UNIVERSITA' DEGLI STUDI DI VERONA

FACOLTA' DI MEDICINA E CHIRURGIA

Dipartimento di Patologia e Diagnostica

SCUOLA DI DOTTORATO DI

Scienze Biomediche Traslazionali

DOTTORATO DI RICERCA IN

Biomedicina Traslazionale

CICLO XXV

TITOLO DELLA TESI DI DOTTORATO

MOLECULAR AND CELLULAR MECHANISMS UNDERLYING COGNITIVE NEUROADAPTATION IN ADDICTION: AN IN VIVO-VITRO INTEGRATIVE APPROACH

S.S.D BIO14/FARMACOLOGIA

Coordinatore: Chiar.mo Prof. Cristiano Chiamulera

Tutor: Chiar.mo Prof. Cristiano Chiamulera

Dottorando:

Dott. Vincenzo Tedesco

Abstract

Tobacco use through cigarette smoking is the leading preventable cause of death in the developed world. The pharmacological effect of nicotine plays a crucial role in tobacco addiction. Nicotine dependence has a huge impact on global health and although several medications are available, including a wide range of nicotine-replacement therapies (NRTs), bupropion, and recently approved nicotinic receptor partial agonist varenicline, at best only about a fifth of smokers are able to maintain long-term (12 months) abstinence with any of these approaches. Thus, there is a need to identify more effective treatment to aid smokers in maintaining long-term abstinence.

Nicotine is positively and negatively reinforcing and leads to the development of "operant conditioning" (motivated behaviour to nicotine consumption) in smokers during the acquisition phase of addiction. Several preclinical and clinical studies have also underlined the importance of non-pharmacological factors, such as environmental stimuli, in maintaining smoking behaviour and promoting relapse. Initially neutral stimuli that are repeatedly paired with a reinforcing drug (e.g. lighter) acquire a new conditioned value (conditioned stimuli, CS) through "Pavlovian conditioning" and become able to elicit craving even in the absence of the drug. Indeed smokers are particularly reactive to smoking/nicotine related CS. This phenomenon is called cue-reactivity and involves a vast array of physiological, psychological and also behavioural responses, such as decrease in heart rate and blood pressure and/or increase in skin conductance and skin temperature, increase in craving and urge to smoke and/or mood change, and also change in smoking behaviour (e.g., latency to smoke, cigarette puff volume and frequency, amount of cigarette consumed and relapse to smoking behaviour). Given the importance of the learned association between stimuli and nicotine in the phenomenon of relapse to nicotine-seeking behaviour, it has been proposed that treatment that disrupts the nicotine-associated memories could act as a pro-abstinent and anti-relapse therapy.

After learning experience, memories are stored by a process called consolidation. Operant conditioning (also called instrumental learning) and Pavlovian conditioning lead to different drug-related memories formation (instrumental memories and Pavlovian memories) responsible for the relapse even after prolonged abstinence. For at least a century it has been a dogma that initially labile memory (short-term memory) is consolidated by the passage of time and become stable and permanent (long-term

memory). However converging evidence from animal and human studies have revealed that memories may return to a vulnerable phase during which they can be updated, maintained and even disrupted. The retrieval of a memory indeed may destabilize the consolidated memory that requires a new process to be maintained. This hypothetical process is called reconsolidation. There is strong evidence that Pavlovian fear memories undergo reconsolidation and it was proposed that interventions to disrupt reconsolidation may help for specific and selective inhibition of fear related memories and, similarly, appetitive memories (i.e., for drug addiction). The disruption of drug-related memories reconsolidation has been proposed as a potential therapeutic target to prevent the CS-induced relapse in ex drug-addicts. Several animal studies have shown that the reconsolidation of some drug-related memories can be disrupted by the administration of an amnestic drug contingently upon retrieval of the memory acting at specific molecular levels (i.e. adrenergic and glutamatergic systems). However it is not known if all memories or only certain kind of memories could be retrieved and reconsolidated or disrupted. To date reconsolidation of instrumental memories is still under discussion and behavioural experiments targeting the pure instrumental memory reconsolidation disruption are needed to clarify this issue.

There are many boundary conditions related to memory retrieval and reconsolidation (i.e., retrieval session duration, memory age and strength). Furthermore memory reconsolidation disruption could only be determined through its absence during reinstatement or renewal test in behavioural studies. This make difficult to interpret the lack of effect of amnestic drugs observed in some circumstances. Infact it is hard to understand if reconsolidation disruption is inhibited by the presence of boundary conditions or by the fact that some memories can not be reactivated and disrupted. From this perspective ex-vivo molecular experiments performed after memory retrieval could directly demonstrate memory reactivation and confirm reconsolidation occurrence supporting behavioural data. It has been demonstrated that zinc finger 268 (Zif268) expression increases in basolateral amygdala after the presentation, in a memory reactivation session, of conditioned stimuli compared to non conditioned. Since Zif268 is also considered a specific marker correlating memory reconsolidation, immunohistochemistry assessment of the expression level of Zif268 in BLA after memory reactivation can help to estabilish if memory reconsolidation is engaged, or not, in our laboratory conditions.

The main objective of the present thesis was to study if it is possible to disrupt

Pavlovian and instrumental nicotine-related memories reconsolidation by β-adrenergic receptor antagonist propranolol, or N-methyl-d-aspartate receptors (NMDARs) antagonist MK-801 respectively. We also verified the feasibility and reliability of Zif268 expression assessment by immunohistochemistry after retrieval of Palovian memory in rodents.

The experimental approach used to address this issue was the laboratory model of nicotine self-administration in rats, based on the paradigms of operant and Pavlovian conditioning to nicotine and nicotine-associated cues. We performed two studies in which the pharmacological treatment (propranolol or MK-801) was associated to retrieval of Pavlovian or instrumental nicotine-related memories. We therefore assessed the effect of these pharmacological treatments on relapse to nicotine seeking behaviour. Retrieval of Pavlovian memories consists in presenting the CS in the absence of US. Retrieval of instrumental memories consists in allowing the animal to press the lever previously paired to nicotine reinforcement, without nicotine infusion. We also performed an immunohistochemistry assay in which the Zif268 level of expression was determined in basolateral amygdala after nicotine CS presentation.

Results showed that propranolol given after retrieval of Pavlovian memories (30 CS presentations) did not reduce the relapse to nicotine seeking behaviour compared to control groups that received vehicle injection in both retrieved or no-retrieved groups. As suggested by Tronson and Taylor (2007), memory extinction (a new learning by which CS previously associated with a reinforcer become newly associated with no outcome) instead of memory reconsolidation, may occur under similar conditions after memory retrieval. The length of the retrieval session is an important determinant of whether reconsolidation or extinction occurs after memory retrieval and it could be the critical factor in extinction and reconsolidation protocols. However there were no differences between retrieved and no-retrieved control groups suggesting that retrieval session was not inducing memory extinction in our conditions. Given that only Pavlovian memories were retrieved in our retrieval session by the CS presentation, it could be that instrumental memories (not retrieved indeed not disrupted) supported lever pressing during reinstatement test. It could also be possible that instrumental memories do not undergo reconsolidation and could not be disrupted. To address this issue we tested the effect of MK-801, known to be more effective against instrumental memory than propranolol, given 30 minutes before the retrieval of instrumental memories. Results showed that instrumental memory retrieval per se increased nicotine-

seeking behaviour in vehicle treated rats. Pre-retrieval MK-801 injection reduced the difference in nicotine-seeking behaviour between retrieval and no-retrieval condition that is observed with vehicle, but did not prevent the relapse to nicotine-seeking behaviour when compared to control groups. This effect suggests a potential role of MK-801 in inhibition of the memory destabilization process instead of reconsolidation disruption. Further experiments in which MK-801 was given after memory destabilization was engaged (i.e. given after memory retrieval) showed that MK-801 prevented the relapse to nicotine-seeking behaviour. Finally immunohistochemistry showed an increased level of Zif268 expression in basolateral amygdala after retrieval of Pavlovian nicotine-related memories. These data confirm the validity and feasibility of immunohistochemistry to assess the expression of molecular markers correlating reconsolidation such as Zif268 after memory retrieval.

In conclusion, our findings suggest that: i) propranolol did not disrupt Pavlovian memory reconsolidation in our conditions, ii) MK-801 given prior to retrieval session could prevent instrumental memory destabilization, but did not disrupt memory reconsolidation in our conditions, iii) MK-801 given after retrieval session disrupted memory reconsolidation in our conditions, iiii) immunohistochemistry is a feasible technique to investigate the expression of molecular markers correlating reconsolidation such as Zif268, thus it can be used to support our future behavioural studies. These data suggest that instrumental memory could be responsible for the lack of effect of some anti-relapse pharmacological treatments and that this kind of memory can be disrupted. New and specific pharmacological intervention, acting at specific molecular mechanisms that underlies reconsolidation of different kind of memories (i.e. Pavlovian but also instrumental memories), could be used as a potential co-adjuvant to current therapeutic interventions for smoking cessation and abstinence maintenance.

1. INTRODUCTION

Tobacco use is the leading global cause of preventable and premature death. It is one of the main causes for a number of chronic diseases, including cancer, lung diseases and cardiovascular diseases. Cigarette smoking is also a risk factor for respiratory tract infections, reproductive disorders, osteoporosis, adverse post-operative events such as delayed wound healing, duodenal and gastric ulcers and diabetes (Vineis et al., 2004). Tobacco use kills nearly 6 million people and causes hundreds of billions of dollars of economic damage worldwide each year. If the current trends continue, by 2030 tobacco will kill more than 8 billion people worldwide each year (World Health Organization Report 2011). Seventy percent of smokers say that they would like to quit, eighty percent who attempt to quit on their own return to smoking within a month, and each year, only three percent of smokers quit successfully.

Smoking-related diseases are a consequence of prolonged exposure to toxins in tobacco smoke; therefore the most dangerous aspect of smoking is that constituents are highly addictive.

Tobacco addiction is reported both in the Diagnostic and Statistical Manual of Mental Disorders, 4th edn. and in the World Health Organization's International Classification of Diseases, version 10.

The criteria for defining drug dependence are the following:

Primary criteria:

- Highly controlled or compulsive use
- Psychoactive effects
- Drug-reinforced behaviour

Additional criteria:

- Addictive behaviour often involves
  -Stereotypic patterns of use
  -Use despite harmful effects
  -Relapse following abstinence
  -Recurrent drug cravings
- Dependence-producing drugs often induce
  -Tolerance
  -Physical dependence
  -Pleasant (euphoriant) effects.

Tobacco dependence fit all the above criteria. It is a behavioural disorder due to chronic exposure to a psychoactive substance, nicotine (Abrams et al., 1999). Importantly, smokers do not just self-administer nicotine while smoking, but they experience the pharmacological effect of nicotine in a context rich of environmental stimuli. Indeed, tobacco addiction arises from an interplay of: i) pharmacological effect of nicotine, ii) psychological and physiological susceptibility of the individual (e.g. genetic predisposition, psychiatric disorder, impulsivity) and, iii) social and environmental influences (including tobacco product and marketing) (Caggiula et al., 2001, Field et al., 2009; Karp et al., 2006; Pomerleau, 1995; Rodriguez et al., 2008).

1.1. Neurobiology of Nicotine

1.1.1. Absorption

Nicotine is an alkaloid that constitutes approximately 0.6–3.0% of the dry weight of tobacco. It is a psychoactive addictive drug. Inhalation of smoke from a cigarette distils nicotine from the tobacco in the cigarette. Smoke particles carry nicotine into the lungs, where it is rapidly absorbed into the pulmonary venous circulation. The nicotine then enters the arterial circulation, rapidly crosses the blood barrier in approximately 7-10 seconds and move into the brain, where it binds to nicotinic cholinergic receptors (nAChR) (Hukkanen et al., 2005).

1.1.2 Nicotinic cholinergic receptors and neuroadaptation

nAChR is a ligand-gated ion channel that normally binds acethylcholine (Albuquerque et al., 2009). It consists in five peptidic subunits: the mammalian brain expresses nine α subunits and three β subunits. Usually the receptor is composed of two α and three β subunits arranged to form a pore (Jensen et al., 2005). The receptor α4β2 is the most abundant and the principal mediator of nicotine dependence. Ligand binding occurs via the α subunit, producing a conformation change that opens the cationic channel and allows sodium and calcium ion influx, after few milliseconds the channel close and become desensitized. In the absence of agonist, the receptor return to the standby stage where it is closed but “activable”. Moreover chronic nicotine exposure increases nicotine or acethylcholine (ACh) binding sites in the brain, a phenomenon known as up-regulation.

When brain nicotine levels decrease, e.g. during abstinence, the up-regulated

receptors return to the standby state leading to a hyperexcitability of cholinergic system. This hyperexcitability is associated with withdrawal effect: the symptoms of craving and withdrawal, indeed, begin in smokers when the up-regulated desensitized α4β2 receptors become responsive during a long period of abstinence, such as overnight. Nicotine binding of these receptors during smoking alleviates craving and withdrawal (Dani & Heinemann, 1996). Smokers regulate the daily amount of cigarette smoking in order to maintain near-complete saturation, and thus desensitization, of the α4β2 receptors. Thus smokers are probably attempting to avoid withdrawal syndrome when maintaining a desensitized state.

1.1.3 Nicotine and neurotrasmitters release

nAChRs are localized mainly at presynaptic level on a number of different type of neurons, such as on glutamatergic, dopaminergic, noradrenergic and gamma aminobutyric acid (GABA) neurons in the ventral tegmental area (VTA), substantia nigra, and striatum (Figure 1). Thus nicotine modulates not only ACh level but also dopamine (DA), glutamate and GABA activity (Albuquerque et al., 1997; Alkondon et al., 1997; Gray et al., 1996; Guo et al., 1998; Ji et al., 2001; Jones et al., 1999; Jones & Wonnacot, 2004; Li et al., 1998; Mansvelder & McGehee, 2000; Marubio et al., 2003; McGehee & Role, 1995; McGehee et al., 1995; Radcliffe & Dani, 1988; Radcliffe et al., 1999; Role & Berg, 1996; Wonnacott, 1997, Yin and French, 2000).

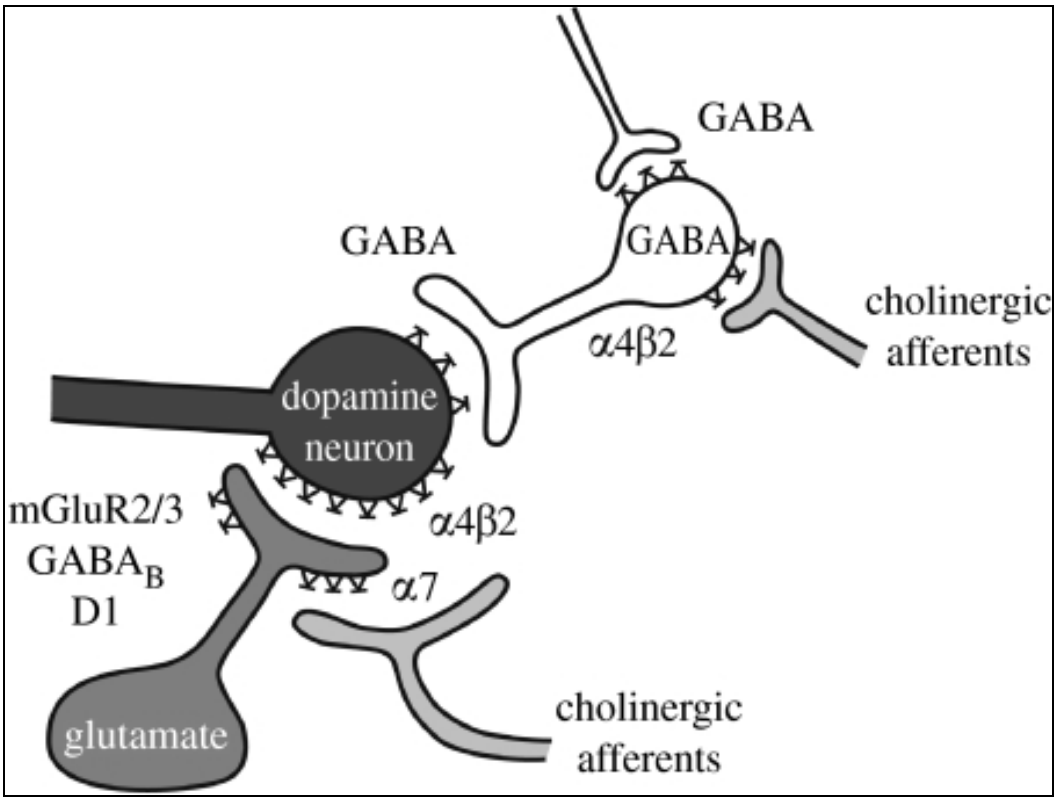

Figure 1. Schematic drawing of dopaminergic, gabaergic, glutamatergic and cholinergic neurons interaction. nAChRs are localized mainly at presynaptic level on glutamatergic, dopaminergic, and gabaergic neurons. Abbreviations in the text, D1 = dopamine receptor 1, mGluR2/3 = metabotropic glutamate receptor type 2 or 3. Image taken from Balfour, 1994.

It is widely accepted that nicotine dependence, similarly to other drugs of abuse (such as cocaine, amphetamine, etc.), arises from nicotine action on dopaminergic neurons in the mesocorticolimbic system. This system is also called reward pathway and involves dopaminergic neurons located in VTA and their projection into the striatum, amygdala, prefrontal cortex and the shell of nucleus accumbens (Figure 2).

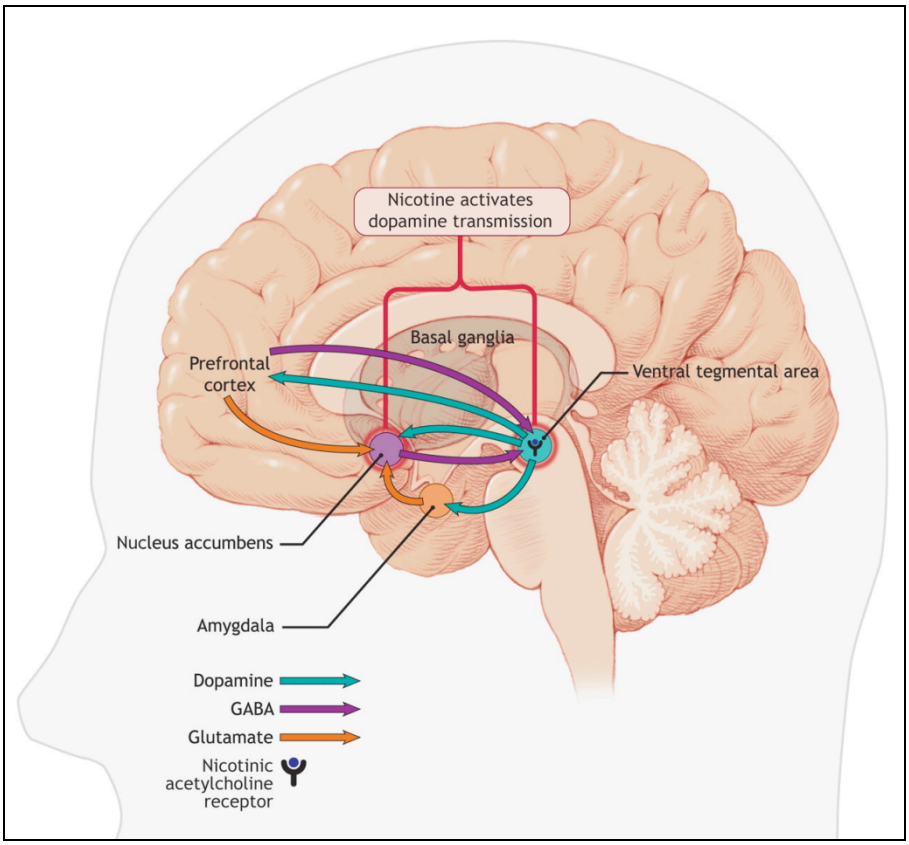

Figure 2. Schematic drawing of mesocorticolimbic pathway, mediating nicotine dependence. Nicotine stimulates nAChR located in the VTA, resulting in release of DA in the nucleus accumbens. Neurons projecting from the prefrontal cortex and amygdala modulate the release of DA in the nucleus accumbens. GABAergic neurons projections modulate DA release in nucleus accumbens and VTA. Image taken from Le Foll & George, 2007.

