Universitat de Barcelona

FACULTAT DE FARMÀCIA

BACHELOR'S DEGREE FINAL PROJECT

Neurobiology of drug addiction:

animal behavioral paradigms, and MDPV as an example of addictive cathinones

Main scope: **Pharmacology and Therapeutics**

Secondary scopes: **Physiology and Physiopathology and Toxicology**

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Abstract

Synthetic cathinones, colloquially referred to as “bath salts” are derivatives of the psychoactive alkaloid found in *Catha edulis*, cathinone. Mephedrone and 2,3-methylenedioxypyrovalerone or MDPV are examples of these amphetamine-like psychostimulants. Its use has increased worldwide in past years, becoming a health and a legal problem.

MDPV and mephedrone as drugs of abuse can induce drug addiction; a chronically relapsing disorder which is defined by a compulsion to take the drug and a loss of control in limiting intake. Here, within the context of neurobiology of drug addiction, mechanisms involved in the development of addiction, including molecular and cellular changes induced in the rewarding circuit after repeated exposure to drugs of abuse are described.

Animal behavioral paradigms are critical to study the reinforcing effects of drugs of abuse. In the present study, conditioned place preference (CPP) paradigm was used to study the reinforcing properties of MDPV in rats. Different doses of MDPV (1, 3, 5 mg/kg s.c.) showed a significant place preference compared with saline controls.

Resum

Les cathinones sintètiques, referides col·loquialment com a sals de bany són derivats de l’alcaloide psicoactiu, catinona, que es troba a la planta *Catha edulis.* La mefedrona i el 3,4-metilenedioxipirovalerona o MDPV són exemples d’aquests derivats amfetamínics. En els últims anys, la seva utilització s’ha vist incrementada a nivell mundial, fent que esdevinguin problemes legals i de la salut.

L’MDPV i la mefedrona com a drogues d’abús poden induir una addicció: un trastorn crònic de recaigudes definit com un desig compulsiu per prendre la substància i una pèrdua del control per limitar la seva administració. Aquí, en el context de la neurobiologia de l’addició a drogues, es descriuen els mecanismes involucrats en el desenvolupament de l’addicció, incloent els canvis a nivell molecular i cel·lular

ocasionats al circuit de la recompensa després de l'administració repetida de drogues d'abús.

Els paradigmes de comportament en animals són crítics per a l'estudi dels efectes reforçants de les drogues d'abús. En aquest estudi, fet en rates, es va utilitzar el paradigma de condicionament de preferència de lloc per estudiar les propietats reforçants de l'MPDV. Les diferents dosis (1, 3, 5 mg/kg s.c.) de MDPV van demostrar diferències significatives de preferència de lloc en comparació amb els animals control.

Integration of the three different scopes

This project is about the neurobiology of addiction (including an example of synthetic cathinone, MDPV) and animal behavioral paradigms. It involves three different scopes: pharmacology and therapeutics, physiology and physiopathology, and toxicology.

As it will be described below, this project is divided in two parts: a bibliographic review and an experimental part. The experiment consists in carrying out a test, the conditioned place preference (CPP) paradigm in order to study the rewarding properties of a drug of abuse, in this case, 3,4-methylenedioxypyrovalerone or MDPV. This experimental part was developed in the Department of Pharmacology and Therapeutic Chemistry and this is why pharmacology and therapeutics is the main scope.

Toxicology scope is shown in the description of synthetic cathinones, particularly MDPV and mephedrone. Both of them are novel recreational drugs and, as substances of abuse, are able to induce addiction. Addiction is a disorder quite common in our society and drugs of abuse are considered chemical substances that may harm user's organisms.

Finally, physiology and physiopathology scope is important to describe the circuits involved in addiction's development. The rewarding circuit and its adaptations, molecular and cellular, due to exposure to drugs of abuse is described in the present study.

1. Introduction

Drug abuse and addiction have negative consequences from people to the society. Drugs of abuse suppose a risk for humans due to the existence of a fast and uncontrollable transition from the research of new experiences and feelings, by consumers, to use and abuse of these substances, which create dependence and, in unfortunate occasions, can finish with overdose or toxicity for chronic use. In Europe, more than 1.6 billion new consumers appear each year.

The market of illicit drugs can be considered just as dynamic as markets for legal products. Designer drugs are synthetic compounds developed to provide rewarding effects similar to illicit drugs of abuse such as amphetamines, cocaine, heroin or marijuana while avoiding existing legislative classification and penalty(1). Often they are just chemically modified at a single position of the original molecule. Illicit laboratories, due to the low purity of cocaine and 3,4-methylendioxymethamphetamine (MDMA), or because of the successful efforts made by governments to prevent the diversion of precursors to manufacture MDMA(2), have realized the need of throwing to the market a new family of drugs of abuse known as “legal highs”. Among these new classes of drugs are the synthetic cathinones.

Synthetic cathinones were first developed for therapeutic purposes, mainly as antidepressants and anorectic agents, but promptly started being abused for their euphoric, stimulant and hallucinogenic effects(1)(3). Cathinone and cathine are alkaloids and the main natural cathinones present in the leaves of the *Catha edulis* plant, commonly known as khat. Khat is a flowering evergreen slow-growing shrub or tree that grows wild in the Horn of Africa and in the Southwest Arabian Peninsula. Peter Forskal, a Swedish botanist, during an expedition to Egypt and Yemen in 1761-1763 described the khat plant. It was identified as a member of the family *Celastraceae*(3).

For centuries, the chewing of fresh khat leaves has been a tradition in local communities for their gratifying stimulant effects. In Yemen, khat chewing is widely

practiced on a daily basis, at the so-called khat sessions, where men chat for several hours, usually after work(3).

The most widely abused synthetic cathinones are mephedrone, methylone and 3,4-methylenedioxyprovalerone or MDPV. All of them are derivatives of cathinone which is an alkaloid and has a similar structure to amphetamine(1).

Synthetic cathinones are a ring-substituted cathinone closely related to phenethylamine family, differing only by a keto functional group attached at the beta carbon on the amino alkyl chain linked to the phenyl ring. This is why they are also known as β-keto amphetamines.

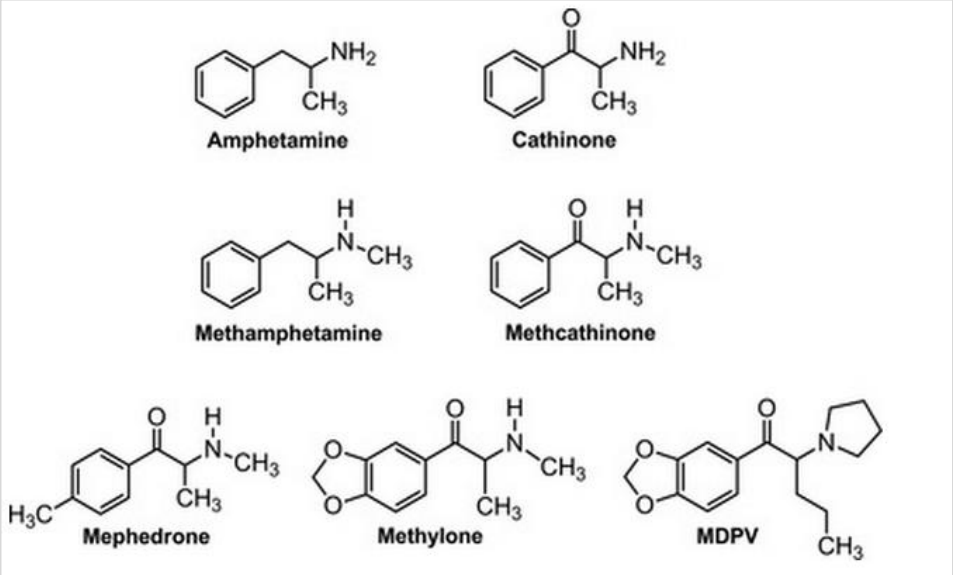

Figure 1. Representation of the structure of cathinone, amphetamine and their derivatives compounds.

