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The role of environmental context in the vulnerability to relapse into heroin and cocaine addiction: a pre-clinical investigation

by
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Publications

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

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1.Abstract

Relapse to compulsive drug-seeking behavior after abstinence is a major problem in the treatment of drug addiction (O'Brien 1997; Stewart 2000); in both human addicts and laboratory animals, after a period of drug withdrawal, reexposures to an addictive drug, a stressful event, or drug-associated environmental cues, often induce drug craving and precipitate relapse to drug-seeking (Jaffe et al. 1989; Carter and Tiffany 1999; Sinha et al. 1999; Shalev et al. 2002). The treatment is further complicated by the fact that drug abuse is rarely limited to a single substance, polydrug use being the norm rather than the exception. In particular, it is a well-documented fact in a number of countries that most heroin addicts also abuse cocaine and vice versa (Leri et al. 2003); such concurrent users are more likely to have poorer treatment outcomes, interrupt treatment programs and to relapse (Broers et al. 2000; Downey et al. 2000; Gossop et al. 2002; Leri et al. 2003). It is important, therefore, to better understand the basis of cocaine and heroin co-abuse and relapse from both pharmacological and ecological point of view, and their reciprocal interactions. From an ecological point of view the preference for one drug or another is widely thought to be a function of local availability, street price, lifestyle, and other socio-cultural factors (Anthony and Chen 2004; Westermeyer 2004; Johnson and Golub 2005; Jofre-Bonet and Petry 2008). It was also proposed that the circumstances immediately surrounding drug taking can modulate drug taking but the evidence is largely anecdotal (Dalgarno and Shewan 1996; McElrath and McEvoy 2002; Stallwitz and Shewan 2004). This is probably due not only to the extreme difficulty of manipulating in a controlled fashion the context of drug taking in our species but also to the strongly held belief that the environmental variables implicated in drug abuse are paramount with cultural or economical factors.

We have recently developed an animal model to study under controlled laboratory conditions the role of setting on drug taking (Caprioli et al. 2007).

To do it we used the intravenous drug self-administration (SA) model, in which laboratory animals typically make a lever press or nose poke to receive contingent drug injections (the premise of this procedure is that drugs of abuse control behavior by functioning as operant positive reinforcers). In our model some rats were transferred to the SA chambers immediately before the SA sessions (Non Resident rats)- a procedure commonly used in most SA studies- whereas other animals were kept at all times in the SA chambers (Resident rats). We have shown an unforeseen dissociation in the effect of environmental context on psychostimulant versus opiate SA: Non Resident rats self-administered more cocaine and amphetamine than Resident rats (Caprioli et al. 2007; Caprioli et al. 2008) and the contrary was found for heroin (Caprioli et al. 2008). Similar results were obtained also when rats were trained to self-administer cocaine and heroin on alternate days or within the same session (Caprioli et al. 2009; Celentano et al. 2009). Interestingly, most human addictcs using both cocaine and heroin, reported similarly to our rats, using heroin at home and cocaine outside the home regardless the drugs were injected or snorted, and regardless the drugs were taken in isolation or with others (Caprioli et al. 2009; Badiani and Spagnolo 2013).

The aim of the research of my Ph.D thesis was to investigate the influence of setting (Non Residents vs Residents) on the ability of different doses of heroin and cocaine priming to reinstate heroin- vs. cocaine-seeking in rats that had been trained to self-administer both drugs and had then extinguished lever pressing behavior (in order to better model the typical pattern of human co-abuse the rats were trained to self-administer cocaine and heroin on alternate days). Specifically, Resident and Non-Resident rats with 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 days (3 hours/session/day). Afterwards, the rats underwent 10 extinction sessions, during which lever pressing resulted in the infusion of vehicle. Finally, after extinction of lever pressing behavior, independent groups of rats were given a

non-contingent intravenous 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. Surprisingly, when given cocaine primings only Non Resident rats exhibited reinstatement of cocaine-seeking and, in contrast, when given heroin primings only Resident rats exhibited reinstatement of heroin-seeking. These results indicate that the setting in which cocaine and heroin are taken can exert a powerful influence on the rewarding effects of these drugs. In particular, it appears that the susceptibility to relapse into drug-seeking behavior after a period of abstinence is substance- and setting-specific. A possible explanation for these results is based on the different central and peripheral non-hedonic effects of opiate versus psychostimulant drugs (for a review, see Badiani 2013). The sedative, parasympathomimetic effects of heroin, for example, may be "appraised" as performance-impairing when in the potentially hostile, non-home environment as opposed to the safe home cage. Hence, heroin would have been appraised as more rewarding by Residents than by Non-Resident rats. In contrast, the arousing, sympathomimetic effects of cocaine may be appraised as anxiogenic at home but not in a more exciting non-home environment, thus making cocaine more rewarding in Non-Residents than in Resident rats. Other pre-clinical and clinical findings, including the lack of pharmacological treatments effective 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 reward and drug addiction (for a review, see Badiani et al. 2011). In particular, the differences between cocaine and heroin illustrated in my research 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.

The present dissertation is organized in other five chapters. The second chapter briefly introduces drug addiction (or substance dependence) in humans and the corresponding animal models. The third chapter contains a review of the neurobehavioral pharmacology of addictive drugs. The fourth chapter summarize the existing, albeit scant literature, concerning the role of context in modulating drug effects, while the fifth chapter summarize the findings obtained in our laboratory concerning the role of circumstances immediately surrounding drug taking in modulating drug effects. Finally the sixth chapter extensively reports on the experimental studies conducted for my dissertation and above summarized.

2. Drug addiction

Substance dependance, or addiction, as currently defined by the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV TR), is a maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by three (or more) of the following, occurring at any time in the same 12-month period:

(1) Tolerance, as defined by either of the following:

a. A need for markedly increased amounts of the substance to achieve intoxication or desired effect.

b. Markedly diminished effect with continued use of the same amount of the substance.

(2) Withdrawal, as manifested by either of the following:

a. The characteristic withdrawal syndrome for the substance.

b. The same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms.

(3) The substance is often taken in larger amounts or over a longer period than was intended.

(4) There is a persistent desire or unsuccessful efforts to cut down or control substance use.

(5) A great deal of time is spent in activities necessary to obtain the substance, use the substance, or recover from its effects.

(6) Important social, occupational, or recreational activities are given up or reduced because of substance use.

(7) The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.

Addiction, therefore, is the final stage of a usage process that moves from drug use to abuse to addiction. Clinically, the occasional but limited use of a

drug with the potential for abuse or dependence is distinct from escalated drug use and the emergence of a chronic drug-dependent state. Addiction, in fact can be defined by its diagnosis, etiology and pathophysiology as a chronic relapsing disorder characterized by the loss of control over drug use, or by the compulsive seeking and taking of drugs despite adverse consequences. The goals of the addicted person become narrowed to obtaining, using, and recovering from drugs, despite failure in life roles, medical illness, risk of incarceration, and other problems. It's caused by the actions of a drug of abuse on a vulnerable brain and generally requires repeated drug exposure. This process is strongly influenced both by the genetic makeup of the person and by the psychological and social context in which drug use occurs. Once formed, an addiction can be a life-long condition in which individuals show intense drug craving and increased risk for relapse (often precipitated by drug, drug-associated stimuli, and stressful events) (Jaffe et al. 1989; Carter and Tiffany 1999; Sinha et al. 1999; Shalev et al. 2002) after years and even decades of abstinence. This means that addiction involves extremely stable changes in the brain that are responsible for these long-lived behavioural abnormalities.

Much of the recent progress in understanding the mechanisms of addiction has derived from the study of animal models of addiction on specific drugs, such as opiates, stimulants and alcohol. While no animal model of addiction fully emulates the human condition, animal models do permit investigation of specific aspects of the process of drug addiction. In the intravenous drug self-administration (SA) model (for reviews, see Shaham et al. 2003; Stewart 2008), animals typically make a lever press or nose poke to receive contingent drug infusions (the premise of this procedure is that drugs of abuse control behaviour by functioning as operant positive reinforcers). In the Conditioned Place Preference (CPP) procedure (for a review, see Aguilar et al. 2009), a particular stimulus environment is paired with the effects of the drug (delivered by the researcher), without the animal having to learn to make a

response to obtain the drug, and a second environment is explicitly paired with the absence of the drug. In the test trial, the animal is allowed, while in a drug-free state, to move freely between the area previously paired with the drug and the unpaired environment. An increase in preference for the drug-associated context serves as a measure of the conditioned rewarding effects. In the Runway model (McFarland and Ettenberg 1997) instead, the speed with which a laboratory animal traverses a long, straight alley for a drug, is considered an index of the animal's motivation to seek the reinforcer. Finally, the drug-discrimination model (for a review, see Koek 2011) is an animal model of the subjective effects of drugs. In this model, laboratory rodents or monkeys are trained to discriminate between a drug state and a non-drug state, or between different drug states. In a typical experiment, a food-restricted animal is trained in a two-lever operant chamber in which the food-reinforced lever differs as a function of whether drug or saline was administered before the session. The model that best resemble the human condition is the drug SA procedure, in which the animals self-administer alone addictive drugs and spontaneously control the own drug intake. More important for this dissertation, the reinstatement model is an animal model of relapse to drug seeking. In the operant-conditioning version of this model (for reviews, see Shaham et al. 2003; Stewart 2008), laboratory animals are first trained to self-administer drugs and after submitted to a period of extinction training (in which lever pressing is not more reinforced but results in vehicle infusions), or simply to the passage of time; finally the presentation of cues explicitly paired with drug delivery during the training, a brief exposure to a stressor or an drug injection all result in increased drug-seeking behaviour. In many respects, the reinstatement phenomena seen in laboratory animals correspond closely with relapse in humans (for a critical review, see Epstein et al. 2006) In both cases, the individuals have a history of drug self-administration, are currently drug-free and not actively seeking the drug before the reinstatement or relapse event occurs. In both cases, drug-seeking responses return after the

precipitating event such as administration of the drug, exposure to drug-related cues, and exposure to stressful events. In the conditioned place preference (CPP) version of the reinstatement model (for a review, see Aguilar et al. 2009), CPP is induced by a drug, extinguished and then induced again by drug priming injections or stressors. It is on this background of renewed drug seeking, or reinstatement, that we are able to begin a search for pharmacological and neurochemical manipulations that can block or attenuate such behaviour.

3. Neurobehavioral pharmacology of addictive drugs

Research done in the last two decades has emphasized the role of shared neural substrates for the behavioral response to addictive drugs (for a review, see Nestler 2004). In particular, it has been shown that virtually all drugs of abuse can increase, albeit via different mechanisms of action, dopamine levels in the terminal regions of the mesotelencephalic dopaminergic system (Di Chiara and Imperato 1988) (Figure 3.1). Cocaine and amphetamine induce dopamine overflow by binding the dopamine reuptake transporter (for reviews, see Johanson and Fischman 1989; Kuczenski and Segal 1994) whereas heroin and morphine facilitate dopaminergic transmission by binding mu-opioid receptors (MOR) in the ventral tegmental area and substantia nigra, hence disinhibiting mesotelencephalic dopamine-releasing neurons (Gysling and Wang 1983; Matthews and German 1984; Johnson and North 1992; Devine et al. 1993).

In this chapter, reviewing the acute and chronic neuropharmacological effects of addictive drugs, and the neural plasticity and neural circuitry where this effects happens, I will show briefly both common and distintive effects of psychostimulants and opioids drugs.

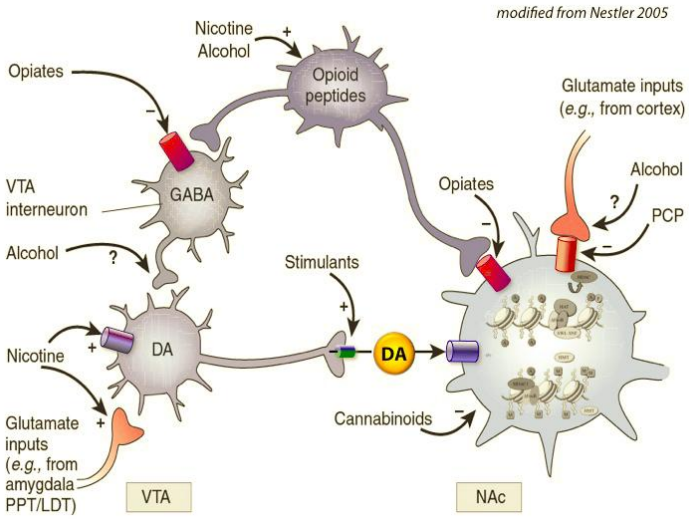

Figure 3.1 Shared circuitry of drug reward

3.1 Acute neuropharmacological effects of psychostimulants and opiates

Psychostimulants produce their psychoactive effects by potentiating monoaminergic transmission through actions on dopamine, serotonin and norepinephrine transporters. Psychostimulants differ in their actions as reuptake-inhibitors versus substrate type-releaser (Fleckenstein et al. 2000; Rothman et al. 2003). Cocaine bind to transporter proteins and interfere with transporter function but is not transported into the nerve terminal. In contrast, amphetamines analogous are classified as “releasers”, due to the fact that they bind to transporter proteins and are subsequently transported into the cytoplasm of the nerve terminal. Releasers elevate extracellular monoamine levels by reversing the process of transporter-mediated exchange, thereby enhancing monoamine efflux (Sulzer et al. 2005). They also increase cytoplasmatic levels of monoamines by interfering with vesicular storage (Rudnick et al. 1993). Typically, releasers are more effective than reuptake-inhibitors in increasing extracellular monoamines because the former increases the pool of neurotransmitters available for release by transporter mediated exchange. Moreover, the effectiveness of releasers in increasing extracellular monoamines is not dependent upon the basal rate of the neurotransmitter release. In contrast, the effectiveness of reuptake-inhibitors is impulse-dependent and, therefore, limited by the tone of pre-synaptic activity.

In addition, psychostimulants differ in their relative affinity for dopamine, serotonin and norepinephrine transporters. Cocaine binds to serotonin transporters with approximately a fivefold greater potency than at dopamine transporters and bind to norepinephrine transporters with approximately threefold lower affinity than dopamine transporters. In contrast, amphetamine, methamphetamine and methylphenidate all have relatively lower affinity for serotonin transporters compared to their affinity for dopamine and norepinephrine transporters. However, the behavioural effects of psychostimulants associated with their rewarding and addictive properties

have been linked primarily to the enhanced dopaminergic activity in the mesocorticolimbic dopamine system (Di Chiara and Imperato 1988).

Dopamine neurotransmitter binds on two families of dopamine receptors termed D1-like or D2-like (Civelli et al. 1991; Schwartz et al. 1992). The D1-like receptors, which include D1 and D5 receptors, are coupled to Gs/Golf protein and thus stimulate adenylate cyclase (AC) to produce the intracellular second messenger 3'-5'-cyclic adenosine monophosphate (cAMP). cAMP in turn activates cAMP-dependent protein kinase (PKA), which phosphorylates numerous substrates, including L-type calcium channels, transcription factors such as cyclic-AMP response-element-binding protein (CREB), and other intracellular signalling components. The D2-like receptors, which comprise D2, D3, D4 receptors, are coupled to Gi/Go protein and thus inhibit AC and also activate an inwardly rectifying potassium channel.

Since the earliest reports by Chang et al. (1988) and Graybiel et al. (1990) it has been known that both psychostimulant and opioid drugs are able to induce the expression of the gene encoding for the Fos protein (c-fos) and other IEGs in a number of forebrain regions, including the caudate nucleus and the NAc (for a review, see Harlan and Garcia, 1998). The interest in this phenomenon is due not only to the fact that IEGs can serve as indicators of neuronal activity (Hughes and Dragunow, 1995; Harlan and Garcia, 1998), but also because they are thought to represent an important initial step in mediating drug experience dependent plasticity (Nestler, 2001; Hyman and Malenka, 2001; Ujike et al., 2002). The IEGs (Immediate-Early Genes) are rapidly (within hours) and transiently expressed in response to a variety of drugs of abuse, and the protein products of these IEGs act as transcription factors that potentially regulate expression of wide variety of other cellular genes (reviewed in Hughes and Dragunow 1995). Acute administration of cocaine has been reported to increase expression of several IEGs in the striatum, including c-Fos (e.g. Graybiel et al. 1990; Young et al. 1991), JunB (Hope et al. 1992) and zif268 (Bhat et al. 1992; Hope et al. 1992). The c-Jun

response to acute cocaine treatment has been reported to be weak (Kosofsky et al. 1995). The most robust c-Fos, JunB and zif268 responses within the caudate putamen nucleus (CPu) were reported to be in the dorsocentral portion, although nearly all regions of the CPu responded. Although the nucleus accumbens (NAc) has been a focus of research on rewarding behaviours, for most of the drugs of abuse, the IEGs response in the NAc has been less robust than that of the dorsal striatum. Common non-striatal brain regions have also been reported to display IEGs responses after psychostimulant. Both cocaine and amphetamine increased expression of one or more IEGs in the cerebral cortex, with the most frequent sites reported to be the cingulate, piriform, and somatosensory cortex. An involvement of glutamate receptors, especially the N-metyl-D-aspartate (NMDA) receptor, in striatal induction of IEGs following acute psychostimulant also appears to be a common mechanism. Blockade of NMDA receptors has been reported to attenuate, if not completely block, the striatal IEGs response to cocaine (Torres and Rivier 1993; Couceyro et al. 1994) and amphetamine (Wang et al. 1994a; Konradi et al. 1996). An involvement of striatal AMPA receptors has also been reported for amphetamine (Wang et al. 1994b). These results are consistent with the reported increased extracellular concentrations of glutamate following psychostimulant (for a review see Kalivas 1995).

Opioid drugs exert their actions by binding to several subtypes of opioid receptors, each characterized by unique distributions (Mansour et al. 1993, 1994 a,b,c), which appears to subserve different physiologic functions. To date, four opioid receptors have been cloned, the MOP (μ = mu for morphine), the KOP (κ = kappa for ketocyclazocine), the DOP (δ = delta for deferens because it was first identified in mouse vas deferens), and the NOP-R (nociceptin/orphanin FQ receptor). While the physiologic role of each of the four receptor types is not yet fully known, it does appear that μ and δ receptors are involved in systems that influence mood, reinforcing effects,

respiration, pain, blood pressure, and endocrine and gastrointestinal function. κ receptors, when activated, can produce endocrine changes and analgesia. In contrast to μ and δ agonists, which are self-administered by animal under experimental conditions, pure κ agonists appear to produce aversive effects in animals (Woods et al. 1987) and dysphoria, rather than euphoria, in human subject (Musacchio et al. 1990). Opioid receptors are coupled to pertussin toxin-sensitive, heterodimeric Gi/Go proteins, which, upon activation, inhibit cAMP pathway. However the action of opioids include the activation of phospholipase C (PLC) (Murthy et al. 1996), the release of calcium from intracellular stores (Jin et al. 1992), the activation of the mitogen-activated protein kinase (MAPK) cascade (Burt et al. 1996; Fukuda et al. 1996; Li and Chang 1996). Also in this case, as previously shown for psychostimulants, the rewarding effect of opioids, are related to the enhanced dopaminergic activity in the mesocorticolimbic dopamine system, although the mechanisms are different; opioids in fact increases dopaminergic neurotransmission in the NAc via the activation of dopamine cells in the VTA, an area that possesses a high density of μ-opioid receptors. This activation results mainly from the inhibition of GABA-ergic interneurons in the VTA (Johnson and North 1992; Bonci and Williams 1997), and then to disinhibition of dopaminergic neurotransmission. Acute morphine treatment also alters the levels of opioid peptides. Prodynorphin mRNA is increased in the NAc after acute treatment (Wang et al. 1999a). Peptides produced from this gene can inhibit morphine-induced dopamine release and produce tolerance to morphine's rewarding effects. As well, levels of orphanin FQ/nociceptin (OFQ/N), is increased in NAc by acute morphine treatment (Romualdi et al. 2002). Thus the acute action of morphine on opioid peptides appears to be compensatory for the increased dopaminergic transmission.

Acute administration of morphine and heroin increased expression of IEGs in several area of the rat brain. Increased expression of the c-fos gene in the striatum, after acute morphine, has been detected with different methods such

as in situ hybridization (Liu et al. 1994; Garcia et al. 1995) and immunocyto chemistry (Liu et al. 1994; Garcia et al. 1996). Within the CPu, a consistent c-Fos response was noted in the dorsomedial region, extending throughout the entire rostral-caudal length of the striatum (Liu et al. 1994; Garcia et al. 1995; Curran et al. 1996). Induction of Fos mRNA expression in the NAc has been reported (Liu et al. 1994; Garcia et al. 1995; Curran et al. 1996; Nye and Nestler 1996). Within the NAc, the shell responded somewhat more than the core (Garcia et al. 1995), and there was no obvious relationship with the distribution of μ opiate receptors (Garcia et al. 1996).

3.2 Chronic neuropharmacological effects of psychostimulants and opiates

The research at the molecular level has led to examining how repeated perturbation of intracellular signal transduction pathways leads to changes in nuclear function and altered rates of transcription of particular target genes. Two transcription factors in particular have been implicated in the plasticity associated with addiction: cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and ΔFosB.

CREB regulates the transcription of genes that contain a CRE site (cAMP response element) within the regulatory regions and can be found ubiquitously in genes expressed in the central nervous system such as those encoding neuropeptides, synthetic enzymes for neurotransmitters, signaling proteins and other transcription factors. CREB can be phosphorylated by protein kinase A (PKA), Ca2+/calmodulin-dependent protein kinase IV, or protein kinases regulated by growth factor–RAS pathways, putting it at a point of convergence for several intracellular messenger pathways that can regulate the expression of genes. CREB is best known for its roles in learning and memory, but it was implicated in drug addiction (Guitart et al. 1992) because its activation was a predictable consequence of up-regulation of the cAMP pathway, one of the best-established adaptations to drugs of abuse.

While acute administration of drugs of abuse can cause a rapid (within hours) activation of IEGs, other transcription factors (isoforms of ΔFosB) accumulate over longer periods of time (days) with repeated drug administration. Biochemically modified isoforms of ΔFosB are induced only slightly by acute drug exposure; however, these ΔFosB isoforms begin to accumulate with ripetute drug administration owing to their high stability. This extraordinary stability resides in the ΔFosB protein *per se* and not in its mRNA, which is relatively unstable, like that of other Fos family members (Carr et al. 1998). As a result, ΔFosB persists in the brain for relatively long periods of time. This phenomenon is a common response to many classes of addictive drugs and animals with activated ΔFosB have exaggerated sensitivity to the rewarding effects of drugs of abuse.

Direct activation of PKA activity – which increases CREB phosphorylation – within the NAc reduces the rewarding effects of cocaine, whereas PKA inhibition has the opposite effects (Self et al. 1998). Similarly, elevation of CREB expression in the NAc decreased cocaine self-administration or over-expression of a dominant-negative CREB in the form of a mutant CREB which acts as a CREB antagonist in the NAc increased cocaine reward (Carlezon et al. 1998). The effects of up-regulation of the cAMP pathway and CREB in the NAc are mediated partly by the opioid peptide dynorphin, which is expressed in a subset of NAc medium spiny neurons; dynorphin causes dysphoria by decreasing dopamine release within the NAc through an action on κ-opioid receptors that are located on presynaptic dopamine-containing nerve terminals in this region (Hyman 1996; Shippenberg and Rea 1997). Moreover, the dysphoria caused by CREB over-expression in the NAc is blocked by κ-opioid receptor antagonists (Carlezon et al. 1998).

Following chronic psychostimulant use, the 35–37 kD isoforms of ΔFosB can be detected, in particular within the NAc. This isoform however is present in several other areas, among which the dorsal striatum, prefrontal cortex (PFC),

amygdala, hippocampus, bed nucleus of the stria terminalis (BNST) and interstitial nucleus of the posterior limb of the anterior commissure (Perrotti et al. 2005, 2008). A work in which ΔFosB was selectively expressed within the dynorphin-containing class of medium spiny neurons in adult mice provides direct evidence that induction of ΔFosB mediates sensitized behavioural responses to cocaine (Kelz et al. 1999).

