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## APPETITIVE MEMORIES AND RELAPSE TO DRUG-USE: INVESTIGATIONS ON EFFECTS OF SELECTIVE DISRUPTION OF MEMORY MECHANISMS IN A RAT MODEL OF NICOTINE DEPENDENCE

S.S.D BIO14/FARMACOLOGIA


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**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.

Several preclinical and clinical studies have 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) 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 response, 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. For at least a century it has been a dogma that initially labile memory (short-term memory) are 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 memory indeed may destabilize the consolidated memories that require a new process to be maintained. This hypothetical process is called reconsolidation. 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 drug-related memories can be disrupted by the administration of an amnestic drug contingently upon retrieval of the memory. Unfortunately most of the compound used in animal studies has serious tolerability and safety issues in humans. Recently Monfils at al and Schiller et al have shown that it is possible to disrupt fear memory reconsolidation and consequently prevent the return of fear by providing CS-extinction training shortly after retrieval of the memory. CS-extinction consists in the repeated presentations of CS (e.g. lighter) in the absence of the unconditioned stimulus US (e.g. drug) leading to a decrease of the previously acquired conditioned response (e.g. smoking behaviour).

The main objective of the present thesis was to investigated whether it is possible to disrupt nicotine related memories reconsolidation by applying CS-extinction after the retrieval of such as memories, and whether this disruption prevent the relapse to nicotine-seeking behaviour in a rat model of nicotine dependence. Furthermore we investigated also whether nicotine-related memories reconsolidation might be pharmacologically disrupted by administering a drug, that have been shown to disrupt memory reconsolidation in previous literature studies (i.e. propranolol or MK-801), at memory retrieval.

The experimental approach used to address this issue was the paradigm of nicotine self-administration in rats, a paradigm based on Pavlovian and operant conditioning to nicotine and nicotine-associated cues. We performed five experiments in which CS-extinction or the pharmacological treatment (i.e. propranolol or MK-801) was associated to different memory retrieval protocols. We therefore assessed the effect of these post-retrieval treatments on relapse to nicotine or food seeking behaviour. Retrieval consists in presenting the CS in the absence of US, a procedure similar to CS-extinction. Since the length of CS exposure (i.e. number of CS presentations) is a crucial factor for reconsolidation or extinction occurrence, different retrieval length (1, 3 or 30 CS presentations) have been presented to retrieve nicotine-related memories.

Results showed that CS-extinction applied after a short retrieval (3 CS presentations) reduced the relapse to nicotine seeking behaviour compared to control groups that did not receive CS-extinction, moreover this effect was not observed when CS-extinction was applied without retrieval. These results suggest that the effect of post-retrieval CS-extinction was specifically due to inhibition of nicotine-related memories reconsolidation. To our knowledge, this is the first evidence of post-retrieval CS-

extinction effect on drug-seeking behaviour. Considering that this is an indirect demonstration of the occurrence of memory reconsolidation process, we would also consider these findings as the first evidence of nicotine Pavlovian memory reconsolidation. On the other hand, no effect of MK-801 or propranolol on nicotine seeking behaviour has been observed. These results are in contrast with other literature data, however methodological issues might explain the contrasting results.

More evidences are needed to confirm that the effect of post-retrieval CS-extinction was due to interference of CS-extinction with reconsolidation process. Further studies will investigate the effect of CS-extinction applied 6 hours after retrieval, a delay time that allows to apply CS-extinction outside the labile phase of memory due to retrieval. Moreover it would be fundamental to identify specific molecular markers of reconsolidation or extinction. To find a selective molecular correlate of reconsolidation will allow to disentangle the point of whether our retrieval protocols are inducing reconsolidation or extinction and will provide further evidence that post-retrieval CS-extinction interfere with reconsolidation of CS-memory. This could also be useful to better understand the lack of effect of MK-801 and propranolol in our experiments.

It has been pointed out by Lee & Everitt (2008) that to successfully reactivate a memory acquired instrumentally (as in our experiments) the CS should be presented contingently upon acquired response. We can then hypothesized that presenting the CS contingently upon response during retrieval session, would lead to a more strong retrieval and destabilization of the memories, and to a stronger effect of CS-extinction and of MK-801 on the reconsolidation of that memory.

Finally, it would be important to assess whether the effect of post-retrieval CS-extinction on nicotine seeking behaviour is persistent, by repeating the test several week after retrieval-CS-extinction procedure.

In conclusion, our findings suggest that the exposure to nicotine CS-extinction, after a short retrieval of the same nicotine CS, may inhibit CS-induced relapse to nicotine-seeking behaviour and may offer a potential co-adjuvant to current therapeutic interventions for smoking cessation and abstinence maintenance.

## CONTENTS

## 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 postoperative 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 3% 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 controller 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 produce
  -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; Pomerlau, 1995; Rodriguez et al., 2007).

## 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 allow sodium and calcium ion influx, after few milliseconds the channel close and become desensitised. 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 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, on 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., 1996; 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)