The backbone of mephedrone, methylone and MDPV is phenethylamine with a ketone group at the β-carbon position. Mephedrone is methylated on the amine group, like methcathinone, and on the aromatic ring of its backbone. For this reason mephedrone is also known as 4-methylmethcathinone. Methylone is also methylated on the amine group of β-ketophenethylamine backbone, but has a methylenedioxy ring attached to the aromatic ring, forming a structure close to MDMA. Methylone due to its structure is also entitled 3,4-methylendioxymethcathinone. MDPV has a methylenedioxy ring attached to the aromatic ring of the β-ketophenethylamine backbone and a

pyrrolidinyl ring and propane attached to α-carbon, instead of a methyl group, forming a structure close to pyrovalerone(1)**(Fig. 1)**. MDPV due to its pyrrolidine ring and the tertiary amino group is a more lipophilic and more potent monoamine uptake inhibitor than other cathinone derivatives. Its structure creates a less polar molecule more able to cross the blood-brain barrier. Moreover, its nitrogen atom forming the tertiary amino group is the responsible of its high solubility in organic solvent(4).

Synthesis of mephedrone was first described in 1929 as stimulant for central nervous system (CNS) by Saem de Burnaga Sanchez(5) and MDPV was synthesized by Boehringer Ingelheim and patented in 1969(6). However, its abuse was not reported until the early 2000s. Methylone, in turn, is a more recent analogue and was patented in 1996(7). After their discovery, the synthetic cathinones were ignored until their abuse as a legal alternative to MDMA was reported on internet drug websites in 2003.

These synthetic cathinones mentioned above are the most common compounds found within “bath salts”, “plant food” and “research chemicals”, slang terms used by manufacturers of these synthetic cathinones in order to circumvent legal repercussions(8). Mephedrone is the most widely abused synthetic cathinone within Europe, whereas MDPV is the most frequently abused within the USA(1).

In 2011, the DEA (Drug Enforcement Administration) placed mephedrone and MDPV and their salts, isomers, and salts of isomers on Schedule I status of the Controlled Substance Act (CSA)(9). This scheduling makes them illegal to manufacture, distribute, possess, import, and export.

Nonetheless, legal regulation of synthetic cathinones is rather difficult to attain success, since they are easily replaced by novel compounds after minor structure modifications. Consequently, for each drug that gets banned, new and more powerful analogues will reach the licit drug markets.

Bath salts contain one or a mixture of synthetic cathinones and are sold under several inexplicit brand names, including “Bloom”, “Bubbles”, “Meow Meow”, “Blue Silk”, “Ivory Wave”, “Purple Wave” and “Vanilla Sky” and labelled as “not for human consumption”(3)(9)(10)(11). Bath salts are purchased locally at convenience stores and

at head- or smartshops, or conveniently over Internet suppliers, being readily accessible, affordable, and technically legal(3). Head shop is a retail outlet specialized in paraphernalia used for consumption of cannabis, tobacco, and legal highs.

Synthetic cathinones are generally sold in form of a white or yellowish amorphous or crystalline powder and are usually found as 200 mg to 10 g packets, or in capsules; tablets are more uncommon(3). Producers and sellers claim to provide synthetic cathinones with over 99% purity. However, analyses of seized and purchased products demonstrate purity of around 95% with adulterants including benzocaine, lidocaine, caffeine, piperazines and paracetamol(1).

These compounds are used via multiple routes including nasal insufflations, oral ingestion, rectal insertion with mucosal absorption, gingival application, inhalation, intramuscular and intravenous, being nasal insufflations and oral ingestion the most common ways(9)(12). Nasal insufflations are done by “snorting” and more specifically by “keying” which consists in dipping a key in powder and then being insufflated(9). Many nasal users experiment agitation because of nasal irritation, leading them to change to oral administration. Oral ingestion is done by swallowing capsules or “bombing” which consists in wrapping the powder in cigarette paper and swallowing it(13).

Reports of “typical” doses for mephedrone are 100 to 200 mg orally, with onset of effects around 30 to 45 minutes and a duration of 2 to 5 hours(12)(14). MDPV seems to present more potent effects, causing effects in 15 to 30 minutes after dosing with a typical oral dose of 10 to 15 mg. The psychoactive effects may last from 2 to 7 hours(14). When MDPV is insufflated with a common dose of 5 to 11 mg, effects are observed in 5 to 20 minutes and they may last from 2 to 3.5 hours. Independently of administration’s route, after coming down from MDPV there are several hours of increased physical and mental stimulation, during which it is difficult to sleep(15).

Studies on the metabolism have shown that mephedrone is N-demethylated to a primary amine, followed by reduction of the ketone moieties to alcohols, subsequently; the tolyl group is oxidized to the corresponding alcohol. Finally, some of

the alcohols are then conjugated via sulfation or glucuronidation and excreted in the urine(16). Metabolism of MDPV begins with the opening of the methylenedioxy ring, followed by demethylation leading to a catechol ring which is subsequently methylated by catechol-O-methyltransferase (COMT). β-keto moiety is also reduced to an alcohol. Finally, some of the metabolites are then conjugated via sulfation or glucuronidation and excreted in the urine, too(17).

Clinical effects reported with mephedrone and MDVP use are similar to sympathomimetic toxicity. Specific case reports and Internet descriptions by users denote effects very similar to cocaine, methamphetamine, and ecstasy(9). Reported subjective effects include mental and physical stimulation, euphoria, increased energy, increased productivity and motivation, empathy, talkativeness, heightened alertness, and sexual arousal(10)(15)(18). Users of synthetic cathinones have also exhibited sweating, palpitations, nausea, vomiting, loss of appetite, headache, tightened jaw muscles, muscle twitching, grinding teeth (trismus and bruxism), dizziness, vertigo, nystagmus, and impaired short-term memory(15)(18)(19). Other neurologic and psychiatric effects like self-mutilation, suicide attempts, and persistent paranoid psychosis have also been described with both acute and chronic use(14). In fact, agitation that can range from mild to severe psychosis is the most commonly reported complication.

Synthetic cathinones were shown to exert their effects by interacting with plasma membrane monoamine transporters, specifically dopamine transporter (DAT), noradrenaline transporter (NET), and serotonin transporter (SERT), resulting in an increased concentration of these biogenic amines in the synaptic cleft(20)(21)(22)(23). However, different affinities toward these transporters are observed between synthetic cathinones. Drugs due to their interactions with monoamine membrane transporters can be classified as substrates like amphetamines or blockers like cocaine. Mephedrone acts as a non-selective inhibitor for all catecholamine transporters and also as serotonin releaser, similar to MDMA(22)(23)(24). Contrarily, MDPV acts as a pure monoamine-selective transporter blocker, with high potency for DAT and NET (50-fold and 10-fold more potent than cocaine), but weak for SERT (10-fold less potent

than cocaine) due to the presence of 3,4-methylenedioxy ring-substitution(21)(25). High concentrations of DA in brain are crucial for addiction and rewarding effects after drugs administration.

Finally, evidences from animal studies support the reinforcing properties and abuse liability of synthetic cathinones, namely mephedrone and MDPV, confirmed by their ability to elicit self-administration patterns in rats. Examining the ability of MDPV to support intravenous self-administration (IVSA) and to lower thresholds for intracranial self-stimulation (ICSS) in rats, Watterson *et al*. demonstrated that the synthetic cathinone MDPV possesses potent reinforcing properties and suggests a high degree of abuse potential in humans(26). Using Conditioned Place Preference (CPP) model, a powerful and direct method to investigate abuse potential, Karlsson *et al*. concluded that mephedrone and MDPV produce CPP suggesting addictive properties(27).