Chronic opioid causes very different, and even opposite, physiological responses from acute opioid use. In accordance many genes show opposite changes in response to acute and chronic morphine treatment. This differential regulation suggests that there is a difference between the immediate, and likely compensatory, response to a single morphine treatment and the longer-term plasticity that results from chronic treatment. Acute morphine decreases the functional activity of the cAMP pathway in many types of neuron. However with continued opiate exposure, functional activity of the cAMP pathway gradually recovers, and increases above control levels following removal of the opiate. These changes in the functional state of the cAMP pathway are mediated via the induction of AC and PKA in response to chronic administration of opiates. Mice with mutations in the *CREB* gene show decreased development of opiate physical dependence, as indicated by an attenuated withdrawal syndrome after administration of an opioid receptor antagonist (Maldonado et al. 1996). Moreover CREB is acutely inhibited by opioid and increased during opioid withdrawal (Guitart et al. 1992; Widnell et al. 1994). Blockade of CREB function within the LC (Locus Coeruleus) reduces the electrophysiological and behavioural markers of opiate physical dependence and withdrawal (Han et al. 2006; Lane-Ladd et al. 1997) and alterations in CREB function within the striatum are also involved in the opioid addiction processes (Chartoff et al. 2003).

Regarding the induction of 35-37 kD isoforms of ΔFosB by chronic morphine, the most dramatic induction was seen in the NAc (in particular the core region), and dorsal striatum. In addition, there have been several reports

of lower levels of ΔFosB induction in certain other brain regions, including PFC, amygdala, ventral pallidum and hippocampus (Nye and Nestler 1996; Perrotti et al. 2005; McDaid et al. 2006; Liu et al. 2007).

3.3 Neural plasticity

ΔFosB expression is the longest-lasting known molecular change in the brain seen in the field of drug exposure. Nevertheless, ΔFosB undergoes proteolysis at a finite rate and dissipates to normal levels within a month or two of drug withdrawal. This means that ΔFosB *per se* cannot mediate the extremely long-lived changes in the brain and behaviour associated with addiction. It's likely that altered expression of genes descrive above, would lead to altered activity of the neurons where such changes occur, and ultimately to changes in neural circuits in which those neurons operate. In fact it's increasingly hypothesized that morphological changes in synaptic structure are the only processes by which the plasticity underlying drug addiction can become near permanent. Data from diverse behavioural experiments with drugs of abuse has implicated specific signalling molecules previously identified as key players in long-term potentiation (LTP) and long-term depression (LTD) at other synapses (Kelley 2004). It is believed that LTP and LTD initiate changes in signalling pathways, and in the synthesis and localization of proteins, which eventually alter the polymerization of actin to affect spine maturation and stability, and ultimately to produce a functional spine (LTP) or retraction of an existing spine (LTD) (for review see Tada and Sheng 2006; Bourne and Harris 2007). Structural plasticity is generally characterized by altered dendrite branching or arborization and by changes in the density or morphometry of dendritic spines. Although the direct behavioural relevance of experience-dependent morphological changes is still under investigation, it is believed that synaptic function is determined not only by the number, but also the size and shape, of each individual spine head.

Studies demonstrate that in vivo administration of cocaine produces long-term changes at excitatory synapses on VTA dopamine neurons (Ungless et al. 2001). To monitor changes in excitatory synaptic strength, the investigators measured the ratio of AMPAR-mediated excitatory postsynaptic currents (EPSCs) to NMDA receptor-mediated EPSCs (the AMPAR/NMDAR ratio), and found that a single exposure to cocaine caused a large increase in this ratio in VTA dopamine cells when measured 24 hours later in brain slices. This drug-induced LTP was prevented when animals were pre-treated with an NMDA receptor antagonist. No change in the AMPAR/NMDAR ratio was observed at hippocampal synapses or at excitatory synapses on VTA GABA-ergic cells, indicating that the effect of cocaine at VTA dopamine cell synapses was specific (Ungless et al. 2001). Although the mutant animals still developed locomotor sensitization to cocaine, conditioned place preference to cocaine was absent, as was their conditioned increase in locomotor activity when placed in the activity box in which they had previously experienced cocaine (Dong et al. 2004). These results are consistent with the idea that drug-induced LTP of excitatory synapses on VTA DA neurons might be necessary for attributing motivational significance to the drug experience or for the learned association between context and drug experience. Surprisingly, after repeated exposure to cocaine, the AMPAR/NMDAR ratios remained at the same level seen 24 hours after a single injection (Borgland et al. 2004). The persistence of the potentiation was also similar in both groups: ratios remained elevated five days after the last cocaine injection but were near control levels after ten days. These results are consistent with the idea that although potentiation of excitatory synapses on VTA DA neurons may initially contribute to the incentive value attributed to the drug experience, adaptations in downstream circuitry are likely to be more important for the longer-lasting behavioural changes associated with addiction.

The rich evidence that sustains the involvement of synaptic plasticity in the development of psychostimulant addiction are in contrast with a relative

modest literature observed for opioid addiction. The demonstration of LTP of inhibitory synapses on VTA dopamine neurons (Nugent et al. 2007) has led researchers to investigate whether or not morphine, which modulates inhibitory function in the VTA, can modulate LTPGABA. Intriguingly, it was found that in vivo morphine administration entirely blocked LTPGABA (Nugent et al. 2007). GABAa synapses in VTA slices from rats that had received morphine 24 h earlier did not exhibit LTP. Pharmacological blockade of GABA-ergic transmission, presumably by enhancing Ca2+ influx by depolarization or NMDA receptor activation, facilitates the induction of LTP at excitatory synapses. The modulation of dopamine transmission in the VTA as a result of the loss of LTPGABA will therefore contribute not only to increased dopamine cell firing and dopamine release, but also to LTP at excitatory synapses as a consequence of morphine exposure (Saal et al. 2003).

There are some important differences between psychostimulans- and opiates-induced synaptic plasticity. First, Robinson and Kolb (1997) found that repeated non-contingent injections of amphetamine in rats induce persistent increases in dendrite branching and spine density in NAc medium spiny neurons and mPFC layer III pyramidal neurons. These findings were extended to cocaine and amphetamine self-administration; by contrast, morphine self-administration had the opposite effect; it causes long-lasting decreases in the complexity of dendritic branching and in the number of dendritic spines in NAc and mPFC (Robinson and Kolb 2004; Russo et al. 2010) (Figure 3.2). Second, studies using *ex vivo* whole-cell electrophysiology have shown that morphine and cocaine differ in their ability to induce LTP and LTD at GABAergic synapses (LTPGABA and LTDGABA) on VTA dopamine neurons. Morphine exerts bidirectional control on such synapses: a single non-contingent injection of morphine in rats abolished both LTPGABA and LTDGABA in brain slices; by contrast, cocaine seems to downregulate the strength of such synapses (Dacher and Nugent 2011; Niehaus et al. 2010). Finally, facilitation of LTP in the mPFC

of rats occurred after withdrawal from repeated cocaine exposure (Huang et al. 2007; Lu et al. 2010) whareas withdrawal from heroin self-administration in rats had no effect on LTP as measured by the AMPA:NMDA ratio in the mPFC (Van den Oever et al. 2008).

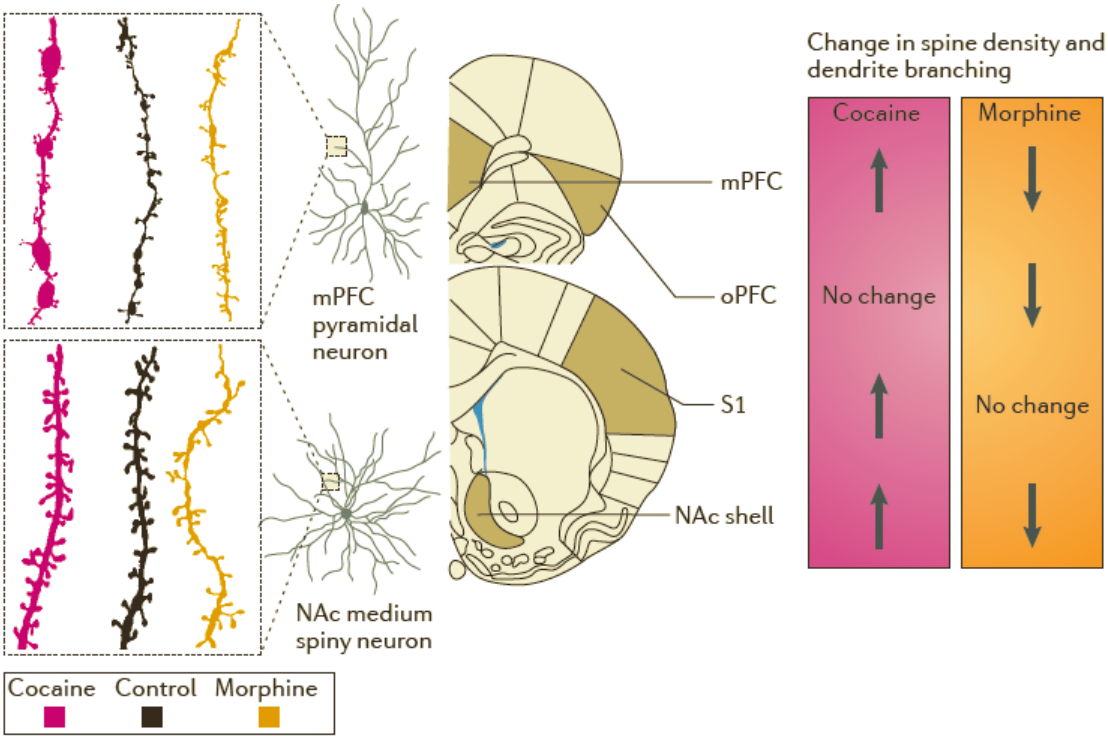

Figure 3.2 Morphine and cocaine have opposite effects on strucural neuroplasticity in the Nac and mPFC (from Badiani et al. 2011; modified from Robinson & Kolb 2004).

3.4 Neurobiological basis of drug reward

The primary neuropharmacological action of psychostimulants responsible for the psychomotor and rewarding effects appears to be on the dopamine systems in the central nervous systems. Lesions of the mesocorticolimbic dopamine system with 6-hydroxydopamine (6-OHDA), have been shown to block amphetamine- and cocaine-stimulated locomotor activity (Kelly et al. 1975;

Roberts et al. 1980). Similar effects have been observed following microinjection of selective dopamine antagonists into the region of the NAc (Pijnenburg et al. 1975). In contrast, disruption of function in the nigrostriatal system blocks the stereotyped behaviour associated with administration of high doses of d-18 amphetamine (Kelly and Iversen 1976). When 6-OHDA lesions are restricted to the striatum itself, such lesions block the intense, repetitive behaviour produced by high doses of amphetamine, and this results in intense locomotor activity (Koob et al. 1984). Thus the terminal regions of the nigrostriatal and mesocorticolimbic dopamine systems appear to mediate different aspects of psychomotor stimulant actions that can have significant implications associated with stimulant abuse. Neurotoxin selective lesions of the mesocorticolimbic dopamine system also block the reinforcing effects of cocaine and amphetamine. Rats trained to self-administer cocaine intravenously and then given a 6-OHDA lesion of the NAc show an extinction-like response pattern and a long-lasting decrease in responding (Roberts et al. 1977, 1980).

The neuronal elements in the region of the VTA and the NAc appears to be the responsible of the activational and reinforcing properties of opioids, and there are both dopamine-dependent and dopamine-independent mechanisms of opioid action (Stinus et al. 1980, 1989; van Ree et al. 1999). Strong evidence for a role of the VTA in the acute reinforcing properties of opioids in nondependent rats comes from place conditioning studies (Bals-Kubik et al. 1993); moreover dopaminergic lesions of the NAc block the acquisition of opioid place preference (Spyraki et al. 1983), and systemic treatment as well as intra-NAc treatment with dopamine antagonists also blocked the development of opioid place preference (Leone and Di Chiara 1987; Longoni et al. 1998). Intracranial self-administration studies have established that the lateral hypothalamus, NAc, amygdala, PAG and VTA all support morphine self-administration (McBride et al. 1999). Intacranial self-administration was established in the lateral hypothalamus and NAc of the rat (Olds 1979) and

amygdala, lateral hypothalamus and PAG in the mouse (Cazala 1990; David and Cazala 1994; David et al. 1998) and the VTA in both rats and mice (Bozarth and Wise 1981; Welzl et al. 1989; David and Cazala 1994; David et al. 1998). In opioid reinforcement, a role for neural elements in the NAc postsynaptic to dopamine afferents became more important with the observation that dopamine receptor blockade and dopamine denervation of the NAc, can eliminate cocaine and amphetamine self-administration (Lyness et al. 1979), but can spare heroin and morphine self-administration (Ettenberg et al. 1982; Pettit et al. 1984; Smith et al. 1985; Dworkin et al. 1988); while systemic administration of dopamine antagonists has been shown to attenuate opioid self-administration (van Ree and Ramsey 1987; Gerrits et al. 1994; Hemby et al. 1996), most of these effects were observed only at doses which affect motor effects or rate of responding (van Ree et al. 1999).

4. The role of context in modulating drug taking

For more than three decades now, research on drug addiction has mostly focused on the cascade of molecular changes stemming from the actions of drugs at specific binding sites in the brain. This approach has been enormously successful in advancing the field, showing, among other things, that virtually all addictive drugs modulate the activity of the mesotelencephalic dopaminergic system, particularly at the level of the nucleus accumbens (NAc), although with different mechanisms of action. Any attempt to reduce the behavioral and psychological effects of drugs to the straightforward consequences of ligand–receptor binding, however, risks overlooking the complexity of drug–environment interaction. Indeed, both clinical and preclinical evidence indicate that drug addiction is a multifactorial disorder in which genetic and environmental variables interact in modulating individual responsiveness to addictive drugs (Figure 4.1) (see Anthony and Chen 2004).

Historically it has been shown that the environment can impinge on the propensity to the abuse drugs in two major ways. First, certain life experiences can make an individual more vulnerable to develop drug addiction or to relapse into drug seeking (conversely, other types of experience may protect an individual from the risk of becoming drug dependent or to relapse after becoming abstinent). Moreover, neutral environmental cues can acquire, through their association with drugs, the ability to trigger drug seeking even after long periods of abstinence; not even a cursory attempt will be made to review here the literature concerning the role played by environmental cues in the expression of drug sensitization (Anagnostaras and Robinson 1996) or in the acquisition of instrumental learning (for a review see Cardinal and Everitt 2004).

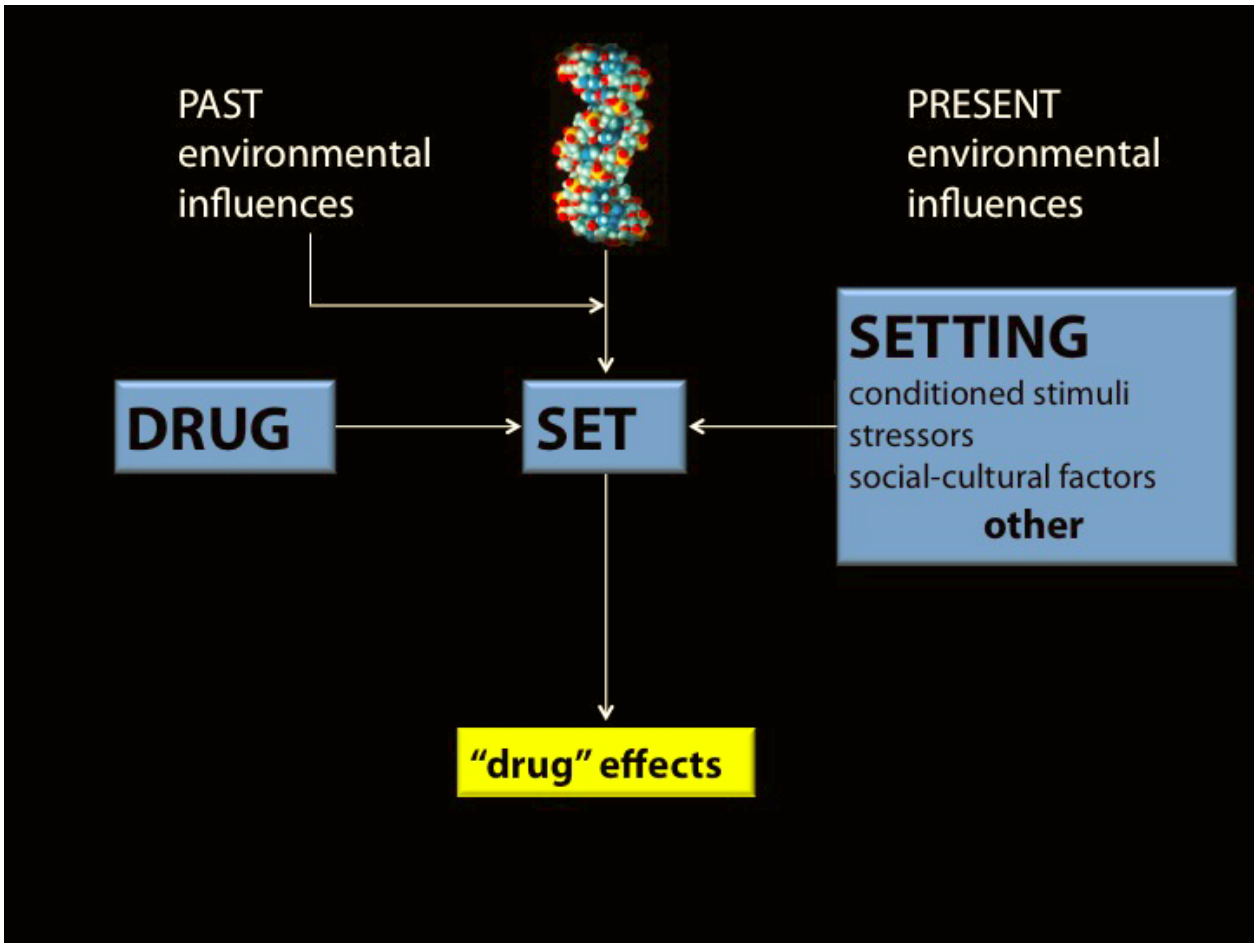

Figure 4.1 Drug-set-setting interaction

4.1 Adverse life experiences

There is a sizeable literature on the association between a history of adverse life experiences and drug addiction. Events as different as sexual abuse/harassment, combat-stress, occupational stress, marriage dissatisfaction, and physical traumas have been linked to the abuse of psychostimulants, opioids, and alcohol (Aro 1981; Triffleman et al. 1995; Richman et al. 1996; Jose et al. 2000; Brady et al. 2001; Clark et al. 2001; Price et al. 2004; Ompad et al. 2005; Brown et al. 2006; Reed et al. 2006). In

particular, there is ample evidence that adverse childhood experiences play an important role in the susceptibility to drug abuse (Dembo et al. 1988; Newcomb and Bentler 1988; Harrison et al. 1997; Dube et al. 2003; Mullings et al. 2004; Fothergill and Ensminger 2006; Osler et al. 2006).

The evidence for a causal relationship between adverse life experiences and addiction in humans, however, has not received comparable support, mostly because of the intrinsic difficulties of conducting controlled studies in our species. The use of animal models represents therefore a major tool for assessing the causal role of stress in drug addiction, as well as for investigating its neurobiological substrates. Several animal models of adverse life experiences have been developed in the last decades. These models vary considerably from the point of view of predictive and construct validity and are characterized by different degrees of ecological and ethological validity.

4.1.1 Early life experiences

Impoverished rearing conditions: Studies concerning the role of rearing conditions in the susceptibility to acquire self-administration behaviour have almost exclusively focused on the comparison between group-housed (GH) and single-housed rats (SH). That is, animals were housed at weaning either individually or in groups of 2–3 per cage for an extended period of time and were later single housed and tested for acquisition of self-administration behaviour. In some experiments the GH environment consisted of a large chamber containing toys, wheels, and other enrichment items (EH). The rationale for these animal models is to somewhat reproduce the impoverished rearing conditions that are thought to increase the vulnerability to drug addiction in humans as well as to assess the putative protective influence of more stimulating rearing environments.

Disappointingly, the results of these studies are not consistent, probably because of differences in experimental design, drug delivery system, type of drug, dosage, etc. In some experiments SH exhibited greater susceptibility to

the reinforcing effects of cocaine than GH rats (Schenk et al. 1987; Boyle et al. 1991). In contrast, other studies reported greater cocaine self-administration in GH than in SH rats (Hill and Powell 1976; Morse et al. 1993; Phillips et al. 1994 a,b). Finally, some studies found no important differences in cocaine self-administration in SH vs GH rats (Bozarth et al. 1989). A series of papers from Bardo's laboratory has shown facilitation of self-administration at medium but not at high doses of amphetamine in SH vs EH rats (Bardo et al. 2001; Green et al. 2002; Stairs et al. 2006). However, no differences in amphetamine self-administration between SH and GH rats were reported by Schenk et al. (1988). Modest facilitation of morphine and heroin self-administration in SH rats relative to GH rats was found by Alexander and colleagues (1978) and by Bozarth and colleagues (1989). To the best of my knowledge there is only one paper (Bardo et al. 2001) that has compared drug self-administration in SH, GH, and EH rats. In this study it was found that amphetamine self-administration in GH rats was intermediate between that of SH and EH rats, suggesting that both social interaction and object novelty contribute to blunt the reward efficacy of amphetamine.

Repeated maternal separation: There is solid evidence that a host of neuroendocrine alterations results from repeated brief maternal separations in rodents (handling). Thus, it has been hypothesized that this manipulation may increase the vulnerability to acquire drug self-administration at adulthood. However, the studies that have employed this model have yielded contradictory findings. Facilitation of cocaine, morphine, and alcohol self-administration, has been reported by some authors (Kosten et al. 2000; Huot et al. 2001; Ploj et al. 2003; Lynch et al. 2005; Vazquez et al. 2005). These effects have been attributed to the ability of neonatal isolation to facilitate psychostimulant-induced dopamine overflow in the striatal complex (Kehoe et al. 1996; Kosten et al. 2005). There is also some evidence of greater alcohol intake in peer-reared than in mother-reared rhesus monkeys (Higley et al. 1991; Fahlke et al. 2000). In contrast, little or no effect of maternal separation

on cocaine and alcohol self-administration has been reported by other authors (Matthews et al. 1999; Jaworski et al. 2005).

4.1.2 Physical stressors

Exposure to a variety of physical stressors has been reported to facilitate drug self-administration, although the literature presents numerous discrepancies. The most studied of these stressors is represented by electrical foot shock, which has been reported to facilitate the self-administration of cocaine (Ramsey and van Ree 1993; Goeders and Guerin 1994; Goeders 2002; Mantsch and Katz 2007), morphine (Shaham and Stewart 1994), and alcohol (Myers and Holman 1967; Anisman and Waller 1974; Volpicelli and Ulm 1990; Fidler and LoLordo 1996). Other physical stressors include tail pinch, which has been shown to facilitate amphetamine self-administration (Piazza et al. 1990), and immobilization, which has been shown to increase alcohol intake (Shaham 1993). Piazza and colleagues have proposed a major role for the HPA axis and glucocorticoid receptors in these effects of physical stressors (Piazza and Le Moal 1998; Marinelli and Piazza 2002; Goeders 2003).

Most important, because of its heuristic value, is the phenomenon of stress-induced reinstatement of drug seeking. In this animal model, developed by Yavin Shaham and Jane Stewart, electric footshock is used to precipitate drug seeking after extinction of responding in animals that had been previously trained to self-administer heroin (Shaham 1993; Shaham et al. 1996; Shaham et al. 2000), cocaine (Erb et al. 1996; Ahmed and Koob 1997; Shalev et al. 2003), or alcohol (Le et al. 1998, 1999, 2006). Drug seeking is indicated by the fact that the animals resume lever pressing on the previously active lever despite the absence of drug reinforcement.