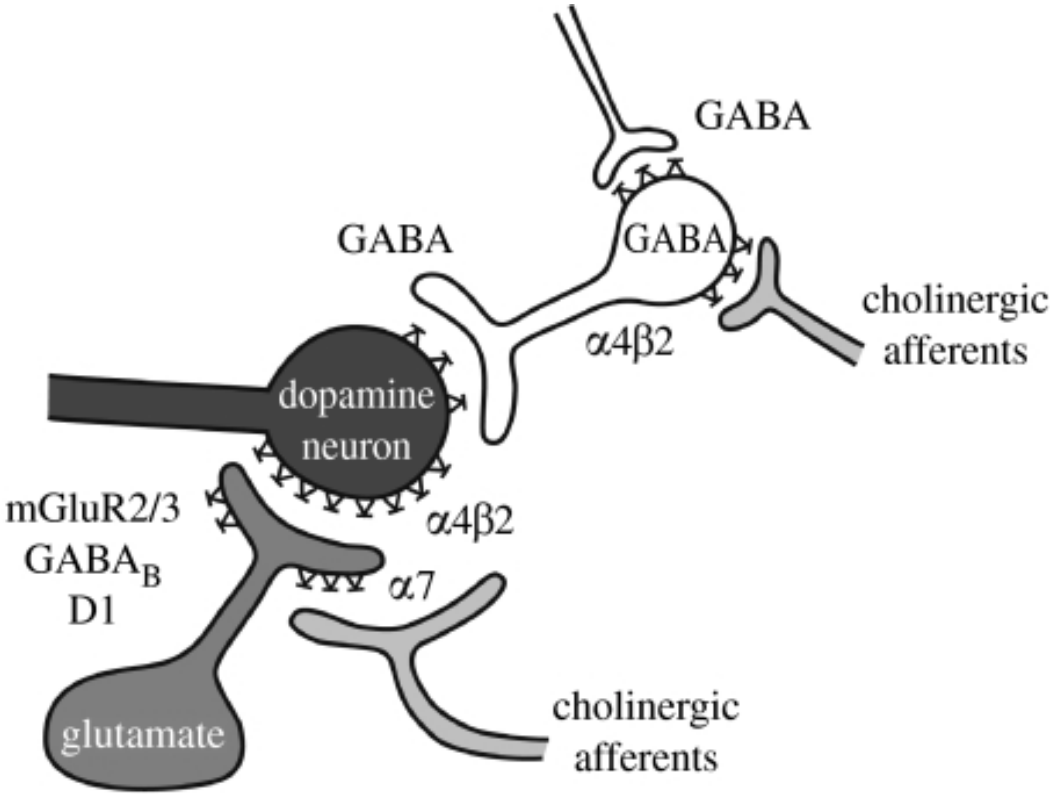


Figure 1: Schematic drawing of dopaminergic, gabaergic, glutamatergic and cholinergic neurons interaction. nAChRs are localized mainly at presynaptic level on glutamatergic, dopaminergic, and gabaergic neurons. Abbreviations in the text, except D1 = dopamine receptor 1, mGluR2/3 = metabotropic glutamate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

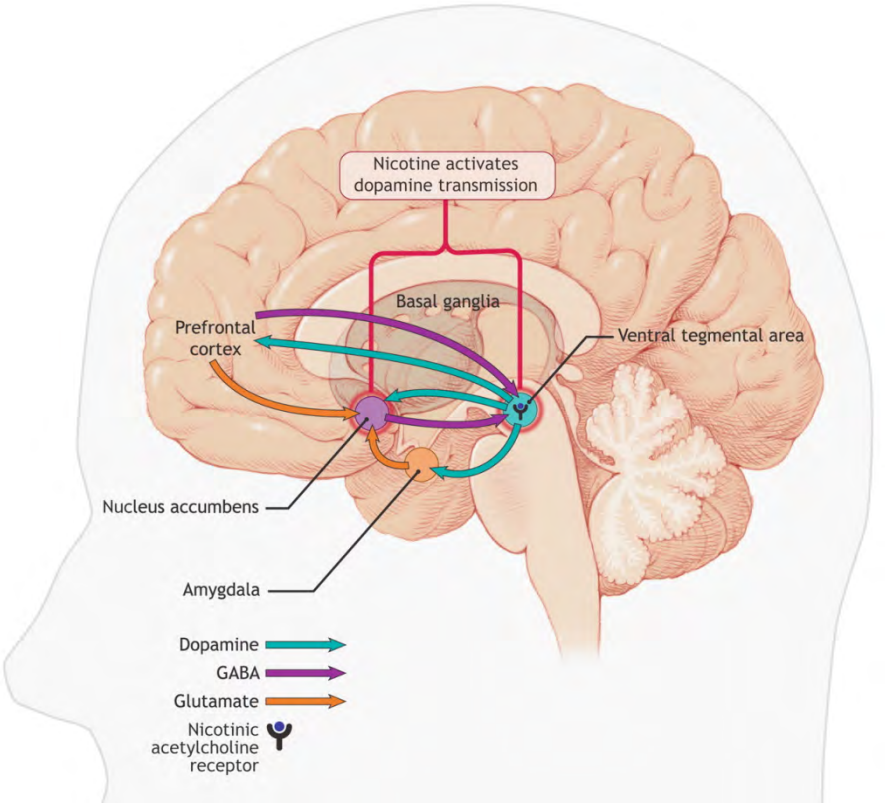


Figure 2: Schematic drawing of mesocorticolimbic pathway, mediating nicotine dependence. Nicotine stimulates nAChR located in the VTA, resulting in release of DA in the nucleus accumbens. Neurons projecting from the prefrontal cortex and amygdala modulate the release of DA in the nucleus accumbens. GABAergic 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 play 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 increases 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 elect to repeat the experience (Rose, 2006). This leads to the next stage where the prolonged exposure to smoke induce a neuroadaptation in the brain, increasing the reinforcing effects of nicotine (Soria, et al., 1996). When CNS nicotine levels ceases abruptly following smoking cessation, it produce 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; 1991) 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 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, 2011). 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 produce a lesser, slower DA release then nicotine with a longer duration if action, Moreover, when varenicline is combined with nicotine, it attenuates nicotine induced DA release in nucleus accumbens (Rolleman 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 subtler compared to other drug. It suggests that the reinforcing effect of nicotine is necessary but not sufficient to explain tobacco dependence (Caggiula, 2001). 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 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 area and pathway 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 influences 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 area 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 activate 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 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 memories, in the phenomenon of relapse to drug-seeking behaviour, it has been proposed that treatment that disrupt the drug-associated memories could act as a pro-abstinent and anti-relapse therapy (Diergaarde, 2008; Tronson &Taylor, 2007; Taylor, 2011). 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 memory are initially in a "labile" form for a short time (short term memory-STM), after which the memory trace is 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 memories have 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 induce a reactivation of the memory trace, that presumably return to a labile state, which initiated another memory process similar to that seen after learning. The processes through which memory are maintained after retrieval is called reconsolidation (Figure 3).

