Department of Physiology and Pharmacology "Vittorio Erspamer"

Ph.D. Program in Pharmacology,

XXVII cycle

In vivo and *in vitro* characterization of a new recreational drug: Benzydamine

*A Dissertation Submitted in Fulfilment of the Requirements for
the Degree of Doctor of Philosophy in Pharmacology*

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Academic year 2013/2014

Content

Chapter 1
Introduction

D rugs misuse is a complex phenomenon that does not appear to have declined significantly in last decades, despite great efforts in terms of repression and therapeutic approaches. Interestingly, the Internet is now playing a key role in shaping how drugs are sold, changing the classic classification of new versus old drugs. Indeed, a growing number of new drugs are now available on the internet drug market, including prescription drugs with psychoactive properties that are easier to obtain than illegal drugs. Among the prescription drugs whose recreational use is on the rise there is Benzydamine (BZY), a non-steroidal anti-inflammatory drug also abused as a *club drug*. BZY abuse was first identified in Brazilian teenagers, and then spread to Poland and Romania, and other European countries (Opaleye et al, 2009; Mota et al, 2010; Babalan et al, 2013; Doksat et al, 2009; Settimi et al, 2012). Today, BZY abuse has become the object of increasing concern in public health also in Italy. It is used because, when taken systemically at high doses, it produces euphoria, excitation, hallucinations, and delirium. According to online forums, BZY causes a long-lasting 'brain-flying' effect similar to LSD. It seems that BZY is often taken in conjunction with tetrahydrocannabinol (THC) and alcohol to obtain an amplified effect. The main goal of the present dissertation is to characterize the reinforcing properties of BZY using the intravenous drug self-administration in the rat and then to investigate the ability of BZY to induce neuroplastic changes in the cortico-accumbens glutamatergic synapses, using slice electrophysiology.

The question is frequently asked: Why does a man become a drug addict? The answer is that he usually does not intend to become an addict but he usually cannot choose to change his behavior. In *Novel with Cocaine*, a mysterious Russian novel first published in 1934 in a *Parisian èmigrè* publications, Mark Levi provides a disturbing account of a young man becoming addicted to cocaine; Vadim, the main character reflects: "*Before I came in contact with cocaine I assumed that happiness was an entity, while in fact all human happiness consists*

of a clever fusion of two elements: (1) the physical feeling of happiness, and (2) the external event providing the psychic impetus for that feeling". This statement highlights the core element of drug addiction. A neutral non-reinforcing stimulus paired repeatedly with drugs use can acquire incentive salience and eventually, after a period of abstinence, it may trigger again drug craving and cause the relapse of the behavior (Stewart et al, 1984; Childress et al, 1988).

To consider a patient dependent of drugs, the American Psychiatry Association requires he to meet at least three of the seven criteria: tolerance and physical dependence reflect physiological habituation to the effects of a drug, whereas other aspects as withdrawal, used larger amounts/longer, repeated attempts to quit/control use, much time spent using, activities given up to use and physical/psychological problems related to use, define the uncontrollable drug abuse.

The human reward system is an extraordinary complex of billions of neurons that guide the behavior toward particular positive objectives, such as eating and procreation. Drug can integrate itself into these crucial mechanisms adapted for our survival, driving compulsive drug-seeking and drug-taking behavior, despite negative consequences. Continued use of drugs induces adaptive changes in the central nervous system that lead to tolerance, physical dependence, sensitization, craving and relapse. Since long time, it has been tried to understand the neuropharmacological adaptation mediating the development and persistence of substance dependence. Early theories of addiction assumed that initial taking drug was driven by the positive reinforcing effects that it produces, but later is the recurrent drive for reward to determine the dependence disorder (Wise, 1980). This idea of positive reinforcement by drugs of abuse has been widely seen as a primary factor behind drug addiction (Gill et al, 1988). However, all drugs, after prolonged use produce the phenomenon of tolerance that lead to compensatory increases of dose and this can exacerbate the neurophysiological alterations in the brain. However, some drugs, such as psychostimulants, can produce an opposing response of tolerance, named sensitization that is an increase in the effects produce by drugs after a prolonged consume (Kalivas & Stewart, 1991; Anagnostaras & Robinson, 1996). Positive and negative reinforcement theories result suitable to explain the development and the maintenance of substances abuse, but they are not able to clarify more complex aspects of the phenomenon of

addiction, as the relapse phenomenon after a period of withdrawal. Currently, three theories are considered more prominent because have endeavored to clarify the complex aspects of the addiction disorder by considering all series of neuroadaptations that are consequence of prolonged abuse. We are talking about the hedonic allostasis theory of Koob and Le Moal, the incentive-sensitization theory of Robinson and Berridge and, finally, the ultimate theories relative to maladaptive learning processes and orbitofrontal cortex impairments in decision making processes.

In their theory, koob and Le Moal suggested that repeated drugs use causes subsequent neuroadaptations that lead to the pathological shift of the drug user's hedonic set point for the efficacy of reinforces and thus add motivational effectiveness to positive drug effects. Allostasis refers to the concept of physiology where an organism must vary all of the parameters of its internal milieu to maintain a state of equilibrium. Drugs produce disequilibrium in brain reward systems for which the individual allostatic processes, or the ability to achieve stability through change, can maintain an apparent stability at a new pathological 'set point'. When the state of dysregulation reached is critical, therefore the organism is not more able to mobilize its own resources, allostasis reaches that point in which normal is considered illness. The state of dysregulation involving the reward system can produce loss of control over drug intake, compulsive use and drug addiction (Koob & Le Moal, 1997; Koob & Le Moal, 2001; Koob & Le Moal, 2005).

Alternatively, Robinson and Berridge argued that drug-seeking and drug-taking behavior are not often motivated by the desire to obtain pleasure or the desire to relieve withdrawal. They suggest that addictive drugs regularly produce long-lasting neuroadaptations in the structures involved in the process of incentive motivation and reward, rendering these hypersensitive ('sensitized'). Notably, they supposed that the brain structures sensitized by the prolonged use of drugs do not directly mediate the euphoric effects of substances (drugs liking), but instead they mediate a component of reward called incentive salience or drugs wanting (Berridge, Venier & Robinson, 1989; Berridge & Valenstein, 1991; Robinson & Berridge, 1993, 2000; Berridge & Robinson, 1995, 1998; Berridge 1996).

More recently, multiple theories attempted to unveil the aspects of drug-induced neuroadaptations that may specifically explain the persistent nature of addictive behaviors: some authors sustain that the transition between the voluntary drug use

to habitual and compulsive consume is caused by the transition, at the neural level, from prefrontal cortical to striatal control (Wise, 2002; Everitt & Robbins, 2005; Volkom et al, 2006); others, support the hypothesis that the changes in the prefrontal cortical activity lead the impairment in behavioral control and decision-making skills (Jentsch & Taylor, 1999; Franklin et al, 2000, 2002; Goldstein & Volkow, 2002) and others Authors consider the overlaps between limbic and cortical area involved in addiction and memory (Hyman & Malenka, 2001) so the behavioral disorders are considered the result in maladaptive associative learning (Di Chiara, 1999).

Humans have used drugs for thousands of years: wine, narcotics, marijuana. But in the 19th century the active substances in drugs were extracted and the use of the so discovered drugs, cocaine, morphine, laudanum, became out of control. By the early 1900s there were an estimated 250˙000 addicts in the United States (Brecher, 1972). The problems of drugs addiction were recognized late, in fact the first national drug law in the United States was in the 1906. Throughout the years, the public's perception of the dangers of specific substances changed. Today, the United States, estimates of the total overall costs of substance abuse exceed $600 billion annually while in the European Union over 80 million adults are estimated to have used alcohol, nicotine, and illicit and prescription drug almost at least once in their life. 73.6 million adults have used cannabis, 141 million have used cocaine, 11.4 million amphetamine and 10,6 million ecstasy. Of this, one-third of adults in Denmark, France and United Kingdom and less than one in 10 in Bulgaria, Greece, Cyprus, Hungary, Portugal, Romania and Turkey. In the most recent data, from 2011, one in four 15- to 16- years old is estimated to have ever used an illicit drug, although prevalence levels vary considerably between countries (European Drug Report, trends and developments, 2014).

During 2013, 81 new psychoactive substances were identified, 29 of which were synthetic cannabinoids, and other 30 compounds did not conform to the readily recognized chemical groups. There were also 13 new substituted phenethylamines reported, seven synthetic cathinones, a tryptamine and a piperazine (European Drug Report, trends and developments, 2014).

In the last few years there has been an incredible change in the context of substances misuse. The revolution, and the consequently expansion, of the drugs

market could be mainly attributed to the new development in the distribution and communication of the recreational substances, probably due to the widespread of drug user communities and forums online in which people share their drug experience. Internet has played a major role in changing the trends of the global

Picture 1 Recreational use of prescription drugs is became a serious problem. National studies show that a teen is more likely to have abused a prescription drug than an illegal street drug. These because many people think prescription drugs are safe because they were prescribed by a doctor. [http://www.troymedia.com/2014/10/02/a-2-step-solution-to-reduce-the-cost-of-prescription-drugs-for-canadians/].

drugs market and today it continues to play a key role in shaping the phenomenon. The increased opportunities to share information online have made available to users, including young and vulnerable people, new pharmaceuticals and research chemicals. Although even now the main drugs used are opioid, psychostimulant and benzodiazepine, anticholinergic effects caused by painkillers, fever reducers and non-steroidal anti-inflammatory drugs (NSAID), such as delirium and hallucinations, support their use for recreational reasons.

Non-medical use of the prescription drugs is an alarming public health problem, because a large amount of pharmaceuticals are taken nowadays not to treat the disease, but to improve some aspect of life and manage ordinary states, mood, sexual performing, body weight and cognitive ability, or, simply, for recreational reasons. This phenomenon is particularly severe among the student, because prescription drugs are widely available, off label prescribing, easy to acquire and are associated with fewer physical, legal and social risks, especially in comparison to illicit street drugs, (Quintero, 2009).

The diffusion of always new substances, sometimes starting with experimentations in close settings, is now a global phenomenon that changes continuously and rapidly, so the available scientific literature is not able to gain sufficient technical

knowledge at the same pace. This is the reason why the European "Psychonaut web mapping project" was born, to monitor the web looking for new drugs, to develop a technical database.

Picture 2 *Psychonaut Web Mapping Project Logo. The Psychonaut Web Mapping Project is a 2-year European Union funded project with the aim of developing a web scanning system to identify and categorize novel recreational drugs/psychoactive compounds, and new trends in drug use based on information available on the Internet. [http://www.psychonautproject.eu/].*

In 2012, the project has led to identification of 412 new substances, classified in different categories:

- Herbal: for example, Salvia Divinorum, a legal hallucinogenic plant.

- Chemicals, such as mephedrone, that is a legal alternative to other stimulants, purchased online as 'plant feeder'.

- Combinations: Spice drugs and synthetic cannabinoid (as JWH-018) are an example.

- Pharmaceutical: BZY, the object of this work, is one example. It is a non-steroidal anti-inflammatory drug (NSAID) abused recreationally for its euphoric and hallucinogenic effects at high doses (500-2000mg).

Chapter 2
Epidemiology

In recent years, abuse of BZY has been popular among teenagers and young adolescents, in Brazil, Romania and Poland but misuse cases are reported for Italy as well.

Data on the adverse effects of BZY are scarce. In 1975 for the first time, Eggers have described few cases of intoxication of BZY in some children, (Eggers et al, 1975). Recently, Gómez-López mentions a case of intoxication in a six-year-old girl who accidentally ingested 500 mg of BZY, suffering visual and tactile hallucinations: she was seeing and feeling flies, worms and birds travelling through her body, biting her and laying eggs. Hallucinations were progressively decreasing and disappeared in 3 hours after hospitalization, (Gómez-López et al,1990). In 2006, Dogan et al, reported another case of BZY poisoning in an 11 years old girl who presented somnolence and visual hallucinations.

Available scientific data about the recreational use of BZY psychoactive proprieties are pretty recent: a first description of the recreational use of this drug was mentioned in a survey with street youth, carried out in 1993. In the same year, a paper was published in which the Authors describe a case report of a 20-years-old man who, using between 400 and 1000 mg of BZY associated with alcohol, described visual alterations and hallucinations after 30 minutes, (Saldanha et al, 1993).

The use of BZY as a club drug is very popular, especially among teenagers, in Brazil, where it is mainly dispensed as tablets (50 mg) and a solution for oral use (3m mg/ml), sold with the commercial name 'benflogin' (a 20 ml bottle costs $3 and 20 tablets cost $4). These drugs can be bought in Brazilian drug stores without the need for a medical prescription. Opaleye et al, (2009) published a cross sectional survey with children and adolescents, between 10 and 18 years old, in street situation, living in 27 Brazilian state capitals. Among the 2807 interviewed, 78 reported having used BZY recreationally at least once in their lifetime; they described a dosage use ranged from 100 and 2000mg, in oral preparation. Some of them reported that they had associated this drug with another substance,

Picture 3 *Trade name with which BZY Hydrochloride is sold in Brazil. Each box of Benflogin contains 20 pills, 50 mg each. Benflogin is also the name used for weekly youth rave party in Rio de Janeiro. [http://anti-inflamatorios.net/anti-inflamatorio-benflogin-beneficios-efeitos-e-mais/].*

commonly alcohol. All of them mentioned the substances by its trade name "Benflogin", which appears as the name of a weekly youth party in Rio de Janeiro that takes place in a privileged economic area, with the slogan "*Shoo boredom now*". Another example is the use of the drug name as title of songs by certain Brazilian bands. Mota et al, in 2010 have published an exploratory essay to present information on the abuse of BZY, evidenced in scientific literature, press releases and on Internet, highlighting the need to strengthen pharmaco-vigilance in Brazil.

BZY preparations are sold over-the-counter in many countries and misuse reports are increasing. It is known that the phenomenon is expanding in Poland, Romania, Turkey, Spain and also Italy.

In 2007, Anand et al. described a case involving a 22 years old polish man with a history of cannabinoid and alcohol abuse who was hospitalized for ingesting 500 mg of BZY, after she found information about its hallucinogenic effects on the internet.

In 2013, it was reported a first case in the Turkish literature about a 22 years old Turkish man hospitalized after taken a high dose of BZY (750 mg) with 500 ml of beer (Babalan et al, 2013).

Normal dose of BZY may cause psychiatric side effects in patients with psychiatric disorders, and overdose may result in chronic psychosis. In 2009, it was reported a case of a 22 year old schizoaffective patient, who had been almost symptom-free for three years. After a leg injury, she ingested 50 mg of BZY. After two days, she had an episode of acute psychosis, with hallucinations, paranoia and dysphoria. The most likely explanation of this case is that BZY exacerbated the psychotic symptoms. After 5 days of withdrawal from BZY, she returned symptom-free (Doksat et al, 2009).

Picture 4 Tantum Rosa is the Italian brand of BZY Hydrochloride. Each sachet contains 500 mg of the active principle. [http://comefare.com/come-usare-il-tantum-rosa/].

From 1977 to 2009, BZY was marketed in Italy by Angelini (with the commercial name of Tantum Rosa), as a pharmacy-supervised drug, that is, a non-prescription drug for which advertising to the public is prohibited. In 2009, Tantum Rosa was re-classified as over-the-counter drugs and advertising to the public was allowed. Unfortunately, one of the advertisements was illustrated by the silhouette of a woman with concentric circles on the abdomen, which may explain at least in part the increase in the number of erroneous oral ingestions of the drug (Settimi et al, 2012).

In the last few years also in Italy some case of recreational use of BZY have been reported. For example, the latest annual report on drug addiction to the Italian Parliament, includes a case report of a 34 year old girl, with a history of multiple substances abuse, hospitalized in Florence after oral ingestion, for recreational reasons, of 500 mg of Tantum Rosa. Another case report concerned a 22 year old man, with a history of cannabis and alcohol abuse, who, after reading on the internet about the psychoactive properties of BZY, decided to try it. (Relazione Serpelloni, 2012).
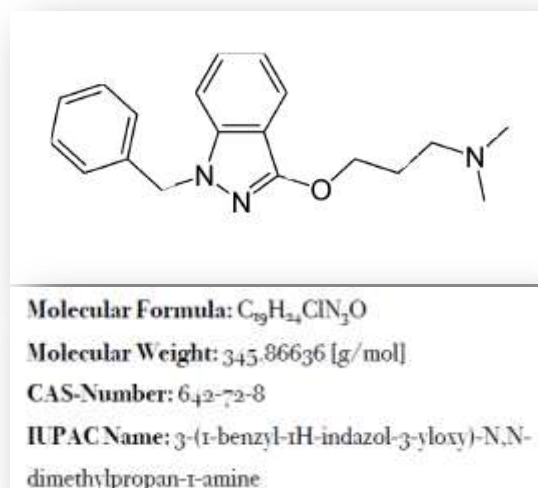
Chapter 3
Pharmacology

Benzydamine [3-(1-Benzyl-1H-indazol-3-yloxy)-N,N-dimethylpropan-1-mine], available as the hydrochloride form, is a non-steroidal anti-inflammatory drug (NSAID) synthesized in Italy by the Angelini research Laboratories in 1964 and marketed in 1966. it has analgesic, anti-inflammatory, antipyretic and antimicrobial properties, providing rapid and extended pain relief. BZY preparations are marketed throughout the world for

Molecular Formula: $C_{19}H_{24}ClN_3O$

Molecular Weight: 345.86636 [g/mol]

CAS-Number: 642-72-8

IUPAC Name: 3-(1-benzyl-1H-indazol-3-yloxy)-N,N-dimethylpropan-1-amine

Picture 5 *Molecular 2D structure of BZY, a benzyl-indazole with analgesic, antipyretic and anti-inflammatory effects. [Benzydamine report; Psychonaut Web Mapping Project].*

the symptomatic treatment of oropharyngeal and gynecological conditions, as a mouthwash, aerosol, dermal cream, vaginal douche, pill, and eye drop. Used in odontostomatology to treat gingivitis, stomatitis, glossitis, aphthous ulcers, dental surgery and oral ulceration due to radiation therapy. In otorhinolaryngology, BZY is used for pharyngitis, tonsillitis, post-tonsillectomy, radiation or intubation mucositis. (Gomez-Lopez et al. 1999).

BZY is available as mouthwash (Tantum Verde) throughout Europe. In the UK it is sold under the trade name Difflam, as Difflam spray, Difflam oral rinse and Difflam cream (Doksat, 2009).

Picture 6 *List of Brands with which BZY hydrochloride is sold in some Countries. [BZY report; Psychonaut Web Mapping Project].*

Country	Proprietary preparations	Multi-ingredient preparations
Austria	Tantum	—
Australia	Difflam	Multi Anti-Inflammatory Cough Lozenges Difflam Dental Difflam-C
Canada	Tantum	—
France	Opalgyne	Hexo-Imotryl
Germany	Tantum	Tantum biotic
Italy	Afloben	Algolisina
	Benzirin	Leucorsan
	Flogaton	
	Ginesal	
	Multum	
	Saniflor Dentifricio	
	Saniflor Vena	
	Tantum	
	Verax	
Netherlands	Tantum	—
South Africa	Andolex	—
	Tantum	
Spain	Fulgium	Bristaciclina Dental
	Rosalgin	Dolosarto
	Tantum	Mentamida
		Prosturol
		Tantum Ciclina
		Viciseptil Otico
Switzerland	Bucco-Tantum	—
	Rhino-Tantum	
	Tantum	
United Kingdom	Difflam	—

Picture 7 *Proprietary and multi-ingredient names of Benzydamine preparations and the countries where they are marketed. [Benzydamine report; Psychonaut Web Mapping Project].*

In Australia it is available as Difflam-C-Alcohol & color free solution, Difflam 3% Gel, Difflam Extra strength Gel 5%, Difflam-C-Solution, Difflam Solution (including Difflam throat spray), Difflam Cream and Difflam Lozenges (Doksat, 2009). BZY is also sold in Eastern Europe, without prescription named Tantum Rosa – as vaginal antiseptic and anti-inflammatory preparation, containing 0.14 g of BZY hydrochloride to be reconstituted with clean water to a 0.1% (1 mg/ml) solution for vaginal enema/instillation.

In Brazil it is sold, by prescription, under the name 'benflogin', while in Pakistan under the name Tantum Capsule (50 mg). In Egypt, BZY is available in a cream and gel named Tantum Fort, while in Canada it is sold as a mouthwash named Novo-Benzydamine. In Turkey it is cheap and sold over-the-counter in all pharmacies as Tantum, Tanflex and Benzidan.

3.1 Chemistry

BZY is fluorescent (excitation 306 nm; emission 362 nm) and is detected fluorometrically in HPLC assays. This absorbance and fluorescence in the ultraviolet region may be associated with a possible pharmacological effect. It has been suggested that BZY and related compounds may reduce the formation of cataract due to absorbance in the UV region or by a molecular interaction with lens protein, reducing the UV damage (Jez JM et al, 1996).

3.2 Pharmacodynamics

BZY, unlike other non-steroidal anti-inflammatory drugs (NSAID), is not ulcerogenic and does not inhibit cyclooxygenase or lipoxygenase. Moreover it has different pharmacological activities consider those of other NSAID. In fact, it is a weak base, unlike aspirin-like non-steroidal anti-inflammatory drugs, (which are acids or are metabolized to acids) and is a weak inhibitor of the synthesis of prostaglandins.

Nevertheless, BZY has several properties which may contribute to its anti-inflammatory activities, because it reduces locally induced inflammation, edema and granuloma formation in animals and demonstrates antipyretic and anti-exudative activities. (White SK, 1988).

BZY has affinity for membrane, with membrane-stabilizing properties and has local anesthetic effects. It inhibits phagocyte degranulation and aggregation and production of reactive oxygen species by phagocytes and leukocyte adhesion to vascular endothelium. Moreover, it has also antithrombotic properties, reduces platelet activation factor (PAF) and inhibits tumour necrosis factor (TNF) properties and the synthesis of the inflammatory cytokine, (Anand et al, 2007).

Finally, BZY possesses also non-specific antibacterial activity at the concentrations used in mouthwashes, in fact, it exhibits activity against bacterial strains resistant to broad-spectrum antibiotics, such as ampicillin, chloramphenicol and tetracycline, at a concentration of 3 mmol/l. This property may prevent the development of secondary bacterial infections following viral-induced pharyngitis. (Quane et al, 1998).
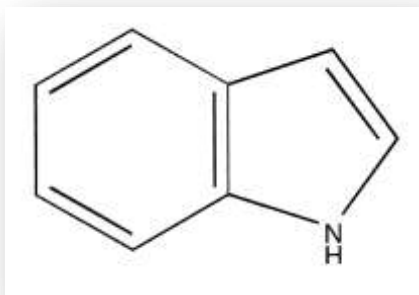
Picture 8 *Indole compound. It has a bicyclic structure, consisting of a six-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring. Molecular formula C_8H_7N; Molecular massa 117.15 g/mol. [Ref. wikipedia].*

When taken at high doses, BZY mimics the effects of the psychedelic compound, LSD, causing visual alterations and hallucinations. The exact mechanism of action for the hallucinations induced is unknown but all of these substances with psychedelic properties, including BZY, are structurally similar to the neurotransmitter Serotonin, because of the presence of the Indole compound (Doksat et al, 2009). Indole is an aromatic heterocyclic compound, consisting of a Benzene ring with six atoms of Carbon and a five-membered nitrogen-containing Pyrrole ring. Indole compound alone has not psychotropic effects, but it represents the central nucleus of different psychedelic substances, such as LSD, Psilocybin and Mescaline.
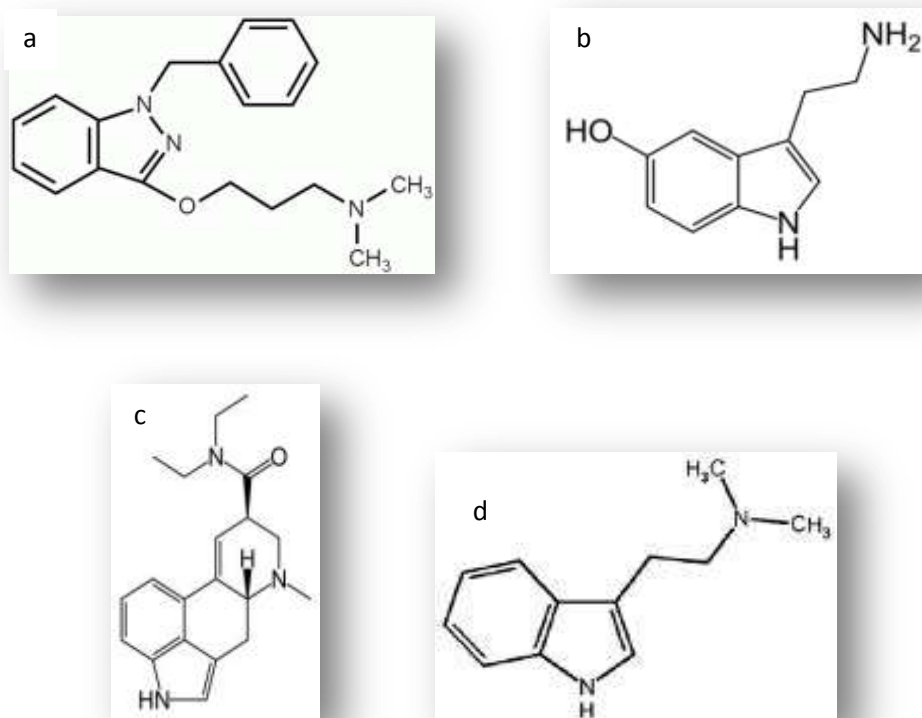
Picture 9 *Molecules contain the indole compound. a. BZY; b. Serotonine; c. LSD; d. Dimethiltriptamine. [Ref. Wikipedia].*

Serotonin is considered an essential neurotransmitter involved in many basic brain functions, linked to mood, sleep, appetite, regulation of the involuntary smooth muscles but also depression and anxiety. Serotonin is thought to exert its relaxing effects on brain by making neurons less likely to fire. It seems that psychedelics exert their effects by an agonist or partial agonist action at serotonin 5HT2A receptors. Early hypothesis support the involvement of 5HT2A receptors in the principal hallucinogenic action of some drugs was suggested by Glennon et al (1983c) based on the results of drug discrimination studies using rats, in which 5HT2A antagonists ketanserin and pirenperone blocked the discriminative stimulus effects of hallucinogens, including LSD. Moreover, it has been demonstrated that ketanserin abolishes virtually all of the psilocybin-induced subjective effects in humans (Vollemweider FX et al, 1998).

5HT2A is a trans-membrane G protein-coupled receptor (GPCRs) member of the 5HT2 receptor sub-family of serotonin, linked to the inositol phosphate (IP) signal transduction (Wainscott et al, 1993)., When activated, 5HT2A stimulates phospholipase C (PLC), which heads a downstream activation of protein kinase C (PKC) as well as an increased release of $Ca2+$ from intracellular stores. Using quantitative auto-radiographic analysis, it has been demonstrated a widespread distribution of 5HT2A receptors in several regions in the brain, including Olfactory Bulb, Nucleus Accumbens, Olfactory Tubercle and the Tractus Solitarius, although a large amount are located in the cerebral cortex (Lopez-Gimenez et al, 1997), as demonstrates the ubiquitous effects of hallucinogens on such complex processes, as cognition, perception and mood, all superior functions that involve the cerebral cortex. Moreover, various studies have demonstrated that activation of cortical 5HT2A receptors, using hallucinogenic drugs or serotonin, heads to a strong increase in glutamate-dependent activity in pyramidal neurons of the prefrontal cortex (PFC) (Aghajanian et al, 1999; Puig et al, 2003; Beique et al, 2007). At the beginning, nevertheless, it was thought that this increase in glutamatergic synaptic activity was produce by a stimulation of glutamatergic thalamo-cortical afferents to the PFC, which express 5HT2A receptors pre-synaptically. However, more recent studies have suggested the direct involvement of post-synaptic 5HT2A receptors on a subpopulation of pyramidal cells in the layer V of the PFC.

However, the biggest serotoninergic nucleus in the brain is the dorsal raphe nucleus, located in the midbrain. Notably, the dorsal raphe nucleus exerts

downstream effects on serotonergic and dopaminergic activity through descending projections that contact the ventral tegmental area (VTA), causing indirectly the activation of 5-HT2A receptors in the medial PFC (mPFC), with consequent increase in the firing rate of 5-HT neurons in the dorsal raphe and dopamine neurons in the VTA (Celada et al, 2001; Vazquez-Borsetti et al, 2009).

A recent study reported that the $5HT_{2C}$ receptor may be involved in the hallucinogenic process (Sanders-Bush and Breeding, 1991). The $5HT_{2A}$ and $5HT_{2C}$ receptors have similar molecular and pharmacological properties and have similar affinities for LSD (Teitler et al, 1987).