A great deal of work has been conducted to elucidate the neural bases of stress-induced reinstatement of drug seeking. A very important role is played by noradrenergic transmission. Indeed, alpha-2 receptor agonists (which inhibit noradrenergic transmission) have been shown to attenuate stress-induced

reinstatement of cocaine, heroin, and alcohol seeking whereas alpha-2 receptor antagonists (which activate noradrenergic transmission) potently reinstate methamphetamine and alcohol seeking (Erb et al. 2000; Highfield et al. 2001; Lee et al. 2004; Shepard et al. 2004). These effects of alpha-2 receptor agonist and antagonists probably depend on the inhibition and activation, respectively, of the lateral tegmental noradrenergic neurons, but not of LC neurons (Shaham et al. 2000).

Another important substrate of stress-induced relapse is represented by extrahypothalamic CRH mechanisms. Indeed, non-selective CRH receptor antagonists, as well as selective CRH1 receptor antagonists, have been shown to attenuate footshock-induced reinstatement of cocaine, heroin, and alcohol seeking whereas the suppression of stress-induced release of circulating corticosterone did not block the ability of footshock or CRH receptor agonist to precipitate reinstatement (Shaham et al. 1997; Erb et al. 1998; Shaham et al. 1998; Le et al. 2000). At least two locations for the CRH mechanisms implicated in stress-induced relapse have been proposed. Some studies have emphasized the importance of CRH-containing projections from the central nucleus of the amygdala to the BNST (Erb et al. 2001). Other studies have demonstrated an important role of stress-induced CRH release in the VTA, where, in cocaine-experienced but not in cocaine-naive rats, CRH acquires control over local glutamate release, thereby regulating the activity of the mesocorticolimbic dopamine system (Wang et al. 2005). This is consistent with the reports of an involvement of NAc D3, GluR1, and GluR2 receptors in stress-induced relapse (Self and Choi 2004; Xi et al. 2004).

One of the most striking aspects of the susceptibility of stressed animals to relapse into drug seeking is its time course. Indeed, the frequency of responding (which is considered an index of the intensity of drug seeking) depends on the duration of withdrawal from the drug before the reinstatement test. The temporal pattern of drug seeking follows an inverted-U shaped curve, increasing over a period of several weeks before declining (Ciccocioppo et al.

2001; Shalev et al. 2001; Grimm et al. 2003), indicating that it depends on the development of long-lasting neuroadaptations. The nature of these neuroadaptations has not been established with certainty. An important role seems to be played by BDNF, a growth factor involved in synaptic plasticity (Lu et al. 2004) via the activation of the ERK pathway (Poo 2001). It has been shown that during withdrawal from cocaine, BDNF expression increases in the mesolimbic system, particularly in the NAc, amygdala, and VTA, where its temporal profile parallels that of drug seeking (Grimm et al. 2003). In addition, withdrawal from cocaine has been found to be accompanied by glutamate-dependent activation of the ERK signalling pathway in the central amygdala (Lu et al. 2005, 2006, 2007).

A thorough discussion of the preclinical validity of reinstatement models can be found in a recent paper by Epstein and colleagues (2006).

4.1.3 Food restriction

A number of studies have investigated the effect of food restriction on drug self-administration of various drugs, including cocaine, amphetamine, ketamine, and phencyclidine (PCP). Most of these studies were conducted by Carroll and colleagues and were concerned with studying the acquisition of drug self-administration in free-feeding vs food-restricted rats or monkeys (Carroll et al. 1981, 1986; Carroll 1982; Carroll and Stotz 1983; Comer et al. 1995 a,b; Campbell and Carroll 2000). It was found that food restriction facilitates the acquisition of self-administration behaviour for most drugs. Similar findings were obtained by others (de la Garza et al. 1981; Oei 1983; Papasava and Singer 1985; Glick et al. 1987; De Vry et al. 1989). This phenomenon is so robust that many authors have incorporated it into their self-administration protocols in order to obtain more reliable and consistent self-administration behaviour. Interestingly, when food-restricted animals are refed self-administration behaviour declines (Carroll and Stotz 1983; Papasava and Singer 1985; Carroll et al. 1986; Comer et al. 1995 a,b), indicating that it is

the state of the animals and not the history of deprivation that characterizes this model.

It is not clear what type of mechanism is responsible for the facilitating effect of food restriction on drug self-administration. Conflicting findings on the role of the HPA axis have been reported (Campbell and Carroll 2001; Carroll et al. 2001). However, it is reasonable to hypothesize that food restriction produces a non-specific enhancement of the motivational state of the animals.

Other studies have shown that acute food deprivation can precipitate, in a manner similar to physical stressors, reinstatement of drug seeking in the rat (Highfield et al. 2002; Shalev et al. 2003, 2006), an effect that is attenuated by central infusions of the hormone leptin (Shalev et al. 2001). Food deprivation-induced relapse, as with other types of stress-induced relapse, appears to depend on the activation of extra-hypothalamic CRH mechanisms because it can be blocked by intracerebroventricular injections of a CRH receptor antagonist (alpha-helical CRH) but not by adrenalectomy, suggesting that corticosterone plays at most a permissive role in this phenomenon (Shalev et al. 2003, 2006)

4.1.4 Social stress

There is robust evidence that male rats exposed to aggression from either same-sex or opposite-sex (lactating females) conspecifics exhibit greater vulnerability to acquire cocaine self-administration relative to rats engaging in non-aggressive social encounters (Haney et al. 1995; Miczek and Mutschler 1996; Tidey and Miczek 1997; Kabbaj et al. 2001). In contrast, it appears that social defeat can reduce alcohol self-administration (van Erp and Miczek 2001; Funk et al. 2005, Croft et al. 2005).

Although the mechanisms responsible for the facilitating effects of social stress on cocaine self-administration in the rat are not known, there is some evidence of an involvement of limbic areas such as the prelimbic and infralimbic cortex, NAc, amygdala, and VTA. Two months after repeated

exposure to social defeat, rats exhibit in fact altered levels of the mRNAs for the transcription factors Fos and zif268 in these brain areas (Miczek et al. 2004; Nikulina et al. 2004; Covington et al. 2005). In contrast, it is unlikely that the HPA axis plays more than a permissive role in this phenomenon, as shown by Covington and Miczek (2005), who found that after a social encounter both "defecate" and "victorious" rats exhibited comparable levels of plasma corticosterone and yet stress-induced facilitation of cocaine self-administration was observed only in the former group.

There are also reports that subordinated cynomologus monkeys living in a hierarchical social group self-administer more cocaine than dominant monkeys, which has been related to reduced expression of D2 receptors in the striatal complex (Morgan et al. 2002; Czoty et al. 2005).

Finally, a particular type of social stress has been described by Ramsey and van Ree (1993), who reported that rats forced to witness other rats receiving foot-shock, or being placed on a hot-plate, self-administered more cocaine than control animals.

4.2 Conditioning

Research done in the last two decades has shown that environmental stimuli paired with drug taking (or with life events capable of affecting drug taking) can acquire, through associative learning, the ability to elicit responses related to the drug experience or even motivate the behaviour directly serving as secondary reinforcers. This type of Pavlovian conditioning has been described in humans since the 1980s, manifesting itself as withdrawal-like symptoms as well as drug craving (Childress et al. 1984, 1986).

The hope of identifying more effective relapse prevention treatments has generated much interest in the neurobiological bases of conditioned withdrawal and craving. The introduction of functional imaging techniques has allowed for the investigation of these phenomenon in vivo and non-invasively

(Grant et al. 1996; Maas et al. 1998; Sell et al. 1999; Volkow et al. 2006). This research has yielded important findings. For example, it has been demonstrated that cocaine addicts watching a video showing cocaine cues exhibited increased dopaminergic transmission in the dorsal striatum (Volkow et al. 2006). These findings are in agreement with the results of studies conducted in rats using in vivo microdialysis (Ito et al. 2002). The concordance of findings between human and animal studies is reassuring because the availability of brain imaging techniques has not made the use of animal models of drug conditioning superfluous. In particular, actual relapse as opposed to self-reported drug craving cannot be investigated, at least presently, using imaging techniques.

Hence, the considerable literature concerning the phenomenon of cue-induced relapse in the rat is of great preclinical importance. Indeed, after the pioneering early work by Stewart and colleagues (de Wit and Stewart 1981; Stewart 1983, 1984), an ever growing number of studies has been showing that drug-paired cues can reinstate drug seeking after extinction of operant responding, in a manner similar to what has already been described for footshock (see above). Interestingly, all types of cues appear to be effective because relapse has been observed after exposure to discrete cues (Davis and Smith 1976; Meil and See 1996), as well as to discriminative (McFarland and Ettenberg 1997; Weiss et al. 2001) and contextual (Crombag and Shaham 2002) cues. These cues acquire conditioned stimulus properties by distinct processes. Discrete cues (e.g., a light, a tone, or both) are paired to a drug's infusion, discriminative cues signal drug availability to animals that are exposed during training to that drug, whereas contextual cues are represented by the self-administration chamber. In the cue-induced reinstatement model, extinction procedures are conducted in the absence of these cues (in the case of contextual cues the rats undergo extinction in different self-administration cages).

Much research effort has focused on the mechanisms responsible for cue-induced relapse (for reviews, see Bossert et al. 2005). Until recently, the neural substrates of cue-induced relapse were thought to be largely independent from those responsible for relapse induced by drug priming and/or stress. Recent evidence, however, indicates that the neurobiological bases of these three types of trigger largely overlap, including the facilitatory involvement of glutamatergic transmission in the VTA (Bossert et al. 2004; Wang et al. 2005) and of dopaminergic transmission in the PFC (McFarland and Kalivas 2001; Capriles et al. 2003; McFarland et al. 2004) and in the amygdala (Alleweireldt et al. 2006). This overlap, however, is not complete. For example, context-dependent relapse has been linked to the activation of glutamatergic receptors in the shell of the NAc (Bossert et al. 2006), whereas the contrary has been found for cue-induced and drug-induced reinstatement (McFarland et al. 2003; Fuchs et al. 2004; Peters and Kalivas 2006).

5. The role of "circumstances" of drug taking in modulating neurobehavioral drug effects

Not all environmental factors capable of affecting the effects of addictive drugs can be conceptualized as adverse life experiences or conditioning. Since the 1960s, albeit sporadically, a number of authors have emphasized the importance of the setting in which drugs are experienced as an important determinant of their behavioral and subjective effects (for example, see Kelleher and Morse 1968; Zinberg 1984; Barrett 1987; Falk and Feingold 1987). A dramatic example of the role of setting is represented by the pattern of use of 3,4-methylenedioxymethamphetamine (MDMA) and ketamine. MDMA- and ketamine-taking are in fact limited almost exclusively to clubs and rave parties (Schifano 2000; Parrott et al. 2004; De Luca et al. 2012). Yet, the evidence concerning the interaction between drugs and environment is largely anecdotal and virtually all published studies deal with the effects of alcohol and cannabis (e.g. Carlin et al. 1972; Lindman 1982; Sher 1985). The only study devoted to amphetamine shows the extreme difficulty of manipulating in a controlled fashion the circumstances associated with the consumption of illicit substances in humans (Zacny et al. 1992). This dearth of information is not unique to the field of human addiction but also applies to the literature on animal models of drug addiction. Generally, until recently, there has been relatively little information about the mechanisms by which environmental setting can modulate drug responsivenss, the available data mostly concerning the role of physical setting. Changes in the physical characteristics of the environment (e.g. size and shape of the cage, type of bedding, etc.) have been shown to have large effect on the behavioral effects of drugs (Ellinwood and Kilbey 1975; Beck et al. 1986; Sullivan et al. 1992; Willner et al. 1992; Einat and Szechtman 1993; Klebaur et al. 2001). There is also some evidence that the presence of novel objects can reduce the self-administration of amphetamine (Klebaur et

al. 2001; Cain et al. 2004) and heat increase that of MDMA (Cornish et al. 2003).

Environmental context, however, can modulate the drug effects independently of its physical characteristics. We (Badiani's Lab) have developed an animal model in which the neurobehavioral response to addictive drugs was studied in rats tested in one of two settings. In this model, some animals were transferred to the test cages immediately before the experimental session (Non Resident Group)- a procedure commonly used in most animal models of drug addiction- whereas other animals were kept in the test cages at all times (Resident Group) (Figure 5.1). Thus, the physical characteristics of the environment in which the animals are tested are virtually identical, with all differences being purely psychological.

In this chapter I will show the results obtained applying this model to drug discrimination, drug sensitization, and drug self-administration procedures. Moreover I will show the environmental modulation on gene expression, and finally a series of human studies conducted by us that confirm the results obtained with animals.

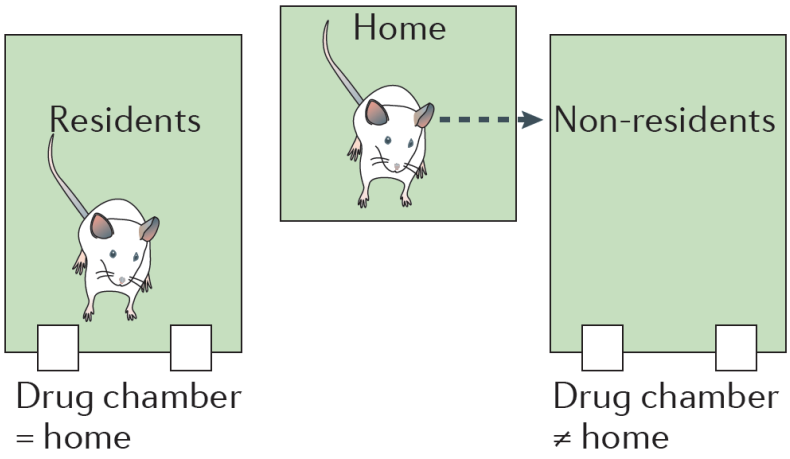

Figure 5.1 Schematic illustration of Non Resident *vs* Resident conditions

5.1 Modulation of drug discrimination

The interoceptive effects of addictive drugs can be altered by environmental context, as shown by a study in which a classical water-reinforced operant conditioning procedure was used to investigate the ability of rats to discriminate low doses of amphetamine from saline (Paolone et al. 2004). In that study it was found that when rats were trained to discriminate amphetamine (0.25 mg/kg, i.p.) from saline in the Resident condition, no animal reached the discrimination criterion. When the same training took place in the Non Resident condition more than half of the rats acquired amphetamine discrimination. Furthermore, we have shown that the effects of environmental context on amphetamine discrimination are dose-dependent. When the training dose of amphetamine was increased from 0.25 to 0.5 mg/kg the facilitatory effect observed in the Non Resident group decreased. The most conservative way to interpret these data is in terms of a leftward shift in the dose–effect curve for drug discrimination produced by experiencing amphetamine under the Non Resident condition.

In preliminary studies based on a very limited number of animals we found that 33% of the Non Resident rats discriminate very low dose of cocaine (1.25 mg/kg, i.p.) vs 0% of Resident rats. Surprisingly the ability of a very low dose of heroin (0.5 mg/kg, i.p.) to provide interoceptive cues is reduced and not enhanced when this drug is experienced in the Non Resident vs Resident condition. It should be noted that these modulatory effects of environment were not accompanied by changes in the ability of the animals to learn and perform the operant routine.

5.2 Modulation of drug-induced psychomotor sensitization

Repeated administration of addictive drugs has been shown to produce behavioral adaptations that are more easily quantified than the state of addiction. In particular, it is well known that repeated exposures to cocaine, amphetamine, morphine and heroin can induce sensitization to the psychomotor activating effects of these drugs (Robinson and Becker 1986; Stewart and Badiani 1993). The interest in the phenomenon of drug-induced psychomotor sensitization derives from the hypothesis that the neuroadaptations responsible for it are similar to those implicated in drug addiction (Robinson and Berridge 1993, 2000, 2003).

Using our model, in the last 10 years we have conducted a series of studies to investigate how the development of psychomotor sensitization to addictive drugs can be modulated by environmental context. In agreement with the notion of shared substrates for activating effects of psychostimulant and opioid drugs, it has been found that psychomotor sensitization to cocaine (Badiani et al. 1995a; Hope et al. 2006), amphetamine (Badiani et al. 1995b; Crombag et al. 1996; Badiani 1997), morphine (Badiani et al. 2000a; Paolone et al. 2003) and heroin (Paolone et al. 2007) are facilitated in rats that are exposed to the activity chambers only for the treatments (Non Resident rats) relative to rats that are kept in the activity chambers at all times (Resident rats). This effect is particularly striking when the treatments are administered via intravenous catheters activated by remote control, so that the animals in the Resident Group are almost completely deprived of any cue that could signal drug delivery. Under these conditions, repeated administration of low doses of amphetamine (Crombag et al. 1996; Browman et al. 1998b; Fraioli e al. 1999; Ostrander et al. 2003), cocaine (Browman et al. 1998b) and morphine (Badiani et al. 2000b) produced robust sensitization only in the Non Resident Group. However, at higher doses psychostimulant drugs produce psychomotor sensitization regardless of environmental context (Browman et al. 1998a, b),

indicating that context does not gate sensitization in an all-or-none way but modulate the ability of drugs to induce the neuroadaptations responsible for this type of behavioural plasticity.

5.3 Modulation of drug self-administration

In these experiments male Sprague-Dawley rats received a catheter in their right jugular vein and after the surgery were assigned to one of two conditions: Resident and Non Resident. Non Resident rats were transferred to the self-administration chambers immediately before the experimental session - a procedure commonly used in most self-administration studies- whereas Resident rats were kept in the self-administration chambers at all times (Figure 5.2).

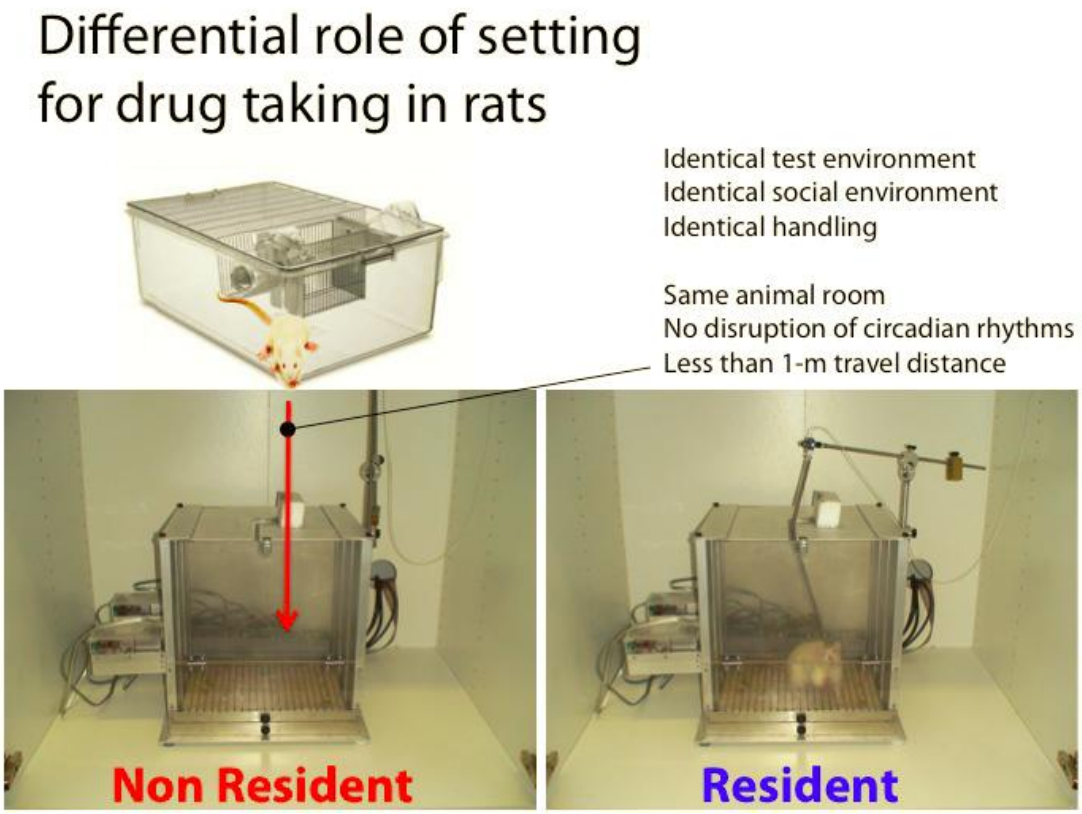

Figure 5.2 Non Resident vs. Resident condition

The following is a summary of the characteristics of the Resident *vs* the Non Resident condition: (1) The SA 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 and, at least initially, novel environment (Non Resident group). (2) The distance travelled by the Non Resident rats during the transfer to the SA chambers was about 1-2 m. Indeed, all animals were kept in the same dedicated testing rooms for the entire duration of the experiments and therefore there was no transport from one room to another and no disruption of circadian rhythmicity. (3) During testing, the SA chambers contained no food or water. The rest of the time the animals had free access to food and water. (4) Both Resident and Non Resident rats were drug naïve before the start of the experiments. (5) All husbandry routines were identical in the 2 groups.

The Figure 5.3 summarizes the results concerning the *acquisition* of drug self-administration under Non Resident vs. Resident condition (it is shown the number of lever pressing on the last training session). Environmental context was devoid of effects on the saline self-administration; so the effects expalined below cannot be attributed to a non-specific state of hyperactivity associated with the arousing properties of the Non Resident condition. Cocaine and amphetamine self-administration were greater in the Non Resident rats than in the Resident rats and the opposite was found for heroin (Caprioli et al. 2007, 2008). Indeed, it appears that dose-effect curve for the acquisition of cocaine and amphetamine self-administration was shifted to the right in the Resident versus the Non Resident condition. In contrast, heroin self-administration was greater in the Resident rats than in the Non Resident rats, with an upward shift of the dose-effect curve. The results of the progressive ratio sessions confirmed the dissociation in the modulatory effects of environmental context on cocaine and amphetamine *vs* heroin SA (Caprioli et al. 2007, 2008).

