely abused despite its known adverse neuropsychological consequences (Parrott et al., 2000; Parrott, 2001). In humans, MDMA induces an entactogenic state with increased self-confidence, emotional excitation and sensorimotor activation, as well as several physiological changes that include hyperthermia and enhancement of blood pressure and heart rate (Liechti et al., 2000; Vollenweider et al., 2002). While the reinforcing properties of MDMA are well documented, its addictive potential is still a matter of debate. However, there is evidence showing that a significant proportion of MDMA users meet several of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM IV) criteria for dependence (Cottler et al., 2001; Stone et al., 2006; Leung and Cottler, 2008). Albeit these data, some aspects of MDMA addictive behaviour such as craving and relapse to drug seeking have not been fully addressed in basic or clinical studies.

MDMA has a complex mechanism of action, which involves an increase in extracellular levels of dopamine (DA), noradrenaline (NE), and serotonin (5-HT) (Green et al., 2003), and differs from other amphetamines in that it primarily affects the serotonergic system by acting on the serotonin transporter (SERT) (Han and Gu, 2006; Trigo et al., 2007). While the involvement of the 5-HT system in the rewarding, hyperlocomotor and hyperthermic effects of MDMA is well established, the relative participation of the different 5-HT receptor (5-HTR) subtypes in these responses is still not well understood. 5-HT$_{1A}$R and 5-HT$_{1B}$R are located both pre- and post-synaptically and have been implicated in MDMA-induced hyperlocomotion, sensitization and tolerance (Green et al., 2003). 5-HT$_{2A}$R and 5-HT$_{2C}$R are located post-synaptically in several brain areas of the mesostriatal and mesocorticolimbic systems of humans and rodents (Lopez-Gimenez et al., 1997; Ito et al., 1998; Andree et al., 1998), where they modulate dopaminergic activity (Navailles et al., 2008; Poras et al., 2002).

Accordingly, an increasing amount of data points to the participation of 5-HT_{2A}R in the addictive properties of psychostimulants. Thus, pre-treatment with the specific 5-HT_{2A}R antagonist, MDL100907 blocked priming- (Fletcher et al., 2007) and cue-induced (Nic Dhonnchadha et al., 2009) reinstatement of cocaine seeking behaviour in rats, without modifying its reinforcing effects in rats and non-human primates (Fantegrossi et al., 2002; Fletcher et al., 2002a; Filip et al., 2006). Contrastingly, MDL100907 partially attenuated responding for S(+)-MDMA, and abolished responding for R(-)-MDMA in non-human primates (Fantegrossi et al., 2002). Moreover, studies in humans using the non-specific 5-$HT_{2A/C}$ antagonist, ketanserin report reductions in MDMA-induced perceptual changes and emotional excitation (Liechti et al., 2000). Although these studies support a role for 5-HT_{2A}R in MDMA-induced reinforcement; there is still a lack of knowledge on how these receptors modulate the reward circuit following MDMA administration, and there is still no data regarding their involvement in relapse to MDMA seeking behaviour.

Pharmacological studies investigating the participation of 5-HT_{2A}R in the locomotor activating effects of MDMA have generated inconsistent results depending on the doses or the animal species used (Fantegrossi et al., 2003; Kehne et al., 1996; Bankson and Cunningham, 2002; Herin et al., 2005). With regards to amphetamine, studies using genetically modified mice have shown that it potentiates locomotor activity in 5-HT_{2A}R knock-out (KO) mice. This sensitized response was related to an enhanced activation of the noradrenergic system in the prefrontal cortex (PFC) (Salomon et al., 2007), implying a close interaction between the 5-HT and NE systems in these effects of amphetamine.

In this study, we used a multidisciplinary approach to investigate the contribution of 5-HT_{2A}R on MDMA behavioural and neurochemical effects. Thus, we evaluated the reinforcing properties of MDMA in 5-HT_{2A}R KO and wild-type (WT) littermates using a self-administration paradigm, and examined instrumental responding for food reward. We also

evaluated MDMA-induced hyperlocomotion in these mice. Furthermore, using *in vivo* microdialysis, we assessed extracellular levels of DA in the nucleus accumbens (NAC), as well as NE and 5-HT levels in the PFC before and after MDMA administration in 5-HT$_{2A}$R KO and WT littermates. In pharmacological experiments, we investigated whether acute blockade of 5-HT$_{2A}$R with SR46349B (eplivanserin) was able to modify cue- and priming-induced reinstatement of MDMA seeking in mice that had previously extinguished MDMA self-administration behaviour.

Material and Methods

Animals

The 5-HT_{2A}R KO and WT littermates used in this study were originally generated at Columbia University (New York, NY) on a 129S6/SvEv background (Gonzalez-Maeso et al., 2003; Gonzalez-Maeso et al., 2007). Subsequently, they were backcrossed over at least ten generations onto the inbred C57BL/6J line. Male and female 5-HT_{2A}R KO and WT mice were genotyped as described by (Fiorica-Howells., et al 2002), and only male mice were used in the studies. Pharmacological experiments were performed in C57BL/6J mice (Charles River, Lyon, France). Mice weighing 25–30 g at the beginning of the experiments were initially housed five per cage in a room with controlled temperature (21 ± 1°C) and humidity (65 ± 10%). Food and drug self-administration experiments took place during the dark cycle, while the other experiments were performed during the light phase. Food was restricted during the food conditioning experiments, but water was provided *ad libitum*. Behavioural tests and animal care were conducted in accordance with the standard ethical guidelines (National Institutes of Health, 1995; European Communities Directive 86/609 EEC) and approved by the local ethical committee (CEEA-PRBB).

Drugs

MDMA hydrochloride was obtained from Lipomed, A.G. (Arlesheim, Switzerland). SR 46349B micronized hemifumarate [(1Z,2E)-1-(2-fluoro-phenyl)-3-(4-hydroxyphenyl)-prop-2-en-one-O-(2-dimethylamino-ethyl)-oxime hemifumarate] was generously provided by Sanofi-Aventis (Montpellier, France). All drugs were dissolved in 0.9 % physiological saline.

Locomotor activity

Locomotor activity was measured using individual locomotor activity boxes (9 × 20 × 11 cm; Imetronic, Lyon, France). The boxes were equipped with two lines of 14 photocells (2 and 6 cm above the floor), and with a fan providing white noise. The boxes were placed in a room with low luminosity (5 lux). Animals received an acute administration of MDMA (20 mg/kg, i.p), and were immediately placed in the locomotor activity boxes. Locomotor activity was measured in 10 min bins for 2 h.

MDMA self-administration procedure

Please see supplement 1.

Food-Maintained Operant Behaviour

Please see supplement 2.

Microdialysis, HPLC analytical procedure and histology

Please see supplement 3.

Reinstatement of MDMA-seeking behaviour

C57BL/6J mice were trained to respond for intravenous infusions of MDMA (0.25 mg/kg/infusion) on a FR1 schedule of reinforcement (see above for methodology). Animals that achieved acquisition criteria (as described above) underwent extinction sessions, which lasted until responding on the active hole decreased to 40 % of the stable acquisition mean during two consecutive days. The cue light associated to drug self-administration was not present during extinction sessions. After reaching extinction, one group of animals received saline 30 min before each of the following reinstatement conditions according to a within-subject design: 1) a priming injection of saline (control relapse) or MDMA (10 mg/kg i.p.) (priming relapse) without the cue light, 2) presentation of the cue light alone (cue relapse), 3) both MDMA-priming plus cue light (cue + priming relapse). The other group, received

eplivanserin (0.5 mg/kg/i.p.) 30 min before each of the reinstatement conditions described for the first group according to a within-subject design: (control relapse, priming relapse, cue relapse, and cue + priming relapse). The dose of eplivanserin was chosen according to previous studies showing that it efficiently blocks 5-HT_{2A}R without modifying locomotor activity in mice (Auclair et al., 2004a; Auclair et al., 2004b; Salomon et al., 2007). Extinction sessions were performed following each reinstatement test, and animals were exposed to the next reinstatement condition only when they reached the same extinction criteria described for the first extinction session.

Statistical Analysis

The locomotor, MDMA self-administration and food-maintained operant responding data were analysed using three-way repeated measures ANOVA. Individual differences were analysed using subsequent post-hoc tests. Discrimination between holes for each day of testing was analysed for dose and genotype separately using a one-way ANOVA. Differences between genotypes in the breaking points to obtain the reward were analyzed using between subjects one-way ANOVAs. Basal extracellular levels of DA, NE and 5-HT were analyzed between genotypes using one-way ANOVA. The effects of MDMA administration on DA, NE and 5-HT outflow were analyzed using a two-way repeated measures followed by the corresponding post-hoc analysis when required. The reinstatement data were analyzed using three-way repeated measures ANOVA with phase and hole as within-subjects factors and treatment (saline or eplivanserin) as between-subjects factors, followed by the corresponding post-hoc analysis when required. In order to compare the different reinstatement conditions in each within-subjects design, a repeated measures ANOVA followed by pairwise comparisons was applied. A two-way between-subjects ANOVA (hole x phase) was additionally used to analyze the effects of eplivanserin on reinstatement of MDMA seeking behaviour.

Results

MDMA-induced locomotor activity in 5-HT$_{2A}$R KO and WT mice

The acute administration of MDMA (20 mg/kg) increased horizontal activity in WT and KO mice with respect to a saline injection (Fig. 1). However, this effect was significantly greater in 5-HT$_{2A}$R KO mice than in WT littermates. Three-way repeated measures ANOVA (treatment x genotype x time) revealed a significant main effect of time [$F_{(11,187)} = 57.407$, $p < 0.001$] and treatment [$F_{(1,17)} = 115.920$, $p < 0.001$], and a significant three-way interaction between treatment, genotype and time [$F_{(11,187)} = 5.839$, $p < 0.001$]. Significant two-way interactions were revealed between treatment and time [$F_{(1,187)} = 20.100$, $p < 0.001$] and genotype and time [$F_{(1,187)} = 5.253$, $p < 0.001$]. Subsequent analysis comparing the effects of MDMA between genotypes at each time point after stimulation revealed a significant increase in horizontal locomotion in KO mice as compared to WT mice during the first 40 min of testing (10-30 min: $p < 0.05$; 40 min: $p < 0.01$) (Fig. 1a). One-way ANOVA comparing the area under the curve between genotypes for each hour of testing, showed a significant increased in horizontal locomotion in KO mice during the first hour in comparison with WT littermates [$F_{(1,17)} = 6.507$, $p < 0.05$], while no differences were found in the second hour [$F_{(1,17)} = 0.36$, NS] (Figure 1b).

MDMA self-administration in 5-HT$_{2A}$R KO and WT littermates

The acquisition and maintenance of operant responding for both doses of MDMA (0.125 and 0.25 mg/kg/infusion) in WT and KO mice are shown in Fig. 2. Three-way repeated measures ANOVA (genotype x hole x day) for the dose of 0.125 mg/kg/inf revealed a significant main effect of hole [$F_{(1, 14)} = 16.690$, $p < 0.001$] and day [$F_{(9,126)} = 2.660$, $p < 0.01$], and a significant two-way interaction between genotype and hole [$F_{(1, 14)} = 5.091$, $p < 0.5$].

Subsequent comparisons between active and inactive responding in WT mice revealed significant differences in discrimination from the fifth to the tenth day of training (days 6-7: $p < 0.05$, days 5, 8-10: $p < 0.01$) (Fig. 2a). The percentage of acquisition in WT mice was of 62.5 %. In KO mice, there were no significant differences between active and inactive responding at any day of testing, and only 20 % of mice reached acquisition criteria at this dose (Fig. 2b).

Three-way repeated measures ANOVA (genotype x hole x day) for the dose of 0.25 mg/kg/inf revealed a significant main effect of genotype [$F_{(1, 16)} = 13.112$, $p < 0.01$], hole [$F_{(1, 16)} = 47.840$, $p < 0.001$], and a significant two-way interaction between genotype and hole [$F_{(1, 16)} = 17.380$, $p < 0.001$], and between hole and day [$F_{(9,144)} = 4.654$, $p < 0.05$]. Comparisons between active and inactive responding for WT mice revealed significant differences in discrimination from the third to the tenth day of training (day 3: $p < 0.05$, days 4-6: $p < 0.01$, days 7-10: $p < 0.001$) (Fig. 2c). The percentage of acquisition in WT mice was of 88.9 %. In contrast, KO mice showed a significant discrimination between holes only on day 7 ($p < 0.001$), and only 25 % of these mice reached acquisition criteria for this dose (Fig. 2d).

In the progressive ratio task, two-way ANOVA between subjects (dose of MDMA x genotype) revealed a significant main effect of genotype [$F_{(1,26)} = 14.630$, $p < 0.01$], but no effect of dose [$F_{(1,26)} = 3.965$, $p = 0.057$] (Fig. 3). Individual comparisons for each dose showed a significant lower breaking point for KO mice with respect to WT at the dose of 0.125 mg/kg/inf ($p < 0.01$), and at the dose of 0.25 mg/kg/inf ($p < 0.05$)

Food maintained operant behaviour in 5-HT_{2A}R KO and WT littermates

The specificity of the effects observed in the MDMA self-administration paradigm were confirmed since both genotypes acquired responding for food in a similar manner. See

supplement 4 and Figure S1 for details.

In vivo microdialysis in 5-HT$_{2A}$R KO and WT littermates

Histological analysis of the brains showed that most probes in the NAC were placed between + 1.34 mm and + 1.54 mm from bregma (Fig.4a), and those in the PFC were placed between + 2.30 mm and + 2.8 mm from bregma (Fig 4b).

Basal extracellular levels of DA in the NAC (Fig. 5a.) were significantly lower in KO mice with respect to WT littermates [$F_{(1,14)} = 49.054$, $p < 0.001$]. On the other hand, basal extracellular levels of 5-HT (Fig. 5b) and NE (Fig. 5c) in the PFC were not significantly different between genotypes.

A robust increase in extracellular levels of DA in the NAC, and of 5-HT and NE in the PFC was observed following an acute challenge with MDMA (20 mg/kg/i.p.; Fig. 6). Extracellular levels of DA in the NAC were increased in both WT and KO mice; although this increase was significantly lower in KO mice. The peak increase in DA was observed 30 min after injection in both WT (8.89 ± 1.6 pg/sample) and KO mice (4.39 ± 0.46 pg/sample, Fig. 6a). Two-way repeated-measures ANOVA (genotype x time after injection) revealed a significant main effect of time [$F_{(11,143)} = 29.117$, $p < 0.001$], genotype [$F_{(11,13)} = 206.839$, $p < 0.001$], and interaction between these two factors [$F_{(11,143)} = 4.547$, $p < 0.001$]. Comparisons between genotypes at each time point following MDMA administration indicated significantly less DA outflow in KO mice from 15 to 75 min and from 135 to 180 min after MDMA injection ($p < 0.05 – 0.01$).

Changes in the extracellular levels of 5-HT in the PFC following MDMA were not significantly different between genotypes (Fig. 6b). The peak increase in 5-HT was observed 30 min after injection in both WT (4.28 ± 0.27 pg/sample) and KO (4.03 ± 0.65 pg/sample) mice. Two-way repeated-measures ANOVA revealed a significant main effect of time after

injection [$F_{(11,143)} = 41.284$, $p < 0.001$], but no significant effect of genotype [$F_{(1,13)} = 0.013$, NS] nor interaction between these two factors [$F_{(11,143)} = 0.440$, NS].

MDMA induced a similar increase in NE levels in the PFC of WT and KO mice (Fig. 6c). The maximum increase was observed 20 min after injection in both WT (4.62 ± 0.92 pg/sample) and KO mice (3.48 ± 0.64 pg/sample). Repeated-measures ANOVA revealed a significant main effect of time [$F_{(9,90)} = 8.353$, $p < 0.001$], after injection but no significant main effect of genotype [$F_{(1,10)} = 0.946$, NS], nor interaction between these two factors [$F_{(9,90)} = 0.565$, NS].

Effects of 5-HT_{2A}R blockade on the reinstatement of MDMA-seeking behaviour in WT mice

Reinstatement of MDMA seeking behaviour evaluated in mice treated with saline or eplivanserin under different conditions is shown in Fig. 7. An overall three-way repeated measures ANOVA (treatment x hole x phase) revealed significant main effects of treatment, hole and phase of study, as well as significant two- and three-way interactions between these factors (see Table 1).

In mice treated with saline (Fig. 7a), active hole responding was compared within phases of the study (control relapse, priming relapse, cue relapse and cue + priming relapse) followed by pairwise comparisons. A significant main effect of phase was revealed [$F_{(8,80)} = 21.073$, $p < 0.001$]. Extinction of responding was achieved with a mean of 13.1 ± 1.2 days. On the last day of extinction, a significant decrease in active responding was observed compared to the mean of the three days meeting the acquisition criteria ($p < 0.01$). No significant differences were observed for active responding between the four different extinction sessions performed before each reinstatement session. A saline administration (control relapse) significantly decreased active responding with respect to the previous extinction session ($p <$

0.01). Similarly, MDMA (10 mg/kg) administration (priming relapse) significantly reduced active responding with respect to the previous extinction session ($p < 0.05$), indicating that under these experimental conditions MDMA priming does not reinstate MDMA seeking behaviour. In contrast, the presentation of the cue light previously paired with MDMA self-administration (cue relapse) or the combination of cue presentation and the priming dose of MDMA (cue + priming relapse) significantly increased active hole responding with respect to the previous extinction session ($p < 0.001$ and $p < 0.01$, respectively). These results show that the cue presentation is the most effective stimulus to induce reinstatement of MDMA seeking behaviour in mice.

In mice treated with eplivanserin (Fig. 7b), active hole responding was compared within phases of the study followed by pairwise comparisons. A significant main effect of phase was revealed [$F_{(8,96)} = 18.102$, $p < 0.001$]. Extinction of responding in this group of mice was achieved with a mean of 12.8 ± 1 days. On the last day of extinction, a significant decrease in active responding was observed compared to the mean of three days meeting the acquisition criteria ($p < 0.001$). No significant differences were observed for active responding between the four different extinction sessions performed before each reinstatement phase. Administration of eplivanserin in mice receiving a saline challenge (control relapse) significantly decreased active responding with respect to the previous extinction phase ($p < 0.01$). The combination of eplivanserin and MDMA (priming relapse) also significantly decreased responding in the active hole with respect to the previous extinction session ($p < 0.01$). On the other hand, eplivanserin pre-treatment before the presentation of the cue light, or before a priming dose of MDMA plus the presentation of the cue light, did not significantly change active responding with respect to each previous extinction session. These results reveal that eplivanserin blocks cue- and cue + priming-induced reinstatement of MDMA seeking behaviour. This effect was confirmed with a between-subjects ANOVA (treatment x

hole) comparing eplivanserin and saline treatment in all conditions (Table 2.) Subsequent analysis showed no significant differences in active or inactive responding between mice treated with saline and eplivanserin during the control relapse phase or the priming relapse phase. In contrast, eplivanserin pre-treatment significantly blocked cue-induced reinstatement (significant differences in active, $p < 0.001$ and inactive $p < 0.05$, responding), and cue + priming-induced reinstatement of MDMA seeking behaviour (significant differences for the active hole, $p < 0.01$).

Discussion

This study shows that MDMA self-administration behaviour is abolished in 5-HT$_{2A}$R KO mice, and that the specific 5-HT$_{2A}$R antagonist, eplivanserin blocks cue-induced reinstatement of MDMA seeking behaviour. Interestingly, our microdialysis data showed lower extracellular levels of DA in the NAC of KO mice before and after an MDMA challenge, whereas extracellular levels of 5-HT and NE in the PFC were similar in KO and WT littermates. These results suggest that a modulatory effect of 5-HT$_{2A}$R on mesolimbic dopaminergic activity could be involved in the behavioural responses induced by MDMA. Conversely, MDMA-induced hyperactivity is potentiated in KO mice, suggesting a differential role for 5-HT$_{2A}$R in the control of locomotor and reinforcing effects of MDMA.

5-HT$_{2A}$R KO mice failed to acquire an operant behaviour to self-administer two different doses of MDMA (0.125 and 0.25 mg/kg/inf), which effectively maintained such behaviour in WT mice. These results reveal that the absence of 5-HT$_{2A}$R activity totally suppresses MDMA reinforcement. Previous pharmacological experiments have shown that acute blockade of 5-HT$_{2A}$R using MDL100907 was partially effective in attenuating S(+)-MDMA and abolished R(-)-MDMA self-administration, in non-human primates (Fantegrossi et al., 2002), and decreased some aspects of MDMA-induced reward in human subjects (Liechti et al., 2000). In contrast, MDL100907 did not affect the ability of d-amphetamine to reduce the threshold required to sustain rewarding brain stimulation in the ventral tegmental area (Moser et al., 1996), nor did it modify the reinforcing potency of cocaine in rats (Fletcher et al., 2002a; Filip et al., 2006) or non-human primates (Fantegrossi et al., 2002). The contrasting results observed in the studies with MDMA compared to amphetamine or cocaine suggest that serotonergic mechanisms mediated via 5-HT$_{2A}$R play a prominent role in the reinforcing properties of MDMA. Indeed, MDMA releases 5-HT more potently than cocaine or amphetamine (Han and Gu, 2006), and it has been shown that 5-HT is crucial for MDMA

reinforcement processes (Trigo et al., 2007). In addition, unlike other psychostimulants, MDMA can also directly stimulate 5-HT_{2A}R, an effect that has been associated with its slight hallucinogenic action (Nichols and Oberlender, 1989). Thus, this receptor seems to play an important role in mediating the reinforcing effects of MDMA, but not those of cocaine or amphetamine.

The mesocorticolimbic DA system, which has been widely related with the reinforcing properties of drugs of abuse (Hyman et al., 2006) including MDMA (Bilsky et al., 1998; Daniela et al., 2004), is known to be regulated by serotonergic neurotransmission (Schmidt et al., 1992; De Deurwaerdère et al., 1998; Gobert and Millan, 1999). 5-HT_{2A}R are localized in several brain structures of the reward circuit such as the ventral tegmental area (VTA), the NAC and the PFC, where they can modulate DA activity (Porras et al., 2002; Bortolozzi et al., 2005). However, acute blockade of 5-HT_{2A}R does not affect basal DA extracellular efflux in the striatum or NAC of rats (Schmidt et al., 1992; De Deurwaerdère and Spampinato, 1999; Porras et al., 2002). In contrast, our in vivo microdialysis experiments revealed that mice constitutively lacking the 5-HT_{2A}R show lower basal levels of DA in the NAC (50 % less). Previous evaluation of the total monoamine content in 5-HT_{2A}R KO mice did not reveal any basal differences with respect to WT mice, probably due to the fact that the experiments were carried out in brain homogenates (Weisstaub et al., 2006). Supporting our data, it has been reported that chronic treatment with MDL100907 reduces the activity of nigrostriatal DA neurons (Sorensen et al., 1993). In addition, our study reveals that 5-HT_{2A}R KO mice showed less MDMA-induced stimulation of DA in the NAC, while 5-HT and NE activation in the PFC was similar to WT mice. These results suggest that the changes observed in DA function following chronic blockade of 5-HT_{2A}R may participate in the impairment of MDMA reinforcement. We have previously shown that serotonin transporter (SERT) KO mice are insensitive to the 5-HT-releasing actions of MDMA and do not self-administer this drug,

while MDMA-enhanced DA levels in the NAC are comparable to WT controls (Trigo et al., 2007). The fact that neither SERT (Trigo et al., 2007) nor 5-HT$_{2A}$R KO mice self-administer MDMA (this study) at doses sustaining operant responding in WT mice argues for the critical involvement of a concurrent 5-HT-DA stimulation in the complete expression of the reinforcing properties of MDMA.