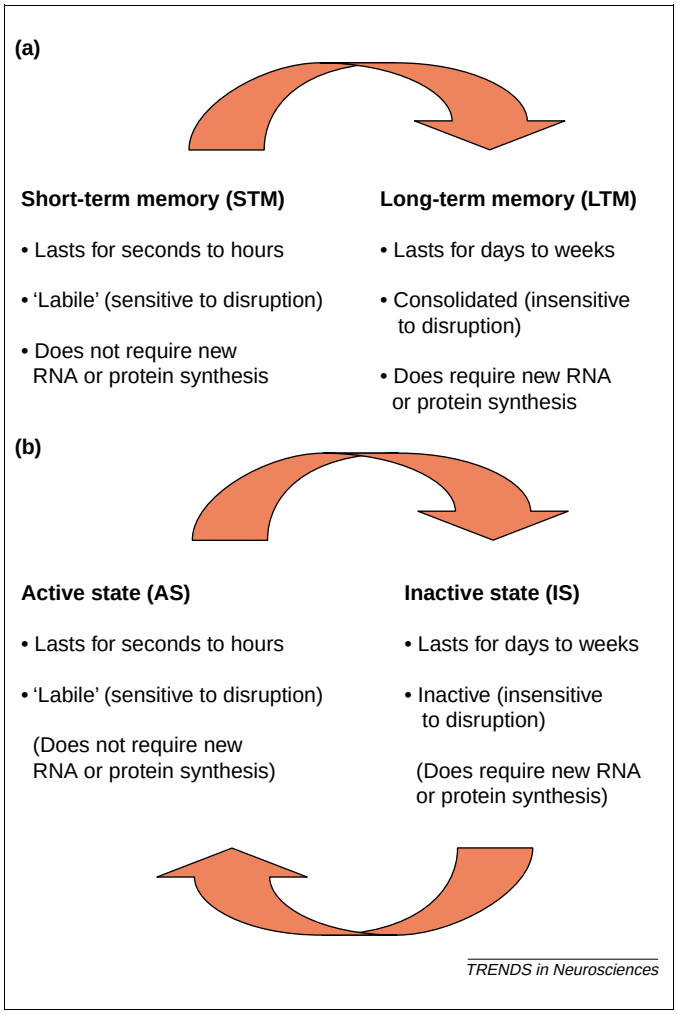


Figure 3: Two model of memory processing. (a) The traditional consolidation memory that stated that a labile short-term memory (STM) and a later, consolidated, permanent long-term memory (b) The memory model proposed by Lewis (1968). The active state (AS) and inactive state (IS) are analogous to STM and LTM, respectively. Memory after learning experience are in AS, then it enter 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 induce a transient labile phase of the memory, the time during which memory trace are labile is called reconsolidation window and persist for several hours after retrieval (Duvarci & Nader, 2004; Nader et al, 2000; 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 (Figure 4).

| Experimental paradigm | Treatment | Animal |
|---|---|---|
| Habituation | Heat shock, and DNQX (antagonist of non-NMDA-type glutamate receptor) | Nematode |
| Auditory fear conditioning | Protein synthesis inhibition, inhibition of kinase activity, and reconsolidation potentiation by protein kinase A activation | Rat |
| Classical fear conditioning | Transient anaesthesia | Medaka (a fish) |
| 'Pavlovian-like' conditioning | Protein synthesis inhibition, sensory block, mRNA synthesis inhibition and blocking bond formation of cell-adhesion molecules | *Hermissenda* |
| Contextual fear conditioning | Protein synthesis inhibition, inducible CREB-knockout and antisense oligodeoxynucleotides | Rat and mouse |
| Context-signal memory | NMDA receptor antagonist | Crab |
| Operant conditioning | RNA synthesis inhibition, and cooling | Snail |
| Appetitive conditioning | Protein synthesis inhibtion | Honeybee |
| Conditioned taste aversion | Protein synthesis inhibition | Rat pups |
| Inhibitory avoidance | Protein synthesis inhibition, glycoprotein synthesis inhibition and antisense oligodeoxynucleotides | Chicks and rats |
| Motor sequence learning | Interference by new learning | Humans |
| Incentive learning | Protein synthesis inhibition | Rat |
| Object recognition | *Zif268*-deficient mouse, and inhibition of kinase activity | Mouse, rat |
| Spatial memory | Protein synthesis inhibition | Mouse and rat |
| Memory for drug reward | Inhibition of the ERK kinase MEK, *Zif268*-deficient knock-in mice and *Zif268* antisense oligodeoxynucleotides | Rat and knock-in mouse |
| Episodic memory | Interference by new learning | Humans |

Figure 4: Example of experimental paradigms and treatment and species involved for studies that reported evidence of reconsolidation process since 2000. (Modified from Nader & Hardt, 2009).

To experimentally demonstrate reconsolidation or the role of a particular molecule in reconsolidation memories must be first consolidated, then reactivated (retrieved) contiguously with some form 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-cue), such as a tone, with a reinforcing stimulus (unconditioned stimulus), such as a foot-shock. Retrieval is induced in a reactivation session, which occur at least 24 hours later and consists in presenting the conditioned stimulus in the absence of unconditioned stimulus. 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 cues 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 a molecular mechanisms underlying 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 NMDARs (N-methyl-d-aspartate receptors). 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 initiate 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 5).

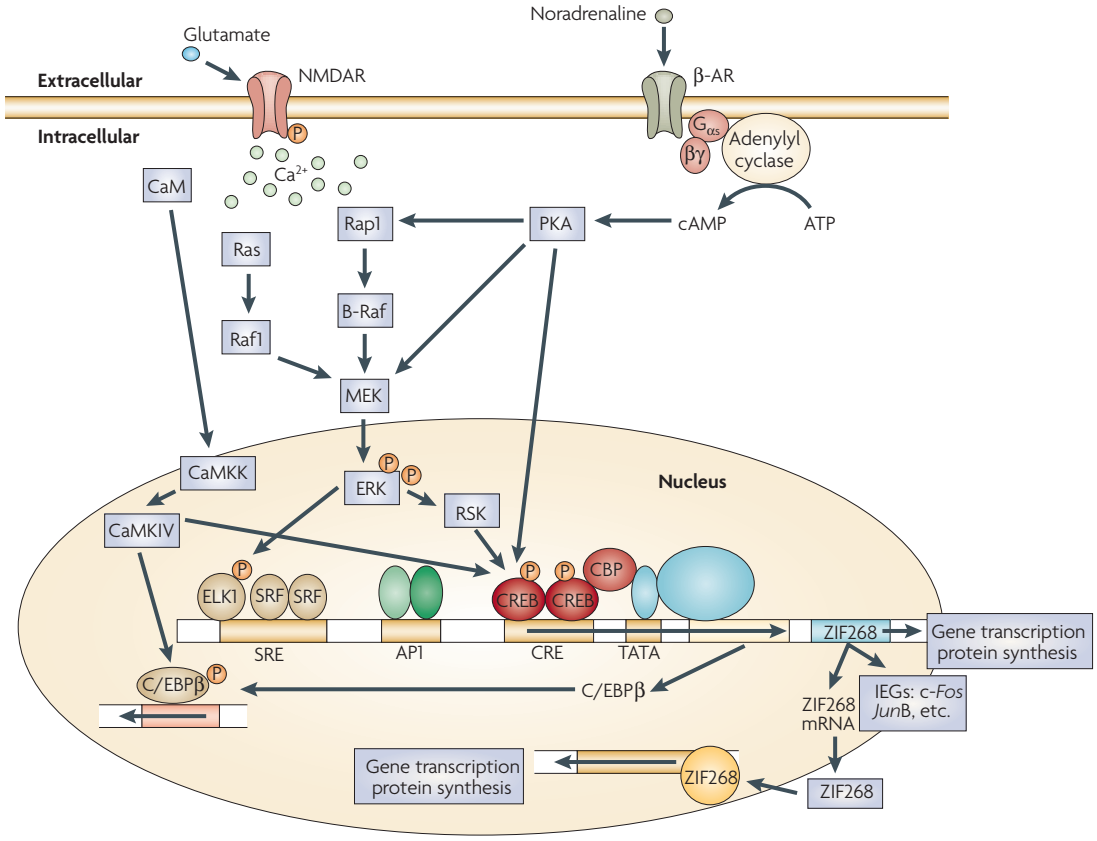


Figure 5: Key molecular mechanisms of memory reconsolidation. Molecular signalling cascades downstream of β-adrenergic receptors (β-AR) and NMDARs (N-methyl-d-aspartate receptors) have been shown to be implicated in reconsolidation. Small GTPases such as Ras, Raf and Rap activated by Ca2+ influx activate 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, 2002). Other authors proposed that