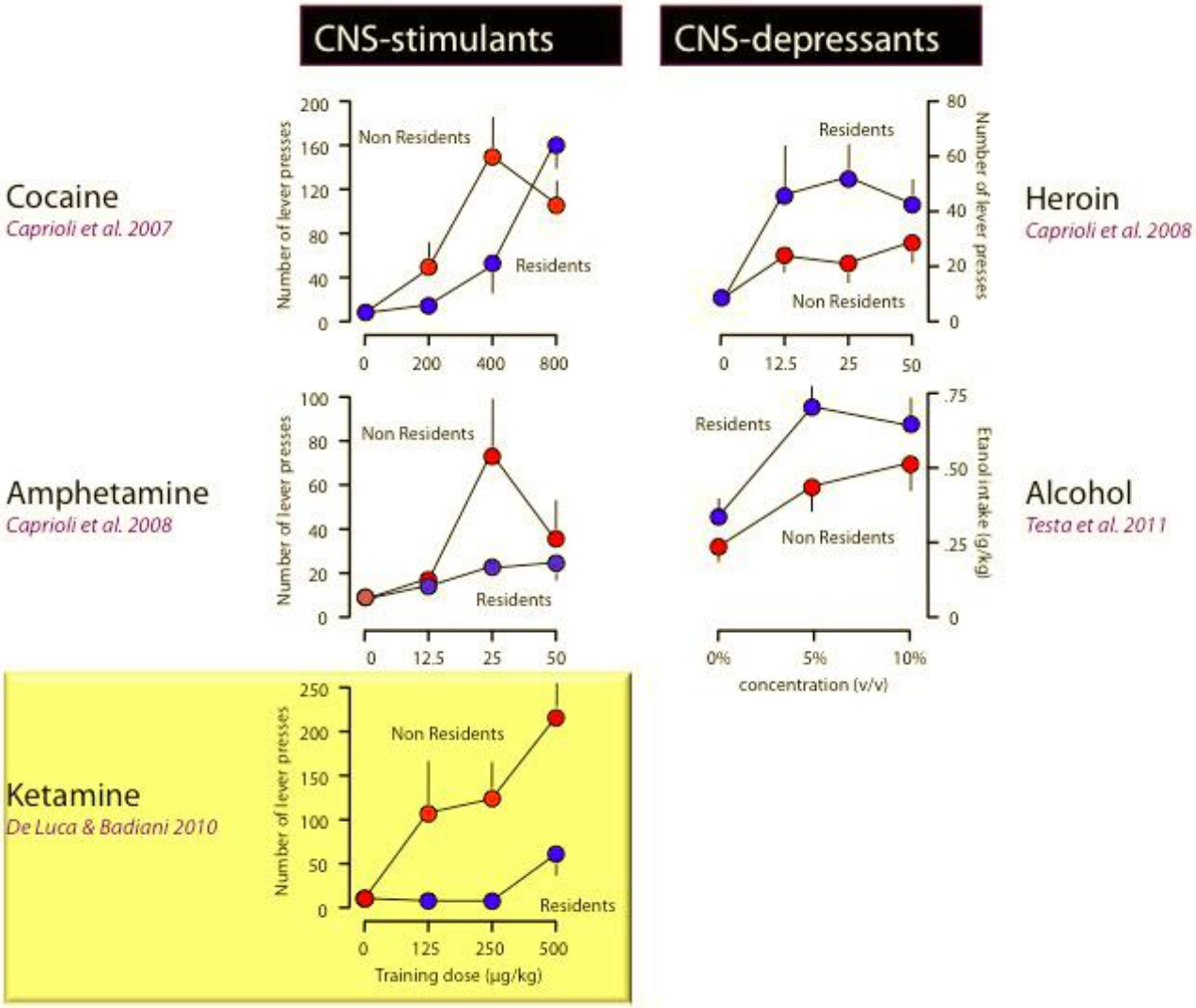

Figure 5.3 Environmental modulation of drug self-administration in the rat

It's interesting to note that ketamine- which, like cocaine, has activating and sympathomimetic effects — is more readily self-administered by rats in the Non Resident environment (De Luca and Badiani 2011); by contrast, alcohol — which, like heroin, initially causes drowsiness and sedation — is more readily self-administered in the Resident environment (Testa et al. 2011). When the rats that had been trained to self- administer amphetamine were shifted (after a 7-day period of rest during which they were kept in their respective home

cages) to heroin self-administration, the context-dependent differences above explained were reported again (Caprioli et al. 2008). Moreover, when rats were given the opportunity to self-administer both cocaine and heroin on alternate days, Non Resident rats took more cocaine relative to heroin than Resident rats (Caprioli et al. 2009; Celentano et al. 2009). Finally, also when rats were given the opportunuty to self-administer cocaine and heroin within the same session, most Non Residents rats preferred cocaine over heroin whereas most Residents rats preferred heroin over cocaine (Caprioli et al. 2009).

These findings were quite surprising for at least two reasons. First, they were partly at odd with previous studies, conducted by us and other authors, concerning drug-induced psychomotor sensitization. In particular, as show above, it had been had reported that Non Resident rats exhibit greater sensitization than Resident rats when repeatedly treated with cocaine, amphetamine, morphine and heroin. The existence of a close relationship between the psychomotor and the rewarding effects of addictive drugs is widely accepted in the literature (Wise and Bozarth 1987). It has even been hypothesized that the neuroadaptations associated with psychomotor sensitization are somewhat similar to those responsible for the development of drug addiction (Robinson and Berridge 1993). Thus, it would be expected that any manipulation capable to facilitate drug- induced psychomotor sensitization would also facilitate drug self-administration. Indeed, this is what we found with cocaine and amphetamine. By contrast, heroin reward was substantially greater in Resident rats relative to Non Resident rats, indicating an unforeseen dissociation between opioid-induced activity and opioid reward.

Second, the dissociation between psychostimulant and opioid reward as a function of the setting of drug taking was quite surprising because the dominant trend, at present, is to emphasize the role of shared substrates in the reward effects of addictive drugs, with particular emphasis on the mesocorticolimbic dopaminergic system (Nestler 2004). However behavioral,

cognitive and neurobiological findings in both laboratory animals and humans indicate important differences between psychostimulant and opioid reward as well as between psychostimulant and opiate addiction (for a recent review, see Badiani et al. 2011). These lines of evidence suggest that the neural substrates of psychostimulant reward differ from those of opioid reward, making it somewhat less surprising that the two classes of drugs would exhibit different interactions with the environment.

5.4 Modulation of gene expression

Research done in the last two decades has emphasized the role of shared neural substrates for the behavioral response to addictive drugs (for a review, see Nestler 2004). In particular, it has been shown that virtually all drugs of abuse can increase, albeit via different mechanisms of action, dopamine levels in the terminal regions of the mesotelencephalic dopaminergic system (Di Chiara and Imperato 1988). Cocaine and amphetamine induce dopamine overflow by binding the dopamine reuptake transporter (for reviews, see Johanson and Fischman 1989; Kuczenski and Segal 1994) whereas heroin and morphine facilitate dopaminergic transmission by binding mu-opioid receptors (MOR) in the ventral tegmental area and substantia nigra, hence disinhibiting mesotelencephalic dopamine-releasing neurons (Gysling and Wang 1983; Matthews and German 1984; Johnson and North 1992; Devine et al. 1993). In turn, dopaminergic mesostriatal and mesoaccumbens transmission has been implicated in both the psychomotor and rewarding effects of addictive drugs (Wise 2004). It is obvious, however, that the modulatory actions of setting on CNS (Cenral Nervous System)-stimulants *vs* CNS-depressants cannot be explained by invoking the modulation of shared neuropharmacological effects, such as the ability to enhance dopamine transmission. Consistent with this logical deduction, we have previously found that the psychomotor activating effects of amphetamine can be modulated by environmental context without

altering amphetamine-induced dopamine overflow in the caudate and in the nucleus accumbens (Badiani et al. 1998, 2000a). A possible lead for an understanding of the neurobiological mechanisms responsible for the modulatory actions of setting on the behavioural effects of drugs, comes from studies indicating that the setting of drug taking can powerfully alter, both quantitatively and qualitatively, the ability of psychostimulants and opiates to induce the expression of immediate early genes (IEGs) in the striatal complex, and in other subcortical areas (Badiani et al. 1998, 1999; Day et al. 2001; Uslaner et al. 2001a,b; Ostrander et al. 2003; Hope et al. 2006). Since the earliest reports by Chang et al. (1988) and Graybiel et al. (1990) it has been known that both psychostimulant and opioid drugs are able to induce the expression of the gene encoding for the Fos protein (c-fos) and other IEGs in a number of forebrain regions, including the caudate nucleus and the NAc (for a review, see Harlan and Garcia 1998). The interest in this phenomenon is due not only to the fact that IEGs can serve as indicators of neuronal activity (Hughes and Dragunow 1995; Harlan and Garcia 1998), but also because they are thought to represent an important initial step in mediating drug experience dependent plasticity (Hyman and Malenka 2001; Nestler 2001; Ujike et al. 2002).

In a series of studies it was found that the effect of cocaine and amphetamine on IEGs expression is very different depending on the circumstances surrounding drug administration (Badiani et al. 1998, 1999; Uslaner et al. 2001a,b, 2003a,b; Day et al., 2001; Ostrander et al. 2003). Although almost every cortical and subcortical structure examined appears to be implicated in the interaction between drugs and environment, the most interesting changes have been found in the striatal complex (notice that the studies discussed below concern non-contingent intraperitoneal administrations of addictive drugs). In the striatum the interaction between psychostimulant drugs and the setting of drug taking appears to be particularly

complex even at a merely quantitative level. In the caudate, the drug treatment outside the home cage of rats, enhanced the effects of amphetamine and cocaine on c-fos expression with a pronounced rostro-caudal gradient. Psychostimulants in home cage have their maximal effects in the mid-caudate and much less effect in the most rostral and caudal portions of the caudate. When administered outside the home (that is, in combination with exposure to a novel environment) a different pattern of gene expression is seen, with Fos mRNA expression progressively increasing from relatively low levels in the rostral caudate to very high levels in the caudal portions of the caudate (Badiani et al. 1998; Uslaner et al. 2001a; Ostrander et al. 2003). In the caudal caudate the combined effect of drug and novelty on Fos mRNA levels was nearly two times greater than what would be predicted by the simple addition of the effects of drugs and novelty alone. These regional differences are not surprising given the complexity of the structural and functional organization of the caudate (for example, see Gerfen 1992; Joel and Weiner 2000; Riedel et al. 2002; Levesque et al. 2003). In a Fos immunohistochemistry study (Paolone et al. 2007), Non Resident condition enhanced the effects of heroin on Fos expression of the caudate nucleus with progressively greater levels in the rostro-caudal direction, similarly to psychostimulants. In the ventral portion of the caudal caudate the net effect of heroin on Fosm RNA levels was nearly 10 times greater than that produced by heroin in home cage. What mechanisms are responsible for the modulatory effects of setting on drug-induced Fos mRNA/Fos expression in the basal ganglia? Regarding the amphetamine, the drug treatment outside home cage, powerfully induces Fos mRNA expression in most cortical areas (Badiani et al. 1998; Uslaner et al. 2001b; Ostrander et al. 2003) and activation of corticostriatal projections has been shown to induce c-fos expression in the striatum (Berretta et al. 1997; Parthasarathy and Graybiel 1997; Sgambato et al. 1997). Thus, Non Resident condition may modulate drug-induced Fos mRNA expression in the caudate via glutamatergic projections from the cortex to the caudate-putamen and to the subthalamic nucleus. In support

of the hypothesis that the modulatory effects of setting could be mediated by glutamatergic mechanisms is the finding that NMDA receptor antagonists reduce amphetamine-induced Fos mRNA expression in the neurons of the indirect pathway (Ferguson et al. 2003), as does transection of corticostriatal fibers (Ferguson and Robinson 2004).

There is evidence that drug, drug history, and environmental context interact in a complex manner in regulating Fos mRNA expression in the mesostriatal circuitry. In a study by Ostrander et al. (2003), Resident and Non Resident rats received repeated administrations of saline or amphetamine and were then challenged with saline or amphetamine. As expected, in most brain regions amphetamine plus "novelty" (that is, amphetamine outside the home cage) produced greater Fos mRNA expression than amphetamine at home, and drug history had no effect. However, within the subthalamic nucleus, substantia nigra pars reticulata, and central nucleus of the amygdala, prior experience with amphetamine outside the home, but not at home, enhanced the effect of the amphetamine challenge on Fos mRNA expression. In contrast, there was a decrease in Fos mRNA expression in amphetamine-pretreated animals, regardless of environmental context, in the ventral portion of the far caudal striatum. Also in the case of heroin, drug, drug history, and environmental context interact in regulating Fos expression (Paolone et al. 2007). Overall, repeated exposures to heroin reduced its ability to induce Fos in the caudate but with important environmental and regional differences. In the postero-ventral caudate the facilitatory effect of "novelty" was maximal in the saline-pretreated animals whereas it was modest in the heroin pretreated animals. By contrast, in the postero-dorsal caudate the modulatory effects of the environment was much greater in heroin-pretreated than in saline-pretreated animals.

We also reported that even a single exposure to low dose of intravenous cocaine (400 µg/kg) or heroin (25 µg/kg) that rats self-administered alone, can enhance Fos mRNA expression in the posterior caudate nucleus; more

important, this Fos mRNA expression depends on the interaction between drug and context (Celentano et al. 2009). In particular heroin exposure induced greater increases in *Fos* expression relative to saline group only in Residents rats, and both in the dorsal and ventral portion of the posterior caudate nucleus (but especially in ventral part). Cocaine, instead, induced greater Fos expression relative to saline group only in the dorsal posteriore caudate and in both Non Resident and Resident rats, but much more in Non Residents. These results are only partially consistent with those obtained with i.p. administrations of much higher doses of cocaine and heroin detailed above. In fact, cocaine-induced Fos mRNA expression in the posterior caudate is facilitated when the treatment is administered outside the home cage both at the doses used to induce psychomotor sensitization (relatively high doses administered intraperitoneally) and at one dose used in self-administration experiments. These neurobiological effects of cocaine agree with the facilitation of both cocaine-induced psychomotor sensitization (Badiani et al. 1995a) and cocaine self-administration (Caprioli et al. 2007) observed in Non Resident rats**.** In contrast, we found evidence of a dissociation in the modulatory actions of environment on the neurobiological effects of high versus low doses of heroin. In the first case heroin-induced Fos expression was greater in the Non Resident than in the Resident group and greater in the dorsal than in the ventral portion of the posterior caudate, whereas the contrary was found with one low dose of heroin self-administered by rats.

Most important, double in-situ hybridization studies of Fos mRNA expression in phenotypically characterized striatal neurons have shown that drug–environment interaction in the basal ganglia involves a qualitative shift in the circuitry engaged, relative to that produced by either drug administration in the home cage or by mere exposure to novelty. Over 90% of striatal neurons are GABAergic neurons projecting either directly or indirectly (via the internal globus pallidus and the subthalamic nucleus) to the output nuclei of basal ganglia (substantia nigra pars reticolata and the external globus pallidus).

When given at home, amphetamine, cocaine, or morphine induce robust Fos mRNA expression almost exclusively in the neurons of the direct pathway, which co-express pre-prodynorphin, substance P, and dopamine D1 receptor mRNAs. In contrast, we found that when psychostimulants are given outside the home, they induce robust Fos mRNA expression in neurons of both the direct and the indirect pathways, which coexpress pre-proenkephalin and D2 receptor mRNAs, (Badiani et al. 1999; Uslaner et al. 2001b, 2003a,b; Hope et al. 2006). Thus, it appears that cocaine and amphetamine engage different neural circuitry depending on the context in which the drugs are administered. In contrast, in a study limited to the postero-dorsal caudate (Ferguson et al. 2004), morphine treatment outside the home exhibited opposite effects on Fos mRNA expression in these two subpopulations, increasing it (but only at high doses) in the striato-nigral neurons while reducing it in the striato-pallidal neurons.

Intriguingly, both cocaine- and amphetamine-induced Fos mRNA expression and heroin-induced Fos expression in the NAc (particularly in the shell subdivision) were modulated by environmental context in a manner opposite to that of the caudate. That is, the net effects of cocaine, amphetamine and heroin were smaller when these drugs were administered outside the home than when were administered at home and no significant rostro-caudal gradient was evident (Badiani et al. 1998; Uslaner et al. 2001a).

Interestingly, Li and colleagues (2004) found that in the NAc core repeated cocaine treatment increased spine density on medium spiny neurons only in the Non Resident group but not in the Resident group (that failed to sensitize). In contrast, cocaine increased spine density in the NAc shell in both groups, that is, independent of sensitization. Furthermore, if the dose of cocaine (and number of treatments) was increased, such that cocaine induced behavioural sensitization even when given in the Resident context, an increase in spine density was now seen in the NAc core.

Additional differences between psychostimulants and heroin were found in the barrel field cortex (BFCx), where exposure to drugs outside the home powerfully induces Fos mRNA and Fos expression (Papa et al. 1993; Badiani et al. 1998; Uslaner et al. 2001b; Ostrander et al. 2003; Paolone et al. 2007). We found that Fos expression in the BFCx was not altered by heroin administered at home whereas it was enhanced when heroin was administered outside the home. This overall effect masked opposite heroin-induced changes in layer IV (where heroin powerfully enhanced Fos expression relative to the control group) versus layers II/III (where heroin reduced Fos expression). Interestingly, pretreatment with heroin in Non Resident rats dramatically altered the effect of heroin on Fos expression in layer IV (where it went from potentiation to suppression) as well as in layers II/III and V/VI (where the suppression became stronger). In contrast, it had been previously reported that amphetamine and cocaine outside the home have relatively little effect on Fos mRNA expression in the BFCx of rats (Badiani et al. 1998; Uslaner et al. 2001a,b; Ostrander et al. 2003) but in these earlier studies no attempt was made to quantify Fos expression in the different cortical layers.

5.5 Role of the HPA axis in the modulatory effects of environmental context

It is a well established fact that the exposure to the Non Resident condition can activate the HPA (Hypothalamic-Pituitary-Adrenal) axis and increase plasma corticosterone levels in the rat (Friedman and Ader, 1967; Badiani et al. 1995c, 1998), an effect that does not necessarily habituate after repeated exposures (Hennessy 1991). Therefore, the possible contribution of the HPA axis to the effects observed in the Non Resident condition described here deserves to be investigated (though we have shown that the modulatory effect of the exposition to the Non Resident condition on amphetamine sensitization is not blocked by the surgical removal of the adrenal glands; Badiani et al. 1995c).

Notice, however, that the neurobiological consequences of elevated levels of corticosterone strongly depend on the context. We have already mentioned that after a social encounter both “victorious” and “defeated" rats exhibit comparable levels of plasma corticosterone but only the latter group exhibits stress-induced facilitation of cocaine self-administration (Covington and Miczek 2005). Other studies confirm that corticosterone plays, at most, a permissive role in the facilitation of cocaine self-administration (Mantsch and Katz 2007) and in the stress-induced reinstatement of cocaine seeking (Erb et al. 1998; Shalev et al. 2003).

Finally, it is important to emphasize that although the term stress has very high face validity there is no consensus on its definition. In particular, the distinction between stress and arousal is often blurred. The results of a study based on the administration of the Stress/Arousal Check List (Mackay et al. 1978) to parachutists and other army personnel, suggest, however that “two distinct responses to a perceived demand are possible. Elevated arousal is associated with a coping response, whilst elevated stress appears to indicate the presence of fear or doubts about coping” (King et al. 1983). It is reasonable to assume that in our studies the exposure to the Non Resident context represented an arousing, but not a stressful, experience for the rats. Indeed, if the exposure to the Non Resident context were a bona fide stressor it would be necessary to conclude that most self-administration experiments described in the literature were conducted under stressful conditions

5.6 The setting of drug taking in human addicts

Our animal studies above detailed have shown differential preferences for CNS-stimulant *vs* CNS-depressants as a function of environmental context. Hence, Badiani and co-workers (Caprioli et al. 2009; Badiani and Spagnolo 2013) decided to adopt a translational approach to investigate the setting of drug taking selected by human addicts.

Addicts co-abusing cocaine and heroin were recruited, among the outpaients of the addiction clinic Villa Maraini in Rome (Italy), to partecipate in a retrospective self-report study. The subjects enrolled in the study met the DSM-IVR Drug Dependence Criteria for cocaine and/or heroin, reported using cocaine and/or heroin at least once a week in the past three months, did not meet DSM-IVR criteria for schizophrenia or any other DSM-IVR psychotic disorder, history of bipolar disorder, or current major depressive disorder, were not under treatment with antipsychotic medications, did not have other medical conditions that would compromise partecipation in the study, and had a fixed address.

The interview was specifically developed to ascertain the physical and social setting in which addicts had taken heroin, cocaine, and heroin plus cocaine ("speedball") in the previous three months. Questions were aimed at assessing, for each drug, whether it was taken: 1) always at home; 2) mostly at home; 3) sometimes at home sometimes outside (50/50); 4) mostly outside the home; 5) always outside the home (to semplify data presentation, the "always at home" and "mostly at home" conditions were collapsed into the "home" condition, and the "always outside the home" and "mostly outside the home" conditions were collapsed into the "outside the home" condition. Outside environments were further classified as: street, park, disco, bar, friend's house and friend's car. The answers about the social setting were classified as alone, with one companion, and with more than two companion. The partecipants were also asked whether the context of drug taking represented a real preference or was the result of pratical constraints related to the route of drug taking (such as the necessity to take heroin at home because of the bulky routines associated to intravenous injection).

Figure 5.4 shows that of all the co-abusers, 70% preferred to take heroin at home whereas 23,1% preferred to take it outside the home; on the other hands 22,5% preferred to take cocaine at home whereas 69,4% preferred to take it outside the home (for the few subjects reported using speed-ball, 59,1%

preferre to take it at home, 31,8% outside the home and 4,1% express no clear preference). The partecipants said that they took the drugs in the preferred setting and not because of pratical constraints related to the route of drug taking: comparable results were obtained in fact with subgroups of co-abusers who injected, snorted or smoked both heroin and cocaine in distinct occasions (see Figure 5.4).

Non-home settings differed between heroin and cocaine: bars and clubs were the preferred settings for cocaine use (57%), whereas street (30%) and friend's car (16%) were the preferred settings for the heroin. It is important to notice that none of the socio-demographic variables had any influence on setting preferences for either cocaine or heroin.

These differences in physical setting did not appear to be a simple outcome of social setting. First, home was the preferred environment for heroin taking regardless whether the drug was taken in isolation or with others; second, considering only the individuals who took the drug in the company of others, there were still differences in the setting for cocaine *vs.* heroin taking.

In summary, three major findings are reported from our human studies. First, addicts co-abusing heroin and cocaine exhibit differential setting preferences for heroin *vs.* cocaine taking: heroin was used preferentially at home, whereas cocaine outside the home. Second, setting preferences were indipendent of the route of drug taking. Third, setting preferences were not a mere consequence of the preference for one social context or the other. The within-subject design of our study makes the findings especially compelling, because the difference in preferred settings for heroin *vs*. cocaine use cannot be attributed to differences in drug availability, peer influence, or other socio-demographic factors.

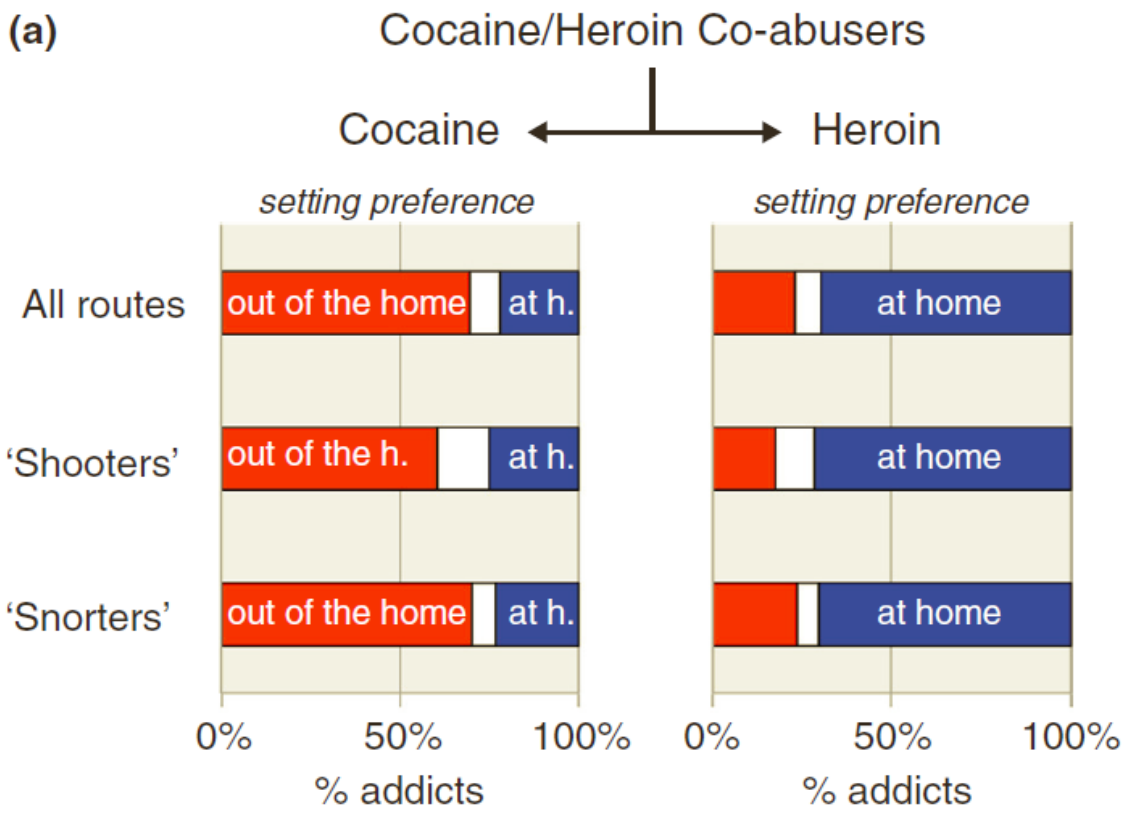

Figure 5.4 Setting preferences of drug taking in human co-abusers (from Badiani 2013)

5.7 An emotional "appraisal" hypothesis of drug reward

What type of explanation can account for the effects of setting on drug taking in both animals and humans? Where and how drug effects meet the setting in the brain? We have previously hypothesized (Caprioli et al 2009; Badiani et al. 2011) that the setting affects drug taking by providing an ecological backdrop against which drug effects are rated as more or less adaptive; recently, Badiani (2013) has further developed this idea, suggesting an emotional "appraisal" hypothesis of drug reward, that could account for the effects of setting on drug taking, and what brain areas might underpin this effect.

