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Effects of social isolation and environmental modulation on the glutamatergic cortico-accumbens synaptic plasticity

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Abbreviations:

aCSF: artificial cerebrospinal fluid

AMPA: α- amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid

BLA: basolateral amygdala

BNST: bed nucleus of the stria terminalis

Ca^{++}: calcium

CaMKII: Ca^{++}/calmodulin-dependent protein kinase II

cAMP: cyclic adenosine monophosphate

DA: dopamine

DAG: diacylglycerol

fEPSP: field excitatory post synaptic potentiation

FR: fixed ratio

GABA: Gamma-aminobutyric acid

GH: grouped house

HFS: high frequency stimulation

IH: isolated housing

IL: infralimbic cortex

K: potassium

LTP: long term potentiation

MAGUK: membrane-associated guanylate kinases

mPFC: medial prefrontal cortex

mGluR2/3: metabotropic glutamate receptor group 2 and 3

MOR: μ opioid receptor

MSN: medium spiny neurons

NAc: nucleus accumbens

NMDA: N-methyl-D-aspartate

NR2b: NMDA2b-containing receptors

PKA: protein kinase A

PKC: protein kinase C

PL: prelimbic cortex

PLC: programmable logic controller

PPR: paired pulse ratio

SA: self administration

SI: social isolation

TARP: transmembrane AMPAR regulatory proteins

USV: ultra sound vocalization

VTA: ventral tegmental area

Abstract

Previous studies in our laboratory have highlighted the importance of the setting in which drug is taken. We used an in vivo animal model in which one group of rats is transferred to the self-administration (SA) chambers during the 3 hour test sessions (Non Resident rats) and a second group literally lives in the SA chambers (Resident rats). Using this model we have seen that the setting modulates heroin self-administration (Caprioli et al. 2008), the choice between heroin and cocaine (Caprioli et al., 2009), the relapse (Montanari et al., 2015), as well as the internal state of the rats (Avvisati et al., 2016). Also the neurobiological effects of heroin are a function of the setting as previously demonstrated (Paolone et al. 2007; Celentano et al. 2009) using in situ hybridization and immunohistochemistry experiments.

Thus, in the present study we have hypothesized the setting may influence also the synaptic plasticity in the cortico-accumbens circuit, areas involved in the impaired ability of addicts to regulate drug seeking, taking and relapse (Jentsch and Taylor, 1999; Luscher and Malenka, 2011; Nestler 2001; Kalivas and Volkow, 2011; Kalivas and O'Brien, 2008; Kalivas et al., 2004; Self et al., 2004). We trained independent groups of rats self administering heroin (25μg/kg/infusion) and saline for 10 sessions (3 hour each). After 14 days of abstinence rats underwent a cue induced reinstatement test. We also exposed single housed rats (IH) to Residence and Non Residence condition and we compared these rats with grouped house rats (GH). Using ex vivo field recordings on parasagittal slice we found that Heroin SA Resident rats showed an impairment in the capability to induce LTP (fEPSP amplitude 140% of baseline) when compared to Heroin SA Non Resident rats (fEPSP amplitude about 160% of baseline). Moreover saline SA, as well, single housed rats showed

an impairment in the capability to induce LTP (fEPSP amplitude about 120% of baseline) when compared with grouped house rats (fEPS amplitude about 160%).

This suggests that 1) cortico-accumbens synaptic plasticity is impaired in isolated rats and 2) heroin produces a recovering in the LTP response in both Non Resident and Resident rats even if Resident rats showed a non complete LTP recovering maybe due to the more rewarding effect of the drug in this environment.

Further elucidations on how drugs of abuse alter cortico-accumbens plasticity will be necessary for the development of new therapies especially studies based on the use of heroin administration since most of the articles in literature have used cocaine.

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1 DRUG ADDICTION

"Drug addiction" or "Substance use disorder" is defined as a chronic, relapsing brain disease characterized by compulsive drug seeking and use, despite harmful consequences (APA, 2000). In the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (APA, 2013) the categories of "Substance abuse" and "Substance dependence" (used in the DSM-4) were replaced with "Substance use disorder". The pathology is measured on a continuum from "mild" to "severe" clinically significant impairment or distress, as reported by the presence of at least two of the following criteria, occurring within a 12-month period:

1. The substance is often taken in larger amounts or over a longer period than it was intended.
2. There is a persistent desire or unsuccessful effort to cut down or control the use of the substance.
3. A great deal of time is spent in activities necessary to obtain the substance, use the substance, or recover from its effects.
4. Craving, or a strong desire or urge to use the substance.
5. Recurrent use of the substance resulting in a failure to fulfil major role obligations at work, school, or home.
6. Continued use of the substance despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of its use.
7. Important social, occupational, or recreational activities are given up or reduced because of the use of the substance.
8. Recurrent use of the substance in situations in which it is physically hazardous.

9. Use of the substance 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.

10. Tolerance, as defined by either of the following:

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

    - A markedly diminished effect with continued use of the same amount of the substance.

11. Withdrawal, as manifested by either of the following:

    - The characteristic withdrawal syndrome for that substance (as specified in the DSM- 5 for each substance).

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

1.1 FROM THE RECREATIONAL DRUG USE TO THE PATHOLOGY

It is useful to compartmentalize the drug taking behaviours into different stages during the progression of the pathology. During these stages different regions of the brain are taken into account. Before getting inside the deep consideration of each stages of the pathology it is extremely important to consider that the majority of the populations that have tried at list one potential drug of abuse once in their lifetime do not develop the pathology and they continue to have a controlled use of the substance. The pathology pops out in 17% of the population (Piazza and Deroche-Gamonet, 2013). In these subjects the genetic and environmental factors, plus obviously the drug, combine (Kreek et al., 2002) modulating the individual propensity to use drugs, passing from a controlled to a compulsive taking. Therefore, the use

of the substance alone is a necessary but not sufficient condition for the diagnosis of the pathology.

1.2 First experiences and the acute effect of the drugs on the reward system

The key component of the reward system is the mesolimbic pathway, originating with dopaminergic cell bodies in the ventral tegmental area (VTA), located in the ventral portion (tegmentum) of the midbrain. These dopaminergic axons project to the nucleus accumbens (NAc), the ventral compartment of the striatum, and extend also into the amygdala, bed nucleus of stria terminalis (BNST), lateral septal area, and lateral hypothalamus. The VTA is close to another dopamine rich area: the substantia nigra. Whereas the substantia nigra projects primarily to the dorsal striatum (via the mesostriatal pathway) and mediates motor activity, the mesolimbic pathway mediates reward (Gardner, 2000).

The acute effect of all addictive drugs (despite their own behavioural effect and pharmacological profile), as well as the natural reward (food, sex, etc.), is the enhancing of extracellular dopamine levels in the reward system and in particular from neuron in the VTA to NAc (Di Chiara and Imperato, 1988) with direct (psychostimulants) or indirect mechanisms (see Heroin).

1.3 From Intermittent to the regular drug use

Chronic and extended expositions to the addicted drugs could cause an alteration in the mesocorticolimbic circuit altering the capability to respond to the natural rewarding and bringing in some subjects to the pathology. As we have told before this alteration is present in a small amount of subjects. Is there something in the brain of these subjects different from the subjects that continue to have a regular use of the drugs?

The progression from initial drug use to addiction may evolve as repeated activation of the mesolimbic pathway associated with persistent drug use heightens the incentive value, or salience, of the drug. As contextual cues become associated and strengthened with repetitive drug administration, a process initially under the purview of the mesolimbic dopaminergic (DA) system progressively incorporates the neurocircuitry involved in emotional memory, obsessive thoughts, stress response, decision making, and behavioural inhibition into the drug experience. These neuroadaptive responses remain engaged even in the absence of ongoing drug use and are thought to be a primary factor in drug relapse

1.4 REPETITIVE DRUG USE AND THE ONSET OF THE PATHOLOGY

Repetitive drug assumptions bring changes in particular brain structures involved in learning and adaptation to important environmental stimuli, whether how to approach rewards, or to avoid dangerous situations (Kelley, 2004; Everitt and Robbins, 2005). These neuroadaptations are long lasting and they persist for a long period and also when the drug is not present in the body anymore. This concept is well summarized in one of the best known slogan of Alcoholic Anonymous: "*Once an alcoholic, always an alcoholic*". This slogan could sound extremely negative but simplifies the concept that even if people haven't used drugs for many years, particular brain regions have changed and are no longer reversible.

One network in which this long lasting neuroadaptations may develop is the Prelimbic (PL)-NAc core. Changes in NAc glutamatergic transmission are thought to encode the transition from occasional use of drugs to the pathological inability to control drug-seeking behaviour (Kalivas and Volkow, 2011; Kasanetz et al., 2010; Peters et al., 2009; Wolf and Ferrario, 2010).

2 "ENVIRONMENT VARIABLES" IN DRUG ADDICTION

Drug addiction is a multifactorial psychiatric disorder in which genetic and environmental factors combine each other modulating the individual propensity to use drugs (Anthony and Chen, 2004).

Environment is a variegate concept including life experiences (both negative and positive) such as social/family interaction, child care or abuse, conditioned stimuli and the psychological value of the setting in which drugs are administered (for a review see Caprioli et al., 2007a). All these variables can affect the propensity to take drug addiction.

2.1 THE SETTING OF DRUG TAKING

To understand the influence of the environment immediately surrounding drug taking, in Professor Badiani's laboratory we have developed an animal model (Caprioli et al., 2007a) in which one group of rats are transferred to the self-administration (SA) chambers during the 3 hour test sessions (Non Resident rats) and a second group literally lives in the SA chambers (Resident rats) (for a further description see Material and Method). Thus, the physical characteristics of the environment for the two groups of rats are the same, meanwhile the psychological value is completely different. The drug SA will take place in a known setting for the Resident Rats; whereas it will be an arousing environment for the Non Resident rats considering the natural arousal peculiar of the rats to examine a non-home environment.

In our opinion, by conducting this banal differentiation between the two settings, we can replicate the human propensity to use different kinds of drugs in different situations. From a translational point of view, retrospective studies in human addicts have shown that certain settings are associated with specific drugs. When heroin and cocaine co-abusers were asked about the circumstances of drug taking, they indicated distinct settings for the two drugs:

heroin being used preferentially at home and cocaine preferentially outside the home (Caprioli et al, 2009; Badiani and Spagnolo, 2013), despite the administration route.

In this way the setting of drug taking acts as an ecological background against which the effects of drugs are appraised (Badiani, 2013): if there is an incompatibility between the drug effects and the central processes required from the environment, the rewarding effects of the drug would be reduced. According to this hypothesis, for example the central and peripheral arousal produced by cocaine would be considered as appropriate to arousing non-home settings but not to the home environment. In contrast, the central and peripheral sedation produced by heroin would be consistent with the safety of the home environment but not to potentially challenging non-home settings (Badiani, 2013).

Specifically, using this model we found that cocaine SA was greater in Non Resident rats than in Resident rats (Caprioli et al., 2007b) whereas the opposite was true for heroin (Caprioli et al., 2008). Also the motivation to self administer drug measured with a progressive ratio procedure is grater in Non Resident for cocaine and in Resident for heroin (Caprioli et al., 2008). During a choice paradigm most Non Resident rats preferred cocaine, whereas Resident rats tended to prefer heroin (Caprioli et al., 2009). Heroin and cocaine also differ in a context dependent manner in their ability to reinstate drug seeking. Heroin priming reinstates the heroin seeking behaviour more in Resident than in Non Resident rats whereas cocaine priming was much more effective in Non Resident than in the Resident rats (Montanari et al., 2015). Finally using ultrasonic vocalizations (USVs) we found that Non-Resident rats emitted more 50-kHz USVs (positive affective states marker) when they self administered cocaine than when self-administered heroin whereas Resident rats emitted more 50-kHz USVs when self-administering heroin than when self-administering cocaine (Avvisati et al., 2016).

Also administering alcohol, we have found a dissociation depending on the setting between Resident and Non Resident (Testa et al., 2011) (considering that at certain dose alcohol is more rewarding at home than outside home for its sedative effect). Finally ketamine shares with psychostimulant drugs the ability to produce (at low doses) tachycardia, increased blood pressure, hyperexcitability and agitation. Coherently with our model we found greater intake of ketamine in Resident than Non Resident rats (DeLuca and Badiani, 2011) and recently in human (DeLuca et al., 2012).

2.2 Social Isolation

The social isolation (SI) perpetuated during the post weaning (also called rearing deprivation) promotes the "isolation syndrome" (Fone and Porkess, 2008): animals start showing symptoms resembling depression and anxiety disorders. Post weaning SI induces changing in the hippocampal synaptic plasticity (Das et al., 2016), in the hippocampal neurogenesis (Cinini et al., 2015) in the VTA synaptic plasticity (Whitaker et al., 2013), in the structure and functioning of prefrontal cortex (PFC) (Biro et al., 2016; DayWilson et al., 2006) and in locomotor activity. It also altered monoamine levels in the brain (Heidbreder et al. 2000; Fone and Porkess 2008; Koike et al. 2009; Dalley et al., 2002). The effect of the isolation is highly dependent on the age of the exposure (Hall, 1998) and few studies exist investigating the cognitive function and no studies have been found on the neurobiological alterations of the adulthood SI a condition called Isolated Housing (IH) (Lu et al., 2003).This is a condition different from the most popular SI after weaning since the PFC maturation is almost completed. Using adult SI it was found that spatial learning (Arranz et al., 2009) working memory (Huang et al., 2011) and fear conditioning (Pibiri et al., 2008) are altered.

SI rats after weaning showed evidences of a higher propensity to self administering different addicted drugs or are more sensitivity to the reinforcing effects of drugs such as

amphetamine (Bardo et al., 2001), cocaine (Hofford et al., 2016) ethanol (Butler et al., 2016; Deehan et al., 2007; Whitaker et al., 2013) and morphine (Alexander et al., 1978) for an extended review see Lu et al., 2003.

Apparently there is just one study investigating the effect of SI after adolescence on cocaine and heroin SA (Bozarth et al., 1989). These authors found an effect of isolation on the rate of initiation of heroin (0.1 mg/ kg/infusion) SA. In the case of heroin SA, the higher intake in the isolated rats may reflect a decrease, rather than an increase, in the reinforcing effects of the drug (Lu et al., 2003).

3 HEROIN

Opioids comprise endogenous and synthetic molecules that bind and activate one of the opioid receptors. Opiates, a subclass of opioids, are derived from the *Papaver somniferum* plant. This subclass includes morphine and codeine, two major metabolites of heroin. Heroin (diacetylmorphine) was first synthesized in 1874 by two English chemists Beckett and Alder Wright. Nevertheless, real interest in the drug began after its subsequent analysis at the Bayer laboratories in Germany. The drug was commercialized in 1898 as an alternative to morphine. Incredibly, the addictive properties of heroin were not recognized until 1910.

At physiological level heroin reduces the brain's responsiveness to changes in carbon dioxide levels and hypoxia, resulting in respiratory depression. It also reduces peripheral vascular resistance (mild hypotension), causing mild vasodilation of the cutaneous blood vessels (flushing), and stimulates histamine release (pruritus). Heroin decreases gastric motility, inhibits the effect of acetylcholine on the small intestine, with consequent constipation. The first experience with opiate can be unpleasant presenting vomiting and nausea but repeated injections produces a warm flushing of the skin and sensations described by users as a “rush” or a sensation of intense pleasure, that begins within 1 minute of the injection and lasts from 1 minute to a few minutes. This rush is followed by a period of sedation that lasts about an hour. The initial rush is likely due to heroin's high lipid solubility and rapid penetration to the brain. The half-life of heroin is 15-30 minutes. Heroin is rapidly converted to 6-monoacetylmorphine and then converted to morphine. Morphine is metabolized by the liver and excreted as a glucuronide product or in its free form by the kidneys.

3.1 NEUROBIOLOGY OF HEROIN

Heroin acts on the endogenous opioid system, a system involved in pain and mood regulation. There are three main families of opioid receptors: μ (MOR), δ and κ receptors. They were discovered in the early 70s by different research groups (Simon et al., 1973; Pert 1973). The endogenous ligands that bind to these receptors are: *enkephalins, dynorphins* and *endorphins*. In general, stimulation of the μ receptors results in analgesia, euphoria, central nervous system depression, respiratory depression. Although none of the endogenous opioids is highly selective for a particular opioid receptor subtype, β-endorphin, enkephalins, and dynorphins have the highest affinity for μ-, δ-, and κ opioid receptors, respectively (Goldstein and Naidu, 1989). The different classes of endogenous opioids have divergent functions, probably related to their differential affinity profiles for the various opioid receptors (Goldstein and Naidu, 1989).

Acute opiates administration indirectly activates DA neurons in the VTA increasing the DA release in the NAc (Di Chiara and Imperato, 1988) through inhibition of GABAergic interneurons (Johnson et al. 1992), activating MOR. MORs are essential for the opioid effect (Matthes et al. 1996). MORs belong to the superfamily of G-protein coupled receptors and in particular to inhibitory $G_{\alpha i}/G_{\alpha o}$. The most relevant neuroanatomical substrates for the opiate dependence and withdrawal are the reciprocal connections within the limbic subcircuit of cortico-striatal circuitry: GABAergic neurons of the NAc, DA neurons of the VTA, and glutamatergic neurons of the PFC (Wise, 1989). MORs are localized postsynaptically on dendrites and cell bodies where they regulate neuronal excitability and transduce receptor activation to down-stream signal transduction pathways, and they can also be localized presynaptically on axon terminals where they inhibit neurotransmitter release via activation of K+ conductance and/or inhibition of Ca2+ conductance (Williams et al., 2001). MORs are

found on glutamatergic and GABAergic terminals and postsynaptically on D1 receptor expressing MSN.

In the following paragraph I will describe how the activation of MOR modulates glutamatergic neurotransmission (for a review see Chartoff and Connery, 2014).

- The acute administration of an opioid in a naïve subject activates MOR and increased extracellular DA in the NAc leading to the suppression of GABA and glutamate release (via inhibition of calcium (Ca^{++}) and activation of potassium (K) conductance and inhibition of cyclic adenosine monophosphate (cAMP). Postsynaptic *N*-methyl-D-aspartate receptor (NMDAR) currents are augmented via MOR-Protein kinase C (PKC) activation. There is no known data on the acute, immediate effects of opioids on α- amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid (AMPAR) in naïve animals.
- During chronic opioids use, the MOR activation leads to a compensatory increase in AC activity so cAMP levels return to normal. Moreover during chronic opioid treatment the inhibitory effect of presynaptic metabotropic glutamate receptor group 2 and 3 (mGluR2/3) to inhibit glutamate release is increased. Surface expression of GluR1 subunits is decreased on medium spiny neurons (MSNs), with no change in total AMPAR subunit expression. Levels and/or function of the NR2A NMDAR subunit are increased, which may contribute to a decreased sensitivity to PKC-mediated NMDAR activation.
- During opioid withdrawal cAMP level increased, extracellular glutamate levels increased, but synaptic transmission may be reduced, enhancing mGluR2/3 autoreceptor function. GABA release is potentiated via augmented cAMP and protein kinase A (PKA). NR2B surface expression is increased, increasing the silent synapses devoid of AMPAR.

4 SYNAPTIC PLASTICITY

Synaptic plasticity is the modification of the strength of the synaptic transmission to preexisting synapses. It has been proposed to play a central role in the brain capability to incorporate experiences into memory (Kauer and Malenka, 2007). Synaptic plasticity plays a key role in the development of neural circuitry in different neuropsychiatric disorders.

Santiago Ramon Y Cajal proposed a theory according to which information would be stored as anatomical changes in the connections between the neurons (Yuste and Bonhoeffer, 2001). Donald Hebb, 50 years later, proposed the "Hebbian learning" theory: associative memories are formed in the brain by a process of synaptic modification that strengthens connections when presynaptic activity correlates with postsynaptic firing (Hebb, 1949). Experimental support arrived when Bliss and Lomo (1973) described this phenomenon in the glutamatergic synapses of the hippocampus. Nowadays, it is clear that synaptic transmission can be enhanced or depressed by modifying the number of neurotransmitters released, the number of postsynaptic receptors and the reuptake of neurotransmitters. These changes could be sustained from milliseconds to hours, days and longer. It has been described an early and a late phase (Nguyen et al., 1994).

Excitatory synapses express a number of different forms of synaptic plasticity, although it is now clear that it is present also on inhibitory synapses. This thesis will focus on glutamatergic synapses. Glutamate is the most important excitatory neurotransmitter in the brain. There are two main receptor categories: mGluR and ionotropic. The mGluR can be grouped in three main families (group I: mGluR1, mGluR5; group II: mGluR2, mGluR3; group III: mGluR4, mGluR6, mGluR7, mGluR8). They modify neuronal excitability through G protein subunits acting on membrane ion channels and second messengers such as cAMP. Three ionotropic receptor ligand gated channels named NMDA, AMPA and kainite have been discovered. These receptors are often colocalized on dendritic spines.

AMPAR are ionotropic glutamate receptors permeable to Na^+ and K^+ cations, and its activation provides the inward current that generates the excitatory synaptic response when the cell is at its resting membrane potential. They are considered the primary postsynaptic mediators of glutamate transmission in the NAc (Cherubini et al., 1988). Trafficking of AMPAR into or out of the synapses determines the level of excitatory synaptic strength and is a major mechanism of plasticity (Malinow and Malenka, 2002).

NMDAR exhibits a voltage dependence considering that it is blocked by extracellular magnesium at negative membrane potentials (Mayer et al, 1984). During depolarization magnesium is removed permitting the ions influx into the cell. It is established that long term potentiation (LTP) in the CA1 region requires activation of NMDARs during postsynaptic depolarization leading to the postsynaptic calcium influx, activating intracellular signalling cascades responsible for the changing in synaptic efficacy (Bliss and Lomo 1973; Luscher et al. 2000).

4.1 SHORT TERM SYNAPTIC PLASTICITY

Short-term synaptic plasticity was studied firstly in Aplysia (Kandel and Tauc, 1965). It is supposed to influence the information processing function of synapses, helping synapses to act as filters. For example, synapses with a low probability of release act as "high-pass filters" (they will facilitate during high-frequency action potential bursts while low-frequency bursts will not be transmitted or less transmitted). In contrast, synapses with a high initial probability of release act as "low-pass filters" (they will depress during high-frequency bursts but facilitate during low-frequency activity) (Abbott and Regehr, 2004).

In electrophysiology short-term synaptic plasticity is induced by the delivering two stimuli with a short time inter stimulus interval as in the Paired Pulse Protocol. Short bursts of activity causes changes in the probability of neurotransmitter release modifying the

biochemical processes of synaptic vesicles exocytosis. A main role in the regulation of this process is played by calcium.

4.1.1 Paired-Pulse Facilitation and Depression

Paired-pulse depression means a decrease in transmitter release and generally occurs in synapses that exhibit a high probability of release at the beginning. It can arise from different causes:

- a transient depletion of the vesicles release from the presynaptic terminal.
- the release of modulatory substances (from the activated presynaptic terminals, postsynaptic cells, or neighbouring cells) that leads to inhibition of the presynaptic release machinery.
- desensitization of ligand-gated receptors, making the target neuron less sensitive to neurotransmitter.

Whereas, *paired-pulse facilitation* is probably due to the residual calcium from the first action potential that contributes to the release during the second stimulation: Likely, additional mechanisms (activation of protein kinases that modulate the presynaptic phosphoproteins) are involved (Magleby and Zengel, 1982).

Both *paired-pulse facilitation* and *depressio*n depends on the recent history of activation of the synapse: synapses that begin with a high probability of release tend to depress their response to the second pulse however, synapses with a low probability of release exhibit an increase in response to the second stimulus.

4.2 Long Term Synaptic Plasticity

Another synaptic modification discovered to process and store information is the *long term synaptic plasticity*. It is well established that different forms of long-term synaptic

plasticity exist and moreover the synaptic strength could be modified amplifying (*Long Term Potentiation*) or reducing (*Long Term Depression*) the strength between synapses.

More recently other forms of synaptic plasticity have been identified including homeostatic plasticity (Turrigiano and Nelson, 2004) and metaplasticity (Abraham and Bear, 1996) but they fall outside the topic of this thesis.

LTP (the main topic of this thesis) consists of an increase in the synaptic strength elicited by brief high frequency stimulation of excitatory afferents. Using electrophysiology different forms of LTP have been identified. The most studied of these forms is LTP NMDAR dependent observed in the CA1 region of hippocampus. NMDARs dependent LTP is propagated following the rules of *associativity* (when a weak input is insufficient for the induction of LTP it can be activated in association with a simultaneous strong input), *input specificity* (once induced, LTP does not spread to other synapses), c*ooperativity*(LTP can be induced either by strong tetanic stimulation of a single pathway, or cooperatively via the weaker stimulation of many pathways. When several weak stimuli are applied to many pathways that converge on a single patch of postsynaptic membrane, the postsynaptic depolarization collectively depolarizes the postsynaptic cell enough to induce LTP), p*ersistence* (lasting from several minutes to many months).

Once Glutamate is presynaptically released, it binds NMDAR on the postsynaptic membrane and it causes the calcium influx. Calcium activates Ca^{++}/calmodulin-dependent protein kinase II (CaMKII) and several other different signal transduction molecules (PKA, Erk/MAPK pathway, Src kinase, PKC) causing the translation of the calcium signal into the long-lasting increase in synaptic strength (Bliss and Lomo 1973; Luscher et al., 2000). There was a great debate concerning the mechanism of the expression of LTP in the past. The main problem was to understand if LTP was expressed postsynaptically as a change of AMPAR

properties or presynaptically, as a change in the probability of transmitter release. This controversy has been resolved, and there is a general consensus on the mechanism of expression of LTP at hippocampal CA1 synapses. It involves an increase in the numbers of AMPARs on the postsynaptic density, driven through changes in AMPAR trafficking (Bredt and Nicoll, 2003; Derkach et al, 2007; Malenka and Nicoll, 1999). To solve the controversy it was useful the discover of silent synapse (Durand et al, 1996; Isaac et al, 1995; Malenka and Nicoll, 1997). Silent synapses are synapses that contain only NMDAR with few or no AMPAR. These synapses do not have postsynaptic activity in response to glutamate. The "un-silencing" process during LTP occurs with the incorporation of AMPARs into the postsynaptic membrane.

Moreover, recently AMPA trafficking has been studied. Recycling endosomes in the dendrites contain a reserve pool of AMPARs that are mobilized during LTP thanks to GTP-binding protein, named Rab11a. AMPARs are not inserted directly into the postsynaptic density but are exocytosed at perisynaptic sites. They then can diffuse in the plasma membrane and be trapped in the perisynaptic sites thanks to MAGUK (membrane-associated guanylate kinases) proteins. PSD-95 (one of MAGUK slot proteins) appears to be particularly important to control the number of AMPAR.

AMPARs do not directly bind to MAGUKs but do it with TARPs (transmembrane AMPAR regulatory proteins).

Much of the work on NMDAR dependent LTP has focused on the mechanisms responsible for its induction and maintenance. The so-called "late phase of LTP" (more than 1 hour after LTP induction) is commonly assumed to depend from the synthesis of different protein and by transcription in the nucleus (Zhou et al, 2006). The signalling to the nucleus required for long-lasting LTP depends on a number of protein kinases including PKA,

CaMKIV, and Erk-MAPK, which activate key transcription factors that may include cAMP response element-binding protein and immediate-early genes (c-Fos and Zif268/Egr-1) (Thomas and Huganir, 2004). Several mRNAs can be found in dendrites, including those of the AMPARs, CaMKII, Arc, and proteins that regulate receptor trafficking (Grooms et al, 2006).