3.3 Pharmacokinetic and bioavailability

Commercially, BZY is available in different formulations (chapter 2) because it can be used orally or topically. Following topical application on skin or mucosa, the absorption is usually less than 10% of total dose and the bioavailability is of 5% with a peak of 0.05 µmol/l after 3 hours. Nevertheless cutaneous application produces slow and prolonged penetration through the tissue: after the application of 100 mg of BZY to human skin, it is reveal in plasma and urine (Andersson et al, 1974).

Differently, following oral administration of 50 mg of BZY hydrochloride in an aqueous solution, gastrointestinal absorption is very fast and almost complete; peak plasma concentrations of 1.5 µmol/l are obtained after 1 hour and half. Approximately 64% of the dose is absorbed in 1h, and complete absorption occurs in 4-6 hours. The high oral absorption is probably related to the high lipid solubility and relatively low systemic clearance (160 ml min-1) which is about one tenth of liver blood flow, while it has an high volume of distribution (110L). In the blood system benzydamine is relatively less likely to bind proteins than other non-steroid anti-inflammatory agents (15-20%), so after oral doses it reaches a very high bioavailability (87%) and the plasmatic half-life is 7-8 h. 55% of total dose is excreted unchanged in the urine while the 45% is metabolized by the liver (for oxidation, dealkylation and coniugation), in inactive metabolites, excreted both in the urine and the faeces. One metabolite, BZY N-oxide, is present in the plasma at a peak concentration of approximately 0.6 µmol/l (Quane et al, 1998).
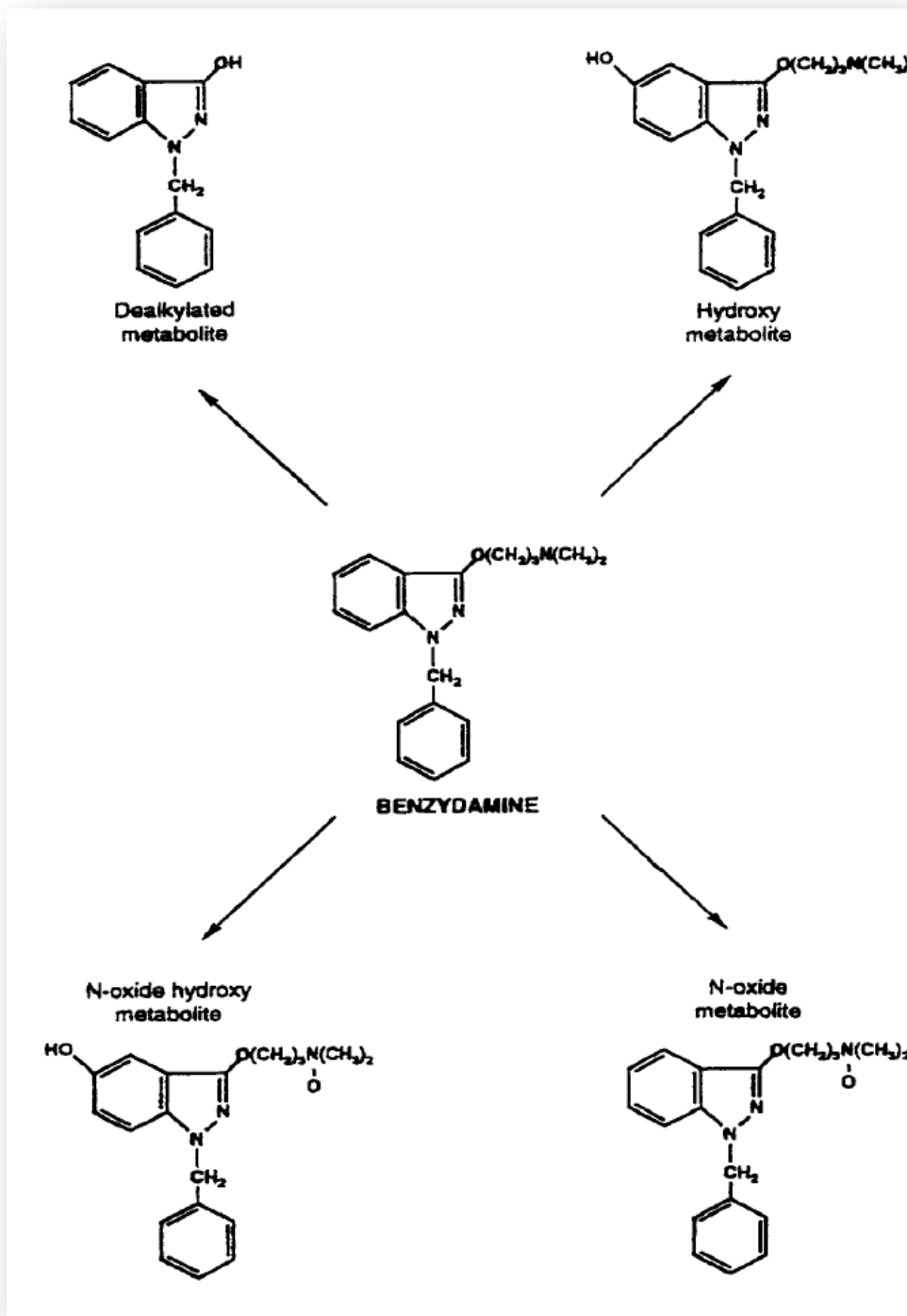
Picture 10 *Metabolites of BZY. The formation of the N-oxide hydroxy metabolite requires two metabolic processes, (Quane et al, 1998).*

3.4 Pharmacological and toxicological effects

Acute poisoning with NSAID is associated with a variety of features: hallucinations, headache, nystagmus, diplopia, tinnitus, dizziness, blurred vision, seizure, dyskinesia, lethargy, loss of consciousness and coma. Hypotension and tachycardia are sometimes present. Vomiting is an important early sign NSAIDs overdose, and abdominal pain, nausea and intestinal bleeding. Granulocytosis, pancytopenia and coagulopathy may also occur.

Recreational use is associated with different desired psychoactive effects: feeling of well-being, euphoria, excitation, disinhibition, talkativeness, feeling of weight loss; and at high doses, delirium and hallucinations (Balaban et al, 2013).

The use of hallucinogenic drugs, also BZY, can induce a variety of powerful perceptual symptoms including kaleidoscopic images, altered shapes and colors and trails of moving objects (Dubois & VanRullen, 2011). Hallucinogenic drugs are considered relatively harmless to self and others compared to heroin and cocaine (Nutt et al, 2010), however they may impair mental functions, causing psychosis (Geyer & Vollenweider, 2008) and sometimes the 'hallucinogen persisting perception disorder' (HPPD), a syndrome colloquially indicated as 'flashbacks' (Abraham and Aldridge, 1993; APA DSM-V 2013). An individual's perception can be severely altered by certain drugs, but effects slowly wear off as a result of its metabolism and excretion. In some individual, however, may undergo prolonged changes in their normal perception that persist for weeks to indefinite time after exposure (Abraham & Duffy, 1996; Lerner et al, 2000). HPPD patients have visual hallucinations in the form of trailing colours, the sensation that something is moving in the peripheral field of vision although there is nothing there, trailing phenomena - moving objects leave trails or after-images, positive after-images (an image that retains the original color), color flashes when lighting is low, colours of increased intensity, haloes surrounding objects, macropsia and micropsia (objects appear respectively larger or smaller than normal), (Erowid org, 2009; Halpern & Pope, 2003). A type of neuron that is possibly involved in the etiology of HPPD is the inhibitory cortical interneuron, which expresses 5-HT2A receptors and releases GABA upon activation (Abraham & Aldridge, 1993). Inhibitory mechanisms are important to suppress neuronal responses when the stimulus is no longer present

and a lack of lateral inhibition may decrease the ability to process edges and thereby result in the perception of halos.

3.5 Recreational use

BZY hydrochloride, an easily accessible and inexpensive NSAID, taken orally at high doses, 500 mg to 3000 mg, is a central nervous system stimulant, rather likely to be abused. It is considered by the users to be a potent substance that gives a bigger sense of reality, in an unpredictable way. The effects are similar to those of LSD, because both are hallucinogenic and are preferred by users to be taken at parties, with friends or a group of people. Frequently, BZY is taken in combinations with other drugs, cannabis, alcohol, Dextromethorphan and amphetamine. BZY is taken principally orally, in the form of tablets or dissolving the content of the sachets (TANTUM® Rosa) in sugar water o in juices for cover the salty taste. Only in rare cases, the content of the sachets is snorted.

A person who takes BZY at high doses may experience several effects, as feeling of well-being, euphoria hallucinate, paranoia, dry mouth and sometimes convulsions. The trip can last up to 8 hours, after that the user becomes tired and quiet, but sleeping is almost impossible.

A psychedelic experience regards the perception of aspects of mind and consciousness previously unknown, the creative exuberance of the mind liberated from its ordinary bondage. Psychedelic states represent an array of experiences, changes of perception, synesthesia, altered states of awareness, mystical states, and occasionally states resembling psychosis, elicited by sensory deprivation as well as by psychedelic substances.

Plants containing hallucinogens substances have been used by tribal societies for medical and religious practices for thousands of years. 'Psychedelic' is an English term derived from the Greek words 'Soul' (Psyche) and 'manifest' (Delos). Jaffe (1990) provided a definition for hallucinogens: "*the feature that distinguishes the psychedelic agents from other classes of drugs is their capacity to reliably induce states of altered perception thought, and feeling that are not experienced otherwise except in dreams or at times of religious exaltation*".

The use of psychedelic began in 1950s after the strong distribution of classical hallucinogen lysergic acid diethylamide (LSD), discovered by Albert Hoffmann. Research into experimental, chemotherapeutic and psychotherapeutic use of psychedelic drugs was conducted over the next 10-15 years, all over the world. Psychedelics use was widely diffused in the past between those people who want to achieve altered state of consciousness and investigate the psyche, because these substances facilitate the psychoanalytic processes. In more recent years these were particularly useful for patients with problems what were difficult to treat, including alcoholics. In fact, in the 1965 there were more than 1000 published clinical studies supporting therapeutic effects in over 40000 subjects. LSD, psilocybin and sporadically ketamine have been reported to have therapeutic effects in patients with anxiety, alcohol addiction and other brain disease (Vollenweider & Kometer, 2010).

Chapter 4

The reward system

Olds and Milner experiments, in 1954, provided some of the first key evidence for the existence of several structures in the brain that, if stimulated electrically, can produce a reinforcing effect; Authors reported that experimental animals were able to develop an operant behavior that had electrical stimulation of these areas as a scheduled consequence. These results have permitted to hypothesized that also drugs of abuse may produce reinforcing effects performing their action in the same regions, binding the receptors for the endogenous chemical messenger: For example, opioids act at opiate receptors, nicotine at nicotinic acetylcholine receptors and phencyclidine at NMDA receptors, and so on. Although drugs of abuse often produce different behavioral effects and have different pharmacological profiles, all share the ability to enhance the extracellular dopamine levels in the nucleus accumbens (Wise, 1996).

Although they account for less than 1% of the total neuronal population (Arias-Carrion et al, 2007), dopaminergic neurons have a profound effect in the adult brain functions. Principally, their activity is involved in the processes of learning and memory (Kandel, 2001) and in reward-related behavior underlying drug addiction (Pessiglione et al, 2006). Dopaminergic cells interconnect the areas that shape the complex system which originates in the substantia nigra pars compacta (SNpc), ventral tegmental area (VTA) and hypothalamus (Arias-Carrion et al, 2007; Bjorklund et al, 2007). Considering the related functions, dopaminergic system was originally considered as four major pathways: mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular (Arias-Carrion et al, 2010), (Picture 11).

The nigro-striatal pathway originates in the substantia nigra pars compacta and extends its fibers into the caudate-putamen nucleus, forms with cortex, thalamus and basal-ganglia, a complex system involved in the control of movement (Smith et al, 2008).
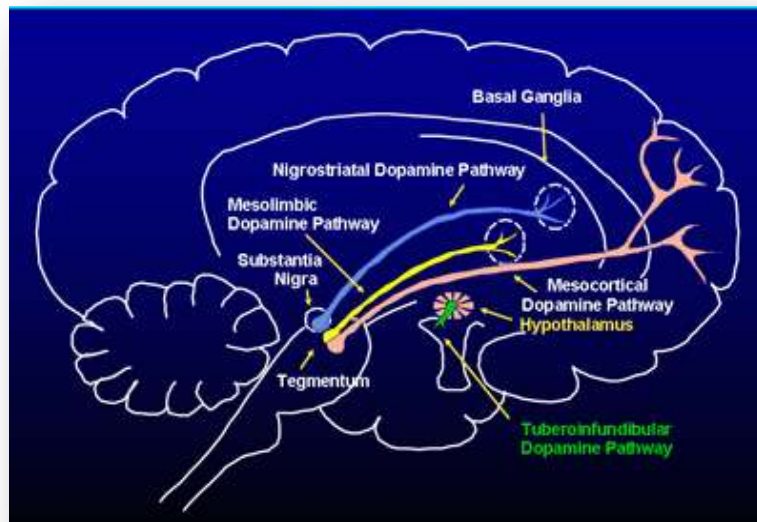
Picture 11 *Dopaminergic pathways, neural pathways in the brain that trasmit the neurotrasmitter dopamine from one regione of the brain to another. In the image are shown the four major dopaminergic pathways: mesolimbic pathway, from ventral tegmental area to the limbic system via the nucleus accumbens; mesocortical pathway, from the ventral tegmental area to the frontal cortex; nigrostriatal pathway from the substantia nigra to the striatum; tuberoinfundibolar pathway, from the hypothalamus to the pituitary gland. [http://www.medscape.org/viewarticle/585155]*

The tubero-infundibolar pathway begins in the arcuate nucleus of the medio-basal hypothalamus (the tubero region) to the median eminence (the infundibolar region). This pathway is involved in the regulation of the secretion of prolactine from the anterior pituitary gland (Porter et al, 1990; Reymond et al, 1985).
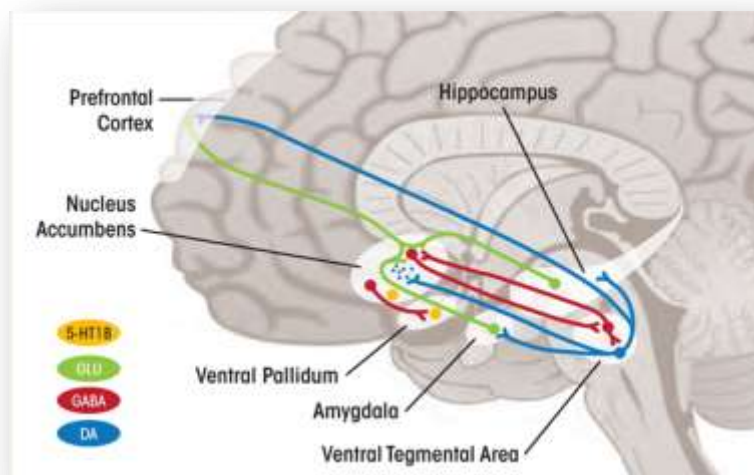
The mesolimbic and mesocortical pathways play a pivotal role in drug reward and pathological neuroadaptations in this circuitry are thought to be implicated in the development of drug addiction (Koob et al, 1992; Robinson & Berridge 1993, 2000; Heimer, 2003; Everitt & Robbins, 2005; Nestler, 2005; Zhang et al, 2006; Feltenstein & See, 2008).

The mesolimbic dopaminergic system includes the projections from the ventral tegmental area to the nucleus accumbens, as well as the olfactory tubercle innervating the septum, amygdala and hippocampus (Oades & Halliday, 1987). On the other hand, the mesocortical dopaminergic system includes ventral tegmental area that projects to the prefrontal, cingulate and perirhinal cortex (Bannon & Roth, 1983). Because of the overlap between these two pathways, they are often considered a unique system, sometimes called mesocorticolimbic dopamine system (Bjorklund & Dunnett, 2007a), (Picture 12).

Mesocorticolimbic circuit is a complex involved in the attribution of incentive value to biologically relevant stimuli, resulting in the production of adaptive behaviors,

crucial for survival (Kelley & Berridge, 2002). Growing experimental evidences suggest that drugs of abuse exert their dominant control over behavior launching this circuitry and altering its functions. Notably, both acute and repeated drugs

Picture 12 *Mesolimbic and mesocortical pathways of dopamine. These two highly conserved systems play a critical role in the assignment of motivational value to biologically relevant stimuli, resulting in the production of the adaptive behaviors. Because of the overlap between these two systems, they are often considered the unique mesocorticolimbic system (Bjorklund and Dunnett, 2007a). [anatomie.lf3.cuni.cz].*

exposure, directly or indirectly, activates dopamine release in the nucleus accumbens and cause long-lasting alterations in the structures of the reward system, particularly ventral tegmental area and nucleus Accumbens (Berke &Hyman, 2000; Henry et al, 1989; Henry & White, 1995; Hu et al, 2002; Nestler, 2004, 2005; Pierce & Kalivas, 1997). The Nucleus Accumbens, that is, the ventral part of the striatum, plays a key role as interface between the limbic system to basal ganglia and extrapyramidal motor system, but also involved in the generation of motivated behaviors and in spatial memory and adaptive processes (Berridge, 2007; Ikemoto, 2007; Nicola, 2007). Notably, it facilitates reward-seeking because act as an interface by integrating dopaminergic reinforcement signals with glutamate-encoded environmental stimuli (Brown et al, 2011; Day et al, 2007; Flagel et al, 2011; Philips et al, 2003; Stuber et al, 2008; Volkow et al, 2003, Schacter et al, 1989; Pennartz et al, 1994).

Nucleus accumbens is generally considered as two distinct sub-regions named shell and core, respectively in the ventro- and dorso- medial position and with different anatomical connectivity. Cells in the core, involved in motor functions, project strongly to the zona compacta of the substantia nigra, while shell, involved in emotions as a transition area of the extended amygdala, projects strongly to the

ventral tegmental area (Alheid & Heimer, 1988; Heimer et al, 1991). Considering the cytochemical aspects of the nucleus accumbens, a different subdivision in other two different compartments is possible: the larger one named *matrix* receives afferents from the primary sensory and motor areas of the cerebral cortex and from a subset of dopaminergic neurons in the substantia nigra pars compacta; additional, it sends projections to the substantia nigra pars reticulata. Within the matrix, somatostatin-containing neuronal processes have been found, as well as neurons positive for choline acetyl-transferase, acetylcholinesterase, muscarinic cholinergic receptors. Binding sites for µ opioid receptors are instead less present.

The other compartment, named *striosome* or *patches*, is much smaller, accounting for only 10% of the neostriatal volume. The striosome receives afferents from a restricted and less easily characterized area in the cortex and from dopaminergic neurons in the substantia nigra, while projecting to the substantia nigra pars compacta. The striosome is relatively poor in acetylcholinesterase, choline acetyltransferase and muscarininc cholinergic receptors and contains a very high concentration of µ opioid receptors (Kawaguchi et al, 1989).

The principal population of neurons in the nucleus Accumbens, GABAergic Medium Spiny Neurons (MSN), account for the 95% of the cells total,) while the remaining 5% consist of a very heterogeneous population of interneurons, including fast spiking interneurons (FS), low spiking interneurons (LS), tonically active cholinergic neurons (TAN) and GABAergic interneurons (Tapper & Bolan, 2004). Medium spiny neurons make synapses with other MSN and cholinergic interneurons but not with fast spiking (FS) interneurons. In turn, all interneurons make synapses principally with MSN (Chuhma et al, 2011).

Nucleus Accumbens, compared to the dorsal striatum, is less ordered (Sesack & Grace, 2010). However, core has an organization similar to that of the dorsal striatum, while shell has an organization which is an hybrid between striatum and amygdala.

Although, traditionally, dopamine has received most attentions as the key player in drug addiction, another considerable body of literature provides evidences for the role of glutamate in the adaptive processes emerging after a prolonged use of drugs: in fact, the activation of the prefrontal cortex is commonly observed in brain imaging studies, in human addicts that are exposed to drug associated stimuli (Goldstein & Volkow, 2002).

Glutamate is the most important excitatory neurotransmitter at the synapses between prefrontal cortex, ventral hippocampus, basolateral amygdala and the midline thalamus to the medium spiny neurons of the nucleus accumbens (Wolf 1998; Kalivas & O'Brien, 2008; Britt & Bonci, 2013; Horne et al, 1990).

Here, we focus on the prefrontal cortex (PFC), a brain area involved in attention, decision making, cognitive flexibility, learning and responses to environmental stimuli (Groenewegen & Uyling, 2000; Miller 2000; Robbins, 2000a; Miller & Cohen, 2001; Dalley et al, 2004). Prefrontal cortex, through efferent projections to the nucleus accumbens, can assimilate both sensory and limbic information to support goal-directed behavior (Robbins & Everitt 1996). it sends glutamatergic projections to the ventral tegmental area, septal areas and basal forebrain (Geisler et al, 2007; Vertes, 2004) while, in turn, VTA sends dopamine outputs to nucleus accumbens, hippocampus and amygdala (Sesack & Pickel, 1990; Gasbarri et al, 1994; Hasue & Shammah-Lagnado, 2002) and others sends cholinergic projections to hippocampus and amygdala (Gaykem et al, 1990; Carlsen et al, 1985). In this way, the prefrontal cortex can exert a modulation on the activity of the nucleus accumbens, hippocampus and amygdala, both directly through the glutamatergic efferent projections and indirectly through the activation/inhibition of dopamine and cholinergic inputs (Robbins 2000b; Gabbott et al, 2005).

The rodent prefrontal cortex is divided in anterior cingulate cortex (dorsomedial portion), prelimbic and infralimbic cortex (ventromedial portion) and orbitofrontal cortex (Franklin & Chudasama, 2012), and all receive the most part of dopaminergic efferents that leave ventral tegmental area dopamine neurons (Tzschentke 2001; Tritsch & Sabatini, 2012). The 80% of the total prefrontal cortex population is represented by excitatory glutamatergic pyramidal projection neurons and GABAergic inhibitory interneurons (Homayoun & Moghaddam, 2007; Card et al, 1999; Beaulieu, 1993). Like other cortical areas, prefrontal cortex is subdivided in six layers with characteristic type of neurons and connections (Mountcastle, 1997; Douglas & Martin, 2004). In particular, the most prominent type of cortical neurons are pyramidal neurons located in layers V and VI and receive projections from ventral tegmental area while projecting to layers II and III and to subcortical areas, including the ventral tegmental area and nucleus accumbens (Carr & Sesack, 2000; Douglas & Martin, 2004; Gabbott et al, 2005). Dopaminergic and glutamatergic synapses shape the so-called 'triads' that are thought to be the

structural basis for the direct dopamine modulation of glutamatergic transmission (Law-Tho et al, 1994).

The brain circuitry involved in drug addiction is complex and prolonged drugs use can underlie deep neural adaptations as consequences of *in vivo* exposure to drugs of abuse (Robinson & Berridge, 1993; Kalivas, 1995; Wolf, 1998; Hyman &Malenka, 2001; Nestler, 2001; Carlezon & Nestler, 2002; Everitt & Wolf, 2002; Hyman et al, 2006). Although neuroadaptations directly related to dopaminergic activation appear crucial for the development of addiction, once it is developed, the impaired ability of addicts to regulate drug seeking and drug-taking behaviors is thought to be dependent of long-lasting neuroadaptations at the prefrontal glutamatergic input to the nucleus accumbens (Luscher & Malenka, 2011; Nestler 2001; Kalivas & Volkow, 2011; Kalivas & O'Brien, 2008; Kalivas et al, 2004; Self et al, 2004). Clinical investigations revealed that the basal activity in the prefrontal cortex is reduced in addicts during withdrawal, but can increase rapidly following presentations of drug-associated cues. Biologically, the presentation of stimuli associated with positive reinforces strongly activate the PFC-accumbens pathway in normal subjects, but in addicts the increase is brusque (Garavan et al, 2000). Imaging studies have shown the association between the impairments in prefrontal cortex and reduction of dopaminergic function suggesting the hypothesis of the involvement of dopamine in the drug-induced neuroadaptations in the prefrontal cortex. Similarly, behavioral studies have demonstrated that blocking AMPA/kainate glutamate receptors in the nucleus accumbens prevents the reinstatement of drug seeking (Cornish & Kalivas, 2000; Di Ciano & Everitt, 2001; Park et al, 2002); moreover, microdialysis estimates of extracellular glutamate reveal an increased release of glutamate in the projections from the prelimbic cortex to the core compartment of the nucleus accumbens during drug seeking (McFarland et al, 2004). Finally, it was demonstrated that repeated *in vivo* psychostimulant exposure increases the number of dendritic spines (Robinson and Kolb, 2004), cell surface expression of AMPARs (Boudreau & Wolf, 2005) and the behavioral response to AMPA infusion in the nucleus accumbens (Pierce et al, 1996; Cornish & Kalivas, 2000; Suto et al, 2004).

The adaptations known in the nucleus accumbens, as consequences of prolonged use of drugs, can be modelled using long-term potentiation (LTP) and long-term depression (LTD) paradigms. It is now accepted that either LTP or LTD are basic

properties of most excitatory and inhibitory synapses throughout the central nervous system and can be used experimentally for studying multiple brain functions (Malenka & Bear, 2004). Notably, using electrophysiological methods in brain slices, different forms of LTP and LTD in the brain have been identified. The two best-understood forms of long term potentiation in the mammalian brain are the NMDAR-dependent LTP and the presynaptic LTP. The former involves both presynaptic and postsynaptic neurons because when glutamate is released the postsynaptic neuron must be depolarized. Therefore, glutamate binds NMDA receptors on the postsynaptic membrane and rises the calcium influx which activates intracellular signaling cascades responsible for the changing in synaptic efficacy (Bliss & Lomo, 1973; Malenka & Bear, 2004; Malenka & Nicoll, 1999; Lynch, 2004; Yuste & Bonhoeffer, 2001; Matsuzaki et al, 2004; Lau & Zukin, 2007; Malenka, 1994; Luscher & Malenka, 2014). Slightly different is presynaptic LTP that does require neither NMDAR receptors activation nor postsynaptic factors, but seems to be initiated by an activity-dependent rise in extracellular calcium within the presynaptic terminals. The cascade is responsible for the increase in glutamate release (Malenka & Bear, 2004; Nicoll & Schimtz, 2005). Three different forms of long-term depression are, instead, identified in the mammalian brain: NMDAR-dependent LTD, metabotropic glutamate receptor-dependent LTD and endocannabinoid-mediated LTD.

NMDA-dependent LTD, like presynaptic LTP, requires repeated activation of the presynaptic neuron at low frequencies but not the postsynaptic activity (Malenka & Bear 2004; Carroll et al, 2001). Conversely, metabotropic glutamate receptor-dependent LTD requires either postsynaptic calcium influx through voltage-gated ion channels and postsynaptic group I mGluR activation (Malenka & Bear, 2004; Ito, 1989). Endocannabinoid-mediated LTD, that recently has received more attention than the others, has been recognized at many glutamatergic and GABAergic synapses: it is well-established that a sustained postsynaptic calcium influx is necessary to trigger the synthesis of the endogenous endocannabinoid that retrogradely bind the presynaptic CB1 receptors and depress neurotransmitter release (Wilson & Nicoll, 2002).

Very little is known about the mechanisms of long-term plasticity in the Accumbens synapses and parallelism between the main forms of LTP and LTD in these pathways are not simple to do. Recently, NMDAR-dependent LTP and LTD and

endocannabinoid-mediated LTD have been reported also in these regions (Thomas et al, 2000; Kombian & Malenka, 1994; Schramm et al, 2002; Robbe et al, 2002; Hoffman et al, 2003). Electrophysiological experiments it has been demonstrated a loss of long-term depression in the cortico-accumbens pathway of rats withdrawn from the self-administration of cocaine (Martin et al, 2006; Moussawi et al, 2009; Knackstedt et al, 2010); in particular the impairment in long-lasting depression endured only in the subpopulation of rats that developed uncontrolled intake (Kasanetz et al, 2010).

Growing evidence suggests the crucial role of the endocannabinoid system in the neuroadaptations induced by the prolonged use of drugs (Gonzalez et al, 2005; De Vries et al, 2005; Parolaro et al, 2005; Rodríguez de Fonseca et al, 2005; Fattore et al, 2005; Ameri et al, 1999; Solinas et al, 2008), probably increasing the hedonic impact of rewarding stimuli (Mahler et al, 2007; Kirkham 2009; Jarrett et al, 2007; Cota et al, 2006; Solinas et al, 2008) by a close interaction with the endogenous opioid system (Cota et al, 2006). It important to underline that drugs derived from cannabis (marijuana and hashish) are the most common illicit drugs that activate mesolimbic neurons and increase the extracellular dopamine level in the nucleus accumbens (Gessa et al, 1998; Tanda et al, 1997).