In order to verify that the effects observed on MDMA self-administration were not due to unspecific motor or learning deficits, we tested 5-HT$_{2A}$R WT and KO mice in an operant food-reinforced task. 5-HT$_{2A}$R KO showed lower active responding for food pellets than WT mice only during the first 3 days of acquisition. Similar levels of responding were observed between genotypes during the remaining training period, and all the KO mice tested reached the acquisition criteria. Moreover, breaking points for food reward evaluated in a PR task were not altered in KO mice. These findings indicate that a constitutive deletion of 5-HT$_{2A}$R does not disrupt instrumental responding or the motivation for food reward, and support a specific role of 5-HT$_{2A}$R in MDMA reinforcement.

Interestingly, the hyperactivity induced by an acute administration of MDMA was potentiated in 5-HT$_{2A}$R KO mice. These findings are in line with studies showing that an acute administration of d-amphetamine also enhances locomotor responses in mice with a constitutive deletion of the 5-HT$_{2A}$R as compared to WT control mice (Salomon et al., 2007). In that study, the behavioural response to d-amphetamine was paralleled by increased extracellular levels of NE in the PFC (Salomon et al., 2007). These authors proposed that 5-HT$_{2A}$R and α1b-adrenergic receptors in the PFC regulate each other under normal conditions to modulate psychostimulant-induced locomotor activity, as well as DA levels in the NAC (Auclair et al., 2004a; Auclair et al., 2004b). In mice lacking 5-HT$_{2A}$R, a partial "constitutive" sensitization to d-amphetamine occurs, related to heightened NE levels in PFC, possibly leading to higher levels of DA in the NAC (Salomon et al., 2007). In our study, we did not

find an enhancement in NE levels in the PFC or DA in the NAC in KO mice compared to WT mice following MDMA administration. These results suggest that NE may not be involved in the MDMA-induced hyperlocomotor effect observed in 5-HT_{2A}R KO mice. Most pharmacological studies using acute administration of 5-HT_{2A}R antagonists have shown a reduction in MDMA-induced locomotor activation in several animal species, although contradictory results have also been reported (Kehne et al., 1996; Bankson and Cunningham, 2002; Fantegrossi et al., 2003; Herin et al., 2005;Ball and Rebec, 2005).

Our studies indicate that locomotor and reinforcing effects induced by MDMA are modulated differently in 5-HT_{2A}R KO mice. These distinct responses could be attributed to the different dosing regimens of MDMA (contingent low doses of MDMA in self-administration vs. non-contingent high intraperitoneal doses in the locomotor studies), or to possible compensatory mechanisms that may have differentially influenced MDMA-induced locomotion and reinforcement. Indeed, 5-HT_{1B} and 5-HT_{2B-2C} receptors have been shown to participate in distinctive aspects of MDMA-related effects (Kehne et al., 1996; Scearce-Levie et al., 1999; Fantegrossi et al., 2002; Fletcher et al., 2002b; Ramos et al., 2005; Doly et al., 2009). Although no changes in mRNA expression for the different 5-HT receptors have been observed in 5-HT_{2A}R KO mice (Weisstaub et al., 2006), a possible 5-HT_{2B}R hyposensitivity has been reported in these mutants probably related to receptor desensitization (Popa et al., 2005). This finding is relevant because a high dose of MDMA induces locomotor hyperactivation in 5-HT_{2B}R KO mice, while MDMA conditioned place preference is abolished (Doly et al., 2009). Therefore, even if 5-HT_{2B}R are discreetly expressed in the adult brain (Leysen, 2004), a possible change in the sensitivity of these receptors could have also contributed to the enhancement of MDMA locomotor responses observed in 5-HT_{2A}R KO mice.

In our pharmacological experiments using WT mice, a priming injection of MDMA was unable to reinstate MDMA seeking behaviour, while the presentation of a cue previously paired with MDMA self-administration did. The result showing that an acute challenge with MDMA failed to reinstate MDMA seeking was surprising since we had previously shown that MDMA could reinstate cocaine seeking behaviour in mice (Trigo et al., 2009). Differences in the mechanisms involved in cocaine and MDMA reinstatement or in mice strains used (CD1 vs. C57BL/6J) could possibly explain these discrepant results. On the other hand, our results showing that cue presentation reinstates MDMA seeking behaviour agree with previous studies performed in rats, where cue presentation alone (Ball et al., 2007) as well as, cue plus MDMA priming also reinstate MDMA seeking behaviour (Schenk et al., 2008). More interestingly, we demonstrate for the first time that selective blockade of 5-HT_{2A}R can effectively prevent cue-induced reinstatement of MDMA seeking behaviour. The ability of eplivanserin to block cue-induced reinstatement can not be explained by changes in locomotion, as previous studies have shown that even higher doses than the one used in this experiment (1 mg/kg/i.p.), do not modify locomotion in mice (Auclair et al., 2004a; Auclair et al., 2004b; Salomon et al., 2007). In agreement, our results show that eplivanserin on its own does not modify unspecific responding on the inactive hole. Therefore, the suppression of cue-induced reinstatement of MDMA seeking behaviour by eplivanserin is due to a selective 5-HT_{2A}R antagonism. In agreement, recent data show that MDL100907 prevented cue-induced cocaine seeking (Nic Dhonnchadha et al., 2009). Our reinstatement studies were carried out after an extended period of extinction in mice which had self-administered MDMA during 10 days. In recent studies, rats withdrawn from acute or chronic treatment with MDMA (Reneman et al., 2002) or cocaine (Carrasco and Battaglia, 2007; Carrasco et al., 2007) show up-regulation of 5-HT_{2A}R. Furthermore, imaging studies in MDMA users report an increase in 5-HT_{2A}R after 30 days of abstinence (Reneman et al., 2002). Thus, the

blockade by eplivanserin of cue-induced reinstatement of MDMA seeking behaviour, may be related to a 5-HT_{2A}R hyperactivity produced after repeated MDMA self-administration in structures involved in reinstatement processes, such as the medial PFC (Ciccocioppo et al., 2001; Bradberry and Rubino, 2004; See, 2005; Van den Oever et al., 2009). Indeed, 5-HT_{2A}R are highly expressed in the PFC (Bortolozzi et al., 2005; McDonald and Mascagni, 2007; Vazquez-Borsetti et al., 2009; Jiang et al., 2009), where they enhance the excitatory cortical output to the VTA, activating DA neurones and increasing DA release in NAC (Bortolozzi et al., 2005; Pehek et al., 2006; Vazquez-Borsetti et al., 2009).

In summary, we show that 5-HT_{2A}R KO mice do not acquire MDMA self-administration behaviour; while they do learn an instrumental response to obtain food pellets, supporting the specific involvement of these receptors in MDMA reinforcing properties. These effects were associated to lower basal and MDMA-stimulated levels of DA in the NAC of 5-HT_{2A}R KO mice. Conversely, MDMA-induced hyperlocomotion was enhanced in 5-HT_{2A}R KO animals, suggesting a different involvement of these receptors in the locomotor effects of MDMA. The results also showed that eplivanserin potently blocked MDMA seeking behaviour. Considering the crucial role of 5-HT_{2A}R in MDMA reinforcement and seeking behaviour, 5-HT_{2A}R antagonists may be a therapeutic option for reducing craving and preventing relapse in patients with addiction to MDMA.

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Statement of Interest

The authors state no conflict of interest.

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Figure Legends

Figure 1. Locomotor effects produced by the acute administration of MDMA (20 mg/kg/i.p.) or saline in 5-HT_{2A}R knockout (KO) (n = 10) and wild-type (WT) (n = 9) mice. Horizontal locomotion **(a)** (average + S.E.M. photocell counts during 120 min) was increased in KO mice to a greater extent than in WT mice. The area under the curve (AUC) in KO and WT mice is shown for each hour after MDMA treatment. The asterisks denote significant differences between WT and KO mice. * $p < 0.05$; ** $p < 0.01$ (one-way ANOVA).

Figure 2. Acquisition of intravenous MDMA self-administration. Animals were trained to respond for the dose of 0.125 mg/kg/infusion (WT: n = 8) **(a)**, (KO: n = 8) **(b)**, and for the dose of 0.25 mg/kg/infusion (WT: n = 8) **(c),** (KO: n = 10) **(d).** The data represent the average number of nose-pokes + S.E.M. in the active and inactive holes in 2-hour sessions during 10 days of training. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ active versus inactive hole (one-way ANOVA).

Figure 3. Breaking points for MDMA at the dose of 0.125 mg/kg/infusion **(a)** and at the dose of 0.25 mg/kg/infusion **(b)** on a progressive schedule of reinforcement in 5-HT_{2A}R knockout (KO) and wild-type (WT) mice. The data represent the average breaking points + S.E.M. achieved in a 2 h session. The asterisks denote significant differences between WT and KO mice. * $p < 0.05$; ** $p < 0.01$ (one-way ANOVA).

Figure 4. Representative coronal brain sections stained with Cresyl violet showing the placement of microdialysis probes in the nucleus accumbens (**a**, bregma +1.42) and the prefrontal cortex (**b**, bregma + 2.34).

Figure 5. Basal extracellular levels of monoamines in 5-HT_{2A}R knockout (KO) and wild-type (WT) mice. DA basal levels in the NAC **(a)** were significantly decreased in KO (n = 9) with respect to WT (n = 6) mice, while 5-HT basal levels in the PFC **(b)** were not significantly different between KO (n = 9) and WT (n = 6) mice. Basal NE levels in the PFC **(c)** were also similar between KO (n = 7) and WT (n = 5) mice **(c).** The data are expressed as the average + S.E.M. pg of 5 baseline values. *** $p < 0.001$ (one-way ANOVA).

Figure 6. Stimulated extracellular levels of monoamines in 5-HT_{2A}R knockout (KO) and wild-type (WT) mice following an acute administration of MDMA (20 mg/kg, i.p.; black arrow). DA levels in the NAC **(a)** were significantly attenuated in KO with respect to WT mice. Neither 5-HT **(b)** nor NE **(c)** levels in the PFC were significantly different between KO and WT mice**.** The data are expressed as the average + S.E.M. pg/sample * $p < 0.05$; ** $p < 0.01$, WT vs. KO mice.

Figure 7. Acquisition (ADQ), extinction (EXT) and reinstatement of MDMA-seeking behaviour in C57BL/6J mice. Reinstatement of MDMA-seeking behaviour was evaluated according to a within-subject design in one group of mice following a priming injection of saline (Sal) (control relapse) or MDMA (10 mg/kg i.p.) (priming relapse) without the cue light, following presentation of the cue light alone (cue relapse), and following both priming plus cue light (cue + priming relapse) (n = 11) **(a)**. In another group of animals MDMA-seeking behaviour was evaluated under the same experimental conditions except that mice were pretreated with eplivanserin (0.5 mg/kg/i.p.) or saline and 30 min later they received MDMA (10 mg/kg, i.p.) or saline (n = 13) **(b)**. Significant differences in responding on the active hole were observed within phases of the study (☆ $p < 0.05$; ☆☆ $p < 0.01$; ☆☆☆ $p < 0.001$). Comparisons between studies confirmed that eplivanserin completely abolished cue-

induced reinstatement of MDMA seeking behaviour ** $p < 0.01$; *** $p < 0.001$ (pairwise comparisons).

TABLE 1. Three-way ANOVA (Treatment X Hole X Phase) for responses in active and inactive holes for eplivanserin and saline treated groups at each phase of the study

	F-Value	*p*-Value
Three-way ANOVA		
Treatment	$F_{(1,22)} = 15.471$	<0.001
Hole	$F_{(1,22)} = 76.312$	<0.001
Phase	$F_{(8,176)} = 2.660$	<0.001
Treatment X Hole	$F_{(1,22)} = 4.642$	<0.001
Treatment X Phase	$F_{(1,22)} = 16.287$	<0.001
Hole X Phase	$F_{(8,176)} = 15.375$	<0.001
Treatment X Hole x Phase	$F_{(8,176)} = 10.250$	<0.001

TABLE 2. Two-Way ANOVAs (Hole X Phase) Comparing Responses in the Active and Inactive Holes for Each Relapse Phase

Two-way ANOVA	Treatment		Hole		Hole X Treatment	
	F-Value	*p*-Value	*F*-Value	*p*-Value	*F*-Value	*p*-Value
Control Relapse						
Sal + Sal vs Epli + Sal	$F_{(1,22)} = 3.321$	N.S.	$F_{(1,22)} = 2.182$	N.S.	$F_{(1,22)} = 0.637$	N.S.
Priming Relapse						
Sal + MDMA vs Epli + MDMA	$F_{(1,22)} = 1.407$	N.S.	$F_{(1,22)} = 16.706$	<0.001	$F_{(1,22)} = 1.834$	N.S.
Cue Relapse						
Sal + Sal vs Epli + Sal	$F_{(1,22)} = 31.447$	<0.001	$F_{(1,22)} = 31.892$	<0.001	$F_{(1,22)} = 20.857$	<0.001
Cue + Priming Relapse						
Sal + MDMA vs Epli + MDMA	$F_{(1,22)} = 13.067$	<0.001	$F_{(1,22)} = 24.738$	<0.001	$F_{(1,22)} = 10.873$	<0.001

Fig. 1

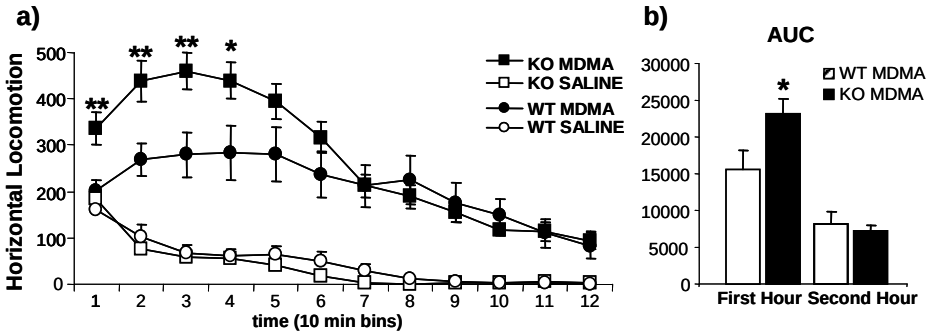

a)
Horizontal Locomotion
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
11
12
time (10 min bins)
**
**
**
*
KO MDMA
KO SALINE
WT MDMA
WT SALINE
b)
AUC
30000
25000
20000
15000
10000
5000
0
*
WT MDMA
KO MDMA
First Hour
Second Hour

Fig.2

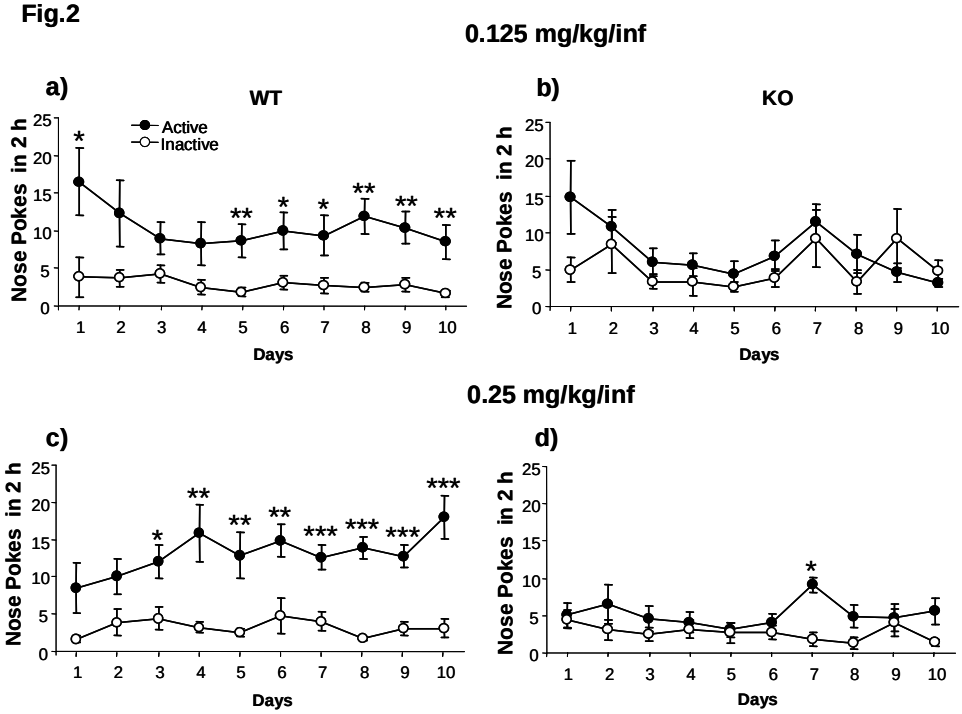

0.125 mg/kg/inf
a)
WT
Active
Inactive
Nose Pokes in 2 h
Days
b)
KO
Nose Pokes in 2 h
Days
0.25 mg/kg/inf
c)
Nose Pokes in 2 h
Days
d)
Nose Pokes in 2 h
Days

Fig. 3

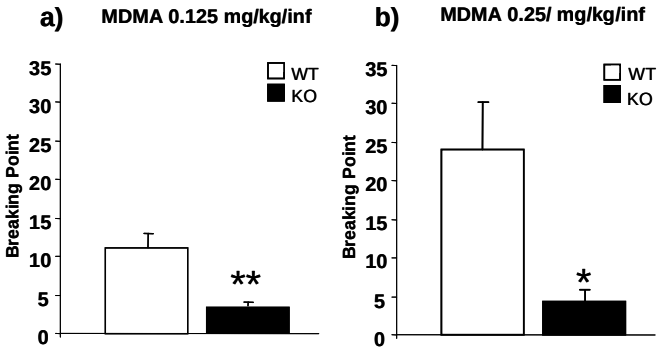
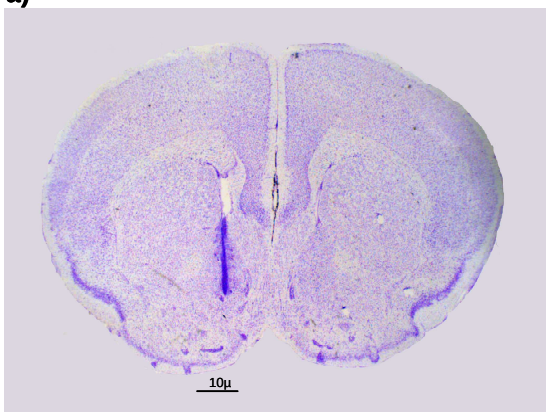

a)
MDMA 0.125 mg/kg/inf
b)
MDMA 0.25/ mg/kg/inf
Breaking Point
WT
KO
35
30
25
20
15
10
5
0
**
*

Fig. 4

a)



b)

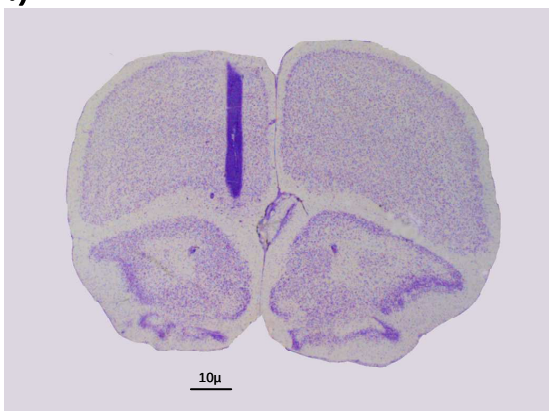


Fig. 5

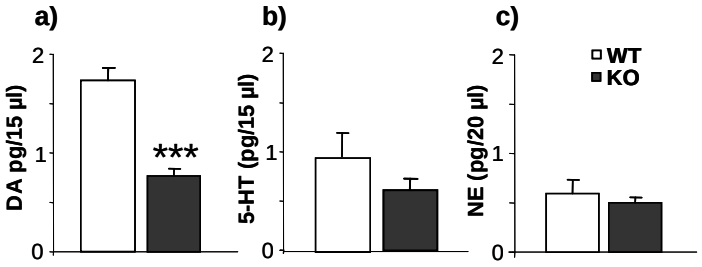


Fig. 6

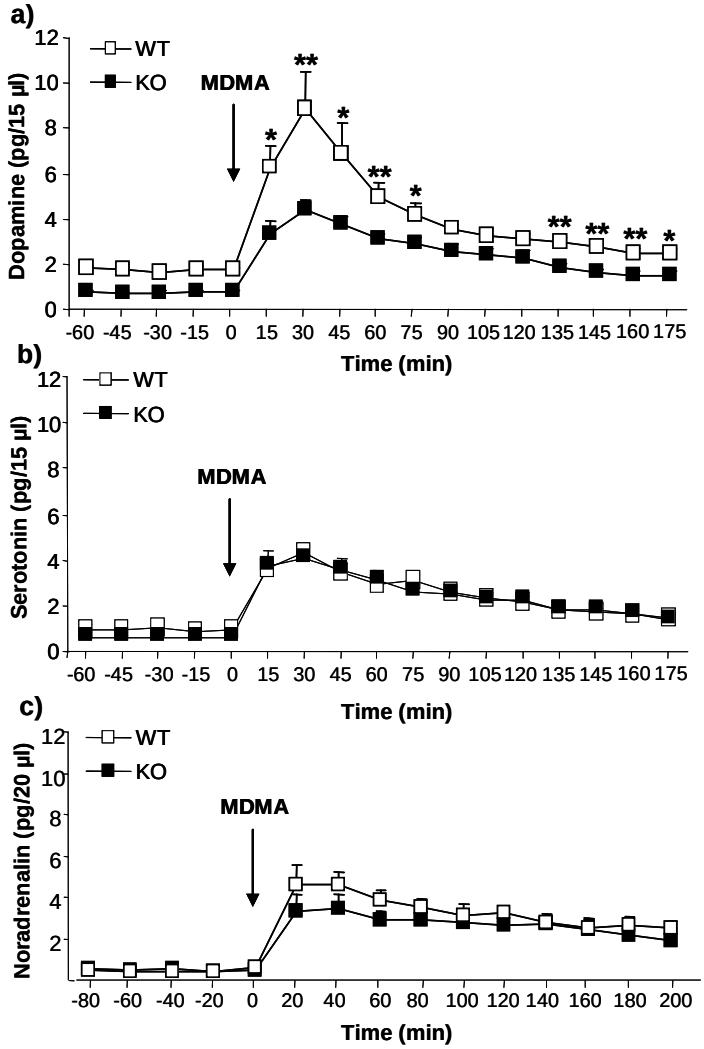
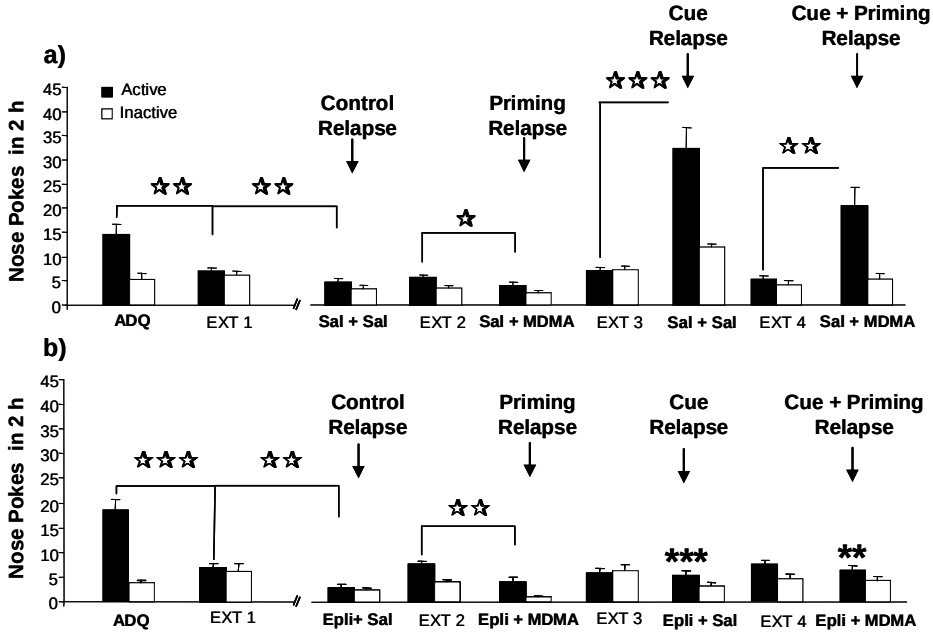

a)
WT
KO
MDMA
Dopamine (pg/15 µl)
Time (min)
b)
Serotonin (pg/15 µl)
c)
Noradrenalin (pg/20 µl)

Fig. 7


a)
Active
Inactive
Nose Pokes in 2 h
Control Relapse
Priming Relapse
Cue Relapse
Cue + Priming Relapse
ADQ
EXT 1
Sal + Sal
EXT 2
Sal + MDMA
EXT 3
Sal + Sal
EXT 4
Sal + MDMA
b)
Nose Pokes in 2 h
Control Relapse
Priming Relapse
Cue Relapse
Cue + Priming Relapse
ADQ
EXT 1
Epli+ Sal
EXT 2
Epli + MDMA
EXT 3
Epli + Sal
EXT 4
Epli + MDMA

CHAPTER 5

Discussion

5.1 Passive versus Active MDMA Administration

Experiments carried out in the last decade have lent support to certain influential theories in the field of memory and addiction, which consider drug addiction as a chronic brain disease that goes beyond the direct effects of the drug. Phenomena such as context-dependent drug overdose, and the relevance of drug-related cues and context for relapse, are some of the factors that have highlighted the importance of conditioning elements in drug seeking behaviour. Therefore, addiction is now recognized as a disease where learning and cognitive disadaptations play a critical role (Hyman, 2005; Hyman et al., 2006; Kelley, 2004; Robbins et al., 2008).