Badiani proposed that not only the “hedonic” drug effect (which is a shared effect of most addictive drugs and probably is indifferent to environmental context), but also both central and peripheral “non-hedonic” drug effects undergo emotional appraisal by the brain and that the resulting information participates in the computation of drug reward**.** Moreover, this emotional appraisal of both “hedonic” and “non hedonic” drug effects depends on the environmental context of drug taking; in fact, the appraisal of a drug could be very different in different setting, hence result much or less rewarding.

The idea that afferent signals from the Autonomic Nervous System (ANS) drive emotional responses was first proposed at the end of the 19th century by James and Lange (James and Lange 1922). It was later shown that the role of visceral information is context-dependent. More important for our hypothesis, Gray et al. have recently confirmed that the incongruous physiological signals (that is, signals at odd with the context) might be more emotionally relevant than congruous signals (Gray et al. 2007, 2012; Critchley 2009).

A possible explanation for our results is based on the different central and peripheral non hedonic effects of CNS-depressants *vs.* CNS-psychostimulants. The sedative and the parasympathomimetic effects of heroin (with reduced heart rate, hypotension, and miosis) (Haddad and Lasala 1987; Thornhill et al. 1989), for example, may be ‘appraised’ as performance-impairing when in the potentially hostile, non-home environment as opposed to the safe home environment; in contrast, the arousing and sympathomimetic effects of cocaine (e.g. increased heart rate, hypertension, and mydriasis) (Billman 1995; Sofuoglu 2009) may be appraised as anxiogenic at home but not in a more exciting non-home environment. Hence, heroin would have been appraised as more rewarding at home whereas cocaine would have been appraised as more rewarding outside the home. Moreover, also other drugs of abuse seems to be evaluate as more or less rewarding on the basis of the environment of drug taking. Ketamine for example- which, like cocaine, has activating and sympathomimetic effects (Hancock and Stamford 1999)- is more readily self-

administered by rats in the Non Resident environment (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 more readily self-administered in the Resident environment (Testa et al. 2011). Similar results were reported in humans: it was show in fact a greater intake of ketamine outside the home than at home (De Luca et al. 2012), and a more rewarding effect of alchohol at home than outside the home in heavy drinkers (Nyaronga et al. 2009).

A crucial component of our model is the amygdaloid complex, which, on the basis of its anatomical and functional characteristics can be subdivided into three major compartments: the basolateral amygdala (BLA), the central amygdala (CeA), and the medial amygdala (Sah et al. 2003). The BLA projects to the prefrontal cortex, CP, and NAcc, whereas the CeA projects to the anterior portion of the Bed Nucleus Stria Terminalis (BNST) and to the hypothalamic and brain stem centers that regulate the activity of the ANS (McDonald 1991; Sah et al. 2003; Salzman and Fusi 2010). While the BLA has been linked to positive affect, particularly in drug reward (Prado-Alcala´ and Wise 1984; Kane et al. 1991; David and Cazala 1994; Stuber et al. 2011) the main role of the CeA appears to be that of monitoring potential threats to the organism and of generating negative affective reaction, such as anxiety and vigilance, as well as appropriate feedback to the ANS (Hitchcock and Davis 1986; Murray 2007; Somerville et al. 2010).

Badiani proposes that the BLA encodes for the “affective value” of drug experience by integrating exteroceptive and interoceptive information, central and peripheral drug effects that ‘tweak’ interoceptive information, and direct actions of the drugs on the BLA. This information is then transferred to the brain regions that directly control goal-directed behavior like the CP and NAc. If interoceptive and exteroceptive information are congruous, the BLA encodes a rewarding experience. On the other side, when the BLA detects a mismatch between interoceptive and exteroceptive information, the positive affective

value of the drug experience will be blunted and the CeA and the anterior BNST will translate the same incongruous information into anxiety and vigilance (Figure 5.5).

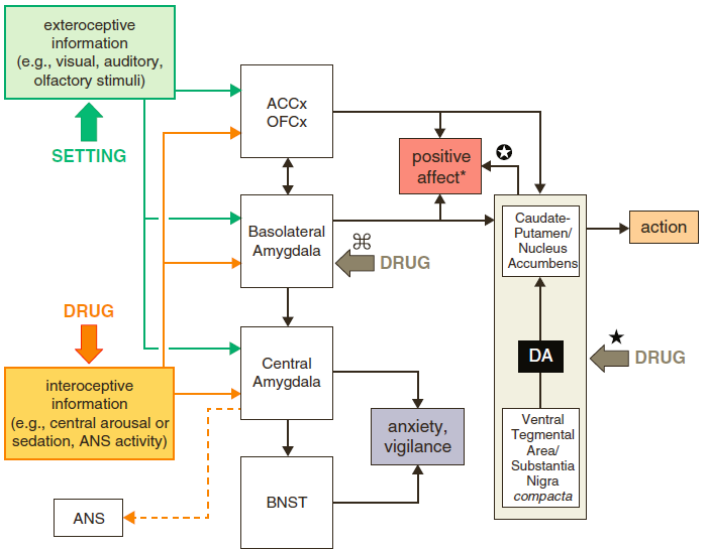

Figure 5.5 Schematic diagram of the emotional appraisal model of drug reward (from Badiani 2013).

Studies based on in situ hybridization of c-fos mRNA (used as an index of neuronal activation) and on in vivo microdialysis (for the quantification of dopamine release) match the major features of our model, at least with respect to psychostimulant drugs (Figure 5.6). Cocaine and amphetamine for example, produced greater c-Fos mRNA expression in the BLA (Day et al. 2001; Ostrander et al. 2003), CP and NAcc (Badiani et al. 1998; 1999; Uslaner et al. 2001b, 2003; Caprioli et al. 2009) in Non Resident than in Resident rats. In contrast, the same drugs produced much greater c-Fos mRNA expression in the CeA and BNST in Resident than in Non Resident rats (Day et al. 2001; Ostrander et al. 2003; this pattern was not found in any other region of the rat brain). We found no differences in amphetamine- induced dopamine overflow in the CP or NAcc as a function of the setting; finally were not found setting differences for c-Fos mRNA expression in the VTA/SNc induced by cocaine and almphetamine (see Figure 5.6).

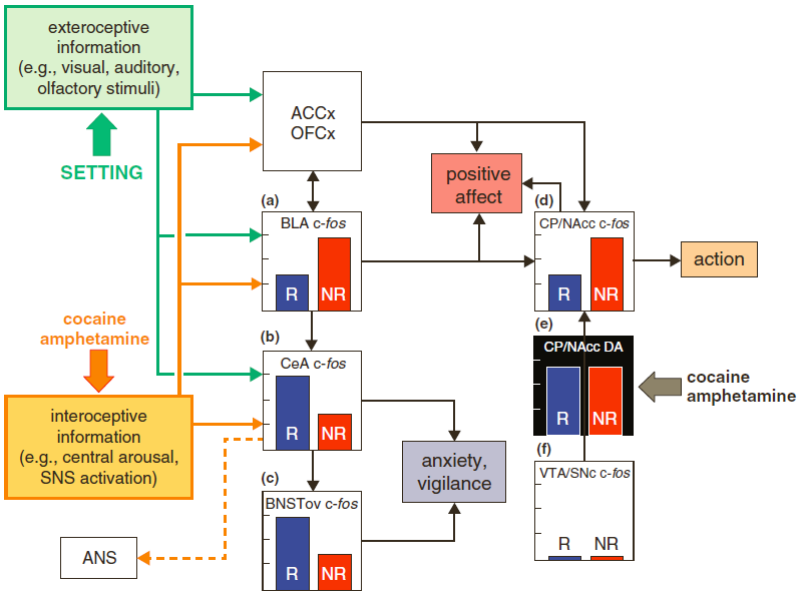

Figure 5.6 Schematic illustration of in situ hybridization and microdialysis findings matching the emotional appraisal model of drug reward (from Badiani 2013).

It is important to emphasize that emotional appraisal does not necessarily entails the conscious evaluation of stimuli (LeDoux 1996, 2012). That is, the fact that heroin is preferentially taken at home and cocaine outside the home should not be seen as the mere expression of an intentional decision to take a depressant drug in a place where one can lie down and an activating drug where one can move around. The results of studies conducted in rats suggest that it is the setting that endows drug effects with emotional valence, rather than the opposite.

5.8 Conclusions and therapeutic implications

It has been know for many years that environmental contexts or places in which drugs are taken play an important role in human addiction. We reviewed

here our findings showing that the setting in which drugs are taken can powerfully influence the discriminative, the psychomotor and the rewarding effects of drugs in both animals and humans. We suggest that to begin understanding the substance-specific nature of environmental influences in drug addiction it is necessary to take into consideration all effects of addictive drugs and not only those that are immediately related to hedonics and incentive salience. In particular, the substance-specific influence of setting on drug reward 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.

6. The role of environmental context in the vulnerability to relapse into heroin and cocaine addiction: a pre-clinical investigation

Abstract

Background: We have recently observed an unforeseen dissociation in the effect of environmental context on cocaine versus heroin self-administration (SA) in rats. Rats that were transferred to the SA chambers only for the test sessions (Non Residents) took more cocaine than rats housed in the SA chambers (Residents). The contrary was found for heroin. The aim of the present study was to investigate the influence of context on the ability of different doses of cocaine and heroin priming to reinstate cocaine- vs. heroin-seeking in rats that had been trained to self-administer both drugs and had then extinguished lever pressing behavior.

Methods: Resident (N=65) and Non-Resident (N=64) 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 days (3 hours/session/day). After extinction of lever pressing behavior, 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 and extinguished lever pressing behavior for both drugs. When given cocaine primings only Non Resident rats exhibited reinstatement of cocaine-seeking and, in contrast, when given heroin primings only Resident rats exhibited reinstatement of heroin-seeking.

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

6.1 Introduction

Relapse to compulsive drug-seeking behavior after abstinence is a major problem in the treatment of drug addiction (O'Brien 1997; Stewart 2000); in both human addicts and laboratory animals, after a period of drug withdrawal, reexposures to an addictive drug, a stressful event, or drug-associated environmental cues, often induce drug craving and precipitate relapse to drug-seeking (Jaffe et al. 1989; Carter and Tiffany 1999; Sinha et al. 1999; Shalev et al. 2002).

The treatment is further complicated by the fact that drug abuse is rarely limited to a single substance, polydrug use being the norm rather than the exception. In particular, it is a well-documented fact in a number of countries that most heroin addicts also abuse cocaine and vice versa, both in untreated individuals and in individuals in treatment, at entry as well as during follow-up (Leri et al. 2003). Concurrent users of cocaine and heroin are more likely to have poorer treatment outcomes, interrupt treatment programs and to relapse (Broers et al. 2000; Downey et al. 2000; Gossop et al. 2002; Leri et al. 2003). Still, in this concurrent users, methadone (Strain et al. 1996; Schottenfeld et al. 1997; Borg et al. 1999; Schottenfeld et al. 2005; Epstein et al. 2009) or buprenorphine (Schottenfeld et al. 1993; Strain et al. 1994, 1996; Montoya et al. 2004; McCann 2008) maintenance has been reported to reduce heroin craving and abuse, but there is no consensus in the literature about the clinical efficacy on cocaine craving and abuse for both methadone (Kosten et al. 1987; Borg et al. 1999; Schottenfeld et al. 2005; Peles et al. 2006; Epstein et al. 2009) and buprenorphine (Kosten et al. 1989; Oliveto et al. 1993; Schottenfeld et al. 1993, 1997; Compton et al. 1995; Montoya et al. 2004; Gerra et al. 2006). Moreover approved medications for the treatment of cocaine dependence are still lacking and cognitive behavioral approaches to drug addiction are moderately effective (Downey et al. 2000; Gossop et al. 2002; Epstein et al. 2009; Vocci and Montoya 2009; Penberthy et al. 2010; Hartzler et al. 2012);

finally there is no consistent evidence for a prolonged efficacy of drug cue-extinction treatment (Taylor et al. 2009; Myers and Carlezon 2012).

It is important, therefore, to better understand the basis of cocaine and heroin co-abuse and relapse from both pharmacological and ecological point of view. From an ecological point of view the preference for one drug or another is widely thought to be a function of local availability, street price, lifestyle, and other socio-cultural factors (Anthony and Chen 2004; Westermeyer 2004; Johnson and Golub 2005; Jofre-Bonet and Petry 2008). It was also proposed that the circumstances immediately surrounding drug taking can modulate drug taking in ways that are not immediately reducible to conditioning (Zinberg 1984; Falk and Feingold 1987), but the evidence is largely anecdotal (Dalgarno and Shewan 1996; McElrath and McEvoy 2002; Stallwitz and Shewan 2004). This is probably due not only to the extreme difficulty of manipulating in a controlled fashion the context of drug taking in our species but also to the strongly held belief that the environmental variables implicated in drug abuse are paramount with cultural or economical factors.

We have recently developed an animal model to study under controlled laboratory conditions the role of setting on drug taking (Caprioli et al. 2007). We used the intravenous drug self-administration (SA) procedure, in which laboratory animals typically make a lever press or nose poke to receive contingent drug injections. In our model some rats were transferred to the SA chambers immediately before the SA sessions (Non Resident rats)- a procedure commonly used in most SA studies- whereas other animals were kept at all times in the SA chambers (Resident rats). We have shown an unforeseen dissociation in the effect of environmental context on psychostimulant versus opiate reward: Non Resident rats self-administered more cocaine and amphetamine than Resident rats (Caprioli et al. 2007, 2008) and the opposite was found for heroin SA (Caprioli et al. 2008). Also when trained to self-administer cocaine and heroin on alternate days, Non Resident rats took more cocaine than heroin in comparison with Resident rats (Caprioli et al. 2009;

Celentano et al. 2009). Moreover, when rats were permitted to self-administer either cocaine and heroin within the same session, most of Non Residents rats preferred cocaine over heroin whereas most Resident rats preferred heroin over cocaine (Caprioli et al. 2009). The euristic relevance of our animal model is indicated by the results of a translational study in which we investigated the environmental and social setting selected by human addicts (co-abusers of cocaine and heroin) to take drugs: most addicts, similarly to ours rats, used heroin at home and cocaine outside the home, regardless the drugs were injected or snorted, and regardless the drugs were taken in isolation or with others (Caprioli et al. 2009; Badiani and Spagnolo 2013).

Inwardly this research framework, the aim of the present study was to investigate the influence of setting (Non Residents vs Residents) on the ability of different doses of heroin and cocaine priming to reinstate heroin- vs. cocaine-seeking in rats that had been trained to self-administer both drugs and had then extinguished lever pressing behavior. We predicted that cocaine primings would have a stronger effect on reinstatement in the Non Resident rats, and, by contrast, heroin primings would have a stronger effect on reinstatement in the Resident rats. In order to better model the typical pattern of human co-abuse, the rats were trained to self-administer cocaine and heroin on alternate days. In fact, although the pattern of co-abuse varies from individual to individual, the majority of co-abusers seems prefer to take cocaine and heroin separately within the same day or on different days (Leri et al. 2005).

6.2 Methods and Materials

6.2.1 Animals, Surgery, and Test chambers

The study was conducted using a total of 185 male Sprague–Dawley rats (Harlan Italy, San Pietro al Natisone, Italy) weighing 250–275 g at their arrival.

Data from 129 rats were analyzed (numbers [*n*] refer to these rats), while other 56 rats were excluded from the analyses because of catheter clogging or breaking (15 rats), sickness or death (6 rats), or because did not acquire SA criterion [at least 2 self-infusions of cocaine and at least 2 of heroin on the last 2 sessions (35 rats)]. Throughout the experiments, the rats were housed and tested in the same dedicated temperature- and humidity-controlled room, 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.). After their arrival, the rats were housed 2 per cage for 10-12 days before the surgery, after which were housed individually (see General Procedures section). Husbandry and 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 Italian Ministry of Health.

Using standard surgical procedures previously described in detail (Caprioli et al. 2007, 2008), double-lumen catheters were inserted into and secured to the right jugular vein of the rats. The distal end of the catheters was externalized through a small incision at the nape of the neck, and connected to an L-shaped 22-gauge cannulae, which were secured to the rat's skull using dental cement and stainless steel screws. Thus, rats with double-lumen catheters received 2 connecting cannulae. 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).

The apparatus consisted of self-administration (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 S.r.l. (Rome, Italy), and custom-developed control software 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 13,3 µl/s).

6.2.2 Experiments

General Procedures: After the surgery, the rats were assigned to 1 of 2 conditions: Resident and Non Resident. 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 animals this was also their home (Resident group) whereas for other animals it represented a distinct and, at least initially, novel context (Non Resident group). Testing began 1 week after the surgery. The experiments included 21 sessions; all test sessions lasted 3 hours and took place during the dark phase, between 12:30 and 16:30 h, 7 days a week. All testing procedures were identical between Resident and Non Resident rats (including the absence of food or water). The catheters were connected, through infusion lines and liquid swivels, to the infusion syringes, 3 hours 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, food and water were

removed from the chambers 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 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% steril saline, and drugs and saline solution were given in 40µl/3s via motorized pump. Like other studies (Carelli and Ijames 2000; Carrera et al. 2000; Tran-Nguyen et al. 2001; Lenoir and Ahmed 2007) no inactive lever was used in the present study; under our conditions inactive responses are negligible.

Training phase (days 1–10): Resident (n=65) and Non Resident (n=64) 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 3-hours sessions (i.e., there were 5 sessions for each drug). Cocaine and heroin were each paired with 1 of the 2 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 lever presses required to obtain a single infusion [Fixed Ratio (FR)] was FR1 for sessions 1-2, FR2 for sessions 3-4, and FR5 for sessions 5–10. 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 40-sec time-out period. The rats were allowed to self-administer a maximum of 100 infusions of cocaine and heroin to minimize the risk of overdosing. When needed, the animals were placed with 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 animals 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 animals that had not spontaneously self-administered at least 1 infusion. These infusions (0.81±0.1 *vs* 0.56±0.18 infusions per session in the Resident *vs* the Non Resident group for cocaine; 0.14±0.05 vs 0.24±0.09 infusions per session in the Resident *vs* the Non Resident group for heroin) have been subtracted from statistical analysis and graphs. Each lumen of double lumen catheters was used the same number of times for either drug, in a counterbalanced manner.

Extinction phase (days 11-20): The day after the end of the training phase, the rats underwent 10 extinction sessions (3-hours/session/day), during which was continued alternating of the lever and cue associated specifically with cocaine and heroin, but lever pressing on FR5 schedule resulted in the infusion of saline solution.

Reinstatement session (day 21): After extinction of drug seeking behavior, the reinstatement session was carried out to assess whether cocaine or heroin primings have been able to restore the search for the same drug in the absence of it. During the reinstatement session (3 hours) the rats were connected to double channel liquid swivels; one channel was connected to cocaine or heroin syringe and the other one to saline syringe. Immediately before the beginning of the reinstatement session, independent groups of rats were given (via motorized infusion pumps) a non-contingent intra venous (i.v.) infusion of cocaine (400, 800, or 1600 µg/Kg) or heroin (25, 50, or 100 µg/Kg). During the session lever pressing on FR5 resulted in the infusion of saline and drug seeking was quantified by counting non-reinforced lever presses. On the reinstatement session, only the lever associated with the drug delivered before the beginning of session was extended and the appropriate cue light was turned on. Six independent groups of rats received just before the start of the session 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=12 for the Resident group; n=13 for the Non Resident Group), 50 (n=10 for the Resident group; n=9 for the Non Resident Group), or 100 µg/Kg (n=11 for the Resident group; n=10 for the Non Resident Group).

6.2.3 Summary of the characteristics of the Resident and Non Resident groups

(1) The SA 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 and, at least initially, novel environment (Non Resident group). (2) The distance travelled by the Non Resident rats during the transfer to the SA chambers was about 1-2 meters. Indeed, all animals were kept in the same dedicated testing rooms for the entire duration of the experiments and therefore there was no transport from one room to another and no disruption of circadian rhythmicity. (3) Immediately before the start of each session Resident rats were briefly handled to remove food and water from the SA cages. (4) Both Resident and Non Resident rats were drug naïve before the start of the experiments. (5) During testing, the SA chambers contained no food or water. The rest of the time the animals had free access to food and water. (6) When necessary, both Resident and Non Resident rats were briefly handled to deliver an infusion (see above). (7) All husbandry routines were identical in the 2 groups.

6.2.4 Catheter patency test

At the end of the experiments, all rats underwent a catheter patency test in which they received 2 i.v. boluses of 40 mg/kg of thiopental sodium (Pharmacia Italia, Milan, Italy), one in each catheter lumens, with a 15-min interval between the two. The rats who did not become ataxic within 5 s from the injection were excluded from data analysis. All the animals included in the analysis were positive to the catheter patency test in both lumens of catheters.

6.2.5 Body weight

At the beginning and the end of the experiments the body weight mean was 337.73 ± 1.51 g and 381.98 ± 1.61 g, respectively; rats were weighed weekly, and the drug infusions were corrected for the weight.

6.2.6 Data Analysis and Statistics

Group differences were assessed with various tests, with significance level set at $p < 0.05$. Detailed information about data analysis and the rationale for the statistical tests are provided below.

Training phase: The lever pressing behavior and the infusion data for each pair of training sessions were analyzed using a 3-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: 5 pairs of sessions) as within-subject factors. When necessary, paired samples t-tests were used.

Extinction phase: Group differences for cocaine- versus heroin-seeking were assesed using a 3-way ANOVAs with context as the between-subject factor and drug lever and session as within subject factors. When necessary, independent or paired samples t-tests were used.

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 session [2 levels: last extinction session (relating to the substance of the drug priming] *vs* reinstatement session)] as within-subject factor. We considered only the 1st hour of both last extinction session and reinstatement session, because most studies have demonstrated the temporary effect of drug priming on drug-seeking behavior. We have carried out a linear regression analysis between the intake of cocaine and heroin during the training and the number of lever presses in the 1°st hour of the reinstatement session. When necessary, paired samples t-tests were used.

6.3 Results

Training phase: The rats rapidly acquired cocaine and heroin SA, as shown in Figure 6.1, adjusting the rate of lever-pressing to the increases in the FR schedule requirements. The ANOVA yielded, for both lever presses and infusions, significant drugxcontext [$F(1,127)= 16.853$, $p< 0.001$, and $F(1,127)=15.885$, $p< 0.001$, respectively], sessionxcontext [$F(4,508)= 6.619$, $p< 0.001$, and $F(4,508)= 4.755$, $p=0.001$, respectively], drugxsession [$F(4,508)=48.339$, $p< 0.001$, and $F(4,508)= 29.282$, $p< 0.001$, respectively] and drugxsessionxcontext [$F(4,508)=10.617$, $p< 0.001$, and $F(4,508)=6.561$, $p< 0.001$, respectively] interactions as well as significant main effects of drug [$F(1,127)=80.462$, $p< 0.001$, and $F(1,127)=72.907$, $p< 0.001$, respectively] , session [$F(4,508)=176.391$, $p< 0.001$, and $F(4,508)=60.322$, $p < 0.001$, respectively] and context [$F(1,127)= 8.469$, $p=0.004$, and $F(1,127)=8.460$, $p= 0.004$, respectively]. Paired samples t-tests revealed for both Non Resident and Resident groups, and for both cocaine and heroin, a significant difference between sessions 1-2 and all the other pairs of sessions (all with the $p< 0.001$).