It has been well established that the activation of mesocorticolimbic DA pathways is associated with drug reward (Di Chiara, 2000), where increased neuronal firing in the VTA (Clarke, 1990; French, et al., 1996) and DA release in the nucleus accumbens (Di Chiara and Imperato, 1988) are neurochemical correlates of psychostimulant self-administration. Laboratory animals self-administer nicotine, indicating that the drug exerts effects on mesocorticolimbic DA neurotransmission in a comparable manner to other psychostimulant drugs of abuse. Supporting a predominant role for enhanced dopaminergic neurotransmission, nicotine concentrations self-administered by rodents and humans also increase DA release in the nucleus accumbens (Imperato, et al., 1986; Nisell, et al., 1994) and activate DA neurons in the VTA (Pidoplichko, et al., 1997). Moreover it has been shown that inhibition of DA release in nucleus accumbens by antagonist drugs attenuates reinforcing properties of nicotine, leading to a decrease in nicotine self-administration in rats (Corrigal & Coen, 1989; Stolerman & Shoaib,

1991.).

As stated above, nicotine also augments both glutamate and GABA release: the former one facilitates DA release, the latter inhibit DA release. Chronic exposure to nicotine induces desensitization of some types of nAChR, but not all. As a results GABA inhibitory action diminishes while glutamate-mediated excitation persists, leading to an increase dopaminergic neurons firing and enhancement in responsiveness to nicotine (Mansvelder & McGhee, 2000; 2002).

Nicotine also affects the release of endogenous opioid peptides. Nicotine binding to nAChR within hypothalamus induces the release of a precursor of ß-endorphin. It is thought to be involved in mood regulation, decrease response to stress, conserve energy and relaxation (Cesselin, 1995).

As far as concerns serotonergic transmission, it has been shown that chronic nicotine exposure produces a selective decrease in the concentration of 5-HT in the hippocampus (Benwell & Balfour, 1979). The effect of this neuroadaptation is still unclear, however, considering the findings that 5-HT deficits have been implicated in depression and anxiety, it may be hypothesized that during chronic nicotine exposure and withdrawal, the decrease in serotonin function plays a role in the onset of negative affective symptoms, such as depressed mood and irritability (Schwartz, 1984).

1.1.4 Nicotine effects and withdrawal

The activation of peripheral nAChRs increases noradrenaline release, with concomitant increase in heart rate, blood pressure, and respiratory rate. Centrally nicotine improves working memory functions, learning and attention; it also induces pleasure and reduces stress and anxiety. At the initial experience it can give nausea/disorientation.

After a first experience of smoking, as a result of pharmacological and non-pharmacological factors, an individual frequently elects to repeat the experience (Rose, 2006). This leads to the next stage where the prolonged exposure to smoke induces a neuroadaptation in the brain, increasing the reinforcing effects of nicotine (Soria, et al., 1996). When CNS nicotine level ceases abruptly following smoking cessation, it produces temporary imbalances in neurological systems before compensatory mechanisms are triggered to restore homeostasis (Lowinson, 2005). This imbalance is associated with unpleasant withdrawal effects such as irritability, headache, nausea, constipation or diarrhoea, falling heart rate and blood pressure, fatigue, drowsiness or insomnia, depression, increased hunger and energy, lack of concentration, anxiety, and

cravings for cigarettes (Benowitz, 1988) which are powerful incentives to take up/relapse smoking again (Hughes, 1992; Hughes et al., 1984; 1990). Thus basis of nicotine addiction is a combination of positive reinforcement of mood and avoidance of withdrawal symptoms. In addition, conditioning has an important role in the development of tobacco addiction.

1.1.5 Neuroplasticity

Neuroplasticity induced by drugs can be considered initially as transient changes that are antecedent to developing a new behaviour (that takes hours to weeks) and, then, stable neuroplasticity that corresponds to persistent information that is retrieved to guide the execution of learned behavior (from weeks to being permanent). Drug addiction is a pathology in mechanisms of brain neuroplasticity that are used to establish the adaptive hierarchy of behaviours that ensure survival (Kalivas and O'Brien, 2008). Thus, enduring drug-induced neuroplasticity establishes a maladaptive orientation to the environment that manifests as the two cardinal features of addiction: i) impaired ability to regulate the drive to obtain and use drugs (ie, relapse), ii) reduced drive to obtain natural rewards.

1.1.6 Pharmacological smoking cessation treatment

First-line pharmacological treatments of tobacco dependence recommended by clinical practice guidelines are nicotine replacement therapy (NRT), bupropion and varenicline (Lerman et al., 2007).

Nicotine replacement therapy (NRT) is the only first-line smoking cessation treatment available without prescription and has increased short-term smoking cessation rates by 50–70% (Rigotti, 2002). NRT reduces the severity of withdrawal symptoms such as anxiety, insomnia, depressed mood, and inability to concentrate (Ford and Zlabek, 2005). Smoking whilst using NRT provides a deterrent, as the high nicotine doses can produce aversive effects such as nausea, palpitations, hypotension, and altered respiration (Frishman, 2007). NRT treatments are available as a nasal spray, chewing gum or transdermal patches. However, despite initial benefits, around 95% of ex-smokers who had undergone transdermal patch NRT relapsed after a period of time (Clinical Practice Guideline Treating Tobacco Use and Dependence 2008 Update Panel, Liaisons, and Staff. U.S.A. Public Health Service report. Am J Prev Med. 2008 35:158-76.).

Bupropion is an antidepressant drug; its primary pharmacological action is thought to be noradrenergic and dopaminergic reuptake inhibition. It binds selectively to DA transporter, but its behavioural effects have often been attributed to its inhibition of noradrenaline reuptake (Balfour, 2001). It also acts as a nAChRs antagonist. Its efficacy might be explained by its antidepressant effect, indeed depression is a withdrawal symptom that reliably predict relapse among abstinent smokers (Hughes, 2007). Moreover, its antagonist-like activity on nAChR decreases the reinforcing effect of nicotine.

Varenicline is nAChR partial agonist. It activates DA reward system with less abuse liability of nicotine. Indeed it produces a lesser, slower DA release than nicotine with a longer duration of action. Moreover, when varenicline is combined with nicotine, it attenuates nicotine induced DA release in nucleus accumbens (Rollema et al., 2007).

Behavioural interventions play an integral role in smoking cessation, either in conjunction with medication or alone. They employ a variety of methods to assist smokers in quitting, ranging from self-help materials to individual cognitive-behavioural therapy. These interventions teach individuals to recognize high-risk smoking situations, develop alternative coping strategies, manage stress, improve problem solving skills, as well as increase social support (Clinical Practice Guideline Treating Tobacco Use and Dependence 2008 Update Panel, Liaisons, and Staff. U.S.A. Public Health Service report. Am J Prev Med. 2008 35:158-76.).

1.2 Psychobiology of tobacco addiction

The severity of nicotine dependence (abuse liability, frequency of consumption, high rate of relapse) is similar to other drug dependence, such as opiates or cocaine. In contrast, the reinforcing properties of nicotine is subtle compared to other drug. It suggests that the reinforcing effect of nicotine is necessary but not sufficient to explain tobacco dependence (Caggiula, 2002). Furthermore several preclinical and clinical studies have underlined the importance of non-pharmacological factors, such as environmental stimuli, in maintaining smoking behaviour and promoting relapse.

1.2.1 Operant Conditioning

Operant conditioning (also called instrumental conditioning or instrumental learning) is a form of learning that occurs through rewards and punishments for behavior and in which an individual's behavior is modified by its consequences; the behaviour may

change in form, frequency, or strength (Skinner, 1938). Operant conditioning is distinguished from Pavlovian conditioning in that operant conditioning deals with the modification of "voluntary behaviour" or “operant behavior”. Operant behavior operates on the environment and is maintained by its consequences. Since nicotine is positively and negatively reinforcing operant behavior in smokers is triggered and maintained by nicotine per se at the beginning but other factors and interactions became important with the passage of time (Chiamulera, 2005; Everitt and Robbins, 2005).

1.2.2 Pavlovian Conditioning

A stimulus that is repeatedly and contingently paired with an unconditioned stimulus (e.g. nicotine effect) acquires a Pavlovian conditioned value (Pavlov, 1927). Thus with regular smoking within a complex individual and social context, smokers associate specific situation, mood or environmental factors with the rewarding effect of nicotine. These smoking-associated stimuli may trigger physiological, psychological and behavioural reactivity in smokers, and it is widely accepted that they can precipitate relapse in ex-addicts (Abrams, 1999; Drummond, 2000; Niaura et al., 1988). There are two classes of conditioned stimuli: proximal discrete cues that become conditioned stimuli (CS) after association to drug effects (e.g. cigarettes, lighter), and distal stimuli that are present in the environmental context (e.g. bar and people around) (Conklin et al., 2008).

1.2.2 Nicotine’s multiple-action

Several studies suggest that in addition to its primary reinforcing properties, nicotine has a second effect that may be important in promoting smoking behaviour. Nicotine is a cognitive enhancer drug and may enhance the salience of other reinforcers, including the CS that has acquired conditioned values by repeated pairing with nicotine effect (Caggiula et al., 2002). Nicotine activates and potentiates information processing at those brain areas and pathways where reinforcement and sensory transmission are integrated into emotional, motivational and cognitive processes that control for smoking behaviour. Smoking behaviour may therefore be maintained by a “multiple-action” effect of nicotine: i) as a primary reinforcement and, ii) as an enhancer of the multiple smoking/smoking-associated stimuli processing. This model may help to explain how nicotine could play a central role in initiation, maintenance and difficulty to stop smoking, despite of its mild reinforcing properties (Chiamulera, 2005).

1.2.3 Cue reactivity

Cue reactivity is the vast array of responses that are observed when addicts or ex-addicts are exposed to drug-related CS (Drummond, 2000). These responses can be: i) physiological, such as decrease in heart rate and blood pressure and/or increase in skin conductance and skin temperature, ii) psychological, such as increase in craving and urge to smoke and/or mood change, iii) and also behavioural, such as cigarette-seeking and change in smoking behaviour (e.g. latency to smoke, cigarette puff volume and frequency, amount of cigarette consumed and relapse to smoking behaviour). Several factors may influence smokers' cue reactivity: type of stimuli (e.g. distal vs. proximal) (Conklin et al., 2008), degree of nicotine dependence (Payne et al., 1996) impulsivity, genetic, comorbidity (Drummond, 2000), contextual factor drug-availability or expectation (Field & Duka, 2001).

Several brain imaging studies have revealed that brain areas of the mesocorticolimbic system are specifically activated in smokers exposed to smoking-associated stimuli, and that these effects may overlap with those induced by nicotine administration. The fact that exposure to smoking cues and nicotine administration activates similar brain patterns suggests a causal relationship between nicotine effect through smoking and development/maintenance of cue reactivity (Yalachkov et al., 2009). Cue reactivity may last in ex-smokers even after years of smoking cessation, and it is the main cause of relapse to smoking behaviour (Shiffman, 2009).

1.3 Nicotine-related memories

Given the importance of the learned association between stimuli and drug, that we can also call drug-associated memory, in the phenomenon of relapse to drug-seeking behaviour, it has been proposed that treatments that disrupt the drug-associated memory could act as a pro-abstinent and anti-relapse therapy (Diergaarde, 2008; Tronson & Taylor, 2007). Instrumental memories, in addition to Pavlovian memories, play an important role in smokers and also in animal models of drug addiction. Therefore there is an increasing interest in investigate the phenomena of drug memories consolidation and reconsolidation.

1.3.1 Reconsolidation theory

Memories are stored after a learning experience through a process called consolidation.

For more than 100 years the idea that once consolidated memories become permanently stored in the wiring of the brain has been a dogma. In the traditional consolidation theory new memories are initially in a "labile" form for a short time (short term memory-STM), after which the memory traces are fixed or "consolidated" into the physical structure of the brain (long term memory-LTM). In 1968 Lewis and colleagues observed that an electroconvulsive shock (an amnestic treatment), provided after the memory has been reactivated by its retrieval, could induce amnesia the following day. Given that amnesia was not produced in the absence of memory reactivation it has been argued that retrieval of memory induced a reactivation of the memory trace, that presumably returns to a labile state, which initiated another memory process similar to that seen after learning. The processes through which memories are maintained after retrieval is called reconsolidation (Figure 3).

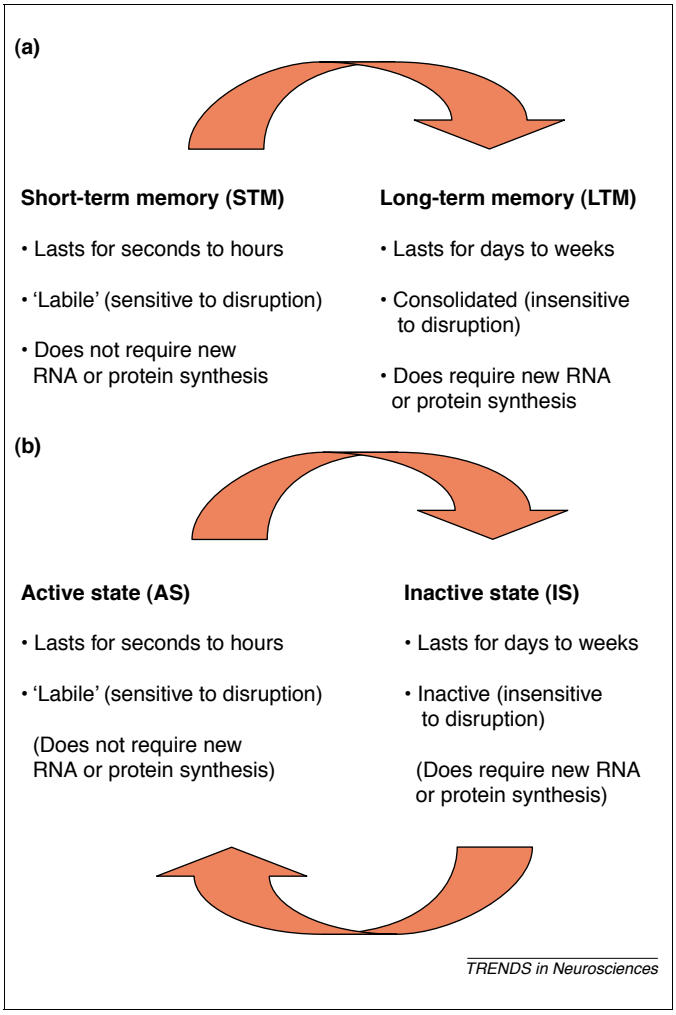

Figure 3. Two models of memory processing. (a) The traditional consolidation memory that stated that a labile short-term memory (STM) switch to a consolidated, permanent long-term memory (LTM). (b) The memory model proposed by Lewis (1968). The active state (AS) and

inactive state (IS) are analogous to STM and LTM, respectively. Memory after learning experience is in AS, then it enters in IS by the passage of time. Retrieval of the memory returns it to the AS (Nader, 2003).

Furthermore it has been shown that amnesia can be induced only if the amnestic treatment, such as the electroconvulsive shock, is given shortly after retrieval (Misanin et al., 1968; Schneider & Sherman; 1968). These findings suggest that retrieval induces a transient labile phase of the memory. The time during which memory traces are labile is called reconsolidation window and persist for several hours after retrieval (Duvarci & Nader, 2004; Nader et al., 2000b; Sara, 2000).

In the past 10 years the study of reconsolidation have been extended to numerous species, including crabs, chicks, honey bees, etc. and to numerous experimental paradigms.

To experimentally demonstrate reconsolidation or the role of a particular molecule in reconsolidation, memories must be first consolidated, then reactivated (retrieved) contiguously with some forms of manipulation. Finally, modification of the memory must be observed.

Reconsolidation is frequently studied using Pavlovian conditioning paradigms, such as fear conditioning. Training consists of pairing a neutral stimulus (conditioned stimulus, CS), such as a tone, with a reinforcing stimulus (unconditioned stimulus, US), such as a foot-shock. Retrieval is induced in a reactivation session, which occur at least 24 hours later and consists in presenting the CS in the absence of US. The manipulation (such as the administration of an amnestic drug) is applied either prior or immediately after the reactivation session. Finally at least 24 hours later the memory is tested by re-presenting the CS and measuring the unconditioned responding, in this case the freezing (measure of fear response), compared with animal in the non-manipulated control group.

Demonstrating reconsolidation not only requires evidence of modification of a previously consolidated memory, but also evidence that in the absence of retrieval or if the amnestic manipulation is applied outside the reconsolidation window, the memory remains unmodified.

To better understand the cellular and molecular mechanisms underlying reconsolidation of particular focus have been the molecular cascades previously demonstrated to be important in memory consolidation and those downstream of therapeutically relevant neurotransmitter targets including β-adrenergic receptors and N-methyl-d-aspartate

receptors (NMDARs). De-novo protein synthesis is required for memory reconsolidation; several animal studies have shown that injection of protein synthesis inhibitor, such as anisomycin, after retrieval of a previously consolidated memory, can disrupt the original memory. It has been shown that the immediate-early genes c-Fos and JunB are activated during, and CCAAT-enhancing binding protein-β (C/EBPβ) is required for, memory reconsolidation. The gene transcription is initiated by the activation of transcription factors such as cAMP response element-binding protein (CREB), zinc finger 268 (Zif-268), ELK1 and nuclear factor kB (NF-kB). These, in turn, are activated by upstream kinase, such as extracellular-regulated kinase (ERK) and protein kinase A (PKA) (for review see Tronson & Taylor, 2007) (Figure 4).

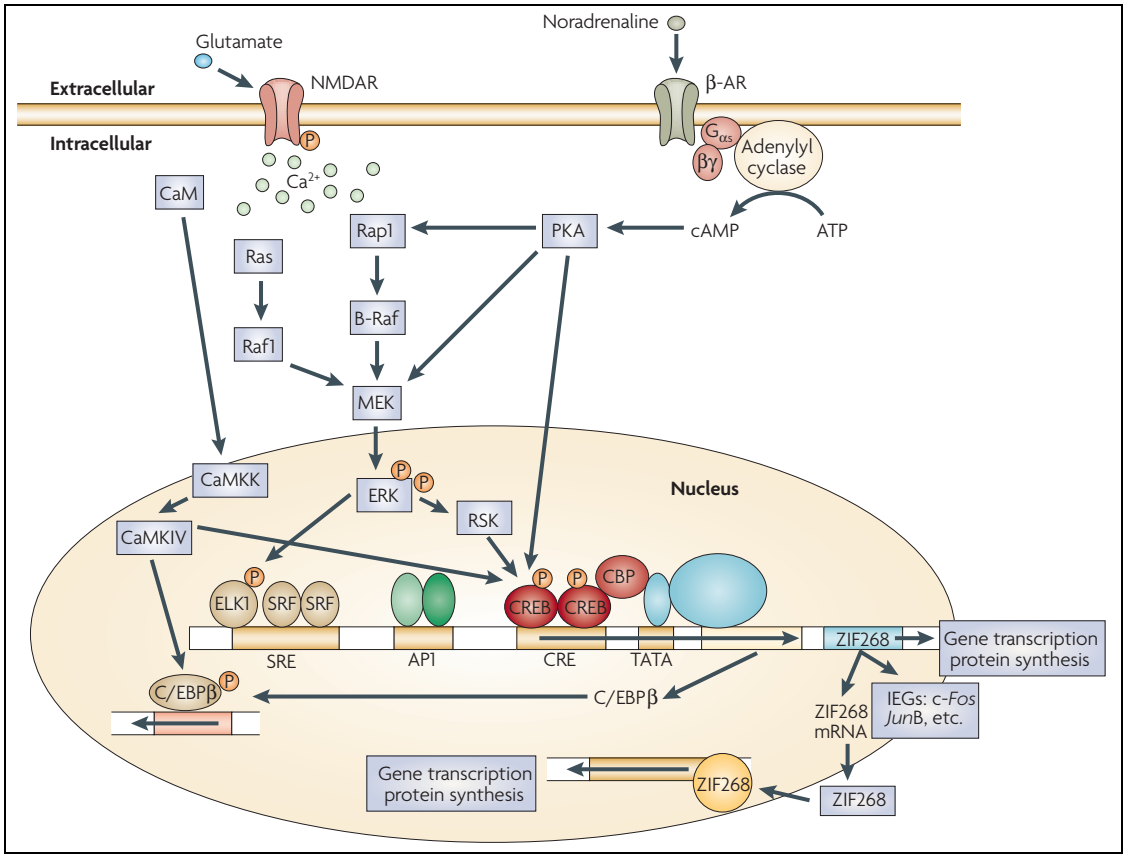

Figure 4. Key molecular mechanisms of memory reconsolidation. Molecular signalling cascades downstream of β-adrenergic receptors (β-AR) and N-methyl-d-aspartate receptors (NMDARs) have been shown to be implicated in reconsolidation. Small GTPases such as Ras, Raf and Rap activated by Ca2+ influx activate the extracellular signal-regulated kinase pathway (ERK). Protein kinase A (PKA) is activated by cyclic AMP (cAMP) and acts directly, or indirectly through ERK and ribosomal protein S6 kinase (RSK), to activate transcription factors including cAMP response element-binding protein (CREB), zinc finger 268 (ZIF268) and ELK1, which then initiate gene transcription. The immediate-early genes c-Fos and JunB are activated during,

and CCAAT-enhancing binding protein-β (C/EBPβ) is required for, memory reconsolidation (image taken from Tronson &Taylor, 2007).