reconsolidation might serve to strengthen memory (Inda et al, 2011; Lee, 2009; Sara, 2000).

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, 2000; Nader et al, 2000; 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*

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 (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, 2008; 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 NMDAR antagonist, 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 (Sandler 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, 2008). 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 NMDAR antagonist D-APV into the basolateral amygdala before a memory reactivation disrupt 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. Also Lee (2005) 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 linked to NMDAR. 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 memory 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 (2008) 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 has 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, 2007a). 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).

Unfortunately the drugs used to disrupt memory reconsolidation in animal cannot be used in human, since they are toxic. The only drug that can be administered in human is propranolol, even if it could lead to side effect, such as orthostatic hypothension, decreased libido, bronchial adverse effects in patients affected by respiratory disease.

Since there is an unmet need of novel interventions to be integrated in the current therapy, a new strategy based on targeting reconsolidation should guarantee efficacy and tolerability.

### *1.3.3 Extinction therapy*

One of the most used cognitive-behavioural therapies for the treatment of anxiety disorder and to prevent the relapse in ex-drug addicts is extinction (also called Cue-Exposure). Extinction consists in the repeated presentation of previously CS in the absence of unconditioned stimulus. It is widely accepted that extinction is a new learning process, through which CS become associated to no US, leading to a decrease of the conditioned response. Extinction does not erase the original associative (CS-US) memory but instead generate a competitive inhibitory memory capable of temporally suppressing the expression of the original conditioned response (Pavlov, 1927; Quirk & Mueller 2008; Pape and Pare, 2010). Indeed original memory and extinguished response may re-emerge under three general condition: i) reinstatement, when US is presented unexpectedly (Pavlov 1927; Rescorla and Heth 1975; Westbrook et al. 2002, de Wit & Steward, 1981, Shaham et al, 1994), ii) renewal, when CS is presented outside the extinction context (Bouton and Bolles 1979) and iii) spontaneous recovery, when a certain amount of time has passed.

Several efforts have been dedicated to enhance the efficacy of extinction. It is widely acknowledged that glutamatergic NMDA receptor is directly involved in the formation of new learning and memories (Walker & Davis, 2002), and in early rodent studies it has been shown that a NMDA partial agonist D-cycloserine (DCS) may facilitate extinction (Liu et al, 2009; Falls et al, 1992; for review see Ganasen et al, 2010). Other compounds may be useful for strengthening or accelerating extinction, as suggested by recent rodent studies. These include fibroblast growth factor, methylene blue, endocannabinoids and yohimbine, N-acetylcysteine (Chatwal et al, 2009; Gonzales-Lima & Bruchey, 2004; Graham and Richarson, 2010; Morris & Bouton, 2007; Zhou & Kalivas, 2008).

### *1.3.4 Extinction and reconsolidation interaction.*

Thus current research for the treatment of neuropsychiatric disorders based upon maladaptive memories, including drug addiction, is focused on the facilitation of extinction and on the disruption of maladaptive memory reconsolidation.

Unfortunately the compounds used in animal studies to block the reconsolidation, such as protein synthesis inhibitor (eg., anisomycin) and NMDAR antagonist (such as MK801) cannot be used in human, given their toxicity and bioavailability constraints. Moreover the efficacy of pharmacological improvement of extinction therapy is

controversial (Marissen et al. 2007).

An important step forward came from Monfils and colleagues in 2009. Capitalizing on reconsolidation as an update mechanism that allow for new information available at the time of retrieval to be integrated in the original memory trace, they hypothesized that proving no-fearful information through extinction training during the labile phase induced by retrieval, would lead to a modification of the original memory, reinterpreting CS as safe and therefore would prevent the CS-induced return of fear. They trained rats to Pavlovian fear conditioning; 24 hours later fear memory was reactivated by a single presentation of CS; 10 minutes, 1 hour, 6 or 24 hours later animal underwent an extinction session while CS was repeatedly presented in the absence of US (non fearful information). The day after they tested the return of fear under reinstatement or renewal conditions and one month later they tested the spontaneous recovery of fear. Results showed that extinction, only when applied within the reconsolidation window (10 minutes or 1 hour after retrieval) but not when applied outside the reconsolidation window (6 or 24 hours after retrieval), interfered with fear memory update and prevented fear conditioned responses such as renewal, reinstatement and spontaneous recovery. Additionally rats that received extinction without retrieval of the CS showed re-emergence of fear under renewal, reinstatement and also spontaneous recovery.

These findings were supported by the work of Schiller and colleagues in 2010. Using human electrodermal fear conditioning model they demonstrated that extinction, applied 10 minutes after retrieval (single CS presentation) of a fear memory, prevented the spontaneous recovery and reinstatement of fear response. Conversely extinction applied 6 hours after retrieval (outside the reconsolidation window) had no effect.

These two studies suggest that a new learning may interfere with memory reconsolidation of the original memory, this notion has received support also from other studies targeting episodic, motor and declarative memories both in human and in laboratory animals. Boccia and co-workers in 2005 showed the exposure to a new learning task could affect the memory reconsolidation in an inhibitory avoidance task in mice. Forcato and colleagues in 2007, 2009 and 2010 demonstrated that new information provided within the reconsolidation window may modify the original declarative memories in human. There are evidences that also episodic memories could be selectively impaired following retrieval (Hupbach et al, 2007; Strange et al, 2010).