Extinction phase: On the extinction sessions (Figure 6.2) the rate of pressing rapidly abated, with a significant effect of session [$F(4,508)=143.622$, $p< 0.001$], drug leverxcontext [$F(1,127)=13.031$, $p< 0.001$] and drug leverxsessionxcontext [$F(4,508)=3.266$, $p= 0.012$] interactions, but not of drug lever [$F(1,127)= 1.116$, $p=0.293$], context [$F(1,127)=0.001$, $p=0.972$], and sessionxcontext [$F(4,508)= 0.101$, $p= 0.982$] or drug leverxsession [$F(4,508)= 0.964$, $p = 0.427$] interactions. Paired samples t-tests revealed for both Non Resident and Resident group, and for both cocaine and heroin lever, a significant difference between 1-2 sessions and the other pairs of sessions (all with the $p< 0.001$); independent samples t-tests showed a difference between Non Resident and Resident rats only for heroin lever and only on sessions 3-4 ($p=0.037$).

Reinstatement session: As showed in Figure 6.3, when given cocaine primings only Non Resident rats exhibited reinstatement of cocaine-seeking, whereas when given heroin primings only Resident rats exhibited reinstatement of heroin-seeking. The ANOVA for the 1st of 3 hours/reinstatement session indicated a significant effect of session [$F(1,117)= 31.565$, $p< .001$], contextxdrug priming [$F(1,117)=6.366$, $p=0.013$] and sessionxcontextxdrug priming [$F(1,117)=10.637$, $p= 0.001$] interactions, but not of context [$F(1,117)=2.780$, $p= 0.098$], drug priming [$F(1,117)=2.716$, $p= 0,102$], dose [$F(2,117)= 0.056$, $p= 0.946$], contextxdose [$F(2,117)=1.085$, $p= 0.341$], drug primingxdose [$F(2,117)=1.728$, $p= 0.182$], contextxdrug primingxdose [$F(2,117)= 0.072$, $p= 0.939$], sessionxcontext [$F(1,117)=0.000$, $p=0.998$], sessionxdrug priming [$F(1,117)= 3.193$, $p= 0.077$], sessionxdose [$F(2,117)= 0.134$, $p= 0.875$], sessionxcontextxdose [$F(2,117)=1.261$, $p= 0.287$], sessionxdrug priming x dose [$F(2,117)= 1.656$, $p= 0.195$] or sessionxcontextxdrug primingxdose [$F(2,117)= 0.128$, $p= 0.880$] interactions. Paired samples t-tests were significant only in Non Residents for cocaine-induced reinstatement (400 µg/kg: $p=0.02$; 800 µg/kg: $p=0.03$; 1600 µg/kg: $p=0.001$) and only in Residents for heroin induced-reinstatement (25 µg/kg: $p=0.04$; 50 µg/kg: $p=0.02$). Among all groups, a correlation between the intake of cocaine and heroin and relapse to cocaine and heroin was not found, except for only one significant correlation between cocaine intake and number of lever presses on cocaine lever for the Resident rats that received a cocaine priming of 1600 µg/Kg [$r^2=0.6$, $p=0.008$]. Note that the significant correlation was expressed for a group that has not performed relapse to drug-seeking.

6.4 Discussion

The major finding of this study was that the context of drug administration differently modulates drug-induced reinstatement of cocaine-seeking *vs* heroin-seeking in rats trained to self-administer both drugs. This finding is in agreement with our previous results. We have reported in fact that cocaine and

amphetamine SA were greater in Non Resident rats than in Resident rats (Caprioli et al. 2007; Caprioli et al. 2008), whereas the opposite was found with heroin SA (Caprioli et al. 2008). Similar results were obtained also when rats were trained to self-administer cocaine and heroin on alternate days or within the same session (Caprioli et al. 2009; Celentano et al. 2009). The neural substrates for these differences are still unknown, but an *in situ* hybridization study conducted using one i.v. SA dose of cocaine and heroin identical to those used here during the training, produced different patterns of Fos mRNA expression in the posterior caudate of the rat brain as a function of context: cocaine produced greater Fos mRNA expression in the Non Resident group than in the Resident group and the opposite was found for heroin (Celentano et al. 2009).

Here we demonstrate that the vulnerability to relapse into drug seeking behavior is substance-specific and setting-specific. Surprisingly, when cocaine primings were given only Non Resident rats exhibited reinstatement of cocaine-seeking and, in contrast, when heroin primings were given only Resident rats exhibited reinstatement of heroin-seeking. It should be noted that these results are not due to correlations between the intake of cocaine and heroin and relapse to cocaine- and heroin-seeking. When the heroin priming was given at the dose of 100 µg/kg, also Resident rats did not exhibited reinstatement of heroin-seeking. We believe that after a period of extinction, this dose could have satisfy the rat's heroin demand; in fact 100 µg/kg of heroin approximately corresponds to the amount of heroin that rats were self-administered the 1st hour of training sessions. On the other side, at all 3 tested doses, Resident rats did not exhibit reinstatement of cocaine-seeking and Non Resident rats did not exhibit reinstatement of heroin-seeking. If we are the unique laboratory that use the so-called "Resident" group, many laboratories (with rats similar to our "Non Residents") have reported that heroin primings induced heroin-seeking (Fattore et al. 2003, 2005; Luo et al. 2004; Lenoir and Ahmed 2007), even when rats were trained to self-administer cocaine and heroin on alternate days (Leri

and Stewart 2001; Leri et al. 2004; Sorge et al. 2005). Interestingly, in 2 of the mentioned above studies, subgroups under conditions similar to ours didn't show heroin-induced reinstatement of heroin-seeking. For example, in the study of Lenoir and Ahmed (2007) rats in the "Short Access" group (which underwent 1 hour SA sessions but took about 3 times more heroin than our Non Resident group) did not show reinstatement of heroin-seeking after 3 i.v. priming doses very similar to those here used. More important, Leri and Stewart (2001) reported that rats trained to self-administer cocaine and heroin on alternate days with the heroin dose of 25 µg/Kg/infusion (the same our dose), did not show relapse to heroin-seeking after extinction. Discrepant results may arise from a combination of different procedural factors, but we believe that in our study the absence of relapse in Non Resident rats after heroin primings is mainly due to the setting of drug taking: in fact, at all 3 tested doses, is not even present a trend to relapse to heroin-seeking. Finally, after cocaine primings we used only cocaine lever and after heroin primings only heroin lever, because previous studies (Leri and Stewart 2001; Leri et al. 2004; Sorge et al. 2005) have shown that in rats trained to self-administer cocaine and heroin on alternate days, the seek for drug after extinction is selectively directed towards the lever corresponding to the substance of priming.

The dissociation between psychostimulant and opioid reward as a function of the setting of drug taking was quite surprising because the dominant trend, at present, is to emphasize the role of shared substrates in the rewarding effects of addictive drugs, with particular emphasis on the mesocorticolimbic dopaminergic system (Nestler 2004). However behavioral, cognitive and neurobiological findings in both laboratory animals and humans indicate important differences between psychostimulant and opioid reward as well as between psychostimulant and opiate addiction (for a recent review, see Badiani et al. 2011). These lines of evidence suggest that the neural substrates of psychostimulant reward differ from those of opioid reward, making it

somewhat less surprising that the two classes of drugs would exhibit different interactions with the environment. A possible explanation for our results is based on the different central and peripheral non-hedonic effects of opiate *vs*. psychostimulant drugs (for a review, see Badiani 2013). The sedative and the parasympathomimetic effects of heroin (with reduced heart rate, hypotension, and miosis) (Haddad and Lasala 1987; Thornhill et al. 1989), for example, may be appraised as performance-impairing when in the potentially hostile, non-home environment as opposed to the safe home environment. In contrast, the arousing and sympathomimetic effects of cocaine (e.g. increased heart rate, hypertension, and mydriasis) (Billman 1995; Sofuoglu 2009) may be appraised as anxiogenic at home but not in a more exciting non-home environment. Hence, heroin would have been appraised as more rewarding at home whereas cocaine would have been appraised as more rewarding outside the home. Also other drugs of abuse seems to be evaluate as more or less rewarding on the basis of the environment of drug taking. For example ketamine - which, like cocaine, has activating and sympathomimetic effects (Hancock and Stamford 1999) - is more readily self-administered by rats in the Non Resident environment (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 more readily self-administered in the Resident environment (Testa et al. 2011). Moreover, most human addicts (co-abusers of cocaine and heroin) reported using heroin at home and cocaine outside the home, regardless the drugs were injected or snorted, and regardless the drugs were taken in isolation or with others (Caprioli et al. 2009; Badiani and Spagnolo 2013). The within-subject design of this study makes the findings especially compelling, because the difference in preferred settings for heroin use compared to cocaine use cannot readily be attributed to differences in drug availability, peer influence or other socio-demographic factors.

In summary we report 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 substance- and setting-specific. 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). In particular, 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.

6.5 Figures

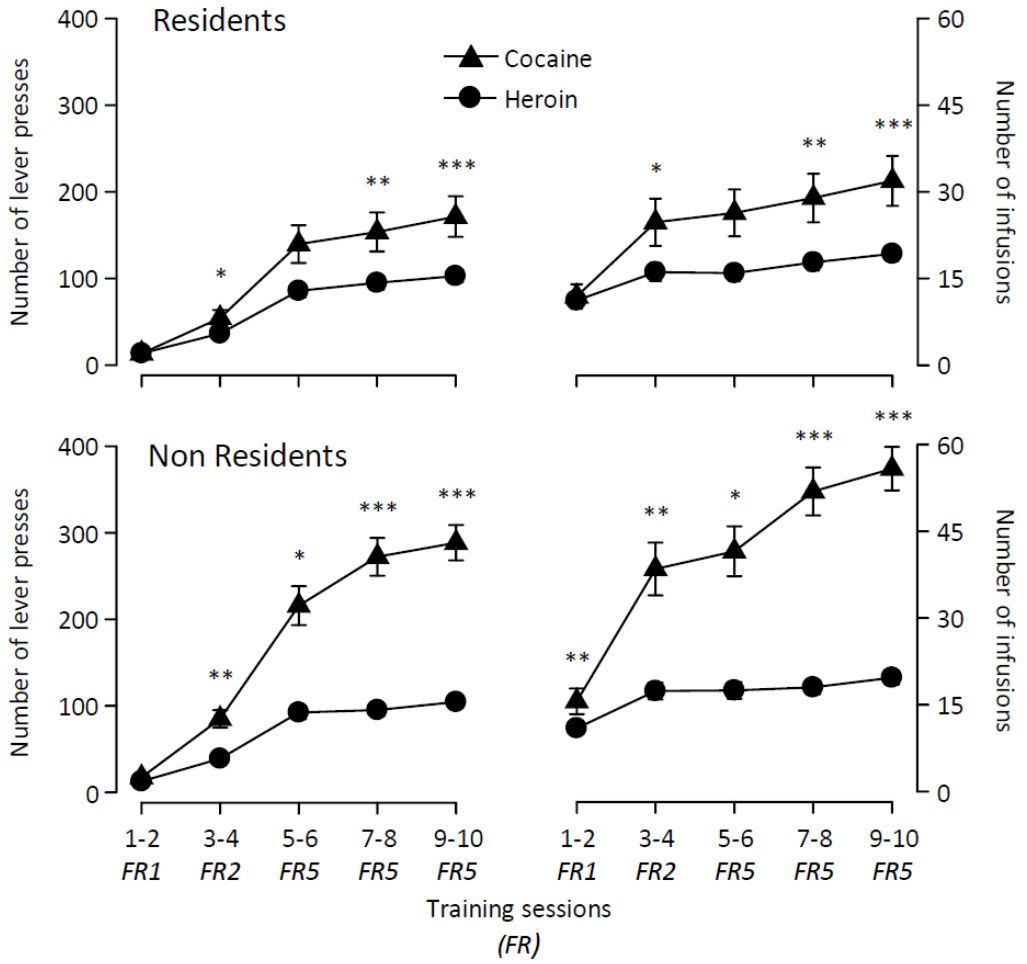

Figure 6.1. Mean (±SEM) number of lever presses and infusions for cocaine (400 µg/kg) and heroin (25 µg/kg) self-administration (SA) during the training phase for the Resident (top panels) and Non Resident (bottom panels) groups. *, **, and *** indicate cocaine versus heroin differences at $p < 0.05$, $p< 0.01$ and $p< 0.001$, respectively.

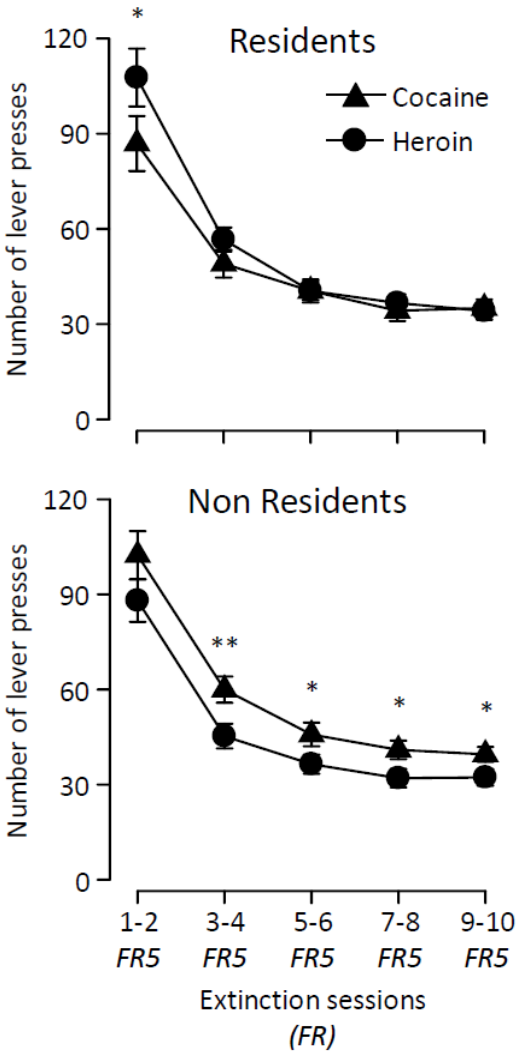

Figure 6.2. Mean (±SEM) number of lever presses on the cocaine- versus heroin-paired lever during the extinction phase for the Resident (top panel) and Non Resident (bottom panel) groups. *, ** indicates cocaine versus heroin differences at $p< 0.05$ and $p<0.01$, respectively.

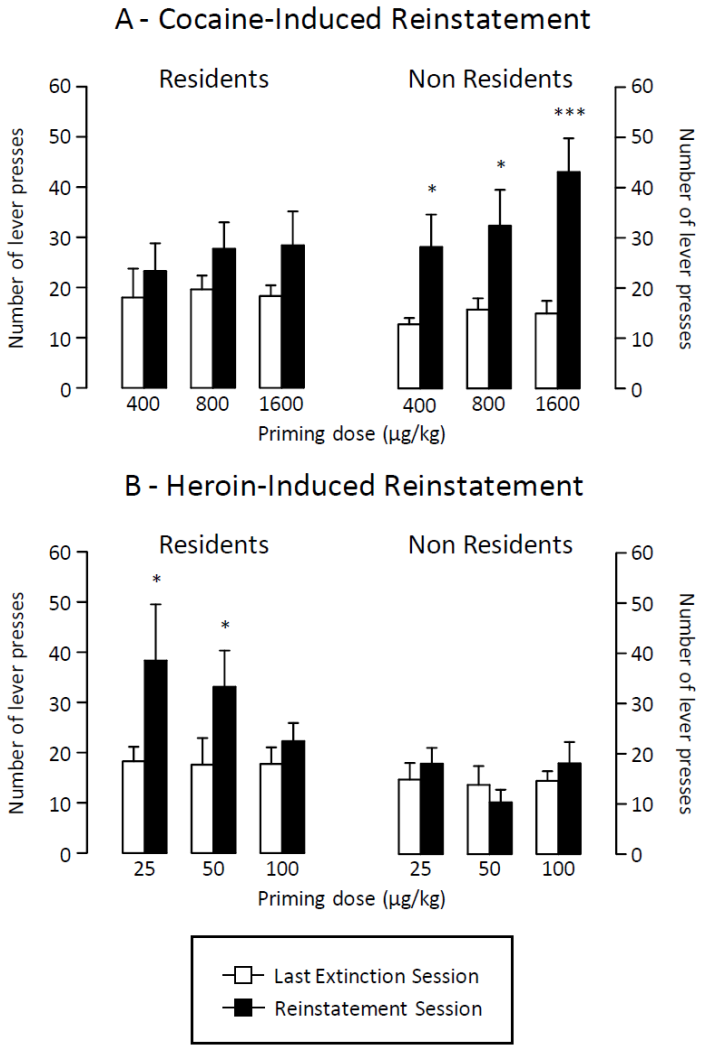

Figure 6.3. The panel A compares for both Resident and Non Residents rats the mean (±SEM) number of lever presses between the 1°st hour of the last cocaine extinction session *versus* the 1°st hour of the reinstatement session after non contingent intra-venous (i.v.) primings of cocaine (400, 800, or 1600 µg/kg). The panel B compares for both Resident and Non Residents rats the mean (±SEM) number of lever presses between the 1°st hour of the last heroin extinction session *versus* the 1°st hour of the reinstatement session after non contingent i.v. primings of heroin (25, 50, or 100 µg/kg). *, and *** indicate extinction *versus* reinstatement session differences at $p < 0.05$ and $p< 0.001$, respectively.

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8. Reprints of published articles

The research concerning the role of setting in drug addiction and in particular in the vulnerability to relapse (Montanari et al. 2014) illustrated in my dissertation is still unpublished; a first draft of the article is just been completed and fully reported in Chapter 6 of this thesis.

I also collaborated to other research projects, the results of which have been published (Orsini et al. 2013; Meringolo et al. 2012) or are in the process of being published (De Luca et al. 2014).

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Partial extinction of a conditioned context enhances preference for elements previously associated with cocaine but not with chocolate

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HIGHLIGHTS

- Exposition to single elements of compound CSs increases CPP for those elements.
- Increased CPP was evident only for cocaine but not chocolate conditioned CSs.
- The findings have implication for learning theory in regard to extinction learning.

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ABSTRACT

Drug-associated stimuli are crucial to reinstatement of drug-seeking after periods of abstinence, representing a central problem in treatment of addiction. The present study investigated the influence of partial extinction of the conditioned context on the expression of conditioned place preference (CPP). Mice of the inbred DBA/2J strain were conditioned with cocaine or chocolate in a context identified by multiple elements (A + B) and subsequently CPP expression was evaluated in a context containing only one element (A or B) or both (A + B). Cocaine- and chocolate-conditioned mice showed CPP in presence of the original compound stimulus. However, cocaine-conditioned mice did not show CPP when tested in A or B context, while chocolate-conditioned mice did show CPP to single element context. After conditioning mice were exposed to extinction training of the context A or B and then tested for CPP 1 and 9 days after the end of the extinction (days 9 and 18). Cocaine-conditioned mice showed CPP 9 days after extinction while chocolate-conditioned mice were relatively insensitive to the extinction procedure on day 1 after extinction, but they did not show CPP for the partial or the original compound 9 days after extinction. Cocaine-conditioned mice not submitted to the extinction training (simple passage of time) or submitted to a Sham-extinction procedure (saline injections and confinement in a new environment) did not show CPP on day 9 or 18. Cocaine-conditioned mice exposed to extinction training showed increased c-Fos expression in several brain areas in comparison to mice exposed to Sham-extinction. The extinction procedure did not specifically reduce behavioral sensitization. The results suggest that extinction training involving only elements of a drug-associated context can result in increased associative strength of those elements.

1. Introduction

Drug addiction is a chronic disease, and relapse in drug seeking after long periods of drug withdrawal is considered one of the main features of drug addiction [1]. Environmental stimuli associated with drug intake have the power to induce drug craving in humans [2,3] and to provoke reinstatement of drug-seeking behavior in preclinical models [4,5].

Extinction of drug-associated cues has been proposed as a mean of reducing the motivational properties of cues to prevent relapse [6,7]. However, clinical studies using extinction therapy have reported little success [8], mainly attributed to the context dependence of extinction learning [9]. Indeed the renewal phenomenon is a clear problem for cue-exposure addiction treatment and it appears to be a strong candidate for explaining why extinction-based treatments so often fail [10] and efforts to the generalizability of the extinction learning both by pharmacological and psychological treatment have been recently made [11–13].

Context refers to a configuration of many different stimuli (including smells, physical environments, interceptive drug states, mood or hormonal states and time of the day) providing the background setting of

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learning [14]. In animal research such compound of contextual stimuli is capable of modulating the extinction and reinstatement of drug-seeking [15,5]. Moreover, contextual stimuli associated with psychostimulant drugs provide a more effective CS and gain more associative control over behavior than discrete stimuli [16].

The conditioned place preference paradigm (CPP) has numerous advantages for the studying of the role of contextual stimuli in the reward process, though preference measures are not a direct model of "addictive" behaviors. Indeed, CPP response is based on a learned association between the contextual conditioned stimuli and the rewarding properties of the unconditioned stimulus (US), which result in animals spending more time in that context due to the reward evoking properties of those contextual stimuli. Unless specifically modified [17,18], a two-compartment CPP procedure is a context-conditioning, where multiple aspects of the environment (including visual, tactile, spatial, and olfactory features) in which drug is experienced enter into association with the drug. Thus, preference expressed in the absence of the drug represents both a conditioned "wanting" of the reward, but also a measure of the stamping-in of stimuli–reward associations. Moreover, preference for the conditioned compartment can be reinstated by addictive drugs following extinction [19].

We have previously reported that mice of the inbred strain DBA2/J (DBA) show high liability to prime-induced reinstatement of extinguished CPP after conditioning with a low cocaine dose and long withdrawal [20]. In the present study we further investigated the phenotype of DBA mice by manipulating the drug-associated contexts after the cocaine conditioning. Context was manipulated by extinction training of partial stimuli following the initial compound conditioning with tactile and visual stimuli. We also extended this investigation of the context role on expression of CPP when compound conditioning was with a natural reward. Finally, the neural substrate of CPP expression after context manipulation was investigated by quantification of c-Fos immunostaining.

2. Materials and methods

A total of 112 male mice of the inbred DBA/2JICo (DBA) strain (Charles River Laboratories, Italy) were purchased at 6 weeks of age and housed to a cage on a 12-h light–dark cycle (lights on at 7:00 A.M.) for 2 weeks before behavioral testing. Experiments were carried out in accordance with the Italian national law (DL 116/92) on the use of animals in research.

2.1. Apparatus

All behavioral testing was performed in four identical boxes formed by two gray lateral polyvinyl chloride (PVC) chambers (15 × 15 × 20 cm) connected by a central alley (15 × 5 × 20 cm). Two sliding doors (4 × 4 cm) connected the alley to the chambers. This apparatus was originally designed to avoid biased preference for any of the chambers [21]. However, in the present study each chamber was differentiated by two compound stimuli: 2 different floor textures (transparent Plexiglas) constituted the A stimuli, and two three-dimensional patterns with triangular bases (5 × 5 × 20 cm) made of black PVC and arranged to shape one of the chamber walls (covering the same pavement surface and not impairing the exploration) constituted the B stimuli. Different AB combinations with the two chambers were counterbalanced across subjects. In all experiments conditioning training was made to the AB compound.