From an evolutionary perspective, it has been argued that reconsolidation may serve as an adaptive update mechanism allowing for new information, available at the time of retrieval, to be integrated into the initial memory representation (Alberini, 2005; Hupbach et al., 2007; Monfils et al., 2009; Nader, 2003). Other authors proposed that reconsolidation might serve to strengthen memory (Inda et al., 2011; Lee, 2009; Sara, 2000). Indeed different from extinction learning, that is a new learning by which CS previously associated with a reinforcer become newly associated with no outcome (Tronson and Taylor, 2007), the reconsolidated/modified memory should persist with the passage of time because the original memory trace is updated.

As stated above, it has been shown in several animal studies that memory could also be disrupted acting on the molecular mechanisms underlying reconsolidation (for review see Tronson and Taylor, 2007; Nader et al., 2000a; Soeter & Kindt, 2011). This offers a potential for the treatment of psychiatric disorders characterized by strong pathogenic memories, such as post-traumatic stress disorders (PTSD), phobias and also drug addiction (Centonze et al., 2005).

1.3.2 Reconsolidation as a potential target in drug addiction treatment: Pavlovian vs. instrumental memories reconsolidation

Drug addiction is increasingly viewed as the endpoint of a series of transitions from initial drug use—when a drug is voluntarily taken because it has reinforcing, often hedonic, effects—through loss of control over this behavior, such that it becomes habitual and ultimately compulsive (Everitt and Robbins, 2005). The change from voluntary drug use to more habitual and compulsive drug use represents a transition at the neural level depending on the drug-induced neuroplasticity in both cortical and striatal structures leading to an improved understanding of associative learning mechanisms that conceive of behavioral output as an interaction between Pavlovian and instrumental learning processes (Dickinson and Balleine, 1994; White and Mc Donald, 2002). Drug addiction is a chronic disorder characterized by a high rate of relapse to drug use among abstinent. One of the main causes of relapse is the exposure to the CS that are associated to drug effect in a Pavlovian manner and influence drug-seeking behaviour and relapse through the memory they evoke and the interaction with

instrumental memories (Milton and Everitt, 2010).

Therefore molecular and neuroanatomical processes involved in the reconsolidation of drugs-associated memories have been proposed as novel targets for the treatment of vulnerability to CS in drug addicts (Tronson & Taylor, 2007; Diergaarde et al., 2008; Taylor et al., 2009; Milton & Everitt, 2010). Mechanistic studies identified receptors, signalling molecules and transcription factors underlying drugs-associated memory reconsolidation (Sadler et al., 2007; Brown et al., 2007; Fricks-Gleason & Marshall, 2008; Itzhak, 2008; Lee & Everitt, 2008a; Milton et al., 2008a, 2008b; Fuchs et al., 2009; Ramirez et al., 2009; Sanchez et al., 2010; Théberge et al., 2010; Wu et al., 2011). These studies have been focused mostly upon two neurotransmitters receptors, known to be involved in the reconsolidation of emotional memories: NMDA subtype of glutamate receptor and β-adrenergic receptor.

It has been shown that NMDARs antagonists, such as MK-801 or D(-)-(2R)-amino-5-phosphonovaleric acid (D-APV), given shortly after retrieval, may inhibit the reconsolidation of drug-associated memory in different Pavlovian conditioning paradigms in rats, such as conditioned place preference produced by cocaine (Kelley et al., 2007), amphetamine (Sadler et al., 2007; Sakurai et al., 2007), and morphine (Zhai et al., 2008); cue-induced reinstatement of alcohol seeking (Von der Goltz at al, 2009); and the acquisition of a new instrumental response for a CS previously paired with cocaine (Milton et al., 2008a). It has been proposed that a reduction in the expression of the immediate-early-gene Zif268 is linked to disruption of memory reconsolidation. Indeed Milton and colleagues found that administration of the NMDARs antagonist D-APV into the basolateral amygdala before a memory reactivation disrupts the reconsolidation of cocaine-associated memory in rats trained to cocaine self-administration and this effect is associated with a reduction in the expression of Zif268. Lee (2005) also showed that an infusion of the Zif268 antisense oligodeoxynuclotides (ASO) into the basolateral amygdala contingently upon retrieval of cocaine-associated memory could disrupt the conditioned reinforcing value of the CS. However it has not yet been investigated through which signalling cascade (e.g. ERK activation or protein kinase A) expression of Zif268 is activated. On the other hand, in rats trained to self-administer cocaine, systemic administration of MK-801 contingently upon retrieval, showed no effect on subsequent cocaine-primed reinstatement of cocaine-seeking behaviour (Brown et al., 2008).

The first evidence of the role of β-adrenergic receptor in the reconsolidation of

appetitive memories had been provided by Diegaarde and colleagues in 2006: in their work they showed that the administration of propranolol, an antagonist of β-adrenergic receptor, contingently upon retrieval, could reduce the context-induced reinstatement of sucrose seeking behaviour in rats trained to sucrose self-administration. Subsequently Milton and colleagues (2008b) showed that the administration of propranolol in rats trained to self-administer cocaine resulted in a retrieval-dependent impairment in the acquisition of a new response for cocaine-conditioned reinforcement, suggesting that reconsolidation of cocaine-associated memories had been disrupted. Moreover it has been shown that propranolol, administered upon retrieval, could disrupt place preference conditioned by cocaine (Bernardi et al., 2006) morphine (Robinson & Franklin, 2007). However propranolol, given at retrieval, failed in reducing cue-induced reinstatement of cocaine seeking behaviour, following forced abstinence, in rats trained to cocaine self-administration (Milton & Everitt 2010). Alberini and collegues highlighted the limited efficacy of Propranolol in memory reconsolidation disruption in particular when instrumental learning is a relevant component of the memory (Muravieva and Alberini, 2010; Milton et al., 2012). There is evidence that protein synthesis is always required for memory retrieval and reconsolidation of young and old fear related memories and that protein PKA activation is required only for CS induced memory retrieval and reconsolidation of young fear related memories but not for motor or older and stronger memories (Kemenes et al., 2006; Inda et al., 2011). These data suggest potential different molecular mechanisms engaged during reconsolidation depending on the age and kind of the memory. The role of PKA in memory reconsolidation of cocaine associated CS has been investigated by Sanchez and collegues (2010). The molecular cascade that involves PKA activation seems to be triggered by β-adrenergic receptor activation and not by NMDARs (Tronson and Taylor, 2007). It could be argued that Propranolol, a β-adrenergic antagonist, preventing PKA activation can disrupt memory reconsolidation only when PKA is needed and recruited. Propranolol limited efficacy in older or instrumental memory could be related to the fact that in the reconsolidation process of these memories PKA is not recruited. NMDARs antagonists (i.e. MK-801) seem to be more effective in reconsolidation disruption of different kind of memory, an effect probably due to the inhibition of key molecules like MEK, by interaction with other kinases than PKA.

1.4 Aim

This research originated from the experimental evidences that reconsolidation of some drug related memories could be disrupted. However, to date it is not known if all memories can be disrupted. A better knowledge of what kind of memory can be disrupted is fundamental in preventing the relapse to drug seeking behaviour.

The aim of this research was to investigate if it is possible to disrupt the reconsolidation of different kind of nicotine-related memories by the application of drugs acting at specific molecular levels such as adrenergic and glutamatergic systems, and whether this disruption prevents the relapse to nicotine-seeking behaviour in a rat model of nicotine dependence.

Since there are many boundary conditions related to memory retrieval and reconsolidation (i.e., retrieval session duration, memory age and strength) that could interphere with the results of behavioural experiments, in this research we also want to validate a cellular and molecular technique that allows a direct demonstration of memory reactivation and reconsolidation occurrence.

Three main issues are addressed:

1. Is a post-retrieval pharmacological treatment, such as propranolol able to disrupt Pavlovian memory reconsolidation of nicotine-related memories and prevent the reinstatement of nicotine-seeking behaviour?
2. Is a pre or post-retrieval pharmacological treatment, such as MK-801 able to disrupt instrumental memory reconsolidation of nicotine-related memories and prevent the reinstatement of nicotine-seeking behaviour?
3. The feasibility and reliability of Zif268 (specific marker correlating memory reconsolidation) expression assessment by immunohistochemistry after memory retrieval in rats.

We assessed whether post-retrieval administration of propranolol, and if pre or post-retrieval administration of MK-801 may reduce reinstatement of nicotine-seeking behaviour when rats were placed back in the training context. We also evaluated Zif268 level of expression in basolateral amygdala, the most important brain region involved in reconsolidation of Pavlovian memories, after retrieval of Pavlovian nicotine-related memories.

We performed three studies:

In ***Study #1*** we assessed the effect of Propranolol 10 mg/kg on reinstatement of nicotine-seeking behaviour in rats trained to nicotine S/A. Retrieval consisted in 30 CS presentations.
In ***Study #2*** we assessed the effect of MK-801 0,01 mg/kg on reinstatement of nicotine-seeking behaviour in rats trained to nicotine S/A. Retrieval consisted in allowing rats to press the lever previously paired to nicotine 20 times.
In ***Study #3*** we evaluated Zif268 level of expression in basolateral amygdala by immunohistochemistry, after retrieval of nicotine Pavlovian memories. Retrieval consisted in 3 CS presentations.

In the study-protocol we included no-retrieved groups and no-treated groups (receiving a vehicle injection after retrieval or no-retrieval). These groups allow to control for the specificity of the treatment (propranolol, MK-801) effect on nicotine Pavlovian or instrumental memories.

	STUDY #1	STUDY #2	STUDY #3
EXPERIMENTAL PARADIGM S/A	Nicotine	Nicotine	Nicotine
RETRIEVAL LENGHT	30 CS presentations	20 lever presses	3 CS presentations
TREATMENTS	Propranolol	MK-801	X

Figure 5. Schematic table of the studies. In the Study #1 we assessed the effect of propranolol 10 mg/kg applied after 30 CS presentations on reinstatement of nicotine-seeking behaviour. In the Study #2 we assessed the effect of MK-801 applied 30 minutes before or 1 hour after the first of 20 lever presses on lever previously paired to nicotine, on reinstatement of nicotine-seeking behaviour. In the Study #3 we evaluated the level of expression of Zif268 2 hours after 3 CS presentations.

1.4.1 Experimental model

The experimental model used was intravenous nicotine S/A in rats, a laboratory model based on operant and Pavlovian conditioning to nicotine and nicotine-associated cues.

The term operant conditioning describes one type of associative learning in which there is a contingency between behaviour and the presentation of a biologically significant event (e.g. reinforcer). A positive reinforcement occurs when a behaviour (lever press) is followed by a stimulus which is appetitive or rewarding (e.g. food or nicotine administration), increasing the frequency of that behaviour (conditioned response). The term Pavlovian conditioning describes the associative learning in which an initially neutral stimulus (e.g. a light) repeatedly paired with an unconditioned stimulus (e.g food or nicotine administration-US) become associated to unconditioned stimulus and acquired a conditioned values (CS) which may elicit the conditioned response (e.g. food or nicotine seeking behaviour) even in the absence of US.

Addiction can not be modelled in animals, at least a whole, however different procedures of operant behaviour can be applied as rodent analogues of addiction's major elements including drug seeking and relapse (Ator and Griffiths, 2003; Sanchis-Segura & Spanagel, 2006). Drug S/A has been widely characterized for all the drugs abused by humans, under different modes of administration. The paradigm has a high analogy to the pathological condition; it allows to study the underlying neurobiological mechanisms, having a high predictive validity for the identification of novel anti-addiction therapies. In our nicotine S/A models rats are placed in a cage, the so-called Skinner box (Figure 6), equipped with two levers, one active and one inactive.

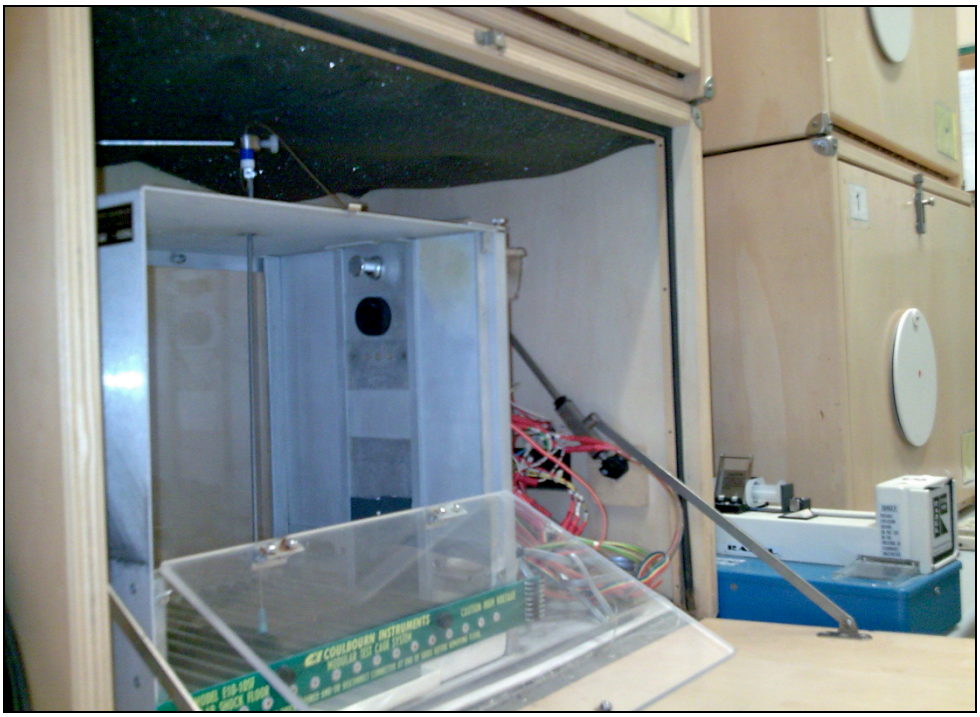

Figure 6. Skinner box photo. The operant chamber is placed in a sound and light-isolating box. It is equipped with two levers (one active and one inactive), a catheter connected to a syringe pump (for nicotine injection) and with a sugar pellet magazine through which sugar pellet are delivered.

Initially rats press the lever by chance. The pressing of active lever results in the administration of sugar pellet or nicotine infusion (rats are previously implanted with an intrajugular catheter) and in presentation of a cue light (CS). Since the sugar pellet or nicotine acts as reinforcement, the lever presses behaviour are repeated and become motivated to seek for food or nicotine infusion (conditioned response). Generally this training phase lasts until rats reach a stable response over at least three consecutive days. Since we were interested in investigating the Pavlovian and instrumental memories, our criterion was: i) the stability of animal behaviour (the value of reinforcements/session did not vary more than 20% between three consecutive sessions) for Pavlovian memory and, ii) an equal number of nicotine sessions (10 nicotine self-administration sessions) for all animals across the entire training phase for instrumental memory in order to have similar memory strength across each experimental group. Once trained the nicotine related memories were retrieved by the non-contingent presentation of CS (Pavlovian memory reactivation) or allowing animals to press the lever previously paired to nicotine without nicotine infusion (instrumental memory reactivation). Manipulation such as propranolol or MK-801 was

provided before or after the retrieval session. Twenty-four hour later the effect of treatment on nicotine related memory was tested by measuring the conditioned response (number of lever presses) when the animals were placed in the context previously associated to nicotine administration (reinstatement).

2. MATERIALS AND METHODS

2.1 Subjects

Male Sprague Dawley rats (Harlan, Italy) 240-260 g were used for these studies. All animal procedures were carried out in accordance with the Principles of laboratory animal care (National Institute of Health publication No.85/23, revised 1985), the European Communities Council Directive of 24 November 1986 (86/609/EEC). The inter-departmental Centre approved these procedures for Laboratory Animal Service and Research of the Verona University, according to art.7 D.L. 116/92 of the Italian Legislation. All efforts were made to minimize animal suffering and to keep the number of animals used as low as possible.

2.2 Drugs

Nicotine hydrogen tartrate (Sigma, Italy) was dissolved in heparinized bacteriostatic saline (0.9% NaCl + 0.9% benzylalcohol + 1 IU/mL heparin) and pH adjusted to 7.4 with NaOH. Nicotine unit doses are expressed as mg of free base/kg of body weight/infusion. Adjustment of nicotine concentration to changes in rat body weight was not needed because rats' body weight was kept stable at 250 g (± 10g). Propranolol hydrocloride (Sigma-Aldrich) was dissolved in saline (0.9% NaCl), while (+)-MK801 hydrogen maleate (Sigma-Aldrich) was dissolved is ultrapure water (Milli-Q). Both propranolol and MK801 were administered via intraperitoneal injection (IP) in a volume of 1 mL/kg, immediately after retrieval (or no-retrieval session), or 30 minutes before, or 1 hour after, the retrieval (or no-retrieval) session respectively. All doses were expressed as salt.

2.3 Surgical procedure

Rats were anaesthetized with 0.5 mg/kg/0.5 mL medetomidine (Domitor®, Pfizer, Italy), 10 mg/kg tiletamine + 10 mg/kg zolazepam (Zoletil 100®, Virbac, Italy; 0.2 mL/kg intramuscular), and then implanted with a silicon catheter (inner diameter 0.30 mm, outer diameter 0.63 mm, Cam Caths, Cambridgeshire, UK) in the right jugular vein. Immediately after surgery, animals were medicated with 5mg/kg/1 mL subcutaneous carprofen (Rymadyl®, Pfizer, Italy) and 25,000,000 IU benzylpenicilline + 1 g/kg dihydrostreptomycin (Rubrocillina Forte®, Intervet, Italy; 1 mL/kg subcutaneous), 0.5 mg/kg/0.1 mL intramuscular atipamezole (Antisedan®, Pfizer,

Italy). Each day after recovery, animals received 0.1 mL i.v. injection of heparin solution (30 IU/mL heparin sodium, Sigma, Italy) before and after the experimental session.

2.4 Study #1

2.4.1 Subjects

Twenty-seven Male Sprague Dawley rats (Harlan, Italy) were individually housed in a temperature controlled environment (19-23 °C) on a 12 hours light–dark cycle with light on at 06:30 p.m. All the experimental procedures were conducted within the dark phase of the light-dark circle. Animals were food restricted to maintain their body weight range between 240-260 g. Food diet (2-4 pellets, for a total of 10-20 g/day) was made available after each experimental session. Animals have ad libitum access to water except during experimental sessions (3 to max 360 minutes/day). Rats were trained or tested once daily.

2.4.2 Apparatus

This study was conducted in eight identical operant conditioning chambers (Coulbourn Instruments, Lehigh Valley, Whitehall, PA, USA) encased in sound-insulated cubicles, equipped with ventilation fans (Ugo Basile, Comerio, Italy). Each chamber was equipped with two levers, symmetrically centred on the frontal panel, and located 12.5 cm apart, 2 cm above the grid floor. The food magazine was situated in an opening in a panel between the two levers, 1 cm above the floor. This opening was closed during nicotine S/A training, retrieval, and reinstatement sessions. A 2 W white house light was located 26 cm above the food magazine and activated during the entire session duration, except during the time-out period (TO, 60 seconds interval after each reinforcement in which levers were inactive). ALP (right or active lever presses) corresponding to Fixed Ratio (FR) values, required by the schedule of reinforcement, produced the delivery of 45-mg sugar food pellet (Bioser, USA) or the activation of the infusion pump (model A-99Z, Razel Scientific Instruments Inc., Stamford, CT, USA), except during the retrieval and reinstatement sessions. Nicotine solution was administered via the infusion pump at the volume of 0.04638 mL during a 1 second period. Nicotine infusion was associated with 1 second illumination of one yellow and one green light emitting diode (LED) centrally placed above the food magazine (CS). Left lever presses (‘inactive lever presses’) did not have any consequence. All types of

lever presses, sugar pellet and infusion deliveries were recorded. Data acquisition and schedule parameters were controlled by med-PC software (Med Associates Inc, Georgia, USA) running on a PC-computer interfaced with the chambers via interface modules (Med Associates Inc.).