Thus, cognitive processes, motivation and expectation of drug consumption can differentially modulate the effects that the drug causes in an individual, including changes in gene-expression and neurotransmitter release (as explained in Section, 1.6). Currently, the intravenous drug self-

administration technique is widely used in laboratory animals to study the abuse liability of drugs. In this paradigm, animals are actively engaged in drug taking since they must learn an operant response to obtain drug infusions according to a set schedule of reinforcement. The processes involved in this behaviour can be differentiated from those taking place during passive drug administration by adding a yoked control subject that receives an infusion of the drug at the same time than the active animal (method explained in Section, 1.3.3.4). We used such a control in two of the articles presented in this thesis to evaluate differential changes in extracellular levels of NAC DA (*article 2*), as well as changes in gene expression (*article 3*) occurring in brain areas relevant to the development of an addictive state following active or passive MDMA administration.

Article 2 was the first publication to report differential changes in extracellular levels of NAC DA 48 hours after the last active or passive MDMA administration. The two main results reported in this work were: 1) basal levels of NAC DA were equally decreased in both contingent and non-contingent mice, when compared to saline treated animals. The lack of differences between passive and active MDMA administration groups suggests that repeated doses of MDMA can result in lower basal DA levels in the NAC, regardless of whether the animal self-administered the drug or not. It may, therefore, be a direct consequence of drug intake; 2) a challenge dose of MDMA during early abstinence increased extracellular levels of NAC DA to a lower extent in mice that previously received MDMA when compared to animals treated with saline. However, this difference was significantly more pronounced in those animals that self-administered the drug than in those that received it passively. These results suggest that different neuroadaptations occur at the level of the dopamine mesolimbic system in animals when the drug is actively administered. Moreover, these neuroadaptations persist in time, at least during early abstinence (48 hours from the last dose), indicating that cognitive processes can modify tolerance to the stimulating effects of a subsequent MDMA challenge. In concordance with our results, studies performed in rats have reported a lower stimulation of NAC DA by d-amphetamine during early abstinence in animals that self-administered this drug when compared to yoked subjects

(Ranaldi et al., 1999; Di Ciano et al., 1996, 1998). These data are in agreement with the learning hypothesis which states that NAC DA functions as a reward prediction error signal (see Section, 1.1.2.2). Therefore, in animals that self-administered MDMA, a subsequent drug injection produces an "expected" effect, which may lead to neuronal adaptations in these animals at the level of mesolimbic DA to counteract the expected effect of the drug. This hypothesis may account for the low stimulation of NAC DA observed in animals that previously self-administered the drug when compared to yoked controls (Day and Carelli, 2007; Day et al., 2007).

In order to further analyse the differences observed between groups following a challenge administration of MDMA, we performed a correlation analysis between the total amount of MDMA intake and the activation of mesolimbic DA in the NAC (see Figure 3, from article 2). We found a negative correlation between these two parameters for both groups, indicating that tolerance to MDMA-induced DA activation is directly proportional to drug intake, and produced by the drug itself. However, the slope of the correlation was much smaller for those animals that had previously self-administered the drug compared to those that had received it passively. Therefore, the learned prediction of the effect of the drug may induce conditioned compensatory mechanisms that make this tolerance effect more pronounced in these animals. Furthermore, as revealed by our autoradiography studies, these differences were not due to possible neurotoxic effects following repeated MDMA administration since we did not observe any change in SERT or DAT binding in the cortex and striatum, respectively. Further experiments are needed to elucidate which mechanisms are involved in this effect and how long these changes persist in time. In summary, expectancy processes may play an important role in modulating pharmacokinetic and pharmacodynamic tolerance to reduce the effect of the drug during active drug-taking, as revealed by other evidence in animals (Siegel et al., 2000; Ramos et al., 2002), and some MDMA users (Parrott, 1998).

It is important to note that the challenge dose of MDMA was administered in a non-contingent manner and in a non-drug associated context, where animals could not predict the administration of the drug. Thus,

our results showing lower MDMA-induced DA activation under these conditions, suggest that the neuroadaptations that took place during self-administration sessions may generate a different set-point of the reward threshold that persists in time, in accordance with the allostasis theory of addiction (see Section, 1.2.1.2). In this sense, human drug addicts show lower interest for natural rewards, and neuroimaging studies using PET have revealed that non-human primates with a history of cocaine consumption exhibit lower NAC activation in response to natural stimuli (Volkow et al., 2002). In future experiments, it would be interesting to evaluate whether repeated self-administration of MDMA induces changes in the motivation for natural rewards in comparison to passive drug-intake.

In order to further investigate the neuroadaptations that take place during active versus passive MDMA administration, we evaluated different gene expression changes in brain reward-related areas (hippocampus, PFC, NAC, and dorsal raphe), following ten days of either active or passive MDMA administration (article 3). When comparing contingent MDMA to yoked MDMA mice, the highest number of statistically significant changes in gene expression were observed in the hippocampus. This result underpins the role of this structure in the contextual associations formed during active MDMA self-administration. On the other hand, no significant changes were observed in the PFC. This was unexpected since the PFC is involved in complex associative learning processes such as those required in the self-administration procedure.

Genes differentially expressed in the hippocampus of mice self-administering MDMA showed a general down-regulation pattern in comparison to yoked-MDMA mice, where genes were mostly up-regulated. Thus, changes in gene expression follow opposite directions depending on whether the drug is actively or passively administered, resulting in a characteristic mirror-image pattern of gene expression (see Figure 5.1). This can be explained by the manifestation of conditioned compensatory responses in the contingent mice to minimize the effect of the drug.

When functional clustering was performed among significant biological categories related to neurological processes, differences were predominantly found in genes related to synapse, transmission of nerve impulse,

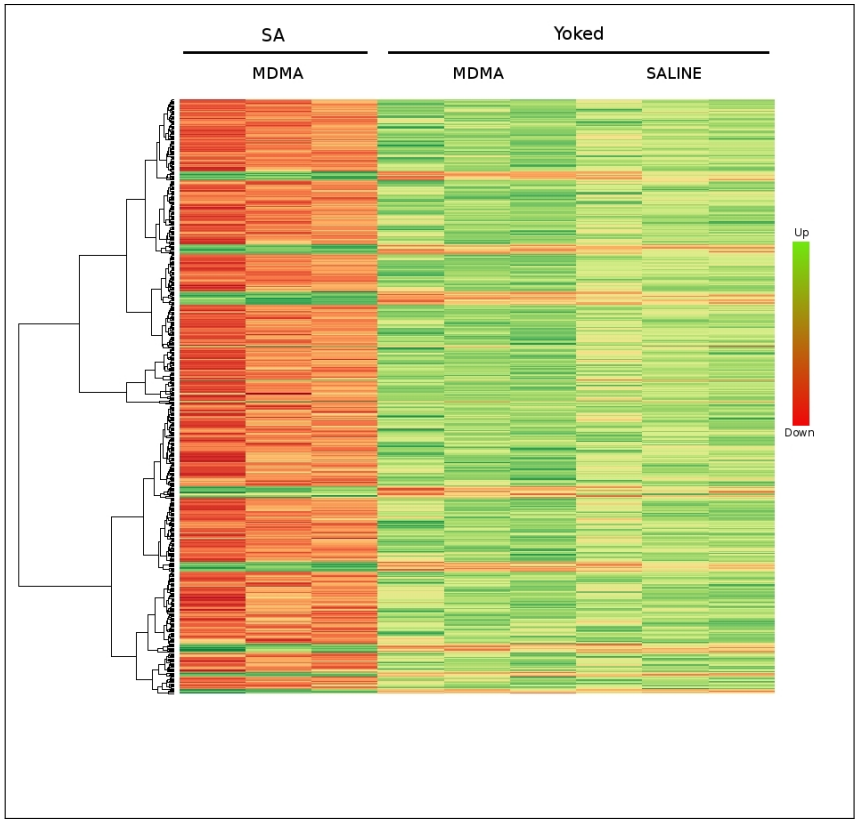


Figure 5.1: **Heat Map: Active *vs* Passive MDMA Administration** Changes in gene expression in the hippocampus after self- or yoked MDMA and saline administration. Transcripts are mainly down-regulated (*red*) in the MDMA self-administration (SA) group, whereas expression is mostly up-regulated *(green)* in yoked groups (unpublished image, from *article 3*, section 4.3, page 92.)

regulation of neurotransmitter levels, cellular morphogenesis and glutamate receptor binding. These results indicate that previously learned self-administration behaviour mainly affected the expression of genes related to plasticity, learning and neurotransmitter release, which may have also resulted from conditioned compensatory responses in the contingent mice. The observed changes in the expression of genes involved in learning and memory processes in the hippocampus such as Ntrk2, Camk2a, Gsk3 and Akt1, among others, were subsequently validated using quantitative real time-polymerase chain reaction (qRT-PCR). Unfortunately, of the 44 selected genes from a total of 537, none were positive. This lack of validation

could be explained by the limited sample size precluding the detection of differences.

In the raphe nucleus, non-statistically significant differences were found between contingent and yoked-MDMA, except for the connective tissue growth factor precursor gene, which to our knowledge, has no specific relevance in this structure. One factor that could have masked the detection of significant differences in these analyses is the strong effect produced by MDMA on the immune system (Connor et al., 2001; Pacifici et al., 2000b,a; de Paula et al., 2009). Indeed, we found that six of the most significant over-represented biological categories involved in cellular stress and immune responses showed differential expression after exposure to either contingent or yoked MDMA versus yoked saline (see Figure 3 from article 3, page 92). This result points to the neurotoxic effects of MDMA, since cellular stress can ultimately result in neuronal toxicity.

Given these observations, we decided to use a more restrictive threshold for expression differences (Log Fold Change over 1.5 or below -1.5), and a less restrictive statistical significance criterion (FDR threshold of 11% instead of 5%). This analysis identified the dorsal raphe nucleus as the brain region showing most relevant functional changes. Five genes of interest were identified in the dorsal raphe nucleus: Camk2a, Ddn, Egr3, Kalrn and Zic1. All of them, except for Zic1, were validated by qRT-PCR and were shown to be significantly up-regulated in contingent mice as compared to the MDMA yoked animals. The differential changes observed in these genes involved in neuroplasticity and neuron remodelling indicate that the raphe nucleus may play an important role in active MDMA self-administration learning behaviour. In agreement, recent data have revived theories involving the serotonergic system in reward related learning (Nakamura et al., 2008). Hence, dorsal raphe nucleus neurons seem to code for the value of the received reward depending on whether it is expected or not (Nakamura et al., 2008).

Of particular interest is the up-regulation observed in contingent mice for the Camk2a gene that encodes the Ca2+/calmodulin-dependent protein kinase II alpha (CaMKIIalpha). This protein mediates activity-dependent synaptic plasticity, and is important for the persistence of LTP and other

forms of neural and behavioural plasticity (Nestler, 2001). Studies using other psychostimulants, such as cocaine have shown differential changes in LTP and the mesolimbic DA system, depending on whether the drug is administered actively or passively. Thus, self-administration of cocaine, but not passive cocaine infusions, produced persistent LTP in the VTA, which was still present after 3 months of abstinence and this alteration was resistant to three weeks of extinction training (Chen et al., 2008). Similarly, cocaine active but not passive administration induces a long lasting (21 days) inhibition of LTD in the NAC core (Martin et al., 2006). Together, these results suggest that only voluntary drug intake can induce long-lasting neuroadaptations in the mesolimbic dopaminergic system. This mechanism may underlie the loop of drug-seeking behaviour and relapse even after long periods of abstinence that characterizes an addictive state.

In our study, when comparing contingent and yoked MDMA mice vs yoked saline mice, we observed also an up-regulation of genes involved in neuroadaptations and synaptic plasticity, indicating an effect of direct exposure to MDMA. The Sgk1 and Sgk3 genes, which encode the serum-glucocorticoid regulated kinase 1 and 3 respectively, were identified in the dorsal raphe nuclei in contingent and yoked MDMA mice versus yoked saline mice. Both genes are involved in memory consolidation processes (von Hertzen and Giese, 2005) and regulate glutamatergic neurotransmission that is critical for the acquisition, consolidation and retrieval of memories (Strutz-Seebohm et al., 2005a,b). This finding is in concordance with previous evidence showing that repeated doses of MDMA dose-dependently impair the acquisition and recall of an active avoidance task in mice (Trigo et al., 2008), and induce memory impairment in MDMA users (Reneman et al., 2000; Fox et al., 2001). These results suggest that active of passive repeated exposure to MDMA, produces changes in glutamatergic transmission that may account for the MDMA-induced cognitive deficits evidenced in animals and humans.

5.2 Involvement of 5-HT$_{2A}$R in MDMA-Induced Reinforcement

The data presented in article 4 show that intravenous self-administration of (+)-MDMA was suppressed in mice lacking 5-HT$_{2A}$R at both doses tested (0.125 and 0.25 mg/kg/inf). These results suggest that 5-HT$_{2A}$R are critically important for MDMA-induced reinforcement. Previous pharmacological studies showed that acute administration of the selective 5-HT$_{2A}$R antagonist, MDL-100907 to non-human primates partially attenuates S(+)-MDMA self-administration, and completely abolishes R(-)-MDMA self-administration (Fantegrossi et al., 2002). Moreover, humans pretreated with the non-selective 5-HT$_{2A}$R antagonist ketanserin reported a decrease in some of the rewarding effects induced by MDMA (Liechti et al., 2000). Interestingly, the 5-HT$_{2A}$R does not seem to play a major role in the reinforcing properties of other drugs of abuse. Pharmacological studies revealed that MDL-100907 failed to affect the ability of amphetamine to reduce the threshold required to sustain rewarding brain stimulation in the VTA (Moser et al., 1996). In addition, this antagonist did not modify the reinforcing potency of cocaine in rats (Filip et al., 2006; Fletcher et al., 2002b), or non-human primates (Fantegrossi et al., 2002). Discrepancies observed in studies using MDMA and other psychostimulants may be due to the fact that MDMA exerts its effects mainly through the serotonergic system, while cocaine and amphetamine act predominantly on the dopaminergic system (Han and Gu, 2006). Moreover, MDMA and MDA can exert a slight hallucinogenic effect that distinguish them from other amphetamines and psychostimulants (Nichols, 1986). The hallucinogenic effects produced by MDMA, mediated by the 5-HT$_{2A}$R (Gonzlez-Maeso et al., 2007), may participate in the behavioural and reinforcing properties of MDMA a mechanism that would not be observed for other psychostimulants.

Our microdialysis data confirmed that 5-HT$_{2A}$R modulate the DA me-

solimbic system. In this respect, 5-HT$_{2A}$R KO mice exhibited lower basal and stimulated DA extracellular levels in the NAC, while 5-HT and NE levels in the PFC were similar to WT mice. This is in concordance with previous studies showing that the activation 5-HT$_{2A}$R, specifically in the PFC, increases DA in the VTA (Bortolozzi et al., 2005; Martín-Ruiz et al., 2001).

Our results also showed a lower MDMA-induced stimulation of NAC DA in 5-HT$_{2A}$R KO compared to WT mice, while no difference in 5-HT or NE in the PFC were observed between genotypes. The lower extracellular levels of mesolimbic DA observed in KO mice support the involvement of dopaminergic neurotransmission in the reinforcing effects of MDMA. Evidence for a dopaminergic component in MDMA induced reward and reinforcement has been previously put forward. Thus, the acute impairment of DA neurotransmission by a systemic DA release inhibitor, prevents the acquisition of MDMA CPP in rats (Bilsky et al., 1998). Similarly, the blockade of DA D1 and D2 receptors differentially disrupts MDMA self-administration in rats (Daniela et al., 2004; Brennan et al., 2009; Shin et al., 2008) (discussed more depth in section 1.4.5). However, previous data showing that SERT KO mice display normal MDMA-induced increase in NAC DA, but do not self-administer MDMA at different doses (Trigo et al., 2007), argues for the critical involvement of 5-HT mechanisms in MDMA reinforcement processes. Together, these data reveal that a concomitant 5-HT-DA stimulation is necessary for the full expression of the rewarding properties of MDMA.

The modulation of mesolimbic DA by other serotonin receptors, and their participation in the rewarding and activating effects of MDMA have been studied recently. In this respect, mice lacking 5-HT$_{2B}$R. Thus, these mice showed blunted MDMA-evoked 5-HT and DA release in the NAC and VTA, as well as no MDMA-induced hyperlocomotion or conditioned place preference (Doly et al., 2009). The authors postulate that the lack of MDMA-induced DA release in NAC seen in 5-HT$_{2B}$R KO mice, may be attributed to the absence of 5-HT release in the VTA, where postsynaptic 5-HT$_{2A}$R have a stimulatory effect (Doly et al., 2008).

The involvement of 5-HT$_{1A}$R in the reinforcing properties of MDMA

can also be put forward since these receptors modulate mesolimbic DA. Hence, the activation of mPFC 5-HT$_{1A}$R enhances the activity of VTA DA neurons and promotes mesocortical DA release (Díaz-Mataix et al., 2005). Moreover, this receptor is colocalized with the 5-HT$_{2A}$R in the PFC pyramidal neurons and activate the same cellular pathways (Amargós-Bosch et al., 2004). However, the 5-HT$_{1A}$R can modulate the DAergic and 5-HTergic systems in opposite direction depending on whether they are located pre- or post- synaptically (Mller et al., 2007).

On the other hand, our study also showed that MDMA-induced hyperactivity is potentiated in KO mice. This contradicts pharmacological studies where pre-treatment with MDL-100907, a selective 5-HT$_{2A}$R antagonist, attenuated hypermotility produced by a high dose of racemic MDMA (Fantegrossi et al., 2003; Kehne et al., 1996), while only peripheral hyperactivity produced by lower doses of MDMA was decreased (Bankson and Cunningham, 2002; Herin et al., 2005). Furthermore, MDL-100907 had an opposite effect on MDMA enantiomers (Fantegrossi et al., 2003). In addition, pre-treatment with the selective 5-HT$_{2A}$R antagonist, eplivanserin, attenuated racemic MDMA-induced locomotor activation (Ball and Rebec, 2005). The discrepancy observed between pharmacological and genetic studies could be related to compensatory mechanisms induced by the chronic absence of these receptors in the KO animals. Although no changes in mRNA expression for the different 5-HT receptors have been observed in 5-HT$_{2A}$R KO mice (Weisstaub et al., 2006), we can not rule out the possibility that other receptors may have participated in the effects observed in these mice.

Changes in D1 and D2 receptors have also been implicated in MDMA-induced hyperlocomotion (Benturquia et al., 2008; Ball et al., 2003). In addition, 5-HT$_{2C}$R also modulate MDMA hyperlocomotion, but in an opposite direction to 5-HT$_{2A}$R, since 5-HT$_{2C}$R antagonists potentiate locomotor activation induced by MDMA (Bankson and Cunningham, 2002). In conclusion, it is likely that all monoamines are involved in MDMA induced hyperlocomotion through a fine coupling mechanism that is not yet fully understood.

Interestingly, d-amphetamine administration enhanced locomotor re-

sponses in mice with a constitutive deletion of the 5-HT$_{2A}$R to a greater extent than in WT control mice. Furthermore, the α1-adrenergic antagonist, prazosin completely blocked the d-amphetamine-induced locomotor response (Salomon et al., 2007). In that study, the behavioural response to d-amphetamine was paralleled by an increase in extracellular levels of NE in the PFC. The authors postulate that 5-HT$_{2A}$R and α1-adrenergic receptors in the PFC regulate each other under normal conditions to modulate psychostimulant-induced locomotor activity, as well as DA levels in the NAC (Auclair et al., 2004a,b). In our study, the behavioural effects of MDMA in 5-HT$_{2A}$R KO mice were similar to those observed with d-amphetamine, however our microdialysis data showed that basal, as well as stimulated NE levels in the PFC of KO mice did not differ from WT mice. We did not, therefore, carry out further experiments to evaluate the interaction between α1-adrenergic and 5-HT$_{2A}$R in the effects of MDMA.

Finally, our results show a dissociation of MDMA-induced hyperactivity and reinforcement in 5-HT$_{2A}$R KO mice. Although these two properties of psychostimulants are often correlated, hyperlocomotion is not always directly proportional to reward/reinforcement (Robledo et al., 1993). Our data support the hypothesis that different neurobiological mechanisms may underlie MDMA-induced hyperlocomotion and reinforcement.

Based on this evidence, it seems that serotonin, through 5-HT$_{2A}$R and their modulation of the dopaminergic system, plays a mayor role in the rewarding and reinforcing properties of MDMA. However, it is important to take into account that monoamines are reciprocally modulated, and following a genetic ablation of a specific receptor, compensatory mechanisms can either account for or mask the results obtained.