Components of the endocannabinoid system, in particular the CB1 receptors, are widely distributed throughout the brain reward circuit, on axons, cell bodies and dendrites (Herkenham et al., 1990, 1991; Tsou et al., 1998; Egertova et al., 2003; Solinas et al., 2008). Once activated, CB1 receptors inhibit both GABAergic and excitatory synaptic transmission in the hippocampus (Hoffman and Lupica, 2000; Sullivan, 1999), prefrontal cortex (Auclair et al., 2000) and nucleus accumbens (Hoffman and Lupica, 2001; Robbe et al., 2001). In a work of 2001, Manzoni and collaborators, gave evidence for a presynaptic localization of CB1 receptors at the excitatory synapses to the medium spiny neurons in the nucleus accumbens. In addition they demonstrated that cannabinoid agonists induced long-term synaptic depression in the nucleus accumbens. By using both extracellular field potential and patch-clamp recordings, Robbe and colleagues (2002) explored the involvement of the endocannabinoid system in the prefrontal cortex-nucleus accumbens synaptic plasticity. They showed that a tetanic stimulation (at biological occurring frequencies) of the cortical glutamatergic afferents induces a long-term

depression in the NAc. Endocannabinoids may exert a retrograde modulation of the synaptic transmission, by the activation of the metabotropic glutamate 5 (mGlu5) receptor which caused an increase of postsynaptic calcium (Zhang et al., 2011; Lerner et al., 2012; Freestone et al., 2013); this deployment of calcium lead to a postsynaptic release of endocannabinoids which retrogradely activated the presynaptic CB1 receptors (Robbe et al., 2002). Once activated, CB1R stimulation suppressed glutamate release either transiently (short term depression, STD) or persistently (long term depression, LTD), (Kano et al., 2009; Ohno-Shosaku et al., 2012; Katona et al., 2012; Castillo et al., 2012; Piomelli, 2014; Gao et al., 2010; Gerdeman et al., 2002; Younts et al., 2013).

I*n vivo* delta (9)-tetrahydrocannabinol (THC) administration reduced the endocannabinoid-mediated long-term depression in the mouse cortico-accumbens synapse (Robbe et al., 2003) maybe due to a reduction of the coupling efficiency of CB1 receptors to Gi/o transduction proteins (Mato et al., 2005).

CB1 receptor activation also modulated cortical excitatory synaptic transmission (Wilkison et al., 1982; Pontzer et al., 1986; Wallace et al, 2003). In vitro studies found that CB1 receptors are differentially expressed, with highest levels of expression in layers II, III and V. In particular, it was reported that cannabinoids can both balance directly GABAergic and glutamatergic transmission, but also increase the spike probability of pyramidal neurons in the layers 2/3 (Fortin et al., 2004; Barbara et al., 2003). Acute treatment with WIN 55, 212-2, suppressed evoked excitatory postsynaptic responses recorded from layer V pyramidal neurons (Auclair et al., 2000). Similar results were obtained by Fortin and Levine, in the 2007: application of WIN 55, 212-2 depressed excitatory but not inhibitory post synaptic currents in the pyramidal neurons of the layer V, while the stimulation of the layer V, but not the layers II and III, evoked cannabinoid-sensitive synaptic currents; these results suggest that only a subset of glutamatergic inputs express cannabinoid receptors. Altogether these studies underlined the involvement of cannabinoid system in the modulation of cortical functions, showing different effects on excitation and inhibition across the layers, in particular layer V that may serve to decrease the efficacy of a subset of excitatory inputs.

Chapter 5

Overview of the techniques

5.1 Self-administration paradigm
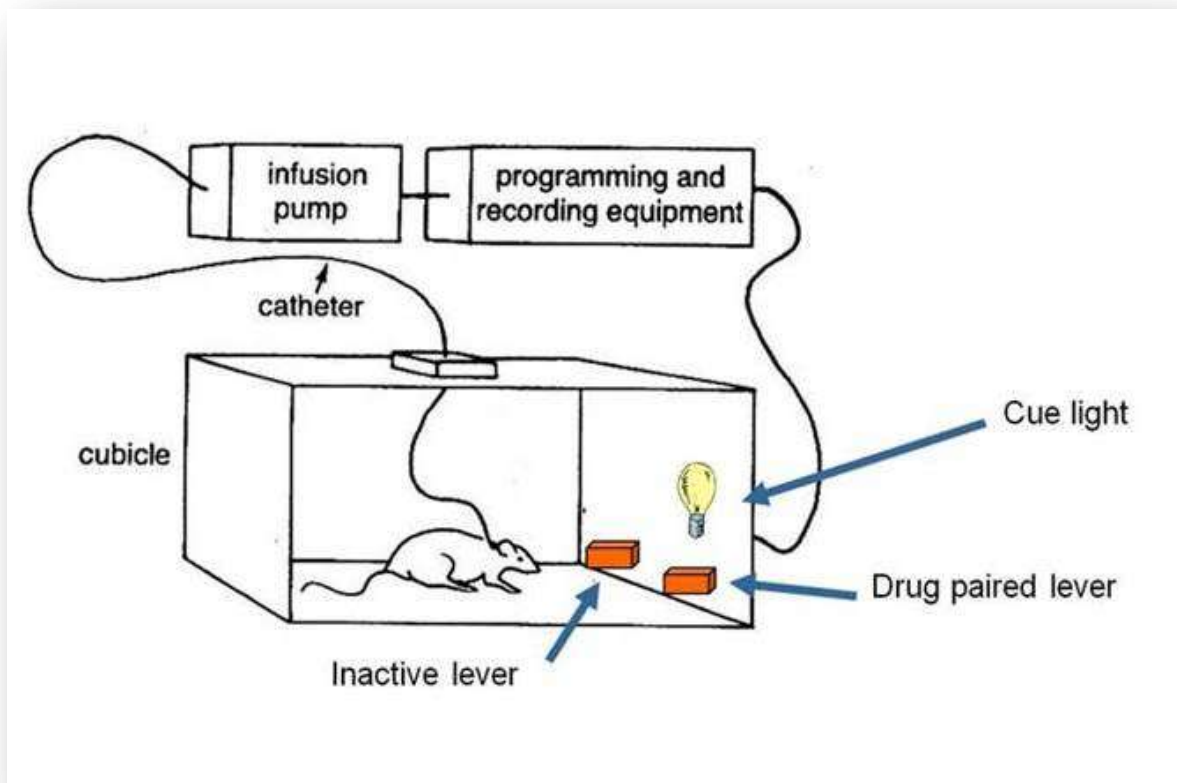
Picture 13 *Animal self-administration is a form of operant conditioning where the reward is a drug, administered remotely through an implanted intravenous catheter. Animals can readily detect the contingency between an instrumental response (in this case, lever press) and the delivery of the drug and respond in an operant manner to obtain the drug. [www.med-association.com; Intravenous Self-Administration of Drugs of Abuse in Mice, Brown et al. 2012]*

The reinforcing properties of substances of abuse are considered to underlie the risk of abuse liability in humans (Balster & Bigelow, 2003; Panlilio & Goldberg, 2007). To investigate these properties have been developed three main animal models of human drug abuse: drug discrimination, conditioned place preference and self-administration paradigms (Ator & Griffiths, 2003; Carter & Griffiths, 2009; Sanchis-Segura & Spanagel, 2006; Tzschentke, 1998). For our study we used the intravenous self-administration model, initially developed in non-human primates by

Weeks and collaborators (1962). Thus, animals can readily detect the contingency between an instrumental response (such as a nose poke or, in our case, lever press) and the delivery of the drug, (in our case, an intravenous infusion of BZY) and respond in an operant manner to obtain the drug (Clark et al, 1961; Weeks, 1962). Therefore, the substance is considered reinforcing if it is able to maintain the self-administration behavior.

Self-administration paradigm appears to be good correspondence between humans and animals in terms of pattern of drug intake: in fact, usually, drugs that are abused by humans maintain responding behavior in animals, while drug without reinforcing properties (as hallucinogenic drugs), that do not maintain the animals behavior, generally are not abused by humans. The advantages to use a self-administration paradigm is the fact that the drug is self-administered instead experimenter-administered; in this way the schedule of reinforcement can be changed and an accurate measurement of the quantities of drug consumed and the pattern of infusions can be get.

To perform self-administration paradigm generally rodents are preferred, although also others species are employed, such as dogs, cats and non-human primates. The most common routes of drug administration are intravenous and oral, but intracerebroventricular, intracranial, inhalation, intragastric and intramuscular has been also used although it is more advisable to use route comparable to that used by humans. For example, intravenous route is used preferably for studies with cocaine and heroin while studies with alcohol typically use an oral route of administration. The advantages using intravenous route is to avoid the problems related to first pass metabolism or taste and to produce rapid increase in blood and brain drug level.

Self-administration model can imply the use of different schedule of reinforcement. In the simple fixed ratio schedule, it is necessary a completion of a fixed number of operant responses (fixed by the experimenter) by the animal to obtain the infusion of drug. The number of ratio is fixed during the session but can be increased between the sessions, during training procedure. There is, in this way, a direct relationship between the actual response and drug delivery. Different schedules can be used for the investigation of different aspects of drug addiction. More complex schedules of reinforcement are the progressive ratio schedule (PR),

(Gardner, 2000; Rowlett, 2000; Richardson & Roberts, 1996) and the choice procedure (Ward et al, 2005).

In the animal self-administration literature, the rates of responding on an operant lever (active lever) that leads to the delivery of the drug are reported; this measure is, in most cases, compared with the responding on a second operant lever (inactive lever) on which responding has no scheduled consequences. Generally, a drug is considered reinforcing if the responding on the active lever significantly exceeds the responding on the inactive lever. In studies where a negative control is included, self-administration is identified as responding for the drug which significantly exceeds responding for the negative control for saline.

Since the self-administration paradigm has been designed, new progresses have been done to investigate the reinforcing properties of drugs: using the binge self-administration model, animals given prolonged access to drug (6 hours); in this way it is possible to understand the propensity of abuse and the escalation pattern of drug intake (Ahmed & Koob, 1998). Another variation from the basic model is the self-administration under punishment that mimics the human compulsive use of drug despite the negative consequences.

However, one must remember that drug-self-administration model measures only one of many factors that can contribute to abuse liability that is drug reinforcement properties. Thus integrating data from pre-clinical and clinical abuse potential models will provide a more accurate assessment of abuse liability risk.

5.2 Extracellular field recordings
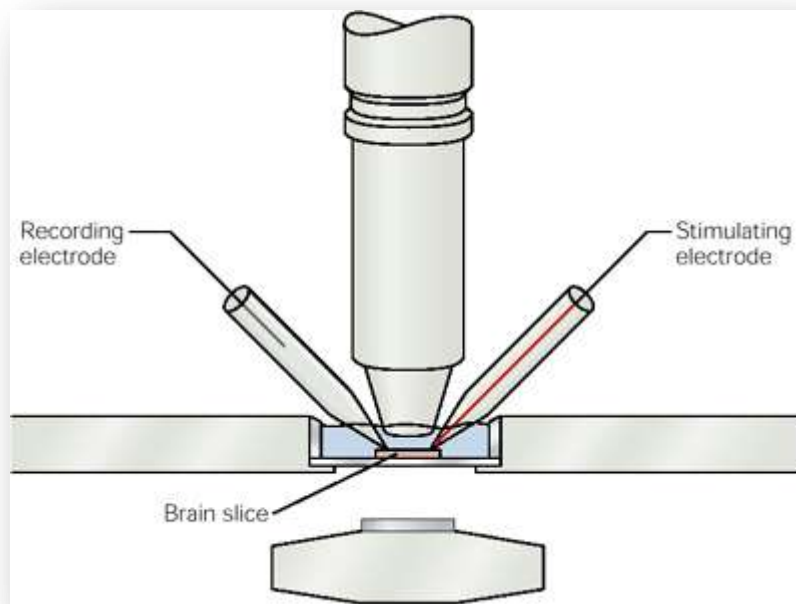
Picture 14 *After recovery, slice is transferred to a recording chamber on the microscope and submerged beneath continuously flowing aCSF. Stimulating- and recording- electrodes are placed on slice in target areas and finally the signal detected by an amplifier and collected. [Gary L. Westbrook " Seizures and Epilepsy" adapted by Konnerth, 1990)]*

Prolonged use of drugs is responsible of the reorganization of neural circuitry in the reward areas resulting in changes in the synaptic strength/plasticity, the best candidates of which are various form of long-term potentiation (LTP) and depression (LTD) (Hyman and Malenka, 2001). The demonstration of synaptic plasticity at excitatory synapses of the mesocorticolimbic dopamine system, the correlative changes in glutamate AMPA receptors expression and single unit responses to glutamate after *in vivo* exposure to drugs of abuse support a role for long-term plasticity in the development of addiction (Zhang et al., 1997; Carlezon and Nestler, 2002; Thomas and Malenka, 2003).

The best method to investigate synaptic activity at the network level is electrophysiology. Different types of electrophysiological recording techniques exist: intracellular (for example patch-clamp), extracellular (field potentials) and planar patch clamp recordings.

In our work, we used field potentials recordings, which allowed the detection of local current sources generated by simultaneous activation of a large number of nearby neurons, within a small volume of nervous tissue, during the electrical stimulation intra-area or of the relative efferents. Evoked action potentials can

produce a voltage changing that can be detected using a recording microelectrode, fill with artificial cerebrospinal fluid (aCSF) and place sufficiently far from local individual neurons. This signal is then low-pass filtered to obtain field potential that can be displayed and recorded on an oscilloscope (Buzsaki et al., 2012).

Therefore, the field is defined as the contribution of all ionic processes because any single excitable membrane can contribute to the extracellular field recordings. Responsible of the strongest currents across the neuronal membranes, fast action potentials are detected as spike in the extracellular medium. However, also the slow fluctuations of glia cells, responsible of non-synaptic events and voltage-dependent regenerative calcium spikes, can be detected and cannot separate in the field recordings (Hirsch et al., 1995; Schiller et al., 2000; Polsky et al., 2004; Larkum et al., 2009Vanhatalo et al., 2004; Hughes et al., 2011; Poskanzer and Yuste, 2011).

Furthermore, it is useful to consider the different contribution of various cell types to the field potentials: a bigger pyramidal neuron with long thick apical dendrites generates strong dipoles then a smaller interneuron or astrocyte. But in those tissues in which the extracellular medium is homogeneous, both the spatial alignment and the temporal synchrony between all neurons and glia cells are important for the generation field potentials (Buzsaki et al.,2003; Linden et al., 2011).

The main properties of the field potentials waveform, amplitude and frequency, depend of the various characteristics of brain tissue. Moreover, the larger distance between the recording electrode and the current source prevents the correct measurement of field potentials.

For decades, extracellular field recording has been considered an efficient method to assess synaptic transmission and plasticity in different brain regions. The advantageous of extracellular recordings, relative to intracellular method, is the healthy stabile recordings for a long period, also several hours, that results useful for plasticity studies.

Chapter 6

Benzydamine self-administration

6.1 Abstract

Rationale Use of illicit drugs by young and vulnerable people is an expanding cultural phenomenon, but in recent years, there has been a dramatic increase in prescription drugs misuse. Some medications, in fact, have psychoactive properties and are sometimes used for recreational reasons and non therapeutical purpose. The abuse of BZY, a non-steroidal anti-inflammatory drug (NSAID), has been object of increasing concern in Brazil and in other Country in Europe, also in Italy. As reported in many drug forums, BZY behaves principally as an hallucinogen. The first aim was to determine BZY abuse liability, assessing the reinforcing properties using an operant self-administration procedures.

Materials and methods Indipendent groups of rats with intravenous catheters were given the possibility to self-administer different doses of BZY (250, 500 and 1000 µg/kg per infusion) under two conditions: naïve or drug-experienced. Naïve animals have not previously subjected to particular experimental situation, while experienced animals have exposed previously to cocaine and heroin, before training with BZY. After training, dose-effect curve (125, 250, 500, and 1.000 µg/kg per infusion) were calculated.

Results We found that BZY has a powerful reinforcing effect and that this effect is greatly facilitated in animals that already had substance experience, having previously self-administered heroin and cocaine, indicating cross sensitization between BZY and other common drugs of abuse.

Conclusion The present study confirms, at a preclinical level, the reinforcing properties of BZY. This results may suggest that also in human, the subjects more prone to BZY consumption are those which had previous experienced other drugs.

6.2 Introduction

BZY, available as the hydrochloride form, is an anti-inflammatory drug, that taken at high dosages of 500 mg to 3000 mg, behaves as a psychoactive substance and a central nervous system stimulant. Considering the users' descriptions found in several online forums, BZY may be principally considered an hallucinogenic drug. Hallucinations may be explained considering the serotonergic hypothesis: it is known in fact that other hallucinogenic drugs as LSD, mescaline, DMT and psilocybin act as agonists of $5HT_{2A}$ receptors present in certain areas of the CNS. Notably, BZY molecule has a structural affinity with serotonin, because the presence of the indole compound, (Opaleye et al, 2009).

Elizado Carlini of CEBRID (Centro Brasileiro de Informações sobre Drogas Psicotrópicas, Departamento de Medicina Preventiva da UNIFESP, Universidade Federal de São Paulo) has proposed a different hypothesis: BZY, taken at high doses, could increase the release of Dopamine in the brain and consequently activate the limbic system. In this way, the emotional experiences could be recall and changed so they are perceived altered, (Mota et al, 2010).

In this framework, it is necessary to consider that the derivatives of amphetamine, as methamphetamine and MDMA, have hallucinogenic properties as LSD, but also reinforcing properties; in fact while there is no evidence for animal self-administration of primary hallucinogenic substances like LSD (Deneau et al, 1969), animals self-administer amphetamine derivatives (Fantegrossi et al, 2002; Lamb et al, 1987; Schenk et al, 2003, 2007; Sannerud et al, 1996).

Here we investigated the reinforcing properties of BZY in an animal model of intravenous self-administration. In particular we wanted to test the capacity of this substance to develop and mantein the self-administration behavior in rats, using both naïve and drug experienced animals. Notably, BZY is frequently used in association to other 'main' drugs as alcohol or THC, or to substitute drugs temporarely not available. Moreover, different cases report of volontary BZY use refers to addict.

6.3 Materials and methods

6.3.1 Animal

The study was conducted using 23 male Sprague Dawley rats (Harlan Italy, San pietro Natisone, Italy) weighing 275 g at their arrival in the laboratory. All rats were housed, two per cage, for 7-10 days before the surgery,in a dedicated temperature- (20±2 °C) and humidity- (70%) controlled rooms, with free access to food and water (except during the test sessions) under a 14 h dark/ 10 h light cycle (light off at 7 am).

All procedurs were in accordance with the Italian Law on Animal Reserch (directive 2010/63/EU of the European Parliament and of the Council) and with the guidelines for the care and use of laboratory animals issued by the Italian Ministry of Health.

6.3.2 Drugs

- **Rompum** 20 mg/ml injectable(Bayer® HealthCare pharmaceuticals): 2.33 mg of muscle relaxant was administered intraperitoneal(each ml contains 23.32 mg xylazine hydrochloride, corrispondent to 20 mg of xylazine equivalent base).

- **Zoletil 100** injectable anaesthetic/sedative (Virbac®, Carros, France). It is a combination of a dissociative anethetic agent, tiletamine hypochloride (50 mg/ml), and a tranquilizer, zolazepam hypochloride 850 mg/ml).Each rat were anesthetized with an intra-peritoneally injection of 0.56 ml/kg of Zoletil.

- **Baytril antibacterial** injectable solution 2.27% for veterinary use (Bayer®, KVP Pharma + Veterinär Produkte Gmbh, Kiel, Germany); it was administered intravenous: 15mg/0.1 ml after surgery and 0.4 mg/ml daily (1 ml injectable solution contains 50 mg enrofloxacin).

- **Heparin sodium** 12500UI/0.5ml (Marvecs Services, Agrate Brianza, Italy). Catheters were flushed daily with 0.1 ml of a sterile saline solution containing 25 IU heparin.

- **Tiopental sodium** (Pharmacia Italia, Milano, Italia).Each rat were administered intravenously with 40 mg/kg.

- **Benzydamine hydrochloride** (Sigma Aldrich®), wasdissolved in isotonic NaCl (0.9% w/w saline in water), and infused at a volume of 40 µl over 4 sec at a dose of 250, 500 and 1000 µg/kg/infusion.

- **Cocaine HCl** (National Institute on Drug Abuse) was dissolved in sterile saline (isotonic NaCl 0.9% w/w saline in water), and infused at a volume of 40 µl over 4 sec at a dose of 400 µg/kg/infusion.

- **Heroin** (diacetylmorphine HCl,(National Institute on Drug Abuse)was dissolved in sterile saline (isotonic NaCl 0.9% w/w saline in water) and infused at a volume of 40 µl over 4 sec at a dose of 25µg/kg/infusion.

6.3.3 Intravenous surgery

Animals were deeply anesthetized with an intraperitoneal injection of Zoletil 100® and Rompum® and an indwelling catheter (SILASTIC® tubing, Plastics One, Roanoke, VA), (0.51 mm inner diameter, 0.94 mm outer diameter, and 11 cm length, sheathed at 3.4 cm from its proximal end by a 5 mm length of heat-shrink tubing), was surgically implanted into the right jugular vein. The catheter was secured to the vein with surgical silk suture and passed subcutaneously and externalized through a small incision at the nape of the neck and connected to an L-shaped 22 Gauge cannula. The cannula was then secured to the rat's skull using dental cement and stainless steel screws. After surgery, the rats were given baytril 0,1 ml intravenously. Catheters were flushed daily with a solution containing baytril and heparin to mantein patency.

6.3.4 Self-administration chambers and experimental context

The apparatus consisted of SA chambers (28.5 cm length, 27 cm width and 32 cm height) made of trasparent plastic (front and rear walls), aluminium (sidewalls and ceiling) and stainless steel (grid floor). Plastic trays covered with pinewood shaving were placed under the grid floors. Each chamber was equipped with two retractable levers, positioned on the left hand wall 12.5 cm apart and 9 cm above

Picture 15 *Self-administration chamber for rats. The chamber is assembled with black aluminium walls and a transparent front door. The chambers employ a stainless-steel grid floor that allows waste to collect in a removable tray. All floor components (including the grid) are removable. Inside the chamber are present two extractable levers. On the right external side of the chamber is fixed a steel arm that supports a swivel, for the infusion line. [http://www.instechlabs.com/Infusion/systems/selfadministration-rat.php]*

the floor, three cue lights (red, yellow and green), positioned above each lever, and a counterbalanced arm holding a liquid swivel.

The SA chambers were placed within sound- and light- attenuating cubicles. Each cage was connected via an electronic interface to a syringe pump (Razel Scientific Instruments, St. Albans, VT, USA), in turn connected to a computer. Chambers, accessories and electronic interfaces were purchesed from EATEL s.r.l. (Rome, Italy) and custom-developed control software from Aries System S.r.l. (Rome, Italy).

The infusion line consisted of a length of silastic tubing protected by a stainless steel spring and connected (through the liquid swivel and another length of silastic tubing) to a syringe positioned on the pump (which was programmed to work at an infusion rate of 10 µL/s.

Picture 16 *Each operant-chamber is placed within a cubicle, connected either to a syringe pump, throughout an electronic interface, and to a computer software. [www.med-associates.com/drug_del/rmouseselfadmin.htm]*

6.3.5 Self-administration procedures

After surgery, the rats were housed in tranparent plastic cages (40 cm length, 24.5 cm widht and 18 cm height) with stainless steel tops and flat bottoms covered with ground cornocob bedding.

Before the start of the experiment the animals were divided and assigned to one of two testing conditions: naïve- and experienced- group. The two groups underwent two different experimental procedures: all animals in the 'naive' group underwent an experimental procedure in which they were trained to self-administered only BZY.

On the contrary, the animals in the 'drug-experienced' group underwent two different training schedules: in the first one they were trained to self-administered cocaine and heroin, while in the second phase, after a period of extinction, they were given the chance to self-administered BZY. This because the virtual totality of BZY recreational use cases are reported to involve individuals with previous substance abuse history, sometimes to substitute other drugs when are absent.

For both groups, training procedure began 1 week after surgery. Immediately before the start of each session, every animal were transferred to the self-administration chamber and their catheters connected to infusion lines filled with the appropriate drug solution. At the end of each session, animals were re-transferred to their standard cages. All test sessions lasted 3 hours and took place during the dark phase, between 9 am and 5 pm, 7 days a week.

During the self-administration sessions the doors of the cubicles were kept closed to prevent noise that could disturb the animal. At the start of each session, two levers were extended in each cage: only one of the two was active and triggered an infusion of BZY delivered over a period of 3 seconds; the second lever had not direct consequences except resetting the counter on the active lever. Within each group, left and right levers were counterbalanced for the active and inactive status. Above the active lever were presented three light cues that were turned on only during extension of the lever. No other light cue was provided.

Picture 17 *Training scheme. Animals of both groups received 14 training sessions. Under the squares are reported the number of consecutive responses required to obtain a single infusion: FR1 to FR5, in the showed correspondence.*

Training procedure for 'naïve' group consisted of fourteen sessions under a fixed ratio schedule progressively incresed from 1 to 5 (days 1-7 FR1, day 8 FR2, day 9 FR3, day 10 FR4, days 11-14 FR5); fixed ratio means that a single infusion was raised only after a specified number of responses.

After the completion of each task, a 40 sec time-out period was presented (i.e. for that no cage element was on, as levers or light cues).

Five minutes after the beginning of each session, animals that had not spontaneously self-administered at least one infusion were placed with their forepaws on the active lever so as to trigger a priming infusion. Priming infusions were administered again at 1 hour to animals that had not spostaneously self-administered at least two infusions during the first 60 min of session and at 2 hours to animals that had not spostaneously self-administered at least two infusions during the first 120 min of session. On FR5 sessions (days 11-14), priming infusions were given only to rats that failed to self-administer at least one infusion within the 0-5 min period. These experimenter-induced lever presses were opportunely subtracted from the total.

The animals were divided, at the beginning of the experiment, in independent subgroups, following three different doses of BZY: 250 µg/kg, n=4; 500 µg/kg, n=4; 1000 µg/kg, n=4. The rats were allowed to self-administer a maximum of 50 infusions of BZY per session.

After the training period, the animals underwent other 4 session (15-18), in which each rat have received the possibility to self-administered (following a latin square design) three additional doses of BZY than the training dose. In this way, all animals were tested for responding to four different dosages (125 mg/kg, 250 mg/kg, 500 mg/kg, 1000 mg/kg). These sessions were conducted under a FR5 schedule of reinforcement and without drug priming administration.

The animals of 'experienced' group underwent two different self-administration procedures. During the training phase, beginning one week after surgery, the animals received the possibility to self-administer cocaine (400.0 μg/kg) and heroin (25.0 μg/kg), made available on alternate days. The two substances were paired with different levers, in a counterbalanced fashion (left vs right, drug on the first session). Training procedure consisted in fourteen sessions under a fixed ratio schedule progressively increased from FR1 to FR5. The animal that had not spontaneously self-administered at least one infusion after 5 minutes from the beginning, two infusions after 60 minutes and three infusions after 120 minutes, had received a priming infusion, as the animals of the 'naive' group. After the training phase, the animals of the experienced group had undergone 10 days of extinction during which they were transferred to the self-administration chambers and their catheter connected to infusion lines, filled only with saline. After this period of twenty four days, the animals were transferred in a different experimental room and had undergone fourteen days of BZY self-administration, with the same protocol received from 'naive' group.

6.3.6 Catheter patency test

After the last test session all rats, of both groups, were undergone a catheter patency test consisting in the administration of 0,1 ml saline with 40 mg/kg Tiopental Sodium (see the section 'Drugs'), in a single intravenous bolus. Rats that did not became ataxic within 5 s after test were excluded from the statistics.

6.4 Data analysis and statistics

In vivo data were analyzed using both three- and four-way mixed ANOVA for repeated measures, with lever (active vs inactive) and session treated as within factors, while dosage and experience (naïve vs drug-experienced) as between factors. Data were analyzed using IBM SPSS 20 statistical software.

Chapter 7

Field recordings

7.1 Abstract

Rationale The recent theories of addiction have focused on particular aspects of drug-induced neuroadaptations that may explain craving, seeking and taking behavior and relapse after a period of withdrawal. Alterations in cortico-accumbens glutamatergic pathway is considered very crucial in the development of drug addiction.

The second aim of our study was to assess if BZY was able to induced long-lasting modification of basal glutamatergic cortico-accumbens transmission directly or by the involvement of other signiling ie the dopaminergic and endocannabinoid system.

Materials and methods Extracellular field recordings were made in parasagittal slices containing both nucleus accumbens and infralimbic cortex, from adult male rats. BZY was applied in the bath perfusion and the field excitatory post-synaptic potentials (fEPSP), elicited by stimulating the glutamatergic cortical afferents at the border of the accumbens, were recorded in the ventral striatum. The D1R antagonist SCH23390 and the CB1R antagonist AM-251 were added at the final concentration to the superfusion medium.