One possibility for a long-term maintenance mechanism of LTP is the structural remodelling of potentiated synapses (Luscher et al, 2000). Spines can undergo rapid shape changes that are influenced by activity (Yuste and Bonhoeffer, 2001). It has been demonstrated that during LTP there are morphological changes such as growth of new dendritic spines, enlargement of spines, and splitting of spines (Abraham and Williams, 2003; Matsuzaki et al, 2004; Yuste and Bonhoeffer, 2001).

Although for over 30 years the spotlight was on NMDAR-dependent LTP, using electrophysiology, other forms of LTP and LTD have been identified in the brain. One of these is the presynaptic LTP. This LTP does not require neither NMDAR receptors activation nor postsynaptic factors, but seems to be due to high frequency tetanic stimulation, which causes a large, activity dependent increase in calcium concentration (Nicoll and Malenka, 1995; Nicoll and Schmitz, 2005; Zalutsky and Nicoll, 1990) within the presynaptic terminals. The cascade is responsible to the increase in glutamate release (Malenka and Bear, 2004; Nicoll and Schimtz, 2005).

Three different forms of long-term depression are identified in the mammalian brain: NMDAR-dependent LTD, metabotropic glutamate receptor-dependent LTD and endocannabinoid-mediated LTD but I will not discuss them in the thesis.

5 THE CORTICO-ACCUMBENS CIRCUITRY

The striatum can be divided in ventral and dorsal portions. The *dorsal striatum* is divided into the dorsomedial and the dorsolateral parts that regulate goal-directed and stimulus–response habitual movements, respectively (Redgrave et al., 2010; Yin and Knowlton, 2006). The majority of the neurons are MSNs, whose activity depends on excitatory inputs from cortical and limbic regions (Sesack and Grace, 2010). MSNs can be divided into two distinct populations: *direct pathway* expressing the D1 DA receptors and projects directly to the midbrain, and the *indirect pathway* which expresses the D2 DA receptors and projects to pallidal nuclei (Kreitzer and Malenka, 2008). These two main populations of MSNs are thought to exert opposing effects on behaviour (Stuber et al., 2011).

The *ventral* portion of the *striatum* also known as *NAc* receives glutamatergic afferents from different cortical and subcortical regions, including PFC (Berendse et al.,1992; Fuller et al., 1987; Stefanik et al., 2012; Stefanik et al. 2016), the basolateral amygdala (BLA) (Kelley et al., 1982), the ventral hippocampus (Britt et al., 2012) the thalamic nuclei (Berendse and Groenewegen, 1990), and the VTA (Gorelova et al., 2012). The NAc can be subdivided in core and shell compartment (Zahm and Brog, 1992).While both nucleus accumbens subregions (NAcore and NAcshell) receive input from all cortical regions, there is strong topographic bias. The NAcore surrounds the anterior commissure and receives glutamatergic input mainly from the PL and the BLA. It has been shown to be implicated in drug induced reinstatement of drug seeking (McFarland and Kalivas, 2001), drug-induced behavioral sensitization (Ferrario et al., 2005; Li et al., 2004) and drug-seeking behavior (Kalivas and Volkow, 2005). NAcshell receives strong glutamatergic input from infralimbic cortex (IL), the ventral hippocampus, VTA, thalamus nuclei and the BLA. The NAcshell plays a critical role in the reinforcing effects of drugs (Di Chiara et al., 2004).

The medial prefrontal cortex (mPFC) in the rat consists of four subdivisions which, from dorsal to ventral, are the medial agranular, the anterior cingulated, the PL, and the IL cortices (Ongur and Price, 2000). mPFC takes part in memory, attention, cognition and regulates behavioural inhibition. mPFC projections to NAc appear to primarily originate from PL (Beckstead et al.,1979).

In this thesis I focused on PL-to-NAcore pathway. This pathway is involved in the execution of drug seeking (Capriles et al., 2003; Kalivas et al., 2005; McFarland et al., 2004; McFarland and Kalivas, 2001; McLaughlin and See, 2003; Millan et al., 2011; Peters et al., 2009, 2008; Rocha and Kalivas, 2010; Stefanik et al., 2012; van den Oever et al., 2008); whereas, IL to NAcshell pathway is responsible for the extinction of drug-seeking behaviour (Peters et al., 2009), appearing to be involved in the learning of extinction rather than simply in the suppression of drug seeking behaviour (LaLumiere et al., 2010). However, studies examining the role of IL inactivation on reinstated drug seeking show inconsistent results, depending on the protocol used (Willcocks and McNally, 2013). Moreover, inactivation of the PL prevents reinstated drug seeking in various animal models, while inactivation of the IL increases cocaine seeking. Interestingly, it has been shown that without a period of extinction training, inactivation of the IL can inhibit drug-seeking behaviour (Koya et al., 2009). Opposite roles for the PL and the IL, such as controlling drug seeking, have been reported for extinction (Peters et al., 2009) as well as cue-induced cocaine seeking (McLaughlin and See, 2003).

5.1 CORTICO ACCUMBENS SYNAPTIC PLASTICITY AFTER HEROIN ADMINISTRATION

Not a lot is known about the mechanisms of long-term plasticity in the accumbens synapses. Recently, NMDAR-dependent LTP and LTD and endocannabinoid-mediated LTD

have been reported in this region (Thomas et al., 2003; Kombian and Malenka, 1994; Robbe et al., 2002).

Using electrophysiological experiments an impairment in the capability to induce LTD and LTP in the cortico-accumbens (core) pathway of rats withdrawn from the SA of cocaine have been found (Martin et al., 2006; Moussawi et al., 2009; Knackstedt et al., 2010); moreover Kasanetz et al. (2010) showed that permanently impaired LTD was found in rats animals that progressively develop the behavioural hallmarks of addiction, whereas LTD is progressively recovered in non addicted rats maintaining a controlled drug intake (Kasanetz et al., 2010).

If similar adaptations in glutamate transmission occur during heroin and cue induced reinstatement of heroin seeking is still unknown. Lalumier and Kalivas (2008) found that the recruitment of the glutamatergic projection from the PL to NAcore is necessary to initiate the reinstatement of heroin seeking. Moreover, it was showed that heroin relapse requires long-term potentiation (LTP)-like increases in synaptic strength in the PFC projection to the NAc. The increased synaptic strength was paralleled by dendritic spine enlargement in accumbens spiny neurons and required up-regulated surface expression of NMDA2b-containingreceptors (NR2B). Blocking NR2B before reinstating heroin-seeking also prevented the induction of LTP-like changes in spine remodeling and synaptic strength, and inhibited heroin relapse (Shen et al., 2011).

Recently, it was shown that LTP and LTD in the core of the NAc following in vivo stimulation of the PL PFC were impaired after heroin SA (Shen and Kalivas, 2013).

Shen et al. (2014) also showed that heroin SA produced long-lasting down regulation of glutamate uptake and surface expression of the transporter GLT-1. This down regulation was associated with spillover of synaptic glutamate to extrasynaptic NMDA receptors within

the NAcore. Ceftriaxone (increase expression of the GLT-1) restored glutamate uptake and prevented synaptic glutamate spillover and cue-induced heroin seeking.

Even if there is a huge literature showing the difference between opiate and psychostimulant (for an extended review see Badiani et al., 2011).there may exist a shared mechanism related to addiction that underpins the deficit in LTP and LTD after chronic use of addictive drugs. Taken together these studies with heroin and cocaine indicate that impaired LTP and LTD in the PFC to accumbens synapses may be a common neuropathology between addictive drugs. The future challenge is to understand if cocaine and heroin deficit in LTP and LTD are due to the same damage in the structure of dendrites and in the dendritic spines of the cells. For instance Robinson and Kolb (2004) demonstrated that rats exposed to cocaine showed increased dendritic branching and increased spine density in both NAc MSN and mPFC pyramidal neurons; by contrast, rats exposed to morphine had both reduced dendritic branching and reduced spine density in these brain regions.

6 EFFECT OF SOCIAL ISOLATION AND ENVIRONMENTAL MODULATION ON THE GLUTAMATERGIC CORTICO-ACCUMBENS SYNAPTIC PLASTICITY

6.1 INTRODUCTION

The setting surrounding drug seeking and taking exerts a powerful modulatory influence on drug reward, seeking, taking and on the likelihood of relapse (priming induced). Using an animal model of environmental modulation in which Resident rats live in SA chamber for all the time and Non Resident rats were transferred in the SA chamber during the three-hour daily session we found that SA is facilitated in Resident than in Non Resident rats (Caprioli et al., 2008). Enhanced motivation (measured by progressive ratio) for heroin SA was exhibited by Resident rather than Non Resident rats (Caprioli et al., 2008). Furthermore, when rats have the possibility to choose between heroin and cocaine during the same session, Resident rats tend to prefer heroin to cocaine (Caprioli et al., 2009). Finally, Resident rats are more vulnerable to relapse into heroin seeking than Non Resident rats after they self-administered cocaine and heroin on alternate days (Montanari et al., 2015). These modulatory effects of the setting can be observed also in human co-abusers. Addicts prefer taking heroin at home and cocaine outside home, regardless the administration route (Caprioli et al., 2009; Badiani and Spagnolo 2013).

Taking into account these data we suppose the setting of drug taking might provide an ecological background against which the central and peripheral effects of drugs are appraised (Badiani, 2013). In the presence of a "mismatch" between the setting and the internal state of the organism, the rewarding effects of the drug would be reduced. According to this hypothesis, the central and peripheral (parasympathomimetic) sedation produced by heroin would be consistent with the safety of the home environment (Resident, match condition) but not with the potentially challenging non-home setting (Non Resident, mismatch condition).

In the present work we wondered if the “match” or “mismatch” conditions affect the capability to induce synaptic plasticity in the cortico-accumbens circuitry after heroin SA and an incubation period. Surprisingly, we found that also the condition of the housing (grouped *vs* isolated) and not only the psychological value of the drug taking setting (Residence *vs* Non Residence) has a powerful impact on the synaptic plasticity in the cortico-accumbens circuitry.

The cortico-accumbens circuitry is known to be implicated in the impaired ability of addicts to regulate drug seeking, taking and relapse behaviours (Jentsch and Taylor, 1999; Luscher and Malenka, 2011; Nestler 2001; Kalivas and Volkow, 2011; Kalivas and O’Brien, 2008; Kalivas et al., 2004; Self et al., 2004) and it is supposed to be the neurobiological substrate in which long lasting neuroadaptations occur, after a drug addiction story, enhancing the likelihood to relapse in the drug seeking behaviour after a period of abstinence (Kalivas, 2009; Luscher and Malenka, 2011). Starting from 1998 when the glutamate homeostasis hypothesis was formulated (for a review Kalivas, 2009) a wide amount of works were conducted studying the dynamics of glutamatergic transmissions in the circuitry from the PFC to the NAc.

Human neuroimaging studies have shown a lower metabolic activity of the PFC in drug addicts compared with controls during the presentation of biologically relevant stimuli (Volkow et al., 2003; Garavan et al., 2000) but the exposition of addicts to environmental stimuli, previously associated with the drug experience, activates the PFC (Goldstein and Volkow, 2002). This activation is correlated with the intensity of reported desire to relapse (Childress et al., 1999; Kalivas and Volkow, 2005).

Similarly, animal models of relapse show an activation of the PL PFC glutamatergic projections into the NAcore. This activation is associated with cue-, stress-, or drug induced

reinstatement of drug seeking (Kalivas, 2009). Using measurements of extracellular glutamate in the NAcore, it was found that the reinstatement of drug seeking is associated with a marked increase in extracellular glutamate, largely deriving from PL PFC afferents (McFarland et al., 2003; LaLumiere and Kalivas, 2008; Gasset al., 2011; Gipson et al., 2013). Moreover Kasanetz et al. (2013) found that transition to addiction is specifically associated with an impairment of mGluR2/3-LTD in this circuitry and that there was a concomitant postsynaptic plasticity of basal synaptic strength.

6.2 MATERIALS AND METHODS

6.2.1 Animals

99 male Sprague Dawley rats (Ebri, Italy), weighing 250–275g at the beginning of the experiment were used in this study. However, we excluded one rat from the SA and electrophysiology analyses because its catheter was clogged, and 3 rats from electrophysiological analyses due to problems with the equipment.

Two months old rats were transferred from EBRI animal facility to a dedicated room with temperature and humidity controlled and 12h light/dark cycle (light of at 7:00 am). They were handled for one week. After one week of adaptation rats were randomly assigned to one of the following conditions:

- the Grouped House (GH) rats (n=19) were stabulated in group of 4 rats in standard transparent plastic cages;
- the Isolation Housing (IH) Resident group (n=8) were single housed in the SA chambers, where they remained for the entire duration of the experiment;
- Isolation Housing (IH) Non Resident rats (n=11) were single housed too in standard transparent plastic cages and transferred to the SA chambers for

three hour daily sessions (note these groups have not been submitted to the drug SA procedures but they have been exposed to the SA cages);

- The Saline SA Resident rats(n=10) were surgery implanted (see below) with a catheter and single housed in the SA chambers, where they remained for the entire duration of the experiment;
- Saline SA Non Resident rats (n=14) were surgery implanted and single housed in standard transparent plastic cages. They were transferred to the SA chambers for three hour daily SA sessions
- Heroin SA Resident rats (n=19) were surgery implanted with a catheter and single housed in the SA chambers, where they remained for the entire duration of the experiment;
- Heroin SA Non Resident rats (n=17) were surgery implanted and single housed in standard transparent plastic cages. They were transferred to the SA chambers for three hour daily SA sessions.

6.2.2 Surgery

After one week of acclimatization rats were anesthetized with Xylazine (Rompun) and Zoletil100 (Virbac) and a catheter was inserted and secured to the right jugular vein of the rats using standard surgical procedures previously described in detail (Caprioli et al. 2007b). The distal end of the catheter was externalized through a small incision at the nape of the neck and connected to a L shaped 22-gauge cannula that was secured to the rat's skull using dental cement and stainless steel screws. Catheters were 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).

6.2.3 Self-administration chambers and experimental context

The apparatus consisted of 8 SA chambers (28.5-cm length, 27-cm width, and 32-cm height) made of transparent plastic (front and rear walls), aluminium (side walls and ceiling), and stainless steel (grid floor). Plastic trays covered with pine wood shaving were placed under the cage 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, two sets of three cue lights (red, yellow, and green), positioned above each lever, and a counterbalanced arm holding a liquid swivel.

The SA chambers were in a sound- and light attenuating cubicles. Each chamber was connected via an electronic interface to a motorized syringe pump (Razel Scientific Instruments, St. Albans, VT, USA) and to a programmable logic controller (PLC; Allen Bradley, Milwaukee, WI, USA). Finally, the PLCs were connected to PCs running control software. Chambers, accessories, and electronic interfaces were purchased from ESATEL (Rome, Italy) and the software from Aries Sistemi (Rome, Italy). The infusion line consisted of a length of Silastic® tubing protected by a stainless steel spring and connected(through the liquid swivel and another length of Silastic® tubing) to a syringe positioned on the pump (which was programmed to work at an infusion rate of 13.3µl/s).

6.2.4 Self-administration and Reinstatement test procedures

GH rats and IH Resident rats were daily handled twice for one minute each time for ten days and then left undisturbed for 14 days until the electrophysiological recordings.

IH Non Resident rats were exposed for ten consecutively days to the SA chamber for three hours each day and then left for 14 days undisturbed in their home cages.

Rats with catheter implantation underwent the following training schedule.

Drug-training schedule: 10 days of 3-hour daily sessions, with FR (fixed ratio) progressively increased from 1 to 5 (days 1-4 FR1, days 5-6 FR2, days 7-10 FR5). During the test two levers were extracted, the active one triggered a heroin infusionof 25 μg/kg upon the completion of the task (counterbalanced right *vs* left) for Heroin Resident and Non Resident groups and a saline infusion for Saline Resident and Non Resident groups; while the pressing on the inactive lever reset the counter on the active *lever*. After the completion of the task a time-out of 40 sec began, during which both the levers were retracted and the cue light turned off. The infusion volume was 40 μl delivered over a period of 3 seconds. Rats which did not spontaneously self administered at least one infusion within 5, 65 and 125 minutes of the session were instructed by the experimenter to place their forepaws on the active lever. After training session a catheter patency test was performed consisting in the administration of Tiopental Sodium. Rats which did not become ataxic within 5 s after Tiopental Sodium administration were excluded from the statistic analysis.

Incubation period: After the training phase rats were left in their home cages undisturbed for 14 days.

Cue induced reinstatement test: Rats underwent 3 or 4 one hour sessions of extinction during which the active lever was exposed without the cue light previously associated. The completion of the task didn't cause any consequences. While the lever pressing behavior was extinguished one hour reinstatement session took place. The active lever was extracted and the cue light turned on. While the animal completed the task (FR5) the light turned off, the lever was retracted and a saline infusion was administered.

6.2.5 Catheter patency test

After the last SA session all rats underwent a catheter patency test in which a single intravenous bolus of Tiopental Sodium was administered. Rats did not became ataxic in 5 s after test were excluded from the statistics.

6.2.6 Electrophysiology procedures: Slice preparation and recording techniques

Slice collection: The day after the end of incubation period animals were anesthetized with halothane (Sigma-Aldrich) and decapitated. The brain was removed and 300 µm thick, parasagittal slices were collected using a Leica 1200 vibrating blame microtome. The cut chamber was filled with the cutting solution (in mM: KCl 2.5, NaH_2PO_4 1.25, $MgSO_4$ 10, $CaCl_2$ 0.5, Choline 120, $NaHCO_3$ 26, Glucose 10) continuously perfused with 95% O_2 – 5% CO_2at room temperature. The slices were then kept in artificial cerebrospinal fluid (aCSF) in a holding chamber for 1 hour at 35°C, then transferred to room temperature for the rest of the experimental day. After being placed into a recording chamber, slices were constantly perfused with aCSF (NaCl 126, KCl 1.25, NaH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, Glucose 10mM saturated with a 95% O_2 – 5% CO_2 mixture) at 32°C (3 ml/min).

Stimulation and recordings: Field excitatory post synaptic potentials (fEPSPs) were elicited placing a glass recording electrode filled with aCSF on the Nacore. PL cortical afferents were stimulated by a 25 µs current dispensed at 0.03 Hz (a sweep every 30 s) through a bipolar stainless steel microelectrode placed at the PL cortex-NAcore border. The $GABA_AR$ antagonist Picrotoxin (100 µM) was added to the perfusion aCSF. Signals were fed into a Multiclamp 700b amplifier (Axon Instruments), filtered at 1 kHz, converted by a Digidata 1322A (Axon Instruments) and analyzed with Clampex.

The stimulus intensity that evoked a fEPSP of 50-60 % of the maximal response (based on the input-output response curve) was chosen to acquire the baseline response (15 minutes).

After the baseline recording, 3(HFS) high frequency stimuli (100 Hz for 1 second each, with 6 seconds intervals) at the stimulation intensity yielding a maximal response were administered. fEPSPs were recorded for the following hour, bringing back the intensity to the baseline value.

Before the train administration and after the one hour recording a paired-pulse protocol was recorded. It consisted of two stimuli delivered within a 120 ms inter-stimulus interval for three times. The paired-pulse ratios (PPRs) were calculated as fEPSP2/fEPSP1 (3 pairs of sweeps average).

6.2.7 Data analysis and statistics

Using IBM SPSS 22 the SA lever pressing behavior and the reinforce datas were analyzed using a mixed ANOVA, with Session as within factors (10 levels), while Drug (2 levels: saline and heroin) and Setting (2 levels: Resident and Non Resident) as between factors. Bonferroni's post hoc analysis was applied when needed.

The Reinstatement test was analyzed taking into account the lever pressing behavior in the last hour of extinction and the one hour cue induced reinstatement test using a 3 way mixed ANOVA: Context (2 levels: Resident and Non Resident) and Substance (2 levels: heroin and saline) as between factors and Reinstatement test (2 levels: extinction and cue induced reinstatement test).

Mixed ANOVA was used to analyze electrophysiological experiments with LTP Time as a within factor (3 levels: the averages of the first, middle and last ten minutes after HFS administration); Context (2 levels: Resident, Non Resident) and Substance (2 levels: heroin, saline) as between factors; Bonferroni's post hoc analysis was applied when needed.

To analyze the difference in the housing condition it has been performed a first mixed ANOVA with LTP Time (3 levels: the averages of the first, middle and last ten minutes after

HFS administration) as within factor and the setting isolated Resident and isolated Non Resident Rats as between factor.

Since there is no difference between IH Resident and Non Resident rats we putted together the data from the two groups and we conducted a mixed ANOVA with LTP Time (3 levels: the averages of the first, middle and last ten minutes after HFS administration) as within factor and the housing condition (2 levels: IH vs GH) as between factor. Bonferroni's post hoc analysis was applied when needed.

Paired pulse data were analyzed using paired samples t-test.

A linear correlation analysis has been used to investigate the relation between the average of the amount of the heroin self administered during the training session and the average of the LTP.

6.3 RESULTS

6.3.1 Self administration

As shown in Fig.1 rats rapidly acquired heroin SA. The ANOVA conducted on Lever Pressing data (Fig. 1a) yielded significant main effects Substance [F(1;56)=7,280; p=0,009]; Sessions [F(9;504)=11,532; p=0.000]. There was also a significant effect of the interaction SubstanceXSessions [F(9;504)=6,704;p=0,000]. Post hoc analysis indicated significant differences between session number 1 and 10, 2 and 5,7,8,9,10; 3 and 5, 7,8,9,10; 4 and 7,8,9,10; 5 and 10; 6and 7, 10.

The ANOVA conducted on the Reinforce data (Fig. 1b), yielded significant main effects of Sessions [F(9;504)=2,495; p=0,009]; Substance [F(1;56)=5,818; p=0,019]. There were also a significant effects of the interactions: SessionsXSubstance [F(9;504)=3,924;p=0,000] and SessionsXSetting [F(9;504)=2,064; p=0,031]. A tendency was reported for the SubstanceXSetting interaction [F(1;56)=3,326;p=0,074]. The post hoc analysis revealed a significant differences between session number 1 and 5,7,8,9; 4 and 5,7,8,9; 5 and 1,4; 6 and 7,8,9; 9 and 10.

Figure 1

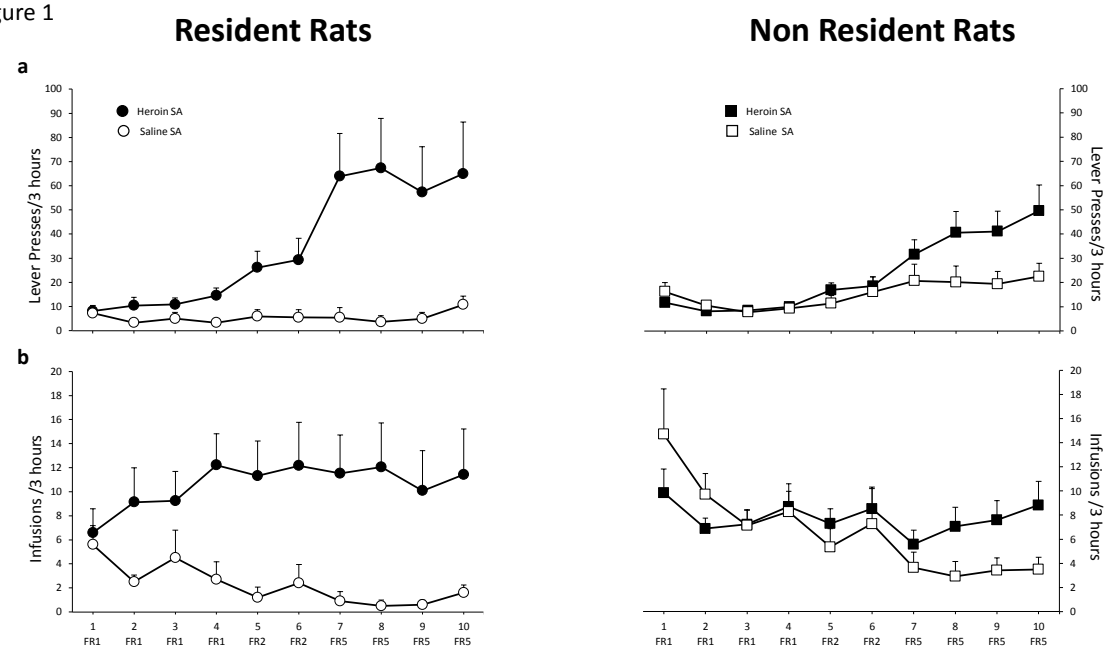

Figure 1 Mean (+SEM) number of lever presses (a) and infusions (b) for heroin (25 µg/kg) (full) and saline (empty) SA during the 10 3h sessions for Resident (dot) and Non Resident (square) independent groups.

6.3.2 Reinstatement

As shown in Fig.2 the lever pressing behaviour during the reinstatement session was greater than during the last hour of the extinction session. The ANOVA conducted on the lever pressing behaviour yielded significant main effects of Substance [F(1;56)=5,271; p=0,025]; Reinstatement test [F(1;56)=21,598;p=0,000]. There was a significant effect of the interaction Reinstatement testXSubstance [F(1,56)=12,859;p=0,001].

Figure 2

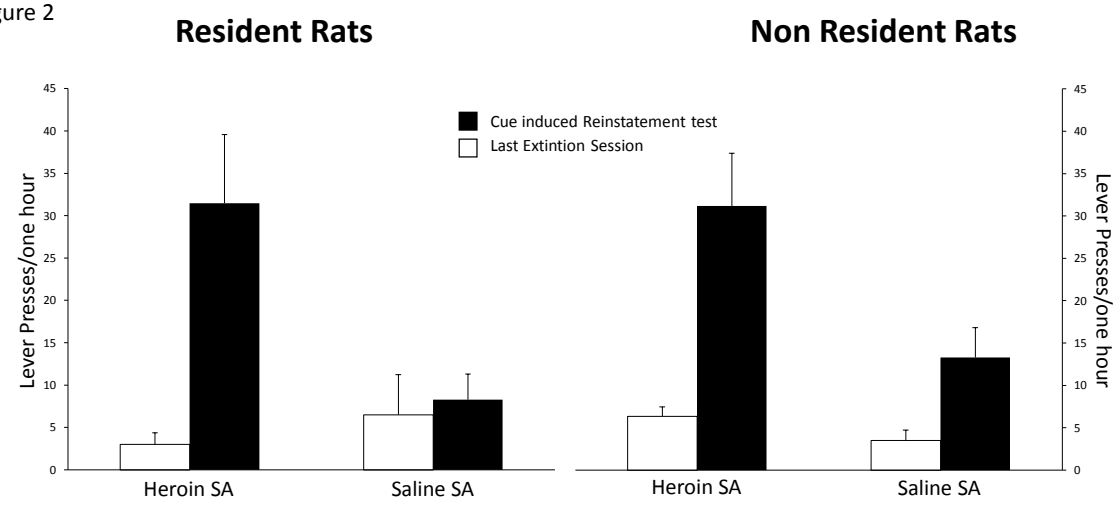

Figure 2 Mean (+SEM) number of lever presses during the first hour of the last extinction session (white bars) versus the cue induced reinstatement session (black bars) in the Resident and Non Resident groups.