Interestingly, Flavell et al. (2011) have recently shown extinction given in conjunction

to retrieval was able to block the reconsolidation of appetitive memory in rats. They have used the paradigm of acquisition of new response for stimuli previously paired to sucrose. In this experimental paradigm rats are initially trained to self-administer sucrose by an instrumental response (e.g. nose-poke), and each sucrose administration is paired with the presentation of a CS, such as a light or a tone. In a second phase, rats are required to acquire a new instrumental response (e.g. lever press) to receive a CS presentation. Therefore the new instrumental behaviour is supported by the conditioned reinforcing properties of the CS. In this study Flavell et al. showed that CS-extinction applied after the retrieval of the CS, inhibited the acquisition of new response in rats trained to self-administer sucrose. This effect was retrieval-dependent since no effect was observed when extinction was applied without previous retrieval of the CS. They hypothesized that extinction applied within the vulnerable phase of the retrieved memory, was interfering with their reconsolidation. However they also pointed out that it was equally plausible that prior retrieval of the memory might facilitate extinction and therefore potentiate its effect, in a similar manner to pharmacological enhancement of extinction. To disentangle this account in a new groups of rats they substitute retrieval with the administration of D-cycloserine (DCS), an NMDA receptor partial agonist, well know to enhance extinction of memory (see paragraph 1.3.3.). Results showed that when rats were injected with DCS, instead of being retrieved, before the administration of extinction there was no effect on subsequent acquisition of new response with conditioned reinforcer. Therefore they argued that the observed post-retrieval extinction effect was due to the interference with reconsolidation of sucrose-related memories. Furthermore Flavell et al investigated the effect on retrieval-extinction procedure on reconsolidation of contextual fear memory. They showed that extinction, only when applied in combination to retrieval, prevented the return of fear in the subsequent test. Further evidence that the effect of extinction was retrieval dependent came from the fact that injection nimodipine, a blocker of L-type voltage-gated calcium channel (LVGCC) known to block the destabilization of memory at retrieval, immediately after retrieval impaired the effect of retrieval-extinction in preventing the return of fear. This result provides further evidence that extinction applied after retrieval inhibits the re-expression of the original memory by the disruption of memory reconsolidation. On the other Flavell and colleagues have also showed that the combination of memory retrieval and extinction did not prevent the return of fear, using the auditory fear conditioning paradigm. However they highlight that some methodological issues might explain the

contrasting results with the original finding of Monfils et al (such as training length).

The molecular mechanism underlying the effect of post-retrieval extinction has been investigated by Clem & Huganir (2010), pairing fear conditioning paradigm and elettrophysiology assay. They trained animal in fear conditioning paradigm, the day after training memory was retrieved 30 minutes before extinction, and renewal and spontaneous tests performed the day after and also 5 days after retrieval-extinction. They observed that, compared to the no-retrieved groups, retrieval-extinction procedure inhibited the return of fear. Subsequently a groups of animal were injected with 1-aminoindan-1,5-dicarboxylic acid (AIDA), a competitive antagonist of AMPA receptor mGluR1, 1 hour before retrieval. Post-retrieval extinction effect in preventing the return of fear was inhibited by the previous administration of AIDA. Thus, they argued that effect of extinction upon retrieval required the mGluR1 activation. In further electrophysiological studies they observed a significant decrease of AMPA receptors – mediated transmission in the retrieved group compared to the no-retrieval group. This decrease was accompanied by the selective removal of synaptic calcium-permeable AMPA (CP-AMPAr) receptors in the lateral amygdala. Moreover the stability of CP-AMPAr was regulated by the activation of mGluR1. Considering post-retrieval extinction effect as a reconsolidation update author suggest that mGluR1 activation is required to update memories, and that mGluR1 could be a potential drug target for preventing the return of fear.

Other studies have shown that the retrieval-extinction paradigm used by Monfils and Schiller was ineffective in preventing the return of fear in fear conditioning paradigm both in human and in laboratory animals. This type of procedure allow to isolate the acquired conditioned Pavlovian conditioned reinforcing properties if CS, from the instrumental component of the conditioning (see below, operant and Pavlovian conditioning).

First evidence came from a preclinical study by Chan and colleagues (2010): they use the same procedure described by Monfils et al (2009) to study the effect of a single CS presentation (retrieval) on the extinction and recovery of conditioned fear response via renewal and reinstatement in fear conditioning paradigm. Conversely to Monfils et al, they found that exposure to retrieval prior to extinction increased responding to that retrieved CS on subsequent test for renewal and reinstatement. The retrieval-extinction procedure has been also tested on remote fear memory (29 days old) in a mouse model of PTSD (Siegmund and Wotjak, 2007a), that compared to that used by Monfils et al,

take in consideration also the non associative component of fear memory (i.e. sensitization process that increase the animal response to harmless stimuli independently from the CS-US association) (Costanzi et, 2011). The main result of this study is that extinction when applied after retrieval of remote fear memory did not persistently attenuate the expression of fear. In a recent paper Pèrez-Cuesta (2009) investigated the effect of retrieval-extinction procedure in memory model of the crab Chasmagnathus (Maldonado, 2002). They trained crabs using the context-signal memory paradigm, and 24 hours later they exposed the crabs to the training context for 15 minutes (short exposure induced retrieval of the conditioned context) and 15 minutes later they exposed the crabs to the same context for an additional 2 hours (long exposure induced extinction). The day after the crab were tested for CS-US memory (test 1), and, if no memory was found, the test was replicated 24 hours later (test 2) to distinguish reconsolidation impairment (supposed to be permanent) and extinction (supposed to be transient). On test 1 no memory recovery has been observed in crabs that receive the retrieval-extinction treatment, on the contrary on test 2 a re-emergence of memory have been noticed. These data suggest that extinction applied after retrieval does not update the original memory trace.

The effect of the combination of retrieval and extinction has been also investigated using the paradigm of morphine-induced conditioned place preference (CPP) (Ma et al. 2011). They showed that repeated retrieval-extinction procedure (across 10 days) suppressed the reinstatement and spontaneous recovery of extinguished CPP. On the other hand no effect was observed when extinction was applied without prior retrieval of the memory. However recovery of the CPP was found in a reinstatement test performed 4 week after the last extinction session. The latter finding suggests that memory trace was not been erased by post-retrieval extinction. It can be hypothesized that extinction applied after retrieval did not affect the reconsolidation of memory under the conditions used in the paper by Ma et al.; otherwise, as suggested by the authors, that reconsolidation blockade did not lead to the erasure of the memory that can re-emerge by the passage of time.