5.3 Involvement of 5-HT$_{2A}$R in Relapse to MDMA-Seeking Behaviour

In *article 4*, we demonstrated for the first time the importance of 5-HT$_{2A}$R in relapse to MDMA seeking behaviour. Thus, the selective blockade of 5-HT$_{2A}$R effectively prevented cue-induced reinstatement of

MDMA-seeking behaviour in C57BL/6J mice (see *article 4*). Previous studies have reported similar findings with other drugs of abuse. For instance, cue-induced relapse to cocaine seeking behaviour was attenuated using the selective antagonist MDL-100907 (Nic Dhonnchadha et al., 2009), and the non selective 5-HT_{2A}R antagonist ariprazole (Feltenstein et al., 2007). Reinstatement of morphine CPP was also blocked by aripiprazole (xia Li et al., 2009). Ariparazol, besides being a 5-HT_{2A}R antagonist, is a 5-HT_{1A}R agonist, and a D2 partial agonist. In both studies, the authors attributed the ability of aripiprazole to block reinstatement to its antagonist effects on the 5-HT_{2A}R.

Hence, it is likely that the 5-HT_{2A}R is involved in the general mechanisms mediating reinstatement of drug seeking behaviour. Accordingly, changes in 5-HT_{2A}R sensitivity have been observed following withdrawal from cocaine (Carrasco and Battaglia, 2007; Carrasco et al., 2007) and MDMA (Reneman et al., 2002) after both acute and chronic treatment, as revealed by SPECT[1] brain imaging. Furthermore, in this latter study, the serotonin depletion observed after MDMA administration was positively correlated with an increase in the number of 5-HT_{2A}R in the rat PFC. Additionally, during early abstinence, MDMA users display a decrease of 5-HT_{2A}R, while 30 days after the last exposure, 5-HT_{2A}Rs are up-regulated to a higher level when compared to control subjects (Reneman et al., 2002). Moreover, these receptors are located in brain areas critical for cue-induced relapse such as the BLA and medial PFC (Ciccocioppo et al., 2001; Weiss et al., 2001). To date, however, there have been no experiments using selective antagonists injected intra-cerebrally, which could have corroborated the specific involvement of these receptors in BLA or PFC in cue-induced relapse.

On the other hand, the excitatory modulation of PFC 5-HT_{2A}R over mesolimbic DA (Bortolozzi et al., 2005; Vázquez-Borsetti et al., 2009), has been suggested as a possible mechanism by which 5-HT_{2A}R could mediate cue-induced relapse. Thus, 5-HT_{2A}R antagonists may represent a possible therapeutic option for preventing cue-relapse in humans. However, as revealed by our microdialysis data, the chronic blockade of these receptors

[1]Single photon emission computed tomography

can induce a decrease in NAC DA basal levels, which could blunt the motivational value of other non-drug related rewards. Nevertheless, in our study we show that KO mice do not exhibit a major motivational impairment either when trained to acquire operant responding for food reward, or in the PR paradigm, a more precise measure of the motivation of the animal to work for a reward.

As explained above, 5-HT$_{2A}$R blockade does not affect cocaine reinforcement (Filip et al., 2006; Fantegrossi et al., 2002; Fletcher et al., 2002a) or morphine reward (xia Li et al., 2009), but does block cocaine (Nic Dhonnchadha et al., 2009), morphine (xia Li et al., 2009) and MDMA (article 4) relapse. These data support a dissociation between the neurobiological mechanisms involved in reinforcement and relapse to drug-seeking, as previously reported (For review see See, 2005, and references therein). Furthermore, this effect seems to be specific for drugs of abuse since eplivanserin administration did not block the reinstatement of responding for cues associated with sucrose self-administration (Nic Dhonnchadha et al., 2009).

Another interesting result of the work presented in *article 4* (see Section 4.4) is that a priming dose of MDMA did not reinstate MDMA-seeking behaviour in C57BL/6J mice. This result was surprising because we found in *article 1* (see Section 4.1, page 73) that MDMA-priming reinstated cocaine-seeking behaviour. This incongruity, may be explained by the fact that different strains of mice where used in both experiments: CD1 mice in *article 1*, and C57BL/6J mice in *article 4*. Accordingly, it has been reported that C57BL/6J mice are more resistant to prime-induced relapse than CD1 mice (Yan and Nabeshima, 2009).

Given that the concomitant consumption of other psychostimulants is quite high in cocaine users, in *article 1* we investigated whether MDMA or d-amphetamine could reinstate cocaine seeking behaviour. Reinstatement of cocaine-seeking behaviour after the acute administration of priming doses of a variety of drugs, including cocaine itself, amphetamine and MDMA, has been previously shown in rats using different experimental procedures (de Wit and Stewart, 1981; Schenk et al., 2008a). However, we found that a priming injection of d-amphetamine failed to reinstate

cocaine-seeking behaviour in CD1 mice. This discrepancy can also be explained by differences in species and in the route of administration of the drug. In general, it is more difficult to observe reinstatement of drug-seeking behaviour induced by a priming dose of the drug in mice than in rats, while cue-induced reinstatement is quite prominent in mice (for review see Yan and Nabeshima, 2009 and references therein).

Our results showing the ability of MDMA to reinstate cocaine seeking behaviour are supported by previous data showing a behavioural cross sensitization to cocaine in rats repeatedly treated with MDMA (Kalivas et al., 1998). Moreover, the effects of MDMA on the dopaminergic system may participate in these effects. In this respect, the systemic administration of DA, but not of 5-HT or NE transporter inhibitors can reinstate cocaine seeking behaviour in rats (Schmidt and Pierce, 2006). However, other studies support the role of the serotonergic system in the reinstatement of cocaine-seeking behaviour, following extinction (Grottick et al., 2000). Thus, MDMA by its action on 5-HT can also reinstate cocaine seeking. Indeed, MDMA binds with higher affinity to the SERT than to the DAT (Rothman and Baumann, 2003; Han and Gu, 2006), and produces a higher release of 5-HT than DA (Schmidt et al., 1987; Koch and Galloway, 1997). Additionally, both MDMA and cocaine inhibit the 5-HT re-uptake mechanism increasing the availability of this neurotransmitter, which has been proposed to play an important role in modulating the discriminative and stimulant effects of both substances (Yamamoto and Spanos, 1988; Rocha, 2003).

Therefore, a general increase in monoaminergic tone might be responsible for the reinstatement of cocaine-seeking behaviour following a priming injection of MDMA in mice, as has been previously suggested for rats (Schenk et al., 2008b). In addition, this study shows that mice models of relapse can be used to evaluate specific factors involved in drug-seeking behaviour. These models are highly relevant since they can be applied to genetically modified mice, which are proven powerful tools in the search for new pharmacological targets for treating drug addiction.

CHAPTER 6

Conclusions

1. MDMA but not d-amphetamine reinstated cocaine-seeking behaviour in mice. This result raises concerns about the specific participation of 5-HT and DA in the discriminative effects of cocaine.

2. Repeated low doses of MDMA produce a decrease in basal extracellular levels of DA in the NAC of mice during early abstinence.

3. A challenge administration of MDMA increases NAC DA extracellular levels to a lower extent in mice previously trained to self-administer MDMA as compared to yoked MDMA and yoked saline mice. Thus, the neuroadaptations that take place during MDMA self-administration can differentially affect the neurochemical response of mesolimbic dopaminergic activity.

4. These neuroadaptive changes were not related to neurotoxicity of DA terminals in the striatum, or to alterations in SERT binding in the cingulate cortex or the hippocampus.

5. MDMA exposure induces the expression of genes related to neurotoxicity, inflammatory and immunological response in PFC, NAC, hippocampus and dorsal raphe.

6. Active and passive MDMA administration induces a different gene expression profile in brain areas involved in the reward circuit.

7. Active but not passive MDMA administration produces changes in the expression of genes involved in learning and memory processes in the hippocampus and dorsal raphe nucleus, brain structures related to learning the contingencies of reward.

8. 5-HT_{2A}R KO mice do not acquire MDMA self-administration behaviour, while they do learn an instrumental response to obtain food pellets, supporting the specific involvement of these receptors in MDMA reinforcing properties.

9. These effects were associated to lower basal and MDMA-stimulated extracellular levels of DA in the NAC of 5-HT_{2A}R KO mice.

10. MDMA-induced hyperlocomotion was enhanced in 5-HT_{2A}R KO animals, suggesting a different involvement of these receptors in the locomotor effects of MDMA.

11. The specific 5-HT_{2A}R antagonist, eplivanserin, potently blocked cue-induced relapse to MDMA-seeking behaviour.

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Appendices

APPENDIX A

Supplementary Material for Article 3

Table S1. One-way ANOVA for active versus inactive hole discrimination in contingent mice self-administering MDMA (0.25 mg/kg/inf).

Probe	*F*- value	*p*- value
Day 1	$F_{(1,17)} = 6.010$	<0.05
Day 2	$F_{(1,17)} = 4.779$	<0.05
Day 3	$F_{(1,17)} = 5.416$	<0.05
Day 4	$F_{(1,17)} = 6.877$	<0.05
Day 5	$F_{(1,17)} = 15.154$	<0.01
Day 6	$F_{(1,17)} = 20.512$	<0.001
Day 7	$F_{(1,17)} = 12.115$	<0.01
Day 8	$F_{(1,17)} = 14.967$	<0.01
Day 9	$F_{(1,17)} = 25.474$	<0.001
Day 10	$F_{(1,17)} = 12.745$	<0.01
Day 11	$F_{(1,17)} = 7.434$	<0.05

Table S2. Genes differentially expressed in hippocampus identified in the contingent-MDMA vs yoked-MDMA and contingent-MDMA vs yoked-saline comparisons after applying a 5% FDR.

Probe	Gene symbol	Gene Name	Contingent-MDMA vs Yoked-MDMA (Fold Change)	Contingent-MDMA vs Yoked-Saline (Fold Change)
Upregulated				
1453578_at	Pter	phosphotriesterase related	1.50	1.53
1451996_at	Tm2d1	TM2 domain containing 1	1.42	1.22
1426857_a_at	Hsdl2	hydroxysteroid dehydrogenase like 2	1.38	1.20
1423711_at	Ndufaf1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1	1.33	1.15
1453468_at	Cep290	centrosomal protein 290	1.39	1.17
1447807_s_at	Plekhh1	pleckstrin homology domain containing, family H (with MyTH4 domain) member 1	1.45	1.27
1422685_at	Exoc4	exocyst complex component 4	1.38	1.18
1430991_at	1810014B01Rik	RIKEN cDNA 1810014B01 gene	1.73	1.49
1436559_a_at	Psmd10	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10	1.33	1.20
1435377_at	2410002O22Rik	RIKEN cDNA 2410002O22 gene	1.43	1.26
1424105_a_at	Pttg1	pituitary tumor-transforming 1	1.35	1.20
1422031_a_at	Zfand6	zinc finger, AN1-type domain 6	1.30	1.21
1444589_at	EG240038	predicted gene, EG240038	1.34	1.24
1428507_at	Hdhd2	haloacid dehalogenase-like hydrolase domain containing 2	1.31	1.16
1431507_a_at	Synj2bp	synaptojanin 2 binding protein	1.31	1.20
1439397_at	Fmn1	formin 1	1.48	1.32
1420620_a_at	Rnf13	ring finger protein 13	1.34	1.22
1444041_at	AU041133	expressed sequence AU041133	1.27	1.17
1453739_at	Tmem126b	transmembrane protein 126B	1.31	1.18
1417653_at	Pvalb	parvalbumin	1.35	1.19
1416867_at	Bet1	blocked early in transport 1 homolog (S. cerevisiae)	1.33	1.25
1438989_s_at	B130021B11Rik	RIKEN cDNA B130021B11 gene	1.27	1.16
1422868_s_at	Gda	guanine deaminase	1.65	1.41
1452007_at	Vamp7	vesicle-associated membrane protein 7	1.34	1.21
1419924_at	Fnip1	folliculin interacting protein 1	1.46	1.37
1418623_at	Rab2a	RAB2A, member RAS oncogene family	1.39	1.35
1419918_at	Tmed7	transmembrane emp24 protein transport domain containing 7	1.53	1.54
1438260_at	Kcnq2	potassium voltage-gated channel, subfamily Q, member 2	1.25	1.35
1426243_at	Cth	cystathionase (cystathionine gamma-lyase)	1.43	1.28
1424912_at	Slc25a17	solute carrier family 25 (mitochondrial carrier, peroxisomal membrane protein), member 17	1.28	1.30
1422753_a_at	Polr3k	polymerase (RNA) III (DNA directed) polypeptide K	1.43	1.27
1423740_a_at	Rbm10	RNA binding motif protein 10	1.25	1.21
1448309_at	Ap3m1	adaptor-related protein complex 3, mu 1 subunit	1.27	1.18
1423127_at	Impa1	inositol (myo)-1(or 4)-monophosphatase 1	1.23	1.20
1419975_at	Scp2	sterol carrier protein 2, liver	1.57	1.63
1447967_at	Tmem69	transmembrane protein 69	1.41	1.41
1423840_at	Ccdc56	coiled-coil domain containing 56	1.31	1.21
1419287_at	Tmem208	transmembrane protein 208	1.26	1.18
1457983_s_at	Rwdd4a	RWD domain containing 4A	1.28	1.22
1424508_at	Ttc5	tetratricopeptide repeat domain 5	1.23	1.22
1447623_s_at	Prkd1	protein kinase D1	1.25	1.28
1435999_at	Spink8	serine peptidase inhibitor, Kazal type 8	1.24	1.30
1459522_s_at	Gyg	glycogenin	1.27	1.20
1439511_at	Cdk7	cyclin-dependent kinase 7 (homolog of Xenopus MO15 cdk-activating kinase)	1.28	1.21
1419153_at	2810417H13Rik	RIKEN cDNA 2810417H13 gene	1.25	1.29
1417263_at	Ptgs2	prostaglandin-endoperoxide synthase 2	1.26	1.23
1435222_at	Foxp1	forkhead box P1	1.26	1.26
1449388_at	Thbs4	thrombospondin 4	1.27	1.36
1423531_a_at	Hnrnpa1	heterogeneous nuclear ribonucleoprotein A1	1.33	1.28
1437729_at	Rpl27a	ribosomal protein L27a	1.44	1.33
1438478_a_at	Ppp3ca	protein phosphatase 3, catalytic subunit, alpha isoform	1.20	1.22

1438251_x_at	Htra1	HtrA serine peptidase 1	1.20	1.42
1436646_at	Scn2a1	sodium channel, voltage-gated, type II, alpha 1	1.59	1.52
1416292_at	Prdx3	peroxiredoxin 3	1.19	1.21
1449799_s_at	Pkp2	plakophilin 2	1.28	1.33
1420982_at	Rbm39	RNA binding motif protein 39	1.29	1.28
1438654_x_at	Mmd2	monocyte to macrophage differentiation-associated 2	1.21	1.22
1438975_x_at	Zdhhc14	zinc finger, DHHC domain containing 14	1.22	1.21
1419913_at	Strap	serine/threonine kinase receptor associated protein	1.40	1.34
1455860_at	Pigh	phosphatidylinositol glycan anchor biosynthesis, class H	1.21	1.22
1437614_x_at	Zdhhc14	zinc finger, DHHC domain containing 14	1.21	1.20
1437418_at	100041799	predicted gene, 100041799	1.18	1.30
1419699_at	Scgb3a1	secretoglobin, family 3A, member 1	1.82	3.29
1456037_x_at	Preb	prolactin regulatory element binding	1.29	1.30
1459840_s_at	Ccdc28b	coiled coil domain containing 28B	1.24	1.24
1442051_at	Hist2h3c1	histone cluster 2, H3c1	1.21	-1.21
1417695_a_at	Soat1	sterol O-acyltransferase 1	1.30	1.36
1436948_a_at	6430550H21Rik	RIKEN cDNA 6430550H21 gene	1.15	1.25
Downregulated				
1418015_at	Pum2	pumilio 2 (Drosophila)	-1.82	-1.54
1422073_a_at	Celsr2	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	-1.72	-1.49
1434908_at	Scaf1	SR-related CTD-associated factor 1	-1.63	-1.42
1441312_at	Cnnm1	cyclin M1	-1.92	-1.63
1436780_at	Ogt	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	-1.76	-1.61
1423561_at	Nell2	NEL-like 2 (chicken)	-1.68	-1.47
1429088_at	Lbh	limb-bud and heart	-1.69	-1.52
1424137_at	Gprin1	G protein-regulated inducer of neurite outgrowth 1	-1.51	-1.40
1435889_at	Mark2	MAP/microtubule affinity-regulating kinase 2	-1.46	-1.30
1438732_at	RP23-448C18.1	novel similar to human Nance-Horan syndrome protein (RP11-262D11.5)	-1.64	-1.48
1448901_at	Cpxm1	carboxypeptidase X 1 (M14 family)	-1.47	-1.27
1430197_a_at	Pitpnm2	phosphatidylinositol transfer protein, membrane-associated 2	-1.83	-1.47
1447412_at	Gm996	gene model 996, (NCBI)	-1.49	-1.38
1416184_s_at	Hmga1	high mobility group AT-hook 1	-1.52	-1.37
1435152_at	Leng8	leukocyte receptor cluster (LRC) member 8	-1.53	-1.22
1434208_at	Rnf169	ring finger protein 169	-1.52	-1.31
1418784_at	Grik5	glutamate receptor, ionotropic, kainate 5 (gamma 2)	-1.56	-1.32
1452650_at	Trim62	tripartite motif-containing 62	-1.51	-1.35
1455701_at	Snx26	sorting nexin 26	-1.55	-1.50
1421053_at	Kif1a	kinesin family member 1A	-1.72	-1.49
1434032_at	Centg3	centaurin, gamma 3	-1.60	-1.23
1451762_a_at	Kif1b	kinesin family member 1B	-1.58	-1.53
1421181_at	Nptxr	neuronal pentraxin receptor	-1.85	-1.78
1419757_at	Pitpnm2	phosphatidylinositol transfer protein, membrane-associated 2	-1.47	-1.35
1437968_at	Grin1	glutamate receptor, ionotropic, NMDA1 (zeta 1)	-1.49	-1.33
1417440_at	Arid1a	AT rich interactive domain 1A (SWI-like)	-1.47	-1.39
1416997_a_at	Hap1	huntingtin-associated protein 1	-1.65	-1.32
1442707_at	Camk2a	calcium/calmodulin-dependent protein kinase II alpha	-1.75	-1.73
1443805_at	Dact3	dapper homolog 3, antagonist of beta-catenin (xenopus)	-1.76	-1.61
1443170_at	Cnnm1	cyclin M1	-1.49	-1.35
1437125_at	Camk2a	calcium/calmodulin-dependent protein kinase II alpha	-1.98	-1.97
1455463_at	Phyhip	phytanoyl-CoA hydroxylase interacting protein	-1.51	-1.32
1436919_at	Trp53i11	transformation related protein 53 inducible protein 11	-1.48	-1.29
1452453_a_at	Camk2a	calcium/calmodulin-dependent protein kinase II alpha	-1.78	-1.77
1422034_a_at	Palm	paralemmin	-1.58	-1.46
1455215_at	C530028O21Rik	RIKEN cDNA C530028O21 gene	-1.46	-1.40
1427763_a_at	Camk2d	calcium/calmodulin-dependent protein kinase II, delta	-1.76	-1.26
1429192_at	Ski	ski sarcoma viral oncogene homolog (avian)	-1.58	-1.39
1423653_at	Atp1a1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	-1.51	-1.58
1435558_at	Bai2	brain-specific angiogenesis inhibitor 2	-1.43	-1.26
1438208_at	Taok2	TAO kinase 2	-1.45	-1.35
1426242_at	Polr2a	polymerase (RNA) II (DNA directed) polypeptide A	-1.69	-1.33
1428054_at	Slc8a2	solute carrier family 8 (sodium/calcium exchanger), member 2	-1.51	-1.81
1460724_at	Ap2a1	adaptor protein complex AP-2, alpha 1 subunit	-1.64	-1.45

1455499_at	Nrxn2	neurexin II	-1.78	-1.82
1455942_at	Fbxl11	F-box and leucine-rich repeat protein 11	-1.46	-1.39
1454759_at	Git1	G protein-coupled receptor kinase-interactor 1	-1.41	-1.34
1417632_at	Atp6v0a1	ATPase, H+ transporting, lysosomal V0 subunit A1	-1.48	-1.30
1427481_a_at	Atp1a3	ATPase, Na+/K+ transporting, alpha 3 polypeptide	-1.79	-1.64
1450903_at	Rad23b	RAD23b homolog (S. cerevisiae)	-1.39	-1.32
1420834_at	Vamp2	vesicle-associated membrane protein 2	-1.43	-1.42
1428986_at	Slc17a7	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	-1.54	-1.41
1423425_at	1300012G16Rik	RIKEN cDNA 1300012G16 gene	-1.47	-1.19
1455771_at	Bzrap1	benzodiazapine receptor associated protein 1	-1.40	-1.17
1435279_at	BC059842	cDNA sequence BC059842	-1.39	-1.20
1459107_at	Kcnh3	potassium voltage-gated channel, subfamily H (eag-related), member 3	-1.38	-1.20
1451808_at	Kcnj4	potassium inwardly-rectifying channel, subfamily J, member 4	-1.36	-1.26
1451475_at	Plxnd1	plexin D1	-1.57	-1.23
1449420_at	Pde1b	phosphodiesterase 1B, Ca2+-calmodulin dependent	-1.40	-1.21
1458441_at	Slc6a17	solute carrier family 6 (neurotransmitter transporter), member 17	-1.40	-1.38
1435762_at	Pacs1	phosphofurin acidic cluster sorting protein 1	-1.38	-1.26
1435026_at	Spock2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2	-1.48	-1.41
1460692_at	Ehmt2	euchromatic histone lysine N-methyltransferase 2	-1.39	-1.26
1451663_a_at	Trim3	tripartite motif-containing 3	-1.37	-1.20
1420752_at	Dtx3	deltex 3 homolog (Drosophila)	-1.41	-1.36
1425760_a_at	Pitpnm1	phosphatidylinositol transfer protein, membrane-associated 1	-1.42	-1.29
1444650_at	Arhgef18	rho/rac guanine nucleotide exchange factor (GEF) 18	-1.43	-1.31
1433765_at	Ube2o	ubiquitin-conjugating enzyme E2O	-1.34	-1.24
1436078_at	Fcho1	FCH domain only 1	-1.46	-1.27
1445475_at	Pak6	p21 (CDKN1A)-activated kinase 6	-1.36	-1.31
1425777_at	Cacnb1	calcium channel, voltage-dependent, beta 1 subunit	-1.38	-1.40
1434270_at	Nptxr	neuronal pentraxin receptor	-1.43	-1.26
1435907_at	Nrxn2	neurexin II	-1.35	-1.19
1434415_at	Dact3	dapper homolog 3, antagonist of beta-catenin (xenopus)	-1.40	-1.30
1450662_at	Tesk1	testis specific protein kinase 1	-1.36	-1.26
1426756_at	Galnt2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2	-1.43	-1.28
1428382_at	Smarcc2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	-1.32	-1.21
1427099_at	Maz	MYC-associated zinc finger protein (purine-binding transcription factor)	-1.50	-1.45
1436094_at	Vgf	VGF nerve growth factor inducible	-1.36	-1.47
1424954_a_at	Pip5k1c	phosphatidylinositol-4-phosphate 5-kinase, type 1 gamma	-1.34	-1.28
1449362_a_at	Mink1	misshapen-like kinase 1 (zebrafish)	-1.36	-1.22
1452267_at	Flywch1	FLYWCH-type zinc finger 1	-1.41	-1.22
1420354_at	Cnnm1	cyclin M1	-1.37	-1.19
1451872_a_at	Neurl	neuralized-like homolog (Drosophila)	-1.41	-1.31
1436381_at	Dlgap3	discs, large (Drosophila) homolog-associated protein 3	-1.36	-1.31
1418078_at	Psme3	proteaseome (prosome, macropain) 28 subunit, 3	-1.64	-1.40
1433662_s_at	Timp2	tissue inhibitor of metalloproteinase 2	-1.39	-1.22
1437226_x_at	Marcksl1	MARCKS-like 1	-1.36	-1.18
1419045_at	Slc25a23	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23	-1.40	-1.40
1424472_at	Nol6	nucleolar protein family 6 (RNA-associated)	-1.33	-1.26
1460262_a_at	1700037H04Rik	RIKEN cDNA 1700037H04 gene	-1.32	-1.18
1437210_a_at	Brd2	bromodomain containing 2	-1.42	-1.24
1423603_at	Zfpm1	zinc finger protein, multitype 1	-1.41	-1.31
1452812_at	Lphn1	latrophilin 1	-1.43	-1.43
1434381_at	Atmin	ATM interactor	-1.35	-1.18
1434610_at	Plec1	plectin 1	-1.37	-1.20
1418570_at	Ncstn	nicastrin	-1.49	-1.28
1452082_at	6430548M08Rik	RIKEN cDNA 6430548M08 gene	-1.40	-1.27
1429719_at	Foxp4	forkhead box P4	-1.43	-1.30
1452379_at	Auts2	autism susceptibility candidate 2	-1.44	-1.36
1434095_at	Unc5a	unc-5 homolog A (C. elegans)	-1.52	-1.31
1449961_at	Rph3a	rabphilin 3A	-1.37	-1.39
1451071_a_at	Atp1a1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	-1.41	-1.39
1416588_at	Ptprn	protein tyrosine phosphatase, receptor type, N	-1.41	-1.25