6.3.3 Electrophysiology on heroin and saline SA rats

The ANOVA indicated a significant main effect of Substance [F(1;53)=9,459;p=0,003]; LTP [F(2;106)=29,98; p<0.000]. Post hoc analysis yielded a significant difference between the average of the first and the middle (p=0,000) and the average of the first ten minutes and the average of the last ten minutes (p=0,000), while there is no significant difference between the middle and the last ten minutes.

PPR

The analysis of the short-term plasticity measured by PPR (Fig. 3d) has shown no differences between the second synaptic response recorded one hour after the HFS administration (fEPSP2) and the one (fEPSP1) recorded before the administration of the train.

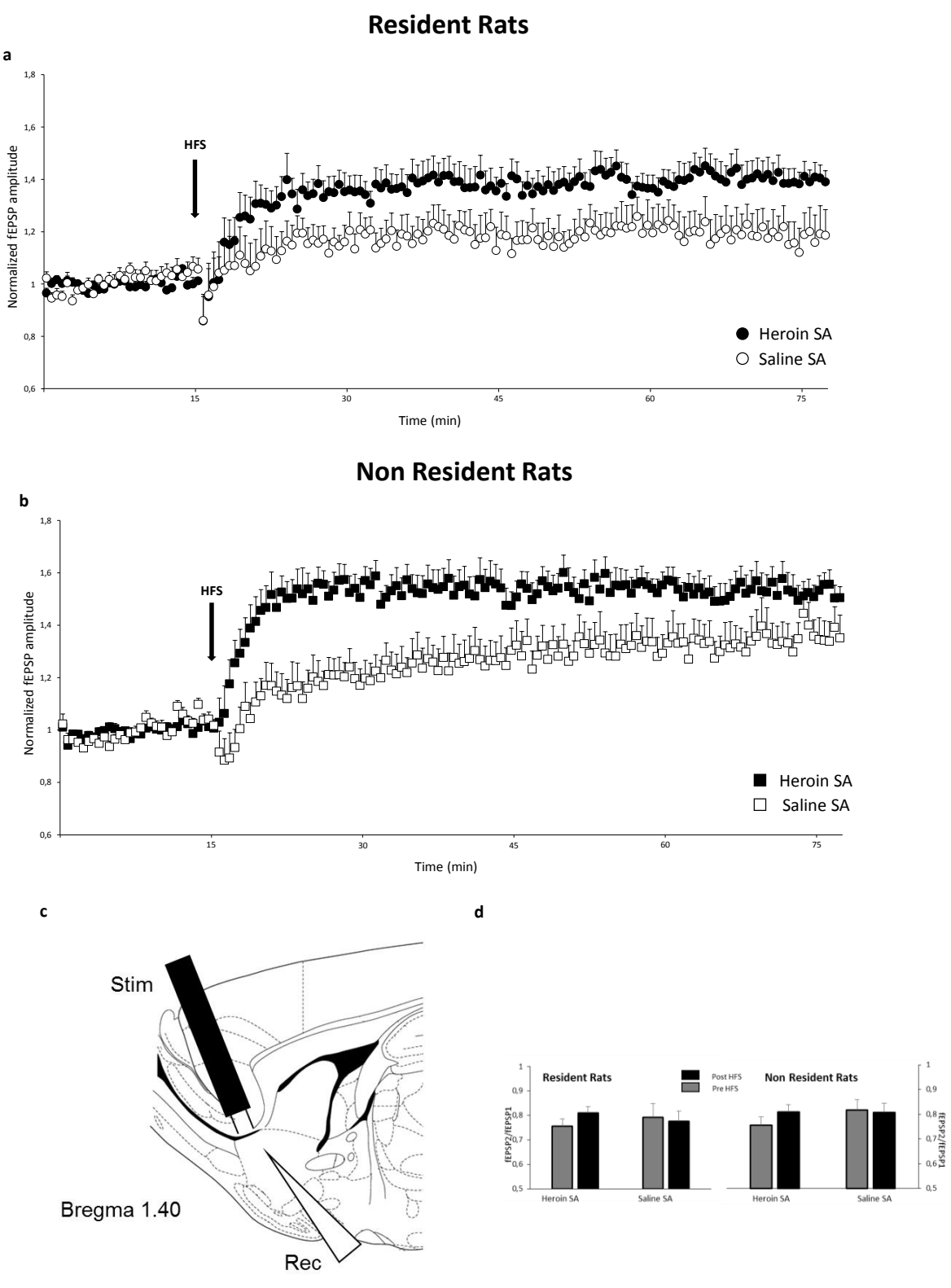

Figure 3 Resident (a) and Non Resident (b) LTP recorded in the NAcore after HFS applied on the PL PFC (c) in animals had previously self administered heroin (full) or saline (empty). No difference in the neurotransmitter release probability has been found using PPR (d).

6.3.4 Electrophysiology performed on GH and IH rats

As reported in Fig. 4a the mixed ANOVA (within factor: 3 levels LTP and between factor: setting IH Resident vs Non Resident rats) has shown a significant main effect of LTP [F(2;40)=5,883;p=0,006]. The post hoc analysis has shown significant differences between the average of the first ten minutes of the LTP and the average of the middle ten minutes of the LTP (p=0,062); as well as a significant difference between the average of the first ten minutes of the LTP and the average of the last (p=0,004) ten minutes of the LTP.

Since there is no difference between IH Resident and Non Resident rats, the electrophysiological data have been plotted together and we conducted a mixed ANOVA (within factor: 3 levels LTP and Housing condition as between factor: IH vs GH). The analysis showed a significant main effect of LTP [F(2,78)=12,603;p=0,000] and Housing [F(1;39)=29,240;p=0,000]. The post hoc analysis showed a significant difference between the average of the first ten minutes of LTP and the average of the 10 middle minutes (p=0,003); as well as between the average of the first ten minutes of LTP and the average of the last ten minutes (p=0,000).

The analysis of the short-term plasticity measured by PPR has shown no differences between the second synaptic response recorded one hour after the HFS administration (fEPSP2) and the one (fEPSP1) recorded before the administration of the train.

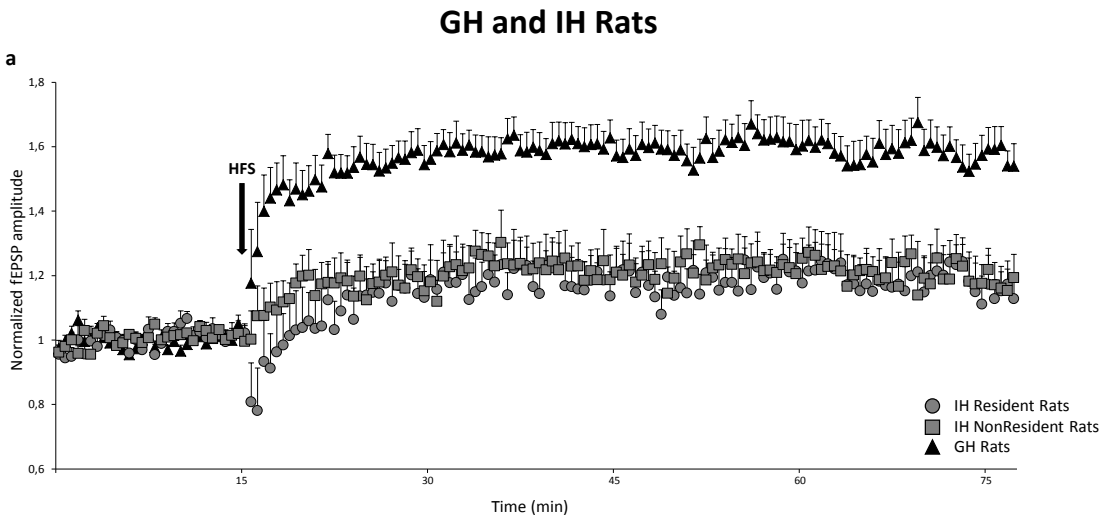

Figure 4 GH rats (black triangle) and IH (grey) Resident (dot) and Non Resident (square) LTP recorded in the NAcore after HFS applied on the PL PFC (Fig 3c).

6.3.5 Correlation analysis

There is no significant correlation (p=0,335) between the average of the amount of the injections during the SA and the average of the LTP as shown in Fig. 5.

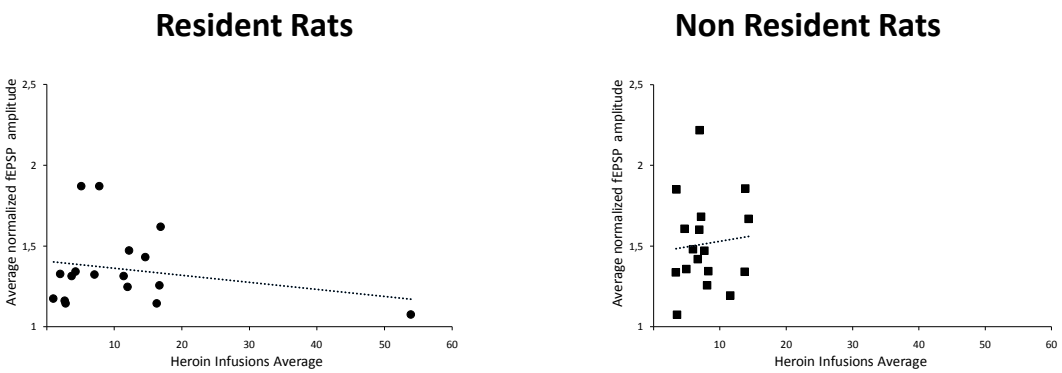

Figure 5 Correlational analysis between the average of the amount of heroin injections and the LTP average in Resident (dot) and Non Resident Rats (square).

6.4 DISCUSSION

The aim of the present study was to examine if the setting in which drugs are administered exerted a powerful influence on the capability to induce synaptic plasticity in the PL PFC-NAcore network (Fig. 3c) after heroin SA, 14 days of abstinence and a cue induced reinstatement test.

Independent groups of rats underwent ten sessions self administering heroin or saline. As we have previously reported (Caprioli et al., 2008) Resident rats showed a tendency to SA more heroin than Non Resident rats (Fig. 1).

After 14 days of incubation rats underwent a cue induced reinstatement test. Resident and Non Resident rats both reinstated the heroin seeking behaviour (Fig. 2). Non Resident rats self administering saline presented a spontaneous response in reinstatement session. Taking into account the difference between the rats that had previously administered saline and the ones which administered heroin, Resident rats presented a stronger response in the cue induced reinstatement test. Moreover, we previously reported (Montanari et al., 2015) Resident rats relapsed more than Non Resident rats in heroin drug seeking. In the present study we have not observed any differences in the Resident and Non Resident reinstatement test. This may be due to the different procedures used in the two studies. First of all in Montanari et al. 2015 we used a priming induced reinstatement test whereas in this study we used a cue induced reinstatement test; moreover in Montanari et al. 2015 rats self administered heroin and cocaine on alternate days while in this study rats only took heroin. Future studies will elucidate this difference.

The day after the reinstatement test ex vivo electrophysiology was performed on cortico-accumbens network (Fig. 3c). The data showed that Saline SA Resident and Non Resident rats have a reduction in the synaptic plasticity of the cortico-accumbens network

(fEPSP amplitude about 120% of baseline) if compared with heroin SA Resident (fEPSP amplitude about 140% of baseline) and Non Resident rats (fEPSP amplitude about 160% of baseline). We speculated that this reduction could be due to the housing condition so we performed cortico-accumbens ex vivo electrophysiology on naïve rats housed in group (GH) or isolated (IH). Isolated rats were also exposed to Resident or Non Resident conditions. We have found that there is no difference in the LTP induction in Resident and Non Resident IH (Fig. 4a) but there is a significant difference between IH (fEPSP amplitude about 120% of baseline) and GH (fEPSP amplitude about 160% of baseline) (Fig. 4).

LTP impairment (showed by IH) is completely recovered in Heroin SA Non Resident rats (fEPSP amplitude about 160% of baseline as GH rats), but not in Resident rats (fEPSP amplitude about 140% of baseline). We hypothesized that this could be due to the "match condition" between the setting and the internal state produced by heroin SA. The central and peripheral (parasympathomimetic) sedation produced by heroin would be consistent with the safety of the home environment (Resident condition) but not with the potentially challenging non-home setting (Non Resident, mismatch condition). The incapability to recover LTP plasticity found in the Resident group after heroin SA could be used as a useful biomarker taking into account that the cortico-accumbens circuitry should be the neurobiological substrate in which long lasting neuroadaptations occur, after a drug story, enhancing the likelihood to favour addiction in the vulnerable subjects (Nestler 2001; Kalivas et al., 2004; Self et al., 2004).

We also investigated the short term synaptic plasticity. The PPR analysis has shown no differences between the second synaptic response recorded one hour after the HFS administration (fEPSP2) and the one (fEPSP1) recorded before the administration of the train indicating that there is no difference in the neurotransmitter release probability (Fig. 3d and 4b).

Moreover, we have found no correlation between the average of the amount of heroin injections and the LTP average in Resident and Non Resident Rats (Fig. 5). Therefore the capability to induce LTP is not due to the mere amount of heroin self administered during the training.

In conclusion the cortico-accumbens network is the neurobiological substrate responsible of the incapability of addicts to regulate drug taking, seeking and relapse (Luscher and Malenka, 2011; Nestler 2001; Kalivas and Volkow, 2011; Kalivas and O'Brien, 2008). It has been hypotesized that this network is the neurobiological substrate in which long lasting neuroadaptations (carachterizing the drug addiction pathology) took place. These neuroadaptations could be the cause of the increased likelihood to relapse and the lost capability to control drug taking showed in addicts (Kalivas, 2009; Luscher and Malenka, 2011). The aim of the present study was to examine how self administering heroin in different setting (Resident versus Non Resident conditions) caused differences in this network. In particular we have shown the setting in which drug is administered can influence the capability to induce LTP regardless the amount of heroin taking. We hypotesized that the difference in the cability to induce synaptic plasticity in the cortico-accumbens circuitry is due to a "match condition" present in the Resident group in which the central and peripheral (parasympathomimetic) sedation produced by heroin would be consistent with the safety of the home environment (Resident condition) but not with the potentially challenging non-home setting (Non Resident, mismatch condition). In this way the presence of a "match" condition between the psychological value of the setting and the effects of the drugs enhanced the rewarding effects of the drug, the likelihood to favour the escalation through the addiction pathology in the vulnerable subjects and the premature presence of biomarker usually associated with the pathology

Surprisingly, we also found that social isolation (GH vs IH) has a powerful impact on the synaptic plasticity in the cortico-accumbens circuitry.

6.5 FUTURE DIRECTIONS

Future studies are necessary to demonstrate if the crystallized synaptic plasticity in the cortico-accumbens circuitry in the Resident group might be a predictive marker which appears in a premature phase yielding a faster escalation towards the pathology.

Further elucidations on how drugs of abuse alter PFC-NAcore plasticity will be necessary for the development of new therapies as well as to establish a causal role between this circuitry and the escalation through the pathology.

It will be useful to increase the number of heroin studies (Kalivas, 2009; Luscher and Malenka, 2011; Kasanetz et al.; 2013) considering that the majority of the studies on drug addiction presented in literature have been done on cocaine. Moreover, the study of the differences among the classes of addictive substances is necessary to shed light on the most appropriate pharmacological and psychological therapies (Badiani et al., 2011). Unitary models of drug addiction must be formulated and validated on the basis of empirical results from studies that include several classes of addictive drugs.

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8 LIST OF PUBLICATIONS

8.1 JOURNAL ARTICLES:

Stendardo E, Avvisati R, Meringolo M, Marinelli S, Badiani A. Synaptic plasticity alterations in cortico-accumbens circuit of heroin self-administered Resident and Non Resident rats.(In preparation).

Avvisati R, Meringolo M, **Stendardo E**, Malavasi E, Marinelli S, Badiani A. Intravenous self-administration of benzydamine in the rat: electrophysiological evidence for a central cannabinoidergic mechanism of action.(Addiction Biology Submitted).

Avvisati R, Contu L, **Stendardo E**, Michetti C, Montanari C, Scattoni ML, Badiani A. Ultrasonic vocalization in rats self-administering heroin and cocaine in different settings: evidence of substance-specific interactions between drug and setting. *Psychopharmacology (Berl)*. 233(8):1501-11. 2016.

Montanari C, **Stendardo E**, De Luca MT, Meringolo M, Contu L, Badiani A. Differential vulnerability to relapse into heroin versus cocaine-seeking as a function of setting. *Psychopharmacology*. 232(13):2415-24. 2015.

Addiction Biology SSA SOCIETY FOR THE STUDY OF ADDICTION

Intravenous self-administration of benzydamine, a non-steroidal anti-inflammatory drug with a central cannabinoidergic mechanism of action

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Keywords:	Benzydamine, NSAID, self-administration
Abstract:	Benzydamine (BZY) is a non-steroidal anti-inflammatory drug (NSAID) used for the topical treatment of inflammations of the oral and vaginal mucosa. Virtually nothing is known about the central pharmacological actions of BZY. Yet, there are reports of voluntary systemic overdosage of BZY in drug addicts, resulting in a euphoric, hallucinatory state. In the present study, we investigated the reward properties of BZY in a rat self-administration paradigm. We found that BZY has a powerful reinforcing effect and that this effect is greatly facilitated in animals that already had substance experience, having previously self-administered heroin and cocaine, indicating cross sensitization between BZY and other common drugs of abuse. We then assessed the effect of BZY on Prelimbic Cortex-to-Nucleus Accumbens (PLCx-NAcc) glutamatergic transmission, using field recordings in rat parasagittal brain slices. BZY dose-dependently reduced both field excitatory post synaptic potential (fEPSP) amplitude and paired pulse ratio, suggesting a presynaptic mechanism of action. Similarly to the in vivo paradigm, also the electrophysiological effects of BZY were potentiated in slices from animals that had undergone cocaine and heroin self-administration. Furthermore, BZY-induced LTD-like responses in the PLCx-NAcc circuitry, were significantly reduced in the presence of the CB1 receptor antagonist AM251. These findings provide firm evidence of the abuse liability of BZY and suggest a possible cannabinoidergic mechanism of action. Further research is needed in order to give insights into the molecular mechanism underlying BZY psychoactive and reinforcing effects,

	to better understand its abuse potential and possibly redefine the toxicological profile of this drug.

Intravenous self-administration of benzydamine, a non-steroidal anti-inflammatory drug with a central cannabinoidergic mechanism of action.

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ABSTRACT

Benzydamine (BZY) is a non-steroidal anti-inflammatory drug (NSAID) used for the topical treatment of inflammations of the oral and vaginal mucosa. Virtually nothing is known about the central pharmacological actions of BZY. Yet, there are reports of voluntary systemic overdosage of BZY in drug addicts, resulting in a euphoric, hallucinatory state. In the present study, we investigated the reward properties of BZY in a rat self-administration paradigm. We found that BZY has a powerful reinforcing effect and that this effect is greatly facilitated in animals that already had substance experience, having previously self-administered heroin and cocaine, indicating cross sensitization between BZY and other common drugs of abuse. We then assessed the effect of BZY on Prelimbic Cortex-to-Nucleus Accumbens (PLCx-NAcc) glutamatergic transmission, using field recordings in rat parasagittal brain slices. BZY dose-dependently reduced both field excitatory post synaptic potential (fEPSP) amplitude and paired pulse ratio, suggesting a presynaptic mechanism of action. Similarly to the *in vivo* paradigm, also the electrophysiological effects of BZY were potentiated in slices from animals that had undergone cocaine and heroin self-administration. Furthermore, BZY-induced LTD-like responses in the PLCx-NAcc circuitry, were significantly reduced in the presence of the CB1 receptor antagonist AM251. These findings provide firm evidence of the abuse liability of BZY and suggest a possible cannabinoidergic mechanism of action. Further research is needed in order to give insights into the molecular mechanism underlying BZY psychoactive and reinforcing effects, to better understand its abuse potential and possibly redefine the toxicological profile of this drug.

Keywords: Benzydamine, cannabinoid receptor type 1, drug abuse, fEPSP, NSAID, self-administration

INTRODUCTION

Benzydamine (BZY) is a non-steroidal anti-inflammatory drug that was synthetized by Angelini's laboratories in 1964 and made commercially available since 1966 for the symptomatic treatment of acute inflammatory states of the oral and vaginal mucosae. A number of pharmaceuticals preparations for topical use are available (e.g., TANTUM® Verde and GineTANTUM® in Italy, Difflam® in the UK, Tanflex® and Benzidan® in Turkey, Flogo-Rosa® in Brazil). Benzydamine is also contained in medications for systemic use (with concentrations of BZY ten times lower than those for topical use), as in the case of Benflogin® in Brazil, which, however, is no longer available on the market.

The mechanisms of action of BZY are complex. The anti-inflammatory effect mainly depends on the inhibition of prostaglandin synthesis, probably due to disruption of calcium mobilization/sequestration at the level the plasma membrane of inflammatory cells (Jeremy et al., 1991). Benzydamine also exhibits a less specific mechanism of action termed 'membrane stabilization', resulting in the inhibition of granule release by neutrophils and lysosomes (Quane et al., 1998). In addition to the anti-inflammatory/analgesic effect, BZY has local anesthetic effects (Sato and Maehara, 1967; Silvestrini et al., 1966a, 1966b).

Relatively little is known about the effects of BZY on the central nervous system. Symptoms such as dizziness, hallucinations, and anxiety have been reported as a consequence of unintended systemic ingestion (Gómez-López et al., 1999; Ballesteros et al., 2009; Settimi et al., 2012; Acar et al., 2014). Furthermore, there is evidence of recreational use of BZY in various countries, including Brazil, Italy, Poland, and Turkey (Nappo et al., 1993; Anand et al., 2007; Opaleye et al., 2009, 2011; Settimi et al., 2012; Balaban et al., 2013; Barwina et al., 2014). In Brazil, for example, BZY has been popular among drug-

experienced street youth since at least the early 1990s, probably due to its low price and ready availability (Nappo et al., 1993). In the following two decades its use spread to Brazil club scene, with the blossoming of theme rave parties (Benflogin® parties) and pop songs titled 'Benzydamine'. Informal self-reports, hosted by internet drug forums and social networks, detail the psychotropic effects of BZY and provide information about route of administration, dosage, and substance preparation from commercial preparations, as well as advice about other psychotropic substances to be assumed with BZY to enhance its pleasurable effects and dampen the undesired ones (Souza et al., 2008). Indeed, BZY seems to be particularly popular among poly-drug users (Anand et al., 2007; Opaleye et al., 2009). We have recently reported BZY use among the outpatients of an addiction clinic (Villa Maraini in Rome, Italy) who co-abused heroin and cocaine (Malavasi et al., 2012).

In the present study, in order to better understand BZY abuse potential, we investigated the ability of this drug to sustain intravenous self-administration in drug-naïve rats. To verify whether pre-exposure to addictive drugs would facilitate the reinforcing effects of BZY, we also investigated BZY self-administration in rats that had been previously trained to self-administer cocaine and heroin (to mimic the drug history of polydrug abusers). Finally, we investigated the electrophysiological effects of BZY on the PLCx-NAcc synapses, which are thought to play an important role in drug reward and drug seeking (Ikemoto and Panksepp, 1999; Kalivas and Volkow, 2005; Kalivas et al., 2009). This circuitry has been shown to undergo neuroplasticity changes following repeated exposure to addictive drugs, which has been implicated in the development of drug addiction (Van den Oever et al., 2012; Quintero 2013; Kalivas et al., 2009).

MATERIALS AND METHODS

Experiment 1: BZY self-administration

Animals and surgery: All the experimental procedures were carried out according the guidelines established by the European legislation (Directive 2010/63/EU) and the Italian legislation (L.D. 26/2014).

A total of 23 male Sprague-Dawley rats (Harlan Laboratories) weighting 250-300 g upon arrival were used. The rats were housed in pairs in transparent plastic cages (40 cm in length, 24.5 cm in width, and 18 cm in height) with stainless steel grid tops and flat bottoms covered with ground corncob bedding, in a temperature (21±1 °C) and humidity (70%) controlled room with a 14-h dark/10-h light cycle (lights off at 0700 hours). The rats were gently handled twice a week for two weeks before undergoing surgical catheterization, following procedures described previously in detail (Caprioli et al., 2007, 2008). On the day of surgery, the rats received an i.p. injection of 2.33 mg of xylazine hydrochloride (Rompun®, Bayer HealthCare) and 0.56 ml/kg of Zoletil 100® (Virbac, Carros, France), containing tiletamine (50 mg/ml) and zolazepam (50 mg/ml). The catheter consisted of 10.5 cm of silicone tubing (0.37-mm inner diameter and 0.94-mm outer diameter) sheathed at 3.4 cm from its proximal end by a 5-mm piece of heat-shrink tubing. The catheter was inserted into the right jugular vein and secured to the surrounding soft tissues with silk thread. Catheter distal end was externalized through a small incision at the nape of the neck and connected to an L-shaped 22-gauge cannula, which was secured to 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). The catheters were flushed daily (1800 hours) with 0.1 ml of sterile saline solution containing 0.4 mg of enrofloxacin and 25

IU heparin (Marvecs Services, Agrate Brianza, Italy). The rats had ad libitum access to food and water throughout the experiment, except during the self-administration sessions. The rats were allowed to recover from the surgery for 7-10 days and were then assigned to either the drug-naïve group (n=12) or the drug-experienced group (n=11) before the start of drug self-administration.

Apparatus: The apparatus consisted of self-administration cages (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 cage was equipped with two retractable levers, positioned on the left-hand wall 12.5 cm apart and 9 cm above the floor, two white cue lights, positioned above each lever, and a counterbalanced arm holding a liquid swivel. Cages and accessories were purchased from ESATEL S.r.l. (Rome, Italy). The self-administration cages were placed within sound- and light-attenuating cubicles. Each cage was connected via an electronic interface to a syringe pump (Razel Scientific Instruments, St. Albans, VT, USA) and to a programmable logic controller (PLC; Allen Bradley, Milwaukee, WI, USA). Finally, the PLCs were connected to PCs running control software developed by Aries Sistemi S.r.l. (Rome, Italy).

Self-administration procedures in the drug-naïve group: The rats in the drug-naïve group underwent fourteen 3-h daily sessions of BZY self-administration. Independent groups (n=4) self-administered one of three infusion doses of BZY (Sigma Aldrich): 250, 500, and 1000 µg/kg (dissolved in 40 µl of sterile saline solution). The sessions took place during the dark phase between 0900 and 1700 hours. During the session both levers were extended, but only one of them (counterbalanced across animals) was 'active' whereas pressing on the

other lever had no scheduled consequences except resetting the counter for the active lever. The number of consecutive presses (on the active lever) required to obtain a single drug infusion (fixed ratio, FR) was progressively increased during training: FR1 on sessions 1-7, FR2 on session 8, FR3 on session 9, FR4 on session 10, and finally FR5 on sessions 11-14. Once the appropriate FR had been reached, a drug infusion was delivered over a period of 3 s and at the same time both levers retracted for a time-out period of 40 s. A white cue light above the active lever was on throughout the session except during the time-out period. Rats that did not spontaneously self-administer at least one infusion within the first 5 min of the session were placed with their forepaws on the active lever, 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 5-60 and 60-120 min. On FR5 sessions (days 11-14) priming infusions were given only to rats that failed to self-administer at least one infusion within the 0-5 min period. These experimenter-induced lever presses were opportunely subtracted from the total. The rats were allowed to self-administer a maximum of 50 infusions within a single session.