2.4.3 Training to lever press

Following a 24 hours food deprivation period, all rats were trained to lever press for food as reinforcement. The final training schedule of reinforcement was FR2. Session duration was 60 minutes. Once trained to lever press for food reinforcement (it required approximately 2 weeks) rats underwent surgery to implant an i.v. cannula.

2.4.4 Training to nicotine self-administration (S/A)

After 7 days of recovery, rats were trained to intravenously self-administer nicotine. Initially the schedule of reinforcement was FR1: nicotine 0.03 mg/kg/infusion, 1 second CS, TO 60 seconds; session duration up to 25 infusions or 180 minutes elapsed. If the animals met the criterion of 25 infusions within the end of daily session, the FR value was increased to FR2 with session duration lasting up to 60 minutes. Rats were considered to reach a stable responding on nicotine S/A under a FR2 schedule of reinforcement when the value of reinforcements/session did not vary more than 20% between three consecutive sessions. Lever pressing during the TO period was also recorded although it did not have any consequence.

2.4.5 Retrieval

After the nicotine S/A, rats underwent to 30-34 days of forced abstinence in their home cage without access to the operant chamber or the experimental room. Then rats were divided into two groups respectively exposed to retrieval (Ret) or not (No-Ret). Both groups were placed for 20 minutes in the training context. The Ret, but not the No-Ret, group was exposed to 30 non-contingent CS presentations (30[FI 40 seconds: 1 second CS]). Immediately after the retrieval session (or no-retrieval) both groups of rats were treated with vehicle (Ret Veh, No-Ret Veh) or propranolol 10 mg/kg (Ret Prop, No-Ret Prop), then returned to their home cage.

2.4.6 Reinstatement

The day after the retrieval (or no-retrieval) session, all the subjects were re-exposed to the training context and CS presentation was made contingent upon responding on ALP (FR2: 1 second CS, no nicotine, session duration 60 minutes).

2.5 Study #2

2.5.1 Subjects

Thirty-five Male Sprague Dawley rats (Harlan, Italy) were individually housed in a temperature controlled environment (19-23 °C) on a 12 hours light–dark cycle with light on at 06:30 p.m. All the experimental procedures were conducted within the dark phase of the light-dark circle. Animals were food restricted to maintain their body weight range between 240-260 g. Food diet (2-4 pellets, for a total of 10-20 g/day) was made available after each experimental session. Animals have ad libitum access to water except during experimental sessions (3 to max 360 minutes/day). Rats were trained or tested once daily.

2.5.2 Apparatus

Behavioural testing was conducted in operant chambers encased in sound-insulated cubicles, equipped with ventilation fans (Med Associates Inc., St Albans, Vermont, USA). Each chamber was equipped with 2 levers, symmetrically centred on the front panel. A 2 W house light was located on the back panel near the chamber ceiling to provide ambient illumination during the entire session duration except during the TO. A fixed number corresponding to FR of ALP produced the activation of the infusion pump (Med Associates Inc.) leading to 1 second nicotine infusion except during TO, retrieval and reinstatement sessions. During the retrieval session active or inactive levers pressure did not have any consequence. All types of lever presses, sugar food pellet deliveries or nicotine infusions were recorded. Data acquisition and schedule parameters were controlled by Med-PC software (Med Associates Inc.).

2.5.3 Training to lever press

Following a 24 hours food deprivation period, all rats were trained to lever press for food as reinforcement. The final training schedule of reinforcement was FR1. Session duration was 60 minutes. Once trained to lever press for food reinforcement (it required approximately 2 weeks), rats underwent surgery to implant an i.v. cannula.

2.5.4 Training to nicotine self-administration (S/A)

After 7 days of recovery, rats were trained to intravenously self-administer nicotine. The schedule of reinforcement was FR1: nicotine 0.03 mg/kg/infusion, TO 60 seconds; session duration up to 12 infusions or 60 minutes elapsed. Rats were trained for 10 consecutive sessions and considered to reach a stable responding on nicotine S/A under a FR1 schedule of reinforcement when the value of reinforcements/session did not vary more than 20% between three consecutive sessions. Lever pressing during the TO period was also recorded, although it did not have any consequence.

2.5.5 Retrieval

After the nicotine S/A phase, rats were divided into two groups respectively exposed to retrieval 20 (Ret20) or no-retrieval (No-Ret). On the retrieval session all the groups were placed in the training context. The Ret20 group was allowed to 20 ALP presses, as well as No-Ret group was exposed to the training context for 1 hour. Both groups (Ret20 and No-Ret) were further divided into sub-groups respectively treated with vehicle or MK-801 0,01 mg/kg. Vehicle or MK-801 was injected 30 minutes before the retrieval session (pre-Ret20 Veh, pre-Ret20 MK-801) or context re-exposure (pre-No-Ret Veh, pre-No-Ret MK-801) in some sub-groups. In other sub-groups of rats MK-801 was injected 1 hour after the 1st active lever press (post-Ret20 MK801) or after 1 hour of context re-exposure (post-No-Ret MK801).

2.5.6 Reinstatement

The day after the retrieval (or No-Retrieval) session, all the subjects were re-exposed to the training context and lever presses were recorded (FR1: no nicotine infusion, session duration 60 minutes).

2.6 Study #3

2.6.1 Subjects

Ten Male Sprague Dawley rats (Harlan, Italy) were individually housed in a temperature controlled environment (19-23 °C) on a 12 hours light–dark cycle with light on at 06:30 p.m. All the experimental procedures were conducted within the dark phase of the light-dark circle. Animals were food restricted to maintain their body weight range between 240-260 g. Food diet (2-4 pellets, for a total of 10-20 g/day) was

made available after each experimental session. Animals have ad libitum access to water except during experimental sessions (3 to max 60 minutes/day). Rats were trained or tested once daily.

2.6.1 Apparatus

Behavioural testing was conducted in operant chambers encased in sound-insulated cubicles, equipped with ventilation fans (Med Associates Inc., St Albans, Vermont, USA). Each chamber was equipped with 2 levers, symmetrically centred on the front panel. A 2 W house light was located on the back panel near the chamber ceiling to provide ambient illumination during the entire session duration, except during TO periods and retrieval session. A fixed number of ALP produced the delivery of 45 mg sugar pellet or 5 seconds illumination of a stimulus light (CS) placed above the ALP with the activation of the infusion pump (Med Associates Inc.) except during the instrumental learning-extinction. During the retrieval session, levers were not available and CS was presented on a Fixed-Interval (FI) 60 seconds time schedule. Inactive lever presses did not have any consequence. All types of lever presses and nicotine infusions were recorded. Data acquisition and schedule parameters were controlled by a Med-PC software (Med Associates Inc.).

2.6.2 Training to lever press

Following a 24 hours food deprivation period, all rats were trained to lever press for food as reinforcement. The final training schedule of reinforcement was FR1. Session duration was 60 minutes. Once training to lever press for food reinforcement (it required approximately 2 weeks), rats underwent surgery to implant an i.v. cannula.

2.6.3 Training to nicotine self-administration (S/A)

After 7 days of recovery, rats were trained to intravenously self-administer nicotine under a schedule of reinforcement of FR1. ALP presses resulted in nicotine 0.03 mg/kg/infusion, 5 seconds CS. Session duration lasted up to 25 infusions or 60 minutes were elapsed. Lever pressing during the 60 seconds TO period was also recorded, although it did not have any consequence. Rats were considered to meet the criteria of nicotine S/A training once they reached the value of 200 ± 15 (mean ± standard error of the mean-S.E.M.) associations between nicotine infusion and CS.

2.6.4 Instrumental learning extinction phase (ILEXT)

Following the nicotine S/A phase, ALP responding was extinguished during an instrumental learning extinction phase. On these daily 60 min sessions, subjects were placed in the operant chamber and responding on either lever had no programmed consequences. Instrumental learning extinction criterion was reached when ALP/session were < 50% of ALP at the first instrumental learning extinction session, for at least three consecutive sessions (Chiamulera et al., 2010). The inclusion in the experimental design of an instrumental learning extinction phase allowed to control for the operant conditioning component of nicotine S/A, and to evaluate the specificity of the CS-induced Pavlovian memory retrieval.

2.6.5 Retrieval

After the ILEXT phase, rats were divided into two groups exposed to retrieval (3 CS presentation; React) or no-retrieval (0 CS presentation; No-React). Both groups were placed in a novel context (CxB: Skinner box with thick blank striped sheets on the wall and a 1 cm grid on the floor) for 3 or 60 minutes and exposed to 3 [FI 55 seconds: 5-seconds CS] or 0 CS presentation respectively.

2.6.6 Immunohistochemistry and Zif268 quantification

Two hours after retrieval (or no-retrieval) rats were rapidly and deeply anesthetized with Pentobarbital 65 mg/Kg (Lipomed AG, Switzerland) and transcardially perfused with heparin 100 UI/L (Sigma Aldrich, Italy) and paraformaldehyde (PFA) 4% in phosphate buffered vehicle solution (PBS). Brains were removed from all the perfused animals and post-fixed in 4% paraformaldehyde-PBS for 2 hours. After three PBS washes, 30 minutes each, brains were cryoprotected in 30% sucrose-PBS for 48–72 hours. Free-floating BLA sections (40 μm) were cut using a sliding microtome and collected in PBS containing 0.1% sodium azide for storage.

Three sections were processed for Zif268 immunoreactivity. After extensive washing in PBS, endogenous peroxidase was neutralized with hydrogen peroxide 0,75% for 10 minutes. Then sections were blocked in a solution of 0.5% Horse Serum (HS, BioWhittaker) and 0.5% Triton X-100 (Sigma Aldrich) in PBS. Slices were then incubated overnight at 4°C in anti-Zif268 antibody (Santa Cruz, rabbit polyclonal, 1:1000) in PBS-0.5% HS-0.5% Triton X-100. Afterwards 5 washes in PBS-0.5% HS-0.5% Triton X-100 sections were incubated 2 hours in anti-rabbit biotinilated antibody

(GE Healthcare, 1:1000). Following two washes in PBS-0.5% HS-0.5% Triton X-100 and three washes in PBS, tissue sections were visualized using VectaStain ABC kit (Vector Laboratories) and developed in DAB peroxidase substrate (Sigma) for 2-3 minutes. Sections were mounted on gelatinated slides, dehydrated with 50, 70, 80, 90, 96% and absolute ethanol. After 5 minutes in xylol slides were cover slipped with Entellan (Merck).

Sections were observed at transmission microscope (Axioskop 2, Zeiss). Two images per section (one from each hemisphere), for a total of six images per animal were acquired by the connected video camera (COHU High Performance CCD camera). Images were acquired with the 10X objective. Quantification of the number of neurons positive to Zif268 was done using the NIH software "Image-J" (www.rsbweb.nih.gov). Intensity threshold, minimum and maximum cell size parameter values were initially determined in an empirical fashion under blind conditions.

2.7 Data Analyses.

ALP responding on reinstatement session was compared among groups in order to test the efficacy of pharmacological treatments (vehicle, propranolol or MK-801) after retrieval or no-retrieval conditions. A two-way ANOVA for retrieval and pharmacological treatment factors was performed on total ALP/60 minutes on reinstatement session for each treatment. The number of ALP/60 minutes was the between subjects dependent variable.

The dependent variable for the immunohistochemistry experiments was the positive neurons count for Zif268. Unpaired Student's t-test for Ret and No-Ret group was performed. Statistical significance was reached for $P \leq 0.05$. All the statistical analysis were performed by using Prism 4 (Graph Pad, U.S.A.).

3. RESULTS

3.1 The Model

3.1.1 Nicotine self-administration acquisition

In Studies #1, #2, #3 rats were trained to self-administer nicotine.

In Study #1 (see experimental design in Figure 13) rats were trained until the number of reinforcements/session did not vary more than 20% between the last three consecutive S/A sessions (criteria of stability). Training to nicotine self-administration lasted 17.1 ± 0.2 sessions (mean ± S.E.M). At stability, the average number of nicotine infusions was 12.5 ± 0.1 (mean ± SEM of the last three self-administration session) (Figure 8, panel B). The average number of nicotine-paired lever (ALP) and inactive lever (IL) presses across the last three sessions were 40.1 ± 0.9 (baseline) and 2.8 ± 0.4 respectively (mean ± SEM). The specificity of nicotine seeking behaviour is confirmed by the discrimination between ALP and IL (Figure 8, panel A).

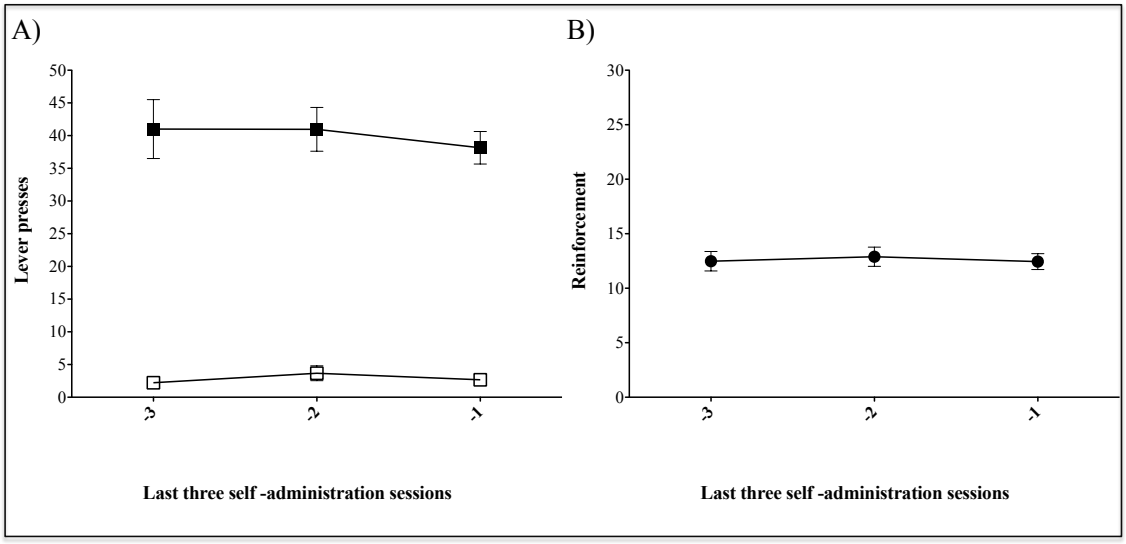

Figure 8. Nicotine self-administration acquisition in Study #1. A) Mean number of ALP and IL (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n=27). Discrimination between ALP and IL can be observed across the last three self-administration sessions. B) Mean number of reinforcement (nicotine infusion) across daily session. Stability of the response can be observed across last three sessions. (ALP: nicotine paired lever; IL: inactive lever).

In Study #2 (see experimental design in Figure 15) all rats were trained for 10 consecutive nicotine self-administration sessions and criteria of stability is also checked as for Study #1. At stability, the average number of nicotine infusions was 10.9 ± 0.3 (mean ± SEM of the last three self-administration session) (Figure 9, panel B). The average number of ALP and IL presses across the last three sessions was 19.5 ± 1.0 (baseline) and 4.5 ± 0.6 respectively (mean ± SEM). The discrimination between ALP and IL provides goal-directed evidence towards nicotine-seeking behaviour (Figure 9, panel A).

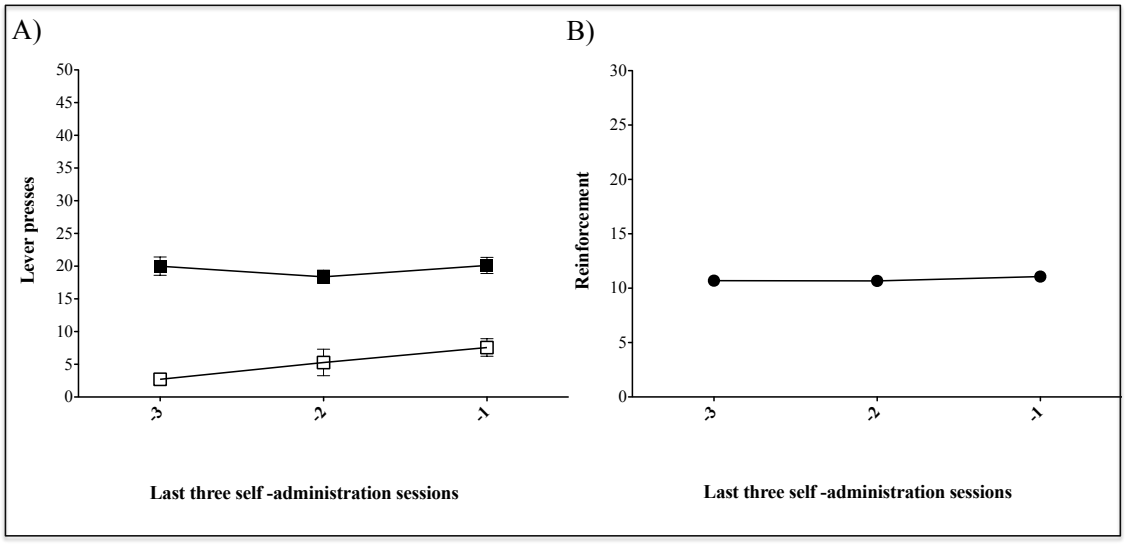

Figure 9. Nicotine self-administration acquisition in Study #2. A) Mean number of ALP and IL (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n=35). Discrimination between ALP and IL can be observed across the last three self-administration sessions. B) Mean number of reinforcement (nicotine infusion) across daily session. Stability of the response can be observed across the last three sessions. (ALP: nicotine paired lever; IL: inactive lever).

In Study #3 (For experimental design see Figure 17) rats were considered to meet the criteria of nicotine S/A training once they reached the value of 200 ± 15 (mean ± S.E.M.) associations between nicotine infusion and CS. Training to nicotine self-administration lasted 13.7 ± 0.5 sessions (mean ± S.E.M). At stability, the average number of nicotine infusions was 16.6 ± 1.1 (mean ± SEM of the last three self-administration session) (Figure 10, panel B). The average number of ALP and IL presses across the last three sessions were 34.1 ± 2.6 and 14.3 ± 0.9 respectively (mean ± SEM). The specificity of nicotine seeking behaviour is confirmed by the discrimination between ALP and IL (Figure 10, panel A).

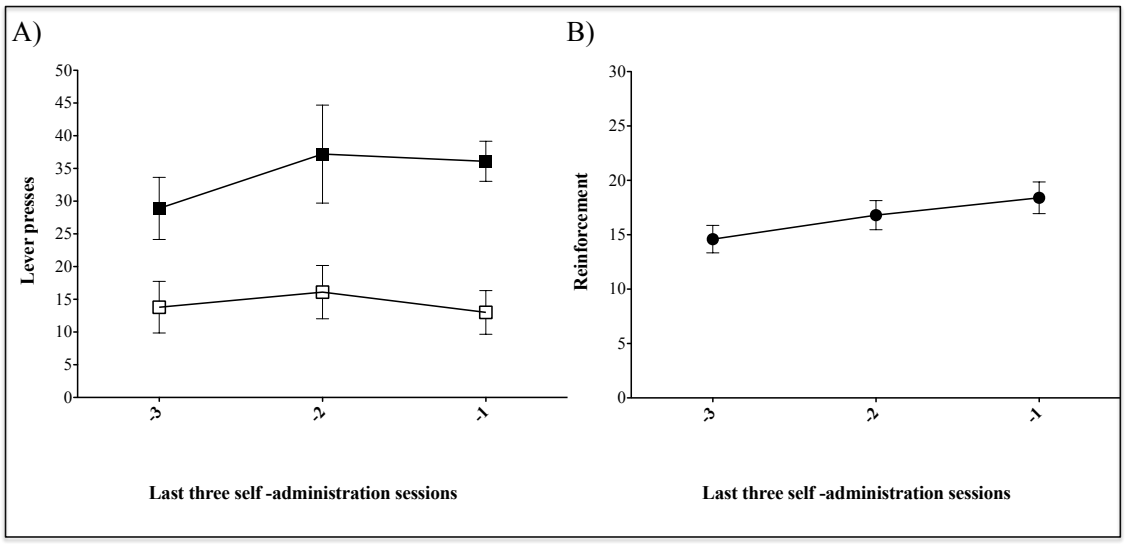

Figure 10. Nicotine self-administration acquisition in Study #3. A) Mean number of ALP and IL (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n=10). Discrimination between ALP and IL can be observed across the last three self-administration sessions. B) Mean number of reinforcement (nicotine infusion) across daily session. (ALP: nicotine paired lever; IL: inactive lever).