2. Objectives

The overall aim of the present study is to get close to drug addiction, and in order to achieve it, the following study is divided in two parts. The first one is a bibliographic review in which the main aim is to describe drug addiction, considering factors which may have influence on drug abuse and mechanisms and pathways involved in addiction. Due to drugs of abuse present different risk of abuse in humans and this risk can be predicted using tests based on behavioral paradigms in animals, description of various animal behavioral paradigms will be addressed in this study.

The second part is an experiment and its aim is to investigate if the synthetic cathinone MDPV induces conditioned place preference in rats. There is not any published study on MDPV and CPP in rats, whereas there are on MDPV in mice and on mephedrone in both, rats and mice.

3. Materials and methods

3.1 Animals and treatment groups

Males Sprague-Dawley rats (Harlan, Spain) aged 5 weeks and weighing 130 to 160 g were used. Animals were housed two per cage under normal indoor temperature and humidity, and maintained on 12 h light/dark cycle from 8:00 am to 8:00 pm. They had free access to food (standard laboratory diet, PANLAB SL, Barcelona, Spain) and drinking water.

All experimental procedures for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia and following the guidelines of the European Communities Council (86/609/EEC). Efforts were made to minimize suffering and reduce the number of animals used.

Rats (n=28) were assigned to one of four treatment groups: saline (saline s.c. + saline s.c.), MDPV 1 mg/kg (saline s.c. + MDPV 1 mg/kg s.c.), MDPV 3 mg/kg (saline s.c. + MDPV 3 mg/kg s.c.), MDPV 5 mg/kg (saline s.c. + MDPV 5 mg/kg s.c.). Before experimentation, all of the animals received two habituation sessions (48 and 24 hours before testing) in order to reduce the novelty and stress linked to handling and injection.

3.2 Drugs

Pure MDPV hydrochloride was synthesized and characterized in the Department of Pharmacology and Therapeutic Chemistry of Faculty of Pharmacy following the protocol described by Aarde et al., (2013)(28) with little modifications. MDPV solutions for injection were prepared in sterile 0.9% sodium chloride (saline) immediately before administration.

3.3 Literature research

In order to conduct a search of articles as extensively as possible, literature explorations were performed using the following electronic database: PubMed. The

key words used were: addiction, 3,4-methylenedioxypyrovalerone OR MDPV, mephedrone OR 4-methylmethcathinone, CPP, self-administration, self-stimulation, synthetic cathinones. Key words were used singly and in combination.

3.4 Conditioned Place Preference (CPP) paradigm

The place conditioning protocol used was non-biased and closed to the one Ciudad-Roberts et al., (2013)(29) followed. The apparatus was composed of three distinct compartments separated by manually operated doors. The central compartment or corridor measured 27 x 10 x 25 cm (w x d x h) and its function consisted in communicating the two pairing sides. The pairing compartments had the same size 20 x 20 x 25 (w x d x h) but different appearance. One compartment had black and white chequered walls with a smooth and shiny floor, whereas the other had white and light blue painted walls and rough floor.

CPP was performed in three phases: preconditioning, conditioning, and test. During preconditioning phase, day 1, animals were placed in the middle of the corridor and had free access and move among the three compartments (corridor and both pairing sides) for 20 minutes. The time spent in each compartment was recorded by computerized monitoring software (Smart 3, Panlab SL, Barcelona, Spain). Animals were expected not to demonstrate preference for any of the pairing compartments.

On days 2, 4, 6 and 8 of conditioning phase, rats were treated with MDPV (1, 3 and 5 mg/kg, s.c.), or saline, 20 minutes before being placed in one of the two compartment for 30 minutes. On days 3, 5, 7 and 9 of the conditioning phase, animals received saline and were placed in the opposite compartment respect where they had received the drug the day before. Animals were exposed to only one compartment per day and treatments were counterbalanced to ensure that some animals received MDPV in one of the two pairing compartments and others in the other one. Control animals received saline every day.

The test phase, day 10, was conducted identically to the preconditioning phase. Animals were placed in the middle of the corridor and had free access to move among the three compartments for 20 minutes. On test day, animals did not receive the

substance. The time spent in each compartment was measured and a preference score was expressed in seconds. This score was calculated as the difference between the times spent in the drug-paired compartment in the test phase minus the time spent in the preconditioning phase.

3.5 Statistical analysis

All data are expressed as means ± standard error of the mean (S.E.M.). Differences between groups were compared using one-way analysis of variance (ANOVA). For all comparisons, *p* values < 0,05 were considered statistically significant. Significant differences were then analyzed by Tukey's post hoc test for multiple means comparisons.

All data were kindly given by PhD Patrícia Muñoz (Department of Pharmacology and Therapeutic Chemistry of Faculty of Pharmacy).

4. Results

4.1 Results from bibliographic research

Addiction is the quintessential complex disorder. It is a process that manifests itself in the uncontrollable, compulsive drug seeking and use, and that persists even in spite of negative health and social consequences(30). So, it is defined specifically as a compulsive pattern of drug-seeking and drug-taking behavior that takes place at the expense of most other activities. It is believed that the development of addiction involves a transition from casual to compulsive patterns of drug use.

Addictive substances induce pleasant states such as euphoria in the initiation phase. Continued use induces adaptive changes in the central nervous system that lead to tolerance, physical dependence, sensitization, craving, and relapse(31).

Theories of addiction have been developed from neurobiologic evidence and data from studies of learning behavior and memory mechanisms. Koob and Le Moal(32) proposed that when a drug is administered, the organism tries to counteract the drug's effects through a vicious circle in which the hedonic set point –point at which pleasure is achieved- is continually changing in response to the administration of the drug.

Robinson and Berridge's(33) explanation for addiction is that drugs are first taken because they are pleasant, but with repeated drug use homeostatic neuroadaptations lead to tolerance and dependence. Then, unpleasant withdrawal symptoms appear after the cessation of use. Compulsive drug taking is maintained to avoid unpleasant withdrawal symptoms. So, addictive drugs are first taken to achieve pleasant drugs "highs", and after addiction, to escape withdrawals "lows".

Robinson and Berridge also emphasized the dissociation between the incentive value of the drug called "wanting" and its pleasurable or hedonic effects "liking"(33). They added that the brain system involved in the reward mechanism becomes hypersensitized to direct effects of the drug and also to the stimuli associated that are not directly attributable to the drug. In fact, environmental stimuli also known as cues,

associated with drug use itself can induce a conditioned response, withdrawal or craving, in the absence of the drug. The hypersensitisation causes pathologic wanting or craving, independently of the presence of withdrawal symptoms and leads to compulsive drug-seeking and drug-taking behavior(33).

4.1.1 Opponent process theory of addiction

Solomon and colleagues(34) described a theory named the opponent process and based on the pleasure/withdrawal view of addiction. This theory describes processes of addiction in graphic ways that allow the transition to addiction to be visualized.

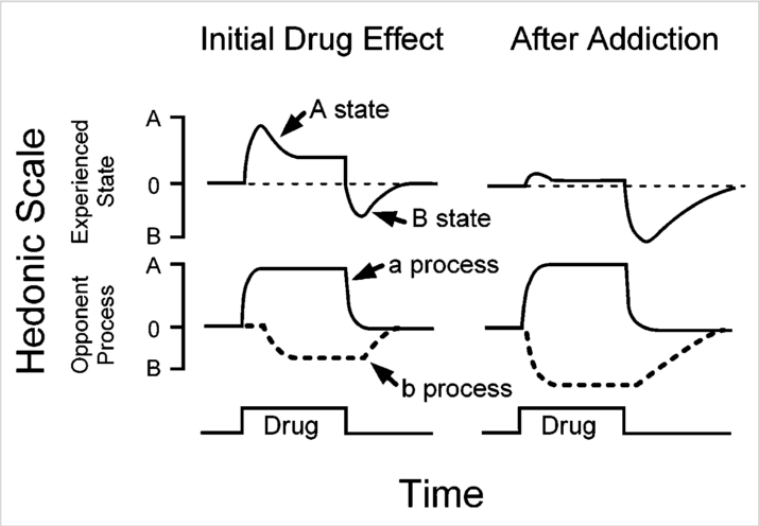

Figure 2. Opponent process model of addiction. Initially A-state is large, followed by a small B-state. However, with repeated drug use, the opponent b-process increases in magnitude and duration, leading to a large B-state(33).