1451864_at	Cacng8	calcium channel, voltage-dependent, gamma subunit 8	-1.64	-1.88
1433712_at	AW555464	expressed sequence AW555464	-1.30	-1.20
1424764_at	Sez6l	seizure related 6 homolog like	-1.30	-1.24
1416279_at	Ap1b1	adaptor protein complex AP-1, beta 1 subunit	-1.30	-1.18
1417423_at	Grina	glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)	-1.36	-1.21
1428910_at	2310022B05Rik	RIKEN cDNA 2310022B05 gene	-1.45	-1.21
1418053_at	Sncb	synuclein, beta	-1.33	-1.27
1449173_at	Mpp2	membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2)	-1.52	-1.46
1418785_at	Mapk8ip2	mitogen-activated protein kinase 8 interacting protein 2	-1.37	-1.25
1424956_at	Ahdc1	AT hook, DNA binding motif, containing 1	-1.41	-1.45
1417709_at	Cyp46a1	cytochrome P450, family 46, subfamily a, polypeptide 1	-1.35	-1.32
1424203_at	Ncln	nicalin homolog (zebrafish)	-1.33	-1.21
1426738_at	Dgkz	diacylglycerol kinase zeta	-1.36	-1.31
1460343_at	Neurl	neuralized-like homolog (Drosophila)	-1.31	-1.20
1415922_s_at	Marcksl1	MARCKS-like 1	-1.38	-1.28
1460210_at	Pkd1	polycystic kidney disease 1 homolog	-1.49	-1.29
1423221_at	Tubb4	tubulin, beta 4	-1.43	-1.45
1424666_at	Gpatch8	G patch domain containing 8	-1.56	-1.45
1450027_at	Sdc3	syndecan 3	-1.31	-1.16
1429204_at	Camk2n2	calcium/calmodulin-dependent protein kinase II inhibitor 2	-1.48	-1.28
1437724_x_at	Pitpnm1	phosphatidylinositol transfer protein, membrane-associated 1	-1.34	-1.22
1438688_at	Srrm2	serine/arginine repetitive matrix 2	-1.45	-1.24
1434825_at	Tnrc18	trinucleotide repeat containing 18	-1.40	-1.36
1426617_a_at	Ttyh1	tweety homolog 1 (Drosophila)	-1.40	-1.26
1436077_a_at	Fcho1	FCH domain only 1	-1.43	-1.20
1452292_at	Ap2b1	adaptor-related protein complex 2, beta 1 subunit	-1.50	-1.58
1423967_at	Palm	paralemmin	-1.45	-1.38
1424757_at	BC018242	cDNA sequence BC018242	-1.42	-1.28
1436306_at	Saps1	SAPS domain family, member 1	-1.34	-1.32
1428819_at	Mapre1	microtubule-associated protein, RP/EB family, member 1	-1.37	-1.18
1421312_a_at	Kifc2	kinesin family member C2	-1.35	-1.27
1445805_x_at	Kcnh3	potassium voltage-gated channel, subfamily H (eag-related), member 3	-1.33	-1.21
1442066_at	C530028O21Rik	RIKEN cDNA C530028O21 gene	-1.58	-1.41
1438686_at	Eif4g1	eukaryotic translation initiation factor 4, gamma 1	-1.67	-1.68
1448807_at	Hrh3	histamine receptor H3	-1.42	-1.25
1452515_a_at	Xylt2	xylosyltransferase II	-1.34	-1.17
1452789_at	Snn	stannin	-1.32	-1.20
1456492_at	9130404D08Rik	RIKEN cDNA 9130404D08 gene	-1.38	-1.19
1450183_a_at	Sh2b3	SH2B adaptor protein 3	-1.34	-1.30
1428723_at	2310047M10Rik	RIKEN cDNA 2310047M10 gene	-1.32	-1.31
1434920_a_at	Evl	Ena-vasodilator stimulated phosphoprotein	-1.36	-1.31
1419640_at	Purb	purine rich element binding protein B	-1.36	-1.20
1420964_at	Enc1	ectodermal-neural cortex 1	-1.65	-1.75
1449273_at	Cyfip2	cytoplasmic FMR1 interacting protein 2	-1.34	-1.20
1449117_at	Jund	Jun proto-oncogene related gene d	-1.33	-1.20
1436014_a_at	Rusc1	RUN and SH3 domain containing 1	-1.29	-1.29
1426493_a_at	Kifc2	kinesin family member C2	-1.32	-1.21
1450147_at	Nptxr	neuronal pentraxin receptor	-1.68	-1.57
1427754_a_at	Dnm1	dynamin 1	-1.47	-1.34
1437183_at	Lrrc4b	leucine rich repeat containing 4B	-1.48	-1.38
1420841_at	Ptprf	protein tyrosine phosphatase, receptor type, F	-1.35	-1.25
1418865_at	Zfp385a	zinc finger protein 385A	-1.32	-1.37
1437400_at	Nedd4l	neural precursor cell expressed, developmentally down-regulated gene 4-like	-1.40	-1.25
1416792_at	Ppm1g	protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	-1.33	-1.21
1434476_at	Crtc1	CREB regulated transcription coactivator 1	-1.31	-1.17
1423502_at	Brd2	bromodomain containing 2	-1.30	-1.18
1429113_at	Prrt2	proline-rich transmembrane protein 2	-1.27	-1.15
1448825_at	Pdk2	pyruvate dehydrogenase kinase, isoenzyme 2	-1.33	-1.21
1424640_at	Arl8a	ADP-ribosylation factor-like 8A	-1.41	-1.34
1460650_at	Atp6v0a1	ATPase, H+ transporting, lysosomal V0 subunit A1	-1.59	-1.48
1422799_at	Bat2	HLA-B associated transcript 2	-1.38	-1.38

1457311_at	Camk2a	calcium/calmodulin-dependent protein kinase II alpha	-1.35	-1.35
1425690_at	B3gat1	beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	-1.38	-1.30
1451992_at	Adrbk1	adrenergic receptor kinase, beta 1	-1.38	-1.29
1450151_at	Zfp316	zinc finger protein 316	-1.35	-1.32
1433802_at	Tmem151a	transmembrane protein 151A	-1.36	-1.36
1420575_at	Mt3	metallothionein 3	-1.64	-1.49
1451841_a_at	Ncor2	nuclear receptor co-repressor 2	-1.32	-1.29
1455140_at	Pitpnm3	PITPNM family member 3	-1.41	-1.39
1418881_at	Necab2	N-terminal EF-hand calcium binding protein 2	-1.39	-1.25
1455143_at	Nlgn2	neuroligin 2	-1.33	-1.25
1453527_a_at	Neurl	neuralized-like homolog (Drosophila)	-1.51	-1.34
1450040_at	Timp2	tissue inhibitor of metalloproteinase 2	-1.34	-1.27
1440128_s_at	Sez6l	seizure related 6 homolog like	-1.42	-1.23
1428708_x_at	Ptms	parathymosin	-1.41	-1.31
1423995_at	Kif1b	kinesin family member 1B	-1.41	-1.33
1417542_at	Rps6ka2	ribosomal protein S6 kinase, polypeptide 2	-1.31	-1.17
1428707_at	Ptms	parathymosin	-1.41	-1.31
1427079_at	Mapre3	microtubule-associated protein, RP/EB family, member 3	-1.35	-1.27
1423087_a_at	Prickle4	prickle homolog 4 (Drosophila)	-1.42	-1.36
1452149_at	Ube3b	ubiquitin protein ligase E3B	-1.27	-1.18
1454051_at	2600011E07Rik	RIKEN cDNA 2600011E07 gene	-1.31	-1.39
1435784_at	Atg9a	autophagy-related 9A (yeast)	-1.39	-1.31
1418181_at	Ptp4a3	protein tyrosine phosphatase 4a3	-1.29	-1.26
1435256_at	Clip3	CAP-GLY domain containing linker protein 3	-1.28	-1.24
1460566_at	Mtap1a	microtubule-associated protein 1 A	-1.29	-1.30
1425369_a_at	Sox10	SRY-box containing gene 10	-1.32	-1.64
1416113_at	Fkbp8	FK506 binding protein 8	-1.33	-1.21
1438241_at	Rgma	RGM domain family, member A	-1.38	-1.33
1455363_at	Bai1	brain-specific angiogenesis inhibitor 1	-1.35	-1.27
1434902_at	Rnf157	ring finger protein 157	-1.39	-1.33
1418794_at	Cds2	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2	-1.35	-1.36
1428095_a_at	C2cd2l	C2 calcium-dependent domain containing 2-like	-1.32	-1.23
1454821_at	B3gat1	beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	-1.28	-1.19
1434930_at	Tpcn1	two pore channel 1	-1.29	-1.19
1452235_at	Man1b1	mannosidase, alpha, class 1B, member 1	-1.34	-1.26
1420619_a_at	Aes	amino-terminal enhancer of split	-1.44	-1.30
1435780_at	Psd	pleckstrin and Sec7 domain containing	-1.35	-1.27
1420720_at	Nptx2	neuronal pentraxin 2	-1.63	-1.58
1449554_at	Tle3	transducin-like enhancer of split 3, homolog of Drosophila E(spl)	-1.46	-1.30
1422044_at	Ndst1	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	-1.63	-1.63
1458443_at	Crtc1	CREB regulated transcription coactivator 1	-1.62	-1.47
1440437_at	Herc1	hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1	-1.46	-1.36
1439748_at	Dpp6	dipeptidylpeptidase 6	-1.41	-1.38
1427110_at	Raver1	ribonucleoprotein, PTB-binding 1	-1.38	-1.28
1427379_at	Pnpla6	patatin-like phospholipase domain containing 6	-1.35	-1.30
1460675_at	Igsf8	immunoglobulin superfamily, member 8	-1.30	-1.26
1453569_s_at	Mark4	MAP/microtubule affinity-regulating kinase 4	-1.32	-1.27
1435196_at	Ntrk2	neurotrophic tyrosine kinase, receptor, type 2	-1.30	-1.22
1418535_at	Rgl1	ral guanine nucleotide dissociation stimulator,-like 1	-1.39	-1.34
1423365_at	Cacna1g	calcium channel, voltage-dependent, T type, alpha 1G subunit	-1.28	-1.25
1450202_at	Grin1	glutamate receptor, ionotropic, NMDA1 (zeta 1)	-1.44	-1.32
1439148_a_at	Pfkl	phosphofructokinase, liver, B-type	-1.30	-1.20
1431213_a_at	LOC67527	murine leukemia retrovirus	-1.54	-1.47
1431214_at	LOC67527	murine leukemia retrovirus	-1.45	-1.45
1450382_at	Nf2	neurofibromatosis 2	-1.32	-1.34
1460365_a_at	Dnm1	dynamin 1	-1.28	-1.23
1451465_at	Ubl7	ubiquitin-like 7 (bone marrow stromal cell-derived)	-1.32	-1.29
1452742_at	Trak1	trafficking protein, kinesin binding 1	-1.34	-1.33
1450228_a_at	Pip5k1c	phosphatidylinositol-4-phosphate 5-kinase, type 1 gamma	-1.53	-1.55
1425711_a_at	Akt1	thymoma viral proto-oncogene 1	-1.46	-1.30
1428852_at	Dock3	dedicator of cyto-kinesis 3	-1.26	-1.18
1426828_at	1300018I17Rik	RIKEN cDNA 1300018I17 gene	-1.28	-1.23
1426791_at	Rusc2	RUN and SH3 domain containing 2	-1.34	-1.23
1429269_at	BC068157	cDNA sequence BC068157	-1.34	-1.31
1452663_at	Svop	SV2 related protein	-1.28	-1.16

1436305_at	Rnf217	ring finger protein 217	-1.32	-1.26
1448320_at	Stim1	stromal interaction molecule 1	-1.26	-1.15
1448790_at	Sema6b	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6B	-1.31	-1.23
1434417_at	Solh	small optic lobes homolog (Drosophila)	-1.26	-1.22
1425659_at	Tom1l2	target of myb1-like 2 (chicken)	-1.50	-1.57
1437861_s_at	Prkce	protein kinase C, epsilon	-1.25	-1.17
1451422_at	Myo18a	myosin XVIIIa	-1.26	-1.20
1450576_a_at	Sf3a2	splicing factor 3a, subunit 2	-1.30	-1.24
1450471_at	Smad3	MAD homolog 3 (Drosophila)	-1.50	-1.46
1415899_at	Junb	Jun-B oncogene	-1.44	-1.27
1451110_at	Egln1	EGL nine homolog 1 (C. elegans)	-1.29	-1.24
1435942_at	Kcnq2	potassium voltage-gated channel, subfamily Q, member 2	-1.30	-1.29
1435105_at	Rnf208	ring finger protein 208	-1.41	-1.29
1426446_at	6430548M08Rik	RIKEN cDNA 6430548M08 gene	-1.31	-1.24
1433460_at	Ttc7b	tetratricopeptide repeat domain 7B	-1.26	-1.26
1436383_at	Cplx2	complexin 2	-1.31	-1.22
1422564_at	Actl6b	actin-like 6B	-1.40	-1.34
1451834_at	Cacnb1	calcium channel, voltage-dependent, beta 1 subunit	-1.46	-1.35
1455622_at	Podxl2	podocalyxin-like 2	-1.44	-1.26
1434643_at	Tbl1x	transducin (beta)-like 1 X-linked	-1.34	-1.21
1439051_a_at	Mark4	MAP/microtubule affinity-regulating kinase 4	-1.33	-1.22
1426227_s_at	Vps37c	vacuolar protein sorting 37C (yeast)	-1.32	-1.25
1450108_at	Kif1a	kinesin family member 1A	-1.35	-1.27
1449888_at	Epas1	endothelial PAS domain protein 1	-1.39	-1.30
1422521_at	Dctn1	dynactin 1	-1.34	-1.21
1430543_at	Clip3	CAP-GLY domain containing linker protein 3	-1.33	-1.30
1422647_at	Ring1	ring finger protein 1	-1.41	-1.29
1434635_at	Rph3a	rabphilin 3A	-1.23	-1.38
1420505_a_at	Stxbp1	syntaxin binding protein 1	-1.27	-1.24
1421789_s_at	Arf3	ADP-ribosylation factor 3	-1.49	-1.48
1428174_x_at	Khsrp	KH-type splicing regulatory protein	-1.24	-1.25
1420669_at	Arnt2	aryl hydrocarbon receptor nuclear translocator 2	-1.37	-1.45
1448019_at	2900006A08Rik	RIKEN cDNA 2900006A08 gene	-1.32	-1.33
1439031_at	Jph4	junctophilin 4	-1.26	-1.28
1427037_at	Eif4g1	eukaryotic translation initiation factor 4, gamma 1	-1.47	-1.54
1421149_a_at	Atn1	atrophin 1	-1.48	-1.45
1452357_at	Sept5	septin 5	-1.29	-1.23
1450733_at	Bicd2	bicaudal D homolog 2 (Drosophila)	-1.29	-1.29
1439042_at	Adcyap1r1	adenylate cyclase activating polypeptide 1 receptor 1	-1.38	-1.39
1418921_at	Cadm3	cell adhesion molecule 3	-1.24	-1.28
1421339_at	Extl3	exostoses (multiple)-like 3	-1.65	-1.71
1450249_s_at	Kif5a	kinesin family member 5A	-1.58	-1.63
1422972_s_at	Kat2a	K(lysine) acetyltransferase 2A	-1.25	-1.19
1429518_at	Faim2	Fas apoptotic inhibitory molecule 2	-1.31	-1.25
1445241_at	Rab11fip4	RAB11 family interacting protein 4 (class II)	-1.44	-1.52
1420901_a_at	Hk1	hexokinase 1	-1.38	-1.25
1460436_at	Ndst1	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	-1.22	-1.16
1454700_at	Lrfn4	leucine rich repeat and fibronectin type III domain containing 4	-1.27	-1.24
1427734_a_at	Dscaml1	Down syndrome cell adhesion molecule-like 1	-1.25	-1.34
1450799_at	Adcyap1r1	adenylate cyclase activating polypeptide 1 receptor 1	-1.28	-1.26
1436621_at	RP23-157O10.7	P140 gene	-1.24	-1.24
1450833_at	Chrm1	cholinergic receptor, muscarinic 1, CNS	-1.30	-1.33
1446265_at	Dnm3	dynamin 3	-1.54	-1.49
1456476_at	Atxn2l	ataxin 2-like	-1.38	-1.28
1421146_at	Rapgef1	Rap guanine nucleotide exchange factor (GEF) 1	-1.39	-1.31
1436845_at	Axin2	axin2	-1.26	-1.31
1448121_at	Wbp2	WW domain binding protein 2	-1.32	-1.22
1435965_at	Cnot3	CCR4-NOT transcription complex, subunit 3	-1.30	-1.24
1418323_at	Fem1b	feminization 1 homolog b (C. elegans)	-1.40	-1.30
1457456_at	Map3k10	mitogen-activated protein kinase kinase kinase 10	-1.31	-1.54
1415702_a_at	Ctbp1	C-terminal binding protein 1	-1.26	-1.19
1420833_at	Vamp2	vesicle-associated membrane protein 2	-1.52	-1.62
1444669_at	Lrfn1	leucine rich repeat and fibronectin type III domain containing 1	-1.29	-1.30
1436513_at	Tanc2	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2	-1.28	-1.22
1421090_at	Epb4.1l1	erythrocyte protein band 4.1-like 1	-1.28	-1.25

1435124_at	EG328644	predicted gene, EG328644	-1.46	-1.39
1437167_at	Tmod2	tropomodulin 2	-1.34	-1.35
1454930_at	Tbcel	tubulin folding cofactor E-like	-1.31	-1.31
1434170_at	Wdr40b	WD repeat domain 40B	-1.26	-1.28
1426989_at	Clstn3	calsyntenin 3	-1.34	-1.35
1448280_at	Syp	synaptophysin	-1.30	-1.30
1427688_a_at	Ptprs	protein tyrosine phosphatase, receptor type, S	-1.68	-1.54
1454848_at	Ppp1r12c	protein phosphatase 1, regulatory (inhibitor) subunit 12C	-1.29	-1.29
1423992_at	Gatad2a	GATA zinc finger domain containing 2A	-1.31	-1.20
1450451_at	Spock2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2	-1.38	-1.31
1435083_at	Ctxn1	cortexin 1	-1.32	-1.32
1454891_at	Cds2	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2	-1.43	-1.42
1455609_at	Cit	citron	-1.29	-1.28
1450854_at	Pa2g4	proliferation-associated 2G4	-1.28	-1.32
1455672_s_at	Cplx2	complexin 2	-1.26	-1.17
1433625_at	5830434P21Rik	RIKEN cDNA 5830434P21 gene	-1.25	-1.22
1428150_at	Coro7	coronin 7	-1.37	-1.30
1416437_a_at	Mapk8ip3	mitogen-activated protein kinase 8 interacting protein 3	-1.44	-1.31
1449552_at	Zfr	zinc finger RNA binding protein	-1.38	-1.37
1449054_a_at	Pcbp4	poly(rC) binding protein 4	-1.36	-1.26
1421255_a_at	Cabp1	calcium binding protein 1	-1.37	-1.25
1435148_at	Atp1b2	ATPase, Na+/K+ transporting, beta 2 polypeptide	-1.24	-1.25
1422522_at	Fxr2	fragile X mental retardation, autosomal homolog 2	-1.29	-1.27
1426888_at	Ehmt2	euchromatic histone lysine N-methyltransferase 2	-1.43	-1.35
1423231_at	Nrgn	neurogranin	-1.27	-1.24
1424040_at	Mtap7d1	microtubule-associated protein 7 domain containing 1	-1.23	-1.23
1425227_a_at	Atp6v0a1	ATPase, H+ transporting, lysosomal V0 subunit A1	-1.49	-1.50
1440031_at	Dact3	dapper homolog 3, antagonist of beta-catenin (xenopus)	-1.44	-1.37
1449055_x_at	Pcbp4	poly(rC) binding protein 4	-1.32	-1.27
1437733_at	Eif4ebp2	eukaryotic translation initiation factor 4E binding protein 2	-1.25	-1.20
1433658_x_at	Pcbp4	poly(rC) binding protein 4	-1.27	-1.19
1435561_at	Erf	Ets2 repressor factor	-1.27	-1.19
1425836_a_at	Limk1	LIM-domain containing, protein kinase	-1.23	-1.32
1431469_a_at	Cxxc5	CXXC finger 5	-1.29	-1.39
1422493_at	Cpox	coproporphyrinogen oxidase	-1.26	-1.26
1449956_at	Prkce	protein kinase C, epsilon	-1.27	-1.22
1421574_at	Rap2a	RAS related protein 2a	-1.27	-1.30
1425116_a_at	Spnb4	spectrin beta 4	-1.25	-1.24
1418518_at	Furin	furin (paired basic amino acid cleaving enzyme)	-1.37	-1.25
1427286_at	D11Bwg0517e	DNA segment, Chr 11, Brigham & Women's Genetics 0517 expressed	-1.31	-1.24
1422984_at	Clip2	CAP-GLY domain containing linker protein 2	-1.44	-1.47
1429190_at	Arsb	arylsulfatase B	-1.31	-1.32
1417708_at	Syt3	synaptotagmin III	-1.40	-1.27
1421154_at	Hcn2	hyperpolarization-activated, cyclic nucleotide-gated K+ 2	-1.25	-1.25
1435199_at	Apc2	adenomatosis polyposis coli 2	-1.55	-1.48
1431253_s_at	Tbc1d9	TBC1 domain family, member 9	-1.37	-1.41
1417330_at	Slc23a2	solute carrier family 23 (nucleobase transporters), member 2	-1.39	-1.39
1455548_at	Dlgap4	discs, large homolog-associated protein 4 (Drosophila)	-1.24	-1.23
1427409_at	March9	membrane-associated ring finger (C3HC4) 9	-1.29	-1.33
1435087_at	Zfp362	zinc finger protein 362	-1.28	-1.19
1431254_at	Kbtbd11	kelch repeat and BTB (POZ) domain containing 11	-1.41	-1.45
1454655_at	Dgkd	diacylglycerol kinase, delta	-1.41	-1.43
1435949_at	Zc3h3	zinc finger CCCH type containing 3	-1.25	-1.27
1437759_at	Pfkp	phosphofructokinase, platelet	-1.41	-1.29
1436665_a_at	Ltbp4	latent transforming growth factor beta binding protein 4	-1.31	-1.40
1422949_at	Nos1	nitric oxide synthase 1, neuronal	-1.42	-1.48
1438164_x_at	Flot2	flotillin 2	-1.28	-1.22
1425735_at	Madd	MAP-kinase activating death domain	-1.23	-1.34
1460568_at	Trim46	tripartite motif-containing 46	-1.25	-1.27
1454769_at	Tatdn2	TatD DNase domain containing 2	-1.34	-1.47
1425061_at	Wasf3	WAS protein family, member 3	-1.25	-1.31
1448656_at	Cacnb3	calcium channel, voltage-dependent, beta 3 subunit	-1.27	-1.27
1425097_a_at	Zfp106	zinc finger protein 106	-1.33	-1.53
1428221_at	Klhdc8b	kelch domain containing 8B	-1.29	-1.27
1435790_at	Olfm2	olfactomedin 2	-1.42	-1.42