3.1.2 Instrumental learning extinction phase

In Study #3 rats underwent an instrumental learning extinction phase in order to extinguish the operant component of conditioning. Both React and No-React group of rats met the criteria of extinguished responding (less than 50% of ALP presses at the first instrumental learning extinction phase session, for three consecutive session): 3.8 ± 0.3 (React) and 5.1 ± 0.5 (No-React) ALP/60 min session (mean ± S.E.M.). The mean number ± S.E.M. of ILP/60 min session was 4.2 ± 0.2 and 3.2 ± 0.5 for React and No-React groups respectively during the last three instrumental learning extinction sessions. The criteria of instrumental learning extinction was met after an average number of 11.2 ± 0.8 sessions (mean ± S.E.M.).

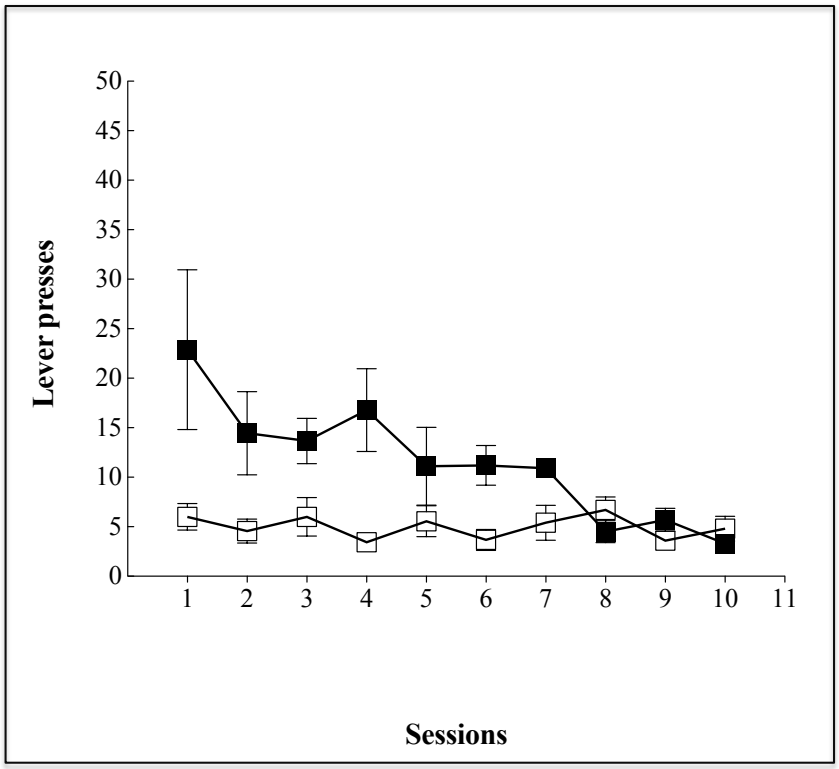

Figure 11. Instrumental learning extinction in Study #3. Mean number of ALP and IL presses (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n= 10). Across last three sessions the number of ALP presses were less than 50% of the number of ALP presses on the first session. (ALP: nicotine paired lever; IL: inactive lever).

3.1.3 Reinstatement

In Studies #1 and #2 a reinstatement session was performed 1 day after memory retrieval. In order to reinstate the nicotine seeking-behaviour, rats were placed back in

the context previously paired with nicotine administration and were exposed to conditioned stimuli (Study #1) or to the context only (Study #2) without nicotine infusion. Reinstatement of nicotine seeking behaviour is revealed by a significantly higher responding/lever presses on lever previously paired with nicotine compared (Figure 12, panel B) to the responding during the last instrumental learning extinction session (Figure 12, panel A).

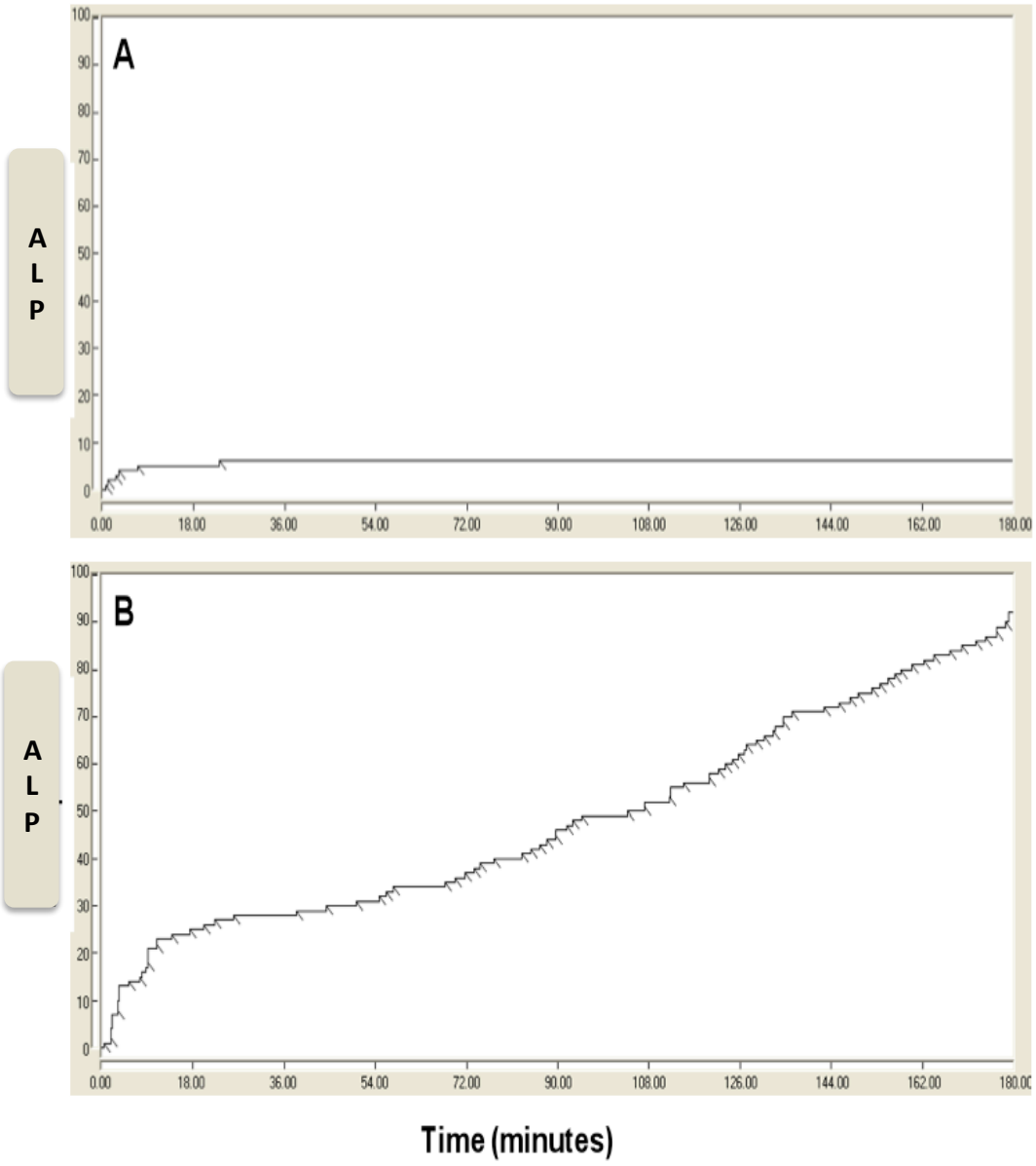

Figure 12. Example of Reinstatement of nicotine-seeking behaviour (animal code: TOM21). Graphs represent number of ALP (ordinates) across minutes (abscissa) during the last instrumental learning extinction session (panel A) and reinstatement session (panel B). Each step represents a lever press. On reinstatement session a reinstatement of nicotine seeking behaviour (lever presses) can be observed.

3.2. The Project

3.2.1. Study #1

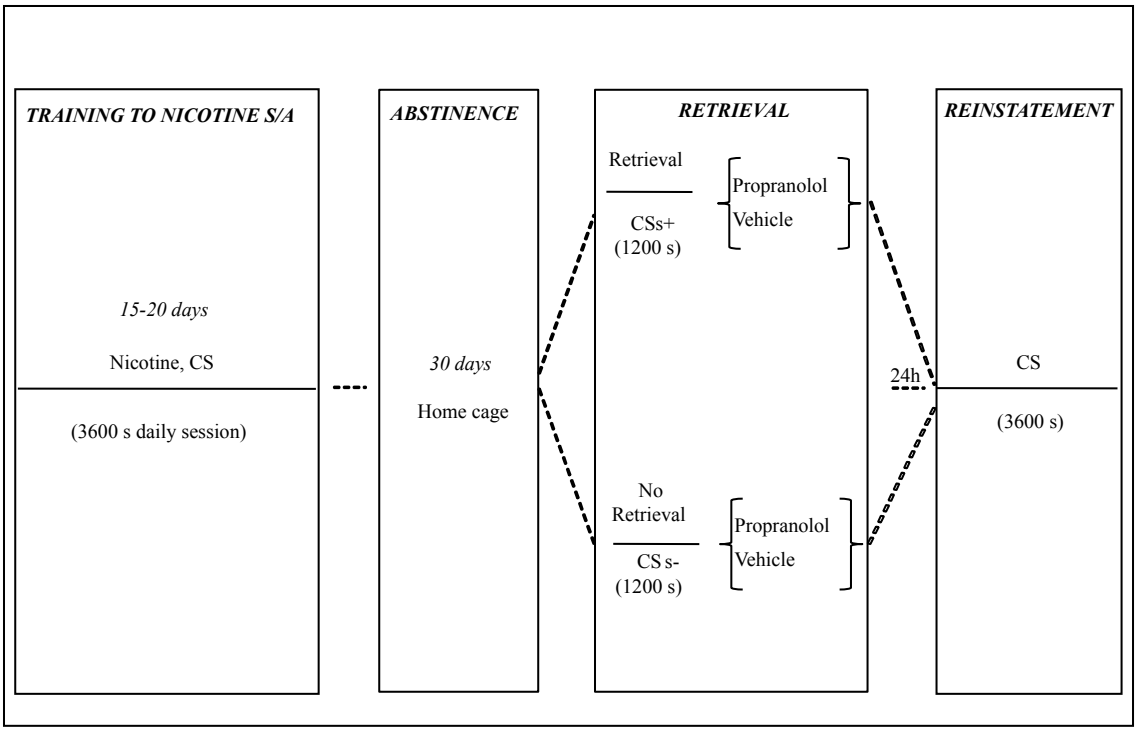

Figure 13. Schematic diagram of experimental design in Study #1. Rats were trained to nicotine self-administration (S/A; approximately 15-20 sessions). One day after the last S/A session they underwent 30 days of forced abstinence in home cage. Then they were divided into two groups that underwent a retrieval or no-retrieval session respectively. Each group was divided into two sub-groups treated with vehicle or propranolol 10 mg/kg immediately after retrieval or no-retrieval. The day after memory was tested in a reinstatement session that consisted in placing the rats in the S/A training context and during which each ALP (nicotine paired lever) press resulted in a CS (conditioned stimuli) presentation.

The total ALP presses at the end of the 60 minutes reinstatement session was 41.7 ± 9.1, 42.5 ± 11.1, 33.7 ± 9.0, 31.4 ± 8.8 (mean ± S.E.M.) respectively for No-Ret Veh (n = 6), No-Ret Prop (n = 6), Ret Veh (n = 8), Ret Prop (n = 7) (Figure 14). Two-way ANOVA for retrieval (ret) and pharmacological treatment (pharm treat) factors showed no statistically significant difference in ALP among groups (ret: $F_{[3,24]} = 0.98$, $p = 0.33$; pharm treat: $F_{[3,24]} = 0.006$, $p = 0.93$; interaction: $F_{[3,24]} = 0.03$, $p = 0.87$). The total IL presses at the end of the 60 minutes reinstatement session did not differ among groups (No-Ret Veh: 6.2 ± 5.4; No-Ret Prop: 2.2 ± 1.6; Ret Veh: 3.0 ± 1.4, Ret Prop: 7.7 ± 6.4; mean ± S.E.M.).

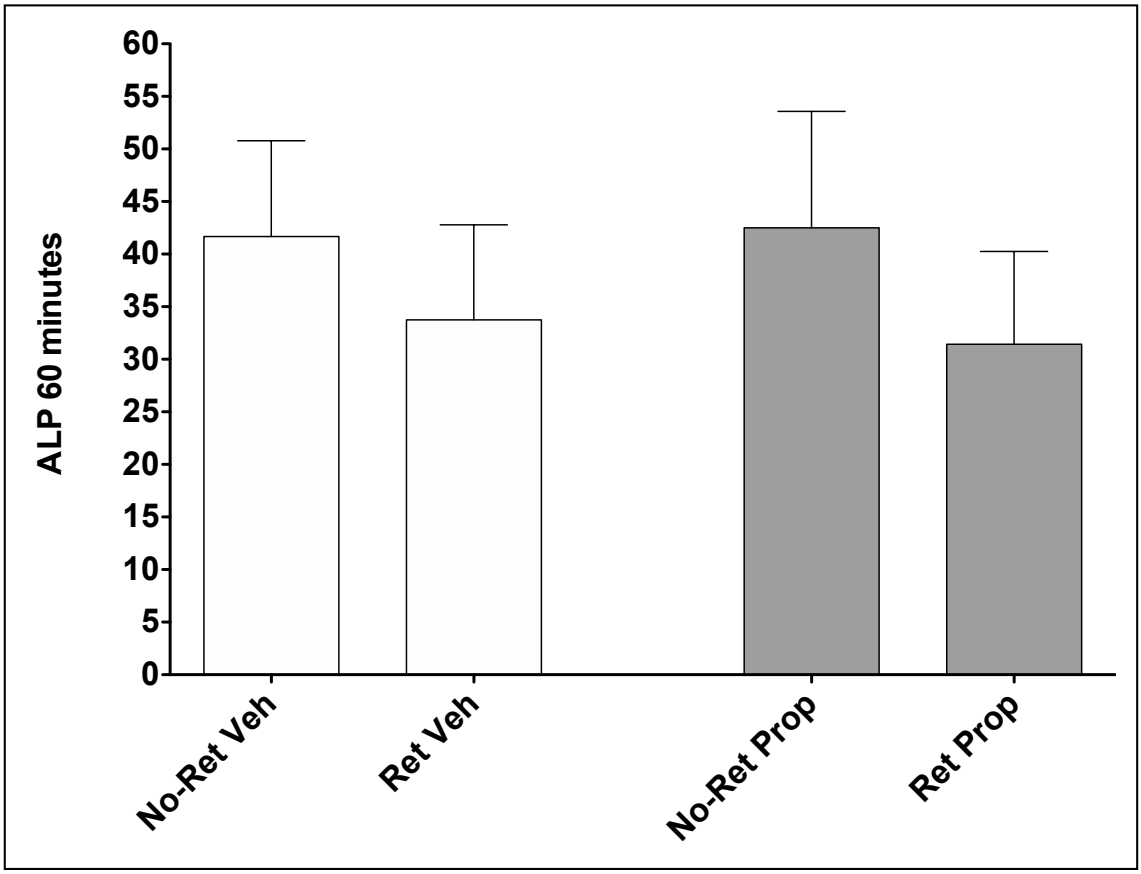

Figure 14. Reinstatement test in Pavlovian memory - Study #1. Propranolol 10 mg/kg had no effect on reinstatement test when given after memory retrieval. Data are expressed as total number of nicotine-paired lever presses, active lever presses (ALP), at the end of reinstatement session (mean ± S.E.M.) for No-Ret Veh group (white column, n = 6), Ret Veh group (white column, n = 8), No-Ret Prop group (grey column, n = 6), Ret Prop group (grey column, n = 7). Two-way ANOVA showed no statistically significant difference.

In summary no difference among groups was observed on reinstatement test. Propranolol 10 mg/kg did not reduce the ALP after memory retrieval or no-retrieval conditions. Propranolol did not prevent reinstatement of nicotine-seeking behaviour.

3.2.2. Study #2

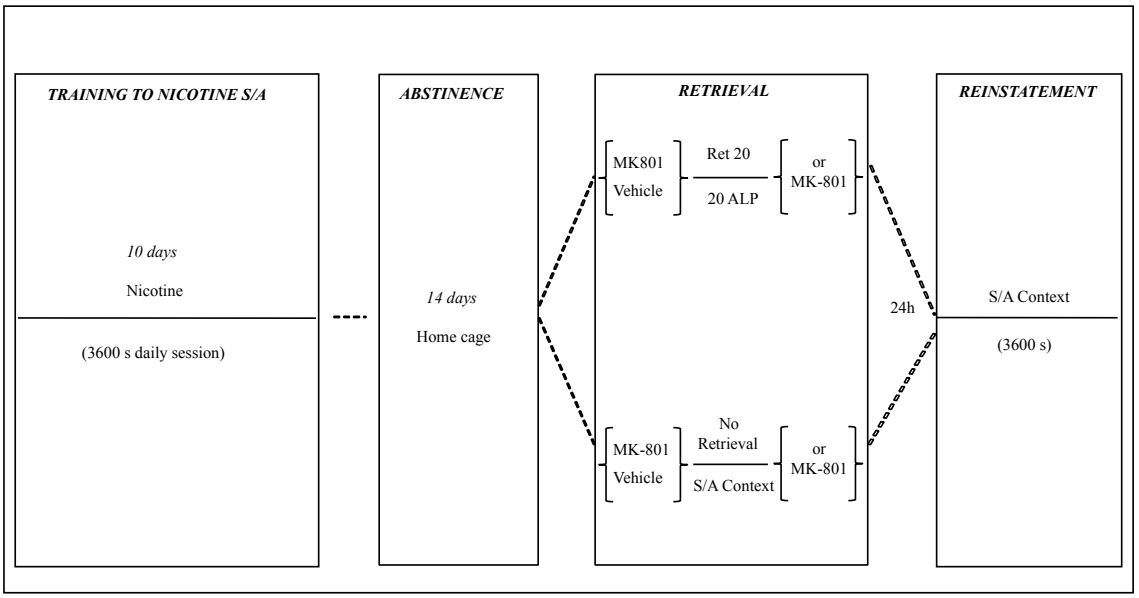

Figure 15. Schematic diagram of experimental design in Study #2. Rats were trained to nicotine self-administration (S/A; 10 sessions). One day after the last S/A session they underwent 14 days of forced abstinence in home cage. Then they were divided into two groups that underwent a retrieval (Ret 20; 20 ALP) or No-Retrieval (S/A context) session respectively. Each group was divided into two sub-groups treated with vehicle or MK-801 0.01 mg/kg 30 minutes before retrieval session. Other two sub-groups received MK-801 1 hour after the first ALP or after 1 hour of S/A context re-exposure on retrieval session. The day after memory was tested in a reinstatement session that consisted in placing the rats in the S/A training context and during which each ALP (nicotine paired lever) and IL (inactive lever) were recorded.

The total ALP presses at the end of the 60 minutes reinstatement session was 12.4 ± 2.8, 30.8 ± 5.8, 20.2 ± 7.0, 22.7 ± 3.1, 30.0 ± 2.8, 16.5 ± 2.2 (mean ± S.E.M.) respectively for pre-No-Ret Veh (n = 5), pre-Ret20 Veh (n = 6), pre-No-Ret MK-801 (n = 5), pre-Ret20 MK-801 (n = 7), post-No-Ret MK-801 (n = 6), post-Ret20 MK-801 (n = 6). Two-way ANOVA for retrieval and pharmacological treatment factors showed statistically significant effect for interaction ($F_{[5,29]} = 7.09$, $p = 0.003$) but not for pharmacological treatment ($F_{[5,29]} = 0.11$, $p = 0.89$) and retrieval ($F_{[5,29]} = 0.52$, $p = 0.47$) when comparing ALP/60 minutes on reinstatement session among pre-No-Ret Veh, pre-Ret20 Veh, pre-No-Ret MK-801, pre-Ret20 MK-801, post-No-Ret MK-801 and post-Ret20 MK-801groups (Figure 16). Bonferroni's post-test did not show significant differences in ALP when comparing pre-No-Ret Veh vs. pre-No-Ret MK-801 ($p>0.05$) and pre-Ret20 Veh vs. pre-Ret20 MK-801 ($p>0.05$). Bonferroni's post-test showed significant

differences in ALP when comparing pre-No-Ret Veh vs. post-No-Ret MK-801 ($p<0.05$) and pre-Ret20 Veh vs. post-Ret20 MK-801 ($p<0.05$) (Figure 16). The total IL presses at the end of the 60 minutes reinstatement session did not differ among groups (pre-No-Ret Veh: 5.8 ± 1.8; pre-Ret20 Veh: 8.0 ± 2.6, pre-No-Ret MK-801: 8.6 ± 3.6, pre-Ret20 MK-801: 5.4 ± 1.6, post-No-Ret MK-801: 6.5 ± 2.0, post-Ret20 MK-801: 2.8 ± 1.1; mean ± S.E.M.).