Behavioral data were collected and analyzed by "Etho Vision" (Noldus Information Technology, Wageningen, The Netherlands), a fully automated video tracking system. The acquired digital signal was then processed by the software to extract the "time spent" (in seconds) and the "distance moved" (in centimeters) in the three compartments of the apparatus.

2.2. Conditioned place preference

The behavioral procedure was previously described [20]. Briefly, on day 1 all mice were free to explore the entire apparatus for 20 min and time spent in the two chambers and the central corridor was recorded and analyzed (pretest). Subjects' assignment to groups in each experiment was counterbalanced such that no bias was shown (time spent in compartment by all mice: unpaired 460.4 ± 13.8; center 286.9 ± 6.8; paired 452.7 ± 11.5). On the following 8 days (conditioning phase), mice were injected and confined daily for 40 min alternatively in one of the two chambers. For each animal, during the conditioning phase, one of the patterns was consistently paired with a saline injection and the other one with a drug injection. Pairings were balanced so that for half of the animals in each experimental group, the drug was paired with one chamber and the other half with the other chamber. On days 1, 3, 5, and 7, all animals received injections of cocaine immediately before starting the conditioning session and on the other days received saline injections immediately before starting the conditioning session. In the present study a modification was added, so that during the training phase the two chambers were identified by a specific AB compound stimulus, consistently paired with a saline or cocaine injection, while in Experiment 2 the two chambers were paired with regular food or milk chocolate (Lindt). In all experiments conditioning training was always made to the AB compound. Testing was conducted on day 10 in drug-free state (or without food/chocolate in Experiment 2) and lasted 20 min similar to the pretest. However, for some mice one element of the conditioned AB compound was removed during the testing phase and never replaced in the following phases, so the animals were not re-exposed to the original AB compound stimulus.

After conditioning and the initial CPP test (TEST), mice were given extinction training by pairing saline (Experiments 1, 3, 4 and 5) or regular food (Experiment 2) with each of the two compartments, once per day over 8 days, similarly to the conditioning training. Extinction training started the day after the CPP test. Mice received extinction training in presence of the same A or B stimuli to which they were previously exposed in the CPP test, thus for example, animals tested for CPP in presence of the only A stimuli received extinction training in presence of the same stimuli. Preference for the originally conditioned chamber was re-evaluated by CPP testing the day after the last saline or food pairing (9 days after the first CPP test, day 9), and once again one week later (18 days after the first CPP test, day 18). In all experiments mice were left undisturbed in the colony room during the week between day 9 and day 18 testing. In Experiments 4 and 5 the extinction training was compared to Sham-extinction condition and mice were injected daily with saline for 8 days between the time points TEST and day 9 (see later description).

In Experiment 2 reinstatement of the original conditioned preference was investigated by an additional test the day after the day 18 test, by replacing into the chambers the original conditioned AB compound stimuli. Such test was not done in the previous experiment, as the increased preference at day 18 would have masked any reinstating effect of the original conditioned compound stimulus. The general timeline diagram of behavioral procedure is illustrated in Fig. 1.

2.3. Tissue preparation

Mice were killed by decapitation 40 min after the end of behavioral testing. Brains were immersion-fixed overnight in 4% paraformaldehyde (4 °C) and cryoprotected in 30% sucrose (4 °C) until they sank (~48 h), then they were frozen with dry ice and cut into 40 μm transversal sections with a sliding microtome. Two adjacent series of sections were collected starting from −1.46 to 1.96 from bregma according to Mouse Brain Atlas [22]. One series was immunostained for Fos protein while the other one was Nissl-stained.

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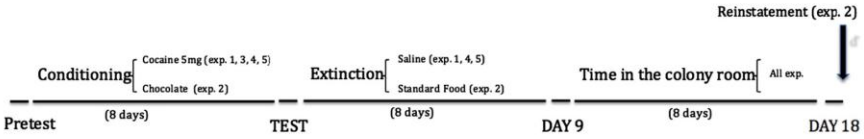

Fig. 1. Timeline diagram of behavioral procedures.

2.4. Immunohistochemistry

Immunohistochemistry was performed as previously described [23]. Fos protein was detected with a rabbit polyclonal antiserum (Ab-5, oncogene, 1:20,000) raised against residues 4–17 of human Fos. Secondary immunodetection was performed with biotinylated antibodies (goat anti-rabbit, Vector Laboratories Inc., Burlingame, CA, USA 1:1000). Peroxidase labeling was performed with standard avidin–biotin procedure (Vectastain ABC elite kit, Vector Laboratories, 1:1000). Metal-enhanced diaminobenzidine (FastDAB, Sigma) was used as the chromogen. Sections from all experimental groups were processed together to avoid batch-dependent staining variability.

2.5. Microscopy and image analysis

Visual examination and digital imaging of immunostained sections were performed with a Nikon Eclipse 80i light-transmission microscope equipped with a Nikon DS-5M CCD camera. For selected brain areas (Fig. 6) two bilateral photomicrographs were taken with the 4× objective and saved in TIFF format. Image analysis of photomicrographs was performed with the public domain software ImageJ (http://rsb.info.nih.gov/ij/) [24] running on Debian GNU/Linux (www.debian.org). First, anatomical boundaries were delineated with the polygon selection tool and saved as region of interests (ROIs) in the ROI manager. ROI determination was performed by an experienced researcher according to Nissl-stained adjacent section and to Mouse Brain Atlas [22]. Second, immunoreactive nuclei were automatically counted in each ROI with a custom-made ImageJ macro based on find maxima algorithm (for source code see http://fiji.sc/cgi-bin/gitweb.cgi?p=imagej.git;a=blob;f=ij/plugin/filter/MaximumFinder.java). The parameters of find maxima algorithm have been tuned to reliably reproduce manual counts in a previous study [23]. Density of immunoreactive nuclei was expressed as n° of nuclei/0.01 mm^2.

2.6. Drug

Cocaine hydrochloride was purchased from Sigma (Milan, Italy). Cocaine was dissolved in saline (0.9% NaCl) and injected intraperitoneally (i.p.) in a volume of 10 ml/kg. All doses were expressed as the salt.

2.7. Data analysis

Time spent in each of the three compartments was used as dependent measure in all CPP experiments. Distance moved inside the paired compartment was used as dependent measure in the behavioral sensitization experiment. The number of immunoreactive cell nuclei was used as dependent measure in the c-Fos experiment.

Statistical analyses were performed by SuperANOVA (Abacus Software, Inc.) and the alpha level of acceptance was 0.05. Post hoc comparisons were performed by Fisher's LSD test whenever significant main effects or interactions were attained. Immunohistochemistry data were analyzed independently for each brain structure.

Data from TEST, day 9 and day 18 were always analyzed separately. For all CPP experiments data were analyzed by mixed-model ANOVAs, with Choice (three levels: unpaired, center, paired) as within factor. For Experiment 1, Stimulus (two levels: A and B) and Treatment (two levels: saline and cocaine) were the between factors. In Experiments 2 and 3 the only between factor variable was Stimulus, while in Experiment 4 the Experience factor (two levels: extinction and Sham-extinction) was added. Locomotor activity data from Experiment 5 were analyzed by the two-factor ANOVA with Pretreatment (two levels: Saline and Cocaine) and Experience (two levels: extinction and Sham-extinction). c-Fos data were analyzed by one-factor ANOVAs for each brain structure, with Experience (two levels: extinction and Sham-extinction) as between factor, with the exception for the amygdala data which were first analyzed by a two-way ANOVA with basolateral complex (two levels: basolateral and lateral nuclei) as within factor and Experience (two levels: extinction and Sham-extinction) as between factor. To determine the degree of association between the CPP and c-Fos induction we calculated the Pearson's product–moment correlation coefficient (r) between the number of cells expressing c-Fos and the degree of preference for the paired compartment, in brain areas where significant effects of extinction were found.

2.7.1. Experiment 1

Thirty-two DBA mice were assigned to this experiment, and after pre-test exposure 2 groups (n = 8) were cocaine conditioned (5 mg/kg, i.p.) and 2 other groups were saline conditioned. All mice were trained to associate chambers identified by the AB compound stimuli. Twenty-four hours after the last conditioning session half of the mice from each treatment condition were tested for CPP with either A or B elements only (TEST), afterwards mice were tested twice for extinction retention (day 9 and day 18) as described in Materials and methods. Another eight DBA mice were added to this experiment to demonstrate that initial CPP was effectively induced but not expressed in presence of partial elements of the original AB compound; these mice were cocaine conditioned as above described, but tested with the whole configuration of the AB stimuli. These data were analyzed separately.

2.7.2. Experiment 2

This experiment was designed to test whether manipulation of contextual stimuli after CPP conditioning had the same effect when a natural reward was the unconditioned stimulus. To this aim, twenty-four DBA mice were trained to associate chocolate with the compound stimulus AB and tested for their place preference with the single elements (A or B) or the whole AB compound (n = 8). After extinction training, mice were then re-tested twice in presence of these same stimuli one (day 9) and two weeks (day 18) after the first CPP test. Contrary to the cocaine conditioned mice chocolate conditioned mice did not show any increased CPP at day 18, thus we decided to also make a test for the reinstatement by re-presenting the original conditioned compound stimulus to all groups. In the previous experiment the increased CPP at day 18 made useless such reinstatement test, as it would had been impossible to distinguish a reinstated preference from the maintenance of the day 18 preference.

2.7.3. Experiment 3

This experiment was aimed at investigating the only effect of time since the last cocaine injection on the CPP observed on day 18. Thus, extinction training was replaced/substituted by a resting time of 8 days in the colony room. Cocaine conditioning and time of testing were identical to previous experiments. Thus, sixteen DBA mice

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trained to associate cocaine (5 mg/kg, i.p.) with the compound stimulus AB, were tested for their place preference with the single elements, and then re-tested twice in presence of the same stimuli one (day 9) and two weeks (day 18) after the first CPP test.

2.7.4. Experiment 4

This experiment had two objectives. First, we were aimed at investigating the role of the manipulation required by the extinction training. Indeed, it is possible that the saline injections of the extinction training might have been an additional "stressful" manipulation, but also that such training caused "frustration" for a thwarted expectation. Second, we were interested in the pattern of c-Fos immunostaining associated with expression of the effect found in Experiment 1. Therefore, in this experiment we compared the preference of mice exposed to the extinction procedure with that of similarly manipulated mice, except for the learning implied in the extinction training. The consistent observation that A or B elements did not induce overshadowing effects obviated the use of this control, and sixteen DBA mice were trained to associate the cocaine dose of 5 mg/kg with the compound stimulus AB and tested only with the B elements. After the first CPP test half of the mice underwent the regular extinction training involving pairing saline injections with the apparatus (extinction), while the other half underwent the same number of saline injections before being placed in a novel cage (Sham-extinction). All mice were then tested twice (day 9 and day 18) as in previous experiments.

Forty minutes after the end of the last CPP test all animals were decapitated and their brains were extracted for subsequent determination of c-Fos immunoreactivity in the following areas: prefrontal cortex (infralimbic, prelimbic, orbitofrontal), nucleus accumbens (core, shell), nucleus caudate (dorso-medial, dorso-lateral, ventro-medial, ventro-lateral), amygdala nuclei (basolateral, lateral, central) and hippocampus (CA1, CA3, dental gyrus).

2.7.5. Experiment 5

This experiment was aimed at investigating whether results of Experiment 1 were associated with sensitization to the psychomotor stimulant effects of cocaine, an indirect index of sensitization of the substrate mediating the motivational attribution to stimuli [25]. Behavioral sensitization was measured by recording locomotor activity in the CPP apparatus in a specifically designed experiment, identical to the CPP procedure for conditioning, extinction and the time points of testing, except for the final test environment (day 18), as we decided to measure locomotor activity expressed in the only cocaine-paired compartment instead of the whole apparatus. Sixteen DBA mice were assigned to this experiment; half were cocaine conditioned (5 mg/kg) and the other half were saline conditioned. After the first CPP test, one of the cocaine-trained groups and one of the saline trained groups underwent the regular extinction training involving pairing saline injections with the apparatus (extinction), and the other two groups received saline injections just before being placed in a novel cage in a different room, in this way the amount and kind of manipulation were similar to the extinction procedure, but no extinction learning was allowed (Sham-extinction). All mice were then regularly tested for extinction retention on day 9 and again on day 18 all mice were challenged with a cocaine prime (5 mg/kg) and immediately confined in the previously cocaine-paired chamber of the CPP apparatus, where locomotor activity was measured for 1 h. Distance moved was taken as dependent variable.

3. Results

3.1. Experiment 1

Results from this experiment are shown in Fig. 2. Mice did not express preference for the compartment identified by the partial elements of the conditioned compound when tested after conditioning, as there was no difference between mean time spent in paired and unpaired regardless of the significant effect of Choice [$F(2,56) = 4.199$; $p < 0.05$]. Also, no differences were found by the post hoc comparison between time spent in paired and unpaired by either saline or cocaine trained mice after the extinction training (day 9), regardless of the significant Choice × Treatment interaction [$F(2,56) = 6.524$; $p < 0.01$].

However, cocaine conditioned mice tested for extinction retention 18 days after the first CPP test, showed a significant preference for the drug-paired compartment regardless of the partial element used to identify it, as revealed by significant Choice × Treatment [$F(2,56) =$

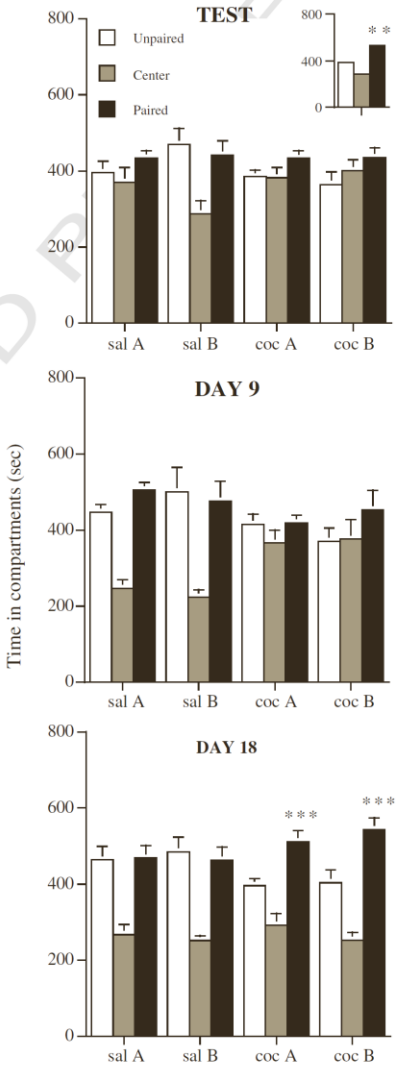

Fig. 2. Place preference response of DBA mice conditioned with cocaine or saline and exposed to partial elements (A or B) of the original conditioned compound stimulus on subsequent CPP testing the day after conditioning (TEST), after the extinction training (day 9) and 9 days after the extinction retrieval test (day 18). Top small panel shows CPP response to the original AB compound (error bars are too low to be shown). Data are expressed as mean time spent (±SEM) in the cocaine paired, center, and unpaired compartments. ***$p < 0.001$; **$p < 0.01$ paired ≠ unpaired.

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3.613; $p < 0.05$] with a significant difference in the time spent in the two main compartments only in the cocaine conditioned groups (cocaine paired vs. cocaine unpaired: $t = -3.516$, $p < 0.001$) independently by the partial element present in the compartments.

ANOVA of preference data of cocaine-conditioned mice tested in presence of the AB compound showed a significant Choice effect [$F(2,14) = 16.084$; $p < 0.001$] and the post hoc comparison revealed difference in time spent in paired vs. unpaired compartments ($t = -3.283$, $p = 0.0054$) (data shown in Fig. 2, small top panel).

Thus, absence of preference for the drug-paired compartment on the first CPP test was most probably due to the reduced strength of the partial elements as conditioned stimuli. These results indicate a late increase of the ability of weak conditioned stimuli to elicit preference.

3.2. Experiment 2

As shown in Fig. 3 all chocolate-conditioned mice showed preference on the first CPP test, as revealed by a significant effect of Choice [$F(2,42) = 22.703$; $p < 0.001$] followed by a post hoc analysis revealing difference in time spent in paired vs. unpaired ($t = -3.454$, $p < 0.01$). After the extinction training the A stimuli group was resistant to the extinction training, as revealed by a significant Choice × Stimuli effect [$F(4,42) = 3.059$, $p < 0.05$] followed by significant difference in the time spent in paired vs. unpaired ($t = -2.152$, $p < 0.05$) in that group. Nevertheless, in the final test (day 18) none of the groups showed preference for the chocolate-paired compartment, and regardless of the significant effect of Choice [$F(2,42) = 49.752$; $p < 0.001$], no differences in time spent in paired and unpaired were found. ANOVA of the reinstatement data revealed a significant Choice effect [$F(2,42) = 42.338$; $p < 0.001$], however post hoc comparison did not identify any difference in mean time spent in paired vs. unpaired.

These results indicate that elements of a compound stimulus associated with a natural reward are not weak conditioned stimuli, and are even resistant to extinction; nonetheless, their ability to elicit preference is lost on late testing.

3.3. Experiment 3

Data obtained in cocaine-conditioned mice not subjected to the extinction training, are presented in Fig. 4. Mice never showed preference for the drug-paired compartment identified by elements of the original conditioned compound. Statistical analyses revealed significant main effects of Choice on TEST [$F(2,28) = 10.618$; $p < 0.001$], day 9 [$F(2,28) = 19.57$; $p < 0.001$] and day 18 [$F(2,28) = 19.976$; $p < 0.001$], but no difference between paired and unpaired on none of the time points tested.

These results support the hypothesis that procedures involved in extinction training are a requirement for the development of the late preference for weak conditioned stimuli, while the mere passage of time is not sufficient.

3.4. Experiment 4

Fig. 5 shows results of DBA mice trained with cocaine and then submitted to extinction training (extinction) or to a similar manipulation (Sham-extinction). The two-way ANOVA revealed a significant effect of Choice [$F(2,28) = 15.424$; $p < 0.001$] but no difference between paired and unpaired. Results of CPP response on the second test (day 9) revealed a significant effect of Choice [$F(2,28) = 28.109$; $p < 0.001$] and Choice × Experience [$F(2,28) = 6.008$; $p < 0.01$]. Paradoxically post hoc comparison of the interaction evidenced that only mice experiencing the extinction training exhibited CPP, as indicated by higher time spent in paired vs. unpaired in these mice ($t = -2.638$, $p = 0.0135$), suggesting an anticipation of the phenotype. This anticipation was indeed confirmed by results of the final test (day 18), which evidenced preference for the drug-paired compartment only in the group submitted to the extinction procedure, as indicated by the significant Choice × Experience interaction [$F(2,28) = 4.942$; $p < 0.05$] and significant differences in the time spent in paired vs. unpaired only in the extinction group ($t = -2.595$, $p = 0.0149$).

These data add support to the view that expression of increased CPP requires the extinction training.

The pattern of c-Fos expression within various brain structures is shown in Table 1, and representative coronal sections from these

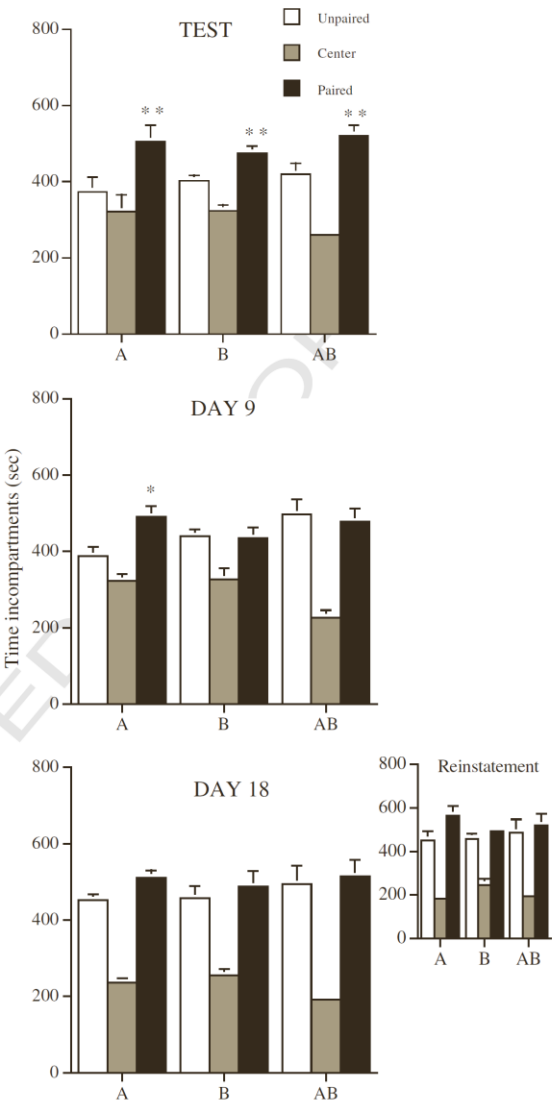

Fig. 3. Place preference response of DBA mice conditioned with chocolate and exposed to partial elements (A or B) and the original conditioned compound stimulus (AB) on the day after conditioning (TEST), after the extinction training (day 9), 9 days after the extinction retrieval test (day 18), and on reinstatement test by exposition to the original conditioned compound stimulus. Data are expressed as mean time spent (±SEM) in the chocolate paired, center, and unpaired compartments. $^{**}p < 0.01$; $^{*}p < 0.05$ paired ≠ unpaired.

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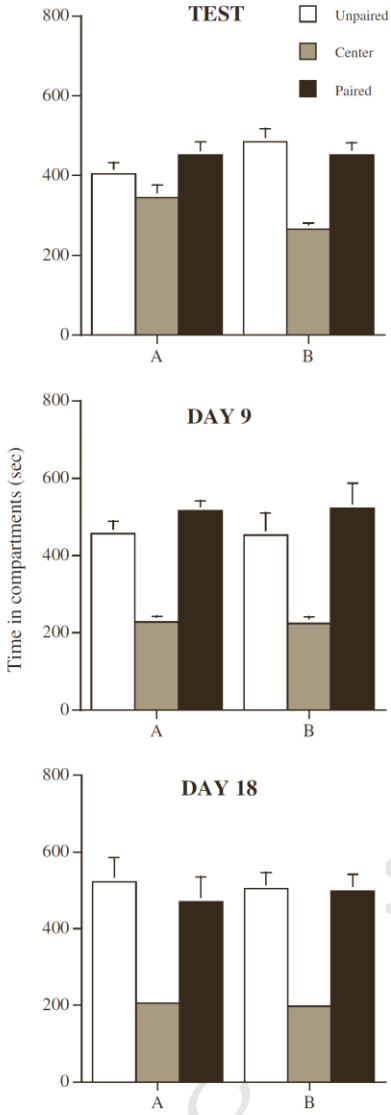

Fig. 4. Place preference response of DBA mice conditioned with cocaine and exposed to partial elements (A or B) of the original conditioned compound stimulus on the day after conditioning (TEST), and 9 days (day 9) and 18 days (day 18) from the first CPP test. Data are expressed as mean time spent (±SEM) in the cocaine paired, center, and unpaired compartments.