Results Upon BZY application, fEPSP resulted in a dose-dependent amplitude reduction in both naïve and drug-experienced rats, although the effects were potentiated in slices from animals that had undergone a previous experience with heroin and cocaine. Also, BZY superfusion right before the HFS train diminished the LTP-like response of NAcc neurons compared to controls. Due to the alteration of short term plasticity we report that BZY induced LTD by a presynaptic mechanism. Furthermore this LTD-like response was significantly reduced by the CB1 receptor antagonist AM 251but not affected by the dopamine D1 receptor antagonist SCH23390.

Conclusion These results provide the first evidence of BZY effect on central nervous system, suggesting the interaction with cannabinoidergic system.

7.2 Introduction

Prolonged drugs use is responsible of synaptic plasticity and aberrant neuroadaptations involved in the uncontrolled drug-seeking and drug taking behavior (Robinson & Berridge, 1993; Kalivas, 1995; Wolf, 1998; Hyman & Malenka, 2001; Nestler, 2001; Carlezon & Nestler, 2002; Everitt & wolf, 2002; Hyman et al, 2006). The drug-dependent reorganization of neural circuitry can be mediated by different mechanisms, included forms of long-term potentiation (LTP) and long-term depression (LTD) (Kalivas, 1995; Wolf, 1998; Clark & Overtone, 1998; Martin et al, 2000; Bear, 1996). All excitatory synapses in the mammalian brain express this form of plasticity, although the mechanisms differ among different brain regions. Notably, LTP and LTD can be expressed at excitatory synapses in the nucleus accumbens and both require the activation of NMDA receptors but not dopamine receptors.

Numerous studies have revealed that while the dopamine system is involved principally in the establishment of drug taking behavior, in a later phase, in which the addiction behavior is developed, drug-seeking and drug-taking behavior are depended on the glutamatergic prefrontal afferents to the nucleus accumbens (see chapter 4). Growing evidence support the involvement of the endocannabinoids system in the neuroadaptations following prolonged drug use. Principally, it is considered crucial the retrograd mechanism by which an increase in intracellular calcium concentration, after a sustained postsynaptic depolarization, is responsible of the release of endogenous endocannabinoids that bind CB1 receptor localized on the presynaptic membrane, with consequent reduction of the neurotransmitter release.

After demonstrating that BZY is able to develop and maintain self-administration behavior, we investigated the *in vitro* effects of BZY of the glutamatergic cortico-accumbens synaptic activity, when it was added at the final concentration to the perfusion aCSF. It was used, added in perfusion fluid, either dopamine antagonist SCH23390 and endocannabinoid antagonist am-251 to investigate the involvment of these two neurotrasmission systems.

7.3 Materials and methods

7.3.1 Animal

The study was conducted using 23 male Sprague Dawley adult rats housed in a dedicated temperature- (20±2 °C) and humidity- (70%) controlled rooms, with free access to food and water, under a 12 h light/ 12 h dark cycle (light on at 7 am).

Drug-experienced rats (n=5, P80/90, previously undergone alternate days cocaine and heroin self-administration) were given 5-7 days of rest in their home cages before they were sacrificed to collect brain slices.

All procedurs were in accordance with the Italian Law on Animal Reserch (directive 2010/63/EU of the European Parliament and of the Council) and with the guidelines for the care and use of laboratory animals issued by the Italian Ministry of Health.

7.3.2 Drugs

- **Halothane** (Sigma Aldrich®).
- **Benzydamine hydrochloride** (30 µM e 100µM, Sigma Aldrich®).
- **Picrotoxin,** GABA$_A$ antagonist (100 µM, Abcam).
- **AM251**, CB1 receptor antagonist (2 µM, Abcam).
- **SCH23390,** D$_1$ receptor antagonist (1 µM, Abcam)

7.3.3 Slice preparation

Each animal was completely anesthetized with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) and decapitated. Parasagittal NAc (Nucleus Accumbens) slices (300 µm thick) were prepared from both hemispheres using a Leica 1200 T vibratome. Throughout the procedure, the tissue was maintained in a room temperature choline cutting-solution (KCl 2,5 mM, NaH2PO4 1,25 mM, MgSO4 10 mM, CaCl2 0,50 mM, Choline 120 mM, NaHCO3 26 mM, Glucose 10 mM), which was bubbled continuously with 95% O2 – 5% CO$_2$. Slices were stored at least 1 hour at 35°C in artificial Cerebro-Spinal Fluid, aCSF (NaCl 126 mM, KCl 1.25 mM,

NaH2PO4 1.25 mM, MgSO4 2 mM, CaCl2 2 mM, NaHCO3 26 mM, Glucose 10 mM), equilibrated with a 95% O2 – 5% CO2 mixture.

7.3.4 Field recordings

After the recovery period, slices were transferred to a recording chamber and submerged beneath continuously flowing (3/3.5 mL/min) aCSF at a temperature of 30-32 °C. Because of the presence of strong GABAergic inhibition on the NAc, picrotoxin (100µM) was added in the aCSF for all experiments.

Extracellular recordings were obtained using glass micropipettes filled with aCSF, from the region of the Nucleus Accumbens core, using the anterior commissure and lateral ventricles as anatomical markers. Field EPSPs (excitatory post-synaptic potentials) were evoked by prelimbic cortical afferents stimulation, (square wave current of 25µs duration erogated at a frequency of 0.03 Hz), placing a bipolar electrodes at the prefrontal Cortex-Accumbens border.

Picture 16 *Parasagittal slices of rat brain (the rat brain atlas of Paxinos and Watson). During field recordings, the recording microelectrode was placed in the nucleus accumbens while the stimulation electrode was placed on the boundary of the accumbens, to stimulate the glutamatergic efferents from the prefrontal cortex.*

All recordings were performed using a Multiclamp 700b amplifier (Axon Instruments), filtered a 2 kHz and digitized at 10 Khz, converted by a Digidata 1322A (Axon Instruments),collected and analyzed using a Clampex 9.2 software.

*Picture **17** Images of electrophysiological setup that has been used for the recordings reported in this work.*

The stimulus was applied every 30 sec. After the stabilization of the evoked fEPSP, control baseline was recorded for 15 minutes, at 50-60 % of the maximal response. After baseline, to test whether BZY could induce *per se* a long-term change of the glutamatergic cortico-accumbens synapses, BZY 30 µM was added at the final concentration to the perfusion aCSF and the amplitude of the fEPSP was recorded for 30 minutes. Slightly different the protocol used for BZY 100 µM: the slice was perfused for 10 minutes with aCSF added with BZY and then the amplitude recorded for 30 minutes in wash condition. This because BZY 100 µM caused a severe depression of the synaptic response. To exclude that this severe depression of the synaptic response could be due to non-specific toxic effect of higher doses of BZY (100 µM), current pulses of higher intensity were delivered at the end of the experiments.

To investigate short-term regulation of neurotransmitter release by BZY, paired stimuli were delivered at 120 ms interstimuls intervals and the paired pulse ratio (PPR) was measured as the ratio of the amplitude of the second on the first postsynaptic response.

7.4 Data analysis and statistics

For electrophysiological experiments, the experimental comparison was between the magnitude of the baseline responses and the magnitude of the responses in BZY. ANOVA for repeated measures was used with time as the within factor. Data from paired pulse experiments were analyzed using paired samples t-test. Data were analyzed using IBM SPSS 20 statistical software.

Chapter 8

Results

8.1 Benzydamine self-administration
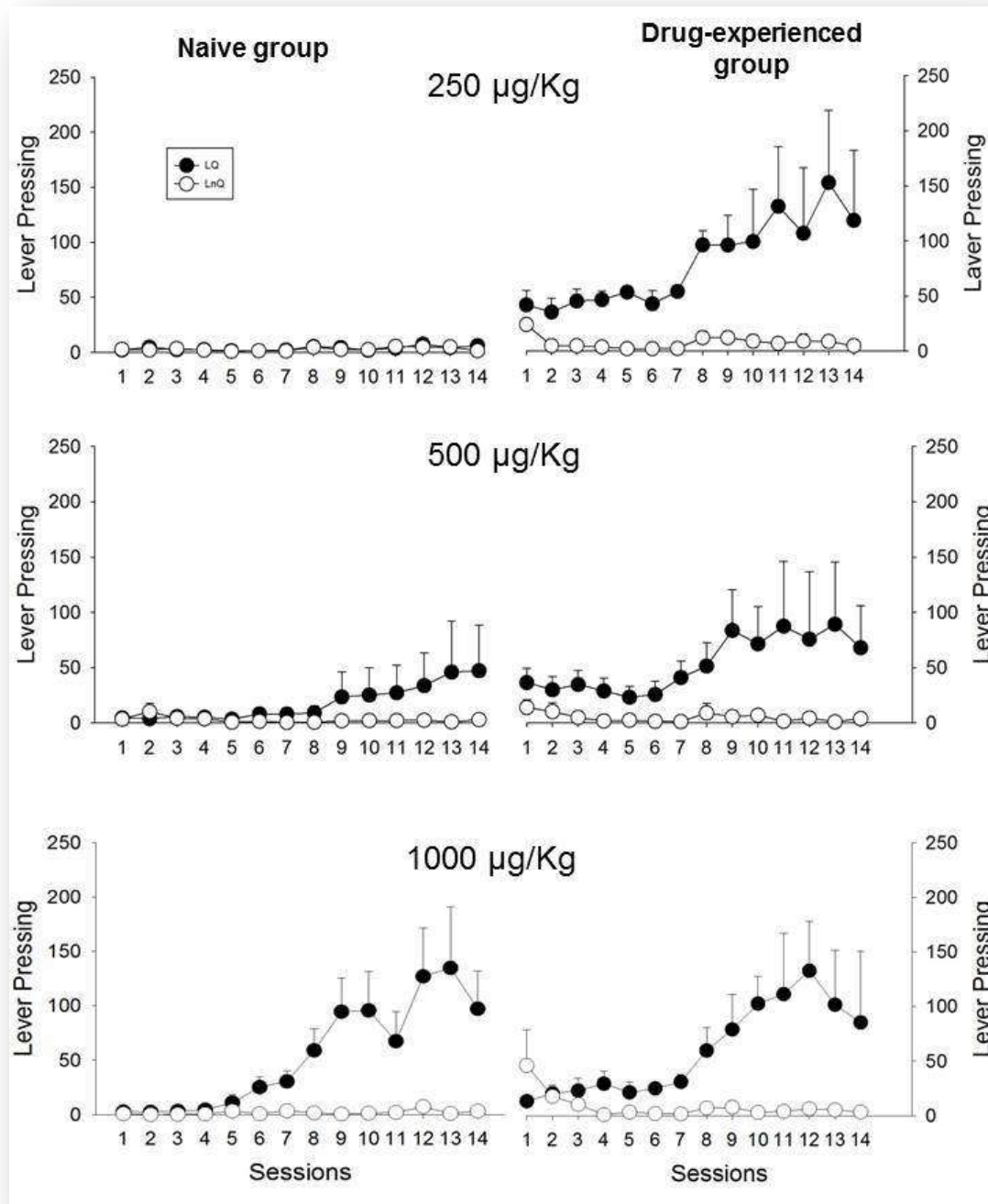
Picture 20 Mean number (±SEM) of lever presses on the active and inactive levers for rats of naïve- and experienced group. Independent sub-groups of rats, for both experimental groups, self-administered 250, 500, or 1000 µg/kg per infusion (top, middle, and bottom panels, respectively). The FR was progressively increased: FR1 (sessions 1–7) FR2 (session 8), FR3 (session 9), FR4 (session 10) and FR5 (sessions 11-14)

For the behavior, the animals of both group, naïve and drug experienced, self-administered three different doses of BZY (250 µg/kg; 500 µg/kg; 1000 µg/kg, during a period of training of 14 days, with an increase of the fixed ratio from 1 to 5 (FR1 1th - 7th days; FR2 8th day; FR3 9th day; FR4 10th day;FR5 11th – 14th day). The rats of both group acquired BZY self-administration behavior, as it's shown in the panels of the picture 20, in which the number of lever pressing on the active lever is higher than the number of presses on the inactive one, as a function of FR. The three-way ANOVA yielded, for both group, significant effects of sessions (naïve [$F_{(5,809)}$ $p<0.001$] drug experienced [$F_{(4,014)}$ $p<0.001$]), lever (naïve [$F_{(8,555)}$ $p<0.017$] drug experienced [$F_{(20,110)}$ $p<0.002$]) and the interaction lever*session (naïve [$F_{(5,940)}$ $p<0.001$] drug experienced [$F_{(5,334)}$ $p<0.001$]). Regarding the interactions session*training dose and lever*session*training dose, the three-way ANOVA (for repeated measure on the factor session) shows, for naïve group, a dose-dependent acquisition of lever pressing behavior (session*training dose [$F_{(2,602)}$ $p<0.001$]; lever*session*training dose [$F_{(2,635)}$ $p<0.001$]), while drug-experienced rats do not display it, working on lever pressing without any statistically significant difference between dose groups.

In the picture 21 we compare the number of lever pressing for both groups, for all doses; a three-way ANOVA (for repeated measures on the variable session) pointed out a significant effect of session [$F_{(9,675)}$ $p<0.001$]. There is not significant session*experience*training dose interaction. As we can observe, at the lower dose (250 µg/kg) only the animals from the drug experienced group show the self-administration behavior, while it is almost absent in the naïve group. Instead, at the higher dose (1000 µg/kg), there is the overlap between the behavior displayed by the animals of the two groups. A four-way ANOVA yielded a significant effect of experience [$F_{1,21}=7.721$; $p=0.013$] and lever*experience interaction [$F_{1,21}=5.629$; $p=0.03$] on the lever pressing behavior. This effects states that the drug experienced rats' operant behavior is more sustained as well as more readily acquired. Variability observed in the late training phase frames a fluctuating self-administration behavior interspersed with days of significantly reduced intake. It's interesting to note that rats which self-administered a great amount of BZY (i.e.

about 20 mg/Kg in a single session) showed hypothermia and hypotaxia at the end of the session, when they were disconnected from the infusion line and returned in their home cages. Unfortunately, we were not equipped to quantitatively measure these parameters. However, days with reduced intake could depend on the stacking of these undesired adverse effects, thus resulting in an intermittent self-administration pattern, particularly clear for the higher dose group.
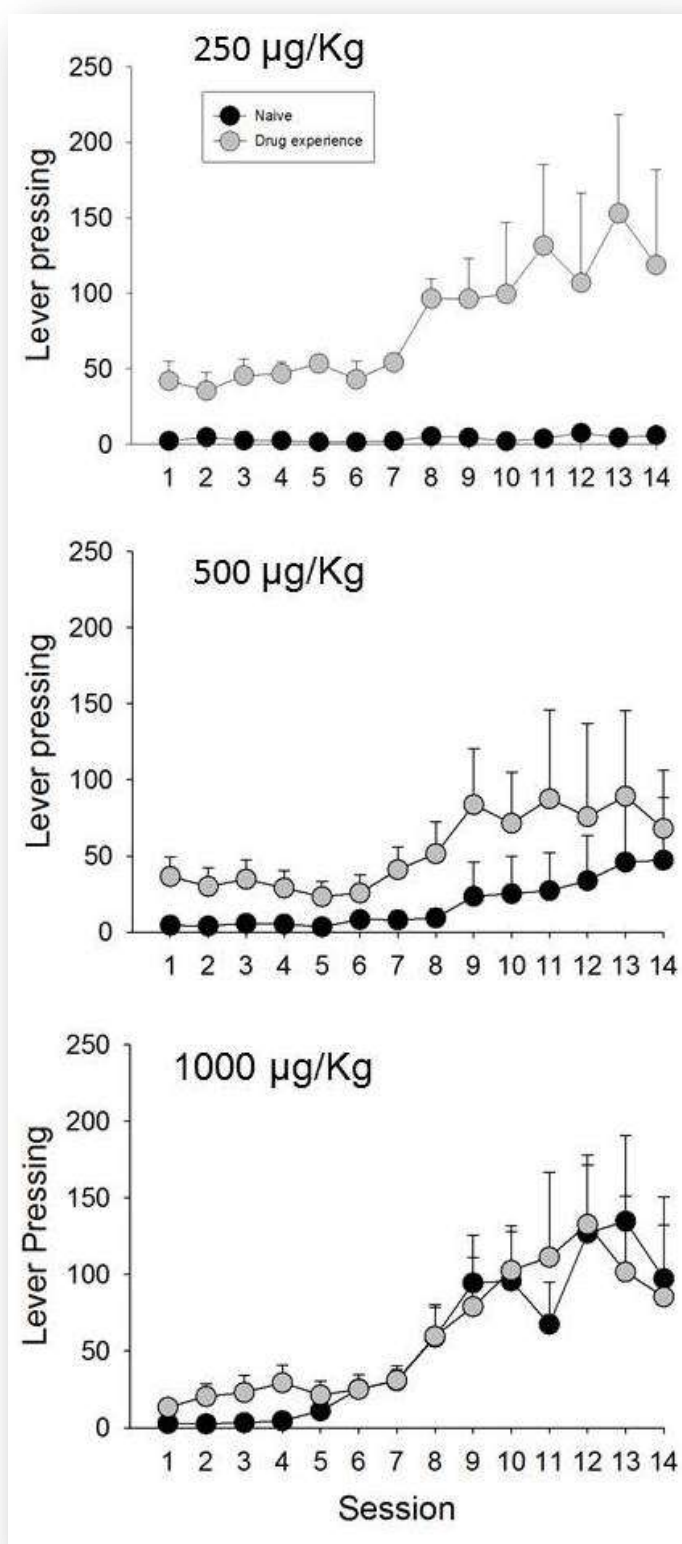
Picture 21 Comparison of mean number (±SEM) of lever presses on the active lever for rats of naïve- and experienced group , self-administered 250, 500, or 1000 µg/kg per infusion (top, middle, and bottom panels, respectively). The FR was progressively increased: FR1 (sessions 1–7) FR2 (session 8), FR3 (session 9), FR4 (session 10) and FR5 (sessions 11-14)
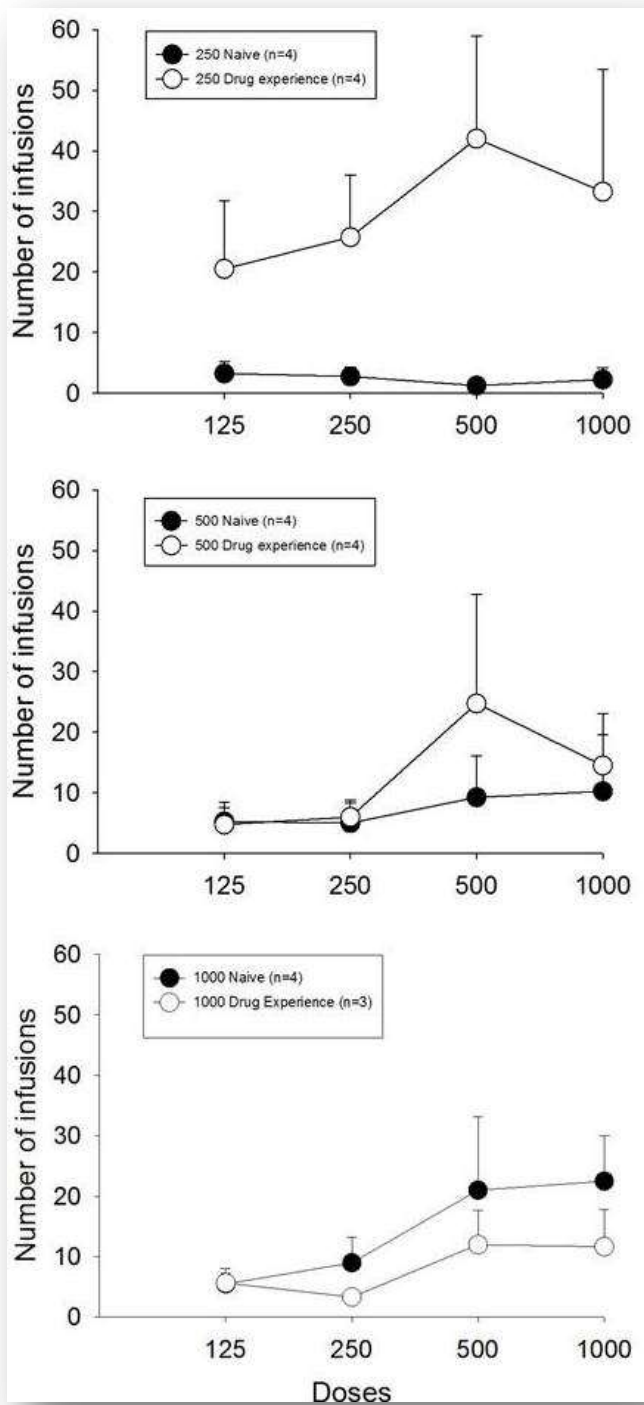
Picture 22 shows the mean number of infusions during the sessions 15-18 for the dose-response curve. Data were analyzed using a three-way ANOVA (with repeated measures as within factor and the experience and the training dose as between factors). The ANOVA conducted on the infusion data indicated significant effects of session [$F_{(3,987)}$ $p<0.013$] but no for interaction session*training dose ($p<0.1$) and interaction session*experience ($p<0.1$).

Picture 22 Dose-response curve for the acquisition of Benzydamine self-administration behavior. Each symbol indicates the mean number (±SEM) of infusions of Benzydamine on a FR5 schedule of reinforcement (i.e., mean values calculated for sessions 15-18)

8.2 Field recordings

Bath application of 30 µM BZY for 30 minutes and 100 µM BZY for 10 minutes dose-dependently decreased cortico-NAcc fEPSP amplitude by 36,1±6,8% (from 0,77±0,06 mV to 0,51±0,09 mV, n=7) and 64,8±5,7% (from 0,75±0,07 mV to 0,27±0,05 mV, n=7), respectively (Fig. 22 a-b).
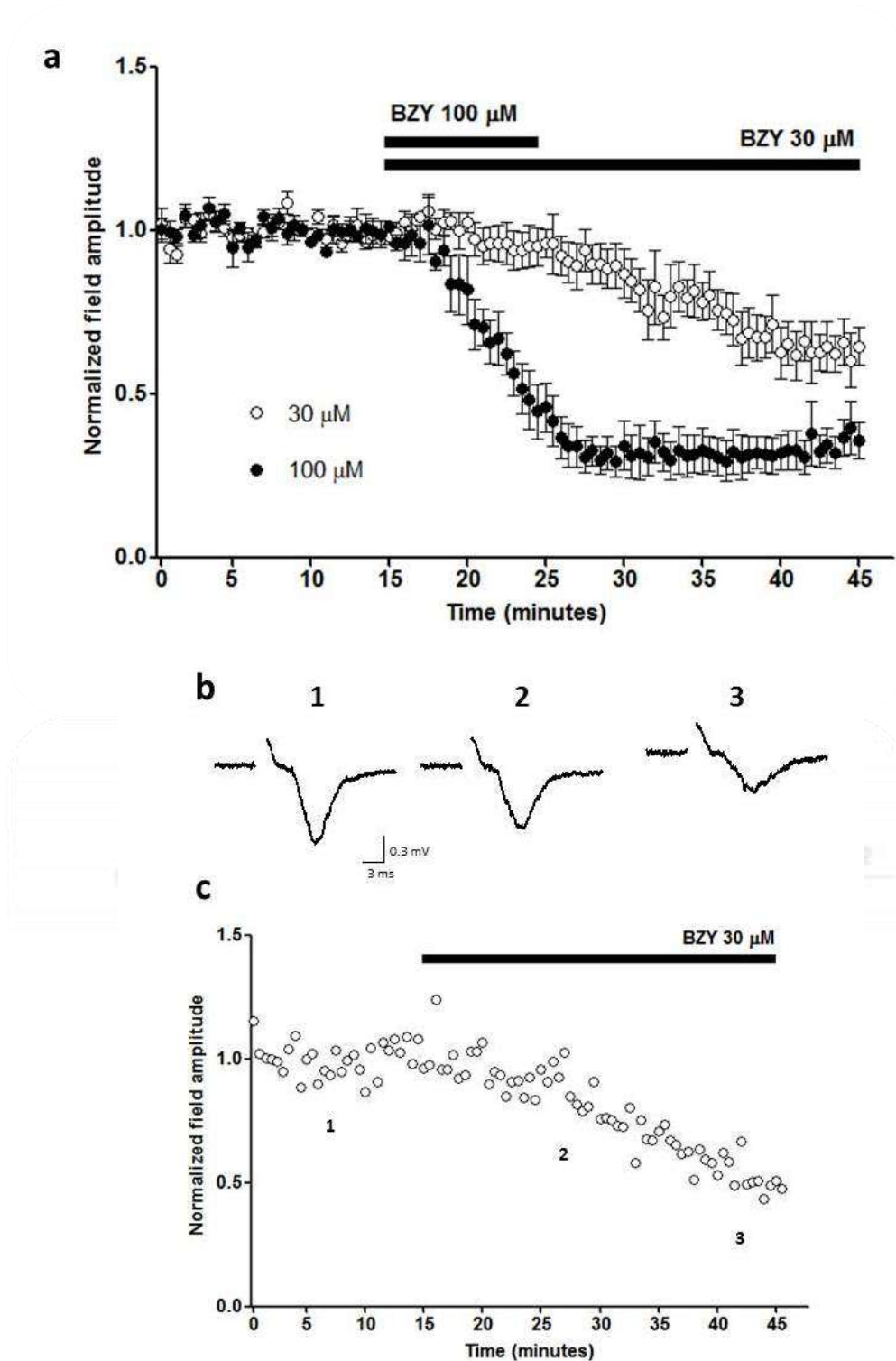
Picture 23 *Effect of BZY perfusion on cortico-accumbens glutamatergic fEPSP. a-b) Sample traces at different time points of the time-course plot of a representative BZY 30 µM.experiment c) Time plot of normalized fEPSP amplitude during perfusion of BZY 30 µM (n=7 slices/3 animals, 30 min) and BZY 100 µM (n=7/3, 10 min).*

Repeated measures ANOVA run on data from minutes 15 to 45 showed a significant main effect of time [$F_{59,708}=20,819$; $p<0,001$], concentration [$F_{1,12}=42,437$; $p<0,001$], and their interaction [$F_{59,708}=4,340$; $p<0,001$]. To exclude that this severe depression of the synaptic response could be due to non-specific toxic effect of higher doses of BZY (100 µM), current pulses of higher intensity were delivered at the end of the experiments. In this experimental condition the depressed fEPSPs recovered to the baseline amplitude values (data not shown).