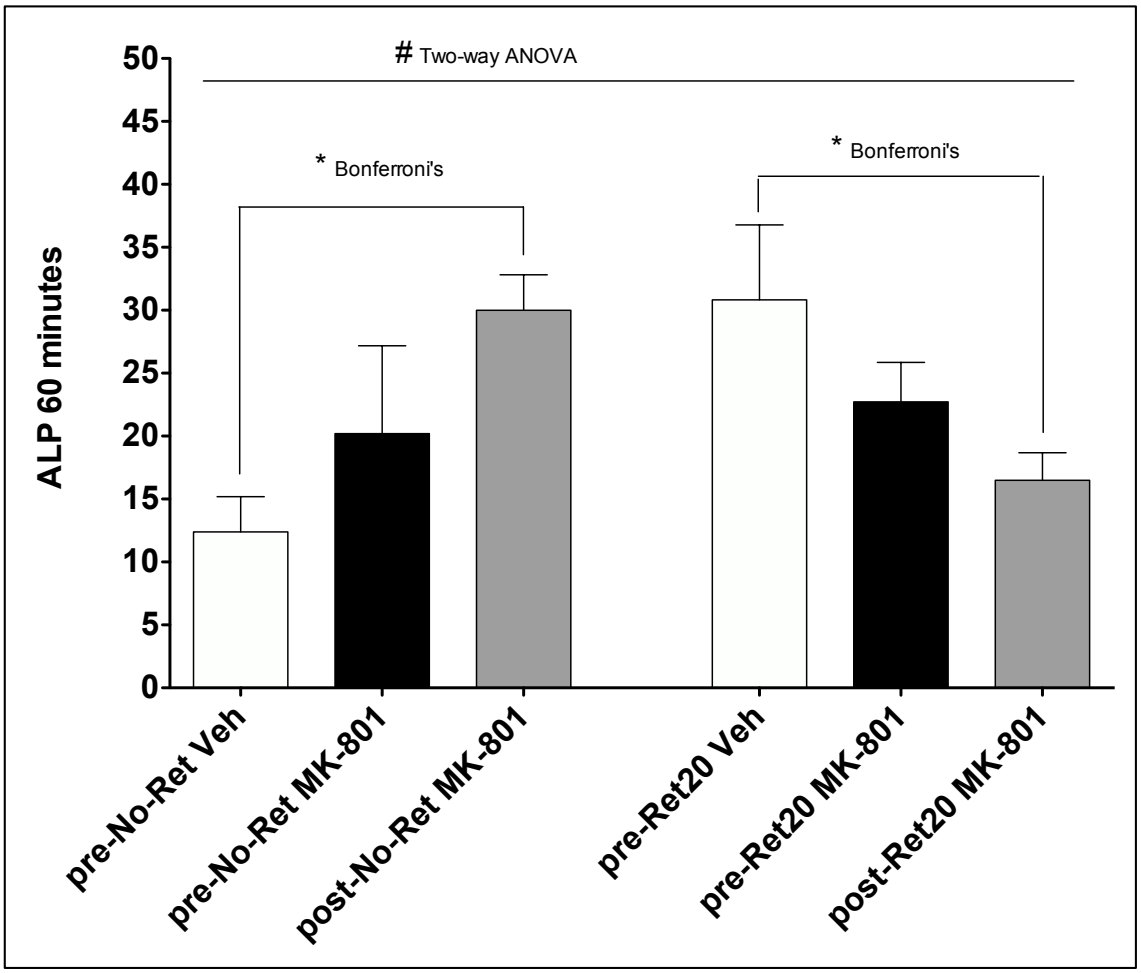

Figure 16. Reinstatement test of instrumental memory - Study #2. Instrumental memory retrieval per se increased ALP when rats were pre-treated with vehicle (pre-Ret20 Veh; white column). Injection of MK-801 0.01 mg/kg prior to retrieval session (pre-Ret20 MK-801; black column) or no-retrieval (pre-No-Ret Mk-801; black column) reduced the difference in the number of ALP between no-retrieval and retrieval condition that is observed with vehicle (black columns vs. white columns), but did not significantly reduce the ALP if compared to control (pre-No-Ret Veh, pre-Ret20 Veh; white columns). Injection of MK-801 0.01 mg/kg after context re-exposure (post-No-Ret MK-801; grey column) induced an increase of ALP if compared to control column (pre-No-Ret Veh; white column) while post-retrieval injection of MK-801 (post-

Ret20 MK-801; grey column) reduced ALP when compared to control column (pre-Ret20 Veh; white column). Data are expressed as total number of nicotine-paired lever presses, active lever presses (ALP), at the end of reinstatement session (mean ± S.E.M.) for pre-No-Ret Veh (white column, n = 5), pre-Ret20 Veh (white column, n = 6), pre-No-Ret MK-801 (black column, n = 5), pre-Ret20 MK-801 (black column, n = 7), post-NoRet MK-801 (grey column, n = 6) and post-Ret20 MK-801 (grey column, n = 6). # = $p<0.05$, two-way ANOVA; * = $p<0.05$, Bonferroni's post-test.

In summary instrumental memory retrieval per se increased the number of ALP after vehicle treatment. Injection of MK-801 0.01 mg/kg prior to retrieval session, or no-retrieval, reduced the difference in the number of ALP between retrieval and no-retrieval condition that is observed with vehicle, but did not significantly reduce the ALP if compared to control (pre-Ret20 Veh, pre-No-Ret Veh). Injection of MK-801 0.01 mg/kg after context re-exposure (no-retrieval condition) induced an increase of ALP if compared to control column (pre-No-Ret Veh) while post-retrieval injection of MK-801 0.01 mg/kg reduced ALP when compared to control column (pre-Ret20 Veh). MK-801 given after, but not prior to retrieval session, prevented reinstatement to nicotine seeking behaviour.

3.2.3 Study #3

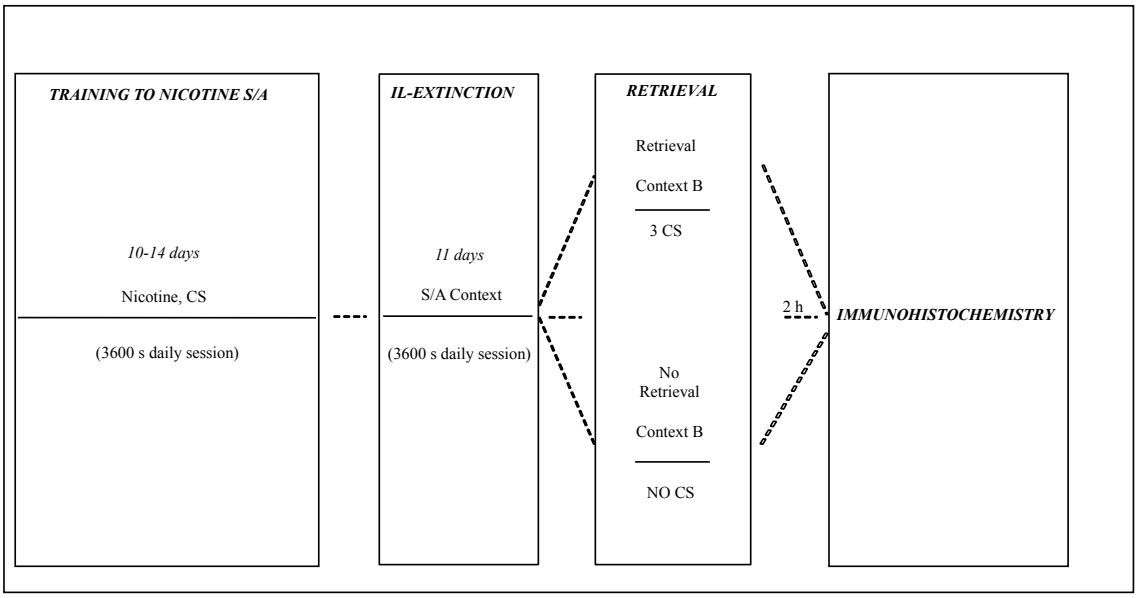

Figure 17. Schematic diagram of experimental design in Study #3. Rats were trained to nicotine self-administration (S/A; approximately 14 sessions) then they underwent an instrumental extinction (IL-EXTINCTION) phase in order to extinguish the instrumental learning component of conditioning (approximately 11 sessions). The day after the end of IL-EXTINCTION rats were divided into two groups that were exposed to 0 (No-Retrieval) or 3 CS (Retrieval) presentations respectively in a different context than the S/A and IL-EXTINCION phases (Context B). Two hours after memory retrieval (or no-retrieval) rats were sacrified and immunohistochemistry to assess the level of Zif268 expression in basolateral amygdala was performed.

The two separate groups of rats (React and NoReact) showed similar behaviour at the end of the nicotine S/A training phase and of the instrumental learning extinction phase. Two hours after reactivation (or no-reactivation) quantification of the number of Zif268 expressing cells was performed by immunohistochemistry (Figure 18, panel a). Reactivation induced an increase in the mean number/mm2 (± SEM) of Zif268 expressing cells in BLA compared to the no-reactivation condition (reactivation: 192.26 ± 24.92; no-reactivation: 103.67 ± 22.71). Student's t-test showed a significant difference between groups (p= 0.0186) (Figure 18, panel b).

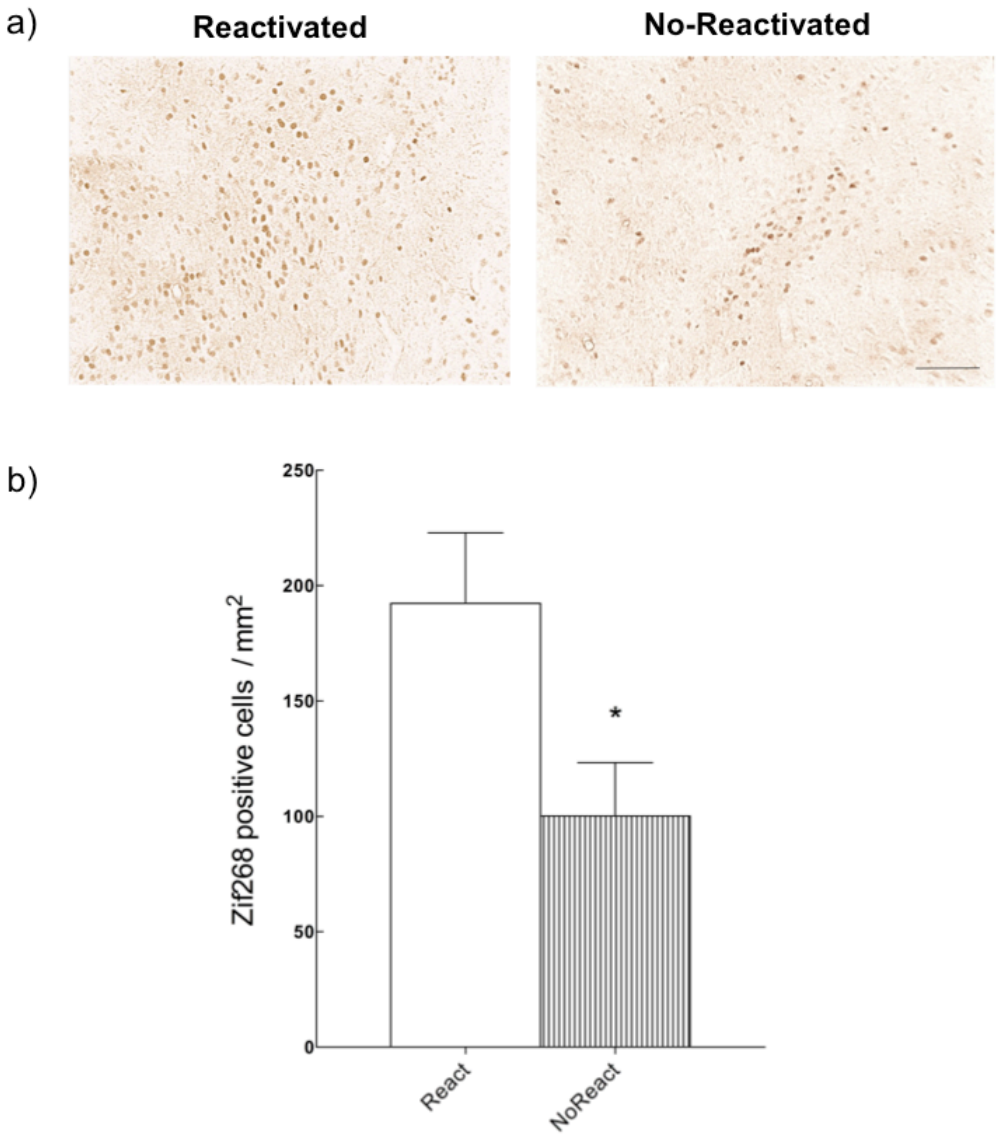

Figure 18. Immunohistochemistry assessment of reactivation-induced Zif268 expression in BLA. Panel a: representative images of transmission microscope sections of the BLA in rats that underwent no-reactivation (No-Reactivated) or reactivation (Reactivated) session. Zeiss Axioskop 2. Objective 10X. Scale bar 100 um

Panel b: number of Zif268 expressing cells / mm^2 in BLA in no-reactivated rats (NoReact) and reactivated rats (React). Data are expressed as mean ± standard error. Three 40 um slices, two pictures (one for each hemisphere) per slice. N=5. *$p<0.05$; Student's t test.

	TREATMENT	RETRIEVAL	ALP/REINSTATEMENT
STUDY #1	Vehicle	0 CS	-
	Propranolol	0 CS	-
	Vehicle	30 CS	-
	Propranolol	30 CS	-
STUDY #2	Vehicle	0 ALP	-
		20 ALP	increase
	Pre-MK-801	0 ALP	-
		20 ALP	-
	Post-MK-801	0 ALP	increase
		20 ALP	decrease
	TREATMENT	RETRIEVAL	Zif268 EXPRESSION
STUDY #3	X	0 CS	-
		3 CS	increase

Figure 19. Summary of results: ALP on reinstatement session and Zif268 expression after memory retrieval. In Study #1, the number of ALP did not vary at any condition. In Study #2, retrieval (20 ALP) increased the number of ALP after vehicle treatment; pre-retrieval injection of MK-801 did not change the number of ALP; post-retrieval injection of MK-801 increased the number of ALP after no-retrieval (0 ALP) and decreased the number of ALP after retrieval (20 ALP) condition. In Study #1 and Study #2 ALP increase or decrease was referred to respective control for each condition. In Study #3, retrieval (3 CS) increased Zif268 expression in amygdala compared to no-retrieval (0 CS) condition.

Box 1 / **"Old" and "new" molecular markers of memory reconsolidation.**

Zif268 and phosphorylated ribosomal protein S6 (rpS6P) expression in rat brain in a fear conditioning laboratory model of memory extinction and reconsolidation.

Memory reconsolidation disruption could be determined only through its absence during reinstatement or renewal test in behavioural studies. There are many boundary conditions related to memory retrieval: i) the length of the retrieval session (shorter session could not reactivate the memory as well as longer session could lead to memory extinction instead of reconsolidation); ii) memory strength and age (retrieval of stronger and/ or older memory could be ineffective); iii) certain kind of memories could not be retrieved and reconsolidated. The assessment of expression of molecular markers correlating reconsolidation (i.e. Zif268; rpS6P) can help to confirm memory reconsolidation occurrence and its disruption in specific laboratory conditions. Aim of these experiments was to verify the feasibility and reliability of Zif268 or rpS6P expression assessment at different stages of retrieval, reconsolidation or extinction in rodents. To address this issue we performed Zif268 or rpS6P immunohistochemistry assay after Pavlovian memory retrieval or extinction in a previously validated fear conditioning laboratory model.

Zif268 vs rpS6P
Zif268 is a standardized and widely used marker correlating reconsolidation. It is usually investigated as mRNA by in situ Hybridization (Hall et al., 2001; Thomas et al., 2002). The nuclear localization of Zif268 protein makes it also a good marker for a quantitative immunohistochemistry assay. The well defined nuclear signal allows to count the number of cells expressing Zif268 simply through the count of the number of positive nuclei. In our laboratory we have already pharmacologically standardized Zif268 immunohistochemistry using vehicle vs. cocaine 15 mg/kg I.P. treated rats.
However Zif268 protein is a product of the immediate early gene Zif268. Although it is accepted as specific marker of reconsolidation under certain conditions one have to consider that immediately early gene expression is the initial step of a "molecular cascade" that could be activated for, and involved in, many other processes.
rpS6P is a new molecular marker taken in consideration in memory reconsolidation field. Recent studies from Hoeffler and Klann (2010) suggest that mammalian target of rapamycin (mTOR) activation is involved in memory consolidation through eIF4F complex formation and in memory reconsolidation through rpS6 phosphorylation. rpS6P is the final step of a "molecular cascade" and could be more specific than an immediate early gene expression in the identification of memory reconsolidation process under certain conditions. In our laboratory we have already pharmacologically standardized immunohistochemistry as a quantitative assay for rpS6P using vehicle vs. ketamine 5 or 10 mg/kg I.P. treated rats. We also performed a double-labeled immunofluorescence as qualitative assay of neuronal rpS6P expression in order to exclude glial expression in our conditions.

Disruption of Pavlovian fear memory through extinction applied after retrieval and molecular correlates
Retrieval of consolidated memories induces a labile phase during which memory can be disrupted or updated. Monfils and collegues (2009) previously showed that fear memory retrieval increases the level of pGluR1 receptor, a calcium-permeable–AMPA receptor (CP-AMPAR), in the lateral amygdala while a second CS presented 1 hour after the retrieval (mimicking extinction learning applied after retrieval) leads to pGluR1 dephosphorylation. A central component of Retrieval-Extinction manipulation (Ret-Ext) induced erasure of fear is the synaptic removal of CP-AMPARs in the lateral amygdala (Clem and Huganir, 2010), a metabotropic GluR1 receptors (mGluR1Rs) dependent mechanism. mGluR1Rs activation has been associated with increased phosphorylation of the mammalian target of rapamycin (mTOR) downstream molecule ribosomal protein S6 (rpS6P; Antion et al., 2008) and mTOR inhibitor rapamycin disrupts reconsolidation of fear memories (Blundell et al., 2008). We tested the effect of retrieval, extinction and Ret-Ext on expression of specific marker correlating reconsolidation Zif268, and a new potential marker that is rpS6P, by immunolocalization in prefrontal cortex, lateral amygdala and hippocampus. Our results showed that retrieval and Ret-Ext, but not extinction, increased Zif268 expression in prefrontal cortex and lateral amygdala. Ret-Ext, but not retrieval or extinction alone, increased the expression of rpS6P in prefrontal cortex and lateral amygdala. Together, these data suggest that: i) reconsolidation is engaged in these laboratory conditions as indicated by Zif268 expression after retrieval; ii) extinction, if applied after retrieval, is incorporated in a memory reconsolidation process as indicated by Zif268 expression after Ret-Ext; iii) rpS6P that is specifically increased after Ret-Ext, but not after retrieval, can not be used as a marker of reconsolidation at least at the investigated time point.