As far as concern the human studies, in 2011 Soeter & Kind pointed out that the electrodermal conditioning used by Schiller and colleagues seems to primarily reflect only the cognitive level (declarative memory) of contingency learning (CS-US association), whereas human strartle potentiation is considered to be a reliable and specific index of fear. In a within-subject (Soeter & Kindt, 2011) and in a between-

subject (Kindt & Soeter in press) studies they tested whether extinction provided during the reconsolidation window prevents the return of the extinguished startle fear response, using a fear conditioning design. ) They found that extinction, provided after retrieval, doesn't affect the startle fear response, skin conductance and US expectancy rating. The Schiller et al study and Soeter-Kindt study diverged in several ways, with most notable differences being the assessment of conditioned responding, i.e. single method (only skin conductance response) vs. multi-method of indexing fear (fear potentiated startle, skin conductance response, US expectancy rating, subjective assessment). The second difference between the two studies consisted in the conditioned stimuli used (geometric figures vs. fear-relevant pictures).

### 1.4 Aim

This research originated from the experimental evidences that drug related memories reconsolidation could be disrupted, and this could be fundamental in preventing the relapse to drug seeking behaviour.

The aim of this research is to investigate if it is possible to disrupt nicotine memory reconsolidation by the application of extinction after retrieval of nicotine-related memory and whether this disruption prevent the relapse to nicotine-seeking behaviour in a rat model of nicotine dependence.

Two main issues are addressed:

1. Is a post-retrieval pharmacological treatment, such as propranolol or MK-801 able to prevent the renewal of food or nicotine-seeking behaviour?
2. Is it possible to disrupt food or nicotine memory reconsolidation by applying post-retrieval extinction of CS (i.e. a cue light) (CS-extinction) previously associated to food or nicotine self-administration (S/A) in rats?

We assessed whether post-retrieval administration of propranolol or MK-801 or extinction, applied in a context (CxB) different from that where food or nicotine S/A and CS association took place (CxA), may reduce renewal of nicotine-seeking behaviour when rats were placed back in CxA. Retrieval consists in presentation of the stimulus (CS, i.e. a light) previously paired with nicotine administration. Since the number of CS presentations at retrieval is a fundamental determinant of whether reconsolidation or extinction occurs (Tronson &Taylor, 2007), we also tested different length of retrieval (i.e. 1, 3, 30 and 300 CS presentations).

We performed five experiments:

In ***Experiment #1*** we assessed the effect of CS-extinction on renewal of nicotine-seeking behaviour in rats trained to nicotine S/A. Retrieval consisted in 30 CS presentations.

In ***Experiment #2*** we assessed the effect of propranolol or CS-extinction on renewal of food seeking behaviour in rats trained to food S/A. Retrieval consisted in 30 CS presentations.

In ***Experiment #3*** we assessed the effect of propranolol or CS-extinction on renewal of nicotine seeking behaviour in rats trained to nicotine S/A. Retrieval consisted in 1 CS presentations.

In ***Experiment #4*** we assessed the effect of three different retrieval lengths (3, 30 or 300 CS presentation) on renewal of nicotine seeking behaviour in rats trained to nicotine S/A.

In ***Experiment #5*** we assessed the effect of MK801 or CS-extinction on renewal of nicotine seeking behaviour in rats trained to nicotine S/A. Retrieval consisted in 3 CS presentations

In the study-protocol we included no-retrieved groups and no-treated groups (receiving just a saline injection after retrieval or no-retrieval), these groups allow to control for the specificity of the treatment (propranolol, MK801 or extinction) effect on food or nicotine Pavlovian memories.

| | EXP #1 | EXP #2 | EXP #3 | EXP #4 | EXP #5 |
|---|---|---|---|---|---|
| EXPERIMENTAL PARADIGM S/A | Nicotine | Food | Nicotine | Nicotine | Nicotine |
| RETRIEVAL LENGHT | 30 CS presentations | 30 CS presentations | 1 CS presentation | 3, 30 0r 300 CS presentations | 3 CS presentations |
| TREATMENTS | CS-extinction | CS-extinction Propranolol | CS-extinction Propranolol | – | CS-extinction MK-801 |

Figure 6: Schematic table of the experiments. In the Experiment #1 we assessed the effect of CS-extinction applied after 30 CS presentations on renewal of nicotine seeking behaviour. In Experiment #2 we assessed the effect of propranolol or CS-extinction applied after 30 CS presentations on renewal of food-seeking behaviour. Experiment #3 we assessed the effect of propranolol or CS-extinction applied after 1 CS presentation on renewal of nicotine-seeking behaviour. Experiment #4 we assessed the effect of different retrieval length on renewal of nicotine-seeking behaviour. Experiment #5 we assessed the effect of MK-801 or CS-extinction applied contingently upon 3 CS presentations on renewal of nicotine-seeking behaviour

### *1.4.1 Experimental model*

The experimental models used are food or intravenous nicotine S/A in rats, a paradigm based on operant and Pavlovian conditioning to food or nicotine and food- or 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 describe 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 cannot 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 studying of the underlying neurobiological mechanisms, as well as having a high predictive validity for the identification of novel anti-addiction therapies. In our food or nicotine S/A models rats are placed in a cage, the so-called Skinner box (Figure 7), equipped with two levers, one active and one inactive.

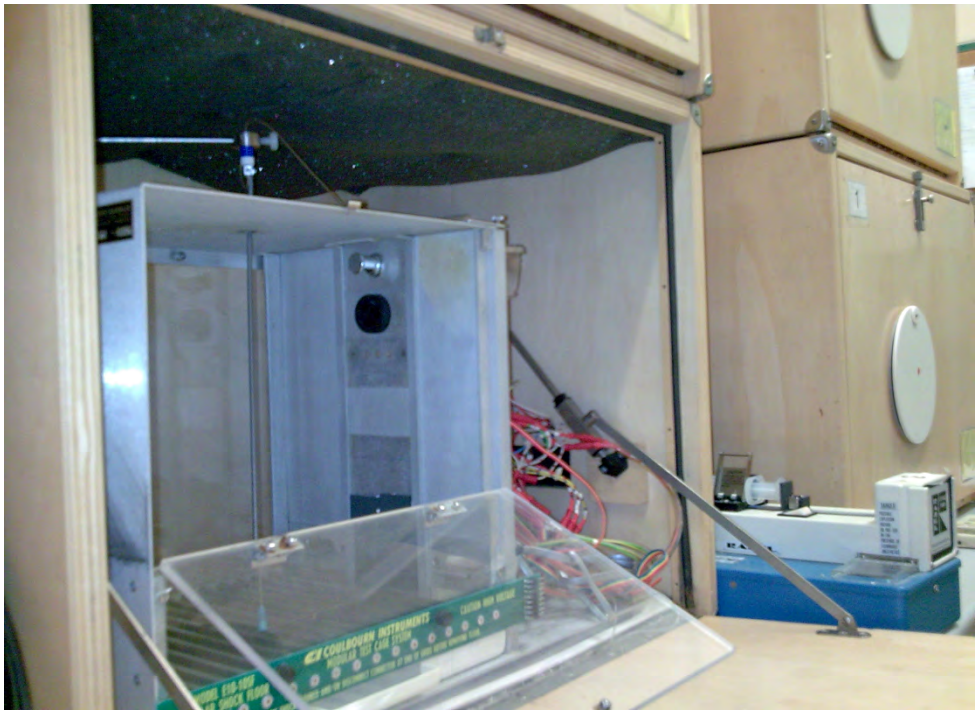


Figure 7: Skinner box photo. The operant chamber is placed in a sound and light-isolating box. It is equipped with two levers (one active and one inactive) a catheter connected to a syringe pump (for nicotine injection) and with a sugar pellet magazine through which sugar pellet are delivered.