The opponent process theory describes that pleasant doses of a drug activate a dose-dependent a-process in brain reward circuits, which in turns triggers activation of a negative or opponent b-process. This process serves to restore homeostasis and bring brain states back to normal. The summation of a- and b- process creates the final experienced state felt by the person. This state is named A-state when the summed effect is pleasantly drug-like what means that a-process surpasses b-process. It is called B-state when it is unpleasantly drug-opposite, what means that b-process surpasses a-process(33)(34)**(Fig. 2)**.

The euphoric high of the drug A-state is directly caused by a-process. B-process is manifested first as mild decay of the drug's high after the initial peak. If the drug is taken again, the b-process is strengthened and manifest as tolerance to drug euphoria, so A-state is reduced. In this state, if the user wants to repeat previous experiences, he or she has to increase the dose. Finally, unpleasant withdrawal is caused when drug effects wear off because the b-process is posited to last longer than the a-process, so the person feels the B-state. Only the b-process is posited to change with repeated drug-taking. In fact, it grows in magnitude and in duration, leading to an experience dominated by the unpleasant symptoms associated with withdrawal. Even though, prolonged abstinence from the drug would decay the b-process, and the ability to reactivate it would return to normal(33)(34).

4.1.2 Factors influencing drug abuse and dependence

There are some factors that may influence on drug abuse and dependence -a cluster of cognitive, behavioral, and physiological symptoms indicating that a person is going to continue using a substance despite having clinically significant substance-related problems(31) :

- Pharmacologic and physicochemical properties of drugs: pharmacologic and physicochemical properties of a drug influence in how a drug is consumed. Liposolubility, for example, facilitates the passage of a drug through the blood-brain barrier (BBB). Contrarily, water solubility promotes the injection of the drug. If the drug is volatile, it will be consumed by inhalation in vapour form, whereas presents resistance to heat, it will be smoked.

  Rapid onset and intensity of effects are characteristics that increase the potential for abuse. Moreover, when a drug has a short half-life it produces more abrupt and intense syndromes of withdrawal -constellation of signs and symptoms that follows the abrupt discontinuation or reduction in the use of a substance or after blockage of the actions of a substance with antagonist- than does a long half-life.

- Personality and psychiatric disorders: personal traits and mental disorders are main conditioning factors in drug addiction. Risk-taking or novelty-seeking features favour the use of addictive drugs. Mental disorders like schizophrenia, bipolar disorder, depression, and attention-deficit-hyperactivity disorder, are associated with an increased risk of abuse.

- Genetic factors: genetic factors which influence the metabolism and the effects of drugs contribute to the risk of addiction.

- Environmental factors: these factors are characteristics in a person's surroundings that increase their likelihood of becoming addicted to drugs. The community, family, school, and friends are environments that may have influence on risk of addiction(35).

4.1.3 Neuropsychopharmacology of reward

A reward is defined as an incentive stimulus that positively reinforces behavior, that is, it increases the probability of the behavior's occurrence due to its pleasure-related effects. Rewards are positive reinforcers, while aversive events, such as electric shocks that elicit an avoidance response, are negative reinforcers and are called punishers. In fact, addictive drugs can act as positive reinforcers when they produce euphoria or as negative reinforcers when they alleviate withdrawal's symptoms or dysphoria(36).

Natural rewards such as food, drink, and sex and drug rewards stimulate the release of dopamine from neurons of the presynaptic ventral tegmental area (VTA) into the nucleus accumbens (NAc), causing euphoria and reinforcement of the behavior. Despite both of them have the same mechanism, in natural rewards there is a rapid adaptive change, or habituation, after a few experiences, and the unexpectedness of the reward seems to be more important in the initial response. In drug rewards, there is not this habituation, and each dose of the drug stimulates the release of dopamine.

Numerous brain regions and neurotransmitters are involved in the reward circuit. However, the most studied among them in relation to drug addiction are mesocorticolimbic and nigrostriatal dopamine systems:

a) The mesolimbic circuit includes projections from cell bodies of the VTA to NAc **(Fig. 3)**. This pathway is known to induce neuronal and behavioral sensitizations associated with rewarding and reward-associated stimuli. Contextual stimuli are represented in different limbic areas, basolateral amygdala and hippocampus, whose glutamatergic projections ascend to the NAc via the medial prefrontal cortex. This pathway has been implicated in memory and conditioned responses linked to craving and the emotional and motivational changes of the withdrawal syndrome(36).

   In order to connect this pathway with the opponent process theory of addiction, Koob and colleagues(32) suggested that the positive a-process is caused by activation of mesolimbic dopamine projections to the NAc and amydala that mediate the acute reinforcing effects of drugs. Repeated drug use, induces tolerance or downregulation in the mesolimbic dopamine system, decreasing the drug A-state.

b) The mesocortical projections ascend from the VTA to the prefrontal cortex, orbitofrontal cortex and anterior cingulated. This circuit is involved in the conscious experience of the effects of drugs, drug craving, and the compulsion to take drugs. It is an essential component of the emotional response network that ensures normal cognitive functioning, in particular working memory and decision making.

c) The nigrostriatal projections connect from the substantia nigra to the dorsal striatum (caudate-putamen), where dopamine is associated with the initiation and execution of habitual behavior, although its role in reward has also been shown.

   Projections from the γ-aminobutiric acid (GABA) neurons of the NAc to the VTA and prefrontal cortex interact with the circuits described above **(Fig. 3)**.

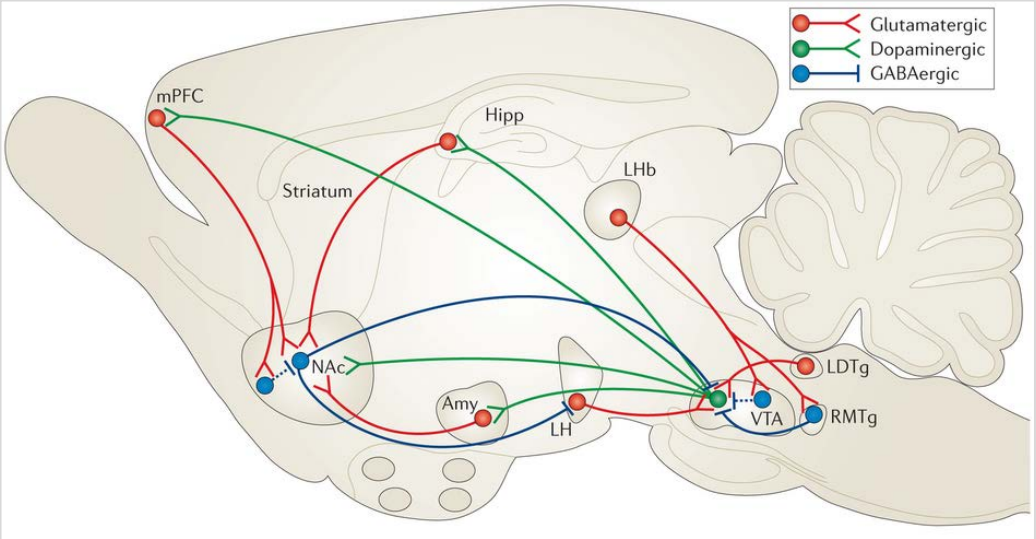

Figure 3. A simplified schematic of VTA-NAc reward circuit in the rodent brain. The primary reward circuit includes dopaminergic projections from the VTA to the NAc. There are also GABAergic projections from the NAc to the VTA. The glutamatergic inputs to VTA control aspects of reward-related perception and memory(37).