4. DISCUSSION

In this project we have investigated whether pre or post-retrieval pharmacological treatments, such as propranolol or MK-801 were able to prevent the reinstatement of nicotine-seeking behaviour in an animal model of nicotine addictive behaviour. We have applied these treatments before or after retrieval of different memories (Pavlovian or instrumental memory). In Study #1, Propranolol was given after Pavlovian memory retrieval (0 or 30 CS presentation) and in Study #2, MK-801 was given before or after instrumental memory retrieval (0 or 20 ALP). In Study #3, the level of Zi268 (a specific marker correlating memory reconsolidation) expression in BLA, was investigated by immunohistochemistry after retrieval of Pavlovian memory (3 CS presentation). The results can be summarized as follows: i) reinstatement of nicotine-seeking behaviour has not been impaired by the administration of propranolol 10 mg/Kg given after Pavlovian memory retrieval; ii) reinstatement of nicotine-seeking behaviour has not been impaired by the administration of MK-801 given prior to instrumental memory retrieval even if the difference in the number of ALP between retrieval and no-retrieval condition that is observed with vehicle has been reduced; iii) reinstatement of nicotine-seeking behaviour has been impaired by the administration of MK-801 given after instrumental memory retrieval; iiii) immunohistochemistry showed an increased level of Zif268 expression in basolateral amygdala after retrieval of nicotine-related Pavlovian memories.

In the first study we were interested to assess whether reconsolidation of nicotine-related Pavlovian memories could be disrupted by the administration of β-adrenergic receptor antagonist propranolol after their retrieval. This is based on the idea that once consolidated, Pavlovian memory can be turned into a labile state through its retrieval, and disrupted by pharmacological treatments acting at noradrenergic system level, before its reconsolidation. The first evidence of the role of β-adrenergic receptor in the reconsolidation of appetitive memories had been provided by Diegaarde and colleagues in 2006: in their work they showed that the administration of propranolol, an antagonist of β-adrenergic receptor, contingently upon retrieval, could reduce the context-induced reinstatement of sucrose seeking behaviour in rats trained to sucrose self-administration. Subsequently it has been shown that propranolol prevented the acquisition of new response for cocaine-conditioned reinforcement (Milton et al., 2008b) and disrupted place preference conditioned by cocaine (Bernardi et al., 2006) and morphine (Robinson

& Franklin, 2007). Therefore we hypothesized that Propranolol given after memory retrieval could also prevent reconsolidation of Pavlovian nicotine-related memories leading to disruption of the conditioned values of CS and resulting in inhibition of nicotine-seeking behaviour reinstatement when CS were presented. Our results showed that Propranolol 10 mg/kg did not reduce the number of ALP on reinstatement test, after memory retrieval or no-retrieval conditions. Propranolol did not prevent CS-induced reinstatement of nicotine-seeking behaviour suggesting that Pavlovian nicotine-related memories reconsolidation had not been disrupted. As reported by Tronson and Taylor (2007), memory extinction (a new learning by which CS previously associated with a reinforcer become newly associated with no outcome) instead of memory reconsolidation, may occur under similar conditions after memory retrieval. The length of the retrieval session is an important determinant of whether reconsolidation or extinction occurs after memory retrieval and it could be the critical factor in extinction and reconsolidation protocols. However there were no differences between retrieved and no-retrieved control groups suggesting that retrieval session was not inducing memory extinction in our conditions. There is evidence that reactivation-dependent amnesia for appetitive memories is determined by the contingency of stimulus presentation (Lee and Everitt, 2008b) and that only associations that were directly reactivated, not those indirectly reactivated underwent reconsolidation in the amygdala of the fear-conditioned rat (Debjec et al., 2006). In Study #1 we have only reactivated Pavlovian nicotine-related memories, through CS presentation without lever presses during retrieval session. Thus it could be that Pavlovian nicotine-related memories were not reactivated because there was not contingency of stimulus. However propranolol, given at retrieval, failed in reducing cue-induced reinstatement of cocaine seeking behaviour, following forced abstinence, in rats trained to cocaine self-administration (Milton & Everitt 2010). Furthermore many authors highlighted the limited efficacy of Propranolol in memory reconsolidation disruption in particular when instrumental learning is a relevant component of the memory (Muravieva and Alberini, 2010; Milton et al., 2012). In addition, Kemenes and collegues (2006) also showed that: i) protein synthesis is always required for memory retrieval and reconsolidation of young and old fear related memories, but ii) PKA activation is required only for CS-induced memory retrieval and reconsolidation of young fear-related memories but not for motor or older and stronger memories. These data reflect potential different molecular mechanisms engaged during reconsolidation depending on the age and kind of the memory. The role

of PKA in memory reconsolidation of cocaine associated CS has also been investigated by Sanchez and collegues (2010). The molecular cascade that involves PKA activation seems to be triggered by β-adrenergic receptor activation and not by NMDARs (Tronson and Taylor, 2007). It could be argued that Propranolol, a β-adrenergic antagonist, preventing PKA activation can disrupt memory reconsolidation only when PKA is needed and recruited (ie. for Pavlovian memory). Propranolol limited efficacy in older or instrumental memory could be related to the fact that in the reconsolidation process of these memories PKA is not recruited. Given that in Study #1 only Pavlovian nicotine-related memories were retrieved in our retrieval session by CS presentation, it could be possible that instrumental memories (not retrieved indeed not disrupted) supported lever pressing during reinstatement test. NMDARs antagonists (i.e. MK-801) seem to be more effective in reconsolidation disruption of different kind of memory, an effect probably due to the inhibition of key molecules like MEK, by interaction with other kinases than PKA. However to date it is not clear if instrumental memory can be reactivated and undergoes reconsolidation or not. Indeed another hypothesis could be that instrumental memories do not undergo reconsolidation and could not be disrupted as suggested by Hernandez and Kelley (2004). To address this issue we tested the effect of the NMDARs antagonist MK-801 0.01 mg/kg given 30 minutes before the retrieval of instrumental memory. Results showed that instrumental memory retrieval per se increased nicotine-seeking behaviour in vehicle treated rats. Injection of MK-801 0.01 mg/kg prior to retrieval session, or no-retrieval, reduced the difference in the number of ALP between retrieval and no-retrieval condition that is observed with vehicle, but did not significantly reduce the ALP if compared to control. Indeed pre-retrieval MK-801 injection reduced the difference in nicotine-seeking behaviour between retrieval and no-retrieval condition that is observed with vehicle, but did not prevent the relapse to nicotine-seeking behaviour when compared to control group. Signaling at NMDARs is known to be important for memory reconsolidation, but although NMDAR-mediated signaling is required for the reconsolidation (restabilization) of conditioned stimulus (CS)–drug (Sadler et al., 2007; Brown et al., 2008; Itzhak, 2008; Milton et al., 2008a; Milton et al., 2012), CS–spatial (Przybyslawski and Sara, 1997), and CS–fear (Pedreira et al., 2002; Lee et al., 2006) memories, antagonism at the GluN2B subtype of NMDAR has been shown to prevent the destabilization of CS–fear memories, thereby protecting them from the effects of amnestic agents (Ben Mamou et al., 2006). Since memory destabilization is the first process engaged after memory reactivation, in order to guide

the memory to its reconsolidation this effect suggests a potential role of MK-801 in inhibition of the memory destabilization process instead of reconsolidation disruption.

We performed other experiments in which MK-801 was given after memory destabilization was engaged (i.e. given after memory retrieval). Injection of MK-801 0.01 mg/kg after context re-exposure (no-retrieval condition) induced an increase of ALP if compared to pre-No-Ret Veh group, while post-retrieval injection of MK-801 reduced the number of ALP when compared to control column (pre-Ret20 Veh). It has been shown that MK-801 impairs reconsolidation, but also blocks extinction of fear memory in rats (Lee et al., 2006). The number of ALP increase observed in post-No-Ret MK-801 group could be due to inhibition of a possible context-induced extinction process, that is engaged in pre-No-Ret Veh group. There was no significant difference in the amount of time spent in the training context among different groups of animals. Thus it could be argued that extinction of the training context was a process engaged in all groups but it was the main memory trace only in no-retrieved groups. When 20 ALP were introduced during retrieval session, instrumental memory reconsolidation was engaged and became the main memory trace. A reasonable explanation could be that pharmacological intervention acts against the main memory trace resulting in, i) ALP increase in post-No-Ret Mk-801 group, where context-induced extinction was the main memory trace and was blocked by MK-801, and ii) ALP reduction in post-Ret20 MK-801, where instrumental memory reconsolidation was the main memory trace and was disrupted by MK-801. Post-retrieval MK-801 injection prevented the relapse to nicotine-seeking behaviour when compared to control groups suggesting that instrumental memory reconsolidation had been disrupted.

There are some limitations on the use of pharmacological agents to interfere with reconsolidation. Milekic and Alberini (2002) showed that in an inhibitory avoidance task, the protein synthesis inhibitor anisomycin did not disrupt memory reconsolidation if the memories were older than 14 days. Further supporting the idea that the age of the initial memory is a relevant factor in the ability of protein synthesis inhibitors to block reconsolidation, Suzuki and colleagues (2004) found that memories less than 3 weeks old were subject to interruption by post-retrieval anisomycin but older memories (8 weeks) were not. Not only is the age of the memory important in predicting success of pharmacological targeting of reconsolidation, but the type of learning paradigm is a key as well. In the fear setting, propranolol administered to rats only works to block the reconsolidation of cued or contextual fear memories (Muravieva & Alberini 2010;

Debiec & LeDoux 2004; Abrari et al. 2008) and does not always appear to affect inhibitory avoidance memories (Muravieva & Alberini 2010). These results suggest that strength or age of the emotional memory, as well as the type of response elicited, could influence the way that reconsolidation paradigms are applied to reduce responding. Furthermore memory reconsolidation disruption could only be determined through its absence during reinstatement or renewal test in behavioural studies. This make difficult to interpret the lack of effect of amnestic drugs observed in some circumstances. Infact it is hard to understand if reconsolidation disruption is inhibited by the presence of boundary conditions or by the fact that some memories can not be reactivated and disrupted. From this perspective ex-vivo molecular experiments performed after memory retrieval could directly demonstrate memory reactivation and confirm reconsolidation occurrence supporting behavioural data. It has been demonstrated that zinc finger 268 (Zif268) expression increased in basolateral amygdala after the presentation, in a memory retrieval session, of conditioned stimuli compared to non conditioned (Thomas et al., 2003). Since Zif268 is also considered a specific marker correlating memory reconsolidation (Lee & Hynds, 2012) we verified the feasibility and reliability of Zif268 expression assessment by immunohistochemistry after memory retrieval in rats. Previous studies conducted in our laboratory demonstrated that 3 CS presentation was sufficient to induce Pavlovian nicotine-related memory retrieval and did not induce extinction. We evaluated Zif268 level of expression in basolateral amygdala, the most important brain region involved in reconsolidation of Pavlovian memories, after retrieval of Pavlovian nicotine-related memories by 3 CS presentation. Immunohistochemistry showed an increased level of Zif268 expression in basolateral amygdala after retrieval of Pavlovian nicotine-related memories compared to no retrieval condition. These data confirm the validity and feasibility of immunohistochemistry to assess the expression of molecular markers correlating reconsolidation such as Zif268 after Pavlovian memory retrieval.

4.1.Conclusion

Our findings suggest that: i) propranolol did not disrupt Pavlovian memory reconsolidation and did not prevent reinstatement of nicotine-seeking behaviour after CS presentation in our conditions, ii) MK-801 given prior to retrieval session could prevent instrumental memory destabilization, but did not prevent reinstatement of nicotine-seeking behaviour and did not disrupt memory reconsolidation in our conditions, iii) MK-801 given after retrieval session prevented reinstatement of nicotine-seeking behaviour and disrupted memory reconsolidation in our conditions, iiii) immunohistochemistry is a feasible technique to investigate the expression of molecular markers correlating reconsolidation such as Zif268, thus it can be used to support our future behavioural studies.

It remains to be addressed if Propranolol given after contingent CS presentation could disrupt Pavlovian memory reconsolidation and prevent reinstatement of nicotine-seeking behaviour. Drug addiction is a chronic disorder characterized by a high rate of relapse to drug use among abstinent. One of the main causes of relapse is the exposure to the CS that are associated to drug effect in a Pavlovian manner and influence drug-seeking behaviour and relapse through the memory they evoke and the interaction with instrumental memories (Milton and Everitt, 2010). We did not investigate if drugs acting at adrenergic level prevent drug-related memory reconsolidation after contingent CS presentation. Thus we can not exclude that contingency of stimulus could reactivate Pavlovian and instrumental memory at the same time rendering both memories, or their interaction, vulnerable to disruption by noradrenergic antagonists. We also did not investigate the effect of Propranolol on younger memories than 30 days old memories. Performing the latter study we could clarify if Pavlovian nicotine-related memories can be differently affected by noradrenergic antagonists depending on their age.

However our data suggest that instrumental memory is a relevant component that could be responsible for the lack of effect of some anti-relapse pharmacological treatments and fortunately we confirmed that instrumental memory can be disrupted acting at specific molecular level that is NMDARs antagonism. Even in this case we did not investigated if instrumental memory older that 14 days was still suscebtible to drugs targeting its reconsolidation. It could be of interest to understand if time is a limiting factor for pharmacological interventions acting against Pavlovian and instrumental nicotine-related memories.

We validated a cellular and molecular technique to directly demonstrate memory reconsolidation occurrence, but we have only determined Zif268 expression after Pavlovian memory retrieval. We should also investigate if the same technique, and the same molecular marker can be used to directly demonstrate instrumental memory reconsolidation. Future studies, supported by the validated immunohistochemistry assessment of Zif268 expression, could thus elucidate if memory reconsolidation occurs after retrieval of different memories and using different protocols. In this way we will be able to understand if the lack of effect of some pharmacological treatments is due to the presence of boundary conditions or to the fact that a different molecular level has to be targeted.

In conclusion our findings suggest that new and specific pharmacological intervention, acting at specific molecular mechanisms that underly reconsolidation of different kind of memories (i.e. Pavlovian but also instrumental memories), could be used as a potential co-adjuvant to current therapeutic interventions for smoking cessation and abstinence maintenance.

5. REFERENCES

Abrams DB (1999) Nicotine addiction: paradigms for research in the 21st century. Nicotine Tob Res 1: S211–S215.

Abrari K, Rashidy-Pour A, Semnanian S, Fathollahi Y(2008) Administration of corticosterone after memory reactivation disrupts subsequent retrieval of a contextual conditioned fear memory: dependence upon training intensity. Neurobiol Learn Mem 89 (2): 178-84.

Alberini CM (2005) Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes? Trends Neurosci 28: 51–56.

Albuquerque EX, Pereira EF, Alkondon, Scott WR (2009) Mammalian Nicotinic Acetylcholine Receptors: From Structure to Function. Physiol Rev 89: 73-120.

Albuquerque EX, Pereira EF, Alkondon M, Schrattenholz A, Maelicke A (1997) Nicotinic acetylcholine receptors on hippocampal neurons: distribution on the neuronal surface and modulation of receptor activity. J Recept Signal Transduct Res 17: 243-266.

Alkondon M, Pereira EF, Barbosa CT, Albuquerque EX (1997) Neuronal nicotinic acetylcholine receptor activation modulates gammaminobutyric acid release from CA1 neurons of rat hippocampal slices. J Pharmacol Exp Ther 283: 1396-1411.

American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, 4th ed., American Psychiatric Association, Washington, DC, 1994.

Antion MD, Hou L, Wong H, Hoeffer CA, Klann E (2008) mGluR-dependent long-term depression is associated with increased phosphorylation of S6 and synthesis of elongation factor 1A but remains expressed in S6K-deficient mice. Mol Cell Biol 28(9): 2996-3007.

Ator N, Griffiths R (1993) Differential sensitivity to midazolam discriminative-stimulus effects following self-administered versus response-independent midazolam. Psychopharmacology (Berl) 110: 1-4.

Balfour DJK (2001) The pharmacology underlying pharmacotherapy for tobacco dependence: a focus on bupropion. Int J Clin Pract 55: 53-57.

Ben Mamou C, Gamache K, Nader K (2006) NMDA receptors are critical for unleashing consolidated auditory fear memories. Nat Neurosci 9: 1237–1239.

Benowitz NL (1988) Drug therapy. Pharmacologic aspects of cigarette smoking and nicotine addition. N Engl J Med 17;319(20): 1318-30.

Benowitz NL, Porchet H, Jacob P (1989) 3rd Nicotine dependence and tolerance in man: pharmacokinetic and pharmacodynamic investigations. Prog Brain Res 79: 279-287.

Benwell ME, Balfour DJ. (1979) Effects of nicotine administration and its withdrawal on plasma corticosterone and brain 5-hydroxyindoles. Psychopharmacology (Berl). 8;63(1): 7-11.

Bernardi RE, Lattal KM, Berger SP (2006) Postretrieval propranolol disrupts a cocaine conditioned place preference. Neuroreport 17: 1443-1447.

Blundell J, Kouser M, Powell CM (2008) Systemic inhibition of mammalian target of rapamycin inhibits fear memory reconsolidation. Neurobiol Learn Mem 90(1): 28-35.

Brown TE, Forquer MR, Cocking DL, Jansen HT, Harding JW, Sorg BA (2007) Role of matrix metalloproteinases in the acquisition and reconsolidation of cocaine-induced conditioned place preference. Learn Mem 14: 214-223.

Brown TE, Lee BR, Sorg BA (2008) The NMDA antagonist MK-801 disrupts reconsolidation of a cocaine-associated memory for conditioned place preference but not for self-administration in rats. Learn Mem 15: 857-865.

Caggiula AR, Donny EC, Chaudhri N, Perkins KA, Evans-Martin FF, Sved AF (2002) Importance of nonpharmacological factors in nicotine self-administration. Physiol Behav 77(4-5): 683-687.

Caggiula AR, Donny EC, White AR, Chaudhri N, Booth S, Gharib MA, Hoffman A, Perkins KA, Sved AF (2001) Cue dependency of nicotine self-administration and smoking, Pharmacol Biochem Behav 70: 515-530.

Centonze D, Siracusano A, Calabresi P, Bernardi G (2005) Removing pathogenic memories: a neurobiology of psychotherapy. Mol Neurobiol 32(2): 123-132.

Cesselin F (1995) Opioid and anti-opioid peptides. Fundam Clin Pharmacol 9(5): 409-433.

Chiamulera C (2005) Cue reactivity in nicotine and tobacco dependence: a "multiple-action" model of nicotine as a primary reinforcement and as an enhancer of the effects of smoking-associated stimuli. Brain Res Brain Res Rev 48(1): 74-97.

Chiamulera C, Tedesco V, Zangrandi L, Giuliano C, Fumagalli G (2010) Propranolol transiently inhibits reinstatement of nicotine-seeking behaviour in rats. J Psychopharmacol. 24(3): 389-95.

Clarke PB (1990) Mesolimbic dopamine activation-the key to nicotine reinforcement? Ciba Found Symp 152:153-162; discussion 162-168.

Clem RL, Huganir RL (2010) Calcium-permeable AMPA receptor dynamics mediate fear memory erasure. Science 330(6007): 1108-1112.

Clinical Practice Guideline Treating Tobacco Use and Dependence 2008 Update Panel, Liaisons, and Staff. A U.S. Public Health Service report. Am J Prev Med.35:158-76.

Conklin CA, Robin N, Perkins KA, Salkeld RP, McClernon FJ (2008) Proximal versus distal cues to smoke: the effects of environments on smokers' cue-reactivity. Exp Clin Psychopharmacol 16(3): 207-214.

Corrigall WA, Coen KM (1989) Nicotine maintains robust self-administration in rats on a limited-access schedule. Psychopharmacology (Berl) 99: 473-478.

Dani JA, Heinemann S (1996) Molecular and cellular aspects of nicotine abuse. Neuron 16: 905–908H.

Debiec J, Doyere J, Nader K, LeDoux JE (2006) Directly reactivated, but not indirectly reactivated, memories undergo reconsolidation in the amygdala. Proc Natl Acad Sci USA 103:3428-3433.

Debiec J, LeDoux, JE (2004) Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. Neuroscience 129: 267-272.

Di Chiara G (2000) Role of dopamine in the behavioural actions of nicotine related to addiction. Eur J Pharmacol 393: 295-314.

Di Chiara G, Imperato A (1988) Drugs of abuse preferentially stimulate dopamine release in the mesolimbic system of freely-moving rats. Proc Natl Acad Sci U.S.A. 85: 5274-5278.

Dickinson A, Balleine B (1994) Motivational control of goal-directed action. Anim Learn Behav 22: 1–18.

Diergaarde L, Schoffelmeer AN, De Vries TJ (2006) Beta-adrenoceptor mediated inhibition of long-term reward-related memory reconsolidation. Behav Brain Res 170: 333-336.

Diergaarde L, Schoffelmeer AN, De Vries TJ (2008) Pharmacological manipulation of memory reconsolidation: towards a novel treatment of pathogenic memories. Eur J Pharmacol 585(2-3): 453-457.

Drummond DC (2000) What does cue-reactivity have to offer clinical research? Addiction 95: S129-S144.