We next investigated short-term regulation of neurotransmitter release by measuring PPRs. In control condition, extracellular pair of stimuli elicited a paired pulse depression, a reduction of the second synaptic response relatively to the first (fEPSP2/fEPSP1) (Fig. 23). BZY (30 µM) decreased PPR (from $0,85\pm0,05$ baseline to $0,59\pm0,03$ after 30 min perfusion; n=7; t=4,978; p=0,003) suggesting a presynaptic site of action. BZY (100 µM) completely abolished the fEPSP in many experiments, thus it was not possible to consider PPR measured in these experiments as meaningful (the resulting ratio yielded PPR values near to 1, data not shown).
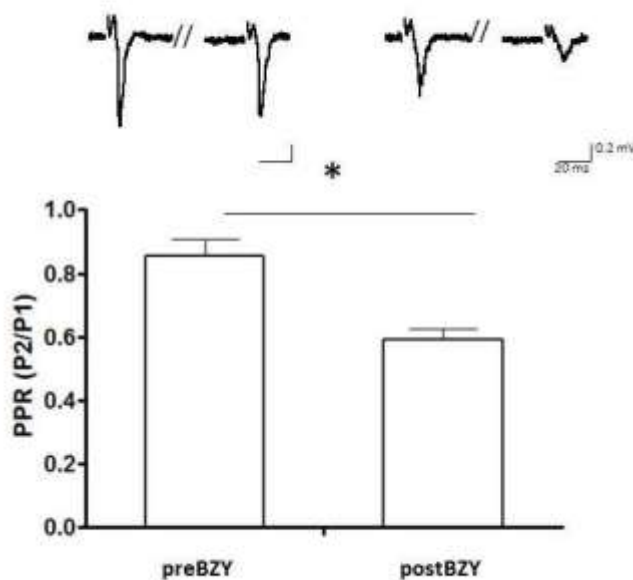

Picture 24 *Paired Pulse Ratio (PPR) values measured right before and after 30 minutes of BZY 30 µM. BZY induced a significant reduction in PPR at cortico-accumbens synapses. Representative traces are displayed above the histogram.*
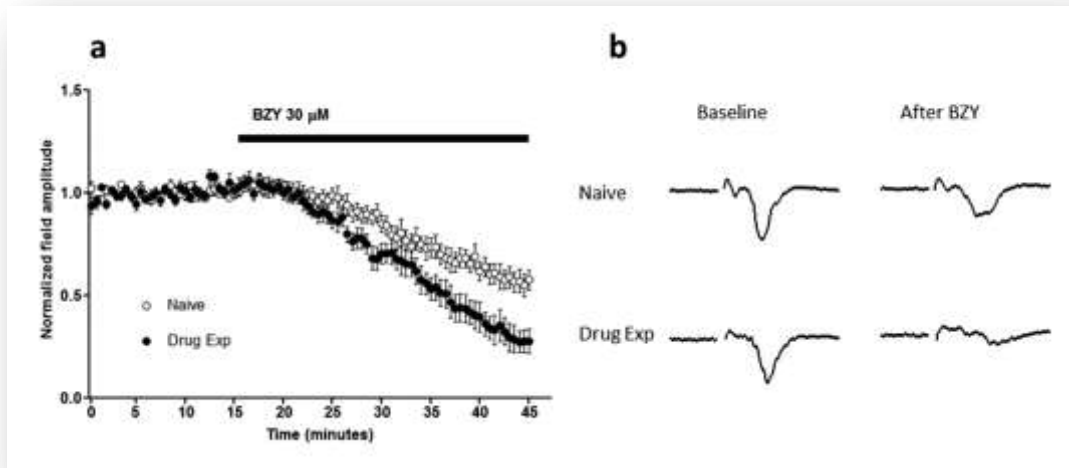
Picture 25 *Electrophysiological effect of BZY is enhanced in drug-experienced rats' brain slices. a) BZY-induced fEPSP depression in brain slices from naïve (n=7 slices/4 animals) and drug experienced rats (n=10/5). b) Representative traces from experiments on brain slices collected from naïve or drug experienced rats. Traces were obtained by averaging 4-5 sweeps at each experimental conditions (baseline and during BZY perfusion). Values are displayed as mean ± SEM.*

The BZY-induced depression of fEPSP was significantly enhanced in drug-experienced animals compared to naïve rats (Fig 24). Two way mixed ANOVA for repeated measures unveiled a significant main effect of experience [$F_{1,21}=9{,}237$; $p=0{,}006$] and experience*time interaction [$F_{59,1239}=3{,}573$; $p=0{,}01$], thus depicting a sensitization of cortico-accumbens synapses to acute effects of BZY perfusion, in drug-experienced rats.
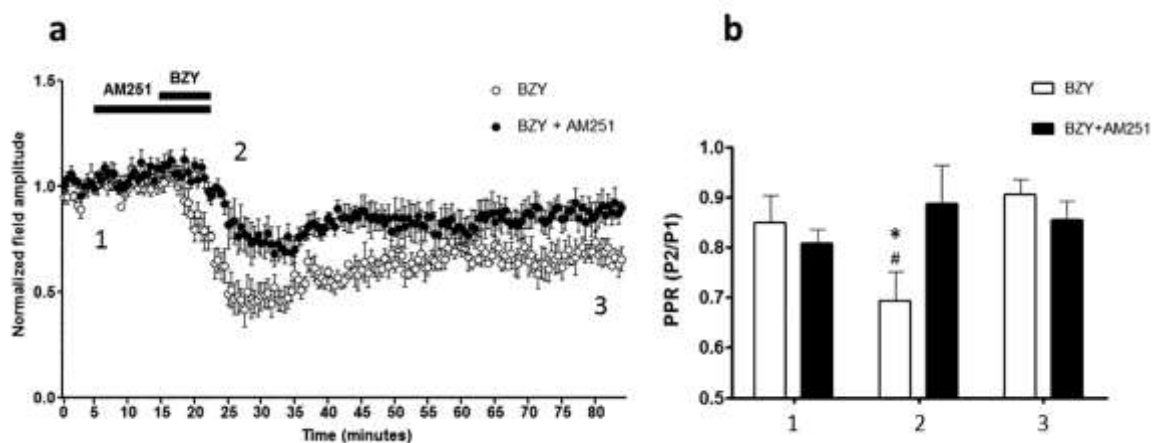
Picture 26 *BZY is able to induce stable LTD at the cortico-accumbens synapses. a) Mean normalized fEPSP amplitude in response to BZY 100 µM perfusion for 8 min (n=5 slices/3 animals) in control condition (open circles) and in the presence of the CB1 antagonist AM251 2 µM (filled circles). Note that the co-perfusion significantly reduces the BZY-induced LTD (n=5/5). b) Paired Pulse Ratio values measured at different time points (as depicted by numbers) show an induction effect that is denied by the presence of CB1 antagonist AM251. * different from BZY baseline (1); # different from BZY end of recording (3). Values are displayed as mean ± SEM.*

As shown in Picture 23, BZY induced a significant reduction in PPR at cortico-accumbens synapses suggesting a presynaptic mechanism. Similarly, endocannabinoids presinaptically reduced glutamate release and mediated LTD at glutamatergic synapses on spiny neurons within the basal ganglia nucleus.

As shown in Picture 25a, bath perfusion of 100 µM BZY for 8 minutes (longer time resulted in a drastic reduction of the fEPSP) reduced fEPSC amplitude by 32,8±2,9% in the last thirty minutes of recordings (from 0,65±0,04 mV to 0,43±0,02 mV; n=5; t=7,265; p=0,002). Interestigly, stable LTD-like response induced by BZY was consistently reduced by the CB1 receptor antagonist AM251 (2 µM) [main effect of treatment, $F_{1,8}=10,472$; p=0,012]. In the presence of AM 251, BZY depressed fEPSP by 14,8±5,8% (from 0,75±0,05mV to 0,65±0,08 mV; n=5; t=1,814; p=0,144) and the BZY-induced reduction of PPR was fully counteracted (from 0,85±0,05 baseline to 0,69±0,06 during BZY alone, t=4,384, p =0,012; from 0,80±0,03 baseline to 0,88±0,07 BZY in AM251, p=0,342). AM251 did not affect PPR per se (0,81±0,03, p=0,825 vs baseline PPR, data not shown). This result suggests that BZY and CB1 agonists may share common mechanisms to inhibit excitatory synaptic transmission. Further experiments will be performed to better characterize BZY/endocannabinoid interaction.

Chapter 9
Discussion

T he present study represents the first investigation of the reinforcing properties of the non-steroidal anti-inflammatory drug BZY in an animal model of drug abuse and the plastic effects on the glutamatergic plasticity in the cortico-accumbens synapses.

Abuse of BZY is a new but not well-known phenomenon, popular in Brazil and in other few Countries and used expecially in the streets context or to substitute other drugs, temporarily not available.

BZY self-administration in the rat model confirms the positive reinforcing effects that support the human abuse. We report that, naïve rats (without any drug experience or previos self-administration training), self-administered BZY only at high doses while at lower doses there is not a significant difference between the number of presses on the active lever versus the inactive one. On the contrary, the animals with a history of self-administration of heroin and cocaine showed a facilitate acquisition of the self-administration behavior of BZY, also at lower dose.

The differences we found at 250 μg/kg. between the groups may be explained considering the previous experience with drugs of abuse. Drugs-experienced animals, in fact, previously acquired self-administration behavior and could be more familiar with the lever pressing task. However, it may be a consequence of the phenomenon of cross-sensitization between cocaine and heroin effects and BZY effects. Generally, sensitization is considered as an enduring increase in the response to drugs as a consequences of the neuroadaptations elicited by repeated exposure (Stewart and Badiani, 1993). Behavioral sensitization that appears to results, at least in part, from the experieced-induced adaptations seems to be a consequence of the motivational value attributed to cue paired with drugs of abuse and also natural rewards (the mesocorticolimbic dopamine system; see Chapter 3). Cross-sensitization is a particular type of sensitization considered the neurobiological process whereby one type of treatment may potentiate the response to another. Several studies have proved the consistency of cross-sensitization phenomenon either between different classes of drugs of abuse and

also between natural reward and psychoactive substances (Robinson and Berridge, 1993; Fiorino and Phillips 1999; Nocjar and Panksepp, 2002; Vitale et al., 2003). Roitman et al. (2002) have demonstrated an increase in dendritic length and branching in nucleus accumbens of animal that had undergone a chronic sodium depletion that were comparable with those previously described in rodent brains sensitized to amphetamine. Moreover, it has been shown that the effects of acute and repeated stress can potentiate the effects of cocaine and other addictive drugs througha cross-sensitization phenomenon: stress, in fact, may increse drug-induced mesocorticolimbic dopamine transmission as well as rewarding and locomotor activating effects of drugs (Lu et al., 2003; Lett, 1989; Capriles and Cancela, 1999; Vezina et al., 2002; Capriles et al., 2003; Saal et al., 2003; Wang et al., 2005). Similaly, it has been reported that pre-exposure to opioids can induce long-lasting sensitization to opioids and psychostimulants and viceversa, pretreatment with amphetamine and cocaine results in long-lasting cross-sensitization between psychostimulants (DuMars et al., 1988; Vandershuren et al., 1997, 1999; Kalivas and Weber, 1988; Pierce and Kalivas, 1995). The behavioral results may suggest that also in human, the subjects more prone to BZY consumption are those which had previous experienced other drugs.

Extracellular field recording revealed that BZY caused a dose-dependent long-term depression (LTD), in both, naïve and drug-experienced rats. In these latters, BZY-mediated effects were greater. possibly due to a cross-sensitization phenomenon (see above). Indeed, we hypothesized that previuos treatment with heroin and cocaine may have caused deep neuroadptations further exacerbated by BZY. Similarly to BZY, dopamine, cocaine and amphetamine reduced the evoked excitatory synaptic responses at prelimbic cortical-nucleus accumbens synapses. This effect was due to the activation of D1 receptors (Nicola and Malenka, 1996) However, in our esperiments, D1-like receptor antagonist SCH23390 did not affect BZY-induced reduction of the evoked responses (data not shown). Likewise, Pennartz and collaborators showed that both LTD and LTP were independent of dopamine receptors, although they used an electrical stimulation of the prelimbic cortical afferents and not a pharmacological procedure (Pennartz et al., 1993; Kombian and Malenka, 1994).

The reduction of the paired pulse ratio (PPR) suggest that the BZY-induced reduction of cortico-accumbens glutamatergic neurotrasmission is mediated by a presynaptic mechanism. It is known that also the endogenous cannabinoids, acting on CB1 receptors, reduce spontaneous excitatory synaptic transmission, thorugh a presynaptic mechanism, by the activation of K^+ conductances (Robbe et al., 2001; Robbe et al., 2002). Coherently, immunohistochemical techniques identified the expression of CB1 receptors on cortical glutamatergic afferents suggesting a presynaptic mechanism. In our study we found that AM-251, a CB1-like receptor antagonist, significantly reduced BZY-induced LTD. BZY may stimulate endocannabinoid synthesis and release, which in turn reduced glutamate release from the cortical terminal. Notably, WIN 55,212,2 induced persistent and long lasting field amplitude depression in the nucleus accumbens (Robbe et al., 2002). Coherently with these observations, amphetamine-evoked acute depression in the amygdala was in large part mediated by CB1 receptors. In fact, CB1 receptor antagonist AM-251 blocked amphetamine-induced LTD, while the same phenomenon was mimicked by WIN 55, 212, 2. (Huang et al., 2003).

Examining the effects of BZY reported in several online forums leap out also hallucinogenic properties of this drug. Therefore, BZY, like other serotoninergic drugs, i.e. LSD, may have a serotoninergic activity throught the $5HT_{2a}$ receptor . This hypothesis is supported by the molecular structure of BZY, in which is presented the indole compound, tipical of the serotonine and other psychoactive molecules. Likewise, BZY share a common molecular structure with several CB1 synthetic agonists (i.e. JWH series compounds), which proved to have hallucinogenic proprieties as well (Forrester, 2012; Harris and Brown, 2013). However, while there is no evidence for animal self-administration hallucinogenic substances, meaning that serotonin may not be reinforcing (Deneau et al., 1969; Yanagita, 1986), we showed that BZY is able to develop and mantain self-administration behavior in rats. Therefore BZY may share a common mechanism with amphetamine derivatives rather than the serotoninergic hallucinogens. Indeed, numerous studies reported self-administration of MDMA in animals (Fantegrossi et al., 2002; Lamb et al., 1987; Schenk et al., 2003, 2007; Sannerud et al., 2006).

In summary, our data demonstrate a BZY-induced long-term depression of PFc to NAcore synapses in mediating BZY self-administration behavior, thereby

supporting the postulated contribution of PFc neuroadaptations in addictive behavior. Specifically, we report that, BZY, like cannabinoids and amphetamine, presynaptically induced long-term depression by the interaction with the endocannabinoid system. In future studies it will be interesting to dissect BZY molecular mechanism for a better understanding of its abuse potential and its toxicological profile. In particular, a further aspect that needs to be investigated is the involvement of metabotropic glutamate receptor (mGlu) subtypes, widely expressed at the glutamatergic afferents to the Nac (Ohishi et al., 1993a; Shigemoto et al., 1993; Manzoni et al., 1997). In particular we will focus on the mGlu 2/3 in view of its key roles in controlling LTD in the hippocampus, amygdala, prefrontal cortex and dorsal striatum (Kahn et al., 2001; Kobayashi et al., 1996; Yokoi et al., 1996; Tzounopoulos et al., 1998; Huang et al., 1997, 1999; Lin et al., 2000; Otani et al., 1999).

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Meringolo M, Brusadin V, De Luca MT, Montanari C, Antonilli L, Nencini P, Badiani A. Induction of morphine-6-glucuronide synthesis by heroin self-administration on the rat. Psychopharmacology (Berl). 2012 May; 221 (2): 195-203.

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Reprints of published articles

The work of my dissertation is not published yet. However, during my PhD I have worked on other research projects, three of them published.

My first work was published on Psychopharmacology (221: 195-203) in the 2012, with the title ' induction of morphine-6-glucuronide synthesis by heroin self-administration in the rat'. In this study we investigated morphine-6-glucuronide synthesis (a metabolite of the heroin) in rats, using a model of intravenous self-administration of heroin.

The second work, 'the role of setting for ketamine abuse: clinical and preclinical evidence', was published on 'Gruyter journals' in the 2012. It is a review on preclinical and clinical evidence of the key role the setting play in ketamine abuse.

In the third work we investigated the influence of setting on the ability of different doses of cocaine and heroin to priming cocaine vs. heroin seeking in rats that had been t rained to self-administer both drugs and had then undergone an extinction procedure.

Induction of morphine-6-glucuronide synthesis by heroin self-administration in the rat

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Maria T. De Luca · Christian L. Montanari ·
Letizia Antonilli · Paolo Nencini · Aldo Badiani

Abstract

Rationale Heroin is rapidly metabolized to morphine that in turn is transformed into morphine-3-glucuronide (M3G), an inactive metabolite at mu-opioid receptor (MOR), and morphine-6-glucuronide (M6G), a potent MOR agonist. We have found that rats that had received repeated intraperitoneal injections of heroin exhibit measurable levels of M6G (which is usually undetectable in this species).

Objective The goal of the present study was to investigate whether M6G synthesis can be induced by intravenous (i.v.) heroin self-administration (SA).

Materials and methods Rats were trained to self-administer either heroin (50 μg/kg per infusion) or saline for 20 consecutive 6-h sessions and then challenged with an intraperitoneal challenge of 10 mg/kg of heroin. Plasma levels of heroin, morphine, 6-mono-acetyl morphine, M3G, and M6G were quantified 2 h after the challenge. In vitro morphine glucuronidation was studied in microsomal preparations obtained from the liver of the same rats.

Results Heroin SA induced the synthesis of M6G, as indicated by detectable plasma levels of M6G (89.7±

37.0 ng/ml vs. 7.35±7.35 ng/ml after saline SA). Most important, the in vitro V_{max} for M6G synthesis was correlated with plasma levels of M6G (r^2=0.78). Microsomal preparations from saline SA rats produced negligible amounts of M6G.

Conclusion Both in vivo and in vitro data indicate that i.v. heroin SA induces the synthesis of M6G. These data are discussed in the light of previous studies conducted in heroin addicts indicating that in humans heroin enhances the synthesis of the active metabolite of heroin and morphine.

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Introduction

In humans and other mammals, heroin is rapidly transformed, after absorption, in 6-monoacetylmorphine (6-MAM), which is further deacetylated to morphine. The metabolism of morphine mainly consists of the glucuronidation to either morphine-3-glucuronide (M3G) or to morphine-6-glucuronide (M6G) (Milne et al. 1996). Heroin metabolites are widely thought to be responsible for the neuropsychopharmacological effects of the parent compound (Gutstein and Akil 2006).

Contrary to M3G, M6G is a potent agonist at mu-opioid peptide receptors (MORs) (Ulens et al. 2001; Penson et al. 2000; Christrup 1997), and there is some evidence that, like heroin, it acts at a MOR-1 splice variant that has little affinity for morphine (Pan et al. 2009). Although M6G is less lipophilic than the parent compound and does not easily cross the blood–brain barrier (Meineke et al. 2002), it

does reach the central nervous system (CNS) and its CNS clearance is significantly lower than systemic clearance (Lotsch 2005; Tunblad et al. 2005). Furthermore, the distribution of M6G in the rat brain is mostly extracellular, suggesting that after morphine administration, M6G concentrations at MOR are not far from those of the parent compound (Stain-Texier et al. 1999). Indeed, there is now substantial evidence that M6G contributes to both the analgesic and toxic effects of morphine. Morphine-6-glucuronide has been shown, for example, to produce analgesia when administered systemically (Romberg et al. 2004, Skarke et al. 2003) and is now under development as a therapeutic agent (Binning et al. 2011). Furthermore, central nervous system-depressant effects produced by repeated morphine administrations in patients with renal failure have been attributed to increased high blood levels of M6G due to impaired excretion (Pauli-Magnus et al. 1999; Peterson et al. 1990). M6G may also be implicated in the well-known individual differences in the responsiveness to morphine. The unusual resistance to morphine overdosing exhibited by some nephropatic patients, for example, has been attributed to a single-nucleotide polymorphism of the MOR gene, resulting in reduced responsiveness to M6G but not to morphine (Lotsch et al. 2002).

There is also some evidence that M6G plays a role in heroin reward (Walker et al. 1999), and thus it is possible that this metabolite is implicated in the natural history of heroin addiction. We have previously found that plasma and urine of heroin addicts contain more M6G and less M3G than those of heroin-naive individuals treated with morphine for pain control (Antonilli et al. 2003a)—which is quite remarkable, given that morphine exposure even at high doses and for long periods of time does not appear to influence M3G or M6G synthesis (Faura et al. 1998; Vermeire et al. 1998; Andersen et al. 2004). This has led us to hypothesize that the increased synthesis of M6G may contribute to the vulnerability to heroin addiction. Of course, this possibility cannot be easily explored in human addicts and calls for the use of animal models.

Intravenous drug self-administration in the rat is widely considered as a robust animal model of drug taking (Markou et al. 1993). Rats are generally thought to produce no M6G (Milne et al. 1996). Yet, relatively small amounts of this metabolite have been detected in adult rats (Wang et al. 2005). Most important, we have shown that repeated non-contingent intraperitoneal (i.p.) injections of high doses of heroin (but not of morphine) can induce the synthesis M6G in the rat (Antonilli et al. 2003b, 2005). Furthermore, microsomal preparations obtained from the livers of heroin-treated rats yielded, when incubated with morphine, measurable quantities of M6G, which was not detectable in microsomal preparations from rats treated with saline (Antonilli et al. 2003b, 2005).

These preliminary findings suggest that the rat may represent a viable model of heroin abuse even with respect to M6G synthesis. The major aim of the present study was to verify this possibility by investigating in vivo and in vitro synthesis of M6G after intravenous (i.v.) heroin self-administration in the rat.

Materials and methods

Animals

The study was conducted using 15 male Sprague–Dawley rats (Harlan Italy, San Pietro al Natisone, Italy) weighing 275 g at their arrival in the laboratory. Notice that one additional rat was tested but was excluded from the analyses because of catheter occlusion. Throughout the experiment, the rats were housed and tested in a dedicated temperature-controlled and humidity-controlled room, with free access to food and water (except during the test sessions) under a 14-h dark/10-h light cycle (lights off at 0700 hours). After their arrival, the rats were housed two per cage for 7–10 days before the surgery. After the surgery, the rats were housed individually. All procedures were in accordance with the Italian Law on Animal Research (DLGS 116/92) and with the guidelines for the care and use of laboratory animals issued by the Italian Ministry of Health.

Surgery

The catheter consisted of 10.5 cm of silicone tubing (0.37-mm inner diameter, 0.94-mm outer diameter) sheathed at 3.4 cm from its proximal end by a 5-mm-long heat-shrink tubing. On the day of surgery, the rats received an i.p. injection of 2.33 mg of xylazine hydrochloride (Rompun®, Bayer HealthCare) and an intramuscular injection of 14,000 IU of benzylpenicillin (Fournier Pharma, S. Palomba, Italy). The rats were then anesthetized with an i.p. injection of 0.56 ml/kg of Zoletil 100® (Virbac, Carros, France), containing tiletamine (50 mg/ml) and zolazepam (50 mg/ml). By using standard surgical procedures, the catheter was inserted into the right jugular vein, so as to reach the right atrium with its proximal end, and was then secured to the surrounding soft tissues with silk thread. The distal end of the catheter was passed subcutaneously in front of the left shoulder, externalized through a small incision at the nape of the neck, and connected to an L-shaped 22-gauge cannula. The cannula was then secured to the rat's skull using dental cement and stainless steel screws. After surgery, the rats were given 15 mg i.v. enrofloxacin (Baytril®, KVP Pharma + Veterinär Produkte Gmbh, Kiel, Germany). Catheters were flushed daily (at

1800 hours) with 0.1 ml of a sterile saline solution containing 0.4 mg of enrofloxacin and 25 IU heparin (Marvecs Services, Agrate Brianza, Italy).

Apparatus

The apparatus consisted of SA chambers made of transparent plastic, aluminum, and stainless steel grid floor. Plastic trays covered with pinewood shaving were placed under the grid floors. Each chamber was equipped with two retractable levers, positioned on the left-hand wall 12.5 cm apart and 9 cm above the floor, with cue lights positioned above each lever and a counterbalanced arm holding a liquid swivel. The SA chambers were placed within sound-attenuating and light-attenuating cubicles. Each cage was connected via an electronic interface to a syringe pump (Razel Scientific Instruments, St. Albans, VT, USA) and to a programmable logic controller (PLC; Allen Bradley, Milwaukee, WI, USA), in turn connected to a PC. Chambers, accessories, and electronic interfaces were purchased from ESATEL S.r.l. (Rome, Italy), and custom-developed control software was from Aries Sistemi S.r.l. (Rome, Italy). The infusion line consisted of a length of silastic tubing protected by a stainless steel spring and connected (through the liquid swivel and another length of silastic tubing) to a syringe positioned on the pump (which was programmed to work at an infusion rate of 10 μl/s).

Procedures

After the surgery, the rats were housed in the SA chambers where they remained for the entire duration of the experiment, which consisted of 20 daily sessions. All test sessions lasted 6 h and took place during the dark phase, between 1000 and 1600 hours, 7 days a week. Testing began 1 week after the surgery. Before the start of each session, the syringe pumps were activated, so as to fill the infusion lines, which were then connected to the catheters. During the 60 s preceding the start of each SA session, food and water were removed from the cage. Self-administered drug infusions and primings consisted of 40 μl of drug solution (or vehicle) and were delivered over a period of 4 s. During the SA sessions, the doors of the cubicles were kept closed. At the start of each session, the two levers were extended and remained extended for the entire duration of the session (except during the time-out periods; see the next paragraph). Only one of the two levers was active: that is, it triggered upon completion of the task an infusion of 50 μg/kg of heroin, whereas the other lever had no direct consequences on heroin infusion. Eleven rats self-administered heroin whereas four rats self-administered saline.

The number of consecutive responses required to obtain on a fixed ratio (FR) schedule a single infusion was raised from FR1 (sessions 1–4) to FR2 (sessions 5–20). Upon

completion of the task, both levers were retracted and extended again after 40 s (time-out). The three lights above the active lever were on when the lever was extended and off when the lever was retracted. No other light cue was provided. Pressing on the inactive lever produced no lever retraction but did reset the counter of the active lever. On the first test session, all animals were placed with their forepaws on the active lever (time 0 min), so as to trigger a priming infusion. Priming infusions were administered again at times 60 and 120 min to animals that had not spontaneously self-administered at least one infusion during time periods 0–60 and 60–120 min, respectively. On sessions 2–7, priming infusions were administered at times 5, 60, and 120 min to animals that had not spontaneously self-administered at least one infusion during time periods 0–5, 5–60, and 60–120 min, respectively. On average, the rats received 0.8 primings per session. No primings were administered on sessions 8–20. The rats were allowed to self-administer a maximum of 100 infusions of heroin per session to minimize the risk of overdosing.

The day after the last SA session, all rats received at 1400 hours a challenge of 10 mg/kg of heroin i.p. (as done in previous studies; Antonilli et al. 2003b, 2005) and after 2 h were sacrificed to obtain blood samples for the quantification of heroin, 6-monoacetylmorphine (6-MAM), morphine, M3G, and M6G (see "Microsomal preparations"), and their livers were excised to obtain microsomal preparations (see "Microsomal preparations").

Microsomal preparations

Liver microsomes were prepared as previously described (Antonilli et al. 2003b). Briefly, tissues were minced and rinsed in ice-cold 1.15% KCl and homogenized in three volumes of 100 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged for 20 min at $9,000 \times g$. The supernatant was further centrifuged for 60 min at $105,000 \times g$. The resulting microsomal pellet was resuspended in 100 mM phosphate buffer containing 0.25 M sucrose.

Glucuronidation assays

The morphine glucuronidation assay was performed as described by Wielbo et al. (1993). Microsomal preparations were resuspended in 100 mM phosphate buffer (pH 7.4) to a final protein concentration of 1.0 mg/ml. Microsomes were preincubated for 20 min in 0.05% deoxycholic acid at 4°C to achieve full enzymatic activity. Morphine concentrations ranged from 0.1 to 4 mM for the calculation of M3G and M6G kinetics. The incubation mixture consisted of 2 mM UDP-glucuronic acid (UDPGA), 100 mM phosphate buffer (pH 7.4), microsomes, and morphine (as

substrate) to a final volume of 0.3 ml. The reaction was started adding UDPGA. Sample and blanks (without UDPGA) were incubated in triplicates at 37°C for 30 min. The reaction was stopped with 0.2 ml of ice-cold acetonitrile, and all samples were kept at 4°C for 15 min; then they were centrifuged for 10 min at 5,800×g.

Sample preparation

Supernatants of incubation and plasma samples underwent solid phase extraction on reversed-phase/strong cation-exchange sorbent Strata-X-C (96-well plates, 30 mg) (Phenomenex, Torrance, CA). Cartridges were conditioned with methanol (0.6 ml) followed by water (0.6 ml) and phosphate buffer (0.01 M pH 3.0, 0.6 ml). The sample (0.1 ml) was applied to the column and absorbed by gravity; then the column was washed with phosphate buffer (0.01 M pH 3.0, 0.6 ml) and dried for 30 s. The analytes were eluted with 0.2 ml of NH$_4$OH 1% in methanol. The eluate was evaporated to dryness at 37°C under a nitrogen stream. The residue was dissolved in 0.2 ml of 5 mM ammonium formate buffer (pH 4.0) and stored at 4°C until LC/MS/MS analysis.

Liquid chromatography and mass spectrometry

The HPLC system consisted of a PerkinElmer 200 Series binary pump and autosampler (PerkinElmer, Norwalk, CT, USA) and an SCIEX API2000MS/MS triple quadrupole mass spectrometer (Applied Biosystem-MDS SCIEX, Thornhill, Ontario, Canada). Incubation and plasma samples were injected onto a LiChroCART® Purospher Star RP-18 column (150×4.6 mm i.d., particle size 5 μm) with a LiChroCART® Purospher Star RP-18 precolumn (4×4 mm, particle size 5 μm; Merck). The mobile phase consisted of a linear gradient (3–80% with respect to acetonitrile) formed by combination of 5 mM ammonium formate buffer in water (pH 4.0, eluent A) and acetonitrile (eluent B). Flow rate of the mobile phase was set at 0.8 ml/min. Heroin, 6-MAM, morphine, M3G, and M6G were detected using multiple reaction monitoring (MRM) in positive ionization mode. Selected ion masses of the protonated precursors and fragmented ions (m/z) were 370.1/268.0, 328.1/165.0, 286.3/201.0, and 462.2/286.0 for heroin, 6-MAM, morphine, M3G, and M6G respectively. Chromatographic peaks were integrated using Analyst™ software (version 1.4.1, SCIEX). The detection limits (LOD) and quantification limits (LOQ) for all analytes were 5 and 10 ng/ml, respectively.

Statistical analyses

Plasma levels of heroin, morphine, 6-MAM, M3G, and M6G were analyzed using two-tailed Student t-tests.

Furthermore, M6G data were also analyzed using the Fisher exact probability test, by classifying the rats as M6G synthesizers versus non-M6G synthesizers (i.e., rats with undetectable plasma levels of M6G).