1432478_a_at	Rnf19b	ring finger protein 19B	-1.26	-1.19
1438730_at	BC028801	cDNA sequence BC028801	-1.42	-1.34
1438262_at	Slc8a2	solute carrier family 8 (sodium/calcium exchanger), member 2	-1.25	-1.28
1449510_at	Zfp467	zinc finger protein 467	-1.33	-1.35
1426263_at	Cadm4	cell adhesion molecule 4	-1.24	-1.22
1455289_at	Ankrd13b	ankyrin repeat domain 13b	-1.20	-1.22
1455369_at	Apba1	amyloid beta (A4) precursor protein binding, family A, member 1	-1.26	-1.37
1423363_at	Sort1	sortilin 1	-1.32	-1.38
1434531_at	Mgat5b	mannoside acetylglucosaminyltransferase 5, isoenzyme B	-1.39	-1.30
1452690_at	Khsrp	KH-type splicing regulatory protein	-1.28	-1.25
1426805_at	Smarca4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	-1.47	-1.38
1451734_a_at	Dbn1	drebrin 1	-1.35	-1.36
1434155_a_at	2310061I04Rik	RIKEN cDNA 2310061I04 gene	-1.22	-1.32
1438017_at	Rusc1	RUN and SH3 domain containing 1	-1.27	-1.32
1452370_s_at	B230208H17Rik	RIKEN cDNA B230208H17 gene	-1.34	-1.29
1427373_at	Amigo1	adhesion molecule with Ig like domain 1	-1.21	-1.25
1456682_at	Lonrf2	LON peptidase N-terminal domain and ring finger 2	-1.31	-1.40
1420924_at	Timp2	tissue inhibitor of metalloproteinase 2	-1.36	-1.33
1421616_at	Grin2a	glutamate receptor, ionotropic, NMDA2A (epsilon 1)	-1.30	-1.22
1420891_at	Wnt7b	wingless-related MMTV integration site 7B	-1.26	-1.38
1418738_at	Scn1b	sodium channel, voltage-gated, type I, beta	-1.27	-1.20
1419155_a_at	Sox4	SRY-box containing gene 4	-1.22	-1.21
1451020_at	Gsk3b	glycogen synthase kinase 3 beta	-1.22	-1.20
1425337_at	Slc12a5	solute carrier family 12, member 5	-1.52	-1.41
1451250_at	Gprin1	G protein-regulated inducer of neurite outgrowth 1	-1.35	-1.32
1417963_at	Pltp	phospholipid transfer protein	-1.44	-1.56
1429290_at	Nptxr	neuronal pentraxin receptor	-1.21	-1.25
1425963_at	Cabp7	calcium binding protein 7	-1.46	-1.35
1428462_at	Ppp2r5e	protein phosphatase 2, regulatory subunit B (B56), epsilon isoform	-1.26	-1.37
1452406_x_at	Erdr1	erythroid differentiation regulator 1	-1.70	-1.69
1424871_s_at	1500031H01Rik	RIKEN cDNA 1500031H01 gene	-1.26	-1.21
1450212_at	Fmnl1	formin-like 1	-1.21	-1.25
1425983_x_at	Hipk2	homeodomain interacting protein kinase 2	-1.24	-1.30
1439767_at	Dlgap2	discs, large (Drosophila) homolog-associated protein 2	-1.32	-1.39
1421961_a_at	Dnajb5	DnaJ (Hsp40) homolog, subfamily B, member 5	-1.26	-1.30
1417658_at	Tbrg4	transforming growth factor beta regulated gene 4	-1.33	-1.38
1458202_at	6330500D04Rik	RIKEN cDNA 6330500D04 gene	-1.28	-1.37
1419650_at	Zfr	zinc finger RNA binding protein	-1.20	-1.24
1459909_at	Nfix	nuclear factor I/X	-1.29	-1.30
1435527_at	Nfic	nuclear factor I/C	-1.22	-1.22
1460237_at	Trim8	tripartite motif protein 8	-1.31	-1.27
1448692_at	Ubqln4	ubiquilin 4	-1.23	-1.31
1418544_at	Kcnip3	Kv channel interacting protein 3, calsenilin	-1.19	-1.20
1424050_s_at	Fgfr1	fibroblast growth factor receptor 1	-1.21	-1.20
1426403_at	Actr1b	ARP1 actin-related protein 1 homolog B, centractin beta (yeast)	-1.21	-1.25
1425679_a_at	Mapk8ip1	mitogen-activated protein kinase 8 interacting protein 1	-1.26	-1.26
1436095_at	Chd5	chromodomain helicase DNA binding protein 5	-1.21	-1.21
1435852_at	Spred3	sprouty-related, EVH1 domain containing 3	-1.24	-1.31
1433468_at	6430527G18Rik	RIKEN cDNA 6430527G18 gene	-1.24	-1.25
1434222_at	Sipa1l1	signal-induced proliferation-associated 1 like 1	-1.26	-1.21
1428123_at	2610528K11Rik	RIKEN cDNA 2610528K11 gene	-1.37	-1.55
1427077_a_at	Ap2b1	adaptor-related protein complex 2, beta 1 subunit	-1.23	-1.36
1428564_at	Zfp579	zinc finger protein 579	-1.25	-1.29
1416568_a_at	Acin1	apoptotic chromatin condensation inducer 1	-1.30	-1.26
1437491_at	Bicd2	bicaudal D homolog 2 (Drosophila)	-1.24	-1.24
1420744_at	Chrnb2	cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)	-1.27	-1.29
1445359_at	Adcy1	adenylate cyclase 1	-1.21	-1.28
1422733_at	Fjx1	four jointed box 1 (Drosophila)	-1.32	-1.31
1420397_a_at	Spen	SPEN homolog, transcriptional regulator (Drosophila)	-1.26	-1.24
1426108_s_at	Cacnb1	calcium channel, voltage-dependent, beta 1 subunit	-1.31	-1.27
1425360_at	Mllt6	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 6	-1.28	-1.28
1435550_at	C430014K11Rik	RIKEN cDNA C430014K11 gene	-1.26	-1.24
1430552_a_at	Sbf1	SET binding factor 1	-1.27	-1.27
1437291_at	2700081O15Rik	RIKEN cDNA 2700081O15 gene	-1.19	-1.25

1456424_s_at	Pltp	phospholipid transfer protein	-1.30	-1.47
1422009_at	Atp1b2	ATPase, Na+/K+ transporting, beta 2 polypeptide	-1.28	-1.31
1421060_at	Mllt1	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 1	-1.53	-1.48
1434374_at	B930006L02Rik	RIKEN cDNA B930006L02 gene	-1.29	-1.31
1451689_a_at	Sox10	SRY-box containing gene 10	-1.24	-1.37
1425885_a_at	Kcnab2	potassium voltage-gated channel, shaker-related subfamily, beta member 2	-1.41	-1.33
1423501_at	Max	Max protein	-1.51	-1.62
1433989_at	Slc6a11	solute carrier family 6 (neurotransmitter transporter, GABA), member 11	-1.24	-1.36
1452669_at	2810012G03Rik	RIKEN cDNA 2810012G03 gene	-1.18	-1.20
1449188_at	Midn	midnolin	-1.35	-1.32
1428319_at	Pdlim7	PDZ and LIM domain 7	-1.30	-1.25
1423334_at	Ergic1	endoplasmic reticulum-golgi intermediate compartment (ERGIC) 1	-1.20	-1.22
1456024_at	Gtf3c1	general transcription factor III C 1	-1.30	-1.36
1454978_at	Ttyh3	tweety homolog 3 (Drosophila)	-1.22	-1.20
1417229_at	Capn1	calpain 1	-1.35	-1.34
1455753_at	C630035N08Rik	RIKEN cDNA C630035N08 gene	-1.34	-1.31
1419031_at	Fads2	fatty acid desaturase 2	-1.20	-1.25
1435664_at	Zfp397	zinc finger protein 397	-1.27	-1.42
1454950_at	B930006L02Rik	RIKEN cDNA B930006L02 gene	-1.26	-1.30
1446543_at	Gcn1l1	GCN1 general control of amino-acid synthesis 1-like 1 (yeast)	-1.30	-1.45
1426692_at	Ccdc97	coiled-coil domain containing 97	-1.22	-1.30
1436897_at	Mfhas1	malignant fibrous histiocytoma amplified sequence 1	-1.28	-1.28
1458685_at	Garnl1	GTPase activating RANGAP domain-like 1	-1.28	-1.43
1451795_at	Tom1l2	target of myb1-like 2 (chicken)	-1.44	-1.44
1448941_at	B4galt2	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 2	-1.27	-1.25
1431137_at	Rusc1	RUN and SH3 domain containing 1	-1.23	-1.26
1450247_a_at	Scamp5	secretory carrier membrane protein 5	-1.30	-1.33
1451592_at	P42pop	Myb protein P42POP	-1.21	-1.35
1453103_at	Ablim1	actin-binding LIM protein 1	-1.25	-1.29
1449290_at	Dpysl5	dihydropyrimidinase-like 5	-1.23	-1.20
1448960_at	Cxxc5	CXXC finger 5	-1.19	-1.42
1417228_at	Capn1	calpain 1	-1.21	-1.26
1420953_at	Add1	adducin 1 (alpha)	-1.21	-1.23
1452921_at	Evi5l	ecotropic viral integration site 5 like	-1.26	-1.25
1439740_s_at	Uck2	uridine-cytidine kinase 2	-1.23	-1.32
1422223_at	Grin2b	glutamate receptor, ionotropic, NMDA2B (epsilon 2)	-1.23	-1.35
1419157_at	Sox4	SRY-box containing gene 4	-1.26	-1.35
1443296_at	Pctk1	PCTAIRE-motif protein kinase 1	-1.21	-1.25
1426849_at	Sec24b	Sec24 related gene family, member B (S. cerevisiae)	-1.29	-1.28
1421933_at	Cbx5	chromobox homolog 5 (Drosophila HP1a)	-1.25	-1.47
1435495_at	Adora1	adenosine A1 receptor	-1.19	-1.25
1450041_a_at	Tub	tubby candidate gene	-1.41	-1.54
1450955_s_at	Sort1	sortilin 1	-1.26	-1.32
1417153_at	Btbd14a	BTB (POZ) domain containing 14A	-1.35	-1.44
1419398_a_at	Reep5	receptor accessory protein 5	-1.22	-1.21
1416845_at	Tmem132a	transmembrane protein 132A	-1.18	-1.21
1426744_at	Srebf2	sterol regulatory element binding factor 2	-1.27	-1.43
1424658_at	Taok1	TAO kinase 1	-1.22	-1.27
1451912_a_at	Fgfrl1	fibroblast growth factor receptor-like 1	-1.24	-1.44
1422145_at	Mgat3	mannoside acetylglucosaminyltransferase 3	-1.38	-1.58
1450439_at	Hcfc1	host cell factor C1	-1.23	-1.26
1421124_at	Cdk5r1	cyclin-dependent kinase 5, regulatory subunit 1 (p35)	-1.32	-1.39
1421298_a_at	Hipk1	homeodomain interacting protein kinase 1	-1.27	-1.30
1437626_at	Zfp36l2	zinc finger protein 36, C3H type-like 2	-1.26	-1.27
1429449_at	Samd4	sterile alpha motif domain containing 4	-1.20	-1.29
1435359_at	BC060632	cDNA sequence BC060632	-1.21	-1.35
1434255_at	Pacs2	phosphofurin acidic cluster sorting protein 2	-1.35	-1.58
1449325_at	Fads2	fatty acid desaturase 2	-1.22	-1.24

Table S3. Significant biological categories related to neurological processes after functional clustering of genes showing differential expression in hippocampus in contingent-MDMA vs yoked-MDMA and contingent-MDMA vs yoked-saline comparisons.

Term	Term ID	Genes	Fraction	P-value
synapse	GO:0045202	24	4.91%	1.45E-08
synapse		17	3.48%	1.32E-06
transmission of nerve impulse	GO:0019226	20	4.09%	4.56E-06
synaptic transmission	GO:0007268	18	3.68%	5.44E-06
synaptosome	GO:0019717	9	1.84%	4.29E-05
regulation of neurotransmitter levels	GO:0001505	11	2.25%	6.15E-05
synaptic vesicle	GO:0008021	9	1.84%	7.19E-05
neuron projection	GO:0043005	13	2.66%	7.23E-05
nervous system development	GO:0007399	32	6.54%	1.18E-04
synapse part	GO:0044456	12	2.45%	1.79E-04
cell morphogenesis	GO:0000902	26	5.32%	3.85E-04
cellular structure morphogenesis	GO:0032989	26	5.32%	3.85E-04
glutamate receptor binding	GO:0035254	4	0.82%	6.32E-04
response to ethanol	GO:0045471	4	0.82%	8.43E-04
cell projection organization and biogenesis	GO:0030030	17	3.48%	1.09E-03
cell part morphogenesis	GO:0032990	17	3.48%	1.09E-03
cell projection morphogenesis	GO:0048858	17	3.48%	1.09E-03
regulated secretory pathway	GO:0045055	8	1.64%	1.11E-03
Postsynaptic cell membrane		9	1.84%	1.25E-03
postsynaptic membrane	GO:0045211	10	2.04%	1.30E-03
visual learning	GO:0008542	5	1.02%	1.31E-03
ionotropic glutamate receptor complex	GO:0008328	4	0.82%	1.41E-03
visual behavior	GO:0007632	5	1.02%	1.56E-03
cell projection	GO:0042995	21	4.29%	1.81E-03
neurotransmitter secretion	GO:0007269	7	1.43%	1.99E-03
cellular morphogenesis during differentiation	GO:0000904	13	2.66%	2.18E-03
neuron morphogenesis during differentiation	GO:0048667	12	2.45%	2.56E-03
neurite morphogenesis	GO:0048812	12	2.45%	2.56E-03
cell projection part	GO:0044463	8	1.64%	2.68E-03
neurite development	GO:0031175	13	2.66%	3.11E-03
neurogenesis	GO:0022008	18	3.68%	4.51E-03
presynaptic membrane	GO:0042734	4	0.82%	4.88E-03
axonogenesis	GO:0007409	11	2.25%	4.97E-03
learning	GO:0007612	6	1.23%	5.05E-03
N-methyl-D-aspartate selective glutamate receptor complex	GO:0017146	3	0.61%	5.36E-03
learning and/or memory	GO:0007611	7	1.43%	5.42E-03
extracellular-glutamate-gated ion channel activity	GO:0005234	4	0.82%	6.57E-03
memory	GO:0007613	4	0.82%	7.05E-03
Glutamate receptor-related	IPR015683	4	0.82%	7.18E-03
ionotropic glutamate receptor activity	GO:0004970	4	0.82%	7.75E-03
neuron development	GO:0048666	13	2.66%	7.93E-03
membrane depolarization	GO:0051899	4	0.82%	8.24E-03
postsynaptic density	GO:0014069	3	0.61%	0.01
regulation of excitatory postsynaptic membrane potential	GO:0060079	3	0.61%	0.02
neuron differentiation	GO:0030182	14	2.86%	0.02
sensory perception of pain	GO:0019233	4	0.82%	0.02
generation of neurons	GO:0048699	15	3.07%	0.02
synaptic vesicle transport	GO:0048489	4	0.82%	0.03
axon	GO:0030424	6	1.23%	0.03
dendritic spine	GO:0043197	3	0.61%	0.03
synapse organization and biogenesis	GO:0050808	5	1.02%	0.03
response to light stimulus	GO:0009416	6	1.23%	0.03
regulation of membrane potential	GO:0042391	5	1.02%	0.03
regulation of postsynaptic membrane potential	GO:0060078	3	0.61%	0.03
dendrite	GO:0030425	5	1.02%	0.04
glutamate signaling pathway	GO:0007215	3	0.61%	0.04
synaptic vesicle exocytosis	GO:0016079	3	0.61%	0.04
axon guidance	GO:0007411	6	1.23%	0.05
response to organic substance	GO:0010033	4	0.82%	0.05
neurotransmitter metabolic process	GO:0042133	4	0.82%	0.05

Table S4. Genes differentially expressed in dorsal raphe nucleus (Log Fold Change below -1.5 or over 1.5) identified in the contingent-MDMA vs yoked-MDMA comparison.

Probe	Gene symbol		Gene Name	Fold Change	P-value	Adjusted P-value
1450826_a_at	Saa3		serum amyloid A 3	-9.76	7.7E-04	0.09
1419764_at	Chi3l3		chitinase 3-like 3	-6.57	1.3E-02	0.13
1422953_at	Fpr2		formyl peptide receptor 2	-3.48	8.6E-04	0.09
1419043_a_at	Iigp1		interferon inducible GTPase 1	-3.35	9.2E-03	0.11
1448881_at	Hp		haptoglobin	-3.32	1.1E-03	0.10
1419042_at	Iigp1		interferon inducible GTPase 1	-3.29	1.5E-02	0.13
1436778_at	Cybb		cytochrome b-245, beta polypeptide	-3.26	2.5E-03	0.10
1416811_s_at	Ctla2a		cytotoxic T lymphocyte-associated protein 2 alpha	-3.00	2.0E-04	0.09
1435906_x_at	Gbp2	*	guanylate binding protein 2	-2.97	7.6E-02	0.25
1448471_a_at	Ctla2a		cytotoxic T lymphocyte-associated protein 2 alpha	-2.84	4.5E-04	0.09
1442707_at	Camk2a		calcium/calmodulin-dependent protein kinase II alpha	2.87	9.9E-03	0.12
1436066_at	Kalrn	*	kalirin, RhoGEF kinase	2.95	3.9E-03	0.10
1423477_at	Zic1		zinc finger protein of the cerebellum 1	3.25	3.7E-02	0.18
1460049_s_at	1500015O10Rik		RIKEN cDNA 1500015O10 gene	3.39	8.9E-02	0.27
1459737_s_at	Ttr	*	transthyretin	3.45	2.3E-01	0.44
1454608_x_at	Ttr	*	transthyretin	3.48	2.3E-01	0.43
1436268_at	Ddn	*	dendrin	3.53	8.2E-03	0.11
1451580_a_at	Ttr		transthyretin	3.78	2.0E-01	0.41
1436329_at	Egr3	*	early growth response 3	4.03	4.9E-04	0.09
1439627_at	Zic1	*	zinc finger protein of the cerebellum 1	4.19	4.5E-02	0.20
1455913_x_at	Ttr	*	transthyretin	4.27	2.2E-01	0.42

* Genes also differentially expressed over Log Fold Change of 1.5 in the contingent-MDMA vs yoked-saline comparison

Table S5. Ventral striatum: Genes differentially expressed in the contingent-MDMA vs yoked-saline and yoked-MDMA vs yoked-saline comparisons after applying a 5% FDR.

Probe	Gene symbol	Gene Name	Contingent-MDMA vs Yoked-Saline (Fold Change)	Yoked-MDMA vs Yoked-Saline (Fold Change)
Upregulated				
1427747_a_at	Lcn2	lipocalin 2	32.83	41.35
1417290_at	Lrg1	leucine-rich alpha-2-glycoprotein 1	4.86	4.19
1455393_at	Cp	ceruloplasmin	3.10	3.30
1418595_at	S3-12	plasma membrane associated protein, S3-12	2.40	2.70
1451006_at	Xdh	xanthine dehydrogenase	2.52	2.17
1418674_at	Osmr	oncostatin M receptor	2.91	3.47
1428352_at	Arrdc2	arrestin domain containing 2	1.97	1.85
1423233_at	Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	2.46	2.90
1417185_at	Ly6a	lymphocyte antigen 6 complex, locus A	2.64	2.68
1448734_at	Cp	ceruloplasmin	2.54	2.61
1419606_a_at	Tnnt1	troponin T1, skeletal, slow	2.20	1.65
1455753_at	C630035N08Rik	RIKEN cDNA C630035N08 gene	1.67	1.64
1426600_at	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member	1.67	1.87
1448688_at	Podxl	podocalyxin-like	1.79	1.89
1439926_at	Heg1	HEG homolog 1 (zebrafish)	1.53	1.62
1449184_at	Pglyrp1	peptidoglycan recognition protein 1	2.64	3.14
1423754_at	Ifitm3	interferon induced transmembrane protein 3	2.72	3.28
1422869_at	Mertk	c-mer proto-oncogene tyrosine kinase	1.43	1.40
1416125_at	Fkbp5	FK506 binding protein 5	1.74	2.17
1450034_at	Stat1	signal transducer and activator of transcription 1	1.69	1.41
1455900_x_at	Tgm2	transglutaminase 2, C polypeptide	2.30	2.53
1435342_at	Kcnk6	potassium inwardly-rectifying channel, subfamily K, member 6	1.51	1.48
1451355_at	Asah3l	N-acylsphingosine amidohydrolase 3-like	2.86	3.65
1432273_a_at	Darc	Duffy blood group, chemokine receptor	1.46	1.56
1421321_a_at	Net1	neuroepithelial cell transforming gene 1	1.40	1.48
1428986_at	Slc17a7	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	3.62	3.59
1423306_at	2010002N04Rik	RIKEN cDNA 2010002N04 gene	1.61	1.47
1424638_at	Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	1.95	2.72
1426368_at	Rin2	Ras and Rab interactor 2	1.36	1.28
1439163_at	Zbtb16	zinc finger and BTB domain containing 16	1.94	2.45
1418586_at	Adcy9	adenylate cyclase 9	1.34	1.51
1437595_at	E030010A14Rik	RIKEN cDNA E030010A14 gene	2.08	2.11
1417495_x_at	Cp	ceruloplasmin	2.33	2.26
1438752_at	A230058F20Rik	RIKEN cDNA A230058F20 gene	1.37	1.69
1417318_at	Dbc1	deleted in bladder cancer 1 (human)	1.32	1.27
1435477_s_at	Fcgr2b	Fc receptor, IgG, low affinity IIb	2.18	2.90
1458233_at	Fryl	furry homolog-like (Drosophila)	1.45	1.77
1452067_at	Naaa	N-acylethanolamine acid amidase	1.33	1.27
1422903_at	Ly86	lymphocyte antigen 86	1.68	1.42
1450850_at	Ezr	ezrin	1.44	1.41
1419699_at	Scgb3a1	secretoglobin, family 3A, member 1	2.60	1.37
1418937_at	Dio2	deiodinase, iodothyronine, type II	1.48	1.39
1417063_at	C1qb	complement component 1, q subcomponent, beta polypeptide	1.48	1.49
1417262_at	Ptgs2	prostaglandin-endoperoxide synthase 2	2.17	2.32
1421571_a_at	Ly6c1	lymphocyte antigen 6 complex, locus C1	1.91	1.96
1430808_at	Tbc1d5	TBC1 domain family, member 5	1.33	1.40
1436763_a_at	Klf9	Kruppel-like factor 9	1.37	1.40
1458459_a_at	E230029C05Rik	RIKEN cDNA E230029C05 gene	1.30	1.34
1437277_x_at	Tgm2	transglutaminase 2, C polypeptide	2.54	2.63
1419483_at	C3ar1	complement component 3a receptor 1	1.54	1.89
1427307_a_at	Dab1	disabled homolog 1 (Drosophila)	1.32	1.54
1417785_at	Pla1a	phospholipase A1 member A	1.45	1.53
1441624_at	Sorbs2	sorbin and SH3 domain containing 2	1.35	1.56
1450033_a_at	Stat1	signal transducer and activator of transcription 1	1.74	1.38
1458203_at	Spire1	spire homolog 1 (Drosophila)	1.32	1.39