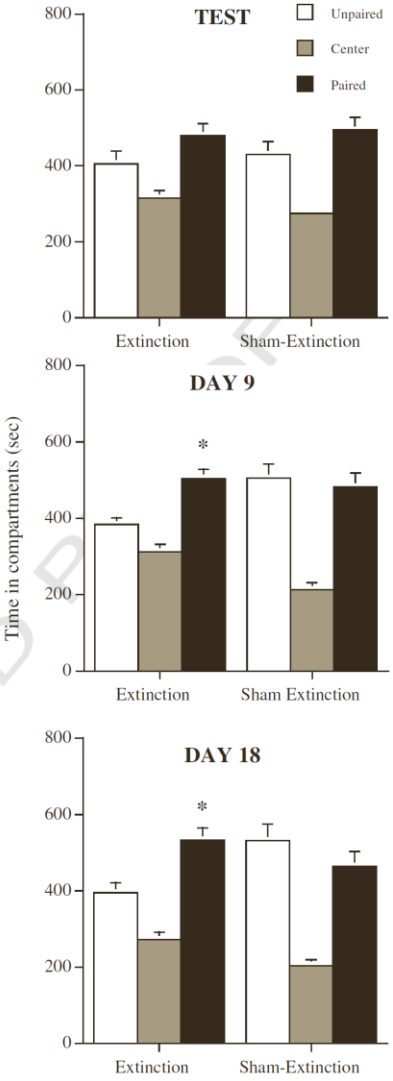

Fig. 5. Place preference response of cocaine conditioned, extinguished (extinction) and un-extinguished (Sham-extinction) DBA mice on the day after conditioning (TEST), and 9 days (day 9) and 18 days (day 18) from the first CPP test. All mice were exposed to the B elements of the original compound stimuli. Data are expressed as mean time spent (±SEM) in the cocaine paired, center, and unpaired compartments. *$p < 0.05$ paired ≠ unpaired.

brain areas are shown in Fig. 6. Significantly greater number of Fos-immunoreactive neurons was observed in the core [$F(1,14) = 7.347$; $p < 0.05$] and shell [$F(1,14) = 9.04$; $p < 0.01$] of the nucleus accumbens, in the dorsomedial striatum [dmCP: $F(1,14) = 9.339$; $p < 0.01$], in the ventromedial striatum [vmCP $F(1,14) = 10.954$; $p < 0.01$], and CA1 of the hippocampus [$F(1,14) = 8.889$; $p < 0.01$] of extinguished mice, as compared to the manipulated ones. The two-way ANOVA of c-Fos expression in the basolateral complex of amygdala (basolateral and lateral nuclei) revealed significant effects of Experience [$F(1,14) = 9.136$; $p < 0.01$] and brain area [$F(1,14) = 58.996$; $p < 0.001$] but no interaction. Individual one-factor ANOVA for each amygdala nucleus evidenced a significant effect of Experience only in the lateral amygdala [$F(1,14) = 8.789$; $p < 0.05$], which was also the only brain area whose activation was positively correlated with place preference behavior ($r = 0.502$; $p < 0.05$).

3.5. Experiment 5

As shown in Fig. 7 all cocaine-conditioned mice showed enhanced locomotor response to the cocaine challenge in comparison with saline-conditioned mice regardless of the experimental condition, as revealed by the significant main effect of Pretreatment [$F(1,20) = 9.575$; $p < 0.01$]. On the other hand, the extinction training reduced cocaine-induced locomotion in both saline- and cocaine-trained

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Table 1
Results of one-way ANOVAs and post hoc (Fisher's LSD test) for each brain region. Levels of c-Fos are shown as mean number of immunoreactive cell nuclei/mm^2 (±SEM). Significance levels refer to differences between extinction and Sham-extinction (* = $p < 0.05$, ** = $p < 0.01$).

	Extinction	Sham-extinction
Frontal cortex		
Infralimbic	107 ± 6.9	111.1 ± 13.8
Prelimbic	64.1 ± 4.9	66.9 ± 10.3
Orbitofrontal	309.4 ± 50.5	246.3 ± 38
Nucleus accumbens		
Core	125.8 ± 8.5*	79.2 ± 14.9
Shell	108.7 ± 8.8**	57.9 ± 14.4
Nucleus caudate		
Dorso-medial	149.5 ± 10.7**	99.5 ± 12.4
Dorso-lateral	27.6 ± 4.0	16.6 ± 3.5
Ventro-medial	124.2 ± 8.5**	76.7 ± 11.9
Ventro-lateral	26.1 ± 6.5	18.1 ± 3.8
Amygdala		
Basolateral	262.4 ± 28.0	178.1 ± 27.7
Lateral	62.7 ± 4.8*	38.9 ± 6.4
Central	152.6 ± 31.1	115.8 ± 26.1
Hippocampus		
CA1	462.9 ± 38.7**	312.8 ± 32.1
CA3	410.8 ± 67.4	268.2 ± 26.2
Dental gyrus	400.6 ± 50.2	343.6 ± 55.2

groups, as revealed by significant effect of Experience [$F(1,20) = 15.47$; $p < 0.001$], thus suggesting the contribution of the extinction inhibitory learning in reducing the conditioned influence of the drug-paired compartment on the behavioral sensitization.

These data do not support the hypothesis of an association between increased CPP and behavioral sensitization expressed by the enhanced locomotor response to cocaine challenge.

4. Discussion

The major finding of the present study is the observation of a lasting increase in preference for individual elements of a drug-associated context following selective extinction training of these elements. This observation is relevant because it reveals a mechanism that could powerfully interfere with effectiveness of cue-exposure treatments of drug addiction. Moreover, the present data strongly support the conclusion that such mechanism might be specific for drugs of abuse.

In the first experiment the two stimuli used to identify the cocaine paired and unpaired compartments of the apparatus were unable to support significant conditioned place preference (CPP) when presented individually (Fig. 2, TEST), possibly due to their weak associative strength. This hypothesis derives from classic observation that conditioned stimuli (CSs) presented in compound elicit stronger response compared to each of the individual CS, implying that their associative strengths combine [26,27]. Two lines of evidence support this interpretation. First, mice expressed significant preference for cocaine-paired compartment when all the conditioned elements were present (see Fig. 2, small top panel). Second, mice preferred the

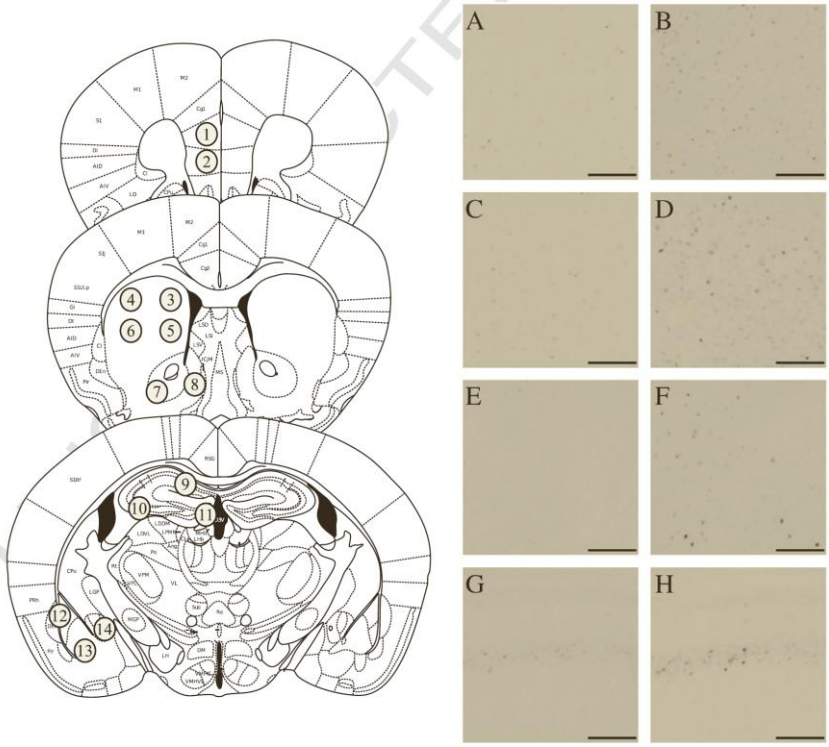

Fig. 6. Upper panel: schematic representation of sample areas subjected to image analysis; (1) pre-limbic cortex, (2) infra-limbic cortex, (3) dorso-lateral striatum, (4) dorso-medial striatum, (5) ventro-lateral striatum, (6) ventro-medial striatum, (7) nucleus accumbens core, (8) nucleus accumbens shell, (9) CA1, (10) CA3, (11) dentate gyrus, (12) lateral amygdala, (13) basolateral amygdala and (14) central nucleus of amygdala. Drawings were adapted from Franklin and Paxinos [22]. Coordinates are given in millimeters from bregma. Lower panel: representative photomicrograph of c-Fos-immunoreactive cell nuclei from mice in the extinction (on the right) and the Sham-extinction groups (on the left) of the core (A, B) and shell (C, D) of nucleus accumbens, in the lateral amygdala (E, F) and CA1 field (G, H) of the hippocampus. Calibration bar 100 μm.

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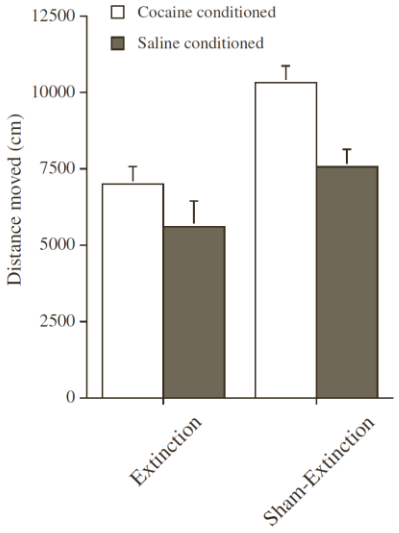

Fig. 7. Locomotor activity response to a cocaine challenge (5 mg/kg, i.p.) in the cocaine paired compartment of extinguished (extinction) and un-extinguished (Sham-extinction) DBA mice previously conditioned with cocaine or saline, on the 18th day after the first CPP test. Data are expressed as mean distance traveled (±SEM) in the only cocaine paired compartment. The ANOVA revealed only the main effects of cocaine conditioning and extinction training.

compartment associated with the same cocaine dose when contextual stimuli were not manipulated between conditioning and extinction training [20]. Moreover, overshadowing of the more salient element over the less salient one cannot account for the present results, as lack of CPP expression was symmetrical [28]. Contrary to what has been reported for the tactile stimuli in CPP paradigm [29–31,17], the two stimuli of the compound (the floor and the pattern) did not differ in their perceptive salience. This could depend on the fact that the geometrical patterns we used in this study (as in the previous ones) had tactile features as well. Indeed, not only were they visual (black against the gray wall) and spatial (different shapes of the equivalent space) stimuli, but they also have a tactile dimension when mice were in their proximity, as they could be "touched" by the whiskers.

Extinguishing an unexpressed behavior may seem paradoxical, however in the present study the extinction was intended as a learning experience more than the decrease of the first conditioned behavior. DBA mice expressed a significant and lasting preference for drug-paired compartment identified by the individual CSs following extinction training (Fig. 2, day 18). This result might be interpreted as a spontaneous recovery after a delay from the extinction, since a CPP was not expressed to A or B to begin with; however we point out that increase in preference was not observed after the mere passage of time (Fig. 4, day 18), nor in mice submitted to a Sham-extinction procedure, as repeated saline injection in an empty cage (Fig. 5, day 18). Moreover, we have previously reported lack of spontaneous recovery of cocaine CPP after extinction at the same time points, when the only cocaine conditioned stimulus was then extinguished [20]. We cannot rule out that continuous retest would have produced different results, because the two protocols have been shown to promote different results [32]. Altogether these observations do not support the hypothesis that increased associative strength of the individual CSs might result from a strain specific liability to spontaneous recovery, or from incubation of motivational value of the CSs due to time of cocaine withdrawal [33].

Data of mice conditioned with the natural reward chocolate revealed many differences compared to cocaine, as shown in Fig. 3. To this regard it is worth pointing out that mice trained for chocolate CPP never experienced restricted feeding. Nonetheless, these animals expressed significant CPP when only elements of the conditioned compound were presented. Extinction training of the individual CSs was only partially effective in reducing their ability to support CPP; however, no sign of conditioned preference for the individual CSs or the original compound stimulus was observable 8 days later. Thus, major differences for the effects of cocaine and chocolate were evident at all stages of the experimental procedure.

The associative strength of the individual chocolate CSs might depend on DBA mice finding chocolate more rewarding than a low dose of cocaine [34,35]. On the other hand, it is well known that cocaine promotes a number of physiological responses that could enter into the compound CS [36]. Therefore, the partial context presented on CPP test could have been more similar to the one originally associated with the reward for the chocolate-trained mice than to the cocaine-trained mice. Both hypotheses may also account for resistance to extinction by the individual chocolate CSs. Nonetheless, lack of CPP 8 days after the end of extinction training does not support the hypothesis that chocolate, in condition of homeostatic balance, promotes strong emotional/motivational arousal. Indeed, high emotional arousal is associated with lasting memory traces [37], whereas the absence of CPP when the original compound is re-presented indicates decay of the association.

Overall, associative strength of cocaine- and chocolate-CSs changed in opposite directions, suggesting that the increased strength observed in cocaine-conditioned mice depends on the specific effect of the addictive drug.

As an initial attempt to explore the involved mechanism, we tested development and expression of sensitization to the psychomotor effects of cocaine. Indeed, behavioral sensitization is a typical effect of addictive drugs, strongly influenced by environmental stimuli and increases with time after the end of drug exposure [38–40,25]. To this aim we conditioned and then extinguished the CPP as in the previous experiments and tested the locomotor response to a cocaine challenge 8 days after the end of the extinction within the drug-paired compartment to avoid interference by choice behavior. The results, shown in Fig. 7, did not support any relationship between the increased CPP for individual CSs and development of behavioral sensitization.

Finally, we evaluated the pattern of c-Fos immunostaining associated with expression of CPP for a single contextual CS. To this aim we compared samples from brains of cocaine-trained mice tested 8 days after the end of extinction training (expressing significant CPP) with samples from mice exposed to the Sham-extinction procedure (not expressing CPP). We found higher c-Fos immunostaining in several brain areas of the CPP expressing compared to the CPP non-expressing animals (Table 1). Differences found in the core and shell compartments of the nucleus accumbens or in the basolateral complex of the amygdala (basolateral + lateral nucleus; BLA) are in line with results obtained by previous studies comparing CPP expressing and non-expressing rodents in different protocols [41–45]. Less evidence is available for CA1 of the hippocampus [44,46] and dorsal caudate [47,48], whereas evidence is reported in the lateral amygdala (LA) nucleus for cue-induced reinstatement of cocaine self-administration [49]. The absence of significant effects of extinction training on c-Fos expression in prelimbic (PL) and infralimbic (IL) cortices is in line with the conflicting results reported in the literature ([41,50,42] but also [48,51,47]), and could support the view that c-Fos expression in these brain areas is not associated with expression of CPP and it does not give information about mechanisms mediating behavioral control by drug-associated stimuli [52,53].

The most interesting result of the immunohistochemical analysis was obtained in the lateral amygdala (LA). Indeed, we found higher level of c-Fos immunostaining in mice submitted to extinction

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training in comparison with those exposed to the Sham-extinction procedure, and a significant positive correlation between c-Fos immunostaining in LA and preference scores. There are no reports on c-Fos levels in the LA of CPP expressing animals, whereas consistent evidences support a major involvement of LA in both food- and drug-induced CPP [54–56]. Moreover, levels of c-Fos expression in LA are generally included in the BLA sample, whereas the correlation between c-Fos and CPP scores was restricted to the lateral nuclei. This selectivity is in line with the role of LA in coding for elemental information [57,58], retrieving of Pavlovian memories [59–61] and controlling the role of contextual elements in associative learning [62]. Thus, the results obtained from LA c-Fos immunostaining suggest that mice expressing CPP when presented with individual CSs are those recalling the specific drug–CS association. If so, then it is conceivable that the repeated exposure to the individual CSs has reconsolidated the specific CS–US association, rendering it independent of the original context. Such hypothesis is supported by evidence of the role of LA in synaptic-specific potentiation of reactivated memories [63], and by evidence that basolateral complex (lateral nucleus in particular) is crucial in reconsolidation of the first acquired association rather than in extinction consolidation [64]. Thus it is possible that our procedure of cocaine conditioning followed by repeated exposure to un-rewarded partial stimuli might had represented the conditions under which the interactive processes of extinction consolidation and reconsolidation are shifted toward a state where reconsolidation is dominant.

Such conclusion would be in line with the absence of higher c-Fos expression in the basolateral nuclei, PL and IL, all known to be crucial in the contextual control on associations [62,65].

A reasonable explanation for the absence of CPP at day 18 in mice exposed to chocolate may be offered by the mechanisms of cocaine-induced neural plasticity in DBA. Indeed, induction of cocaine sensitization in mice from this inbred strain is critically dependent on adrenal hormones [66,67]. Moreover, cocaine exposure promotes a strain-specific pattern of hormone-dependent brain plasticity in these mice [68]. It is unlikely that exposure to a known palatable food in free-fed animals is associated with significant activation of the hypothalamus–pituitary–adrenocortical (HPA) axis. Therefore it is reasonable to suggest that brain plasticity promoted by the HPA activation is required for the reconsolidation of specific drug–cue association in the course of partial extinction of the conditioned context.

5. Conclusion

DBA mice are characterized by relevant endophenotypes of liability to addiction such as trait-impulsivity and dopamine D2 receptor functioning [69–74]. Moreover, they are also highly susceptible to prime-induced reinstatement of an extinguished CPP [20]. The present results while confirming their vulnerability, also suggest that this may depend on the emergence of the reconsolidation over the extinction learning, the two processes normally competing for their expression following memory re-activation [75].

In conclusion, the present results suggest that high individual risk to relapse into drug seeking might be associated with dysfunctional response to treatments based on cue-exposure, and point at such therapy as a potential relapse-precipitating event for those vulnerable subjects. This alert is also supported by clinical reports suggesting caution to provide cue-exposure therapy not in combination with other treatments, as it might be detrimental instead of beneficial [76].

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ORIGINAL INVESTIGATION

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

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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.

Keywords Drug addiction · Drug abuse · Opiates · Morphine-3-glucuronide · Morphine-6-glucuronide · M3G · M6G · Liver microsomes · Microsomal preparations

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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 × *g*. The supernatant was further centrifuged for 60 min at 105,000 × *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_4OH 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 (GraphPad 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

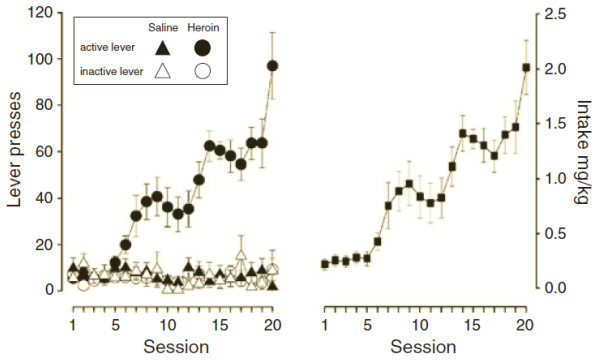

Fig. 1 Number of presses on the active vs. inactive lever (means ± SEM) for rats self-administering saline or heroin (50 μg/kg) on an FR1 (sessions 1–4) and then FR2 (sessions 5–20) schedule of reinforcement

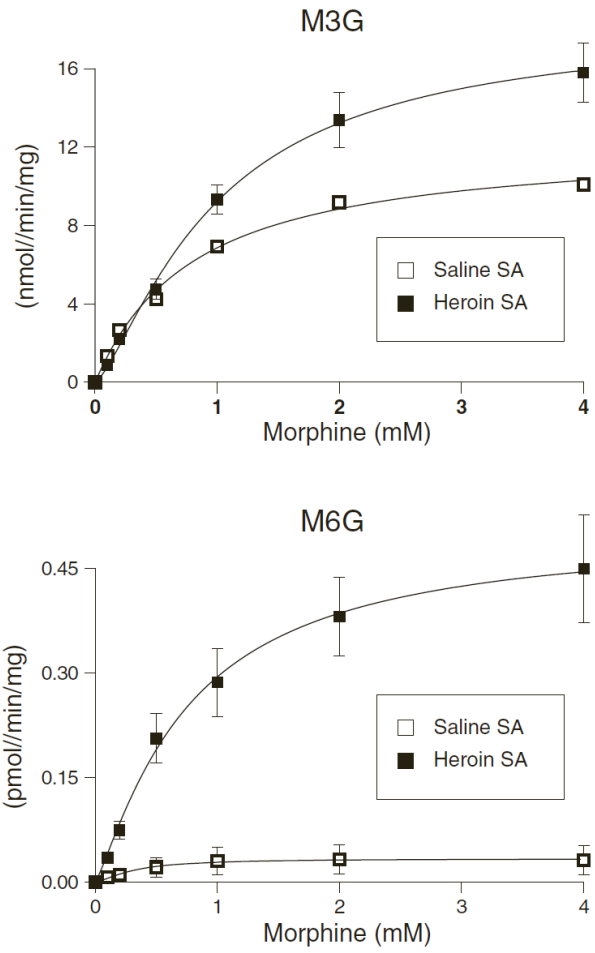

Fig. 2 Kinetic of M3G and M6G formation by microsomal preparations obtained from the liver of rats treated with saline or heroin and incubated with increasing concentrations of morphine. Each data point is an average of triplicate determination ± SEM

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).

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 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).

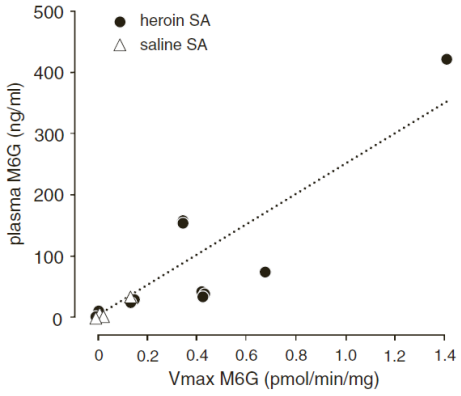

Fig. 3 Regression of plasma levels of M6G over in vitro V_{max} of M6G formation by liver microsomes

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.

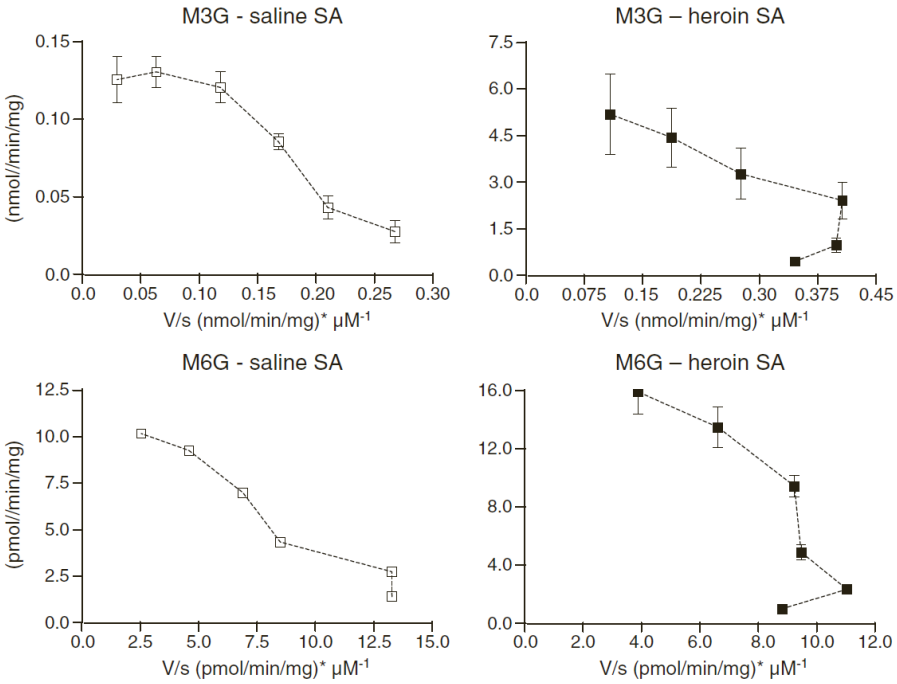

Fig. 4 Eadie–Hofstee plots of M3G and M6G formation by microsomal preparations from rats that had self-administered saline or heroin. From the same data of Fig. 2

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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