Self-administration procedures in the drug-experienced group: The rats in the drug-experienced group were first trained to self-administer cocaine and heroin on alternate days for 14 consecutive sessions (following procedures previously described in detail by Caprioli et al., 2009 and Montanari et al., 2015) and then underwent 10 extinction sessions, during which lever pressing had no scheduled consequences. After a 5-day period of rest in their home cages the rats were trained to self-administer one of three infusion doses of BZY (n=3-4), as described above for drug-naïve rats.

Experiment 2: Slice electrophysiology

Animals: Acute brain slices were obtained from 18 drug-naïve and 5 drug-experienced male Sprague-Dawley rats of the same age of those used in the BZY self-administration experiment (P60-90 for drug-naïve rats and P80-100 for drug-experienced rats). Drug-experienced animals underwent alternate cocaine and heroin self-administration training as detailed in the previous experiment and were then given 5-7 days of rest in their home cage before being anesthetized for brain slice collection. Notice that the rats used in the electrophysiology experiments did not receive BZY self-administration training.

Slice collection: Animals were deeply anesthetized with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane, Sigma-Aldrich) and decapitated. The brain was rapidly removed and 300 µm thick, parasagittal slices were obtained using a Leica 1200T vibratome, and immersed in a cutting solution containing (in mM): KCl 2.5, NaH_2PO_4 1.25, $MgSO_4$ 10, $CaCl_2$ 0.5, Choline 120, $NaHCO_3$ 26, Glucose 10. The slices were then incubated in artificial cerebrospinal fluid (aCSF) in a holding chamber at 32°C for the first 60 min and at room temperature thereafter. Each slice was transferred to a recording chamber constantly perfused with aCSF (31-32°C, 3-4 ml/min) with the following composition (in mM): NaCl 126, KCl 1.25, NaH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, Glucose 10. All solutions were saturated with a 95% O_2 – 5% CO_2 gas mixture.

Stimulation and recordings: Field excitatory post synaptic potentials (fEPSPs) were recorded by placing a glass recording electrode, filled with aCSF, in the Core of the Nucleus Accumbens (NAcc-Core). Prelimbic cortical afferents were stimulated by a 25 µs square wave current delivered at 0.03 Hz (a sweep every 30 s) through a bipolar stainless steel microelectrode placed at the border between PLCx and NAcc. The $GABA_AR$ antagonist

picrotoxin (100 µM) was added to the perfusion aCSF due to presence of strong GABAergic inhibition in the NAcc-Core. Signals were fed into a Multiclamp 700b amplifier (Axon Instruments), filtered at 1 kHz, converted by a Digidata 1322A (Axon Instruments), acquired and analyzed using Clampex 9.2 software.

The stimulus intensity that evoked a fEPSP of 50-60% of the maximal response was chosen to acquire the baseline response (15 min) for all experiments. For short term plasticity experiments, a fEPSP of ~50% of the maximum was obtained and two stimuli were delivered with a 120 ms inter-stimulus interval (ISI). Paired Pulse Ratio (PPR) was calculated as fEPSP2/fEPSP1 (3 sweeps average) right before and after BZY bath-perfusion. For all experiments, BZY was added at the final concentration to the perfusion aCSF. Preliminar experiments showed that at the concentration of 30 µM BZY was effective in decreasing the PLCx-NAcc fEPSP by about 50% after 30 min of exposure, whereas at the concentration of 100 µM fEPSP was virtually abolished. Thus, whereas in the acute perfusion experiments the exposure time was 10 minutes (Fig. 3), in the long term plasticity study the exposure time to 100 µM BZY was reduced to 8 min (Fig. 5).

Data analysis and statistics

Data were analyzed using IBM SPSS 20 statistical software. *In vivo* data were analyzed using a 4-way mixed ANOVA with between-subject factors drug experience (drug naïve vs. drug-experienced) and dose (3 levels) and with repeated measures on the factors lever (active vs. inactive) and session (14 levels). Electrophysiological data were analyzed using ANOVAs with repeated measures on the factor time. Data from paired pulse experiments were analyzed using paired samples t-test.

RESULTS

BZY self-administration

Figure 1 illustrates the reinforcing effects of BZY on lever pressing as a function of session and drug experience. The acquisition of BZY self-administration was greatly facilitated in drug-experienced rats relative to drug naïve rats. A 4-way ANOVA indicated a significant main effect of lever [$F_{1,17}$=30.008; p<0.001], session [$F_{13,221}$=8.646; p=0.001], and drug experience [$F_{1,21}$=7.721; p=0.013]. There were also lever*session [$F_{13,221}$=10.511; p<0.001] and lever*drug experience [$F_{1,21}$=5.629; p=0.03] interactions. Furthermore, a 3-way ANOVA of the number of infusions yielded a main effect of session [$F_{13,221}$=2.672; p=0.014] and of drug experience [$F_{1,17}$=14.564; p=0.001], as well as session*drug experience [$F_{13,221}$=3.183; p=0.004] and session*training dose [$F_{26,221}$=2.119; p=0.016] interactions (data not shown).

Electrophysiological recordings

Bath applications of BZY at concentrations of 30 µM for 30 min or 100 ⍰M BZY for 10 min decreased PLCx-NAcc fEPSP amplitude by 36.1±6.8% (from 0.77±0.06 mV to 0.51±0.09 mV, n=7) and 64.8±5.7% (from 0.75±0.07 mV to 0.27±0.05 mV, n=7), respectively (Fig. 2 and 3). Repeated measures ANOVA run separately for 30 µM and 100 ⍰M experimental groups on the fEPSP amplitude from the time period 15-45 showed a significant effect of time for both the 30 µM [$F_{60,360}$=7.954; p<0.001] and the 100 ⍰M concentration[$F_{60,360}$=18.774; p<0.001]. Furthermore, 2-way repeated measures mixed ANOVA conducted on data from the time period 15-25 min yielded significant main effects of time [$F_{20,240}$=9.763; p<0.001], concentration [$F_{1,12}$=25.583; p<0.001], and a time*concentration interaction [$F_{20,240}$=5.253;

p<0.001]. To exclude that this severe depression in synaptic transmission could be due to non-specific toxic effects of BZY, pulses of higher current intensity were delivered at the end of each experiment. Under these experimental conditions the depressed fEPSPs recovered to the baseline amplitude values (data not shown).

We also performed short-term plasticity experiments by measuring PPRs to assess if BZY-mediated effects are due to alterations of neurotransmitter release probability. In control conditions, the second synaptic response (fEPSP2) was smaller than the first (fEPSP1) (Fig. 2d), a phenomenon termed paired pulse depression. Benzydamine perfusion (30 µM) further decreased PPR (from 0.85±0.05 baseline to 0.59±0.03 after 30 min perfusion; n=7; t_6=4.978; p=0.003), suggesting a presynaptic mechanism of drug action (Creager et al., 1980; Wu and Saggau, 1994). Due to the deep depression of the fEPSP induced by 100 µM BZY (Fig. 3), PPR data collected from these experiments were not included in the analyses.

The fEPSP depression induced by BZY was significantly enhanced in drug-experienced animals compared to naïve rats (Fig. 4). Two-way mixed ANOVA for repeated measures unveiled a significant main effect of experience [$F_{1,21}$=9.237; p=0.006] and experience*time interaction [$F_{59,1239}$=3.573; p=0.01], indicating sensitization of PLCx-NAcc synapses to acute effects of BZY perfusion, in drug-experienced rats.

As shown in figure 5a, bath application of 100 µM BZY for 8 min induced long term depression of fEPSP amplitude with a peak of -60.1±5.0% at min 25-35 (from 0.55±0.04 mV to 0.22±0.03 mV; n=9; t_8=9.984; p<0.001), indicating that BZY can cause long-term changes of PLCx-NAcc synaptic connectivity. Longer time of BZY perfusion often resulted in the complete suppression of the fEPSP (Fig. 3). The fEPSP amplitude did not fully recover during

the wash out (from 0.55±0.04 mV baseline to 0.36±0.03 mV at time 70-80 min; t_8=6.347; p<0.001) and was still reduced (-33.4±4.5%) at the end of recordings. This stable LTD-like response was consistently reduced by the co-application of the CB1 receptor antagonist AM251 ($F_{1,19}$=18.635; p<0.001; Fig. 5A). In the presence of AM251 2 µM, BZY-induced fEPSP depression was smaller than in the BZY group both at the depression peak (-35.8±5.7% vs. -60.1±5.0%; n=12; t_{19}=3.071; p=0.006) and at the end of recordings (-13.9±3.5% vs. -33.4±4.5%; t_{19}=3.420; p=0.003). Repeated measures mixed ANOVA on PPR data showed a significant time point*treatment interaction [$F_{2,40}$=5.548; p=0.007]. The reduction of PPR by BZY (from 0.85±0.03 baseline to 0.73±0.04 in the BZY group; t_8=2.664; p=0.012) was fully counteracted by AM251 co-perfusion (from 0.86±0.05 baseline to 0.93±0.07 in the BZY+AM251 group; t_{11}=1.577; p=0.143; Fig 5B). AM251 did not affect PPR *per se* (0.93±0.06, p=0.175 vs baseline PPR, data not shown). These results suggest that BZY requires the presence of available CB1 receptors to long-term and fully inhibit excitatory synaptic transmission at PLCx-NAcc-Core synapses.

DISCUSSION

We report here three major findings. First, we found that BZY, a non-steroidal anti-inflammatory drug, is self-administered intravenously by rats and that the reinforcing effects of BZY are greatly enhanced by a history of heroin and cocaine self-administration. Second, we found that BZY induces LTD-like plasticity at PLCx-NAcc synapses. Third, we found that the latter effect was significantly reduced by the CB1 receptor antagonist AM251, suggesting a cannabinoidergic mechanism of BZY action.

The finding that BZY is reinforcing in the rat sheds a light on the abuse liability of medications containing BZY. Abuse of BZY is particularly well documented in Brazil (Nappo et al., 1993; Opaleye et al., 2009, 2011) but there have also been case reports from other countries, including Italy (Malavasi et al., 2012; Settimi et al., 2012), Poland (Anand et al., 2007) and Turkey (Balaban et al., 2013). Most cases of voluntary systemic overdosage of BZY concerned individuals with a history of substance abuse. Our study demonstrates that BZY acts as a positive reinforcer in both naïve rats and in drug-experienced rats. Notably, pre-exposure to cocaine and heroin facilitated the acquisition of BZY self-administration at lower unit doses, which were not effective in naïve rats.

Our study also reveals short and long-term changes of the excitatory synaptic transmission in the Prelimbic Cortex to Nucleus Accumbens circuitry elicited by BZY. In particular, acute BZY reduced PLCx-NAcc glutamatergic neurotransmission via a presynaptic mechanism. Similarly to the in vivo drug effects, BZY-induced LTD was potentiated in rats that had previously self-administered cocaine and heroin. Interestingly, previous studies have shown that cocaine and d-amphetamine can also depressed excitatory neurotransmission at the same synapse via dopamine receptors type 1 (Nicola et al, 1996). Yet, BZY-mediated glutamatergic effects do not appear to be dependent on dopaminergic mechanisms since the selective D1 antagonist SCH23390 did not affect BZY-induced reduction of fEPSP (data not shown).

The LTD of glutamatergic transmission induced by BZY seems to rely at least partially on the presence of available CB1 receptors on the glutamatergic presynaptic terminals. Indeed, the absence of BZY-induced changes in the paired pulse ratio in slices perfused with the CB1R antagonist AM251, suggested that CB1Rs took part in the induction of this

pharmacological form of long term synaptic plasticity. The mechanism by which BZY interacts with CB1 receptors remains unclear. BZY might act either as a direct agonist onto CB1 receptors or by stimulating endocannabinoid synthesis and release, both of which in turn reduce glutamate release from the cortical terminal (Domenici et al., 2006). Notably, WIN 55,212,2 is able to induce persistent and long lasting fEPSP amplitude depression in the NAcc (Robbe et al., 2002). Nevertheless, the fact that AM251 dampens but not abolishes BZY-induced LTD response does not allow us to exclude other components of this LTD that may rely on other neurotransmitters or neuromodulators. As far as long term plasticity is concerned, a similar outcome was obtained for amphetamine which has been found to induce a CB1-dependent LTD in the rat amygdala (Huang et al., 2003). It is intriguing to compare the similar synaptic actions of amphetamine and BZY with their stimulant properties. As reported in many drug forums, BZY behaves as a stimulant other than a hallucinogen. While there is no evidence for animal self-administration of primary hallucinogenic substances like LSD, mescaline (Deneau et al., 1969) and DOM (Yanagita, 1986), several reports describe animal self-administration of amphetamine derivatives which have both hallucinogenic and psychostimulant effects like MDMA and similar molecules (Fantegrossi et al., 2002; Lamb and Griffiths, 1987; Schenk et al., 2003, 2007; Sannerud et al., 1996). Thus, drugs acting selectively on serotonin receptors do not seem to be reinforcing, whereas substances that are active on multiple monoamine systems sustain operant behavior in animals. Taken together, these observations strongly suggest that BZY has a mixed effect on the CNS, with the probable involvement of several neurotransmitter systems.

The effects of CB1R on BZY-induced synaptic plasticity provide a potential mechanism for the abuse potential of BZY. Interestingly, the chemical structure of BZY shares some features (like the presence of a benzoyl indole) with several CB1 synthetic agonists (i.e. JWH series compounds), which were shown to possess hallucinogenic proprieties as well (Forrester, 2012; Harris and Brown, 2013).

In conclusion, these data provide the first empirical evidence of BZY abuse liability and its effect on central nervous system, suggesting that it can interact with cannabinoidergic signaling in brain structures critically involved in drug reward. Our findings suggest that individuals with a history of substance abuse might be more prone to develop BZY abuse not only as a consequence of a general predisposition to experiment with psychoactive substances, but also because of a specific pharmacological cross-sensitization to its rewarding effects. This could be of particular relevance for the management of detention facilities or rehabilitation clinics, considering that an addict might consume BZY (o similar substances) as a substitute drug without even being noticed by supervisors, thus unpredictably compromising health conditions along with therapy efficacy. Further research is needed in order to better dissect BZY effects on glutamatergic PLCx-NAcc transmission, understand its abuse potential, and possibly redefine the toxicological profile of this drug.

Funding and Disclosure

Author contribution

The study was designed by AB, SM, MM, RA, EM, and ES. RA, MM, and ES conducted the experiments under the supervision of AB and SM. Data analysis was conducted by RA, AB, and SM. RA and AB drafted the manuscript, which was critically reviewed by SM. All authors approved final version for publication. This work was supported by a grant from Sapienza University of Rome (C26A12LN24) and from Strategic Development Funds (SA027-05) from the University of Sussex.

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Figure 1

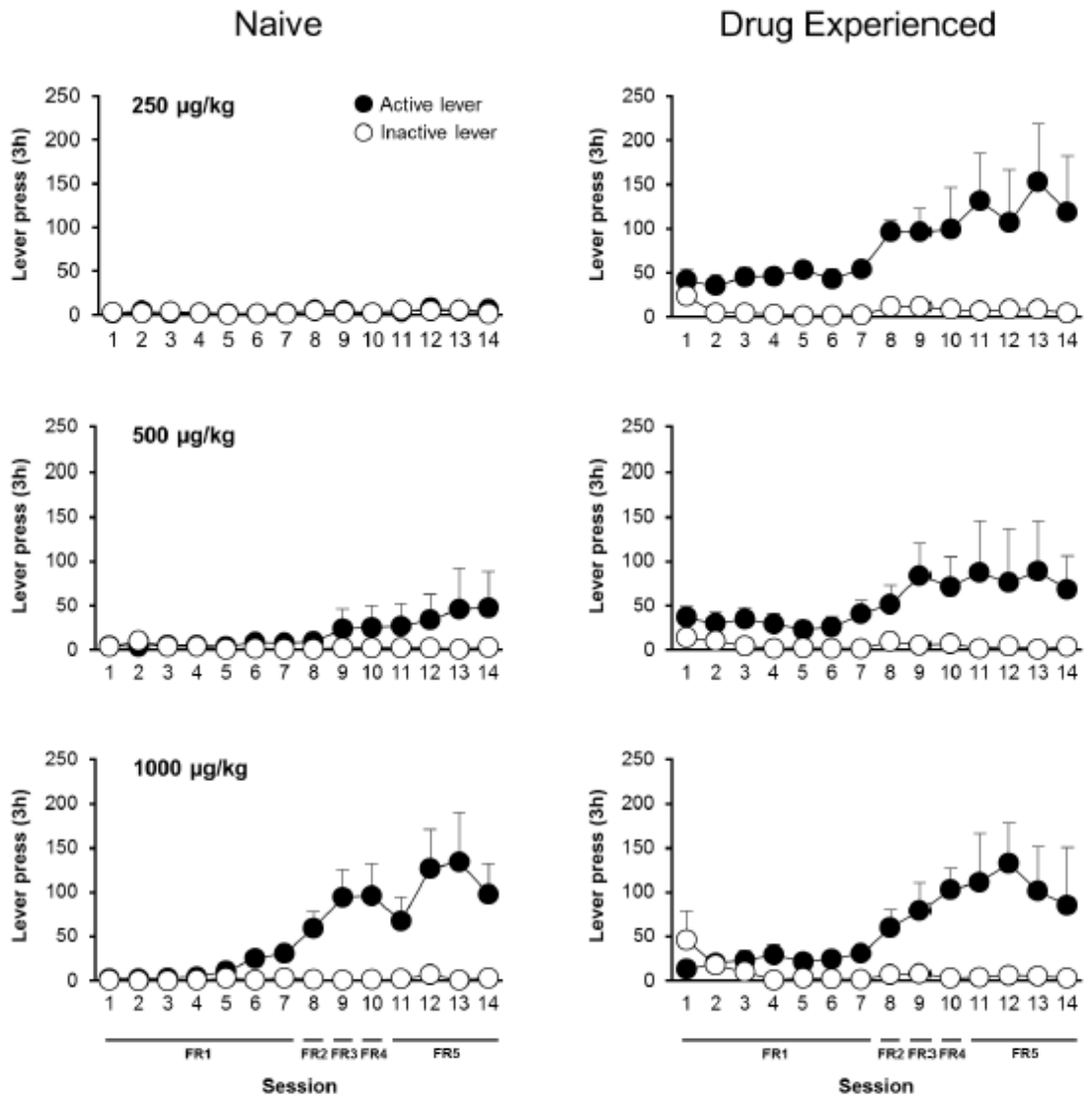

Naive
Drug Experienced
250 µg/kg
500 µg/kg
1000 µg/kg
Active lever
Inactive lever
Lever press (3h)
0
50
100
150
200
250
1 2 3 4 5 6 7 8 9 10 11 12 13 14
FR1
FR2 FR3 FR4
FR5
Session

Figure 2

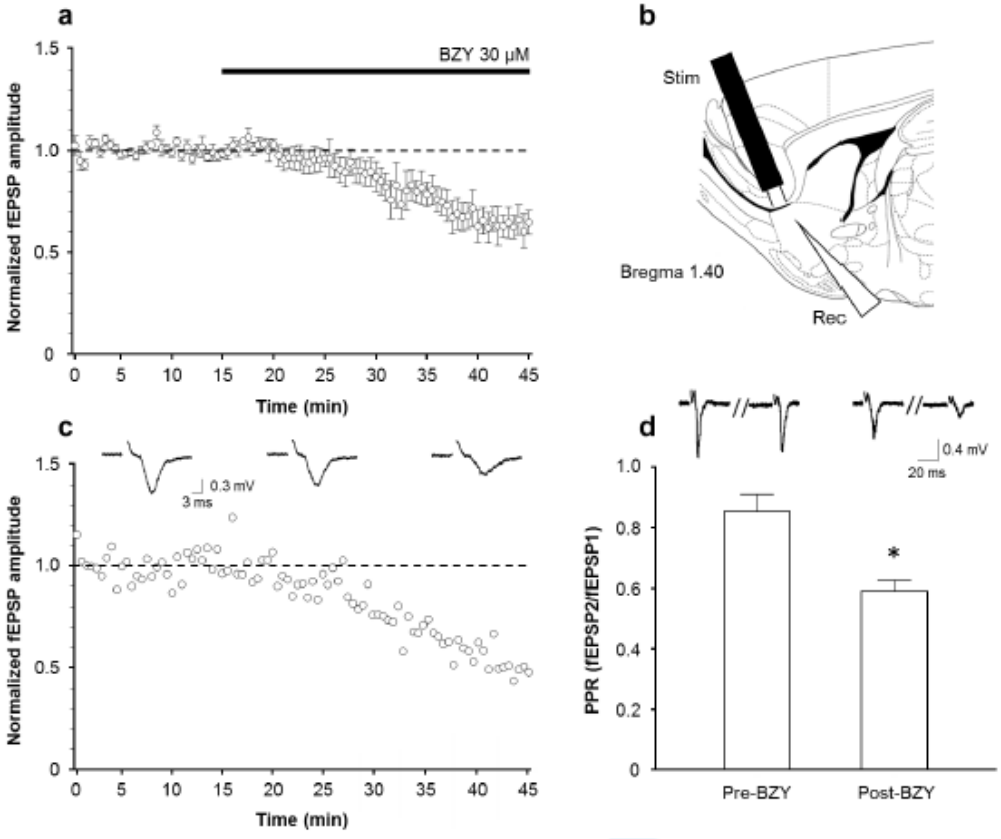
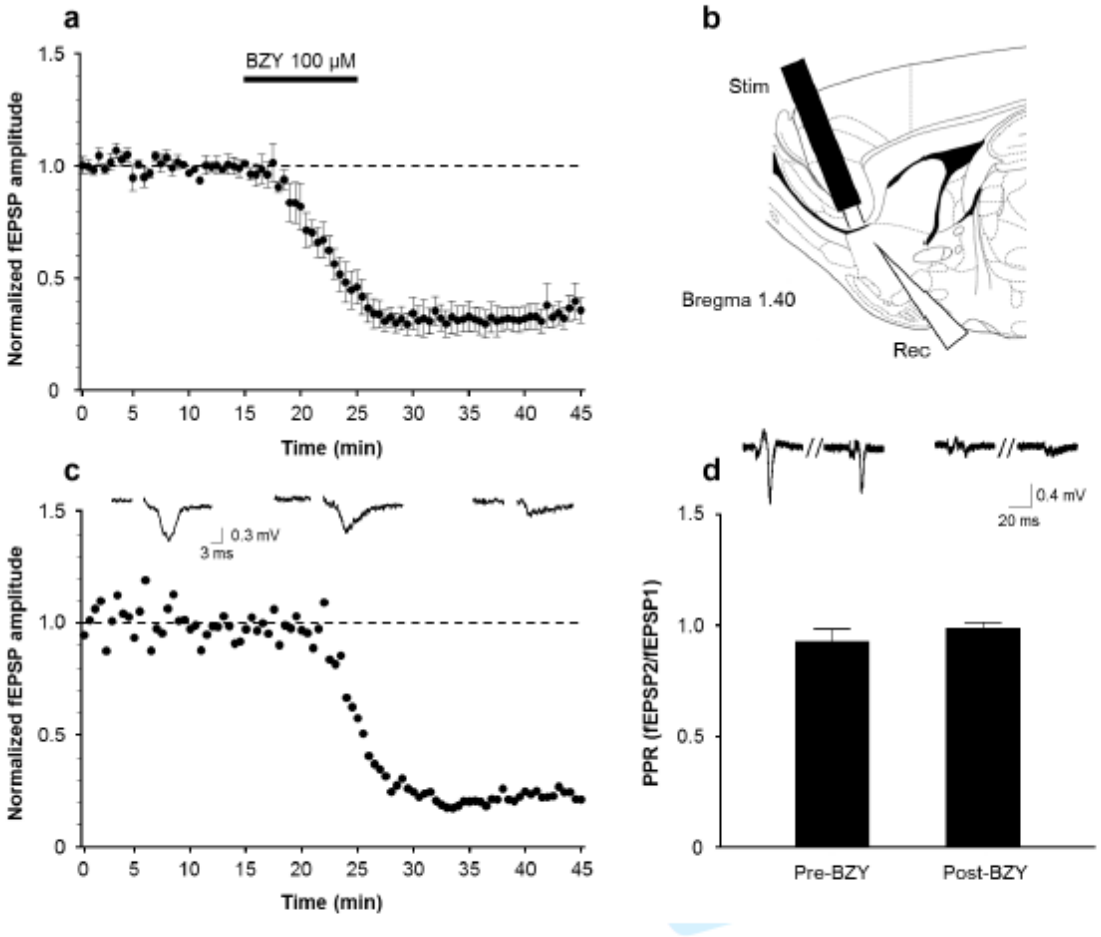

a
BZY 30 μM
Normalized fEPSP amplitude
Time (min)
b
Stim
Bregma 1.40
Rec
c
0.3 mV
3 ms
Normalized fEPSP amplitude
Time (min)
d
0.4 mV
20 ms
PPR (fEPSP2/fEPSP1)
Pre-BZY
Post-BZY
*

Figure 3


a
BZY 100 μM
Normalized fEPSP amplitude
Time (min)
b
Stim
Bregma 1.40
Rec
c
0.3 mV
3 ms
Normalized fEPSP amplitude
Time (min)
d
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20 ms
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Pre-BZY
Post-BZY

Figure 4

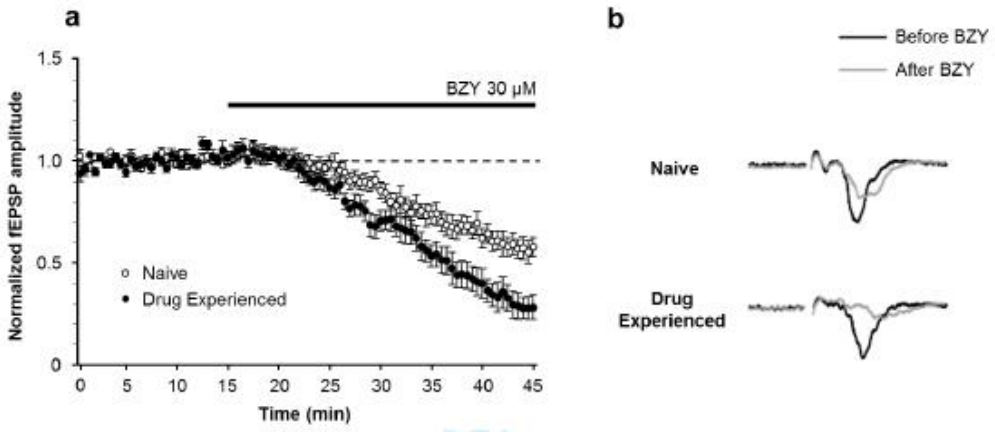
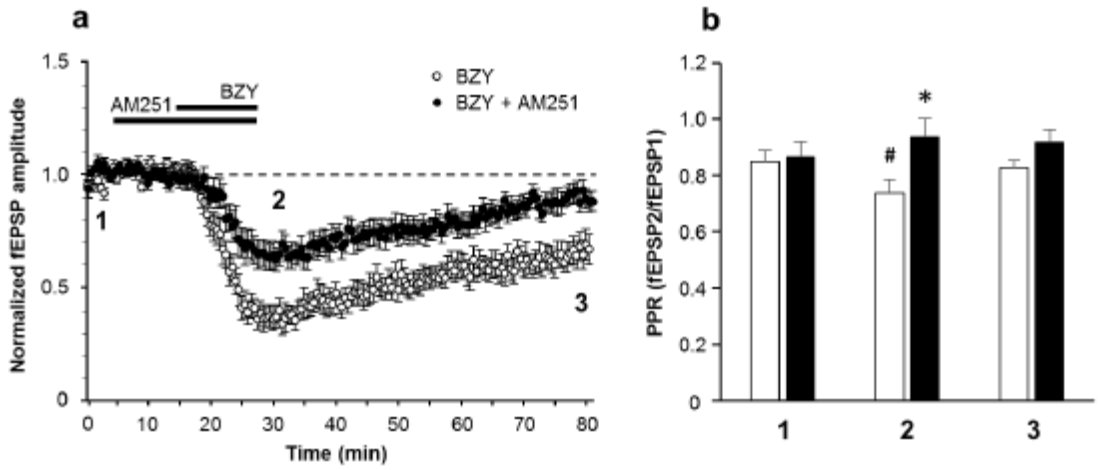


Figure 5



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ORIGINAL INVESTIGATION

Ultrasonic vocalization in rats self-administering heroin and cocaine in different settings: evidence of substance-specific interactions between drug and setting

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Abstract
Rationale Clinical and preclinical evidence indicates that the setting of drug use affects drug reward in a substance-specific manner. Heroin and cocaine co-abusers, for example, indicated distinct settings for the two drugs: heroin being used preferentially at home and cocaine preferentially outside the home. Similar results were obtained in rats that were given the opportunity to self-administer intravenously both heroin and cocaine.
Objectives The goal of the present study was to investigate the possibility that the positive affective state induced by cocaine is enhanced when the drug is taken at home relative to a non-home environment, and vice versa for heroin.
Methods To test this hypothesis, we trained male rats to self-administer both heroin and cocaine on alternate days and simultaneously recorded the emission of ultrasonic vocalizations (USVs), as it has been reported that rats emit 50-kHz USVs when exposed to rewarding stimuli, suggesting that these USVs reflect positive affective states.
Results We found that Non-Resident rats emitted more 50-kHz USVs when they self-administered cocaine than when self-administered heroin whereas Resident rats emitted more 50-kHz USVs when self-administering heroin than when self-administering cocaine. Differences in USVs in Non-Resident rats were more pronounced during the first self-administration (SA) session, when the SA chambers were completely novel to them. In contrast, the differences in USVs in Resident rats were more pronounced during the last SA sessions.
Conclusion These findings indicate that the setting of drug taking exerts a substance-specific influence on the ability of drugs to induce positive affective states.