4.1.4 The leading role of dopamine

Dopamine is a neurotransmitter associated with the reinforcing effects of drugs of abuse and may have a key role in triggering the neurobiological changes associated with addiction. Despite the fact that each drug of abuse binds to different initial protein target, all of them increase the extracellular concentration of dopamine in the NAc(38). For example, stimulants increase dopaminergic transmission in the NAc, directly. Opiates increase the same transmission, but indirectly. They inhibit GABAergic interneurons in the VTA, which desinhibits VTA dopamine neurons. Opiates also act on opioid receptors on NAc neurons, but in this case directly. Nicotine seems to stimulate directly nicotinic cholinergic receptors on VTA dopamine neurons and indirectly receptors on glutamatergic nerve terminals that innervate the dopamine cells. Alcohol, in its turn, promotes $GABA_A$ receptors function what may inhibit GABAergic terminals in VTA like opiates. Alcohol also may inhibit glutamatergic terminals that innervate NAc neurons(39)**(Fig. 4)**.

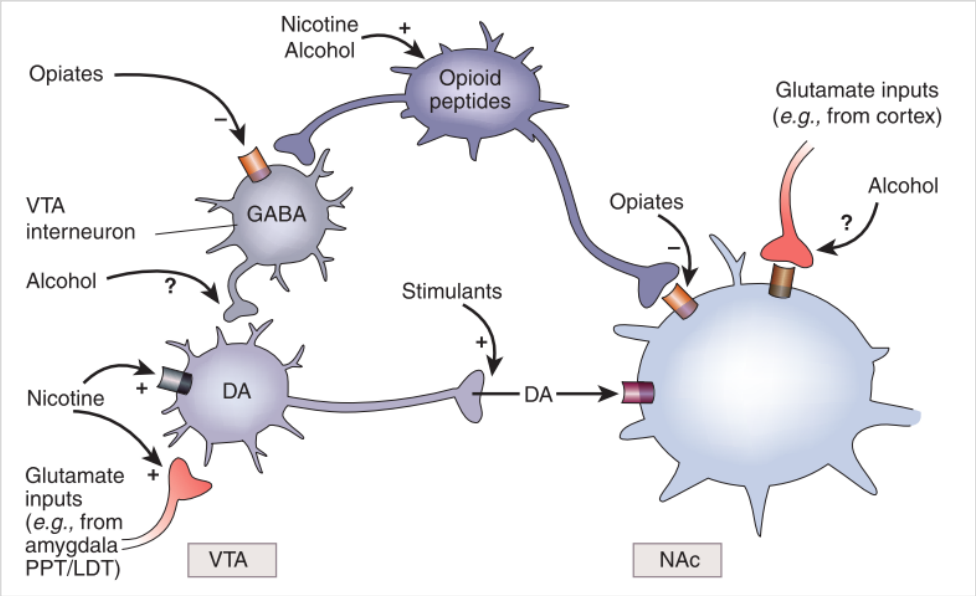

Figure 4. Highly simplified scheme of converging acute action of drugs of abuse on the VTA-NAc.(39)

Dopamine is responsible of diversity effects because modulates different brain regions (limbic, cortical, and striatal). For example it mediates the hedonic consequences of a reinforcing stimulus, promotes associative learning about the stimulus or anticipates its rewarding effects(40). It has been described that when there is a substantial decrease in dopamine levels in NAc, the withdrawal syndrome is experimented(41).

There are five different subtypes of dopamine receptors, but they are classified only in two groups: D1-like receptors group which includes subtypes D1 and D5, and D2-like receptors group, which contains D2, D3 and D4. All of them are G-protein-coupled receptors. D1-like receptors activate adenylyl cyclase (AC), whereas D2-like receptors inhibit the enzyme(31). G proteins are trimeric structures composed of two functional units: α subunit that catalyzes GTPase activity (converts GTP to GDP) and β-γ subunits. Both subunits can activate or inhibit enzymes such as adenylyl cyclase or phospholipase C that synthesize cyclic adenosine monophosphate (cAMP), inositol triphospahte and diacylglycerol. These second messengers can activate protein kinases, which phosphorylate and regulate ion channels. In addition, these protein

kinases induce pharmacological effects and produce changes in transcription factors such as CREB (cAMP-responsive element-binding protein) and ΔFosB(31).

4.1.5 Common circuit-level adaptations

All drugs increase dopaminergic transmission to the NAc after acute administration. After chronic exposure to drugs of abuse, some adaptations in dopamine function are also produced(39). Despite the fact that there are many different drugs of abuse with their dosing regimens and routes of administration, a common adaptation scheme can be described.

Chronic exposure to any drug of abuse impairs dopamine system, which can be seen as a homeostatic response to repeated drug activation of the system, in other words, tolerance. Moreover, after chronic exposure, baseline levels of dopamine function are reduced, and normal rewarding stimuli may be less effective. In addition, chronic drug exposure seems to sensitize the dopamine system, with greater increases in dopaminergic transmission occurring in response to the drug in question and to drug-associated cues. Sensitization is one of the neurobiologic mechanisms involved in craving and relapse(31).

Another adaptation to chronic exposure to drugs of abuse is cortical "hipofrontality", which means that there is a reduced baseline activity of several regions of frontal cortex, such as prefrontal cortex, anterior cingulated cortex, and orbitofrontal cortex. This brain region, frontal cortex, controls executive function, including working, memory, attention and behavioral inhibition. It is also important in controlling individual's response to environmental stimuli.

It has been demonstrated that chronic exposure to drugs of abuse causes complex changes in these frontal cortical regions and their glutamatergic outputs, which are implicated in the profound impulsivity and compulsivity that characterizes a state of addiction(38)(42).

Chronic drug states are also associated with changes in corticotropin releasing factor (CRF) system. When a withdrawal syndrome appears due to any drug of abuse,

it leads to activation of CRF-containing neurons in the amygdale(43). These neurons are involved in fear and other aversive states and innervate many forebrain and brainstem regions. It is known that activation of these neurons during drug withdrawal mediates the negative emotional symptoms, many of the somatic symptoms that occur upon drug withdrawal, and may contribute to drug craving and relapse(39).

In addition, CRF can be considered as an example of "opponent process", which means that it serves to counteract all changes induced in the brain by drugs and drives withdrawal symptoms when drug's administration is discontinued(42).

CREB, a transcription factor is responsible of *CRF* gene regulation. Therefore, the hyperactivity of central CRF pathways upon precipitation of drug withdrawal which reflects molecular adaptations in amygdala neurons can be produced by CREB's induction(32).

4.1.6 Common cellular and molecular adaptations

Chronic exposure to drugs of abuse causes numerous common adaptations at the cellular and molecular level in the VTA-NAc and other brain reward regions. There are too many adaptations to describe here comprehensively; so, only those related to CREB and ΔFosB will be presented.

After chronic exposure to many drugs of abuse, including amphetamines, so do MDPV and mephedrone, transcription factor CREB is activated. The rise of CREB in the NAc and, to a lesser extent, in the VTA, has been linked to reduced drug-induced reinforcement. In fact, overexpression of CREB in the accumbens inhibits the rewarding effects of pshychoestimulants, mu opioids and biological rewards. Alcohol and nicotine, by contrast, reduce CREB activity, at least in the NAc(39).