1424727_at	Ccr5	chemokine (C-C motif) receptor 5	1.34	1.24
1433428_x_at	Tgm2	transglutaminase 2, C polypeptide	2.43	2.54
1416041_at	Sgk1	serum/glucocorticoid regulated kinase 1	1.64	1.88
1455754_at	Lmo3	LIM domain only 3	1.43	1.51
1430675_at	2900055J20Rik	RIKEN cDNA 2900055J20 gene	1.32	1.47
1448620_at	Fcgr3	Fc receptor, IgG, low affinity III	1.75	2.04
1423395_at	Tsnax	translin-associated factor X	1.33	1.29
1423854_a_at	Rasl11b	RAS-like, family 11, member B	1.41	1.57
1446190_at	Dclk1	doublecortin-like kinase 1	1.48	2.15
1417496_at	Cp	ceruloplasmin	1.94	1.82
1459223_at	B930095G15Rik	RIKEN cDNA B930095G15 gene	1.47	1.41
1428909_at	A130040M12Rik	RIKEN cDNA A130040M12 gene	2.64	2.84
1423619_at	Rasd1	RAS, dexamethasone-induced 1	1.82	1.88
1417494_a_at	Cp	ceruloplasmin	1.89	2.10
1449731_s_at	Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.52	1.78
1449289_a_at	Phf20l1	PHD finger protein 20-like 1	1.74	1.98
1440586_at	B430203I24Rik	RIKEN cDNA B430203I24 gene	1.32	1.45
1417381_at	C1qa	complement component 1, q subcomponent, alpha polypeptide	1.41	1.37
1451716_at	Mafb	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	1.30	1.41
1422264_s_at	Klf9	Kruppel-like factor 9	1.38	1.57
1435906_x_at	Gbp2	guanylate binding protein 2	11.09	8.47
1452428_a_at	B2m	beta-2 microglobulin	1.72	1.98
1417460_at	Ifitm2	interferon induced transmembrane protein 2	2.33	3.14
1423125_at	Dclk1	doublecortin-like kinase 1	1.37	1.51
1451941_a_at	Fcgr2b	Fc receptor, IgG, low affinity IIb	1.86	2.42
1431118_at	6720427H10Rik	RIKEN cDNA 6720427H10 gene	1.27	1.26
1426397_at	Tgfbr2	transforming growth factor, beta receptor II	1.41	1.53
1458111_at	C530043G21Rik	RIKEN cDNA C530043G21 gene	1.32	1.46
1423584_at	Polr2b	polymerase (RNA) II (DNA directed) polypeptide B	1.87	2.32
1434270_at	Nptxr	neuronal pentraxin receptor	1.92	1.94
Downregulated				
1433607_at	Cbln4	cerebellin 4 precursor protein	-1.57	-1.63
1440413_at	A830006F12Rik	RIKEN cDNA A830006F12 gene	-1.39	-1.42
1440803_x_at	Tacr3	tachykinin receptor 3	-1.57	-1.70
1421379_at	Zfp354b	zinc finger protein 354B	-1.40	-1.54
1446681_at	BB086117	expressed sequence BB086117	-1.41	-1.50
1427300_at	Lhx8	LIM homeobox protein 8	-1.75	-1.87
1421428_at	Slc5a7	solute carrier family 5 (choline transporter), member 7	-1.42	-1.63
1437029_at	Tacr3	tachykinin receptor 3	-1.59	-1.82
1437268_at	Lancl3	LanC lantibiotic synthetase component C-like 3 (bacterial)	-1.37	-1.36
1417110_at	Man1a	mannosidase 1, alpha	-1.32	-1.26
1443997_at	Gprin2	G protein regulated inducer of neurite outgrowth 2	-1.50	-1.36
1450319_at	Gabrb2	gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2	-1.40	-1.76
1454032_at	Neto2	neuropilin (NRP) and tolloid (TLL)-like 2	-1.48	-1.74
1447825_x_at	Pcdh8	protocadherin 8	-1.33	-1.40
1444058_at	Dzip3	DAZ interacting protein 3, zinc finger	-1.32	-1.38
1418688_at	Calcr	calcitonin receptor	-1.91	-1.56

Table S6. Frontal cortex: Genes differentially expressed in the contingent-MDMA vs yoked-saline and yoked-MDMA vs yoked-saline comparisons after applying FDR 5%.

Probe	Gene symbol	Gene Name	Contingent-MDMA vs Yoked-Saline (Fold Change)	Yoked-MDMA vs Yoked-Saline (Fold Change)
Upregulated				
1427747_a_at	Lcn2	lipocalin 2	52.79	55.75
1417290_at	Lrg1	leucine-rich alpha-2-glycoprotein 1	5.64	4.93
1419282_at	Ccl12	chemokine (C-C motif) ligand 12	2.97	2.17
1451355_at	Asah3l	N-acylsphingosine amidohydrolase 3-like	3.59	4.32
1451006_at	Xdh	xanthine dehydrogenase	3.13	3.01
1423233_at	Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	2.99	3.22
1449184_at	Pglyrp1	peptidoglycan recognition protein 1	7.95	6.91
1417185_at	Ly6a	lymphocyte antigen 6 complex, locus A	3.27	3.32
1455393_at	Cp	ceruloplasmin	4.58	4.47
1449227_at	Ch25h	cholesterol 25-hydroxylase	3.43	4.52
1417500_a_at	Tgm2	transglutaminase 2, C polypeptide	3.39	3.41
1417063_at	C1qb	complement component 1, q subcomponent, beta	1.78	1.90
1416125_at	Fkbp5	FK506 binding protein 5	2.33	2.87
1419599_s_at	Ms4a6d	membrane-spanning 4-domains, subfamily A, member 6D	2.29	3.21
1423584_at	Polr2b	polymerase (RNA) II (DNA directed) polypeptide B	2.68	2.82
1450033_a_at	Stat1	signal transducer and activator of transcription 1	1.94	1.59
1433933_s_at	Slco2b1	solute carrier organic anion transporter family, member 2b1	1.84	1.87
1418674_at	Osmr	oncostatin M receptor	2.77	3.36
1431008_at	0610037M15Rik	RIKEN cDNA 0610037M15 gene	4.01	6.16
1449009_at	Tgtp	T-cell specific GTPase	5.86	6.63
1424923_at	Serpina3g	serine (or cysteine) peptidase inhibitor, clade A, member 3G	5.92	6.25
1418240_at	Gbp2	guanylate binding protein 2	10.32	9.46
1449401_at	C1qc	complement component 1, q subcomponent, C chain	1.75	1.88
1417381_at	C1qa	complement component 1, q subcomponent, alpha	1.65	1.66
1423754_at	Ifitm3	interferon induced transmembrane protein 3	3.48	3.72
1433428_x_at	Tgm2	transglutaminase 2, C polypeptide	2.55	2.78
1448620_at	Fcgr3	Fc receptor, IgG, low affinity III	1.81	2.26
1426509_s_at	Gfap	glial fibrillary acidic protein	3.18	3.09
1438672_at	Parvb	parvin, beta	1.72	1.88
1419043_a_at	Iigp1	interferon inducible GTPase 1	7.60	8.34
1452352_at	Ctla2b	cytotoxic T lymphocyte-associated protein 2 beta	2.44	3.84
1417793_at	Iigp2	interferon inducible GTPase 2	3.24	3.03
1426600_at	Slc2a1	solute carrier family 2 (facilitated glucose transporter),	1.96	2.24
1428909_at	A130040M12Rik	RIKEN cDNA A130040M12 gene	3.18	3.69
1419042_at	Iigp1	interferon inducible GTPase 1	5.54	6.45
1421571_a_at	Ly6c1	lymphocyte antigen 6 complex, locus C1	2.30	2.62
1416811_s_at	Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	3.48	5.93
1435906_x_at	Gbp2	guanylate binding protein 2	8.30	7.72
1437277_x_at	Tgm2	transglutaminase 2, C polypeptide	2.50	2.91
1418536_at	H2-Q7	histocompatibility 2, Q region locus 7	2.92	5.04
1421267_a_at	Cited2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	2.15	1.87
1435477_s_at	Fcgr2b	Fc receptor, IgG, low affinity IIb	2.23	3.26
1418187_at	Ramp2	receptor (calcitonin) activity modifying protein 2	1.77	1.64
1418826_at	Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	2.65	3.87
1417292_at	Ifi47	interferon gamma inducible protein 47	3.13	3.72
1432273_a_at	Darc	Duffy blood group, chemokine receptor	1.62	1.57
1417141_at	Igtp	interferon gamma induced GTPase	4.18	4.04
1459740_s_at	Ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	2.00	2.28
1426508_at	Gfap	glial fibrillary acidic protein	3.30	3.06
1417876_at	Fcgr1	Fc receptor, IgG, high affinity I	1.67	1.70
1418595_at	S3-12	plasma membrane associated protein, S3-12	2.41	3.44
1434329_s_at	Adipor2	adiponectin receptor 2	1.65	1.65
1457758_at	Eny2	enhancer of yellow 2 homolog (Drosophila)	1.98	1.68
1424254_at	Ifitm1	interferon induced transmembrane protein 1	2.69	2.41
1437968_at	Grin1	glutamate receptor, ionotropic, NMDA1 (zeta 1)	1.78	1.62

1417013_at	Hspb8	heat shock protein 8	1.58	1.41
1417821_at	D17H6S56E-5	DNA segment, Chr 17, human D6S56E 5	2.18	2.75
1418296_at	Fxyd5	FXYD domain-containing ion transport regulator 5	1.85	1.86
1451715_at	Mafb	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	1.61	1.43
1417494_a_at	Cp	ceruloplasmin	2.38	2.50
1448688_at	Podxl	podocalyxin-like	1.79	1.94
1417109_at	Tinagl1	tubulointerstitial nephritis antigen-like 1	1.88	1.62
1448890_at	Klf2	Kruppel-like factor 2 (lung)	2.44	2.85
1450696_at	Psmb9	proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2)	2.04	2.01
1429843_at	Grrp1	glycine/arginine rich protein 1	1.60	1.58
1449731_s_at	Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.80	2.12
1451335_at	Plac8	placenta-specific 8	3.39	6.33
1448471_a_at	Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	3.01	4.95
1452055_at	Ctdsp1	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 1	1.55	1.47
1418701_at	Comt1	catechol-O-methyltransferase 1	1.68	1.60
1428016_a_at	Rasip1	Ras interacting protein 1	1.86	1.90
1448306_at	Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.93	2.60
1427844_a_at	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	1.88	1.95
1425545_x_at	H2-L	histocompatibility 2, D region	2.21	2.95
1448188_at	Ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	1.98	2.20
1423593_a_at	Csf1r	colony stimulating factor 1 receptor	1.46	1.38
1429954_at	Clec4a3	C-type lectin domain family 4, member a3	1.79	2.38
1422557_s_at	Mt1	metallothionein 1	1.59	1.62
1435386_at	Vwf	Von Willebrand factor homolog	2.76	4.24
1440926_at	Flt1	FMS-like tyrosine kinase 1	1.53	1.43
1420643_at	Lfng	LFNG O-fucosylpeptide 3-beta-N-	1.79	1.69
1448756_at	S100a9	S100 calcium binding protein A9 (calgranulin B)	4.84	9.37
1437595_at	E030010A14Rik	RIKEN cDNA E030010A14 gene	2.13	2.30
1426599_a_at	Slc2a1	solute carrier family 2 (facilitated glucose transporter),	1.66	2.10
1425336_x_at	H2-K1	histocompatibility 2, K1, K region	2.32	3.42
1455332_x_at	Fcgr2b	Fc receptor, IgG, low affinity IIb	1.67	2.16
1451784_x_at	H2-D1	histocompatibility 2, D region locus 1	2.13	2.89
1436763_a_at	Klf9	Kruppel-like factor 9	1.61	1.58
1449110_at	Rhob	ras homolog gene family, member B	1.64	1.56
1417460_at	Ifitm2	interferon induced transmembrane protein 2	2.32	3.10
1436277_at	Rnf207	ring finger protein 207	1.67	1.86
1419100_at	Serpina3n	serine (or cysteine) peptidase inhibitor, clade A, member 3N	2.09	2.16
1455900_x_at	Tgm2	transglutaminase 2, C polypeptide	2.03	2.45
1420653_at	Tgfb1	transforming growth factor, beta 1	1.96	2.09
1417495_x_at	Cp	ceruloplasmin	2.31	2.44
1422962_a_at	Psmb8	proteasome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7)	2.11	2.41
1418059_at	Eltd1	EGF, latrophilin seven transmembrane domain containing 1	1.79	2.02
1456772_at	Ncf1	neutrophil cytosolic factor 1	1.53	1.51
1435124_at	EG328644	predicted gene, EG328644	1.54	1.54
1449851_at	Per1	period homolog 1 (Drosophila)	1.63	1.74
1451115_at	Pias3	protein inhibitor of activated STAT 3	1.50	1.46
1448825_at	Pdk2	pyruvate dehydrogenase kinase, isoenzyme 2	1.48	1.43
1451255_at	Lsr	lipolysis stimulated lipoprotein receptor	1.68	1.68
1451767_at	Ncf1	neutrophil cytosolic factor 1	1.48	1.67
1415712_at	Zranb1	zinc finger, RAN-binding domain containing 1	1.61	1.51
1449289_a_at	Phf20l1	PHD finger protein 20-like 1	1.73	1.98
1441972_at	6230424C14Rik	RIKEN cDNA 6230424C14 gene	1.73	1.83
1418131_at	Samhd1	SAM domain and HD domain, 1	2.12	2.77
1449620_s_at	Adcy9	adenylate cyclase 9	1.39	1.36
1436613_at	Coro6	coronin, actin binding protein 6	1.55	1.93
1427746_x_at	H2-K1	histocompatibility 2, K1, K region	2.08	2.94
1426025_s_at	Laptm5	lysosomal-associated protein transmembrane 5	1.59	1.51
1418825_at	Irgm1	immunity-related GTPase family M member 1	2.84	3.16
1451931_x_at	H2-L	histocompatibility 2, D region	2.04	2.75
1434380_at	Gbp6	guanylate binding protein 6	1.94	2.12

1429184_at	100042856	predicted gene, 100042856	2.83	3.53
1426004_a_at	Tgm2	transglutaminase 2, C polypeptide	1.96	2.11
Downregulated				
1437801_at	Morf4l1	mortality factor 4 like 1	-1.63	-1.49
1437729_at	Rpl27a	ribosomal protein L27a	-1.57	-1.56
1422168_a_at	Bdnf	brain derived neurotrophic factor	-1.65	-1.64
1438648_x_at	1190003M12Rik	RIKEN cDNA 1190003M12 gene	-1.53	-1.44
1423796_at	Sfpq	splicing factor proline/glutamine rich (polypyrimidine tract	-1.45	-1.50
1436387_at	C330006P03Rik	RIKEN cDNA C330006P03 gene	-1.70	-1.83
1447669_s_at	Gng4	guanine nucleotide binding protein (G protein), gamma 4	-1.51	-1.43
1438540_at	Col25a1	collagen, type XXV, alpha 1	-1.66	-1.65
1432432_a_at	Rab3c	RAB3C, member RAS oncogene family	-1.54	-1.68
1453578_at	Pter	phosphotriesterase related	-1.59	-1.50
1438624_x_at	Hs3st2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2	-1.52	-1.70
1449494_at	Rab3c	RAB3C, member RAS oncogene family	-1.50	-1.64

Table S7. Dorsal raphe nucleus: Genes differentially expressed in the contingent-MDMA vs yoked-saline and yoked-MDMA vs yoked-saline comparisons after applying a 5% FDR.

Probe	Gene symbol	Gene Name	Contingent-MDMA vs Yoked-Saline (Fold Change)	Yoked-MDMA vs Yoked-Saline (Fold Change)
Upregulated				
1427747_a_at	Lcn2	lipocalin 2	26.09	52.40
1416041_at	Sgk1	serum/glucocorticoid regulated kinase 1	3.09	4.22
1422903_at	Ly86	lymphocyte antigen 86	1.83	1.63
1419699_at	Scgb3a1	secretoglobin, family 3A, member 1	3.52	1.92
1417290_at	Lrg1	leucine-rich alpha-2-glycoprotein 1	4.78	4.04
1448734_at	Cp	ceruloplasmin	2.73	3.22
1451355_at	Asah3l	N-acylsphingosine amidohydrolase 3-like	2.55	3.65
1436543_at	Gtpbp10	GTP-binding protein 10 (putative)	1.54	1.33
1423233_at	Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	2.21	2.81
1417496_at	Cp	ceruloplasmin	2.17	2.54
1417381_at	C1qa	complement component 1, q subcomponent, alpha polypeptide	1.57	1.90
Downregulated				
1422705_at	Pmepa1	prostate transmembrane protein, androgen induced 1	-1.60	-1.54
1421163_a_at	Nfia	nuclear factor I/A	-1.57	-1.75
1425886_at	Fev	FEV (ETS oncogene family)	-1.85	-1.51
1428860_at	4930572J05Rik	RIKEN cDNA 4930572J05 gene	-1.56	-1.66
1451124_at	Sod1	superoxide dismutase 1, soluble	-1.69	-1.88

Table S8. Hippocampus: Genes differentially expressed in the contingent-MDMA vs yoked-saline and yoked-MDMA vs yoked-saline comparisons after applying a 5% FDR.

Probe	Gene symbol	Gene Name	Contingent-MDMA vs Yoked-Saline (Fold Change)	Yoked-MDMA vs Yoked-Saline (Fold Change)
Upregulated				
1427747_a_at	Lcn2	lipocalin 2	25.03	36.39
1448620_at	Fcgr3	Fc receptor, IgG, low affinity III	2.26	2.49
1451006_at	Xdh	xanthine dehydrogenase	2.46	2.26
1417290_at	Lrg1	leucine-rich alpha-2-glycoprotein 1	3.84	4.03
1438251_x_at	Htra1	HtrA serine peptidase 1	1.42	1.18
1416041_at	Sgk1	serum/glucocorticoid regulated kinase 1	2.08	1.99
1431406_at	Agxt2l1	alanine-glyoxylate aminotransferase 2-like 1	2.97	3.14
1427345_a_at	Sult1a1	sulfotransferase family 1A, phenol-preferring, member 1	2.02	1.87
1434773_a_at	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member	1.70	1.80
1451355_at	Asah3l	N-acylsphingosine amidohydrolase 3-like	2.91	4.14
1418595_at	S3-12	plasma membrane associated protein, S3-12	2.35	2.52
1445866_at	Mast4	microtubule associated serine/threonine kinase family member	1.37	1.41
1417063_at	C1qb	complement component 1, q subcomponent, beta polypeptide	1.65	1.85
1424671_at	Plekhf1	pleckstrin homology domain containing, family F (with FYVE	1.45	1.27
1417130_s_at	Angptl4	angiopoietin-like 4	1.60	1.59
1422903_at	Ly86	lymphocyte antigen 86	1.79	1.54
1427891_at	Gimap6	GTPase, IMAP family member 6	1.65	1.86
1448167_at	Ifngr1	interferon gamma receptor 1	1.37	1.38
1436905_x_at	Laptm5	lysosomal-associated protein transmembrane 5	1.52	1.35
1435342_at	Kcnk6	potassium inwardly-rectifying channel, subfamily K, member 6	1.51	1.48
1435477_s_at	Fcgr2b	Fc receptor, IgG, low affinity IIb	2.26	3.10
1418674_at	Osmr	oncostatin M receptor	2.49	2.92
1418135_at	Aff1	AF4/FMR2 family, member 1	1.43	1.42
1426599_a_at	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member	1.67	1.89
1455332_x_at	Fcgr2b	Fc receptor, IgG, low affinity IIb	1.75	2.08
1448688_at	Podxl	podocalyxin-like	1.56	1.77
1428352_at	Arrdc2	arrestin domain containing 2	1.82	1.98
1417381_at	C1qa	complement component 1, q subcomponent, alpha polypeptide	1.54	1.75
1418826_at	Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	2.54	3.48
1417185_at	Ly6a	lymphocyte antigen 6 complex, locus A	2.49	2.82
1417495_x_at	Cp	ceruloplasmin	1.87	2.13
1433428_x_at	Tgm2	transglutaminase 2, C polypeptide	2.53	2.47
1455900_x_at	Tgm2	transglutaminase 2, C polypeptide	2.51	2.27
1420088_at	Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.55	1.54
1417494_a_at	Cp	ceruloplasmin	1.86	2.08
1431248_at	5031426D15Rik	RIKEN cDNA 5031426D15 gene	1.27	1.22
1442700_at	Pde4b	phosphodiesterase 4B, cAMP specific	1.34	1.23
1448617_at	Cd53	CD53 antigen	1.55	1.59
1421571_a_at	Ly6c1	lymphocyte antigen 6 complex, locus C1	2.10	2.31
1437277_x_at	Tgm2	transglutaminase 2, C polypeptide	2.67	2.50
1449227_at	Ch25h	cholesterol 25-hydroxylase	2.40	2.34
1451941_a_at	Fcgr2b	Fc receptor, IgG, low affinity IIb	2.11	2.61
1417876_at	Fcgr1	Fc receptor, IgG, high affinity I	1.47	1.58
1452352_at	Ctla2b	cytotoxic T lymphocyte-associated protein 2 beta	2.40	3.47
1434380_at	Gbp6	guanylate binding protein 6	2.35	2.29
1416811_s_at	Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	3.02	5.18
1441799_at	6030422H21Rik	RIKEN cDNA 6030422H21 gene	1.63	1.79
1417346_at	Pycard	PYD and CARD domain containing	1.75	1.68
1417496_at	Cp	ceruloplasmin	1.84	1.80
1449009_at	Tgtp	T-cell specific GTPase	4.40	4.76
1432273_a_at	Darc	Duffy blood group, chemokine receptor	1.40	1.65
1445091_at	Atr	ataxia telangiectasia and Rad3 related	1.30	1.22
1424254_at	Ifitm1	interferon induced transmembrane protein 1	2.28	2.25
1429642_at	Anubl1	AN1, ubiquitin-like, homolog (Xenopus laevis)	1.57	1.74
1429413_at	Cpm	carboxypeptidase M	1.29	1.23
1455393_at	Cp	ceruloplasmin	2.33	2.94