Keywords Ultrasonic vocalizations · USVs · Drug abuse · Cocaine · Heroin · Self-administration · Emotion · Environment · Context · Setting · Reward · Affect

Introduction

Previous experiments have shown that the setting of drug taking exerts a powerful influence on the rewarding effects of heroin and cocaine and that this influence is substance-specific. Cocaine self-administration (SA), for example, is greatly facilitated when rats self-administer the drug in an environment that is distinct from the home environment (Non-Resident rats) relative to rats for whom the SA chamber is also the home environment (Resident rats) (Caprioli et al. 2007a, b). Non-Resident rats also exhibit greater motivation for cocaine SA than Resident rats, as indicated by progressive ratio reinforcement schedule procedures. In contrast, Resident rats self-administer more heroin than Non-Resident rats and also exhibit greater motivation in break-point procedures (Caprioli et al. 2008). Furthermore, Non-Resident rats tend to prefer cocaine to heroin in a choice procedure, whereas Resident rats tend to prefer heroin to cocaine (Caprioli et al. 2009). Finally, Non-Resident rats are more vulnerable to relapse into

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cocaine seeking (in response to cocaine primings administered after a period of extinction) than Resident rats, whereas Resident rats are more vulnerable to relapse into heroin seeking than Non-Resident rats (Montanari et al. 2015). These modulatory effects of setting are not unique to laboratory rats but can be observed also in humans. Translational studies in heroin and cocaine co-abusers have shown that addicts prefer the home setting for heroin use and non-home settings for cocaine use, regardless of the route of drug taking (Caprioli et al. 2009; Badiani and Spagnolo 2013).

The mechanisms responsible for the substance-specific influence of setting on drug taking are not known. It has been proposed that the setting of drug taking might provide an ecological background against which the central and peripheral effects of drugs are appraised (Badiani 2013). In the presence of a "mismatch" between the setting and the internal state of the organism, the rewarding effects of the drug would be reduced. According to this hypothesis, the central and peripheral (sympathomimetic) arousal produced by cocaine would be appraised as appropriate to arousing non-home settings but not to the home environment. In contrast, the central and peripheral (parasympathomimetic) sedation produced by heroin would be consistent with the safety of the home environment but not to potentially challenging non-home settings.

In the present study, we used the ultrasonic vocalizations (USVs) emitted by rats self-administering heroin and cocaine, as an index of the affective state of the rats. Indeed, 50-kHz USVs are emitted in response to rewarding stimuli, such as intraspecific play (Knutson et al. 1998), hetero-specific play/tickling (Burgdorf and Panksepp 2001; Mällo et al. 2007; Panksepp and Burgdorf 2000, 2003; Schwarting et al. 2007; Wöhr et al. 2009), sex (McGinnis and Vakulenko 2003; White et al. 1990; Bialy et al. 2000), food (Burgdorf et al. 2000), electrical stimulation of the medial forebrain bundle (Burgdorf et al. 2000), and exposure to addictive drugs (Ahrens et al. 2009; Knutson et al. 1999; Natusch and Schwarting 2010; Wintink and Brudzynski 2001; Wright et al. 2010; Barker et al. 2010; Maier et al. 2010). In contrast, 22-kHz USVs are emitted in association with exposure to aversive stimuli, such as electrical footshock (Lee et al. 2001; Koo et al. 2004) and predators (Blanchard et al. 1991, 1992), drug withdrawal (Covington and Miczek 2003; Mutschler and Miczek 1998; Vivian and Miczek 1991), defensive or submissive postures during intraspecific aggression (Lore et al. 1976; Portavella et al. 1993; Thomas et al. 1983), and chronic pain (Calvino et al. 1996). Thus, it has been proposed that 22-kHz USVs reflect negative internal affective states of the lab rat, whereas 50-kHz USVs reflect positive affective states (see Barker 2010).

Materials and methods

Animals

A total of 32 male Sprague–Dawley (Harlan Laboratories) rats weighting 250–280 g at the beginning of the experiment were used. Four rats were excluded from the analysis because they did not reach the SA criterion (at least two infusions per session during the last six sessions of SA for at least one substance). One rat died during the experiment. The rats were housed and tested in the same dedicated temperature- and humidity-controlled rooms (21 ± 1 °C; 70 %), with free access (except during the test sessions) to food and water under a reverse 14-h dark/10-h light cycle (lights off at 7:00 a.m.). The rats were gently handled twice a week for 2 weeks before undergoing catheterization surgery.

Catheter surgery

On the day of surgery, the rats received an i.p. injection of 2.33 mg of xylazine hydrochloride (Rompun®, Bayer HealthCare) and 0.56 ml/kg of Zoletil 100® (Virbac, Carros, France), containing tiletamine (50 mg/ml) and zolazepam (50 mg/ml). The catheter consisted of two pieces of silicone tubing of 10.5 cm (0.37-mm inner diameter, 0.94-mm outer diameter) sheathed and held together at 3.4 cm from their proximal end by 5-mm-long heat-shrink tubing. By using standard surgical procedures, this double-lumen catheter was inserted into the right jugular vein and secured to the surrounding soft tissues with silk thread. Catheter distal ends were externalized through a small incision at the nape of the neck and connected to two L-shaped 22-gauge cannulae, which were secured to 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 with 0.1 ml of a sterile saline solution containing 0.4 mg of enrofloxacin and 25 IU heparin (Marvecs Services, Agrate Brianza, Italy).

Self-administration procedures

After the surgery, the rats were assigned to the Resident or Non-Resident group. Resident rats were housed in the SA chamber throughout the experiment, whereas Non-Residents were housed in standard polycarbonate cages and were transferred to SA chambers only for the daily self-administration session (for more detail on apparatus and housing procedures, see Caprioli et al. 2007a). The catheters of Resident rats were connected to the infusion lines 3 h before the start of the SA session. The rats were trained to self-administer cocaine (400 μg/kg per infusion) and heroin (25 μg/kg per infusion) on alternate days for 14 consecutive daily sessions (3 h per

session). These drug doses (dissolved in sterile saline) were selected on the basis of previous studies (Caprioli et al. 2007a, b; 2008; 2009; Celentano et al. 2009). For half of the rats, the starting drug was heroin and for the other half it was cocaine, and each drug was paired with one of the two levers in a between-subject counterbalanced manner. At the beginning of each session, the appropriate lever was extended and the relative cue light was switched on. Completion of the task on the lever resulted in the delivery of the infusion (40 μl) over a 3-s period and in the retraction of the lever and the switching off of the cue light for a 40-s timeout period. The rats that did not spontaneously self-administer at least one infusion within the first 5 min of the session were placed with their forepaws on the lever to prime an infusion. This was repeated at times 60 and 120 min for rats that did not self-administer at least one infusion in time periods 5–60 and 60–120 min. These priming infusions were not included in data analysis. The schedule requirement to obtain an infusion was progressively increased from fixed ratio 1 (FR1) to FR5 according to the following schedule: FR1 on sessions 1–6, FR2 on sessions 7–8, and FR5 on sessions 9–14. The lever alternation continued on sessions 15–16, but upon completion of the task (FR5), the rats received a saline injection.

Ultrasonic vocalizations

Ultrasonic vocalizations were recorded at baseline condition (3 min in a clean polycarbonate cage), during the period 0–30 min of the first two and the last two SA sessions, and again during the two sessions of saline SA (see Fig. 1). Avisoft UltraSoundGate condenser microphones capsule CM16 and Avisoft Recorder software (Version 3.2) were used. The recording settings included sampling rate at 250-kHz, 16-bit format. The recordings were processed using Avisoft SASLab Pro (Version 4.40) and a fast Fourier transformation (FFT). Spectrograms were generated with an FFT length of 1024 points and a time window overlap of 75 % (100 % Frame, Hamming window). The spectrogram was produced at a frequency resolution of 488 Hz and a time resolution of 1 ms. A lower cutoff frequency of 15 kHz was used to reduce background noise.

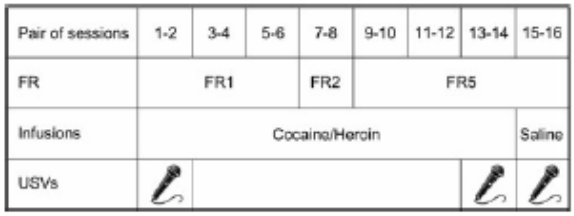

Fig. 1 Outline of the experiment. The microphones indicate the sessions during which USVs were recorded. Please note that during saline self-administration, the alternation between cocaine- and heroin-paired cues and lever position was maintained

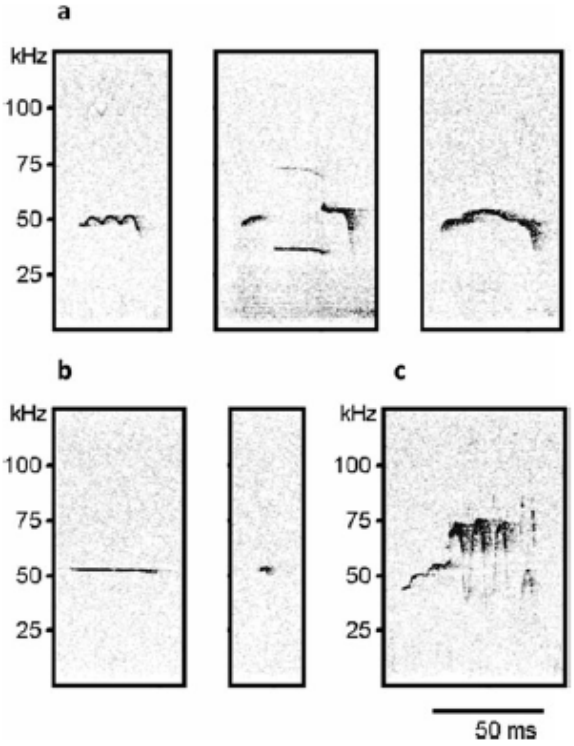

Fig. 2 Representative spectrograms for three main categories of 50-kHz USVs. **a** Frequency-modulated calls are defined as vocalizations continuously or discretely modulated, with a mean slope >0.2 kHz/ms or with one or more pitch-jumps in them, which is an instantaneous change in frequency. **b** Fixed frequency calls have no modulation, with a mean slope of less than 0.2 kHz/ms. **c** Trills vocalizations are characterized by a rapid, massive frequency excursion, either alone or in combination with other calls

Distinct calls were identified on the basis of USV-free intervals ≥50 ms. Each call was visually and acoustically identified by a trained observer and assigned to 1 of 15 categories (Wright et al. 2010), which were then further classified into three main categories, based on previous literature (Brudzynski 2015): (1) "frequency-modulated" (FM) calls, characterized by a continuous or discrete frequency modulation (≥0.2 kHz/s), in either one or two or more directions; (2) "fixed frequency" calls, which were substantially flat USVs (mean change in frequency ≤0.2 kHz/s; (3) "trills" defined as rapid, massive frequency oscillations (including their combinations with vocalizations from other categories); and (4) 22-kHz calls. Representative spectrograms for these USV categories are reported in Fig. 2.

Statistics

Self-administration data were analyzed with a three-way mixed ANOVA with repeated measures on the factor drug

(cocaine vs. heroin) and the factor session, and with setting as a between-subject factor. When the sphericity assumption was violated, Greenhouse–Geisser correction was adopted. Post hoc *t* tests for paired (when confronting lever pressing behavior on pairs of session from the same group) or unpaired samples (when confronting lever pressing behavior on sessions for the same substance between groups) were used to assess differences between sessions. Ultrasonic vocalization data were analyzed, due to high individual variability and lack of normal distribution, using Wilcoxon signed-rank tests for each subcategory. A drug preference score was obtained by calculating the ratio of USV emitted in response to cocaine versus heroin for each animal, after logarithmic normalization: $\log_{10}[(USV_{coc}+1)/(USV_{hero}+1)]$. Two-way mixed ANOVA was run on these data followed by post hoc one-tailed *t* tests, as the direction of change was clearly predicted on the basis of the working hypothesis. Data from three rats (two Non-Residents, one Resident) during the first session were lost due to hardware malfunctioning. Analysis was conducted using IBM SPSS 21.0 statistical software.

Separate analyses were conducted on the 50-kHz calls emitted immediately before (10 s) and immediately after the first ten infusions for sessions 13–14 and 15–16. Given the design of our study, there was large between- and within-subject variability in the number of cocaine, heroin, and saline infusions, as well as in their temporal distribution. Therefore, these data were analyzed using descriptive statistics only (see Figs. 7 and 8), as they were not suitable to inferential statistics.

Results

Self-administration

As illustrated in Fig. 3, cocaine SA and heroin SA were affected in a different manner by the setting. A three-way mixed ANOVA for repeated measures indicated significant main effects of session [$F_{6,150}=56.700$; $p<0.001$] and drug [$F_{1,25}=23.754$; $p<0.001$], and drug × setting [$F_{1,25}=5.818$; $p=0.024$], session × drug [$F_{6,15}=16.176$; $p<0.001$], and drug × session × setting [$F_{6,150}=4.290$; $p=0.012$] interactions. Virtually identical results were obtained analyzing earned infusions, with significant main effects of session [$F_{6,150}=14.328$; $p<0.001$] and drug [$F_{1,25}=17.347$; $p<0.001$], and drug × setting [$F_{1,25}=5.230$; $p=0.031$], and session × drug [$F_{6,15}=6.786$; $p<0.001$] interactions. No group differences were found for the saline SA sessions. The bottom panel of Fig. 2 illustrates the ratio of cocaine to heroin infusions. Two-way mixed ANOVA shown a main effect of setting ($F_{1,25}=10.294$; $p=0.004$).

Ultrasonic vocalizations

Figures 4 and 5 illustrate the number of 50-kHz USVs emitted during the first 30 min of drug SA for sessions 1–2 and 13–14. Overall, Non-Resident rats produced more USVs than Resident rats. However, Non-Resident rats emitted more USVs in response to cocaine than in response to heroin, especially during sessions 1–2 (Fig. 4), when they produced about twice as many USVs for cocaine as for heroin ($p=0.039$; $r=0.42$). In contrast, Resident rats emitted more USVs in response to heroin than to cocaine, especially during sessions 13–14 (Fig. 5), when they produced about three times as many USVs for heroin as for cocaine ($p=0.044$; $r=0.39$). Figures 4 and 5 also illustrate the log-normalized ratios of cocaine-induced over heroin-induced USVs, further indicating that Non-Resident rats vocalize more in response to cocaine than to heroin during the early SA sessions, whereas Resident rats vocalize more in response to heroin than to cocaine during the last SA sessions. Bottom panels in Figs. 4 and 5 show the drug preference score (calculated as described in the "Materials and methods" section) for Resident and Non-Resident rats. A two-way mixed ANOVA for repeated measures conducted on these data indicated a main effect of session ($F_{1,22}=5.256$; $p=0.032$) and of setting ($F_{2,44}=4.006$; $p=0.025$). Post hoc *t* tests revealed a significant difference between Residents and Non-Residents at both early ($t_{24}=-1.732$; $p=0.048$) and late training ($t_{25}=-1.790$; $p=0.043$), but not for saline self-administration ($t_{25}=-0.597$; $p=0.556$). Furthermore, the average of the scores of Non-Residents is significantly different from 0 for early training ($t_{13}=2.081$; $p=0.031$), whereas Resident rats' scores differ from zero for late training ($t_{12}=-2.267$; $p=0.022$).

The differential modulatory influence of setting on cocaine- versus heroin-induced USVs was critically dependant on the actual infusion of heroin or cocaine because it was not observable when the rats were exposed to the conditioned stimuli associated to drug infusion, as during saline SA on sessions 15–16 (Fig. 6). This phenomenon is even more evident when the calls emitted immediately before or after each infusion are considered. Figure 7 compares the frequency of preinfusion calls (10 s before infusion) for the first ten infusions of cocaine or heroin, on sessions 13–14, to that for the first ten infusions of saline, on sessions 15–16. Figure 8 illustrates a similar comparison for the calls emitted in the 40 s after each infusion. In the Resident group, the rats vocalized much more before and after heroin infusion than after saline infusion, whereas the call frequency for cocaine was similar to that for saline. In contrast, Non-Resident rats vocalized more before and after cocaine infusion than after saline infusion, whereas the call frequency for heroin was similar to that for saline.

There was no significant correlation between the number of heroin or cocaine infusions and the number of calls in any

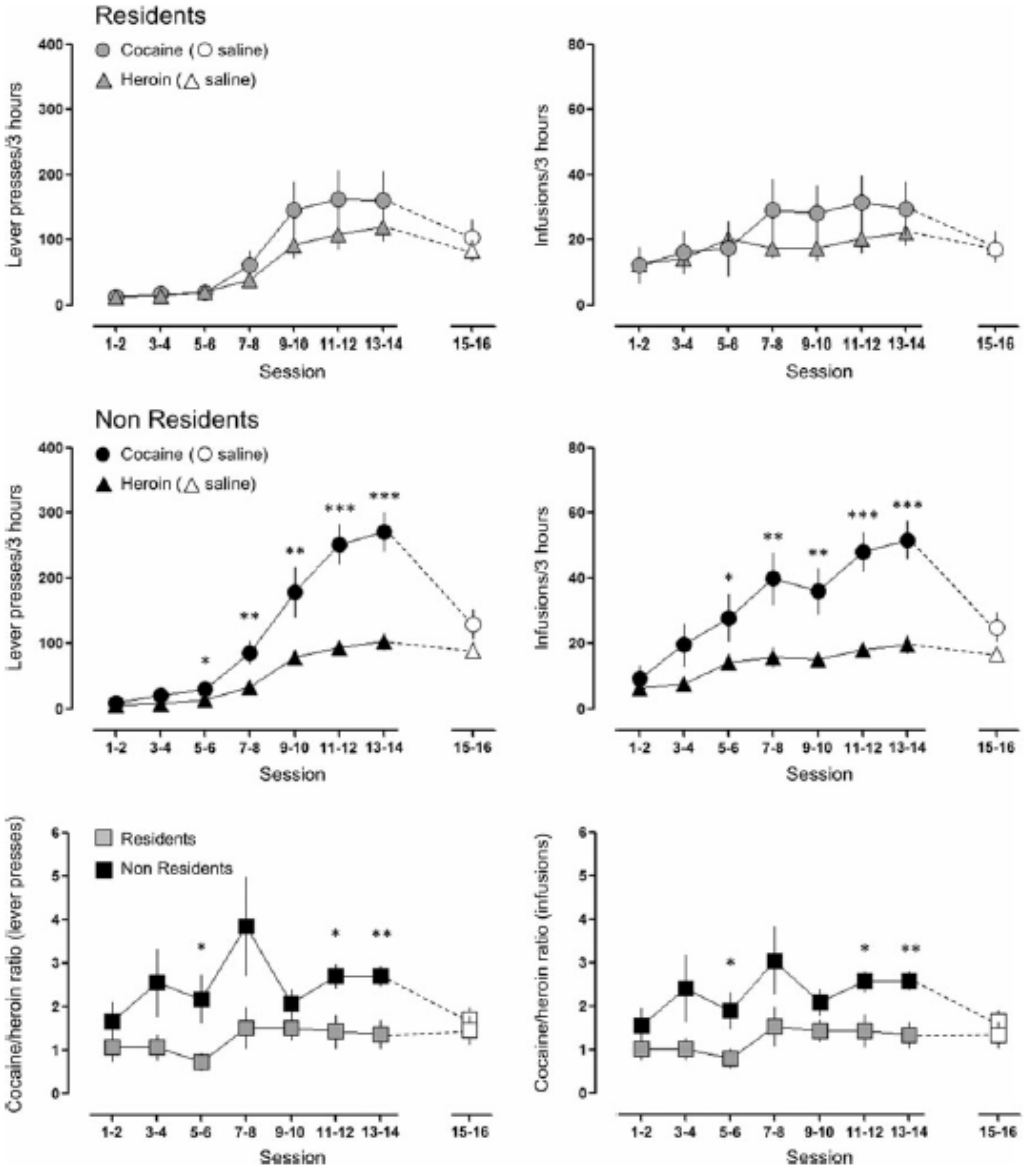

Fig. 3 Self-administration of cocaine, heroin, and saline in Resident versus Non-Resident rats. *Left panels* illustrate the number of lever presses (means ± SEM) and *right panels* the number of infusion (means ± SEM) for each pair of sessions (see "Materials and methods" section). *, **, and *** indicate significant differences ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively) between cocaine and heroin

session for either the Resident or the Non-Resident rats (all p values ≥0.2; data not shown).

Table 1 illustrates the number of 50-kHz USVs for each category. In Non-Resident rats, cocaine elicited more frequency-modulated calls relative to heroin during sessions 1–2 ($p = 0.05$, $r = 0.40$). In contrast, Resident rats emitted more frequency-modulated ($p = 0.032$, $r = 0.42$) and trills ($p = 0.043$, $r = 0.39$) USVs in response to heroin relative to cocaine during sessions 13–14.

The rats emitted very few 22-kHz calls (about 1 % of all recorded calls). The majority of these 22-kHz calls (181 out of 200) were emitted by a single Non-Resident rat on the first session of heroin SA.

Discussion

We report here three main findings. First, we found that the positive affective state (as indicated by 50-kHz USVs)

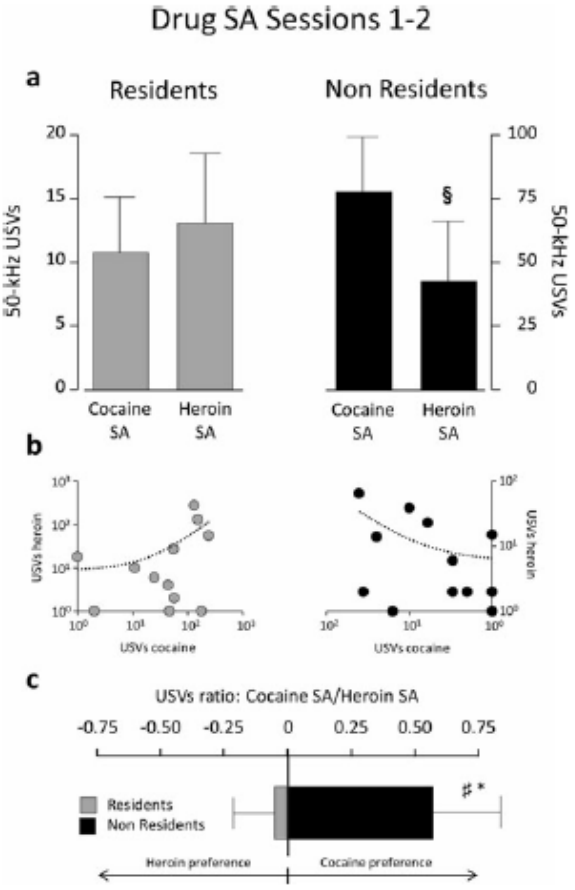

Fig. 4 Fifty-kilohertz USVs emitted by Non-Resident and Resident rats during the first 30 min of drug SA for sessions 1–2. **a** Total number of calls (means ± SEM) for cocaine versus heroin SA. **b** Scatterplots of calls emitted during cocaine versus heroin SA. Each dot represents a single rat. **c** Preference score is calculated as the ratio of log-transformed calls emitted during cocaine versus heroin SA. § indicates significant difference ($p \leq 0.05$) between cocaine and heroin. * indicates significant difference ($p \leq 0.05$) between Residents and Non-Residents. # indicates significant difference ($p \leq 0.05$) from 0

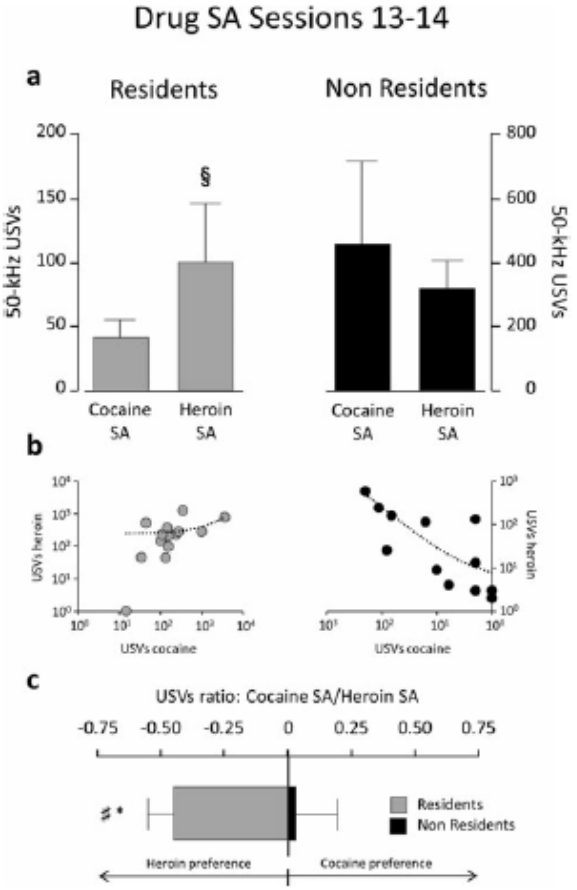

Fig. 5 Fifty-kilohertz USVs emitted by Non-Resident and Resident rats during the first 30 min of drug SA for sessions 13–14. **a** Total number of calls (means ± SEM) for cocaine versus heroin SA. **b** Scatterplots of calls emitted during cocaine versus heroin SA. Each dot represents a single rat. **c** Preference score is calculated as the ratio of log-transformed calls emitted during cocaine versus heroin SA. § indicates significant difference ($p < 0.05$) between cocaine and heroin. * indicates significant difference ($p \leq 0.05$) between Residents and Non-Residents. # indicates significant difference ($p \leq 0.05$) from 0

induced by cocaine versus heroin SA is modulated in a substance-specific manner by the setting of drug taking. On the basis of previous studies (Caprioli et al. 2007a, b; 2008; 2009), we hypothesized that heroin is more rewarding than cocaine when self-administered in a familiar home environment, whereas cocaine is more rewarding when self-administered outside the home. Overall, the findings reported here are in agreement with this hypothesis.