The main steps involved in CREB-mediated gene transcription include dimerization, binding at response elements in DNA, and phosphorylation(43). CREB is a transcription factor that binds as dimmers to the cAMP-response element (CRE), a specialized stretch of DNA that contains the consensus nucleotide sequence TGACGTCA. CRE sites are found within the regulatory region of numerous genes; if a

promoter contains CREs, then it could be subject to regulation by CREB, depending on several tissue-specific factors(44).

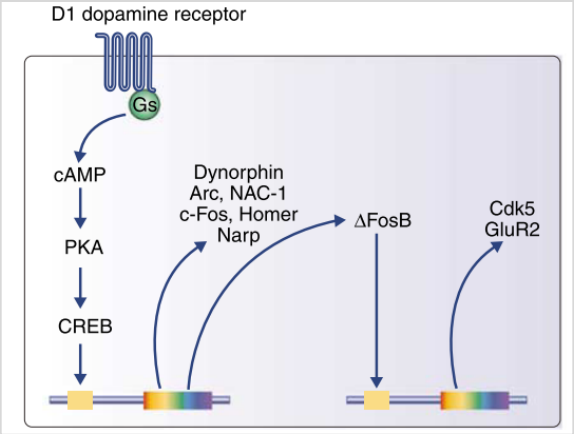

Figure 5. Dopamine D1 receptor-dependent signaling in the NAc hypothesized to underlie the transition from social use to enduring vulnerability to relapse(45).

As described in point 4.1.4, when dopamine D1-like receptors are stimulated, AC is activated and accumulation of cAMP increases which causes liberation of the catalytic subunits of cAMP-dpendent protein kinase (PKA). The free catalytic subunit enters to the cell nucleus and phosphorylates CREB. Phosphorylation of CREB activates a cascade of events that involves recruitment of associated proteins such as CREB-binding protein (CBP) and assembly of a larger transcriptional complex.

There are various phosphorylation sites on the CREB protein that differentially regulate CREB activity. For example, PKA, Ca^{2+}/calmodulin dependent kinase (CaMK)IV and MAPK-activated ribosomal S6 kinases (RSKs), each phosphorylate CREB at serine 133, which stimulates the recruitment of CBP and leads to the activation of gene transcription. Contrarily, CaMKII phosphorylates CREB at serine 142, which promotes the dissociation of the CREB dimer and thereby reduces gene transcription(44).

Increased CREB function, after exposure to drugs of abuse, appears to cause tolerance and dependence, adaptations commonly associated with the development and maintenance of addictive behaviors(44). *Homer*, c-*Fos*, *ΔFosB*, *NAC*-1, and *preprodynorphin* are some examples of genes implicated in addiction and their transcription is promoted by CREB(45) **(Fig. 5)**. Preprodynorphin, NAC-1, and Homer,

contribute to the compensatory effect increasing CREB to reduce the value of drug reward. Increased dynorphin, for example, inhibits the activity of dopamine cells and presynaptic dopamine release. So, increased dynorphin expression is associated with aversive or depressive-like effects such as those that often accompany drug withdrawal(44).

The increase in many transcriptional regulators and immediate early genes, such as cFos, Arc, and Homer, decreases after repeated exposure. In contrast, ΔFosB accumulates in dopamine-terminal fields in the cortex and striatum. This accumulation occurs in response to chronic administration of drugs of abuse, and in response to repeated biologically motivating stimuli. There is considerable evidence that ΔFosB accumulation within NAc neurons contributes to state of sensitization(46). Nestler et al., (2005) (39) hypothesized that induction of ΔFosB mediates many shared aspects of drug and natural addictions by regulating a set of common target genes.

ΔFosB is a member of the Fos family of transcription factors, which dimerize with a member of the Jun family to form activator protein-1 (AP-1) transcription factor complexes. These complexes then bind to AP-1 sites which are consensus sequence, TGAC/GTCA present in the regulatory regions of many genes(41).

Some of the genes regulated by ΔFosB may be compensatory and serve to limit drug reinforcement, and maybe drug-seeking, like genes regulated by CREB. For example, the induction of *Cdk5*, cyclin-dependent kinase-5, phosphorylates the dopamine-regulated phophatase DARPP-32, thereby preventing its phosphorylation and activation by PKA. Cdk5 promotes nerve cell growth and mediates(47) an enduring increase in dendritic spine density in accumbens spiny cells during extended abstinence from chronic psychostimulant administration(45).

On the other hand, the induction of other genes by ΔFosB promotes drug reward and many studies indicate that overexpression of ΔFosB increases drug reward. The induction of *GluR2* in the shell of the accumbens is one of the ΔFosB gene regulation examples that would promote drug reward. GluR2 is a particular AMPA

glutamate receptor subunit and its expression is increased in NAc upon overexpression of ΔFosB.

The induction of GluR2 reduces the electrical excitability of NAc neurons, as GluR2-containing AMPA channels show reduced overall conductance and reduced Ca^{2+} permeability. This reduction could mediate enhanced reward mechanisms by, for example, making the neurons more sensitive to inhibition by subsequent drug exposure(41)(46). Another example is suppression of dynorphin expression which contribute to the enhancement of reward(45)(46) .

4.1.6.1 Role of the BDNF-TrkB signalling in dopaminergic neurons exposed to addictive drugs

Another dopamine-dependent change in protein synthesis that appears particularly important in establishing physiological as well as drug-induced neuroplasticity is a rise in brain-derived neurotrophic factor (BDNF)(45). Neurotrophic factors like BDNF that bind to the tropomyosin-related kinase B (TrkB) receptor were shown to be important in the development of the central nervous system and in shaping neuronal morphology of dopamine neurons and other brain circuits(48).

BDNF expression can be increased by psychostimulants in VTA dopaminergic neurons. These increases consolidate and persist over time during abstinence and during extinction of drug self-administration and in craving incubation paradigms(48).

The main intracellular pathways activated by BDNF-TrkB signalling are the MEK-ERK or MAPK-ERK, the PI3K-Akt-mTORC1, the PLCγ-DAG-PKC/Ca^{2+}, and NFkB pathways, all involved in cell survival and growth(49). These pathways are not only activated by BDNF, but also by G-protein coupled receptors, as described above. Collo et al., (2013) demonstrated that phosphorylation in both MEK-ERK and Akt-mTORC1 pathways is critical for structural plasticity, because pretreatments with selective inhibitors for ERK, PI3K and mTORC1 block the increase of soma size and dendritic arborization produced by psychostimulants(50).

Structural plasticity includes hiperplastic and hypoplastic phenomena, which mean, the increase or decrease of number and size of morphologically defined components of the neuron. Both hyperplastic and hypoplastic phenomena have been produced in the brain reward circuit by drugs of abuse. Psychostimulants are associated with hyperplastic phenomena, whereas opiates with hypoplastic(48). Morphine, for example, reduces BDNF expression in VTA neurons and low BDNF levels are associated with reduced soma size. Moreover, after local infusion with BDNF, normalizes soma size(49). In contrast, stimulants increase dendritic branching and spine number in VTA dopamine neurons and NAc medium spiny neurons. This structural plasticity is associated with activation of ERK in both regions and with activation of PLCγ in the NAc(49).

4.1.7 Neurobiology, animal behavioral paradigms

Various animal behavioral paradigms have been used to study the neuronal substrates involved in addiction, especially euphoria and rewarding effects. Self-stimulation, self-administration and conditioned place preference are examples of animal behavioral paradigms(31). For some drugs, such as synthetic cathinones, the risk of abuse in humans can be predicted using tests based on behavioral paradigms in animals.

4.1.7.1 Intracranial self-stimulation (ICSS)

Intracranial self-stimulation is one family of experimental procedures that has been used to assess abuse liability of stimulants and other drugs. In ICSS, subjects are trained to lever press for pulses of brain stimulation delivered via microelectrodes implanted in brain regions such as VTA, VTA's projections to NAc, prefrontal cortex, and hypothalamus. ICSS produces a positive reward when a threshold of stimulation is achieved(35).