Duvarci S, Nader K (2004) Characterization of fear memory reconsolidation. J Neurosci 24: 9269-9275.

Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci 8(11):1481-9.

Field M, Duka T (2001) Smoking expectancy mediates the conditioned responses to arbitrary smoking cues. Behav Pharmacol 12(3): 183-194.

Field M, Duka T, Tyler E, Schoenmakers T (2009) Attentional bias modification in tobacco smokers. Nicotine Tob Res 11(7): 812-22.

Ford CL, Zlabek JA (2005) Nicotine replacement therapy and cardiovascular disease. Mayo Clin Proc 80(5): 652-656.

French ED, Dillon K, Ali SF (1996) Effects of ibogaine, and cocaine and morphine after ibogaine, on ventral tegmental dopamine neurons. Life Sci 59(12): PL199-205.

Fricks-Gleason AN, Marshall JF (2008) Post-retrieval beta-adrenergic receptor blockade: effects on extinction and reconsolidation of cocaine-cue memories. Learn Mem 15: 643-648.

Frishman WH (2007) Smoking cessation pharmacotherapy-nicotine and non-nicotine preparations. Prev Cardiol 10(2 Suppl 1): 10-22.

Fuchs RA, Bell GH, Ramirez DR, Eaddy JL, Su ZI (2009). Basolateral amygdala involvement in memory reconsolidation processes that facilitate drug context-induced cocaine seeking. Eur J Neurosci 30: 889-900.

Gray R, Rajan AS, Radcliffe KA, Yakehiro M, Dani JA (1996) Hippocampal synaptic transmission enhanced by low concentration of nicotine. Nature 383: 713-716.

Guo JZ, Tredway TL, Chiappinelli VA (1998) Glutamate and GABA release are enhanced by different subtypes of presynaptic nicotinic receptors in the lateral geniculate nucleus. J Neurosci 18: 1963-1969.

Hall J, Thomas KL, Everitt BJ (2001). Cellular imaging of zif268 expression in the hippocampus and amygdala during contextual and cued fear memory retrieval: selective activation of hippocampal CA1 neurons during the recall of contextual memories. J Neurosci 21(6): 2186-93.

Hoeffer CA, Klann E (2010) mTOR signaling: at the crossroads of plasticity, memory and disease. Trends Neurosci 33(2): 67-75.

Hughes JR (1992) Tobacco withdrawal in self-quitters. J Consult Clin Psychol 60(5): 689-697.

Hughes JR (2007) Effects of abstinence from tobacco: etiology, animal models, epidemiology, and significance: a subjective review. Nicotine Tob Res 9(3): 329-339.

Hughes JR (2007) Effects of abstinence from tobacco: valid symptoms and time course. Nicotine Tob Res 9(3): 315-327.

Hughes JR, Hatsukami DK, Pickens RW, Krahn D, Malin S, Luknic A (1984) Effect of nicotine on the tobacco withdrawal syndrome. Psychopharmacology (Berl) 83(1): 82-87.

Hughes JR, Higgins ST, Hatsukami D (1990) Effects of abstinence from tobacco: a critical review, in: Koslowski LT , Annis HM, Cappell HD, Glaser FB, Goodstadt MS, Israel Y, Kalant H, Sellers EM, Vingilis ER (Eds.), Research Advances in Alcohol and Drug Problems, vol. 10, Plenum, New York, 1990: 317 – 398.

Hernandez PJ, Kelley AE (2004) Long-term memory for instrumental responses does not undergo protein synthesis-dependent reconsolidation upon retrieval. Learn Mem 11(6):748-54. Epub 2004 Nov 10.

Hukkanen J, Jacob P 3rd, Benowitz NL (2005) Metabolism and disposition kinetics of nicotine. Pharmacol Rev 57(1): 79-115.

Hupbach A, Gomez R, Hardt O, Nadel L (2007) Reconsolidation of episodic memories: a subtle reminder triggers integration of new information. Learn Mem 14, 47-53.

Imperato A, Mulas A, Di Chiara G (1986) Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats. Eur J Pharmacol 132: 337-338.

Inda MC, Muravieva EV, Alberini CM (2011) Memory retrieval and the passage of time: from reconsolidation and strengthening to extinction. J Neurosci 31: 1635-1643.

Itzhak Y (2008) Role of the NMDA receptor and nitric oxide in memory reconsolidation of cocaine-induced conditioned place preference in mice. Ann N Y Acad Sci 1139: 350-357.

Jensen AA, Frølund B, Liljefors T, Krogsgaard-Larsen P (2005) Neuronal nicotinic acetylcholine receptors: structural revelations, target identifications, and therapeutic inspirations. J Med Chem 48(15): 4705-45.

Ji D, Lape R, Dani JA (2001) Timing and location of nicotinic activity enhances or depresses hippocampal synaptic plasticity. Neuron 31: 131-141.

Jones HE, Garrett BE, Griffiths RR (1999) Subjective and physiological effects of intravenous nicotine and cocaine in cigarette smoking cocaine abusers. J Pharmacol Exp Ther 288(1): 188-197.

Jones IW, Wonnacott S (2004) Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. J Neurosci 24(50): 11244-11252.

Kalivas PW, O'Brien C (2008). Drug addiction as a pathology of staged neuroplasticity. Neuropsychopharmacology 33(1):166-80.

Karp I, O'Loughlin J, Hanley J, Tyndale RF, Paradis G (2006) Risk factors for tobacco dependence in adolescent smokers. Tob Control 15(3): 199-204.

Kelley JB, Anderson KL, Itzhak Y (2007) Long-term memory of cocaine-associated context: disruption and reinstatement. Neuroreport 18: 777–780.

Kemenes G, Kemenes I, Michel M, Papp A, Müller U (2006) Phase-dependent molecular requirements for memory reconsolidation: differential roles for protein synthesis and protein kinase A activity. J Neurosci. 2006 26(23): 6298-302.

Kindt M, Soeter M (2011) Reconsolidation in a human fear conditioning study: A test of extinction as updating mechanism. Biol Psychol (in press).

Lee JL (2009) Reconsolidation: maintaining memory relevance. Trends Neurosci 32(8): 413-420.

Lee JL, Di Ciano P, Thomas KL, Everitt BJ (2005) Disrupting reconsolidation of drug memories reduces cocaine-seeking behavior. Neuron 47: 795–801.

Lee JL, Everitt BJ (2008a) Appetitive memory reconsolidation depends upon NMDA receptor-mediated neurotransmission. Neurobiol Learn Mem 90(1): 147-154.

Lee JL, Everitt BJ (2008b) Reactivation-dependent amnesia for appetitive memories is

determined by the contingency of stimulus presentation. Learn Mem 15(6): 390-3.

Lee JL, Hynds RE. (2012) Divergent cellular pathways of hippocampal memory consolidation and reconsolidation. Hippocampus doi: 10.1002/hipo.22083.

Lee JL, Milton AL, Everitt BJ (2006) Reconsolidation and extinction of conditioned fear: inhibition and potentiation. J Neurosci 26(39): 10051-6.

Lerman C, LeSage MG, Perkins KA, O'Malley SS, Siegel SJ, Benowitz NL, Corrigall WA (2007) Translational research in medication development for nicotine dependence. Nat Rev Drug Discov. 6(9): 746-762.

Li X, Rainnie DG, McCarley RW, Greene RW (1998) Presynaptic nicotinic receptors facilitate monoaminergic transmission. J Neurosci 18(5): 1904-1912.

Lowinson JH, Ruiz P, Millman RB, Langrad JG (2005) Substance abuse: a comprehensive textbook. United States: Lippicott Williams &Wilkins.

Mansvelder HD, McGehee DS (2000) Long-term potentiation of excitatory inputs to brain reward areas by nicotine. Neuron 27(2): 349-357.

Mansvelder HD, McGehee DS (2002) Cellular and synaptic mechanisms of nicotine addiction. J Neurosci 53: 606–617.

Marubio LM, Gardier AM, Durier S, David D, Klink R, Arroyo-Jimenez MM, McIntosh JM, Rossi F, Champtiaux N, Zoli M, Changeux JP (2003) Effects of nicotine in the dopaminergic system of mice lacking the alpha4 subunit of neuronal nicotinic acetylcholine receptors. Eur J Neurosci 17(7): 1329-1337.

Mc Gehee DS, Heath MJS, Gelber S, Devay P, Role LW (1995) Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. Science 269: 1692- 1696.

Mc Gehee DS, Role LW (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annual Review of Physiology 57: 521-546.

Milekic MH, Brown SD, Castellini C & Alberini CM (2006) Persistent disruption of an established morphine conditioned place preference. J Neurosci 26: 3010–3020.

Milton AL, Everitt BJ (2010) The psychological and neurochemical mechanisms of drug memory reconsolidation: implications for the treatment of addiction. Eur J Neurosci 31: 2308–2319.

Milton AL, Lee JL, Butler VJ, Gardner R, Everitt BJ (2008a) Intra-amygdala and systemic antagonism of NMDA receptors prevents the reconsolidation of drug-associated memory and impairs subsequently both novel and previously acquired drug-seeking behaviors. J Neurosci 28: 8230–8237.

Milton AL, Lee JL, Everitt BJ (2008b) Reconsolidation of appetitive memories for both natural and drug reinforcement is dependent on {ß}-adrenergic receptors. Learn Mem 15: 88–92.

Milton AL, Merlo E, Ratano P, Gregory BL, Dumbreck JK, Everitt BJ (2013) Double Dissociation of the Requirement for GluN2B- and GluN2A-Containing NMDA Receptors in the Destabilization and Restabilization of a Reconsolidating Memory. J Neurosci (3): 1109-15.

Milton AL, Schramm MJ, Wawrzynski JR, Gore F, Oikonomou-Mpegeti F, Wang NQ, Samuel D, Economidou D, Everitt BJ (2012) Antagonism at NMDA receptors, but not beta-adrenergic receptors, disrupts the reconsolidation of Pavlovian conditioned approach and instrumental transfer for ethanol- associated conditioned stimuli. Psychopharmacology (Berl.) 219(3): 751-761.

Misanin JR, Miller RR, Lewis DJ (1968) Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. Science 160: 554–555.

Monfils MH, Cowansage KK, Klann E, LeDoux JE (2009) Extinction-reconsolidation boundaries: key to persistent attenuation of fear memories. Science 324(5929): 951-5.

Muravieva EV, Alberini CM (2010) Limited efficacy of propranolol on the reconsolidation of fear memories. Learn Mem 17(6): 306-313.

Nader K, Hardt O (2009) A single standard for memory: the case for reconsolidation. Nat Rev Neurosci 10: 224–234.

Nader K, Schafe GE, Le Doux JE (2000a) Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. Nature 406: 722-726.

Nader K, Schafe GE, LeDoux JE (2000b) The labile nature of consolidation theory. Nat Rev Neurosci 1: 216–219.

Nader K (2003) Memory traces unbound. Trends Neurosci 26(2): 65-72.

Niaura RS, Rohsenow DJ, Binko JA, Monti PM, Abrams DB, Pedraza M (1988) The relevance of cue reactivity to understanding alcohol and smoking relapse. J Abnorm Psychol 97(2): 133-152.

Nisell M, Nomikos GG, Svensson TH (1994) Infusion of nicotine in the ventral tegmental area or the nucleus accumbens of the rat differentially affects accumbal dopamine release. Pharmacol Toxicol 75(6): 348-52.

Nisell M, Nomikos GG, Svensson TH (1994) Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. Synapse 16(1): 36-44.

Pavlov IP (1927) Conditioned Reflexes: an investigation of the physiological activity of the cerebral cortex. Oxford University Press, London, UK.

Payne TJ, Smith PO, Sturges LV, Holleran SA (1996) Reactivity to smoking cues: mediating roles of nicotine dependence and duration ofdeprivation. Addict Behav 21: 139-154.

Pedreira ME, Pérez-Cuesta LM, Maldonado H (2002) Reactivation and reconsolidation of long-term memory in the crab Chasmagnathus: protein synthesis requirement and mediation by NMDA-type glutamatergic receptors. J Neurosci 22: 8305–8311.

Pidoplichko VI, DeBiasi M, Williams JT, Dani JA (1997) Nicotine activates and desensitizes midbrain dopamine neurons. Nature 390(6658): 401-404.

Pomerleau OF (1995) Individual differences in sensitivity to nicotine: implications for genetic research on nicotine dependence. Behav Genet 25(2): 161-177.

Przybyslawski J, Sara SJ (1997) Reconsolidation of memory after its reactivation. Behav Brain Res 84: 241–246.

Radcliffe KA, Dani JA (1988) Nicotinic stimulation produces multiple forms of increased glutamatergic synaptic transmission. J Neurosci 18: 7075-7083.

Radcliffe KA, Fisher JL, Gray R, Dani JA (1999) Nicotinic modulation of glutamate and GABA synaptic transmission of hippocampal neurons. Ann N Y Acad Sci 868: 591-610.

Ramirez DR, Bell GH, Lasseter HC, Xie X, Traina SA, Fuchs RA (2009) Dorsal hippocampal regulation of memory reconsolidation processes that facilitate drug context-induced cocaine-seeking behavior in rats. Eur J Neurosci 30: 901-912.

Rigotti NA (2002) Clinical practice. Treatment of tobacco use and dependence. N Engl J Med 346(7): 506-512.

Robinson MJ, Franklin KB (2007) Central but not peripheral beta-adrenergic antagonism blocks reconsolidation for a morphine place preference. Behav Brain Res 182: 129-134.

Rodriguez D, Tercyak KP, Audrain-McGovern J (2008) Effects of inattention and hyperactivity/impulsivity symptoms on development of nicotine dependence from mid adolescence to young adulthood. J Pediatr Psychol 33(6): 563-575.

Role LW, Berg DK (1996) Nicotinic receptors in the development and modulation of CNS synapses. Neuron16(6): 1077-1085.

Rollema H, Chambers LK, Coe JW, Glowa J, Hurst RS, Lebel LA, Lu Y, Mansbach RS, Mather RJ, Rovetti CC, Sands SB, Schaeffer E, Schulz DW, Tingley FD 3rd, Williams KE (2007) Pharmacological profile of the alpha4beta2 nicotinic acetylcholine receptor partial agonist varenicline, an effective smoking cessation aid. Neuropharmacology 52(3): 985-994.

Rose JE (2006) Nicotine and non-nicotine factors in cigarette addiction. Psychopharmacology (Berl) 184(3-4): 274-285.

Sadler R, Herzig V, Schmidt WJ (2007) Repeated treatment with the NMDA antagonist MK-801 disrupts reconsolidation of memory for amphetamine-conditioned place preference. Behav. Pharmacol 18: 699-703.

Sakurai S, Yu L, Tan SE (2007) Roles of hippocampal N-methyl-D-aspartate receptors and calcium/calmodulin-dependent protein kinase II in amphetamine-produced conditioned place preference in rats. Behav Pharmacol 18(5-6): 497-506.

Sanchez H, Quinn JJ, Torregrossa MM, Taylor JR (2010) Reconsolidation of a cocaine-associated stimulus requires amygdalar protein kinase A. J Neurosci 30: 440-4407.

Sanchis-Segura C, Spanagel R (2006) Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. Addict Biol 11(1): 2-38.

Sara SJ (2000) Retrieval and reconsolidation: toward a neurobiology of remembering. Learn Mem 7: 73-84.

Schneider AM, Sherman W (1968). Amnesia: a function of the temporal relation of footshock to electroconvulsive shock. Science 159: 219-221.

Schwartz RD, Lehmann J, Kellar KJ (1984) Presynaptic nicotinic cholinergic receptors labeled by [3H]acetylcholine on catecholamine and serotonin axons in brain. J Neurochem 42(5): 1495-1498.

Shiffman S (2009) Responses to smoking cues are relevant to smoking and relapse. Addiction 104 (10): 1617-1618.

Skinner BF, (1938) The behavior of Organisms: An Experimental Analysis. New York: Appleton-Century.

Soeter M, Kindt M (2011) Disrupting reconsolidation: pharmacological and behavioral manipulations. Learn Mem 18(6): 357-366.

Soria R, Stapleton JM, Gilson SF, Sampson-Cone A, Henningfield JE, London ED

(1996) Subjective and cardiovascular effects of intravenous nicotine in smokers and non-smokers. Psychopharmacology (Berl) 128(3): 221-226.

Stolerman IP, Shoaib M (1991) The neurobiology of tobacco addiction. Trends Pharmacol Sci 12(12): 467-73.

Suzuki A, Josselyn SA, Frankland PW, Masushige S, Silva AJ & Kida S (2004) Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci* 24: 4787-4795.

Taylor JR, Olausson P, Quinn JJ, Torregrossa MM (2009) Targeting extinction and reconsolidation mechanisms to combat the impact of drug cues on addiction. Neuropharmacology 56 (Suppl 1): 186-195.

Theberge FR, Milton AL, Belin D, Lee JL, Everitt BJ (2010) The basolateral amygdala and nucleus accumbens core mediate dissociable aspects of drug memory reconsolidation. Learn Mem 17: 444-453.

Thomas KL, Arroyo M, Everitt BJ (2003) Induction of the learning and plasticity-associated gene Zif268 following exposure to a discrete cocaine-associated stimulus. Eur J Neurosci 17(9): 1964-1972.

Thomas KL, Hall J, Everitt BJ (2002) Cellular imaging with zif268 expression in the rat nucleus accumbens and frontal cortex further dissociates the neural pathways activated following the retrieval of contextual and cued fear memory. Eur J Neurosci 16(9): 1789-96.

Tronson NC, Taylor JR (2007) Molecular mechanisms of memory reconsolidation. Nat Rev Neurosci 8(4): 262-275.

Vineis P, Alavanja M, Buffler P, Fontham E, Franceschi S, Gao YT, Gupta PC, Hackshaw A, Matos E, Samet J, Sitas F, Smith J, Stayner L, Straif K, Thun MJ, Wichmann HE, Wu AH, Zaridze D, Peto R, Doll R (2004) Tobacco and cancer: recent epidemiological evidence. J Natl Cancer Inst 96(2): 99-106.

Von der Goltz C, Vengeliene V, Bilbao A, Perreau-Lenz S, Pawlak CR, Kiefer F, Spanagel R (2009) Cue-induced alcohol-seeking behaviour is reduced by disrupting the

reconsolidation of alcohol-related memories. Psychopharmacology (Berl.) 205: 389-397.

White NM, McDonald RJ (2002) Multiple parallel memory systems in the brain of the rat. Neurobiol Learn Mem 77: 125–184.

Wonnacott S (1997) Presynaptic nicotinic ACh receptors. Trends Neurosci 20: 92-98.

Wu P, Xue YX, Ding ZB, Xue LF, Xu CM, Lu L (2011) Glycogen synthase kinase 3β in the basolateral amygdala is critical for the reconsolidation of cocaine reward memory. J Neurochem 118(1): 113-125.

Yalachkov Y, Kaiser J, Naumer MJ (2009) Brain regions related to tool use and action knowledge reflect nicotine dependence. J Neurosci 29(15): 4922-9.

Yin R, French ED (2000) A comparison of the effects of nicotine on dopamine and non-dopamine neurons in the rat ventral tegmental area: an in vitro electrophysiological study. Brain Res Bull 51(6): 507-514.

Zhai, H, Wu P, Chen S, Li F, Liu Y, Lu L (2008) Effects of scopolamine and ketamine on reconsolidation of morphine conditioned place preference in rats. Behav Pharmacol 19: 211-216.

ACKNOWLEDGMENTS

I acknowledge the Director of the PhD School in Translational Biomedical Science Professor Guido Fumagalli, and the Coordinator of the PhD course in Translational Biomedicine Professor Cristiano Chiamulera for the support given during this research project.

I would like to thank all of my colleagues at the Neuropsychopharmacology Lab, Meningeal Stem Cell Lab (Department of Public Health and Community Medicine, University of Verona) and Psychology Lab (Department of Psychology, University of Texas at Austin).

Thanks to Alessia Auber and Nazeema Sheerin for the help in performing the last behavioural experiments, and thanks to Marijana Kusalo and Valeria Berton for their suggestions regarding the cellular and molecular experiments.

I also would like to thank my friend Thomas Zandonai, that actually did not give me any scientific support, but I know that I must mention his name if I want to keep going our relationship that has been fundamental for my personal growth.

Two biggest thanks go to my supervisor Professor Cristiano Chiamulera and to my second “supervisor” Marzia Di Chio, for their teaching, for the support given to me during these last eight years, and because together with my family they made me who I am.