Second, in agreement with previous reports (Barker et al. 2010; Browning et al. 2011; Ma et al. 2010; Maier et al. 2012; Reno et al. 2013), we found that cocaine SA facilitates the emission of 50-kHz USVs and that this phenomenon is temporally related to drug infusion, as indicated by the fact that the call frequency was higher in the periods immediately before and after the infusions relative to the rest of the session.

Third, we report here for the first time that heroin increases 50-kHz USVs and that, as for cocaine, this effect is temporally linked to drug infusion. To the best our knowledge, no previous study has examined the emission of 50-kHz USVs in rats self-administering heroin, or even morphine (which, in any case, has a pharmacological profile distinct from that of heroin; e.g., Antonilli et al. 2005).

We have previously reported that rats tend to self-administer more cocaine when the setting of drug taking is distinct from the home environment (Non-Resident rats) relative to when the SA chamber is also the home environment (Resident rats) (Caprioli et al. 2007a). In contrast, Resident rats tend to self-administer more heroin than Non-Resident rats (Caprioli et al. 2008). We also conducted experiments in which rats were trained, as in the present study, to

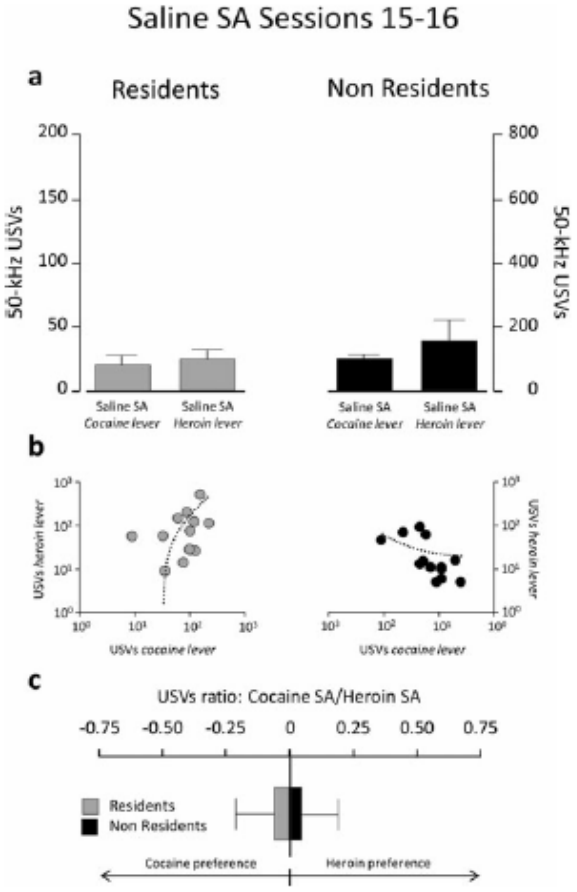

Fig. 6 Fifty-kilohertz USVs emitted by Non-Resident and Resident rats during the first 30 min of saline SA for sessions 15–16. **a** Total number of calls (means ± SEM) when exposed to cocaine-paired versus heroin-paired cues. **b** Scatterplots of calls emitted when exposed to cocaine-paired versus heroin-paired cues. **c** Preference score, for cocaine versus heroin, is calculated as the ratio of log-transformed calls emitted when exposed to cocaine-paired versus heroin-paired cues

self-administer cocaine and heroin (at the same dosages used here) on alternate days (Caprioli et al. 2009; Celentano et al. 2009; Montanari et al. 2015). Under such conditions, Non-Resident rats took much more cocaine than Resident rats whereas the two groups self-administered more or less the same amount of heroin, suggesting that the two drugs affected the intake of one another. Virtually identical results were reported here (see Fig. 2). We have previously discussed in detail the possible reasons for the differential reinforcing effects of cocaine and heroin as a function of setting (Caprioli et al. 2007b; Badiani 2013; Badiani and Spagnolo 2013). For example, although the relationship between the reinforcing and the discriminative effects of addictive drugs is a controversial issue (e.g., Gossop 2001), it is interesting to notice that the setting can affect in opposite directions cocaine and heroin discrimination (Paolone et al. 2004; Caprioli et al. 2007b), much in the same way it affects the self-administration of these two drugs. Thus, it is possible that when a drug is more easily discriminated, it also becomes more easily reinforcing. Another possibility is that the differences in the reinforcing effects of cocaine and heroin as a function of setting depend on differences in the hedonic properties of the two drugs. Heroin might be more reinforcing at home than outside the home because it induces a more positive affective state in the former setting than in the latter, and vice versa for cocaine. To investigate this hypothesis, we used USVs as an index of the emotional state of the rat. Research done in the past 25 years has shown that rats use USVs to communicate their emotional state to other conspecifics (for a review, see Brudzynski 2015). In particular, it has been shown that rewarding stimuli, including drug of abuse, can enhance the emission of 50-kHz USVs (Mutschler et al. 2001; Barker et al. 2010; Maier et al. 2010; Browning et al. 2011; Mahler et al. 2013). Thus, it has been proposed that these USVs may be used as an index of positive affective states in the rat (Knutson et al. 2002).

In the present study, we found major effects of setting and drug SA on the emission of USVs. First of all, Non-Resident rats emitted about ten times more 50-kHz USVs than Resident rats during both drug SA and saline SA. The most likely explanation for this finding is the heightened state of arousal produced by the transfer to a novel test environment (see Maier et al. 2010). Second, the number of USVs greatly increased over sessions in both Resident and Non-Resident rats. Sensitization of USV emission after repeated exposure to addictive drugs has been reported previously (Mu et al. 2009). Third, and most important, we found that the rate of USVs emitted during drug SA was modulated in a substance-specific manner by the setting. Specifically, the ratio of cocaine-induced to heroin-induced USVs was greater in Non-Resident than in Resident rats. The modulatory influence of setting on the emission USVs during cocaine and heroin SA was dependent on the presence of these drugs because it was no longer observable when the rats were shifted to saline SA.

The results summarized above are consistent with a hypothesis discussed in detail in previous papers (Badiani 2013; Badiani and Spagnolo 2013). Briefly, it was proposed that a drug is perceived as less rewarding when its peripheral and central effects are at odds with the setting of drug taking, that is, when there is a mismatch between setting and drug effects. The sympathomimetic, arousing, and activating effects of cocaine (or amphetamine), for example, would be experienced as unsuitable to a safe, non-challenging, domestic environment. In contrast, the drowsiness and sedation produced by heroin would be experienced as unsuitable to an exciting, novel environment. A similar line of reasoning would apply not only to psychostimulants and opiates. We have shown that Non-Resident rats take much more ketamine (which, like cocaine, has activating and sympathomimetic effects; Hancock and Stamford 1999) than Resident rats (De

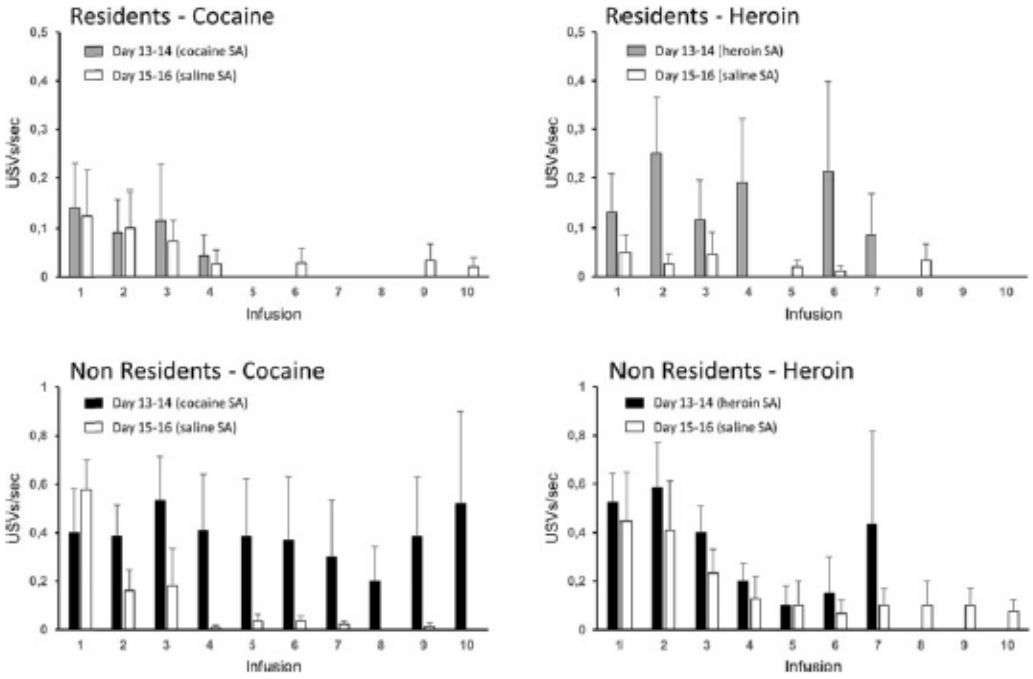

Fig. 7 Pre-infusion calls. Rate of 50-kHz USVs (means ± SEM) in the 10 s before each of the ten first infusions on sessions 13–14 (heroin or cocaine infusions) versus sessions 15–16 (saline infusions). Due to great individual variability in number and timing of earned infusions, only descriptive statistics are displayed for this dataset (see "Materials and methods" section)

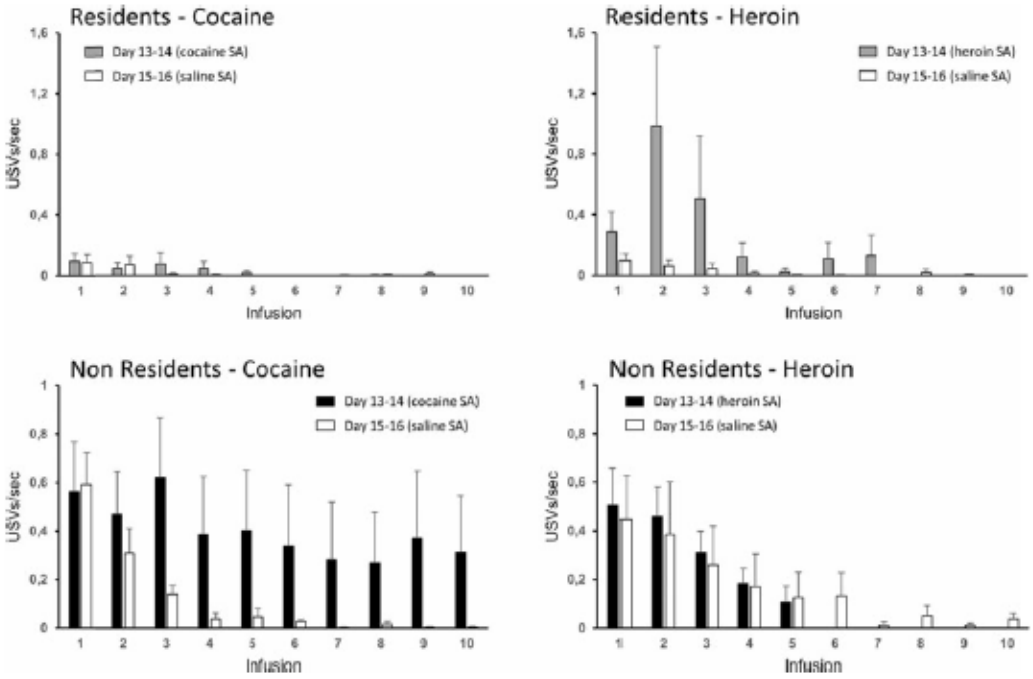

Fig. 8 Post-infusion calls. Rate of 50-kHz USVs (means ± SEM) during the 40 s after each of the ten first infusions on sessions 13–14 (heroin or cocaine infusions) versus sessions 15–16 (saline infusions). Due to great individual variability in number and timing of earned infusions, only descriptive statistics are displayed for this dataset (see "Materials and methods" section)

Table 1 Number (means ± SEM) of trills, frequency-modulated (FM) calls, and fixed frequency (FF) 50-kHz USVs (see text), emitted by Non-Resident and Resident rats during the first 30 min of drug SA (sessions 1–2 and 13–14) and saline SA (sessions 15–16)

		Residents		Non-Residents		All	
Session	USV category	Cocaine lever	Heroin lever	Cocaine lever	Heroin lever	Cocaine lever	Heroin lever
1–2 drug SA	Trills	0.58 ± 0.49	1.33 ± 1.33	8.33 ± 3.70	2.92 ± 1.59	4.18 ± 1.78	2.125 ± 1.03
	FM	4.92 ± 2.22	3.33 ± 1.70	* 45.17 ± 14.13	24.25 ± 13.71	* 25.4 ± 7.66	13.79 ± 7.10
	FF	5.58 ± 1.87	8.67 ± 3.63	24.00 ± 5.93	16.00 ± 8.38	15.96 ± 3.90	12.33 ± 4.53
	Total	10.23 ± 3.91	13.33 ± 5.70	* 78.36 ± 20.65	43.17 ± 23.57	45.55 ± 12.59	28.25 ± 12.26
13–14 drug SA	Trills	1.92 ± 0.81	* 16.62 ± 9.00	# 58.71 ± 29.70	68.00 ± 32.83	31.37 ± 16.12	43.26 ± 17.97
	FM	21.08 ± 10.28	*,# 53.31 ± 23.65	# 299.29 ± 186.02	## 182.71 ± 48.55	# 165.33 ± 98.68	## 120.41 ± 29.94
	FF	14.69 ± 6.13	28.31 ± 13.76	100.86 ± 42.73	66.07 ± 14.71	59.37 ± 23.52	47.89 ± 10.58
	Total	37.69 ± 16.94	*,# 98.23 ± 45.61	# 458.86 ± 255.88	# 316.79 ± 90.55	## 256.07 ± 136.90	## 211.55 ± 55.20
15–16 saline SA	Trills	1.38 ± 0.94	3.23 ± 2.60	14.43 ± 4.92	42.43 ± 20.02	8.15 ± 2.84	23.55 ± 10.96
	FM	7.92 ± 2.97	6.77 ± 3.02	44.14 ± 7.58	69.64 ± 28.73	26.70 ± 5.43	39.37 ± 15.94
	FF	9.62 ± 4.37	13.85 ± 5.09	38.57 ± 7.32	58.29 ± 28.35	24.63 ± 5.12	36.89 ± 15.27
	Total	18.92 ± 7.99	23.85 ± 8.16	97.14 ± 15.78	170.36 ± 75.91	59.48 ± 11.73	99.81 ± 41.41

*Significant differences ($p \leq 0.05$) between heroin- and cocaine-induced calls; #, ## significantly more ($p \leq 0.05$ and $p \leq 0.01$, respectively) drug-induced calls on sessions 13–14 relative to the corresponding saline session (sessions 13–14)

Luca and Badiani 2011), whereas Resident rats take more alcohol (which like heroin causes, at least initially, drowsiness and sedation; Morean and Corbin 2010) than Non-Resident rats (Testa et al. 2011).

The mismatch hypothesis would also account for an intriguing result of the present study, that is, for the fact that the modulatory effect of setting on the emission of USVs during drug SA changed in a substance-specific manner over time. Resident rats exhibited in fact no significant differences in the number of USVs emitted during heroin versus cocaine SA on sessions 1–2, whereas they emitted about three times more USVs during heroin SA relative to cocaine SA on sessions 13–14. It is possible that the first exposure to the testing procedures, including cue light presentation, lever extension, and drug infusion induced a certain degree of arousal, which waned with repeated testing. In contrast, Non-Resident rats emitted twice as many USVs during cocaine versus heroin SA on sessions 1–2, whereas there were no significant differences on sessions 13–14. It is possible that this was due to the repeated exposure of Non-Resident rats to the SA chamber, which might have blunted, but not erased, the relative novelty of the setting. However, it should be noted that when the analysis was limited to the USVs emitted immediately before or after drug infusion (Figs. 7 and 8), Non-Resident rats vocalized more before/after cocaine infusion than after saline infusion, whereas the number of peri-infusion calls for heroin was similar to that for saline.

While the mismatch hypothesis predicted greater rewarding effects of heroin in Resident versus Non-Resident rats and of cocaine in Non-Resident versus Resident rats, it did not necessarily predict greater aversive effects of heroin in Non-Resident versus Resident rats and of cocaine in Resident versus Non-Resident rats. In any case, under the testing conditions of the present study, the rats emitted very few 22-kHz USVs, which are thought to reflect aversive states (Blanchard et al. 1991, 1992; Calvino et al. 1996; Covington and Miczek 2003; Koo et al. 2004; Lee et al. 2001; Lore et al. 1976; Mutschler and Miczek 1998; Portavella et al. 1993; Thomas et al. 1983; Vivian and Miczek 1991). Interestingly, the majority of the very few 22-kHz calls recorded in our study (181 out of 200) were emitted by a single Non-Resident rat on the first session of heroin SA.

What are the neurobiological mechanisms responsible for the differential influence of settings on the emission of heroin- and cocaine-elicited calls? It has been previously shown that the intravenous administration of heroin and cocaine at doses identical to those used in present experiments differentially activate dorsal striatum neurons in Resident versus Non-Resident rats (Celentano et al. 2009). Given the role of the striatal complex in the production of 50-kHz USVs (Barker 2010), further studies are necessary to investigate whether this differential neuronal activation is at least in part responsible for the findings reported here.

In conclusion, the present study shows that the setting of drug administration modulates in a substance-specific manner not only the reinforcing and interoceptive effects of cocaine versus heroin (as shown in previous studies) but also the ability of these drugs to induce positive affective states, at least as reflected by 50-kHz USV. In particular, we have shown that a given setting of drug taking can modulate in opposite manner all aspects of heroin versus cocaine reward: intake (Caprioli et al. 2007a, 2008, 2009; Celentano et al. 2009), motivation

(progressive ratio procedures; Caprioli et al. 2007a, 2008, 2009; Celentano et al. 2009), choice (Caprioli et al. 2009), drug discrimination (Paolone et al. 2004; Caprioli et al. 2007b), and affect (present study). It is important to notice that the setting does not influence all drug effects in the same way. We have previously shown that repeated administrations of heroin or morphine produce greater psychomotor sensitization in Non-Resident than in Resident rats (Badiani et al. 2000; Paolone et al. 2003, 2007), as previously reported for amphetamine and cocaine (Badiani et al. 1995a, b; Crombag et al. 1996; Browman et al. 1998). That is, psychomotor sensitization and rewarding effects can be modulated in opposite directions by the setting, and this opposite modulation has been observed to occur in parallel (e.g., Caprioli et al. 2008). Furthermore, some effects of drugs do not appear to be susceptible to the manipulation of setting investigated here. Tolerance to the analgesic effect of morphine, for example, develops in exactly the same way in Resident and Non-Resident rats (Paolone et al. 2003).

The effects of setting on cocaine versus heroin reward may explain the findings of studies conducted in human addicts, showing distinct setting preferences for cocaine versus heroin use (Caprioli et al. 2009; Badiani and Spagnolo 2013), and in rat models of drug relapse, showing differential vulnerability to cocaine versus primed reinstatement of drug seeking after a period of ext (Montanari et al. 2015). Taken together, these findings indicate the importance of taking into account the substance-specific aspects of drug use and misuse (Badiani et al. 2011; Badiani 2013).

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Compliance with ethical standards

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

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ORIGINAL INVESTIGATION

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

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Abstract
Rationale Previous studies have shown that the effect of setting on drug-taking is substance specific in both humans and rats. In particular, we have shown that when the setting of drug self-administration (SA) coincides with the home environment of the rats (resident rats), the rats tend to prefer heroin to cocaine. The opposite was found in nonresident rats, for which the SA chambers represented a distinct environment.
Objectives The aim of the present study was to investigate the influence of setting on the ability of different doses of cocaine and heroin to prime cocaine- versus heroin-seeking in rats that had been trained to self-administer both drugs and had then undergone an extinction procedure.
Methods Resident (N=62) and nonresident (N=63) rats with double-lumen intra-jugular catheters were trained to self-administer cocaine (400 μg/kg/infusion) and heroin (25 μg/kg/infusion) on alternate days for 10 consecutive daily sessions (3 h each). After the extinction phase, independent groups of rats were given a noncontingent 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 nonreinforced lever presses.
Results All resident and nonresident rats acquired heroin and cocaine SA. However, cocaine primings reinstated cocaine-seeking only in nonresident rats, whereas heroin primings reinstated heroin-seeking only in resident rats.
Conclusions We report here that the susceptibility to relapse into drug-seeking behavior is drug-specific and setting-specific, confirming the crucial role played by drug, set, and setting interactions in drug addiction.

Keywords Addiction · Drug abuse · Environment. Relapse · Reinstatement · Self-administration

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Introduction

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

interesting in the light of the growing interest in the substance-specific aspects of drug reward and drug abuse (Badiani et al. 2011; Marchant et al. 2014; Peters et al. 2013).

We have previously found that addicts who co-abuse heroin and cocaine prefer distinct settings for the two drugs. Most addicts reported using heroin preferentially at home and cocaine preferentially outside the home (Badiani and Spagnolo 2013; Caprioli et al. 2009). Even the choice of specific nonhome settings (pubs, clubs, friends, parks, etc.) differed between the two drugs (Badiani and Spagnolo 2013). Preclinical studies have shown that the setting can exert a substance-specific influence on drug-taking not only in humans but also in laboratory rats (Caprioli et al. 2007a, 2008, 2009; Celentano et al. 2009; De Luca and Badiani 2011; Testa et al. 2011). In these studies, rats with intra-jugular catheters were given the opportunity to self-administer the drug intravenously in one of two distinct settings. Some rats were transferred to the self-administration (SA) chambers immediately before the test sessions and therefore took the drug outside the home (nonresident rats). Other rats were kept at all times in the SA chambers, and therefore, they took the drug at home (resident rats). In a manner reminiscent of the human condition, cocaine was more vigorously self-administered by nonresident rats than by resident rats (Caprioli et al. 2007a, 2009), whereas the opposite was found for heroin SA (Caprioli et al. 2008, 2009). When trained to self-administer both cocaine and heroin on alternate days, nonresident rats took more cocaine than heroin relative to resident rats (Caprioli et al. 2009; Celentano et al. 2009). Most importantly, we found that the setting influenced even drug choice. When rats were permitted to self-administer either cocaine or heroin within the same session, most nonresident rats preferred cocaine to heroin, whereas most resident rats preferred heroin to cocaine (Caprioli et al. 2009).

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

Material and methods

Animals

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

Surgery

After their arrival, the rats were housed two per cage for 10–12 days before the surgery, after which were housed individually. Using standard surgical procedures previously described in detail (Caprioli et al. 2007a, 2008), double-lumen catheters were inserted into and secured to the right jugular vein of the rats. The distal end of the catheter was externalized through a small incision at the nape of the neck and connected to two L-shaped 22-gauge cannulae (one for each lumen) that were secured to the rat's skull using dental cement and stainless steel screws. Each catheter lumen was flushed daily with 0.1 ml of sterile saline solution containing 0.3 mg gentamycin and 12.5 IU heparin (Marvecs Services, Agrate Brianza, Italy).

Apparatus

The apparatus consisted of SA chambers (28.5-cm length, 27-cm width, and 32-cm height) made of transparent plastic (front and rear walls), aluminum (sidewalls and ceiling), and stainless steel (grid floor). Plastic trays covered with pinewood shaving were placed under the cage floors. Each chamber was equipped with two retractable levers, positioned on the left-hand wall 12.5 cm apart and 9 cm above the floor, two sets of three cue lights (red, yellow, and green), positioned above each lever, and a counterbalanced arm holding a liquid swivel. The SA chambers were placed within sound- and light-attenuating cubicles. Each chamber was connected via an electronic interface to a motorized syringe pump (Razel Scientific Instruments, St. Albans, VT, USA) and to a programmable logic controller (PLC; Allen Bradley, Milwaukee, WI, USA). Finally, the PLCs were connected to PCs running control software. Chambers, accessories, and electronic interfaces were purchased from ESATEL (Rome, Italy) and custom-developed control software from Aries Sistemi (Rome, Italy). The infusion line consisted of a length of silastic tubing protected by a stainless steel spring and connected (through the liquid swivel and another length of silastic tubing) to a syringe positioned on the pump (which was programmed to work at an infusion rate of 13.3 μl/s).

Experiments

General procedures After the surgery, the rats were assigned to one of two conditions: resident (N=62) and nonresident (N=63). The rats in the resident group were single housed in the SA chambers, where they remained for the entire duration of the experiment. Nonresident rats were single housed in standard transparent plastic cages (40-cm length, 24.5-cm width, and 18-cm height, with stainless steel tops and flat bottoms covered with ground corncob bedding) and immediately before the start of each session were transferred to the SA chambers. The drug-

taking context was therefore physically identical for all rats, but for some rats, this was also their home (resident group), whereas for other rats, it represented a distinct and, at least initially, novel context (nonresident group). All other husbandry routines were identical in the two groups. Notice that throughout the experiments, the rats were housed and tested in the same dedicated temperature- and humidity-controlled room (i.e., there was no transport from one room to another and no disruption of circadian rhythmicity) with ad libitum access to food and water (except during the test sessions) under a 14-h dark/10-h light cycle (lights off at 0700 hours).

Testing began 1 week after the surgery, and all testing procedures were identical between resident and nonresident rats (including the absence of food or water). The experiments included 21 sessions; all test sessions lasted 3 h and took place during the dark phase, between 1230 and 1630 hours, 7 days a week. The catheters were connected, through infusion lines and liquid swivels, to the infusion syringes, 3 h before the start of each session for resident rats and immediately before the start of sessions for nonresident rats. During 60 s preceding the start of each session, resident rats were briefly handled to remove food and water from the SA cages, and the infusion pumps were activated so as to fill the catheters with the drug or saline solution. The doors of the cubicles were closed for the duration of the session and left open at all other times. At the end of each session, food and water were given back to the resident rats, and nonresident rats were returned to their home cages.