The animal, after having learnt that when it presses a lever a positive reward's effect is produced, will continue pressing the lever looking for the rewarding effects. However, ICSS can be modified with administration of drugs of abuse that are expected to produce a positive reinforcement. When a drug of abuse that produces

positive reinforcement is administered, the number of lever's taps will be reduced because there is a reduction in stimulation's threshold. The animal has to press less times the lever to achieve the same positive reward's effects due to the drug.

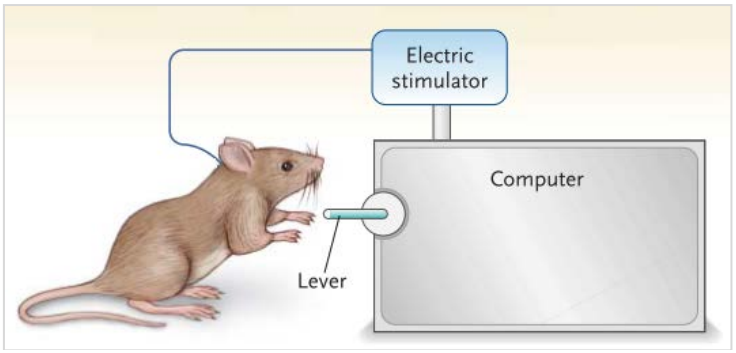

Figure 6. Representation of ICSS paradigm. Animals are trained to press a lever to receive intracranial current in brain-rewarding loci(31).

So, the paradigm consists on administrating a drug of abuse to a subject, and observing if the threshold of stimulation is modified or not. If the threshold is reduced, it will indicate that the drug is a positive reinforcer.

4.1.7.2 Intravenous self-administration (IVSA)

This paradigm consists in conditioning an animal to self-administrate a drug of abuse intravenously with a catheter when a lever is pressed. The catheter is placed into the jugular vein and is connected to an infusion pump which has the drug's solution: when the animal presses the lever, a switch of a programme, which is connected to the pump and regulates the drug's flow, is activated and the drug administrated. There are different kinds of administration, being the intermittent, which means that the drug is only administrated after a particular number of taps on the lever, the most common(35).

This test is conducted in a specific chamber equipped with two levers located on one wall and a food pellet receptacle placed between them. At the beginning of the test procedures, animals have restricted diet and they have to learn that pressing one lever, a sucrose pellet is delivered. During this training, the other lever is inactive. Approximately 24 hours after sucrose training, acquisition phase in "fixed ratio" (FR1)

begins. Fixed ratio means that the animal has to press only once the lever to get the drug's dose or the sucrose pellet. This phase consists on daily sessions of 2 hours self-administration. In this case, a particular volume of drug of abuse is administrated through the catheter when the other lever, not the one which delivers sucrose pellet, is pressed(26).

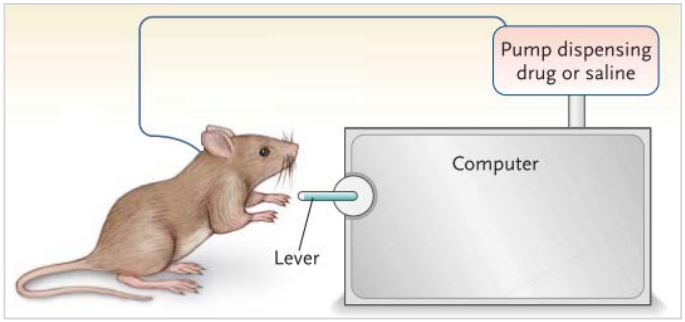

Figure 7. Representation of IVSA. Animals are trained to press a lever to obtain a drug or saline administration(31).

Following ten days of 2 hours IVSA sessions, a progressive ratio (PR) schedule is conducted to study the reinforcement efficacy of the drug. During the PR tests, the number of lever presses required to get a single infusion is determined by an equation described by Richardson and Roberts, 1996 (26): responses per reinforce delivery = $5 \times e^{(\text{injection number} - 0.2)} - 5$. In PR experiments, the break point or final point is also valued. This point is a reflex of the motivation generated by the addictive substance. The break point is considered to be achieved when animals do not emit any lever presses for 2 hours.

4.1.7.3 Conditioned place preference (CPP)

The conditioned place preference paradigm is a standard preclinical behavioral model used to study the rewarding and aversive effects of drugs. The basic characteristic of this test is the association of a particular environment with drug treatment, followed by the association of a different environment with the absence of the drug(51).

A large number of designs and apparatuses are used in this model, but a common variation consists of a three-compartment chamber with the outer compartments designed to have different characteristics. To achieve this difference,

walls can be painted with different colours, black vs white, flooring can be different as well, horizontal grid vs. cross-grid.

The central compartment has not any special characteristics and lets the animal move from one compartment to the other one; is like a corridor. This compartment is not paired with the drug. Moreover, between compartments there are gates which can be opened or closed in order to allow the animal to pass freely or to hold the animal in a specific compartment.

The paradigm consists of a training phase and a test phase. During the training phase, an injection of a drug with rewarding or aversive properties is administered to an animal and then is placed into one of the outer compartments for several minutes. On the following day, the animals are injected with the drug's vehicle and placed into the opposite compartment. Usually, these daily sessions alternate between drug and vehicle for 2 or 3 days each.

After training phase, the test session is conducted. In this session all gates are opened so the animal can move freely around all the apparatuses. This session consists of placing the animal in the middle of the central compartment and recording the time the animal spends in each compartment during the session.

A conditioned place preference (CPP) is found if the animal spends more time in the drug-paired compartment versus the vehicle-paired compartment. Contrarily, if the animal spends more time in the vehicle-paired compartment versus the drug-paired compartment, then is considered a conditioned place aversion (CPA).

As described, the conditioned place preference and self-administration paradigms measure the rewarding properties of drugs. However, some differences exist between them. First, although CPP and IVSA are sensitive to the rewarding effects of many of the same drugs, some drugs produce CPP but may not be self-administered, while others are self-administered but do not produce CPP. Second, the mechanisms that mediate drug-induced CPP and self-administration of a drug may be different. Third, there is an important difference between these two models in methodological procedures; IVSA requires surgical implantation of a catheter. Finally, IVSA studies

have been conducted in monkeys, rats, mice, and pigeons, whereas CPP studies have only been conducted in rats and mice(51).

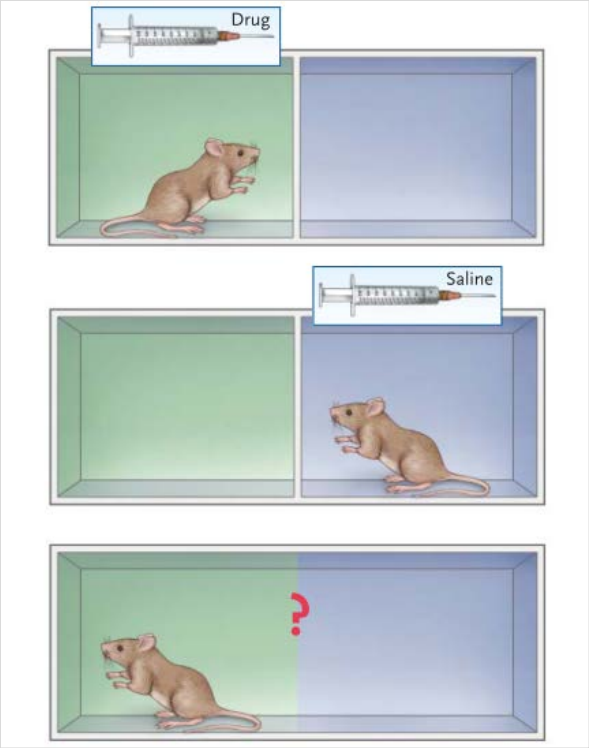

Figure 8. Representation of CPP. A positively reinforcing effect of the drug is apparent if the mouse spends more time in the environment in which the drug was administered(31).