1450033_a_at	Stat1	signal transducer and activator of transcription 1	1.61	1.48
1459857_at	Usp32	ubiquitin specific peptidase 32	1.35	1.33
1438157_s_at	Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.61	1.70
1415996_at	Txnip	thioredoxin interacting protein	1.86	1.87
1425428_at	Hif3a	hypoxia inducible factor 3, alpha subunit	1.36	1.44
1449401_at	C1qc	complement component 1, q subcomponent, C chain	1.42	1.75
1415997_at	Txnip	thioredoxin interacting protein	1.59	1.69
1460511_at	Pkp2	plakophilin 2	1.30	1.25
1450234_at	Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C	1.69	2.11
1448591_at	Ctss	cathepsin S	1.40	1.59
1448471_a_at	Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	3.09	5.25
1448306_at	Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.59	1.89
1417821_at	D17H6S56E-5	DNA segment, Chr 17, human D6S56E 5	1.91	2.20
1419647_a_at	Ier3	immediate early response 3	1.89	2.26
1427256_at	Vcan	versican	1.24	1.21
1417793_at	Iigp2	interferon inducible GTPase 2	2.86	2.73
1428223_at	Mfsd2	major facilitator superfamily domain containing 2	1.37	1.44
1437595_at	E030010A14Rik	RIKEN cDNA E030010A14 gene	1.70	1.88
1437726_x_at	C1qb	complement component 1, q subcomponent, beta polypeptide	1.58	1.62
1435906_x_at	Gbp2	guanylate binding protein 2	8.10	7.57
1418240_at	Gbp2	guanylate binding protein 2	7.49	6.91
1425156_at	Gbp6	guanylate binding protein 6	1.75	1.91
1417292_at	Ifi47	interferon gamma inducible protein 47	2.94	3.53
1426004_a_at	Tgm2	transglutaminase 2, C polypeptide	1.64	1.76
1417822_at	D17H6S56E-5	DNA segment, Chr 17, human D6S56E 5	1.82	1.94
1425362_at	Hrbl	HIV-1 Rev binding protein-like	1.20	1.31
1438822_at	100041799	predicted gene, 100041799	1.32	1.44
1448734_at	Cp	ceruloplasmin	1.82	2.06
1417141_at	Igtp	interferon gamma induced GTPase	4.01	4.12
1429235_at	Galntl2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	1.53	1.59
1418554_at	Gpr182	G protein-coupled receptor 182	1.59	1.68
1426600_at	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member	1.45	1.85
1456014_s_at	Trpt1	tRNA phosphotransferase 1	1.63	1.91
1434366_x_at	C1qb	complement component 1, q subcomponent, beta polypeptide	1.42	1.51
1438805_at	Ccnd3	cyclin D3	1.27	1.43
1439276_at	Adar	adenosine deaminase, RNA-specific	1.20	1.24
1445897_s_at	Ifi35	interferon-induced protein 35	1.68	1.59
1429184_at	100042856	predicted gene, 100042856	2.58	3.13
1419043_a_at	Iigp1	interferon inducible GTPase 1	4.82	5.54
1452428_a_at	B2m	beta-2 microglobulin	1.59	1.82
1451335_at	Plac8	placenta-specific 8	2.84	3.83
1433837_at	8430408G22Rik	RIKEN cDNA 8430408G22 gene	1.55	2.00
1417460_at	Ifitm2	interferon induced transmembrane protein 2	1.85	2.33
1426970_a_at	Ube1l	ubiquitin-activating enzyme E1-like	1.23	1.28
1433935_at	AU020206	expressed sequence AU020206	1.61	1.88
1459548_at	Spire1	spire homolog 1 (Drosophila)	1.22	1.25
1418536_at	H2-Q7	histocompatibility 2, Q region locus 7	2.58	4.30
1456772_at	Ncf1	neutrophil cytosolic factor 1	1.32	1.53
1441228_at	Apold1	apolipoprotein L domain containing 1	1.74	2.56
1448397_at	Gjb6	gap junction protein, beta 6	1.38	1.41
1448181_at	Klf15	Kruppel-like factor 15	1.29	1.37
1429946_at	2610301F02Rik	RIKEN cDNA 2610301F02 gene	1.26	1.30
1417500_a_at	Tgm2	transglutaminase 2, C polypeptide	1.85	2.26
1429745_at	DXBay18	DNA segment, Chr X, Baylor 18	1.25	1.27
1449556_at	H2-T23	histocompatibility 2, T region locus 23	1.83	2.08
1422046_at	Itgam	integrin alpha M	1.27	1.35
1419599_s_at	Ms4a6d	membrane-spanning 4-domains, subfamily A, member 6D	2.23	3.27
1418340_at	Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide	1.58	1.81
1428942_at	Mt2	metallothionein 2	1.68	1.88
1455899_x_at	Socs3	suppressor of cytokine signaling 3	1.96	2.47
1428306_at	Ddit4	DNA-damage-inducible transcript 4	1.32	1.46
1450696_at	Psmb9	proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2)	1.55	1.71
1436033_at	BC031353	cDNA sequence BC031353	1.17	1.22

1423754_at	Ifitm3	interferon induced transmembrane protein 3	1.87	2.70
1418059_at	Eltd1	EGF, latrophilin seven transmembrane domain containing 1	1.48	1.84
1418892_at	Rhoj	ras homolog gene family, member J	1.69	1.88
1419042_at	Iigp1	interferon inducible GTPase 1	3.90	4.73
1453976_at	4432414F05Rik	RIKEN cDNA 4432414F05 gene	1.57	1.91
1419684_at	Ccl8	chemokine (C-C motif) ligand 8	1.36	1.69
1448756_at	S100a9	S100 calcium binding protein A9 (calgranulin B)	4.32	5.25
1452975_at	Agxt2l1	alanine-glyoxylate aminotransferase 2-like 1	1.63	1.80
1424948_x_at	H2-K1	histocompatibility 2, K1, K region	1.62	2.31
1422962_a_at	Psmb8	proteasome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7)	2.01	2.30
1425336_x_at	H2-K1	histocompatibility 2, K1, K region	1.78	2.77
1416656_at	Clic1	chloride intracellular channel 1	1.43	1.49
1455314_at	Lpp	LIM domain containing preferred translocation partner in lipoma	1.22	1.34
1452730_at	Rps4y2	ribosomal protein S4, Y-linked 2	1.22	1.26
1422476_at	Ifi30	interferon gamma inducible protein 30	1.65	2.02
1427041_at	BC013712	cDNA sequence BC013712	1.23	1.25
1416612_at	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	1.53	1.92
1425225_at	Fcgr4	Fc receptor, IgG, low affinity IV	1.69	2.24
1441462_at	Dock4	dedicator of cytokinesis 4	1.28	1.32
1428909_at	A130040M12Rik	RIKEN cDNA A130040M12 gene	1.89	2.40
1434372_at	AW112010	expressed sequence AW112010	2.82	3.28
1428332_at	Pik3ip1	phosphoinositide-3-kinase interacting protein 1	1.19	1.31
1448748_at	Plek	pleckstrin	1.29	1.50
1444681_at	Erc2	ELKS/RAB6-interacting/CAST family member 2	1.53	1.61
1417961_a_at	Trim30	tripartite motif-containing 30	1.51	1.69
1416226_at	Arpc1b	actin related protein 2/3 complex, subunit 1B	1.35	1.74
1449455_at	Hck	hemopoietic cell kinase	1.59	2.21
Downregulated				
1425369_a_at	Sox10	SRY-box containing gene 10	-1.64	-1.24
1448960_at	Cxxc5	CXXC finger 5	-1.42	-1.20
1423530_at	Stk32c	serine/threonine kinase 32C	-1.45	-1.31
1460173_at	Lasp1	LIM and SH3 protein 1	-1.35	-1.18
1421199_at	Dlg2	discs, large homolog 2 (Drosophila)	-1.48	-1.31
1454213_at	Armc9	armadillo repeat containing 9	-2.51	-3.15
1421200_at	Dlg2	discs, large homolog 2 (Drosophila)	-1.69	-1.44
1421399_at	Insm1	insulinoma-associated 1	-1.44	-1.45
1418356_at	Mpst	mercaptopyruvate sulfurtransferase	-1.32	-1.22
1433812_at	Lix1l	Lix1-like	-1.40	-1.30
1432383_a_at	Armc9	armadillo repeat containing 9	-1.74	-1.86
1420931_at	Mapk8	mitogen-activated protein kinase 8	-1.84	-1.61
1442319_at	Usp4	ubiquitin specific peptidase 4 (proto-oncogene)	-1.73	-1.86
1444396_at	Trp53inp2	transformation related protein 53 inducible nuclear protein 2	-1.33	-1.31
1422331_at	Pou3f3	POU domain, class 3, transcription factor 3	-1.47	-1.40
1417155_at	Mycn	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	-1.24	-1.35
1427112_at	Ttl	tubulin tyrosine ligase	-1.28	-1.21
1421493_a_at	Rgs20	regulator of G-protein signaling 20	-1.40	-1.31
1455172_at	AU020094	expressed sequence AU020094	-1.33	-1.27
1426960_a_at	Fa2h	fatty acid 2-hydroxylase	-1.29	-1.29
1419429_at	Cntfr	ciliary neurotrophic factor receptor	-1.39	-1.31
1445387_at	Senp6	SUMO/sentrin specific peptidase 6	-1.44	-1.60
1431413_at	Ramp1	receptor (calcitonin) activity modifying protein 1	-1.31	-1.29
1444599_at	Herc4	hect domain and RLD 4	-1.56	-1.63
1426640_s_at	Trib2	tribbles homolog 2 (Drosophila)	-1.24	-1.28
1422258_at	Chrm3	cholinergic receptor, muscarinic 3, cardiac	-1.27	-1.30
1430177_at	Ube2b	ubiquitin-conjugating enzyme E2B, RAD6 homology (S.	-1.25	-1.28
1426641_at	Trib2	tribbles homolog 2 (Drosophila)	-1.25	-1.26
1421306_a_at	Hdac9	histone deacetylase 9	-1.30	-1.43
1421866_at	Nr3c1	nuclear receptor subfamily 3, group C, member 1	-1.32	-1.36
1429284_at	Mobkl2b	MOB1, Mps One Binder kinase activator-like 2B (yeast)	-1.26	-1.30
1423152_at	Vapb	vesicle-associated membrane protein, associated protein B and	-1.20	-1.38
1442051_at	Hist2h3c1	histone cluster 2, H3c1	-1.21	-1.46

APPENDIX B

Supplementary Material for Article 4

The International Journal of Neuropsychopharmacology

Supplement 1

MDMA self-administration studies

Animals were anesthetized with an intraperitoneal (i.p.) injection of a ketamine/xylazine mixture (5:1; 0.10 ml/10 g; Sigma-Aldrich, Spain) and then implanted with an indwelling intravenous (i.v.) silastic catheter in the right jugular vein, as previously described (Trigo et al., 2007). Animals were pretreated with meloxicam (5 mg/kg; Boehringer-Ingelheim, Spain) subcutaneously (s.c.) for analgesia. After surgery, mice were housed individually for the remainder of the experiments. In order to avoid clots and infection, they were flushed through the catheter during 5 days with 0.02 ml of a solution containing heparin (30 UI/ml) and cefazoline (50 mg/ml, Chiesi, Spain) in 0.9 % sodium chloride. The patency of the catheters was evaluated at the end of the experiment by injecting 0.1 ml of thiopental (5 mg/ml) through the catheter. If prominent signs of anaesthesia were not apparent within 3 s of the infusion, the mouse and its corresponding data were removed from the experiment.

Different groups of 5-HT_{2A}R KO and WT littermates were trained to self-administer two different doses of MDMA (0.125, and 0.25 mg/kg/infusion delivered in a volume of 23.5 µl over 2 s) on a fixed ratio 1 (FR1) schedule of reinforcement in 2 h sessions. Responses in the active hole resulted in one MDMA infusion, while nose poking in the inactive hole had no programmed consequences. Sessions were conducted 6 days per week during 10 days. A stimulus light, located above the active hole, was paired with the delivery of the drug. Daily sessions started with a priming infusion of MDMA at the selected training dose for each animal. Each infusion was followed by a 5 sec time-out period during which an active nose-poke had no consequence. Stable acquisition of MDMA self-administration behaviour was achieved when all of the following conditions were met: 1) less than 20% deviation from the mean of the total

number of reinforcers earned in three consecutive sessions (80% stability); 2) at least 65% responding on the active hole; and 3) a minimum of 5 reinforcers earned per session. After FR1 training, all mice were tested on a 3 h progressive ratio (PR) schedule reinforced by the training dose. In this paradigm, the response requirement to earn an infusion escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The breaking point, defined as the last ratio completed before drug seeking behaviour extinguished was determined.

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Supplement 2

Food-Maintained Operant Behaviour

Naive mice were partially food-deprived (95% of their *ad libitum* weight) and trained in operant boxes to respond for standard laboratory food pellets on a FR1 schedule of reinforcement, as previously described (Soria et al., 2005). A 10 sec time-out period was established after each reinforcer, and the session finished once 100 pellets were delivered or after 1 hour had elapsed, whichever occurred first. The criteria for the acquisition were achieved when mice maintained stable responding with less than 20% deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80 % of stability), with at least 75 % responding on the active hole, and a minimum of 10 reinforcers per session. Subsequently, mice were tested on a 2 h PR task using the escalation of the response requirement to earn a reward described in the previous paragraph.

Supplement 3

Microdialysis, HPLC analytical procedure and histology

Mice were anesthetized with a ketamine/xylazine mixture (5:1, 0.1ml/10g body weight, i.p.) and placed in a stereotaxic apparatus with a flat skull (Franklin and Paxinos 1997). Analytical probes (CMA7, 1 mm, CMA Microdialysis, Stockholm, Sweden) were directly implanted in the NAC (AP: + 1.5; ML: ± 0.8; DV: - 4.8 mm from bregma) to measure extracellular levels of DA, or (CMA7, 2 mm; Microdialysis, Stockholm, Sweden) in the PFC (AP: - 2.6 mm; ML: ± 0.4 mm; DV: - 3.0 mm from bregma) to determine 5-HT and NE. Probes were fixed to the skull with dental cement. One day after probe implantation, mice were habituated to the experimental environment overnight. The following morning, a Ringer solution was pumped through the dialysis probe (NaCl: 148 mM, KCl: 2.7 mM, $CaCl_2$:1.2 mM and $MgCl_2$: 0.8 mM, pH 6.0) at a constant rate of 1 µl/min. A period of 1 h was allowed for stabilization before the collection of 5 baseline samples. Mice were subsequently challenged with MDMA (20 mg/kg, i.p.), and collection of samples (every 15 min for DA and 5-HT and 20 min for NE) continued for an additional period of 3 h.

Dialysate samples were injected without any purification into a high-performance liquid chromatography (HPLC) system that consisted of a pump linked to an automatic injector (Agilent 1100, Palo Alto, California), and a coulometric detector (Coulochem II, ESA Inc., Chelmsford, Maryland) with a 5011A analytical cell. DA and 5-HT were quantified as previously described (Robledo et al., 2004). Briefly, for DA and 5-HT a reverse-phase C18 column was used (Zorbax SB C18, 3.5 µm, 150 x 4.6 mm, Agilent Technologies). For DA, the first electrode was fixed at -100 mV and the second electrode at + 300 mV, and the composition of the mobile phase was 50 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.65 mM octyl sodium sulphate and 15 % methanol, pH

3.5. For 5-HT, the first electrode was fixed at -50 mV and the second electrode at + 300 mV, and the composition of the mobile phase was 50 mM sodium acetate, 0.1 mM EDTA, 0.65 mM octyl sodium sulphate and 22 % methanol, pH 5.0. For NE, a reverse-phase C8 column was used (Zorbax SB C8, 3.5 µm, 150 x 4.6 mm, Agilent Technologies). The first electrode was fixed at + 125 mV and the second electrode at -175 mV, and the composition of the mobile phase was 0.1 M sodium acetate, 0.3 mM EDTA, 1.8 mM octyl sodium sulphate and 22 % methanol, pH 5.5. The flow rate for all assays was set at 1 ml/min. The sensitivity of the assay for DA was 1 pg/15 µl, 0.5 pg/15 µl for 5-HT, and 1 pg/20 µl, for NE.

At the end of the experiments, mice were sacrificed and brains were cut using a cryostat in 20 µm serial coronal sections, which were then processed with Cresyl Violet (Sigma-Aldrich, Spain) and observed under a microscope. Only those mice with correct probe placements were used in the study.

Supplement 4

Food maintained operant behaviour in 5-HT_{2A}R KO and WT littermates

All KO mice tested reached the acquisition criteria, whereas seven out of eight WT mice tested acquired the behaviour (Fig. S1a). Although KO mice responded on the active lever less than WT littermates at the beginning of training, both genotypes showed similar responding in the active hole from the fourth to the tenth day of testing. In agreement, the average time required for achieving the acquisition criteria was not significantly different between genotypes (WT: 5.3 ± 0.7 days; KO: 4.8 ± 0.5 days). Three-way repeated measures ANOVA (genotype x hole x day) showed a significant main effect of hole [$F_{(11,187)} = 254.680$, $p < 0.001$] and day [$F_{(1,17)} = 5.536$, $p < 0.001$], and significant interactions between genotype and hole [$F_{(11,187)} = 5.086$, $p < 0.05$], and hole and day [$F_{(1,17)} = 6.128$, $p < 0.001$]. Comparisons between genotypes for active nose poke responding revealed significant differences on days 2 and 3 of testing ($p < 0.01$). In order to evaluate whether WT and KO mice showed differences in the motivation to obtain food, a PR schedule of reinforcement was performed. One-way ANOVA did not reveal significant differences between genotypes [$F_{(1,16)} = 0.606$, N.S.] in the breaking points reached (Fig. S1b).

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Fig. S1

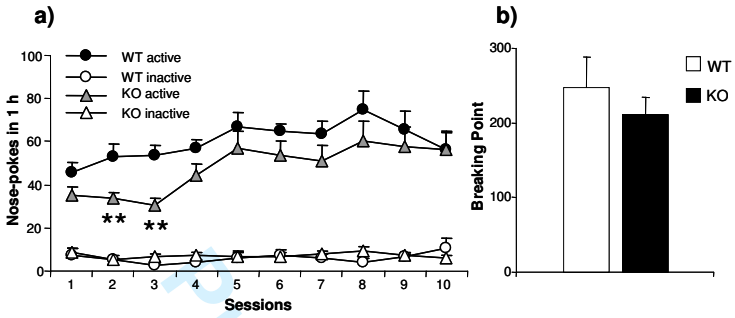

Figure S1. Acquisition of operant responding for food in 5-HT_{2A}R knockout (KO) (n = 8) and wild-type (WT) (n = 9) mice during ten days of training. The data represent the average number of nose-pokes + S.E.M. in the active and the inactive holes in 1 h sessions **(a).** The asterisks denote significant differences between WT and KO mice. ** $p < 0.01$ (one-way ANOVA). The breaking points achieved by WT and KO mice under a progressive ratio schedule of reinforcement are shown in **(b).**

APPENDIX C

List of Abbreviations

TH tyrosine hydroxylase

NAC nucleus accumbens

CS conditioned Stimulus

CR conditioned reinforcer

CR conditioned response

UR unconditioned response

US unconditioned stimulus

PET positron emission tomography

PFC prefrontal cortex

mPFC medial prefrontal cortex

5-HT serotonin

5-HIAA 5-hydroxyindoleacetic acid

5-HT$_{2A,2B,2C,1A}$R serotonin receptor 2A,2B,2C,1A

PET positron emission tomography

VTA ventral tegmental area

AA arachidonic acid

VMAT vesicle monoamine transporter

IP3 inositol phosphates

MAO$_A$ monoamine Oxidase A

MAO$_B$ monoamine Oxidase B

S stimulus

R response

qRT-PCR quantitative real time-polymerase chain reaction

ACh acetylcholine

LTP long-term potentiation

DRN dorsal raphe nucleus

DA dopamine

NE noradrenaline

H histamine

MDA 3,4-methylenedioxyamphetamine

MDMA 3,4-methylenedioxymethamphetamine

DAT dopamine transporter

NET noradrenaline transporter

SERT serotonin transporter

HMMA 4-hydroxy-3-methoxymethamphetamine

HHA 3,4-Dihydroxyamphetamine

CYP2D6 cytochrome P450 isoenzyme debrisoquine 4-hydroxylase

HHMA 3,4-dihydroxymethamphetamine

ROS reactive oxygen species

OFC orbital prefrontal cortex

D1 dopamine receptor 1

D2 dopamine receptor 2

MAPK mitogen-activated protein kinase

ERK extracellular signal-regulated kinases

INDEX

GLOSSARY

Allostasis	Equilibrium or stability achieved outside of the normal *homeostatic* range.
Conditioned Reinforcer (CR)	A previously neutral stimulus that acquires its reinforcing properties (positive or negative) by pairings with other, generally primary, reinforcers such as food, drugs, sex or electric shock.
Conditioned stimulus (CS)	A previously neutral sensory stimulus that will be able to elicit approach/avoidance behaviour depending on the nature of the used unconditioned stimuli pared with it.
Contingency	A consistent temporal relationship between two (or more) events that reduces the uncertainty of the subsequent event. For example, a situation in which a tone (CS) always occurs at the same time as a shock (US).

Dependence	An adaptive state that develops in response to repeated drug administration and is unmasked by *withdrawal* which occurs when drug taking stops.
Discriminative stimulus	A stimulus in the presence of which a response is reinforced according to a schedule of reinforcement. For example, drug cues can act as discriminative stimuli for behavioural responding that is maintained by drug reinforcement.
Entactogenic	Coined by David E Nichols as an alternative to *empathogen*, it describes a chemical agent that induces feelings of "empathy". The word was chosen to avoid possible negative connotations from $\pi\alpha\theta o\varsigma$ (pathos, suffering in ancient Greek).
Homeostasis	Dynamic equilibrium achieved by coordinated physiological self-regulated processes that maintain stability while adjusting to changing conditions.
Incentive	A stimulus that elicits approach behaviour (positive incentive) or withdrawal behaviour (negative incentive).
Negative reinforcer	An event that when omitted or terminated increases the probability of the response on which it is contingent.
Neuroplasticity	The ability of the brain to adapt and change over time. This plasticity underlies all types of memory formation.

Operant	A response on which the presentation of a reinforcer is contingent, such as lever pressing. Such behaviour is either called "instrumental" in obtaining a goal ("outcome" or "reinforcer"), or else it is a voluntary action. The learning of such behaviour is called instrumental conditioning.
Pavlovian Conditioning	Involves the pairing of a neutral sensory stimulus, the conditioned stimulus (CS), with a US in a temporally contingent manner. Learning occurs as the previously neutral stimulus obtains predictive value for the coming reward based on repeated pairings of the CS and the US. Eventually, this novel cue is able to evoke a response that is often topographically similar to that produced by the US itself. The learned response that the CS elicits is called the conditioned response.
Positive reinforcer	An event that increases the probability of a response on which it is contingent. For example, drug infusions increase the probability of lever pressing for the drug.
Reward	Hedonic neural representation.
Sensitization	An increase in the potency and /or efficacy of a drug in producing a particular response following its repeated administration.
Tolerance	The diminishing effect of a drug after repeated administration at the same dose, or to the need of a dose increase in order to produce the same effect.

Unconditioned responses	Innate responses such as salivation, approach, and consumption.
Unconditioned stimuli (US)	Biologically relevant stimuli such as food, water, or sexual stimuli which are able to evoke innate or unconditioned responses. Drugs of abuse are also generally considered unconditioned stimuli.