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

Training phase (days 1–10) Resident and nonresident rats were trained to self-administer cocaine (400 μg/kg/infusion) and heroin (25 μg/kg/infusion) on alternate days for 10 consecutive daily 3-h sessions (i.e., there were 5 sessions for each drug). Cocaine and heroin were each paired with one of two retractable levers and a cue light (red or green); the starting drug was counterbalanced within groups, and the assignment of levers and cues was counterbalanced for both drugs. At the start of each session, only the lever associated with the drug to be self-administered on that session was extended, and the appropriate cue light was turned on. The number of consecutive lever presses required to obtain a single infusion (fixed ratio (FR)) was 1 for sessions 1–2 (FR1), FR2 for sessions 3–4, and FR5 for sessions 5–10. After each infusion, the cue light was turned off and the lever retracted. After a 40-s time-out period, the cue light was turned on and the lever extended again. The rats were allowed to self-administer a maximum of 100 infusions of cocaine or heroin to minimize the risk of overdosing. To facilitate the acquisition of drug SA and only when necessary, the rats were primed by placing their forepaws on the lever, so as to trigger one infusion. During sessions 1–4, infusions were administered at times 5, 65, and 125 min to rats who had not spontaneously self-administered at least one infusion during time periods 0–5 min, 5–65 min, and 65–125 min, respectively. On sessions 5–10, infusions were given, if necessary, only at 5 min to rats that had not spontaneously self-administered at least one infusion. These infusions (0.81±0.1 vs. 0.56±0.18 infusions of cocaine per session in residents vs. nonresidents; 0.14±0.05 vs. 0.24±0.09 infusions of heroin per session in residents vs. nonresidents) were excluded from statistical analyses and did not count toward the satisfaction of SA criterion. Each lumen of double lumen catheters was used the same number of times for either drug, in a counterbalanced manner. Like in other studies (e.g., Carelli and Ijames 2000; Carrera et al. 2000; Lenoir and Ahmed 2007; Tran-Nguyen et al. 2001), no inactive lever was used in the present study; under our testing conditions, inactive responses are negligible.

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

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

Catheter patency test

At the end of the experiment, all rats underwent a catheter patency test in which they received two i.v. boluses of 40 mg/kg of thiopental sodium (Pharmacia Italia, Milan, Italy), one in each catheter lumen, with a 15-min interval between the two. The test was considered positive if the rat became ataxic within 5 s after the injection. Rats were included in the analysis only if the test was positive for both lumens of the catheter.

Data analysis and statistics

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

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

Reinstatement session The effect of drug primings was assessed using four-way ANOVAs with context (two levels: nonresidents vs. residents), drug priming (two levels: cocaine vs. heroin), and dose (three levels: low, medium, and high dose) as between-subject factors and priming (two levels: last extinction session for the lever paired to the drug used for priming vs. reinstatement session) as within-subject factor.

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

The relationship between drug intake and vulnerability to relapse was investigated using linear regression analysis with cocaine or heroin intake during training as the independent variable number of lever presses during the reinstatement session as the dependent variable.

Results

Training phase As shown in Fig. 1, the rats rapidly acquired cocaine and heroin SA. The ANOVA conducted on drug infusion data yielded significant main effects of drug [$F(1, 123)=78.498$, $p\leq0.0001$], session [$F(4,492)=60.799$, $p\leq 0.0001$], and context [$F(1,123)=7.844$, $p=0.006$]. There were also significant drug × context [$F(1,123)=14.354$, $p\leq0.0001$], session × context [$F(4,492)=7.975$, $p=0.006$], session × drug [$F(4,492)=60.316$, $p\leq0.0001$], and session × drug × context [$F(4,492)=13.029$, $p\leq0.0001$] interactions. Figure 2 illustrates the ratio of cocaine to heroin infusions. Resident rats took about 1.5 as many infusions of cocaine as of heroin, whereas in nonresident rats, this ratio increased to more than 3. The ANOVA yielded a significant effect of context [$F(1, 123)=7.549$, $p=0.007$] but not of session ($p=0.234$), and there was no session × context interaction ($p=0.203$). Given that there was no session × context interactions, pairwise comparisons were inappropriate (thus were not reported in Fig. 2). Notice, however, that they indicated significant differences for sessions 3–4 and 5–6 ($p\leq0.05$) and for sessions 7–8 and 9–10 ($p\leq0.0001$).

Extinction phase During the extinction sessions (Fig. 3), the rate of pressing rapidly decreased, as indicated by a main effect of session [$F(4,492)=144.601$, $p\leq0.0001$], with a session × drug lever × context interaction [$F(4,492)=2.586$, $p=0.036$]. Pairwise comparison indicated significant differences in pressing on the cocaine-paired lever versus heroin-paired lever in the nonresident group but not in the resident group. In addition, there were significant group differences in pressing on the heroin-paired lever during sessions 1–4.

Reinstatement session Overall, lever pressing during the reinstatement session was greater than during the first hour of the relative extinction session. However, there were important differences in the ability of heroin and cocaine to trigger relapse as a function of setting. As shown in Fig. 4, when given cocaine primings, only nonresident rats exhibited reinstatement of cocaine-seeking, whereas when given heroin primings, only resident rats exhibited reinstatement of heroin-seeking. The ANOVA indicated a significant main effect of priming [$F(1,113)=31.254$, $p\leq0.0001$] but not of context ($p=0.091$), drug ($p=0.116$), or dose ($p=0.988$). Most importantly, there was a priming × context × drug interaction [$F(1, 113)=10.320$, $p=0.002$], but not priming × context ($p=0.965$), priming × drug × dose ($p=0.183$), or priming × context × drug × dose ($p=0.898$) interactions. Simple main effect analyses indicated a significant priming effect of heroin in the resident group ($p=0.002$) and of cocaine in the nonresident group ($p\leq0.0001$). In contrast, there was no significant priming effect of heroin in the nonresident group ($p=0.617$), whereas cocaine priming in the resident group only approached significance ($p=0.074$). Given that there was no effect of dose and no interaction between dose and the other factors, pairwise comparisons were inappropriate (thus were not reported in Fig. 3). Notice, however, that they indicated a

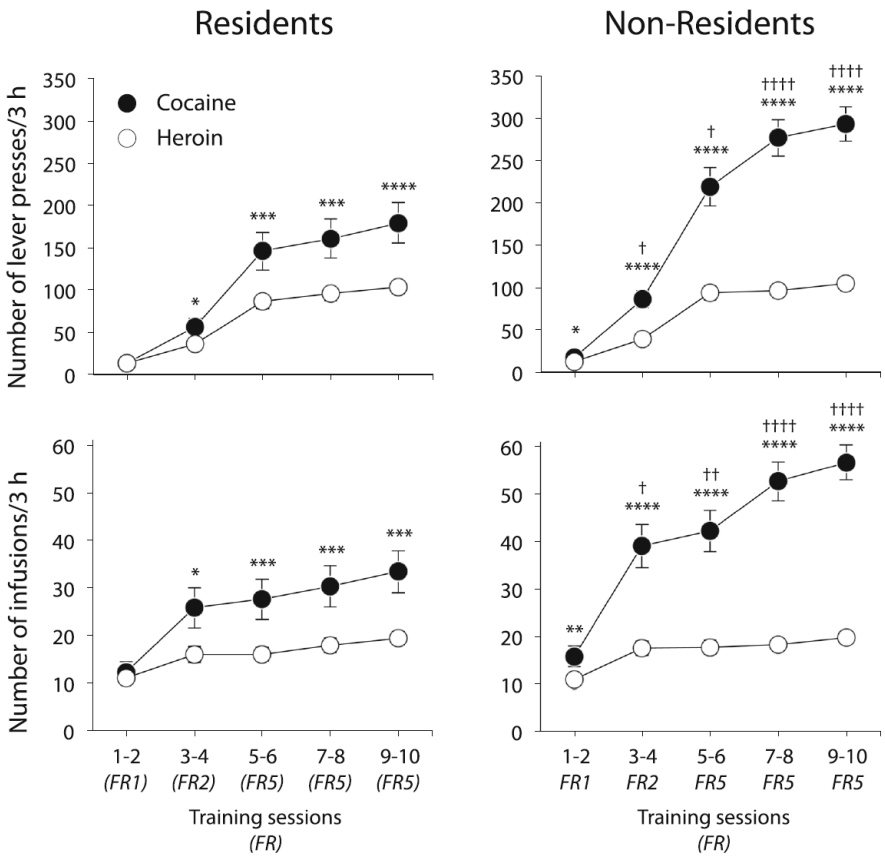

Fig. 1 Mean (±SEM) number of lever presses and infusions for cocaine (400 μg/kg) and heroin (25 μg/kg) self-administration (SA) during the training phase for the resident and nonresident groups. Significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.01$, and ****$p \leq 0.001$) differences in cocaine versus heroin SA. Significant (†$p \leq 0.05$, ††$p \leq 0.01$, and ††††$p \leq 0.0001$) differences between the resident and the nonresident group

significant priming effect for the high ($p = 0.001$), medium ($p \leq 0.05$), and low ($p \leq 0.05$) dose of cocaine in the nonresident group, but not in the resident group, and a significant priming effect for the high and medium doses of heroin in the resident group, but not in the nonresident group.

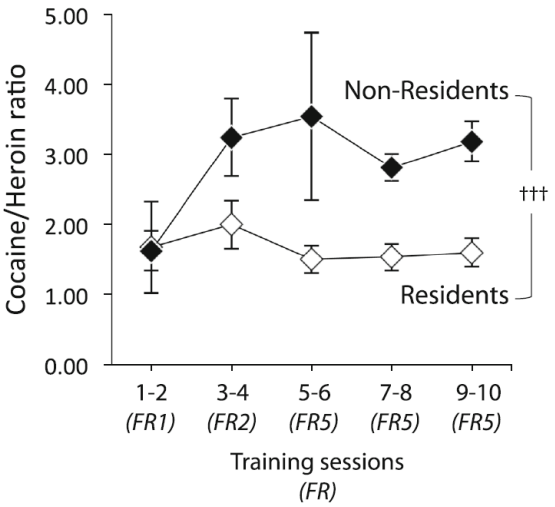

Fig. 2 Mean (±SEM) ratio of cocaine to heroin infusions during training (see Fig. 1) for the resident and the nonresident group. Significant (†††$p \leq 0.001$) main effect of context

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

Discussion

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

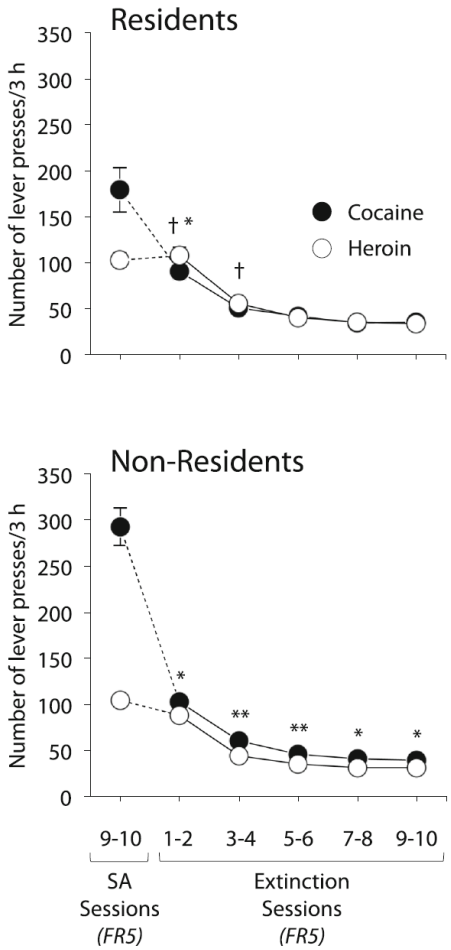

Fig. 3 Mean (±SEM) number of lever presses on the cocaine- versus heroin-paired lever during the extinction phase for the resident and nonresident groups. Significant (*$p \leq 0.05$ and **$p \leq 0.01$) differences in lever pressing for the cocaine-paired versus the heroin-paired lever. Significant (†$p \leq 0.05$) differences between the resident and the nonresident group

Setting and drug reward

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

The ability of context to influence drug reward is not unique to humans. We have previously shown that even in laboratory rats, the preference for cocaine versus heroin depends on the setting (Caprioli et al. 2007a, b, 2008, 2009; Celentano et al. 2009). The procedures used in these earlier studies were identical to those used here. Some rats were housed in the SA chambers (resident rats), whereas other rats were transferred to the SA chambers only for the testing (nonresident rats). Nonresident rats self-administered more cocaine and amphetamine than resident rats (Caprioli et al. 2007a, 2008), whereas resident rats self-administered more heroin than nonresident rats (Caprioli et al. 2008). We also conducted experiments in which rats were trained to self-administer cocaine and heroin on alternate days (Caprioli et al. 2009; Celentano et al. 2009). In this case, nonresident rats took more cocaine than resident rats, and the cocaine/heroin intake ratio was greater in nonresident than in resident rats, whereas there was little or no difference in the intake of heroin. Virtually identical results were obtained here (see Fig. 1), suggesting that in nonresident rats, cocaine SA facilitated heroin SA. More generally, it appears that there were reciprocal influences in the intake of the two drugs, because in rats that had access to both drugs (see, in addition to the present study, Caprioli et al. 2009 and Celentano et al. 2009), the speed of acquisition and the intake of cocaine and heroin were much greater than in rats that had access to only one of the two drugs (see Caprioli et al. 2007a, b, 2008).

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

Drug addiction is a chronic relapsing disorder, as indicated by the propensity to resume drug use after a period of abstinence (American Psychiatric Association 2013). The determinants of relapse are complex and include factors such as cognitive and emotional states, stress levels, social pressure, and psychiatric comorbidity (see Bradley et al. 1989). Among the external stimuli that can lead to relapse, a great deal of attention has been placed on the role of discrete cues or contexts associated with drug use (Crombag et al. 2008; O'Brien et al. 1992).

Also, exposure to small amounts of the drug (such as a puff from a cigarette, a sip of alcoholic beverage, or a snort of cocaine) can trigger relapse (Jaffe et al. 1989; Ludwig et al. 1974). Interestingly, Leri and Stewart (2001) have shown that

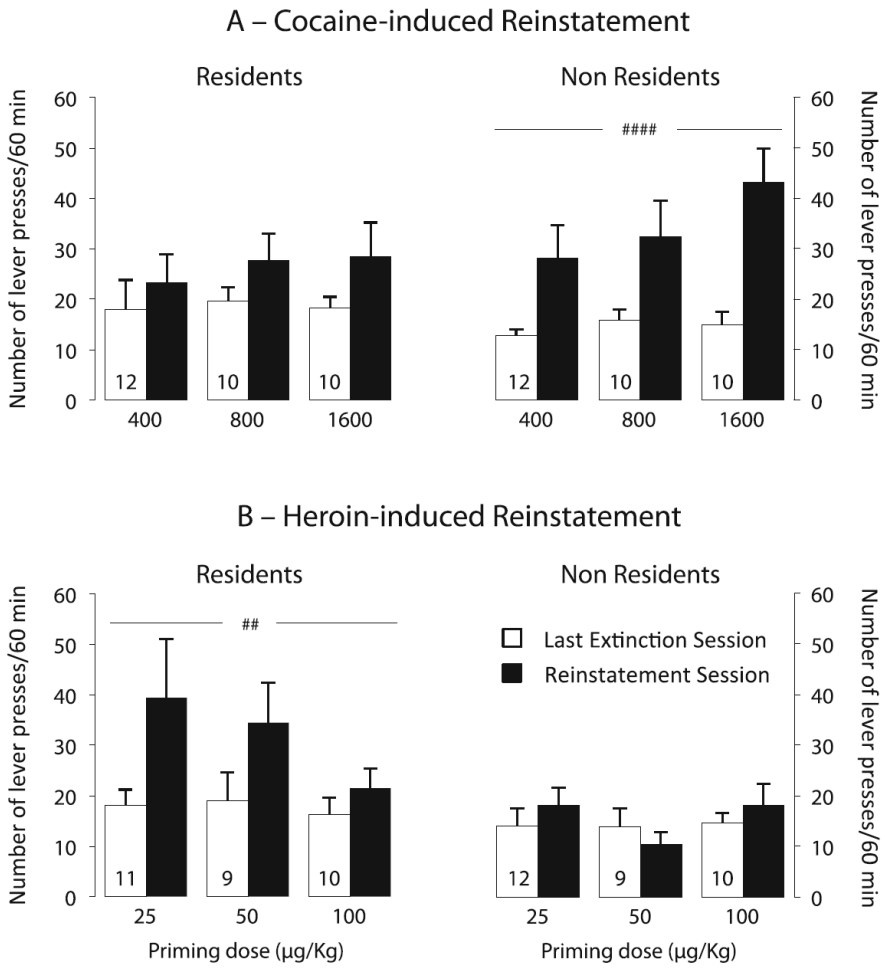

Fig. 4 Mean (±SEM) number of lever presses during the first hour of the last extinction session (*white bars*) versus the reinstatement session (*black bars*) in the resident and nonresident groups. At the beginning of the reinstatement session, independent groups of rats (*N* values are indicated by the *numbers within the white bars*) received a noncontingent intravenous (i.v.) infusions of one of three doses of cocaine (*top panels*) or heroin (*bottom panels*). Significant (##$p \leq 0.01$ and ####$p \leq 0.0001$) main effect of priming

this phenomenon is substance-specific. Rats that had been trained to self-administer both heroin and cocaine relapsed into heroin-seeking when primed with the former and into cocaine-seeking when primed with the latter. The present study indicates that drug-induced relapse is not only substance-specific but also setting-specific. Cocaine primings reinstated cocaine-seeking only in nonresident rats, whereas heroin primings reinstated heroin-seeking only in resident rats (except at the dose of 100 µg/kg). It should be noted that these results were not due to differences in drug SA during the training phase, as there was no correlation between the magnitude of lever pressing during the reinstatement phase and drug intake during the training phase.

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

The fact that heroin primings did not reinstate heroin-seeking in our nonresident rats appears to be at odds with the findings of previous studies that investigated heroin-primed reinstatement under similar housing conditions

(Fattore et al. 2003, 2005; Lenoir and Ahmed 2007; Leri and Stewart 2001; Leri et al. 2004; Luo et al. 2004; Sorge et al. 2005). These apparent discrepancies may be due to procedural differences, including the amount of drug self-administered during training, the route of priming administration, and the extinction procedures. However, when the experimental procedures were similar to those used here, heroin failed to reinstate heroin-seeking in nonresident rats. Lenoir and Ahmed (2007), for example, reported reinstatement of heroin-seeking in "long access" nonresident rats (which during training self-administered about 26 times more heroin than our rats), whereas the rats in the "short access" group (which during training self-administered about two times more heroin than our rats) did not exhibit reinstatement of heroin-seeking after priming doses of heroin very similar to those used here. Leri and Stewart (2001) found that nonresident rats trained to self-administer, on alternate days, cocaine and heroin (the latter at the dose of 25 μg/kg) did not show relapse into heroin-seeking after heroin primings.

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

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

Conclusions

The present study adds critical evidence to a growing body of data indicating fundamental differences between psychostimulant and opioid reward, as well as between psychostimulant and opiate addiction (Badiani et al. 2011; Badiani 2014). For the past 3 decades, the dominant trend in addiction research has been to emphasize the role of shared substrates of drug reward, with particular emphasis on the mesocorticolimbic dopaminergic system (Nestler 2004). However, both animal and human studies suggest that this "unitary" approach cannot fully account for the complexity of drug effects. Studies in rats (Ettenberg et al. 1982; Gerrits and Van Ree 1996; Pettit et al. 1984) have shown, for example, that chemical lesion or pharmacological blockade of the mesolimbic dopaminergic system severely impairs cocaine but not heroin self-administration. Furthermore, two [^{11}C] raclopride PET studies in humans (Daglish et al. 2008; Watson et al. 2014) have shown that heroin can produce subjective "high" without activation of the dopaminergic system. These lines of evidence indicate that the neural substrates of opiate reward do not completely overlap those of psychostimulant reward, making it somewhat less surprising that the same settings could influence in opposite directions the responsiveness to these two classes of drugs.

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

In summary, we reported here that the setting in which cocaine and heroin are taken can exert a powerfully influence on reward effects of these drugs. In particular, it appears that the susceptibility to relapse into drug-seeking behavior is, under certain conditions, substance-specific and setting-specific. Our results also suggest that heroin and cocaine addiction should be seen as distinct entities. Other pre-clinical and clinical findings, including the lack of pharmacological effective treatments for both cocaine and heroin addiction, support the notion that much is to be gained by taking in due account the substance-specific aspects of drug addiction (for a recent review, see Badiani et al. 2011). The differences between cocaine and heroin here illustrated might have important implications for therapy, suggesting, for example, that cognitive-behavioral approaches should be tailored so as to allow the addict to anticipate, and cope with, the risks associated in a substance-specific manner to the various environmental settings of drug use.

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

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8.2 MEETING COMMUNICATION:

Stendardo E, Avvisati R, Meringolo M, Marinelli S, Badiani A. Synaptic plasticity alterations in cortico-accumbens circuit of heroin self-administered Resident and Non Resident rats. (Verona, EBPS 2015).

Avvisati R, **Stendardo E**, Meringolo M, Marinelli S, Badiani A. Benzydamine self-administration in the rat: electrophysiological evidence for a central mechanism of action (Verona, EBPS 2015).

Avvisati R, Contu L, **Stendardo E**, Montanari C, Scattoni ML, Badiani A. Substance-specific influences of setting on drug reward: An ultrasonic vocalization study in rats self–administering heroin or cocaine. (Washington, SFN 2014).

Montanari C, De Luca MT, **Stendardo E**, Meringolo M, Contu L, Badiani A. The role of environmental context in the vulnerability to relapse into heroin and cocaine addiction: a pre-clinical investigation. (LaRochelle, EBPS 2013).

EBBS European Brain and Behaviour Society | ebps | Joint Meeting
Italy - Verona, Gran Guardia Palace
September, 12-15 2015

SYNAPTIC PLASTICITY ALTERATIONS IN THE CORTICO-ACCUMBENS CIRCUIT OF HEROIN SELF-ADMINISTERED RESIDENT AND NONRESIDENT RATS

Stendardo, Emiliana (1) | Avvisati, Riccardo (1) | Meringolo, Maria (1) | Merinelli, Silvia (2) | Badiani, Aldo (1,3)

We have previously reported the setting of drug self-administration has a powerful influence on drug intake(Caprioli et al. 2008). Rats that lived in the self-administration (SA) chamber (ResidentRats) tended to self-administer more heroin than rats that were exposed to the SA chamber only during testing (NonResidentRats living in a distinct home cage). Also the neurobiological effects of heroin are a function of setting. In situ hybridization and immunohistochemistry experiments have shown that the effects of heroin on the expression of FosmRNA and Fos in the reward regions of the brain are very different in Resident vs. NonResidentRats (Paolone et al. 2007; Celentano et al. 2009). Fos is a transcription factor that is thought to be implicated in the early stages of drug-induced neuroplasticity. Thus, we hypothesized that the setting may influence heroin-induced long term plasticity.In the present study, we used ex vivo electrophysiological recordings to investigate the effects of heroin SA on long term cortico-accumbens synaptic plasticity as a function of setting. Both Resident and NonResident rats were trained to self-administer heroin (25µg/kg/infusion) for 10sessions (3h each).After 14 days of abstinence from heroin the rats were killed and their brains excised to obtain parasagittal slices for field recordings. We found that after tetanic stimulation, LTP was greater in NonResidentRats (fEPSP amplitude about 160% of baseline) than in ResidentRats (140% of baseline). This suggests that cortico-accumbens (presumably inhibitory) inputs are reduced when the rats self-administer heroin in their home environment relative to a non-home setting and may explain why ResidentRats tend self-administer more heroin than NonResidentRats.

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Presenter and Poster Info

Stendardo Emiliana | stendardoe@gmail.com
Sunday, 13th September

9 FELLOWSHIP:

- Since November 2016 I have been working at Neurocentre Magendie Bordeaux France.
- From 1.11.2015 to 1.03.2016 I worked at University of Sussex, Brighton UK under Professor Hans Crombag supervision winning ERASMUS+ Student Mobility for Traineeship fellowship. In particular I was interested in studying the role of the BLA during cocaine and heroin choice using in vivo optogenetic technique in Non Resident rats.

  Protocol: Role of BLA in cocaine and heroin taking and choice

  Animals

  Male Sprague-Dawley rats, weighing 250–275 g at their arrival will be used in this study. The rats will be housed two per cage in a dedicated temperature and humidity controlled room with free access (except during self-administration sessions) to food and water under a 12 hours dark/light cycle (lights off at 7:00 AM).

  Surgery and BLA excitotoxic lesions

  After 1 week from arrival all rats will be anaesthetized with ketamine hydrochloride (120 mg/kg and xylazine (12 mg/kg) and implanted with a double lumen intravenous jugular catheter (15 cm) exited dorsally between the scapulae of the rats.

  Catheter implantation: After sterilization and shaving, a 2 cm incision will be made on the back of the rat in order to accommodate the base of the catheter with the two cannulas. Connective tissue will be forced to make space for the catheter base below the skin. A second incision will be made on the clavicle above the jugular vein. Tubing from the base of the catheter will be pulled through the incision on the back and be brought close to the jugular vein by passing the tubing under the skin just over the right shoulder. The end of the catheter tubing will be inserted into the right jugular

vein. Catheters will be flushed daily with 0.1 ml of a sterile saline solution containing 12.5 IU heparin

BLA lesions will be performed on the same day of catheters implantation for one group of rats and after self-administration training (day 11) for a second group. Rats will be positioned in a stereotaxic frame. Lesions will be performed with2 injection of 0.3 µl each of quinolinic acid (Sigma) dissolved in 0.1M phosphate buffer (PB; composition 0.07 M Na2HPO4, 0.028M NaH2PO4 in double-distilled water) to a concentration of 0.09M injected bilaterally using the following coordinates: anteroposterior (AP) -2.5; -2.8 mm, mediolateral (ML) ± 4.8 mm, dorsoventral (DV) -8.3 mm. AP and ML coordinates will be measured from bregma, DV will be measured from dura. Toxin will be infused using a 28-gauge stainless steel cannula attached with a polyethylene tubing to a 1 µl syringe (Hamilton) mounted on a infusion pump. At each site the cannula will be left in place for 2 minutes following 3 minutes infusion to allow diffusion away from the injection site. BLA sham lesion will be made similarly using vector for both groups. After surgery rats will be housed individually in standard Plexiglas cages.

Training and choice self-administration

Training will be done during the dark cycle in self-administration chambers enclosed in sound and light attenuating cubicles and equipped with two retractable levers and with cue lights positioned above each lever.