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 5 seconds presentation of a cue light (CS). Since the

sugar pellet or nicotine act 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 are interested in investigating the Pavlovian memories, our criterion is a certain number of CS-US associations across the entire training phase (200±15) in order to have similar memory strength across each experimental group. Once trained the food or nicotine related memories are retrieved by the non-contingent presentation of CS. We chose to apply the retrieval and post-retrieval treatment in a context other then the training one in order to mimic human real life situation, in which smoking cessation treatment is generally applied in a context (e.g., hospital) other then the context where drug administration occurs. Manipulation such as propranolol, MK801 or CS-extinction is then provided within the reconsolidation window. It has been reported that one hour after retrieval, memory is labile and susceptible to disruption or updating (Monfils et al, 2009). Extinction consists is repeated presentation of CS contingently upon lever presses in the absence of food or nicotine delivery. This phase lasts until the behaviour has been extinguished (no lever presses for at least 30 minutes). Twenty-four hour later the effect of post-retrieval treatment (i.e. propranolol or MK801 or CS-extinction) on food or nicotine related memory are tested by measuring the conditioned response (number of lever presses) when the animals are placed in the context previously associated to food or nicotine administration (renewal) and are presented with the CS.

## 2. MATERIALS AND METHODS

### *2.1.Subjects.*

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. 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 has 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 heparinised 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 the retrieval (or no-retrieval) session respectively. All doses were expressed as salt.

## 2.3 Experiment #1.

### *2.3.1 Apparatus*

This experiment 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 tow 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, CS-Extinction and renewal 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 TO (60 seconds interval after each reinforcement in which levers are inactive). Right lever presses (nicotine paired lever-NPL) corresponding to FR values, required 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, CS-extinction and renewal sessions. Nicotine solution was administered via the infusion pump at the volume of 0.04638 mL during a 1-s period. Nicotine infusion was associated with 1second illumination of one yellow and one green light emitting diode (LED) centrally placed above the food magazine. 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.).

### *2.3.2. Training to lever press*

Following a 24-h 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 training to lever press for food reinforcement (it required approximately 2 weeks), rats underwent surgery to implant an i.v. cannula.

### *2.3.3. 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 of one i.v. injection of heparin solution (30 IU/mL heparin sodium, Sigma, Italy) before and after the experimental session.

### *2.3.4. 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 FR 2 with session duration lasting up to 60 minutes. . Rats were considered to reach a stable responding on nicotine S/A under a FR 2 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.

### *2.3.5 Retrieval*

After the food S/A phase, rats were divided into two groups respectively exposed to retrieval (Ret) or not (No-Ret). Both groups were placed for 20 minutes in a novel context (CxB; Skinner box with thick blank striped sheets on the wall and a 1 cm grid on the floor). The Ret, but not the No-Ret, group was exposed to 30 non-contingent CS presentations (30[FI 40 s: 1 second CS]).

### *2.3.6 Treatment*

After retrieval phase both groups were then returned to the home cage. One hour later all subjects t were moved in the CxB and underwent a CS-extinction session in which the schedule was FR2: 1 second CS. There was no nicotine delivery as consequence of NPL presses. Session duration lasted up to extinction of responding on NPL (no NPL presses for 30 minutes), or after 6 hours elapsed.

### *2.3.7 Renewal*

The day after the retrieval and the post-retrieval CS-extinction, all the subjects were re-exposed to CxA (the context previously associated to nicotine S/A), and CS presentation was made contingent upon responding on FPL (renewal session): FR1: 1 second CS, no nicotine, session duration 180 minutes.

## 2.4 Experiment #2

### *2.4.1 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 Time-Out (TO) periods and retrieval session. A fixed number (fixed-ratio-FR) of right lever (food paired lever-FPL) presses produced 1-second illumination stimulus light (CS) placed above the FPL and the delivery of 45 mg sugar food pellet (Bilaney Consultants Ltd., UK) except during CS-extinction and renewal sessions and CS-extinction. During the retrieval session, levers were not available and CS was presented on a Fixed-Interval (FI) 60 seconds time schedule. Left lever presses (inactive lever presses-ILP) did not have any consequence. All types of lever presses and sugar pellet deliveries were recorded. Data acquisition and schedule parameters were controlled by Med-PC software (Med Associates Inc.).

### *2.4.2. 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 FR2. Session duration was 60 minutes.

### *2.4.3. Training to food self-administration (S/A)*

Rats were then trained to slightly different FR2 schedule of reinforcement in which two FPL presses resulted in the delivery of 45 mg sugar pellet and also in 1-second CS presentation. After sugar pellet delivery a TO period of 60 seconds starts during which each lever presses was recorded but had no consequences. Session lasts up to 25

reinforcements or 30 minutes. This training phase lasted up to 17 sessions in order to have duration comparable to that of nicotine self-administration training phase.

### *2.4.4 Retrieval*

After the food S/A phase, rats were divided into two groups respectively exposed to retrieval (Ret) or not (No-Ret). On the retrieval session, both groups were placed for 20 minutes in a novel context (CxB; Skinner box with thick blank striped sheets on the wall and a 1 cm grid on the floor). The Ret, but not the No-Ret, group was exposed to 30 non-contingent CS presentations (30[FI 40 s: 1 second CS]). Both groups were further divided into three sub-groups, respectively treated with propranolol (Prop) or saline (Veh) or exposed to CS-extinction (CS-Ext).

### *2.4.5 Treatment*

Immediately after the retrieval phase Ret/Prop and No-Ret/Prop groups received an IP injection of propranolol 10 mg/Kg, while Ret/Veh and No-Ret/Veh groups received an IP injection of saline. After retrieval phase Ret/CS-Ext and No-Ret/CS-Ext groups were placed back in their home cage for 1 hour, then they were moved in the CxB and underwent a CS-extinction session in which the schedule was FR2: 1 second CS. There was no food delivery as consequence of FPL presses. Session duration lasted up to extinction of responding on FPL (no FPL presses for 30 minutes), or after 6 hours elapsed.