The saturation curves for the formation of M3G and M6G by liver microsomes leveled off at the highest morphine concentrations. K_m (mM), V_{max} (nmol/min/mg protein), and Hill coefficient of M3G and M6G formation were estimated using nonlinear regression analysis (Graph-Pad Prism 3; GraphPad Software Inc., San Diego, CA, USA).

A Hill coefficient greater than 1 indicates that an enzymatic reaction does not follow Michaelis–Menten kinetics; that is, there is positive cooperation in the catalytic activity. In the presence of data satisfying the normality test, group differences for K_m, V_{max}, and Hill coefficient were investigated using one-way ANOVAs. When appropriate, Fisher post hoc test was used for pairwise comparisons. The K_m and V_{max} values in Exp. 2 were analyzed using nonparametric statistics (Kruskal–Wallis ANOVA and Dunn's pairwise multiple comparison procedure) because these data failed the normality test ($p=0.004$ and $p=0.004$, respectively).

Results

Figure 1 illustrates the number of lever presses on the active vs. the inactive lever for rats self-administering heroin or saline. During the 20 sessions, the rats self-administered a total amount of 18.26±1.88 mg/kg of heroin.

Table 1 illustrates the plasma levels of heroin, morphine, 6-MAM, M3G, and M6G in rats that had self-administered heroin vs. saline. As predicted, rats that had self-administered heroin exhibited sizeable plasma levels of M6G, in contrast to the negligible levels seen in rats that had self-administered saline ($p=0.052$). Indeed, 91% of heroin rats exhibited detectable levels of M6G versus 25% of saline rats (Fisher exact probability test, $p=0.033$). Plasma levels of M3G were about 50% greater in the heroin SA group than in the saline SA group, but this difference was not significant ($p=0.34$).

Figure 2 and Table 2 illustrate the kinetics of in vivo M3G and M6G synthesis when hepatic microsomal preparations were incubated with morphine. Consistent with the in vivo data, negligible amounts of M6G were synthesized in vitro by the microsomal preparations obtained from rats that had self-administered saline (V_{max} and K_m could be calculated only in one rat). In contrast, a significant amount of M6G was synthesized by the microsomal preparations from rats that had self-administered heroin. As illustrated in Fig. 3, the synthesis of M6G appeared to be the result of positive enzymatic
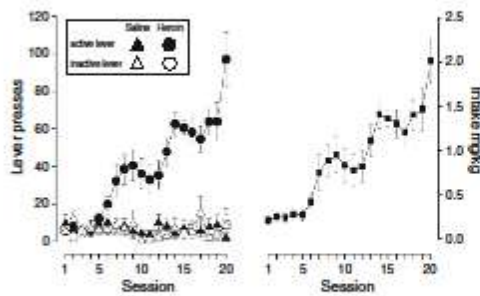
Fig. 1 Number of presses on the active vs. inactive lever (means ± SEM) for rats self-administering saline or heroin (50 µg/kg) on an FR1 (sessions 1–4) and then FR2 (sessions 5–20) schedule of reinforcement

cooperation (Hill coefficient = 1.90±0.34). Most important, the in vitro V_{max} for M6G synthesis was correlated with plasma levels of M6G (r^2=0.78, $p<0.001$) (Fig. 3) and with the amount of heroin self-administered during training (r^2=0.41, $p=0.01$). Thus, it is not surprising that there was also a significant correlation between M6G levels and the amount of heroin self-administered during training (r^2=0.31, $p=0.035$).

As illustrated in Fig. 4, the curve of M3G formation in the microsomal preparation obtained from saline SA rats was in agreement with standard Michaelis–Menten kinetics (Hill coefficient = 1.00±0.09) whereas positive enzymatic cooperation was evident in the case of rats that had self-administered heroin (Hill coefficient = 1.40±0.17; $p=0.053$ vs. saline). Positive enzymatic cooperation for M3G synthesis was independent of positive enzymatic cooperation for M6G synthesis, as indicated by the lack of correlation between the respective Hill coefficients (r^2= 0.03, $p=0.63$). The in vitro V_{max} of M3G synthesis was about 50% greater in the heroin SA group than in the saline SA group ($p=0.022$), but there was no correlation between the V_{max} of M3G synthesis and plasma levels of M3G. The in vitro K_m of M3G synthesis was also greater in the heroin SA group than in the saline SA group, but this difference only approached significance ($p=0.056$).
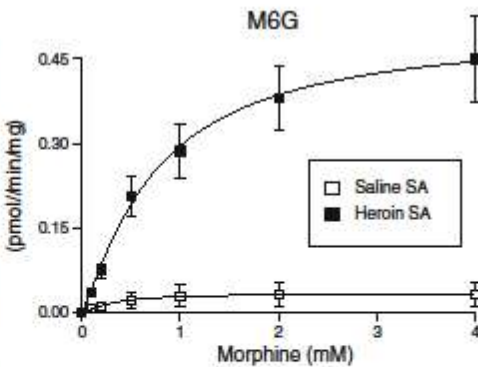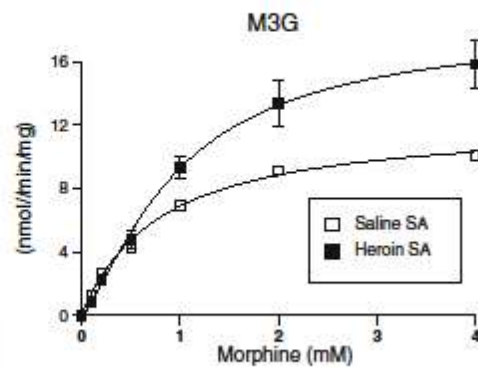
Fig. 2 Kinetic of M3G and M6G formation by microsomal preparations obtained from the liver of rats treated with saline or heroin and incubated with increasing concentrations of morphine. Each data point is an average of triplicate determination ± SEM

Discussion

In the present study, we investigated the synthesis of M6G, an active metabolite of heroin and morphine and a powerful MOR agonist, in a rat model of heroin abuse. We found that

Table 1 Mean (± SEM) plasma levels (nanograms per milliliter) of heroin, 6-MAM, morphine, M3G, and M6G (ad their sum) in samples obtained 2 h after a single i.p. injection of 10 mg/kg of heroin, administered the day after the last of 20 sessions of heroin or saline self-administration

	Heroin	6-MAM	Morphine	M3G	M6G*	Total
Saline	17.00±17.00	170.72±115.11	121.42±35.99	330.92±167.46	7.35±7.35	646.38±158.99
Heroin	9.03±9.03	46.81±29.37	178.25±80.92	534.82±97.89	90.00±36.84	858.77±126.38

*$p<0.05$ vs. saline

Table 2 Kinetics of morphine glucuronidation in microsomal preparation obtained from livers excised 2 h after a single i.p. injection of 10 mg/kg of heroin, administered the day after the last of 20 sessions of heroin or saline self-administration (same rats of Table 1)

	M3G				M6G			
	K_m (mM)	V_{max} (nmol/min/mg)	V_{max}/K_m	Hill coeff.	K_m (mM)	V_{max} (pmol/min/mg)	V_{max}/K_m	Hill coeff.
Saline	0.82±0.16	12.11±1.32	16.48±3.50	1.00±0.09	0.31[a]	0.13[a]	0.42[a]	1.5[a]
Heroin	0.97±0.21	18.31±2.07*	21.30±2.04	1.40±0.17*	0.57±0.16	0.39±0.12	0.79±0.14	1.55±0.36

Data are expressed as means ± SEM

coeff. coefficient

*$p < 0.05$ vs. saline

[a] V_{max} and K_m of M6G synthesis could be calculated only in one saline rat

heroin SA powerfully induced the synthesis of M6G both in vivo (as indicated by detectable plasma levels) and in vitro (in microsomal preparations, obtained from the rats' livers, incubated with morphine).

These findings appear to be at odds with the notion that rats produce no M6G (Milne et al. 1996). However, we have previously shown that M6G can be induced by repeated non-contingent i.p. administrations of heroin (Antonilli et al. 2003b, 2005), and there is evidence that adult rats can synthesize M6G even under basal conditions (Wang et al. 2005). Microsomal preparations, obtained from the livers of these rats, yielded, when incubated with morphine, significant concentration of M6G (which was absent in the microsomal preparations obtained from saline-treated rats). However, in these earlier studies the heroin pretreatment consisted of high i.p. doses of heroin (10 mg/kg×10). Here we show that M6G is formed in even larger amounts in rats self-administering heroin i.v. These elevated plasma levels of M6G were clearly the result of increased synthesis, as indicated by the correlation between plasma levels of M6G and microsomal M6G synthesis in vitro. This conclusion is further supported by the results of other in

Fig. 3 Regression of plasma levels of M6G over in vitro V_{max} of M6G formation by liver microsomes

vitro experiments with isolated rat hepatocytes. When hepatocyte cultures were pre-incubated for 72 h with heroin and then incubated with morphine, significant amounts of M6G were produced, as opposed to cultures pre-incubated with vehicle (Graziani et al. 2008).

The results obtained with heroin SA do not completely overlap with those obtained with non-contingent i.p. administrations of heroin. In particular, there were two major discrepancies. First, in vitro synthesis of M3G was reduced after repeated i.p. injections of heroin (Antonilli et al. 2005; Graziani et al. 2008) but not after heroin SA. Second, M3G and M6G synthesis followed standard Michaelis–Menten kinetics (Hill coefficient = 1) in microsomal preparations obtained from the rats that had received repeated i.p injections of heroin (Antonilli et al. 2005) but not in those obtained from the rats that had self-administered heroin. The reasons of these discrepancies are not clear, as there are many differences in drug regimen between the two procedures (e.g., route of administration, self-administration vs. non-contingent administration, drug amount, etc.).

The mechanisms responsible for the ability of heroin SA to modulate morphine glucuronidation are not known. These effects were not mimicked by methadone nor blocked by naltrexone, suggesting MOR-independent mechanism(s) of action (Antonilli et al. 2005; Graziani et al. 2008). Furthermore, the fact that similar results were obtained with liver microsomes (Antonilli et al. 2005) and isolated hepatocytes (Graziani et al. 2008) indicates that heroin can alter morphine glucuronidation by acting directly on the liver. Interestingly, we found here that the variability in the V_{max} of M6G synthesis by liver microsomes accounted for about 80% of the variance in plasma levels of M6G. Finally, Hill coefficients greater than 1 for the synthesis of M3G and M6G indicate enzymatic cooperativity. Taken together these data suggest that heroin acted at a post-translational level by inducing homodimerization or heterodimerization of UGTs. This hypothesis requires further investigation.
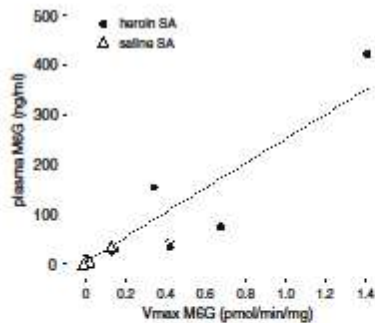
Fig. 4 Eadie–Hofstee plots of M3G and M6G formation by microsomal preparations from rats that had self-administered saline or heroin. From the same data of Fig. 2
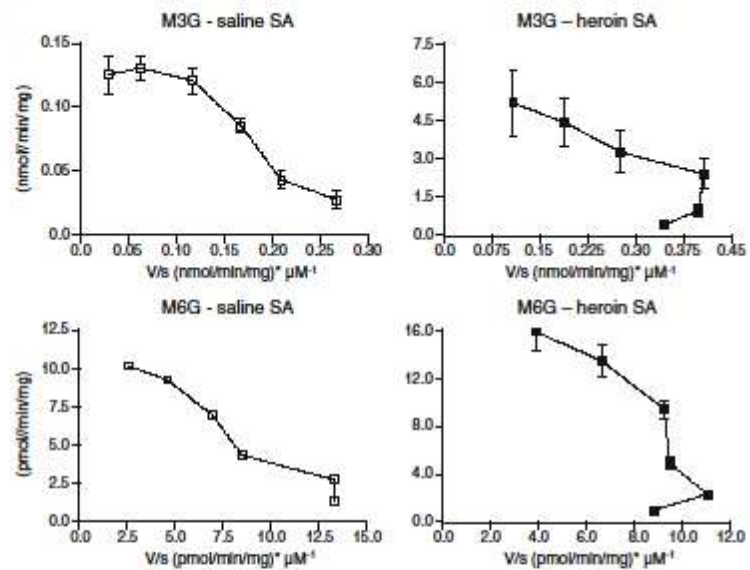
In addition, it is possible that the effects of heroin exposure on morphine glucuronidation depended on changes in the expression of genes encoding for UGTs. We have recently found that 72-h exposure of rat hepatocytes to heroin reduces the expression of both UGT1A1 and UGT1A6 genes, whereas the expression of the UGT2B1 gene was significantly enhanced (unpublished data). It is not yet clear how heroin elicits these changes in the expression of UGTs genes. The most plausible targets of heroin action are the ligand-activated transcription factors that regulate the expression of a wide array of enzymes involved in detoxification, including UGTs. Although there is no direct evidence of an action of heroin on these transcriptional factors, nuclear opioid binding sites associated with regulatory protein kinase C have been identified by Ventura et al. (2003) in cardiac cells. More recently, it has been found that morphine enhances the expression of TNFα in astrocytes and microglia by facilitating translocation of the NF-κB class of transcription factors from the cytoplasm to the nucleus (Sawaya et al. 2009). More experiments are then required to explore the possibility that heroin modulates UGTs expression in the liver by interacting with nuclear transcriptional factors.

The results reported here show that intravenous heroin self-administration can induce M6G synthesis even in the rat. This suggests that the increase in the plasma M6G concentration previously observed in human addicts (Antonilli et al. 2003a)

was not a mere epiphenomenon in the natural history of heroin addiction. What are the possible implications of this finding?

Morphine-6-glucuronide does not easily cross the blood–brain barrier (Meineke et al. 2002), but its distribution in the brain is mostly extracellular, suggesting that its concentrations at MOR are not far from those of parent compounds (Stain-Texier et al. 1999). After intracerebroventricular or intrathecal injection, M6G has been reported to be one to two orders of magnitude more potent than morphine, with respect to its analgesic and ventilatory effects (Paul et al. 1989; Gong et al. 1991; Frances et al. 1992). The greater potency of M6G has been attributed to greater efficacy in activating the MOR (Osborne et al. 2000; Ulens et al. 2001) or to its actions at a unique MOR subtype. The existence of a MOR-1 subtype with greater affinity for M6G than for morphine was first proposed by Rossi et al. (1995a) and was later confirmed by others (Brown et al. 1997; Mantione et al. 2002). Experiments using antisense probes or knockout mice have demonstrated the existence of splice variants of MOR-1 with differential affinity for morphine versus heroin and M6G (Rossi et al. 1995b; Matthes et al. 1996; Sora et al. 1997; Loh et al. 1998; Schuller et al. 1999; Unterwald et al. 1999; Pan et al. 2009). In addition to being a potent MOR agonist, M6G exhibits a much longer half-life than heroin or morphine. The delay between peak plasma concentrations and

analgesic effects in humans, for example, is 2–3 h for morphine versus 7 h for M6G (Lotsch 2005). Hence, the effects of M6G may largely outlast those produced by the parent compounds. In particular, having the same pharmacological profile of heroin, M6G may significantly contribute to the short-lived reinforcing effects of the former, which have been long known to differ from those of morphine (Fraser et al. 1961; Martin and Fraser 1961). Pharmacological antagonism of M6G has been shown to block heroin self-administration (Walker et al. 1999). It follows that all conditions leading to increased synthesis of M6G might play a role in the development of heroin addiction.

In conclusion, the present findings may have important implications for the study of heroin addiction in humans. The exact relationship between the changes in M6G synthesis and the development of addiction, if any, remains to be determined. For example, it is possible that the induction of M6G synthesis represents a mere consequence of repeated exposure to heroin. We are now conducting experiments to investigate the existence of a causal relationship between individual variability in the ability to synthesize M6G and the propensity to develop heroin addiction.

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The role of setting for ketamine abuse: clinical and preclinical evidence

Abstract: Drug abuse is often seen as a unitary phenomenon, partly as a result of the discovery over the past three decades of shared mechanisms of action for addictive substances. Yet the pattern of drug taking is often very different from drug to drug. This is particularly evident in the case of 'club drugs', such as ketamine. Although the number of ketamine abusers is relatively small in the general population, it is quite substantial in some settings. In particular, ketamine abuse is almost exclusively limited to clubs and large music parties, which suggests a major role of context in modulating the reward effects of this drug. This review focuses on recent preclinical and clinical findings, including previously unpublished data, that provide evidence that, even under controlled conditions, ketamine reward is a function of the setting of drug taking.

Keywords: cocaine; context; drug abuse; drug addiction; environment; hallucinogens; heroin; ketamine; opiates; psychostimulants; setting.

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Introduction

Ketamine (ketamine chlorhydrate; CI-581) was synthesized by Calvin Stevens in 1962 at the Parke-Davis Laboratories in Michigan. The new drug was chemically related to phencyclidine (PCP) but presented clear advantages in terms of toxicity relative to the parent drug (Domino et al., 1965). Recovery (the 'emergence' period) from PCP-induced anesthesia is, in fact, associated with unwanted side effects, including confusion, unpleasant dreams and hallucinations (Siegel, 1978). Although ketamine also produces an emergence syndrome in 15%–40% of subjects (Dillon et al., 2001), its shorter half-life makes it more acceptable than PCP. Because of its relatively favorable safety profile, ketamine rapidly became an anesthetic of choice for the American army during the Vietnam War. The dissociative effects of ketamine (that is, its ability to induce a lack of responsive awareness to the environment) were particularly useful in the battlefield. Today, ketamine is still widely used as an anesthetic in developing countries and in remote rural areas of developed countries, such as Australia, because of the minimal equipment requirements for its administration. In addition, ketamine remains the most widely used anesthetic in veterinary medicine.

Ketamine's role in pain management goes beyond its use as a general anesthetic. Ketamine also has analgesic properties, preventing pain 'wind-up' [that is, the sensitization of neurons in the posterior horns of the spinal cord to pain stimuli (Sunder et al., 2008; Morgan and Curran, 2011)], and at low doses (0.1–0.5 mg/kg/h) produces a local anesthetic effect that is particularly useful in neuropathic pain (Correll et al., 2004; Lynch et al., 2005; Morgan and Curran, 2011). Ketamine has also been used in intensive care for the management of prolonged epileptic seizures (Fujikawa, 1995). Other potential medical uses of ketamine are currently under investigation. In particular, ketamine is being tested for the treatment of anti-depressant-resistant mood disorders and for heroin and alcohol addiction (Krupitsky and Grinenko, 1997; Krystal, 2007; Aroni et al., 2009; Li et al., 2010; Vollenweider and Kometer, 2010).

Another interesting aspect of the pharmacology of ketamine concerns its psychotomimetic effects. Indeed, some effects of ketamine resemble the symptoms of acute psychosis (Adler et al., 1999). This has triggered research aimed at increasing our understanding of schizophrenia and at developing new therapies (Adler et al., 1999; Carpenter, 1999). The ketamine 'model' of schizophrenia is still the pharmacological model with the greatest face validity (Morgan and Curran, 2011).

The present review is not concerned with the medical uses of ketamine, nor with the ketamine model of psychosis, but centers exclusively on the recreational misuse of

ketamine. Ketamine is very popular with some people for its ability to produce hallucinations and an internal state similar to a trance. Indeed, ketamine is taken mainly in club settings, which indicates a major role of context in modulating the reward effects of this drug (Curran and Morgan, 2000; Joe Laidler, 2005; Degenhardt and Dunn, 2008). Thus, the review will focus on the role of context in ketamine abuse and, in particular, on recent experimental work conducted in rodents and humans.

Mechanisms of action of ketamine

Ketamine, like PCP, binds the N-methyl-D-aspartate (NMDA)-receptor complex at a site located within the channel (PCP-binding site). The excitatory amino acids glutamate, aspartate, and glycine are the endogenous agonists at the NMDA receptor. Activation of NMDA receptors results in the opening of the channel with increased transmembrane flux of Na^+, K^+ and Ca^{++}, and the depolarization of the neuron. Ketamine and PCP act as non-competitive NMDA receptor antagonists at the NMDA receptor.

Commercially available ketamine is a racemic mixture of two enantiomers. The S-enantiomer is the more potent of the two, with an anesthetic potency approximately three to four times that of R-ketamine. This correlates to the higher binding affinity for the PCP-site of the NMDA receptor. The psychotropic effects of ketamine are mainly caused by the S-enantiomer, although sub-anesthetic doses of R-ketamine have been shown to induce a state of relaxation (Engelhardt, 1997; Vollenweider et al., 2000).

The principal metabolite of ketamine, nor-ketamine, is pharmacologically active. Its binding affinity to the NMDA receptor and its anesthetic properties are approximately one-third of the parent compound, contributing significantly to the analgesic effect of ketamine (Shimoyama et al., 1999). The plasma levels at which ketamine analgesia is achieved are 0.15 µg/ml following intramuscular administration and 0.04 µg/ml after oral administration. This difference may be explained by the greater relative contribution of nor-ketamine after oral relative to intramuscular administration.

The anesthetic and analgesic effects of ketamine and PCP are not surprising given the role of NMDA receptors in the transmission of sensory inputs at the spinal, thalamic, limbic and cortical levels. Ketamine interferes not only with the perception of pain per se, but also with the emotional response to pain and with the formation of pain-related memories (Green and Johnson, 1990; Bergman, 1999; Sprenger et al., 2006). The analgesic effects of

ketamine may depend, in part, on its agonist properties at mu-opioid receptors located at the spinal and supra-spinal level (Fink and Ngai, 1982; Crisp et al., 1991; Sarton et al., 2001). Ketamine was also found to potentiate the activation of mu-opioid receptors by opioid agonists (Gupta et al., 2011). Furthermore, ketamine has been shown to prevent the development of morphine tolerance (Gonzalez et al., 1997) and suppress morphine withdrawal syndrome in experimental settings, probably by acting at the level of the nucleus accumbens (Ji et al., 2004).

Other effects of ketamine may be due to its actions on the catecholaminergic systems, notably on dopamine (DA) transmission (White and Ryan, 1996; Smith et al., 1998; Vollenweider et al., 2000). It has been shown that ketamine stereo-specifically increases DA efflux in the nucleus accumbens and in the prefrontal cortex by mobilizing the DA storage pool to releasable sites (Hancock and Stamford, 1999). In addition, ketamine has been shown to block DA reuptake (Hancock and Stamford, 1999) and activate D2 receptors (Kapur and Seeman, 2002). These dopaminergic effects may be implicated in the euphorigenic, addictive and psychotomimetic properties of ketamine. The initial ketamine-induced DA overflow in the prefrontal cortex undergoes tolerance after repeated administrations, whereas the increase in extracellular 5-hydroxyindole acetic acid (a serotonin metabolite) levels undergoes sensitization. This suggests that the balance between dopamine and serotonin neurotransmission in the prefrontal cortex may change after repeated exposure to ketamine (Lindefors et al., 1997). Ketamine also acts as an agonist at α- and β-adrenergic receptors (Bevan et al., 1997). Finally, ketamine has been shown to act as an antagonist at central muscarinic receptors and as an agonist at σ-receptors (Anis et al., 1983; Izquierdo et al., 1995; Bergman, 1999).

The psychotropic effects of ketamine can be observed in the presence of plasma concentrations ranging from 50 to 300 ng/ml and with regional brain concentrations higher than 500 ng/ml (Hartvig et al., 1995; Bowdle et al., 1998; Oranje et al., 2000).

Ketamine abuse

The non-medical use of ketamine dates from the late 1960s, when the drug began spreading from the Parke-Davis Laboratories in Michigan to other states, particularly Florida, where it was sold as a hallucinogen with names such as 'mean green' and 'rockmesc' (i.e., 'rock mescaline') (Jansen, 2004). Ketamine use remained relatively

rare in Europe until the 1990s, when it appeared on the 'rave' scene as an adulterant to ecstasy (3,4-methylene-dioxy-N-methylamphetamine; MDMA) tablets (Dalgarno and Shewan, 1996). Other street names of ketamine are 'Special K', 'Vitamin K', 'K', 'Kit-kat', 'Keets', 'super acid', 'cut valium', and 'jet'.

Users report that ketamine is easier to take than other hallucinogenic drugs such as LSD and that its hallucinogenic effects are more manageable, owing to a predictable dose-response curve of effects and a relatively short half-life (Dillon et al., 2003; Wolff and Winstock, 2006).

Ketamine is highly lipophilic and can be taken through various routes of administration: intranasal (snorting), intramuscular, oral, intravenous, subcutaneous and inhalatory (smoking). Snorting represents the most popular route of administration. Dosing devices for snorting ketamine are called 'bullets' or 'bumpers' (Chakraborty et al., 2010). Ketamine is often snorted in combination with other drugs ('trail mix'), such as methamphetamine, cocaine (the so-called 'Calvin Klein'), sildenafil citrate or heroin (Tellier, 2002). Other popular drug combinations are taken orally (e.g., ketamine and MDMA) or via smoking (ketamine and cannabis).

At low doses, ketamine induces distortion of time and place, hallucinations and bizarre dissociative effects. According to many users, the most appealing effects of ketamine are represented by 'melting into the surroundings', 'visual hallucinations', 'out-of body experiences', and 'giggliness' (Stewart, 2001). At higher doses, ketamine induces more severe dissociation, commonly referred to as 'K-hole', with the users experiencing intense detachment to the point that their perception appears to be completely divorced from their previous reality. Some users enjoy the experience of a K-hole and describe themselves as 'psychonauts' (see below), whereas others strongly dislike the resulting decrease in sociability (Dillon et al., 2001).

According to the 2012 Ketamine Critical Review Report by the Expert Committee on Drug Dependence of the World Health Organization (WHO), in developed countries, street ketamine comes from two main supply sources: hospitals and veterinary clinics on the one hand, and illegal import from developing countries on the other. In the past, hospitals and veterinary clinics represented the main source of ketamine. This sort of supply is the most appreciated by consumers, as quality control is guaranteed by the pharmaceutical industry. Increasing regulatory control has made it more and more difficult, but not impossible, to obtain medical ketamine. Presently, street ketamine is mostly obtained from countries where it is still easily available, mainly China and India (Jansen, 2004).

Geographical distribution of ketamine abuse

As indicated by the 2010 United Nations World Drug Report, ketamine abuse is a global phenomenon with large geographical variation. In Hong Kong, for example, ketamine is thought to be the single most abused illicit drug, coming mostly from mainland China. However, although China produces massive amounts of ketamine, reliable estimates for the prevalence of ketamine abuse are not available. As of today, five Chinese factories are officially licensed to produce ketamine, but there are reports of illicit production on an industrial scale. In 2009, Chinese authorities reported the seizure of two illicit laboratories producing 8.5 million tons of the immediate precursor of ketamine.

Despite the efforts of several research groups, little is known of the epidemiology of ketamine abuse in other countries (WHO Expert Committee on Drug Dependence, 2012). Topp et al. (2004) describe the Australian Illicit Drug Reporting System (IDRS) and the feasibility of monitoring market trends for 'party drugs'. The trial demonstrated that the system can successfully monitor the market for widely used drugs, such as ecstasy, whereas it is much less sensitive in monitoring the markets for drugs that are used by small proportions of the total population, such as ketamine and other 'club drugs'.

In the USA, according to an official 2009 NIDA publication, an estimated 1%–2% of 10th–12th graders reported having used ketamine (Johnston et al., 2009). More uncertain are the numbers for Europe. Reports of widespread recreational use of ketamine in the UK began to appear in the literature from the early 1990s (Jansen, 1993; Dalgarno and Shewan, 1996). Estimates suggest an increase in the number of ketamine users from approximately 85,000 in 2006/2007 to approximately 113,000 in 2008/2009 (Hoare, 2009). Additional evidence of the growing recreational use of ketamine in the UK has been provided by others (Measham et al., 2001; Moore, 2004; Copeland and Dillon, 2005; Moore and Measham, 2008).

France is another country where ketamine use appears to be significant. As detailed in the 2010 France National Report to the European Monitoring Centre for Drugs and Drug Dependence (EMCDDA 2010c), the Centres d'Accueil et d'Accompagnement à la Réduction des Risques pour Usagers de Drogues (CAARUD) found that among the most striking changes in drug use and method of use there in the years 2008–2009 was the spreading of ketamine misuse outside the alternative party scene (see below). More than 7% of addicts

referred to the CAARUDs reported recent use of ketamine (approx. 5% reported daily use). In the general population of 17 year-olds, ketamine use was estimated at 0.6% (0.8% in males and 0.4% females). Furthermore, according to the same report, although in previous years the first encounter with ketamine was almost a chance event, this drug is now actively desired and sought out by new users. That is, ketamine is in the process of becoming a 'first experimentation' substance for some individuals.