Days 1-10 Training Phase: rats will be transferred to the self-administration chambers immediately before the start of each testing session. The rats will undergo 10 consecutive daily 3-hour sessions to self-administer cocaine (400 µg/kg/infusion) and heroin (25 µg/kg/infusion) on alternate days (Caprioli et al., 2009). Cocaine and heroin will be each paired with one of the two retractable levers and a cue light (red or

green); the assignment of levers and cues will be counterbalanced for heroin and cocaine. At the start of each session, only the lever associated with the drug to be self-administered on that session will be extended and the appropriate cue light will turn on. The number of consecutive lever presses required to obtain a single infusion (Fixed Ratio) will be FR1 for sessions 1-4; FR2 for sessions 5 and 6; FR5 for sessions 7–10. After each infusion, the cue light will be turned off and the lever retracted for 40 second (time-out). Rats will not self-administer an average of 3 infusions of cocaine and 3 infusion of heroin on FR5 sessions will be excluded.

Days 11-16: On day 11 rats without lesions will have surgery and 5 days recovery. Other rats will be left undisturbed for 6 days in their cages.

Days 17-23 Choice Experiment: rats will undergo seven choice session (3 hour each). During the choice sessions, both levers will be extended and cue lights turned on. Rats will have the opportunity to self-administer either cocaine or heroin (at the same doses used during training) on an FR5 reinforcement schedule. Pressing on the left lever will reset the counter of the right lever, and vice versa. Once the animal will complete the FR5 on one of the levers, the drug will be administered and both levers will be retracted for a 10 min time out, after which the levers will be extended and the lights turned on for the next trial. Thus, the rats could self-administer a maximum of 17 infusions/session. Heroin will be delivered through the anterior lumen on 1 day and through the posterior lumen on the following day; the reverse sequence will be used for cocaine (Caprioli et al., 2009).

Perfusion and Histology

After behavioural testing, rats will be anesthetized and perfused using 0.9% saline followed by 4% paraformaldehyde (40 g powdered PFA in 1 1 phosphate buffer). The brains will be removed and post-fixed in 4% PFA for 2 h following which they will be

left overnight in 20% phosphate buffered sucrose solution. Sections will be cut at 60 µm and mounted on gelatine-treated slides which will be then stained with Cresyl Violet. Finally, injection sites and lesion extent will be mapped using a rat brain atlas with standardized coordinates (Paxinos & Watson, 1998).

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- From 18.05.2015 to 30.09.2015 I won the **Unipharma-Graduates Project** training fellowship and I worked at Sussex Neuroscience Building (University of Sussex, Brighton, UK) under Prof. Hans S. Crombag supervision. Specifically, the research involved training and development of in vivo optogenetics approaches in rodents. Practical training consisted of the following:
    - ✓ Construction of bilateral optical-fibers for centeal implanatation in rats and/or mouse
    - ✓ Brain-region specific expression of light-sensitive protein channels using AAV virus-mediated transfection
    - ✓ Implementation with behavioural (conditioning) preparations to optogenetically activate or inhibit brain circuitries.

Erasmus+

ERASMUS+ Programme, Key Action 1 – Student Mobility for Traineeship
A.A. 2014/2015

UNIPHARMA-GRADUATES PROJECT

Scientific coordinator:
Prof. Luciano Saso, School of Pharmacy and Medicine, Sapienza University of Rome

SCIENTIFIC REPORT

(May/September 2015)

"Optogenetic protocol to investigate neural circuits in an animal model of drug self-administration"

Trainee

Tutor/supervisor

University of Sussex, Brighton (UK)

28/10/2015

Optogenetics

Optogenetics (from Greek *Optikós* meening "visible"), Coined *Nature Methods*' 'Method of the Year' in 2010, is a neurobiological technique that uses light to activate and/or inhibited neural circuit that have been genetically modified to express light sensitive ion channels (opsin) with millisecond resolution. This technique passed many of the technical limitations of traditional techniques used in neuroscience research thanks to spatio-temporally precise causal control over cellular signalling.

This technology most commonly involves three core features (Fig.1):

- microbial opsins, members of an ancient gene family adapted from algae and archaebacteria, with each gene encoding a distinct protein that directly elicits electrical current across cellular membranes in response to light,
- methods for targeting specific opsin gene expression to defined cellular elements in the brain,
- methods for guiding precisely timed light to specific brain regions, cells or parts of cells while the experimental subject carries out behavioural tasks.

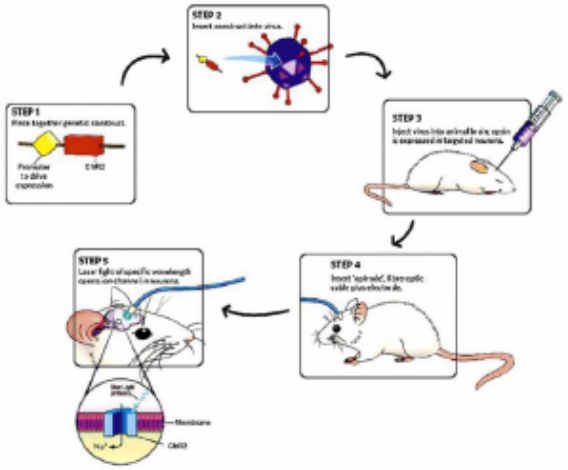


Figure 1.Schematic diagram of optogenetic techniques.

Microbial opsin variant

A number of optogenetic light sensitive ion channel are now available for both excitation and inhibition of neural circuits for use both in vitro and in vivo (Yizard et al., 2011). The first light gated proteins that have found utility in optogenetics were the bacteriorhodopsins, the halorhodopsins and the channelrhodopsins (Fig.2). Bacteriorhodopsins (the first-discovered members of this family, which pump protons out of the cell) and halorhodopsins (which pump chloride ions into the cell) are typically inhibitory in neural systems, hyperpolarizing current make it harder for neurons to fire action potentials; in contrast, channelrhodopsins allow positively

charged ions to flow freely through the opsin pore and so tend to be depolarizing and excitatory (Zhang et al., 2011).

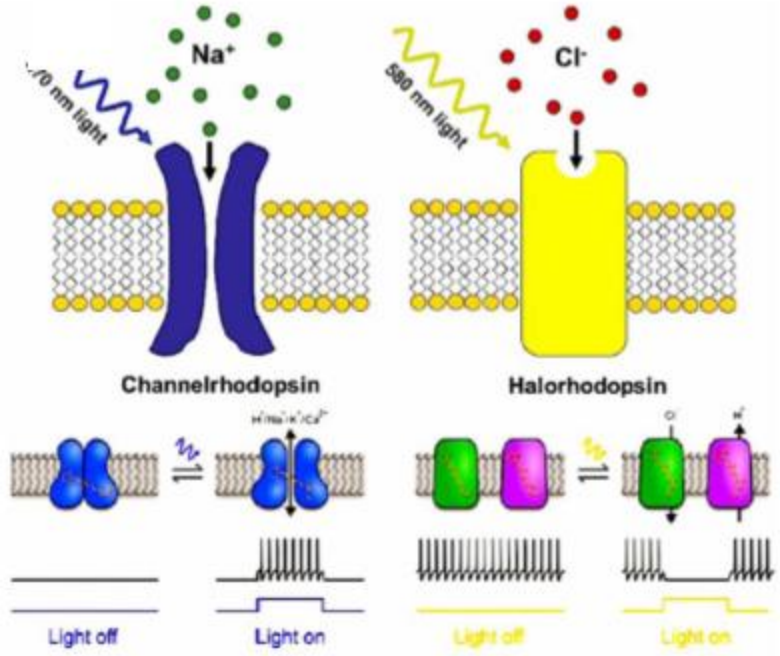
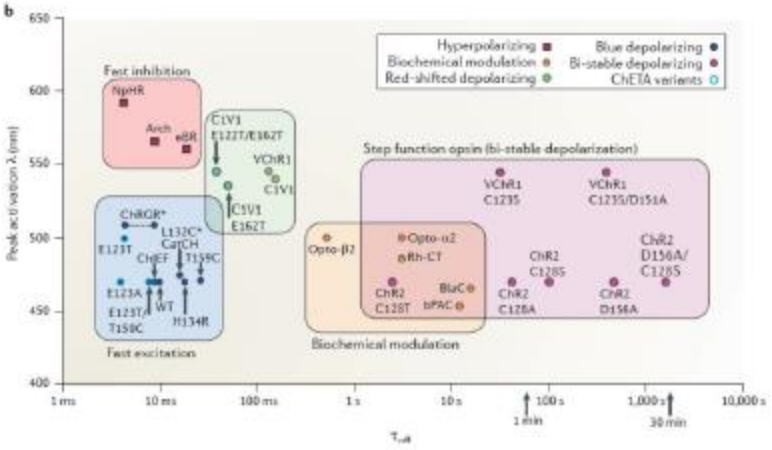


Figure 2.Light sensitive proteins Channelrhodopsin and Holorhodopsin.

However, the expansion of these protein families with faster kinetics, bistable properties, altered ion conductances and shifted color-response properties (Fig.3) now provides greater flexibility in experimental design and more powerful and refined manipulations (Tye & Deisseroth, 2012).

Figure 3. Microbial proteins characterized in terms of wavelenght activation spectra and decay kinetics.



Delivering genes coding for light sensitive proteins into target cells

Optogenetics employs the basic principles of gene therapy. Upon obtaining the exogenous gene (e.g. ChR2) along with a specific promoter sequence, it is re-packaged into an appropriate vector and introduced into the target cell (Fig. 1). Subsequently, cellular mechanisms transcribe and translate the inserted gene, resulting in the synthesis of ChR2 protein, which is then incorporated into the host cell membrane. These proteins are activated by illumination and thus, cellular activity can be altered by controlling the light switch.

There are three main methods for delivering of genes coding for light-sensitive proteins into target cells. Targeted cellular expression can be achieved by using specific promoters, recombinase based conditional systems, delivering a location specific injection, or by restricting activation through targeted light delivery (Fenno et al., 2011).

- Transfection: refers to non-viral methods of introducing the gene into host cells. This includes electroporation, DNA microinjection, liposomal transfection, and calcium phosphate precipitation. Such methods are relatively safe as it does not involve using infectious biological agents (Lin et al., 2012), However, two significant limitations are its effectiveness in achieving a high level of expression on the host cell as well as practicality issues for use in vivo.
- Viral transduction is currently the most popular method used. This technique involves using a recombinant virus containing for example the ChR2 gene, which is injected into a specific region on the gene and hence expressed either selectively or broadly. A multitude of viral vectors, including adenoviruses, adenoassociated viruses (AAV), retroviruses and lentiviruses, have been used experimentally. By using viral vectors, precise cells can be targeted by using specific promoters. However, one significant limitation of using viral delivery is that there is an upper limit to the length of the gene used. The promoter chosen needs to be small (less than 4 kb), precise, and strong (Fenno et al. 2011), and therefore this downside can be circumvented by using Credriver animals and Cre-dependent viruses. Another limitation is that viral transduction could lead to undesirable side effects such as triggering an adverse immune response, causing unspecific systemic dissemination of viral vectors, or potentially even lead to an overexpression of the ChR2 gene.
- Transgenic or knockin animals. The first example involves the generation of a transgenic ChR2-YFP (fusion protein) expressing mouse line under the control of the Thy1 promoter (Arenkiel et al., 2007). One feature of this method is that the

transgene expression increases with animal maturation, contributing to the high level of ChR2 expression in an adult mouse. Furthermore, Techniques involving the use of transgenic animals have an advantage over that involving viral transduction in that there are no viral payload limitations and larger promoters can be used which confer higher control over transgene expression. Additionally, it allows for a more widespread expression of ChR2 in various locations within the brain. One limitation is the inability for blue light to penetrate into deeper regions, such as deep brain tissues (Flusberg et al., 2005).

Training goal

During my training at University of Sussex (Brighton, UK) under the supervision of Professor Hans S. Crombag I provide a protocol for constructing implantable optical fibers, bilateral patch cables and also for virus injection. We chose the implantable optical fibers (instead of optical fiber inserted acutely) because they are permanently inserted into brain tissue and fixed to the skull, reducing tissue damage and greatly enhancing experimental throughput. Implantable optical fibers also ensure that the same tissue is repeatedly stimulated or inhibited over multiple behavioural sessions, whereas optical fibers inserted acutely can potentially stimulate or inhibit different regions of tissue on the basis of movement inside the guide cannula. Furthermore, as each acute experiment requires a new fiber per behavioural session, there is the possibility that brain tissue can be damaged with repeated insertion of an optical fiber. Sparta et al., 2011 observed less tissue damage at the implantation site of optical fibers on the basis of histological examination after prolonged behavioural testing and light emission from the implantable fibers was not substantially diminished over time (Fig. 4).

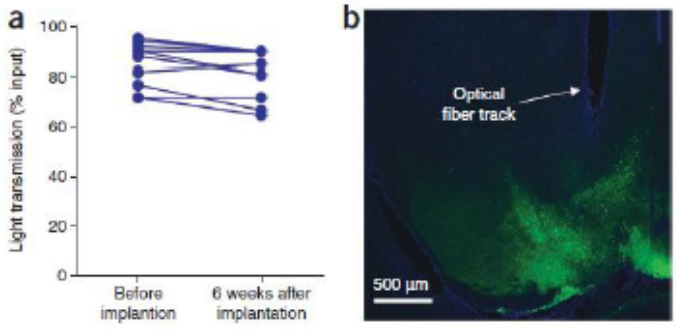


Figure 4. a)Light transmission from implantable optical fiber before and after implantation. b)Typical demage associated with the implantation of an optical fiber secured for 8 weeks.

Construction of implantable optical fiber

- Strip 25 mm of 200 µm core fiber using a micro-stripper while keeping the fiber attached to the fiber spool. Cut the fiber from the spool using diamond knife. Leave 10 mm of unstripped fiber (Fig. 5a).
- Prepare heat-curable epoxy. Insert the ferrule into the vice with the convex side pointing down. To test the viability of the ferrule, insert the stripped end of the fiber into the ferrule. The fiber should slide into the ferrule bore without obstruction. Remove the stripped fiber from the ferrule. Add one drop of epoxy to the flat end of the ferrule. Wipe any excess epoxy off. If epoxy dries to the side of the ferrule, interfacing the implanted optical fiber to the patch cable will be difficult. Insert the stripped end of the fiber into the ferrule and thread it through, leaving an extra 5 mm of stripped fiber exposed. Cure the epoxy bead using a heat. Epoxy will turn black/dark purple when fully cured (Fig. 5b).
- Score the fiber at the convex end of the ferrule with diamond knife. Tap the fiber off with your finger (Fig. 5c). Score the fiber exactly at interface of the ferrule.
- Polish the convex end of the ferrule using a hemostat to hold the ferrule securely in place (Fig. 5d). To polish, make 20 rotations on each grade of polishing paper (four grades total). Polish in this order: 5, 3, 1, 0.3 µm. The ferrule needs to be perpendicular to the polishing paper. Polishing in a vigorous motion may damage the fiber core.
- Secure the remaining unstripped fiber under a piece of Scotch tape secured to a flat surface. Score the stripped fiber with a diamond knife to the desired length (1–2 mm greater than the ventral coordinates of the brain region of interest; Fig. 5e). When scoring the fiber, hold the diamond knife perpendicular to the fiber. Score in one direction in a single motion. Do not cut the fiber completely with the diamond knife. This will damage the fiber core. Use a hemostat to pull on the ferrule with the stripped end remaining secured in the Scotch tape. To ensure an even break, apply pressure to the end of the Scotch tape. The fiber should snap at the scored length. For further precision, examine the scored end of the optical fiber under the surgical microscope to determine whether there is any damage to the fiber core.
- Store the implantable fibers in a box in which the fiber is unobstructed (Fig. 5f).

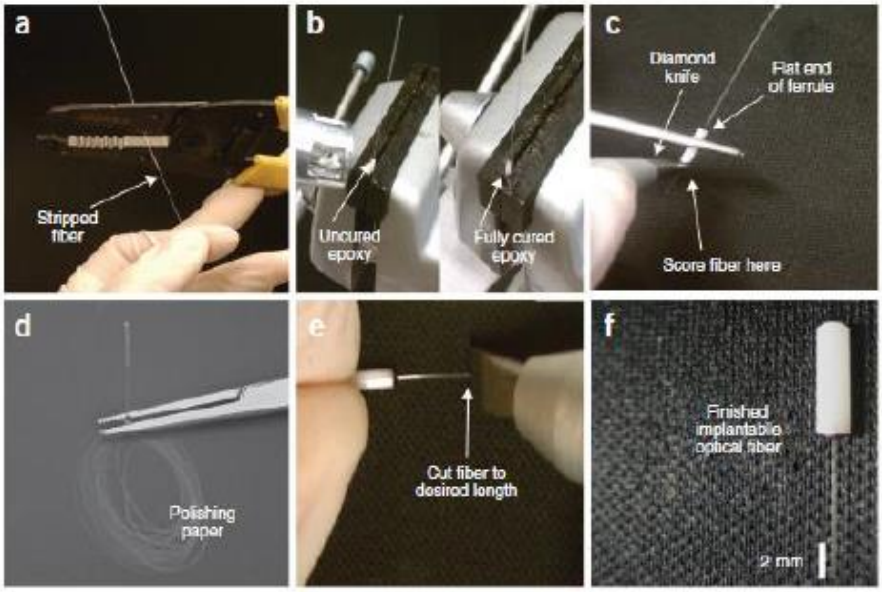


Figure 5. Construction of implantable optical fiber.

Construction of patch cables and bilateral patch cables

- Putting the optical fiber in the furcation tubing: cut the 50µm core multimode optical fiber and furcation tubing to the desired length (approximately 200–300 cm). Cut the furcation tubing 50 mm shorter than the length of the optical fiber. Thread the optical fiber through the furcation tubing (Fig. 6a).
- Strip 25 mm of the optical fiber at both ends (Fig. 6b).
- Insert the multimode FC MM ferrule assembly, ferrule end down, into the vice. Apply epoxy until the back end of the ferrule is filled to the top (Fig. 6c). Before adding the epoxy to the ferrule, insert the stripped fiber into the ferrule to ensure that the bore diameter is correct. Thread the stripped end of the fiber through the ferrule opening until it stops (where the unstripped portion of the fiber starts; Fig. 6c). You will observe the stripped fiber sticking out of the ferrule end. Heat the epoxy with the heat gun.
- Another ferrule is epoxied at the other end of the patch cable: slide the protective boot over the opposite end of the optical fiber and attach it to the ferrule. Score the excess fiber at the end of the FC connector with a diamond knife and tap the excess fiber off with your finger

(Fig. 6d). Score exactly at the opening of the ferrule's FC end. Leaving exposed fiber will damage the fiber core.

- Polish the FC end of the ferrule. To polish, insert the FC end of the ferrule into the polishing disc and make 20 figure 8 patterns on each grade of polishing paper. Polish in this order: 5, 3, 1, 0.3 μm (Fig. 6e).
- Attach and polish the ferrule as described before.
- Place a 2-cm piece of heat-shrink tubing over the interface of the furcation tubing and the ferrule to prevent light emission.
- To increase structural integrity of the patch cable, place a bead of 5-min epoxy on the exposed fiber between the ferrule and the furcation tube (Fig. 6f).
- Store patch cables until the test light output of the patch cable.

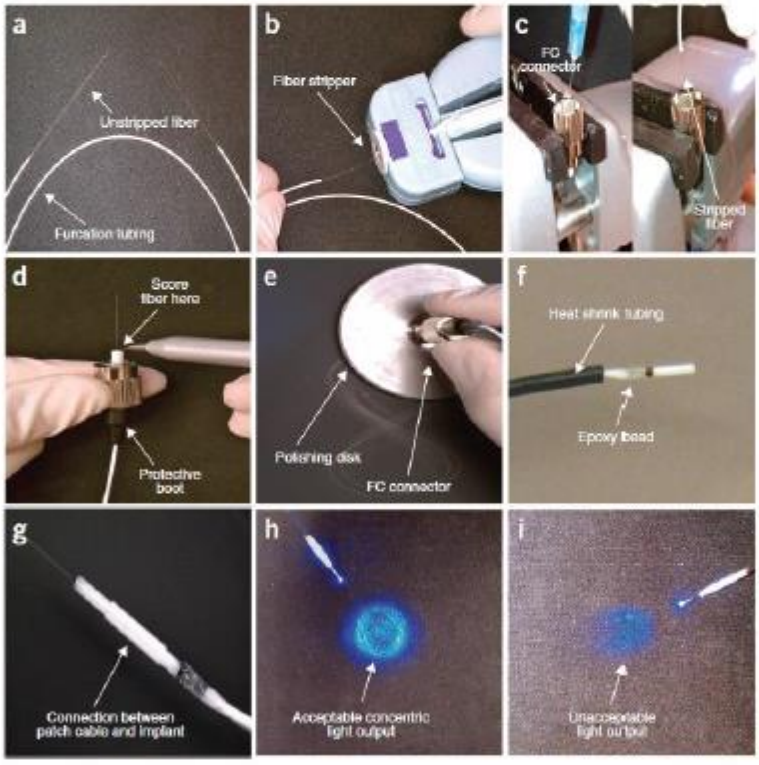


Figure6. Construction of patch cables for use in in vivo optogenetic experiments

Measuring light output of implantable optical fibers and patch cables

Light output from the implant should be more than 70% of the input light from the patch cable. Connect the FC connector of the patch cable into the FC port on the coupler of the laser. Turn the laser driver on and wait 15 min for laser stabilization. The patch cable should emit a bright, concentric circle of light (Fig. 6 h, i). If the light is not concentric, adjust the coupling between the optical fiber and the laser. If no improvement in light output is observed with the coupler adjustment, make a new patch cable. Measure the intensity of the light output through the patch cable using the optical power meter. Set the wavelength on the power meter to match that of the laser being used. Position the ferrule attached to the end of the patch cable directly above the centre of the sensor. An ideal 50 μmcore patch cable should have light loss not exceeding 30% and should emit a concentric circle of light. For example, if the laser output is 20 mW, the patch cable output should be between > 14 mW. If you are using a bilateral patch cable, output should be equivalent on both sides.

Once an acceptable patch cable is constructed, decrease the intensity of the laser driver until 10 mW of light output through the fiber is maintained. Insert approximately 3 mm of the 127 μm ferrule of the patch cable into the ceramic split sleeve. Insert the implantable optical fiber into the remaining space available of the ceramic split sleeve so that the two ferrules are in physical contact with each other (Fig. 6 g). Make sure the implantable optical fiber makes direct contact with the end of the 127-μm ferrule patch cable. Use the window on the sleeve to determine connectivity. Measure the light intensity through the implantable optical fiber using the optical power meter (Fig. 6h, i). An ideal implantable optical fiber should have < 30% light loss and emit a concentric circle of light. As the patch cable is set to 10 mW, an acceptable implantable optical fiber should emit between 7 and 9 mW of light. Record the percent of light transmission for each implantable optical fiber.

Virus injection and Implantation of the optical fibers in the rats' brain

Anesthetize and shave rat's head. Place the rat in the stereotaxic frame. Apply ophthalmic ointment to eyes to prevent drying and wipe the surgical area with Betadine and alcohol. Use a scalpel to make a 1–1.5-cm incision through the midline of the scalp. By using the forceps, pull back the skin into four quadrants to ensure a large working surgical surface. Identify Bregma and Lambda. Level the skull using the anterior-posterior tilt and medial-lateral tilt on the stereotax. To level, place the tip of drill on Bregma and measure the dorsal-ventral (D/V) coordinates. Move the arm to Lambda and record the D/V measurement of the drill back on Bregma. Move the drill ± 1.0 mm laterally and record the D/V measurements. Move the drill to the appropriate coordinates and

drill the skull. Once the skull is removed, use a 26-gauge syringe tip to break through the dura without damaging the cortex. The viral vectors can be injected through a small diameter Hamilton syringe glass needle mounted onto a micro-pump such as the WPI Ultra Micro Pump III, which is directly attached to a stereotactic frame, targeted to the brain region of interest; the use of fine needles minimizes the extent of tissue damage (Zhang et al., 2010). Once the virus is injected, drill two holes into the skull to insert the anchoring screws that will be used to secure the head cap. Attach the stereotaxic adapter for implantable optical fibers to the stereotaxic arm. Insert 2 mm of the implantable optical fiber into the stereotaxic adapter. Align the implantable optical fiber such that it is perpendicular to the skull. Lower the stereotaxic arm with the implantable optical fiber into the brain region of interest. When inserting the optical fiber in the brain, advance the fiber ventrally at a rate of 2 mm/min. Apply the head cap cement evenly around the implantable optical fiber. Use a spatula to smooth the head cap into a sphere. Leave at least 4–5 mm of the ferrule of the implantable optical fiber exposed (i.e., not covered by head cap cement) to ensure that the fiber can be interfaced with patch cable. Let the head cap dry until the cement is completely cured. Place the optical fiber protectors over the ferrule on the implantable fiber. Place the mouse into a clean cage over a heating blanket until the mouse fully recovers from anesthesia. Monitor the mouse's body weight and visually inspect it daily to ensure proper recovery from surgery. The mouse will be ready for behavioural experimentation once its body weight returns to pre surgery levels and when opsin expression is sufficient for the manipulation of neural circuits. Depending on the promoter used to drive opsin expression, this can take approximately 2–4 weeks for optimal expression in cell bodies and approximately 4–6 weeks for optimal expression in cell fibers and terminals.

Future studies: Optogenetics inhibition of heroin seeking in rats

The aim of my PhD thesis is to observe the possible difference in the synaptic plasticity of the cortico-accumbens circuit in Resident and Non Resident rats (Caprioli et al. 2007) that self-administer heroin. To do that we performed ex vivo extracellular field recordings. A stimulant bipolar electrode was placed on the infra-limbic prefrontal cortex and a recording glass pipette was placed on the Nucleus Accumbens core.

After this training period I will be able to perform in vivo optogenetic (Fig. 7) on the same experimental design. In particular I would like to examine the roles of the prelimbic cortex (PL), the nucleus accumbens core and the PL projections to the NAcore during cue induced reinstatement test using inhibitory optogenetics with an adeno-associated virus containing halorhodopsin or archaerhodopsin in rats that had previously self-administered heroin. Our last challenge will be to

improve our protocol to perform self-administration and laser stimulation in the same experimental session. These experiments could shed light on the hypotheses regarding the involvement of the projection from the prelimbic cortex (PL) to the core sub compartment of the nucleus accumbens (NAcore) in the relapse to drug seeking (Kalivas et al., 2005).

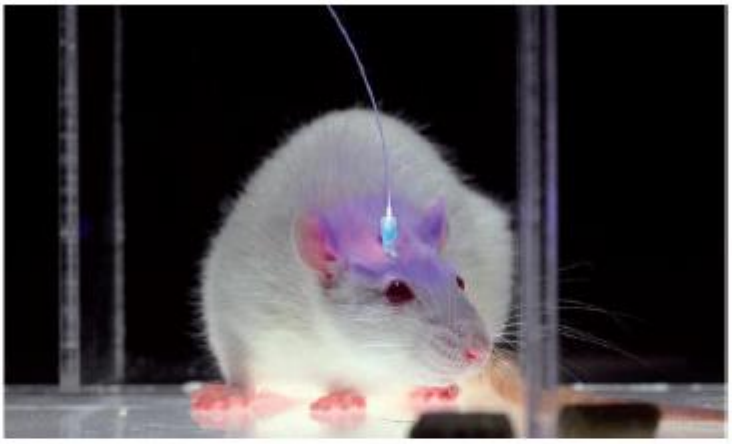


Figure 7. In vivo optogenetic experiment.

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