4.2 Results from experimental procedure

4.2.1 3,4-methylenedioxyprovalerone (MDPV) and Conditioned Place Preference

The CPP paradigm was used to study the effect of different doses on the rewarding properties of MDPV.

During pre-conditioning phase rats were placed in the middle of the corridor and were allowed free access to the entire apparatus for 20 minutes. The relative time spent in each compartment was calculated. Rats that spent more than 60 % of the time in one compartment, so less than 40% in the other one were excluded from test.

Time spent in each compartment was recorded during preconditioning phase and in test. The preference score was calculated by subtracting time in the drug-paired compartment during the pretest from time spent in the drug-paired compartment during the test. Means ± SEM of relative time in drug-paired compartment are shown in **Table 1**.

Treatment	Relative Time Pretest (PT)	Relative Time Test (T)	Preference Score (T-PT)
Saline	650,26 ± 36,27	543,84 ± 76,94	-176,06 ± 60,61
MDPV 1mg/kg	567,35 ± 62,03	727,97 ± 46,70	160,62 ± 23,18
MDPV 3mg/kg	577,82 ± 32,77	785,46 ± 36,15	207,64 ± 57,89
MDPV 5mg/kg	590,32 ± 34,29	735,27 ± 63,70	144,94 ± 66,55

Table 1. Relative time spent in drug-paired compartment during the pretest and during the test. The preference score is also shown. Values are means ± S.E.M.

Significant treatment effect was found between groups when the preference score was compared [$F_{3,24}$ = 10,28 ; p < 0,001]. *Post hoc* analysis revealed that 1, 3 and 5 mg/kg MDPV showed a significant place preference when compared with saline-treatment controls. These results are shown below **(Fig. 9)**.

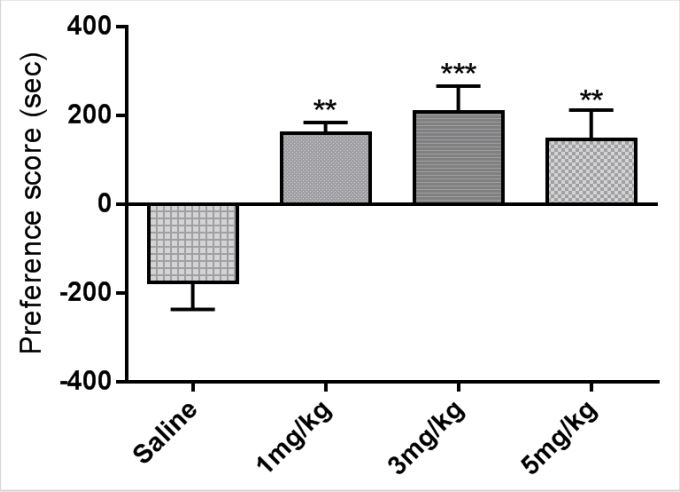

Figure 9. Effects of different doses of MDPV in the CPP assay in rats. Data are expressed as preference score. Values are means ± S.E.M. **p<0,01 ***p<0,001 compared to saline; n=7 in all groups.

5. Discussion

Drug addiction is a chronically relapsing disorder that has been characterized by compulsion to seek and take the drug, loss of control in limiting intake, and emergence of a negative emotional state, such as dysphoria, anxiety, reflecting a motivational withdrawal syndrome when access to the drug is prevented(52). Drug use is initiated primarily to obtain the excitatory actions of addictive drugs on brain reward system(53).

Drug addiction has aspects of both impulse control disorders and compulsive disorders. First ones are characterized by an increasing sense of tension or arousal before committing an impulsive act and are largely associated with positive reinforcement mechanisms. On the other hand, compulsive disorders are characterized by anxiety and stress before committing a compulsive repetitive behavior and are largely associated with negative reinforcement mechanisms and automaticity. Therefore, collapsing the cycle of impulsivity and compulsivity results on a composite addiction cycle composed of three stages: binge/intoxication, withdrawal/negative effects, preoccupation/anticipation, in which impulsivity usually dominates at the early stages and impulsivity combined with compulsivity at the later stages(52).

The development of addiction involves a transition from casual, in fact drug use is initiated primarily to obtain the excitatory actions of addictive drugs on brain reward system, to compulsive patterns of drug use. This transition to addiction is accompanied by many drug-induced changes in the brain and associated changes in pshychological functions(33).

Most of these drug-induced changes, cellular and molecular, have been shown throughout diverse neuronal populations within the brain reward circuit. For example, the transcription factor CREB has been implicated in addiction. Activation of CREB in the nucleus accumbens and several other regions by drugs of abuse mediates certain aspects of drug addiction(44). Some other genes, are transcripted as a result of CREB

binding to DNA, as *ΔFosB, Cdk5, GluR2, dynorphin and Bdnf*, whose proteins ultimate the adaptation to drug exposition.

As described above, ICSS, IVSA and CPP are the most common animal behavioral paradigms used to study the abuse potential of a substance. Watterson et al., (2012) evaluated the abuse potential of MDPV by assessing its ability to support IVSA and to lower thresholds for ICSS in rats(26).

They chose IVSA method due to the high degree correspondence between drugs that can have addictive potential in humans and drugs that function as reinforcers in IVSA procedures in animals. Their study revealed a positive relationship between MDPV doses and reinforce efficacy. This relationship was demonstrated during PR testing, and breakpoints for MDPV reinforcement at the lowest dose (0,1mg/kg) tested were similar to those for the same those of methamphetamine, a drug known to exhibit addictive properties.

When a substance produces lowering of ICSS threshold is generally accepted to be due to the facilitation of brain reward functioning, providing a direct measure of the hedonic and rewarding properties of drugs of abuse. In addition, nearly all abused stimulants including cocaine, amphetamine and methamphetamine lower ICSS threshold. Watterson et al., (2012) results revealed that MDPV lowers ICSS threshold across a wide range of doses compared with the vehicle.

To our knowledge, there were not published any results of which were the effects of MDPV on conditioned place preference in rats. Karlsson et al., (2014) demonstrated that MDPV produce a significant preference shift at all doses tested (0.5, 2, 5, 10, 20 mg/kg) compared with the control mice(27). In fact, their CPP data showed that MDPV is more or equally potent than amphetamine in their tested interval.

Our results revealed that MDPV induces CPP at all doses tested (1, 3, 5 mg/kg) compared with the control rats. Doses tested are very low compared to those used with cocaine (20 mg/kg) to achieve same effects. In addition, there is no linear

relationship between dose and effect, but it is known the lack of a CPP dose-response function for a lot of reinforcers, such as cocaine.

So, taken together these results it is suggested that MDPV possesses a strong potential for compulsive use and can thereby be a risk of abuse in humans.

6. Conclusions

- Drug addiction is a chronic, relapsing disorder in which compulsive drug-seeking and drug-taking behavior persists despite serious negative consequences.
- Each drug of abuse, despite their many distinct actions in the brain, converges in producing some common actions. Prominent among these actions is the activation of mesolimbic dopamine system also known as the rewarding circuit.
- The development of addiction involves a transition from casual to compulsive patterns of use. After repeated exposure to a drug of abuse, molecular and cellular changes are progressively being induced.
- There are two transcription factors, in particular, that have been implicated in addiction: CREB and ΔFosB.
- ICSS, IVSA and CPP are three of the most common animal behavioral paradigms used to study the abuse potential of a substance and its rewarding properties.
- MDPV induces CPP at lower doses compared to cocaine. So, MDPV addictive properties are higher than cocaine's.
- MDPV as an example of synthetic cathinones possesses a strong potential for compulsive use and can be a risk of abuse in humans. MDPV is an amphetamine's derivative and is currently under legal control in some states.

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