An increase in the recreational use of ketamine has been observed in other European countries as well (EMCDDA, 2011). Eight out of the 29 EMCDDA participating countries provided some information about ketamine use (in addition to the UK and France: Czech Republic, Denmark, Hungary, Ireland, Italy, and the Netherlands). The 2010 Czech Republic National Report to the EMCDDA (EMCDDA, 2010b), for example, included data from the 2009 Safer Party Tour project (which provided preventive and harm reduction services at 14 summer festivals) indicating that the lifetime use of ketamine among Safer Party affiliates was 10.8%. Similar data were contained in the 2010 The Netherlands National Report to the EMCDDA (EMCDDA, 2010d), which indicated an 8.5% prevalence of ketamine use among participants in large scale parties (raves) and 4.1% among visitors of clubs and discotheques.

However, the quality of the reports from the different participating countries was not homogeneous, and it is not easy to understand to what extent the lack of data in a report reflects little or negligible ketamine abuse or simply a lack of reliable information. For example, the 2010 Austria National Report to the EMCDDA (EMCDDA, 2010a) makes no mention of ketamine, but the 2007 report (EMCDDA, 2007) details the findings of a survey carried out on the spot at free techno and Goa-like events, according to which 23% of all participants used ketamine (Baumgartner, 2007). This was also the case for the reports of other countries.

Demographics of ketamine abuse

The large majority of ketamine users have a significant history of polydrug use, often confined to parties, more rarely as part of daily use activity. Generally, the first use of ketamine occurs in a group at a rave. Indeed, all available evidence shows that ketamine abuse is, at least initially, framed within the context of rave parties. Raves are parties with loud, electronic 'techno-rock' music, laser light shows and all-night dancing held in clandestine locations, including warehouses, nightclubs and farm fields (Weir, 2000). They first became popular in the UK and the USA in the late 1980s and have since spread to other countries.

Many of the early ketamine users were jobless and without a fixed residence, their lifestyles being focused mainly on drug consumption and on the organization of raves. More recently, ketamine use has moved beyond the context of raves and has become popular in youth clubs whose clients have been in contact with the rave culture. In parallel with the spread of ketamine to mainstream discos and clubs, the social profile of users has become less marginal.

Reynaud-Maurupt et al. (2007a) argue that, among ketamine users, four 'affinity groups' can be identified on the basis of distinct socio-demographic profiles and different levels of consumption: 'alternative', 'urban', 'clubbing' and 'selected'.

The 'alternative' group is composed by counterculture enthusiasts with a hedonistic tinge. The setting of ketamine taking is represented by rave and free parties. It has been argued that this group can be further subdivided into Ravers and Travellers. Ravers come from the rave and 'teknival' culture. They consume ketamine by sniffing and are socially functional. The Travellers' lifestyle focuses on drug consumption and parties. They live in unstable conditions and frequently experience problems derived from drug consumption. Travellers often inject ketamine.

The 'urban party' group is music-oriented, and its habitat is represented by live music bars. Individuals in this group are well integrated at a social level and are characterized first and foremost by their fondness for music. This group includes the highest percentage of students.

The 'clubbing' group is composed by hedonists who devote a substantial portion of their budget to partying and buying clothes. Their habitat is represented by clubs playing electronic music. The 'gay friendly' establishment belongs to this particular affinity group.

The 'selected' group is composed by individuals who attend invitation-only or sponsored-entry bars or clubs requiring 'smart dress' attires. The standards of living of these users are quite high, and there is very little overlap with the other groups. The selected group frequents locations usually accessed through coopting and cultivates a chic and hip image.

Finally, a particular population of ketamine users is represented by the 'psychonauts'. The term psychonautics is of recent coinage and has entered scholarly literature even more recently (e.g., Ott, 2001). Psychonauts may consume ketamine and other psychedelic drugs

('entheogens'), to induce an altered state of conscious-
ness, thereby facilitating the 'exploration' of the psyche.
As their main goal is introspection, psychonauts use
ketamine in quiet places and usually by injection. Pres-
ently, psychonauts represent a minority of ketamine
users (Newcombe, 2008).

The setting of ketamine use

Environment plays an important role in modulating indi-
vidual responsiveness to addictive drugs (Caprioli et al.,
2007a; Badiani et al., 2011). For example, adverse life
experiences (e.g., sexual abuse/harassment, combat-
stress, occupational stress and other forms of social and
physical stress) can facilitate the initiation and the devel-
opment of drug abuse and then of drug addiction and, by
acting acutely, can precipitate drug seeking after a period
of abstinence (Aro, 1981; Triffleman et al., 1995; Richman
et al., 1996; Brady et al., 2001; Clark et al., 2001; Price et al.,
2004; Ompad et al., 2005; Brown et al., 2006; Reed et al.,
2006). Another way the environment can affect drug
taking is represented by drug-associated cues that can
trigger drug seeking even after prolonged abstinence
(Childress et al., 1984, 1986).

   Also in the case of ketamine abuse, context appears
to play a major role. As discussed above, ketamine abuse
is, in fact, prevalent among individuals participating
in music and dance events at nightclubs or rave parties
(Curran and Morgan, 2000; Joe Laidler, 2005; Degenhardt
and Dunn, 2008). This anecdotal evidence has recently
received support from animal and human studies, which
will be reviewed below, along with unpublished data that
will be presented here for the first time.

Setting of ketamine use: pre-clinical studies

Preclinical research concerning the role of context in drug
addiction has focused mostly on the ability of environ-
mental stimuli to act as stressors or as drug cues. However,
context has been shown to affect drug taking in ways that
are not easily attributable to stress or conditioning. For
example, the presence of novel objects has been found to
reduce the intake of amphetamine (Klebaur et al., 2001),
and high temperatures can increase the intake of 3,4-meth-
ylenedioxymethamphetamine (Cornish et al., 2003). Even
nonphysical, apparently negligible differences in the

setting can powerfully alter drug-taking behavior, as indi-
cated by a series of studies in which rats were trained to
self-administer heroin or cocaine under two deceptively
similar environmental conditions. Some rats were trans-
ferred to the self-administration chambers immediately
before the experimental sessions (non-resident rats), a
procedure commonly used in most self-administration
studies. Other rats were kept in the self-administration
chambers at all times (resident rats). Thus, the physical
characteristics of the self-administration environment for
resident vs. non-resident rats were virtually identical,
all differences being purely a function of familiarity. As
illustrated in the top panels of Figure 1, we found that
psychostimulant drugs, such as cocaine and ampheta-
mine, were self-administered more by non-resident rats
than by resident rats, whereas the opposite occurred with
heroin self-administration (Caprioli et al., 2007b, 2008;
Celentano et al., 2009). The influence of setting on drug
taking was particularly striking in experiments in which
rats with double-lumen catheters were repeatedly given
the opportunity to choose between two drugs within the
same session (Caprioli et al., 2009). In fact, most non-
resident rats chose cocaine over heroin, whereas resident
rats tended to prefer heroin.

   We have hypothesized that the setting may affect drug
taking by providing an ecological backdrop against which
drug effects are appraised as more or less 'adaptive' (Cap-
rioli et al., 2009; Badiani et al., 2011). Briefly, we proposed
that the sedative, inward-looking effects of heroin would
be experienced as suitable to a safe, non-challenging
home environment, whereas the sympathomimetic, acti-
vating, performance-enhancing effects of cocaine would
be more appropriate to arousing, exciting contexts (this
hypothesis will be discussed in more detail at the end of
this review). On the basis of this initial hypothesis, we
speculated that drugs producing effects somewhat similar
to those of psychostimulants or opiates would also inter-
act with the environment in a similar manner. Indeed,
we found that ethanol, which, at least at certain doses,
depresses the central nervous system similar to opiates,
was ingested in greater amounts by resident rats than by
non-resident rats (Testa et al., 2011).

   Most important, we also predicted that the intrave-
nous self-administration of ketamine (first reported by
Collins et al., 1984) would be greater in non-resident rats
than in resident rats. The effects of ketamine are particu-
larly complex, also in relation to the dose, and include,
in addition to 'dissociative' anesthesia: tachycardia,
increased blood pressure, ataxia, hyper-excitability, agita-
tion, acute psychotic episodes, unpleasant vivid dreams,
hallucinations and impaired cognitive function. However,
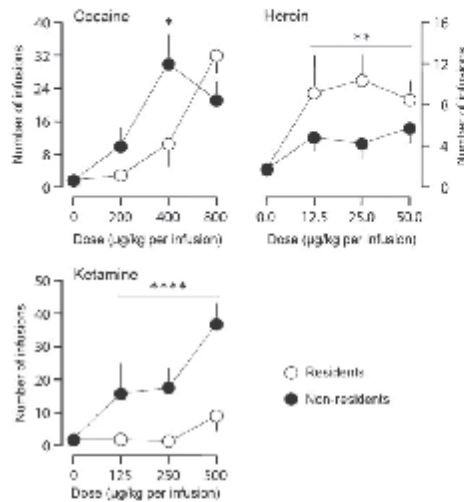
Figure 1 Drug taking as a function of setting in rats.
This figure is based on previously published data (Caprioli et al., 2007b for cocaine; Caprioli et al., 2008 for heroin; De Luca et al., 2011 for ketamine) and illustrates the mean (±SEM) number of infusions on the last day of the training phase (FR5 schedule of reinforcement) as a function of the setting. The data for each infusion dose were obtained in independent groups of rats (n=11–15 for cocaine; n=12–16 for heroin; n=6–10 for ketamine). Resident rats were housed in the self-administration chambers. Non-resident rats were transferred to these chambers only for the test sessions (3 h each). Asterisks indicate significant differences (*p<0.05; **p<0.01, ***p<0.0001) between resident and non-resident groups. For details of the statistical analyses, see the original publications.

at the doses used for recreational purposes, some, but by no means all, of the physiological, behavioral and subjective effects produced by ketamine are similar to those produced by psychostimulant drugs, e.g., tachycardia, increased blood pressure, hyper-excitability and agitation. Thus, it was reasonable to assume that these effects would be experienced as more appropriate to (or less aversive in) a non-home vs. a home environment, as previously reported for cocaine and amphetamine (Caprioli et al., 2007a,b, 2008). (More difficult to speculate on is how the setting affected the appraisal of other effects of ketamine, such as hallucinations and ataxia.)

There is a partial overlap between ketamine and psychostimulants also with regard to the mechanisms of action, as ketamine has been reported to increase dopamine efflux and reduce dopamine uptake in the nucleus accumbens (Hancock and Stamford, 1999). Finally, the fact that ketamine abuse in humans is associated with

clubs or rave parties (Curran and Morgan, 2000; Joe Laidler, 2005; Degenhardt and Dunn, 2008) also leads us to predict greater preference for ketamine in non-resident than in resident rats.

Consistent with our hypothesis, we found that ketamine intake was much greater in non-resident rats than in resident rats (De Luca and Badiani, 2011). The bottom left-hand panel of Figure 1 illustrates the dose-response curve for ketamine self-administration. Non-resident rats acquired ketamine self-administration at all training doses, whereas resident rats self-administered only the highest dose of ketamine (500 μg/kg), but still four times less than non-resident rats (De Luca and Badiani, 2011).

The role of setting in ketamine (Parke-Davis, Detroit, MI, USA) self-administration is also indicated by the results of an experiment in which rats were given the opportunity to choose between ketamine and heroin (S.A.L.A.R.S., Como, Italy) within the same session. These findings are reported here for the first time. The experimental procedures were similar to those described by Caprioli et al. (2009). Briefly, 14 male Sprague-Dawley rats (Harlan, Italy), weighing 250–275 g at their arrival, were housed and tested in the same dedicated temperature and humidity-controlled rooms, with free access (except during the test sessions) to food and water under a 14-h dark/10-h light cycle (lights off at 7:00 am). After the surgery, the rats were housed individually. All procedures were in accordance with the Italian Law on Animal Research (DLGS 116/92) and with the guidelines for the care and use of laboratory animals issued by the Italian Ministry of Health. Using standard surgical procedures, the rats received double-lumen catheters connected with cannulas secured to the rat's skull, as described by Caprioli et al. (2009). At the end of the experiments, all rats underwent a catheter patency test in which they received two i.v. boluses of 40 mg/kg of thiopental sodium (Pharmacia Italia, Milan, Italy), one in each catheter, with a 15-min interval between the two. No rat failed the test, that is, all rats became ataxic within 5 s after thiopental. The testing apparatus (ESATEL S.r.l., Rome, Italy), described in detail in previous papers (Caprioli et al., 2009), consisted of self-administration chambers placed within sound- and light-attenuating cubicles and equipped with two retractable levers, two light cues positioned above each lever and a counterbalanced arm holding a liquid swivel. Each lever was connected via an electronic interface to a syringe pump (Razel Scientific Instruments, St. Albans, VT, USA). Personal computers controlled the chambers, via Programmable Logic Controller (Allen Bradley, Milwaukee, WI, USA), using control software developed by Aries Sistemi S.r.l. (Rome, Italy).

During the training phase, the rats were assigned to one of two conditions: resident and non-resident. Resident rats were housed in the self-administration chambers, where they remained for the entire duration of the experiment. Non-resident rats (n=8) were housed in standard cages and were transferred to the self-administration chambers immediately before the start of each testing session.

Resident rats (n=6) were connected, through liquid swivels, to the infusion lines 3 h before the start of each session. During the 60 s preceding the start of each session, food and water were removed from the chambers, and the infusion pumps were activated so as to fill the catheters with the drug solution. Immediately before the start of each session, non-resident rats were transferred to the self-administration chambers, and their catheters were connected to the infusion lines. At the end of each session, food and water were given back to the resident rats and non-resident rats were returned to their home cages. The rats were trained for 10 consecutive daily 3-h sessions to self-administer ketamine (250 µg/kg/infusion). Ketamine was alternatively paired with one or the other of the two levers, according to a counterbalanced design. That is, for some rats, ketamine was paired with the left lever on sessions 1, 3, 5, 7 and 9, whereas pressing on the right lever had no programmed consequences; the opposite occurred on sessions 2, 4, 6, 8 and 10. For other rats, the sequence was inverted. After each infusion, the cue light was turned off, and the lever retracted. The cue light was turned on and the lever extended again after a time-out (TO) period. Both the fixed ratio (FR, i.e., number of consecutive lever presses required to obtain a single infusion) and the TO period progressively changed during training, to habituate the rats to obtain an infusion every 10 min. The FR increased from FR1 (sessions 1–2, with a 40-s TO, and sessions 3–4, with a 60-s TO), to FR2 (sessions 5–6, with a 2-min TO, and sessions 7–8, with a 3-min TO), and finally to FR5 (sessions 9–10, with a 5-min TO, and sessions 11–12, with a 10-min TO). The goal was to reach, by the end of the training phase, the same reinforcement schedule used during the subsequent choice phase.

During the choice sessions, some rats were given the opportunity to choose between ketamine and heroin, each paired with one of the two levers. Other rats instead received ketamine regardless of the chosen lever (that is, the 'choice' was between ketamine and ketamine). Both levers were available simultaneously at time 0 min and then again 10 min after each infusion. The doses of ketamine and heroin were progressively increased during 12 choice sessions (3-h each). During sessions 1–4, rats had a choice between 250 µg/kg ketamine and either 25 µg/kg

heroin or 250 µg/kg ketamine. During sessions 5–8, rats had a choice between 500 µg/kg ketamine and either 50 µg/kg heroin or 500 µg/kg ketamine. During sessions 9–12, rats had a choice between 1000 µg/kg ketamine and either 100 µg/kg heroin or 1000 µg/kg ketamine. At the end of the experiments, all rats underwent a catheter patency test using thiopental, as described above.

The following is a synopsis of the environmental conditions of resident and non-resident rats: 1) the self-administration environment was physically identical for all rats, but for some animals this was also the home environment (resident group), whereas for other animals it represented a distinct non-home environment (non-resident group); 2) immediately before the start of each session, the resident rats were briefly handled to remove food and water from the chamber; 3) during testing, the self-administration chambers contained no food or water; 4) the distance traveled by the non-resident rats during the transfer to the self-administration chamber was about 1 m (that is, all animals were kept in the same dedicated testing room for the entire duration of the experiments, and therefore there was no transport from one room to another); and 5) all other husbandry routines were identical in the two groups.

In summary, the differences in setting between resident and non-resident rats were of a purely 'psychological' nature. Yet, these apparently negligible differences were capable of altering drug preferences in a substantial manner.

During training, resident rats took much less ketamine than non-resident rats (data not shown), in agreement with the findings by De Luca et al. (2011). Furthermore, when, during the choice phase, resident rats had access to ketamine on both levers, they took very little of it, regardless of the infusion dose, whereas non-resident rats worked for ketamine on both levers, in a dose-dependent manner (Figure 2). An analysis of variance (ANOVA) limited to ketamine intake indicated, in fact, a significant effect of setting [$F_{(1,13)}=6.53$, $p=0.024$] and a significant setting × dose interaction [$F_{(2,26)}=9.47$, $p<0.001$]. In contrast, when resident rats had the opportunity to choose between ketamine and heroin, they eagerly took heroin, but not ketamine, following a dose-dependent pattern. The ANOVA yielded a significant effect of drug [$F_{(1,2)}=6.75$, $p=0.016$] and a drug × dose interaction [$F_{(2,4)}=22.93$, $p=0.006$]. This indicates that the lower propensity of resident rats to self-administer ketamine was drug-specific and did not reflect a general inability to acquire drug-reinforced instrumental behavior. In contrast, non-resident rats that were given the choice between ketamine and heroin took, overall, about the same amount of the two drugs [$F_{(1,3)}=0.42$,

$p=0.56$], although, at the two highest doses, non-resident took about 60% (K50 vs. H500) and 35% (K100 vs. H1000) more ketamine than heroin. We investigated here a very limited combination of drug doses. It is quite possible that at certain doses (e.g., 50 µg/kg of ketamine vs. and 1000 µg/kg of heroin). Non-resident rats would have expressed a more robust preference for ketamine over heroin. Notice, however, that the most remarkable aspect of the present results (as well as of those reported in our previous papers) does not lie with the drug preferences of resident rats per se or non-resident rats per se, but with the comparison between the two groups, as this comparison indicates that the reinforcing effect of a given dose of a given drug changes as a function of the 'psychological' setting in which the drug is taken.

Setting of ketamine use: clinical studies

The fact that certain settings were able to modulate in opposite directions cocaine (or amphetamine or ketamine) vs. heroin self-administration in rats (Caprioli et al.,

Figure 2 Drug preferences as a function of setting in rats. This figure illustrates the mean (±SEM) number of drug infusions in resident (n=6) vs. non-resident (n=8) rats with double-lumen catheters that were repeatedly given the choice between two drug rewards. For some rats, the choice was between identical doses of ketamine (left-hand panels). Other rats had a choice between ketamine and heroin. Asterisks indicate significant differences (*$p<0.05$) between heroin and ketamine. For details of the statistical analyses, see the text.

2007b, 2008, 2009; Celentano et al., 2009) indicated an unforeseen dissociation in the reinforcing effects of different classes of addictive drugs, which is not compatible with unitary models of drug reward (for an in-depth discussion of this issue, see Badiani et al., 2011).

The heuristic relevance of these animal findings is indicated by the results of translational studies in which we investigated the setting of drug taking in human addicts (n=79) who co-abused heroin and cocaine (Caprioli et al., 2009). As illustrated in Figure 3, the majority of addicts reported using heroin always or mostly at home and cocaine always or mostly outside the home. Participants were cocaine and heroin addicts recruited among the out-patients of an addiction clinic (Villa Maraini, Rome, Italy) who: 1) met the DSM-IVR drug dependence criteria for cocaine and/or heroin; 2) reported using heroin and/or cocaine [either drug for the Retrospective Reports study, both drugs for the Momentary Ecological Assessment (EMA) study] at least once a week over the past 3 months; 3) did not meet the DSM-IVR criteria for schizophrenia or any other DSM-IV psychotic disorder, history of bipolar disorder or current major depressive disorder; 4) were not under treatment with antipsychotic medications; 5) did not have cognitive impairment severe enough to preclude informed consent or valid self-reporting; 6) did not have other medical conditions that would compromise participation in the study; and 7) had a fixed address. Approximately 74% of participants reported injecting heroin exclusively, or mostly, at home, whereas approximately 22% preferred to take it exclusively, or mostly, outside the home. The opposite was true for cocaine. A small number of subjects did not express a clear preference for home vs. non-home environments (these individuals were indicated as "50/50" in Figure 3). Virtually identical results were obtained when the analysis was limited to individuals who took both drugs either intravenously or intranasally, indicating that the choice of the setting was not driven by the route of drug taking. We have recently confirmed these results in a study using the EMA technique (Spagnolo et al., 2011).

We used a similar approach to investigate the setting of ketamine use in humans. Preliminary data from this study (n=19) are reported here. In agreement with the findings obtained in rodents, most ketamine users reported taking the drug outside the home rather than at home (Figure 3, right-hand panel). The specific non-home settings of ketamine use were: parties (100%), raves (40%), Goa-like parties (30%), friends' place (30%) and rave festivals (20%). (Notice that each subject could indicate more than one setting.) In the process of conducting this study, we became aware of two previous papers reporting similar

Figure 3 Setting preferences for heroin, cocaine and ketamine use in humans, as indicated by retrospective reports. The data for cocaine and heroin use in addicts co-abusing the two substances (n=79) were published previously (Caprioli et al., 2009). The ketamine data (n=19) are reported here for the first time.

findings. Dillon et al. reported that home was the preferred setting of ketamine use in 16% of cases, vs. 47% at dance and rave parties, 26% at clubs, 10% at the homes of friends and 1% at pubs (Dillon et al., 2001, 2003). Reynaud-Maurupt et al. (2007b) also investigated the circumstances of ketamine use and found that the last dose was taken in private home settings in 35% of cases (notice that this survey did not distinguish between the homes of users and the homes of their friends) vs. non-home environments in 65% of cases (23% at free parties, 14% at techno festivals, 6% at squat parties, 5% at rave parties and 5% in clubs).

Conclusions

We have previously hypothesized that environment influences the reward effects of drugs as a result of the appraisal of drug effects in relation to the surrounding stimuli (Caprioli et al., 2009; Badiani et al., 2011). Each addictive drug produces a distinctive constellation of desired and undesired effects, which may or may not partly overlap with those of other drugs. Some of these effects may be largely indifferent to an environmental context, whereas other effects would be more appropriate (or less inappropriate) in certain settings. The activating, performance-enhancing effects of cocaine and amphetamine, for example, would be experienced as more suitable to an exciting, relatively novel environment than to a home environment. In contrast, the sedative, inward-looking effects of heroin would be experienced as more appropriate to a safe, non-challenging home environment. That is, we hypothesize that the setting might affect drug choice by providing an ecological backdrop against which drug effects are appraised as more or less 'adaptive'. It is important to emphasize that emotional

appraisal does not necessarily entail the conscious evaluation of stimuli (see, for example, LeDoux, 1996, 2012). Thus, the fact that heroin is preferentially taken at home should not be seen as a mere expression of an intentional decision to take a 'downer' where you can 'slouch on the sofa'. It would be difficult to envisage such a mental process in the case of our resident rats, not only because attributing conscious planning to rats would be questionable at best. Indeed, resident rats did not have a choice between different settings but simply adapted their behavior to the context by taking less cocaine (or amphetamine or ketamine) and more heroin relative to non-resident rats.

As previously discussed, one of the reasons for predicting that the self-administration of ketamine, like that of cocaine and amphetamine, would be facilitated in non-resident rats relative to resident rats, was based on the existence of some similarities in the behavioral, physiological and neurochemical effects of ketamine and psychostimulant drugs. Of course, another major reason for predicting greater preference for ketamine in non-resident than in resident rats was the anecdotal evidence that ketamine abuse in humans is associated with clubs or rave parties (Curran and Morgan, 2000; Joe Laidler, 2005; Degenhardt and Dunn, 2008). Remarkably, the findings of human studies (in addition to the data presented here, see Dillon et al., 2001 and Reynaud-Maurupt et al., 2007a) coincided very closely with the results obtained in the rat. In our study, only a minority of users (about 10%) reported using ketamine exclusively, or mostly, at home. These users probably correspond to the 'psychonauts', who are known to titrate the dose to produce an internal state that is not compatible with social gatherings and requires instead quiet environments.

The clinical and pre-clinical findings reviewed here confirm the anecdotal evidence of a major role of setting

for ketamine use. In particular, the study conducted in rats under controlled conditions indicate that the physical environment may affect ketamine reward at a very fundamental level, independent, at least in part, of social interactions. Furthermore, these and other findings (see, for example, Badiani et al., 2011) challenge that notion that drug reward (and more in general reward *tout court*) represents a unified phenomenon, almost invariant of the specific psychopharmacological profile of the various drugs. Much can be learned about the neurobiological underpinning of drug reward by taking into consideration the emotional appraisal of the specific effects produced by each drug within the context of the surrounding environment.

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Differential vulnerability to relapse into heroin versus cocaine-seeking as a function of setting

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Title Page

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Title: Differential vulnerability to relapse into heroin versus cocaine-seeking as a function of setting

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Conflict of interest

The authors report no biomedical financial interests or potential conflicts of interest.

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Abstract

Rationale Previous studies have shown the effect of setting on drug taking is substance specific in both humans and rats. In particular, we have shown that when the setting of drug self-administration (SA) coincides with the home environment of the rats (Resident rats), the rats tend to prefer heroin to cocaine. The opposite was found in Non-Resident rats, for whom the SA chambers represented a distinct environment.

Objectives The aim of the present study was to investigate the influence of setting on the ability of different doses of cocaine and heroin to priming cocaine vs. heroin seeking in rats that had been trained to self-administer both drugs and had then undergone an extinction procedure.

Methods Resident (N=62) and Non-Resident (N=63) rats with double-lumen intra-jugular catheters were trained to self-administer cocaine (400 µg/kg/infusion) and heroin (25 µg/kg/infusion) on alternate days for 10 consecutive daily sessions (3 h each). After the extinction phase, independent groups of rats were given a non-contingent intra-venous (i.v.) infusion of heroin (25, 50, or 100 µg/kg) or cocaine (400, 800, or 1600 µg/kg) and drug seeking was quantified by counting non-reinforced lever presses.

Results All Resident and Non-Resident rats acquired heroin and cocaine SA. However, cocaine primings reinstated cocaine-seeking only in Non-Resident rats whereas heroin primings reinstated heroin-seeking only in Resident rats.

Conclusions We report here that the susceptibility to relapse into drug seeking behavior is drug-specific and setting-specific, confirming the crucial role played by drug, set, and setting interactions in drug addiction.

Keywords Addiction, drug abuse, environment, relapse, reinstatement, self-administration

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Introduction

Human addicts who had become abstinent often relapse into drug-seeking even after long periods of abstinence. Vulnerability to relapse is in fact one of the defining characteristics of addiction (DSM-5). Relapse can be triggered by a variety of stimuli, such as stressors, drug-associated cues or contexts, and exposure to small amounts of the drug itself (Brown et al. 1995; Jaffe et al. 1989; Ludwig et al. 1974; O'Brien et al. 1992; Sinha 2001). This phenomenon can be reproduced in animal models. Rats that have been trained to press a lever to self-administer a drug rapidly decrease lever pressing when the drug is withdrawn but then resume pressing upon presentation of certain stimuli (Crombag et al. 2008; De Wit and Stewart 1981; Shaham et al. 2003). Conditioned stimuli, contextual stimuli, and stressors have all been shown to reinstate drug-seeking after a period of abstinence. Also non-contingent administration of small amounts of the drug (priming) can precipitate relapse. Interestingly, Leri and Stewart (2001) have shown that under certain conditions the priming effect of drugs is substance-specific. They trained rats to self-administer heroin and cocaine, using a two-lever apparatus in which each lever was paired to one of the two drugs, and found that when "primed" with cocaine or heroin the rats exhibited drug-seeking behavior directed to the appropriate lever. This phenomenon is particularly interesting in the light of the growing interest in the substance-specific aspects of drug reward and drug abuse (Badiani et al. 2011; Marchant et al. 2014; Peters et al. 2013).

We have previously found that addicts who co-abuse heroin and cocaine prefer distinct settings for the two drugs. Most addicts reported using heroin preferentially at home and cocaine preferentially outside the home (Badiani and Spagnolo 2013; Caprioli et al. 2009). Even the choice of specific non-home settings (pubs, clubs, friends, parks, etc.) differed between the two drugs (Badiani and Spagnolo 2013). Preclinical studies have shown that the setting can exert a substance-specific influence on drug taking not only in humans but also in laboratory rats (Caprioli et al. 2007a, 2008, 2009; Celentano et al. 2009; De Luca and Badiani 2011; Testa et al. 2011). In these studies, rats with intra-

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jugular catheters were given the opportunity to self-administer the drug intravenously in one of two distinct settings. Some rats were transferred to the self-administration (SA) chambers immediately before the test sessions, and therefore took the drug outside the home (Non-Resident rats). Other rats were kept at all times in the SA chambers, and therefore they took the drug at home (Resident rats). In a manner reminiscent of the human condition, cocaine was more vigorously self-administered by Non-Resident rats than by Resident rats (Caprioli et al. 2007a, 2009) whereas the opposite was found for heroin SA (Caprioli et al. 2008, 2009). When trained to self-administer both cocaine and heroin on alternate days, Non-Resident rats took more cocaine than heroin relative to Resident rats (Caprioli et al. 2009; Celentano et al 2009). Most important, we found that the setting influenced even drug choice. When rats were permitted to self-administer either cocaine and heroin within the same session, most Non-Residents rats preferred cocaine to heroin whereas most Resident rats preferred heroin to cocaine (Caprioli et al. 2009).

The goal of the present study was to investigate whether the setting would exert a substance-specific influence on the vulnerability to relapse into drug-seeking after a period of abstinence.

Material and Methods

Animals

In the present study we used a total of 185 male Sprague–Dawley rats (Harlan Italy, San Pietro al Natisone, Italy) weighing 250–275 g at their arrival. However, some rats were excluded from the analyses because: i) their catheters clogged or broke (15 rats); ii) they were sick (6 rats); iii) they did not satisfy the SA criterion (>2 infusions of cocaine and >2 infusions of heroin in the last 2 sessions; 39 rats; notice that among these rats there were 10 rats that vigorously self-administered heroin but not cocaine and 3 rats that vigorously self-administered cocaine but not heroin).

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Surgery

After their arrival, the rats were housed two per cage for 10-12 days before the surgery, after which were housed individually.

Using standard surgical procedures previously described in detail (Caprioli et al. 2007a; Caprioli et al. 2008), double-lumen catheters were inserted into and secured to the right jugular vein of the rats. The distal end of the catheter was externalized through a small incision at the nape of the neck, and connected to two L-shaped 22-gauge cannulae (one for each lumen) that were secured to the rat's skull using dental cement and stainless steel screws. Each catheter lumen was flushed daily with 0.1 ml of sterile saline solution containing 0.3 mg gentamycin and 12.5 IU heparin (Marvecs Services, Agrate Brianza, Italy).

Apparatus

The apparatus consisted of SA chambers (28.5-cm length, 27-cm width, and 32-cm height) made of transparent plastic (front and rear walls), aluminum (sidewalls and ceiling), and stainless steel (grid floor). Plastic trays covered with pinewood shaving were placed under the cage floors. Each chamber was equipped with 2 retractable levers, positioned on the left-hand wall 12.5 cm apart and 9 cm above the floor, 2 sets of 3 cue lights (red, yellow, and green), positioned above each lever, and a counterbalanced arm holding a liquid swivel. The SA chambers were placed within sound- and light-attenuating cubicles. Each chamber was connected via an electronic interface to a motorized syringe pump (Razel Scientific Instruments, St. Albans, VT, USA) and to a programmable logic controller (PLC; Allen Bradley, Milwaukee, WI, USA). Finally, the PLCs were connected to PCs running control software. Chambers, accessories, and electronic interfaces were purchased from ESATEL (Rome, Italy), and custom-developed control software from Aries Sistemi (Rome, Italy). The infusion line consisted of a length of silastic tubing protected by a stainless steel spring and connected (through the liquid swivel and another length of

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silastic tubing) to a syringe positioned on the pump (which was programmed to work at an infusion rate of 13.3 µl/s).

Experiments

General Procedures: After the surgery, the rats were assigned to one of two conditions: Resident (N=62) and Non-Resident (N=63). The rats in the Resident group were single housed in the SA chambers, where they remained for the entire duration of the experiment. Non-Resident rats were single housed in standard transparent plastic cages (40-cm length, 24.5-cm width, and 18-cm height, with stainless steel tops and flat bottoms covered with ground corncob bedding) and immediately before the start of each session were transferred to the SA chambers. The drug-taking context was therefore physically identical for all rats but for some rats this was also their home (Resident group) whereas for other rats it represented a distinct and, at least initially, novel context (Non-Resident group). All other husbandry routines were identical in the 2 groups. Notice that throughout the experiments, the rats were housed and tested in the same dedicated temperature- and humidity-controlled room (i.e., there was no transport from one room to another and no disruption of circadian rhythmicity) with ad libitum access to food and water (except during the test sessions) under a 14-h dark/10-h light cycle (lights off at 7 a.m.).

Testing began 1 week after the surgery and all testing procedures were identical between Resident and Non-Resident rats (including the absence of food or water). The experiments included 21 sessions; all test sessions lasted 3 h and took place during the dark phase, between 12:30 and 16:30 hours, 7 days a week. The catheters were connected, through infusion lines and liquid swivels, to the infusion syringes, 3 h before the start of each session for Resident rats and immediately before the start of sessions for Non-Resident rats. During the 60s preceding the start of each session Resident rats were briefly handled to remove food and water from the SA cages and the infusion pumps were activated so as to fill the catheters with the drug or saline solution. The doors of the cubicles were closed for the duration of the session and left open at all

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other times. At the end of each session, food and water were given back to the
Resident rats and Non-Resident rats were returned to their home cages.

   The drugs were dissolved in 0.9% sterile saline, and drugs and saline
solution were given in 40µl/3s via motorized pump.

   Training phase (days 1–10): Resident and Non-Resident rats were trained to
self-administer cocaine (400 µg/kg/infusion) and heroin (25 µg/kg/infusion) on
alternate days for 10 consecutive daily 3-hours sessions (i.e., there were 5
sessions for each drug). Cocaine and heroin were each paired with one of two
retractable levers and a cue light (red or green); the starting drug was
counterbalanced within groups and the assignment of levers and cues was
counterbalanced for both drugs. At the start of each session, only the lever
associated with the drug to be self-administered on that session was extended
and the appropriate cue light was turned on. The number of consecutive lever
presses required to obtain a single infusion (Fixed Ratio = FR) was 1 for
sessions 1-2 (FR1), FR2 for sessions 3-4, and FR5 for sessions 5–10. After each
infusion, the cue light was turned off and the lever retracted. After a 40-second
time-out period, the cue light was turned on and the lever extended again. The
rats were allowed to self-administer a maximum of 100 infusions of cocaine or
heroin to minimize the risk of overdosing. To facilitate the acquisition of drug
SA and only when necessary, the rats were "primed" by placing their forepaws
on the lever, so as to trigger one infusion. During session 1-4, infusions were
administered at times 5, 65, and 125 min to rats who had not spontaneously self-
administered at least 1 infusion during time periods 0–5 min, 5–65 min, and 65–
125 min, respectively. On sessions 5–10, infusions were given, if necessary,
only at 5 min to rats that had not spontaneously self-administered at least 1
infusion. These infusions (0.81 ± 0.1 *vs.* 0.56 ± 0.18 infusions of cocaine per
session in Residents *vs.* Non-Residents; 0.14 ± 0.05 *vs.* 0.24 ± 0.09 infusions of
heroin per session in Residents *vs.* Non-Residents) were excluded from
statistical analyses and did not count towards the satisfaction of SA criterion.
Each lumen of double lumen catheters was used the same number of times for
either drug, in a counterbalanced manner. Like in other studies (e.g., Carelli
and Ijames 2000; Carrera et al. 2000; Lenoir and Ahmed 2007; Tran-Nguyen et

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al. 2001) no inactive lever was used in the present study; under our testing conditions inactive responses are negligible.

Extinction phase (days 11-20): The day after the end of the training phase, the rats underwent 10 extinction sessions on FR5 (3 h each), during which the levers and cues associated to, respectively, cocaine and heroin were presented on alternate days, in a manner similar to the training phase, but with the important difference that the completion of the FR5 schedule triggered an infusion of the vehicle and not of the drug solution.

Reinstatement session (day 21): The reinstatement session (1 h) was carried to assess the ability of a non-contingent i.v. priming of cocaine (400, 800, or 1600 µg/Kg) or heroin (25, 50, or 100 µg/Kg,) to restore drug-seeking in independent groups of rats. The rats were connected to double channel liquid swivels; one channel was connected to a syringe containing the appropriate drug solution and the other one to saline syringe. Immediately before the beginning of the session, the infusion pump was briefly activated to deliver the drug priming. Only the lever and cue light associated with the drug used for the priming was made available during each session. As during the extinction sessions, lever pressing resulted in the infusion of saline (on an FR5 schedule). Drug seeking was indicated by non-reinforced lever pressing. Immediately before the start of the session, six independent groups of rats received the following doses of cocaine: 400 (N=12 for both the Resident and the Non-Resident group), 800 (N=10 for both the Resident and the Non-Resident group), or 1600 µg/Kg (N=10 for both the Resident and the Non-Resident group); other six independent groups of rats received instead the following doses of heroin: 25 (N=11 for the Resident group; N=12 for the Non-Resident Group), 50 (N=9 for both the Resident and the Non-Resident group), or 100 µg/Kg (N=10 for both the Resident and the Non-Resident group).

Catheter patency test

At the end of the experiment, all rats underwent a catheter patency test in which they received 2 i.v. boluses of 40 mg/kg of thiopental sodium (Pharmacia Italia,

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Milan, Italy), one in each catheter lumen, with a 15-min interval between the two. The test was considered positive if the rat became ataxic within 5 s after the injection. Rats were included in the analysis only if the test was positive for both lumens of the catheter.

Data Analysis and Statistics

Training phase: The lever pressing behavior and the infusion data for each pair of training sessions were analyzed using a three-way ANOVAs with context (2 levels: Non-Residents *vs*. Residents) as a between-subject factor and drug (2 levels: cocaine *vs*. heroin) and session (5 levels: one for each of the 5 pairs of sessions) as within-subject factors. The hypothesis that Non-Resident rats would take more cocaine relative to heroin than Resident rats was tested by comparing the ratios of cocaine to heroin taking using a two-way ANOVA with context as a between-subject factor and session as a within-subject factor. The ratios of cocaine to heroin infusions were calculated in individual rats. Rarely, some rats failed to reach the Fixed Ratio (FR) for cocaine or heroin and received no infusions; in this case, we assigned a value of 1 to make it possible to calculate the ratio.

Extinction phase: Group differences for cocaine- versus heroin-seeking were assessed using a 3-way ANOVAs with context as the between-subject factor and drug lever and session as within subject factors.

Reinstatement session: The effect of drug primings was assessed using a 4-way ANOVAs with context (2 levels: Non-Residents *vs*. Residents), drug priming (2 levels: cocaine *vs*. heroin) and dose (3 levels: low, medium and high dose) as between-subject factors, and priming (2 levels: last extinction session for the lever paired to the drug used for priming *vs*. reinstatement session) as within-subject factor.

When appropriate, follow-up simple effect ANOVAs and independent or paired samples t-tests were used.

The relationship between drug intake and vulnerability to relapse were investigate using linear regression analysis with cocaine or heroin intake during

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training as the independent variable number of lever presses during the reinstatement session as the dependent variable.

Results

Training phase: As shown in Fig. 1, the rats rapidly acquired cocaine and heroin SA. The ANOVA conducted on drug infusion data yielded significant main effects of drug [$F_{(1,123)}=78.498$, $p \leq 0.0001$], session [$F_{(4,492)}=60.799$, $p \leq 0.0001$], and context [$F_{(1,123)}=7.844$, $p=0.006$]. There were also significant drug x context [$F_{(1,123)}=14.354$, $p \leq 0.0001$], session x context [$F_{(4,492)}=7.975$, $p=0.006$], session x drug [$F_{(4,492)}=60.316$, $p \leq 0.0001$], and session x drug x context [$F_{(4,492)}=13.029$, $p \leq 0.0001$] interactions. Figure 2 illustrates the ratio of cocaine to heroin infusions. Resident rats took about 1.5 as many infusions of cocaine as of heroin, whereas in Non-Resident rats this ratio increased to more than 3. The ANOVA yielded a significant effect of context [$F_{(1,123)}=7.549$, $p=0.007$] but not of session ($p=0.234$), and there was no session x context interaction ($p=0.203$). Given that there was no session x context interactions, pairwise comparisons were inappropriate (thus, were not reported in Fig 2). Notice, however, that they indicated significant differences for sessions 3-4 and 5-6 ($p \leq 0.05$), and for sessions 7-8 and 9-10 ($p \leq 0.0001$).

Extinction phase: During the extinction sessions (Fig. 3) the rate of pressing rapidly decreased, as indicated by a main effect of session [$F_{(4,492)}=144.601$, $p \leq 0.0001$], with a session x drug lever x context interaction [$F_{(4,492)}=2.586$, $p=0.036$]. Pairwise comparison indicated significant differences in pressing on the cocaine-paired lever versus heroin–paired lever in the Non-Resident group but not in the Resident group. In addition, there were significant group differences in pressing on the heroin-paired lever during sessions 1-4.

Reinstatement session: Overall, lever pressing during the reinstatement session was greater than during the first hour of the relative extinction session. However, there were important differences in the ability of heroin and cocaine to trigger relapse as a function of setting. As shown in Fig. 3, when given cocaine primings only Non-Resident rats exhibited reinstatement of cocaine-

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seeking, whereas when given heroin primings only Resident rats exhibited reinstatement of heroin-seeking. The ANOVA indicated a significant main effect of priming [$F(1,113)=31.254$, $p \leq 0.0001$] but not of context ($p=0.091$), drug ($p=0.116$), or dose ($p=0.988$). Most important, there was a priming x context x drug interaction [$F(1,113)=10.320$, $p=0.002$], but not priming x context ($p=0.965$), priming x drug x dose ($p=0.183$), or priming x context x drug x dose ($p=0.898$) interactions. Simple main effect analyses indicated a significant priming effect of heroin in the Resident group ($p=0.002$) and of cocaine in the Non-Resident group ($p \leq 0.0001$). In contrast, there was no significant priming effect of heroin in the Non-Resident group ($p=0.617$), whereas cocaine priming in the Resident group only approached significance ($p=0.074$). Given that there was no effect of dose and no interaction between dose and the other factors, pairwise comparisons were inappropriate (thus, were not reported in Fig 3). Notice, however, that they indicated a significant priming effect for the high ($p=0.001$), medium ($p \leq 0.05$), and low ($p \leq 0.05$) dose of cocaine in the Non-Resident group, but not in the Resident group, and a significant priming effect for the high and medium doses of heroin in the Resident group, but not in the Non-Resident group.

Finally, there was no correlation between the amount of cocaine or heroin self-administered during training and the rate of lever pressing during reinstatement.

Discussion

We report here that vulnerability to relapse into drug-seeking is critically dependent on the setting of drug use. Rats were trained to self-administer heroin and cocaine, using a two-lever apparatus in which each drug was paired to one of the two levers, and were then subjected to an extinction procedure during which lever-pressing was no longer reinforced. Non-contingent administration of low doses of heroin triggered drug-seeking (as indicated by non-reinforced lever pressing) only in rats that had acquired self-administration behavior in the

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home environment (Resident group) whereas cocaine triggered drug-seeking only in rats that had acquired self-administration behavior outside the home environment (Non-Resident group).

Setting and drug reward

The role of context in drug abuse has been a topic of research since the late 1960s (Kelleher and Morse 1968). A great deal of this research has focused on the role of associative learning mechanisms that are thought to underlie the ability of discrete and contextual stimuli to trigger craving and drug-seeking (Crombag et al. 2008). However, environmental stimuli may modulate drug reward in other, more basic ways. It has long been noted, for example, that the circumstances of drug taking can exert a direct influence on the behavioral and subjective response to drugs of abuse (Zinberg 1984). In the past few years we have investigated a particular aspect of this environmental modulation. We have shown that addicts who co-abuse heroin and cocaine tend to use distinct settings for the two drugs. Most addicts reported using heroin at home and cocaine outside the home (Badiani and Spagnolo 2013; Caprioli et al. 2009). Setting preferences appeared to be independent of the route of drug taking, as they were evident in individuals using the same route for both drugs (Badiani and Spagnolo 2013; Caprioli et al. 2009). Even the specific non-home settings differed between heroin and cocaine (Badiani and Spagnolo 2013). Distinct setting preferences have been reported also for alcohol and ketamine users (De Luca et al. 2012; Dillon et al. 2003; Nyaronga et al. 2009).

The ability of context to influence drug reward is not unique to humans. We have previously shown that even in laboratory rats the preference for cocaine versus heroin depends on the setting (Caprioli et al. 2007a, 2007b, 2008, 2009; Celentano et al. 2009). The procedures used in these earlier studies were identical to those used here. Some rats were housed in the SA chambers (Resident rats) whereas other rats were transferred to the SA chambers only for the testing (Non-Resident rats). Non-Resident rats self-administered more cocaine and amphetamine than Resident rats (Caprioli et al. 2007a, 2008)

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whereas Resident rats self-administered more heroin than Non-Resident rats (Caprioli et al. 2008). We also conducted experiments in which rats were trained to self-administer cocaine and heroin on alternate days (Caprioli et al. 2009; Celentano et al. 2009). In this case, Non-Resident rats took more cocaine than Resident rats and the cocaine/heroin intake ratio was greater in Non-Residents than in Residents rats, whereas there was little or no difference in the intake of heroin. Virtually identical results were obtained here (see Fig. 1), suggesting that in Non-Resident rats, cocaine SA facilitated heroin SA. More generally, it appears that there were reciprocal influences in the intake of the two drugs, because in rats that had access to both drugs (see, in addition to the present study, Caprioli et al. 2009 and Celentano et al. 2009) the speed of acquisition and the intake of cocaine and heroin were much greater than in rats that had access to only one of the two drugs (see Caprioli et al. 2007, 2008).

Drug-induced reinstatement ("priming") of drug-seeking

Drug addiction is a chronic relapsing disorder, as indicated by the propensity to resume drug use after a period of abstinence (DSM-5). The determinants of relapse are complex and include factors such as cognitive and emotional states, stress levels, social pressure, and psychiatric comorbidity (see Bradley et al. 1989). Among the external stimuli that can lead to relapse, a great deal of attention has been placed on the role of discrete cues or contexts associated with drug use (Crombag et al. 2008; O'Brien et al. 1992).

Also exposure to small amounts of the drug (such as a puff from a cigarette, a sip of alcoholic beverage, or a snort of cocaine) can trigger relapse (Jaffe et al. 1989; Ludwig et al. 1974). Interestingly, Leri and Stewart (2001) have shown that this phenomenon is substance-specific. Rats that had been trained to self-administer both heroin and cocaine relapse into heroin seeking when primed with the former and into cocaine seeking when primed with the latter. The present study indicates that drug-induced relapse is not only substance-specific but also setting-specific. Cocaine primings reinstated cocaine-seeking only in Non-Resident rats whereas heroin primings reinstated heroin-seeking only in

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Resident rats (except at the dose of 100 μg/kg). It should be noted that these results were not due to differences in drug SA during the training phase, as there was no correlation between the magnitude of lever pressing during the reinstatement phase and drug intake during the training phase.

Given that the only difference between the extinction sessions and the reinstatement session was represented by the drug, it would be reasonable to assume that the differences in drug-seeking during the reinstatement session were due to the priming effect of cocaine or heroin per se. However, it should be noted that the rate of lever pressing during the extinction phase (Fig. 3) remained relatively high, compared to previous reports (Caprioli et al. 2009; Celentano et al. 2009). This might have been due to the fact that, different from our previous studies, during the extinction sessions of the present study the rats were exposed to additional esteroceptive (light) and interoceptive (vehicle infusion) cues, which may have acted acting as secondary reinforcers. Furthermore, during the first few sessions of the extinction phase we found significant differences in responding on the heroin-paired lever, but not on the cocaine-paired lever, as a function of setting, as well as greater responding on the cocaine-paired lever versus the heroin-paired lever in the Non-Resident group. Thus, it cannot be excluded that during the reinstatement session there was an interaction between drug cues and drug priming.

The fact that heroin primings did not reinstate heroin seeking in our Non-Resident rats appears to be at odds with the findings of previous studies that investigated heroin-primed reinstatement under similar housing conditions (Fattore et al. 2003, 2005; Lenoir and Ahmed 2007; Leri and Stewart 2001; Leri et al. 2004; Luo et al. 2004; Sorge et al. 2005). These apparent discrepancies may be due to procedural differences, including the amount of drug self-administered during training, the route of priming administration, and the extinction procedures. However, when the experimental procedures were similar to those used here, heroin failed to reinstate heroin-seeking in Non-Resident rats. Lenoir and Ahmed (2007), for example, reported reinstatment of heroin seeking in "Long Access" Non-Resident rats (which during training self-administered about 26 times more heroin than our rats) whereas the rats in the

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"Short Access" group (which during training self-administered about 2 times more heroin than our rats) did not exhibit reinstatement of heroin-seeking after priming doses of heroin very similar to those used here. Leri and Stewart (2001) found that "Non-Resident" rats trained to self-administer, on alternate days, cocaine and heroin (the latter at the dose of 25 µg/Kg) did not show relapse into heroin-seeking after heroin primings.

Another apparently puzzling result is represented by the dose-effect curve for heroin-induced relapse. Although the statistics indicated no context x dose interaction, mere inspection of Fig. 3 shows that lever pressing after 100 µg/kg heroin was more or less the same of that observed on the last extinction session. However, it is quite possible that this dose could have satisfied the rats' requirements of heroin, as it approximately corresponded to the amount of heroin voluntarily taken by the rats during the first hour of self-administration during training (see Fig. 1).

It is important to emphasize here that we are not arguing that relapse cannot be triggered by cocaine at home and by heroin outside the home in absolute terms. Rather, we argue that, under certain conditions, individual vulnerability to the rewarding effects of addictive drugs, including their ability to precipitate relapse, is sensitive to the circumstances surrounding drug taking.

Conclusions

The present study adds critical evidence to a growing body of data indicating fundamental differences between psychostimulant and opioid reward, as well as between psychostimulant and opiate addiction (Badiani et al. 2011; Badiani 2014). For the past three decades the dominant trend in addiction research has been to emphasize the role of shared substrates of drug reward, with particular emphasis on the mesocorticolimbic dopaminergic system (Nestler 2004). However, both animal and human studies suggest that this "unitary" approach cannot fully account for the complexity of drug effects. Studies in rats (Ettenberg et al. 1982; Gerrits and Van Ree 1996; Pettit et al. 1984) have shown, for example, that chemical lesion or pharmacological blockade of the

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mesolimbic dopaminergic system severely impairs cocaine but not heroin self-administration. Furthermore, two [^{11}C]raclopride PET studies in humans (Daglish et al. 2008; Watson et al. 2013) have shown that heroin can produce subjective "high" without activation of the dopaminergic system. These lines of evidence indicate that the neural substrates of opiate reward do not completely overlap those of psychostimulant reward, making it somewhat less surprising that the same settings could influence in opposite directions the responsiveness to these two classes of drugs.

What type of explanation can account for the effects of setting on drug taking and on drug seeking reported here? It has been proposed (Badiani 2013; Badiani and Spagnolo 2013) that the setting provides a sort of ecological backdrop against which drug effects are appraised as a function of their peripheral and central effects. Each addictive drug produces a distinctive constellation of central and peripheral effects, which may or may not partly overlap with that of other drugs. While some of these effects (e.g., euphoria) may be "neutral" to the context, others would be "felt" as more appropriate (or less inappropriate) to certain settings. The drowsiness and sedation produced by heroin, for example, would be experienced as suitable to a safe, non challenging, domestic environment, whereas the sympathomimetic, activating, performance-enhancing effects of cocaine and amphetamine would be more appropriate to arousing, exciting contexts. Initial evidence in support of this hypothesis comes from our studies showing that ketamine, which, like cocaine, has activating and sympathomimetic effects (Hancock 1999) is preferentially taken outside the home by humans (De Luca et al. 2012; Dillon et al. 2003) and rats (De Luca and Badiani 2011). By contrast, alcohol, which, like heroin, initially causes drowsiness and sedation (Johnson and Ait-Daoud 2005; Morean and Corbin 2010), is taken preferentially at home by humans (Nyaronga et al. 2009) and rats (Testa et al. 2011).

In summary, we reported here that the setting in which cocaine and heroin are taken can exert a powerfully influence on reward effects of these drugs. In particular, it appears that the susceptibility to relapse into drug-seeking behavior is, under certain conditions, substance-specific and setting-specific.

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Our results also suggest that heroin and cocaine addiction are distinct entities. Other pre-clinical and clinical findings, including the lack of pharmacological effective treatments for both cocaine and heroin addiction, support the notion that much is to be gained by taking in due account the substance-specific aspects of drug addiction (for a recent review, see Badiani et al. 2011). The differences between cocaine and heroin here illustrated might have important implications for therapy, suggesting, for example, that cognitive-behavioral approaches should be tailored so as to allow the addict to anticipate, and cope with, the risks associated in a substance-specific manner to the various environmental settings of drug use.

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Figure Captions

Figure 1. Mean (±SEM) number of lever presses and infusions for cocaine (400 µg/kg) and heroin (25 µg/kg) self-administration (SA) during the training phase for the Resident and Non-Resident groups. *, **, ***, and *** indicate significant ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.01$, and $p \leq 0.001$, respectively) differences in cocaine vs. heroin SA. †, †† and †††† indicate significant ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$, respectively) differences between the Resident and the Non-Resident group.

Figure 2. Mean (±SEM) ratio of cocaine to heroin infusions during training (see Fig. 1) for the Resident and the Non-Resident group. ††† indicates a significant ($p \leq 0.001$) main effect of context.

Figure 3. Mean (±SEM) number of lever presses on the cocaine- versus heroin-paired lever during the extinction phase for the Resident and Non-Resident groups. * and ** indicate significant ($p \leq 0.05$ and $p \leq 0.01$, respectively) differences in lever pressing for the cocaine-paired vs. the heroin paired lever. † indicates significant ($p \leq 0.05$) differences between the Resident and the Non-Resident group.

Figure 4. Mean (±SEM) number of lever presses during the first hour of the last extinction session (white bars) *versus* the reinstatement session (black bars) in the Resident and Non-Resident groups. At the beginning of the reinstatement session, independent groups of rats (N values are indicated by the numbers within the white bars) received a non-contingent intra-venous (i.v.) infusions of one of three doses of cocaine (top panels) or heroin (bottom panels). ## and #### indicate significant ($p \leq 0.01$ and $p \leq 0.0001$, respectively) main effect of priming.
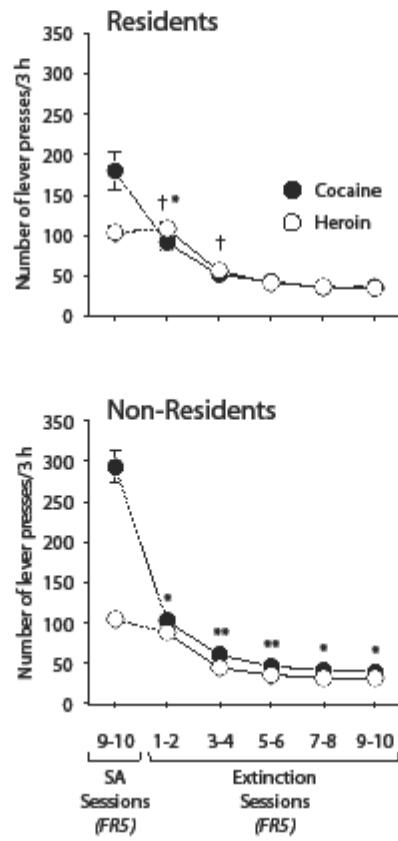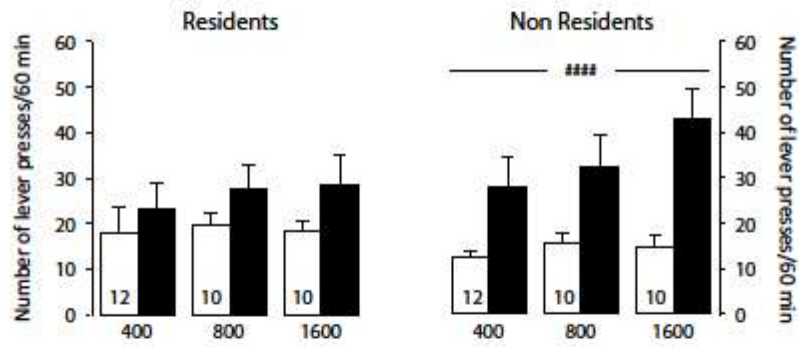
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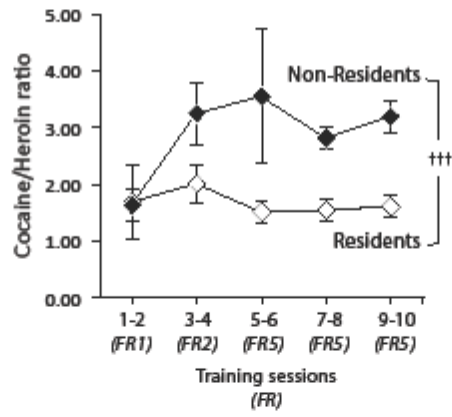
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A – Cocaine-induced Reinstatement

B – Heroin-induced Reinstatement