### *2.4.6 Renewal*

The day after the retrieval and the post-retrieval treatment, all the subjects were re-exposed to CxA (the context previously associated to food S/A), and CS presentation was made contingent upon responding on FPL (FR1: 1 second CS, no sugar pellet). Session duration was 180 minutes.

## 2.5. Experiment #3.

### *2.5.1 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 Time-Out (TO) periods and retrieval session. A fixed number (fixed-ratio-FR) of right lever (nicotine paired lever-NPL) presses produced 1 second illumination of a stimulus light (CS) placed above the FPL, and the delivery of 45 mg sugar food pellet (Bilaney Consultants Ltd., UK) or the activation of the infusion pump (Med Associates Inc.) except during CS-extinction and renewal sessions. During the retrieval session, levers were not available and CS was presented on a Fixed-Interval (FI) 60 seconds time schedule. Left lever presses (inactive lever presses-ILP) did not have any consequence. All types of lever presses and sugar food pellet deliveries were recorded. Data acquisition and schedule parameters were controlled by Med-PC software (Med Associates Inc.).

### *2.5.2. 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 FR2. 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.

### *2.5.3. 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 of one i.v. injection of heparin solution (30 IU/mL heparin sodium, Sigma, Italy) before and after the experimental session.

### *2.5.4. 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 FR 2 with session duration lasting up to 60 minutes. . Rats were considered to reach a stable responding on nicotine S/A under a FR 2 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 (Ret) or not (No-Ret). On the retrieval session, both groups were placed for 11 seconds in a novel context (CxB; Skinner box with thick blank striped sheets on the wall and a 1 cm grid on the floor). The Ret, but not the No-Ret, group was exposed to 1 non-contingent CS presentation. Both groups were further divided into three sub-groups, respectively treated with propranolol (Prop) or saline (Veh) or exposed to CS-extinction (CS-Ext).

### *2.5.6 Treatment*

Immediately after the retrieval phase Ret/Prop and No-Ret/Prop groups received an IP injection of propranolol 10 mg/Kg; while Ret/Veh and No-Ret/Veh groups received an IP injection of saline. After retrieval phase Ret/CS-Ext and No-Ret/CS-Ext groups were placed back in their home cage for 1 hour, then they were moved in the CxB and underwent a CS-extinction session in which the schedule was FR2: 1 second CS. There was no nicotine delivery as consequence of NPL presses. Session duration lasted up to extinction of responding on NPL (no NPL presses for 30 minutes), or after 6 hours elapsed.

### *2.5.7 Renewal*

The day after the retrieval and the post-retrieval treatment, all the subjects were re-exposed to CxA (the context previously associated to nicotine S/A), and CS presentation was made contingent upon responding on NPL (renewal session): FR1: 1 second CS, no sugar pellet, session duration 180 minutes.

## 2.6 Experiment #4.

### *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 Time-Out (TO) periods and retrieval session. A fixed number (fixed-ratio-FR) of right lever (nicotine paired lever-NPL) presses produced the 1-second illumination of a stimulus light (CS) placed above the NPL, and delivery of 45 mg sugar food pellet (Bilaney Consultants Ltd., UK) or the activation of the infusion pump (Med Associates Inc.) except during CS-extinction and renewal sessions. During the retrieval session, levers were not available and CS was presented on a Fixed-Interval (FI) 60 seconds time schedule. Left lever presses (inactive lever presses-ILP) did not have any consequence. All types of lever presses and sugar food pellet deliveries were recorded. Data acquisition and schedule parameters were controlled by aMed-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 FR2. 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. 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 of one i.v. injection of heparin

solution (30 IU/mL heparin sodium, Sigma, Italy) before and after the experimental session.

### *2.6.4. 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. NPL presses resulted in nicotine 0.03 mg/kg/infusion, 5 seconds CS. Sixty seconds TO period was included. Session duration lasted up to 25 infusions or 60 minutes were elapsed. Lever pressing during the 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 (Standard Error of the Mean-S.E.M.) associations between nicotine infusion and CS (i.e., total number of nicotine reinforcement/CS associations = 200).

### *2.6.5. Instrumental learning extinction phase (ILEXT)*

Following the nicotine S/A phase, NPL 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 NPL presses/session were < 50% of NPL at the first instrumental learning extinction session, for at least three consecutive sessions (Chiamulera et al., 2010). The inclusion in the study protocol 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- extinction effect on nicotine Pavlovian conditioning.

### *2.6.6 CS presentation*

After the ILEXT phase, rats were divided into four groups exposed to 3 (3CS), 30 (30CS), 300 (300CS) or 0 (0CS) presentations respectively. All groups (3CS, 30CS, 300CS and 0CS) 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, 30, 30, 300 and 60 minutes respectively, and exposed to 3, 30, 300 or 0 CS presentation respectively (3, 30 or 300 [FI 55 seconds: 5-seconds CS]).

### *2.6.7 Renewal*

The day after the CS presentation session, all the subjects were re-exposed to CxA (the context previously associated to nicotine Self-Administration), and CS presentation was made contingent upon responding on NPL (renewal session): FR1: 5-seconds CS, no nicotine infusion, session duration 180 minutes.

## 2.7. Experiment #5.

### *2.7.1 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 Time-Out (TO) periods and retrieval session. A fixed number (fixed-ratio-FR) of right lever (nicotine paired lever-NPL) presses produced the 1-second illumination of a stimulus light (CS) placed above the NPL, and delivery of 45 mg sugar food pellet (Bilaney Consultants Ltd., UK) or the activation of the infusion pump (Med Associates Inc.) except during CS-extinction and renewal sessions. During the retrieval session, levers were not available and CS was presented on a Fixed-Interval (FI) 60 seconds time schedule. Left lever presses (inactive lever presses-ILP) did not have any consequence. All types of lever presses and sugar food pellet deliveries were recorded. Data acquisition and schedule parameters were controlled by a Med-PC software (Med Associates Inc.).

### *2.7.2. Training to lever press*

Following a 24-h 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 training to lever press for food reinforcement (it required approximately 2 weeks), rats underwent surgery to implant an i.v. cannula.

### *2.7.3. 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 of one i.v. injection of heparin solution (30 IU/mL heparin sodium, Sigma, Italy) before and after the experimental session.

### *2.7.4. Training to nicotine self-administration (S/A)*

After 7 days of recovery, rats were trained to intravenously self-administer nicotine under a schedule of reinforcemen of FR1. NPL presses resulted in nicotine 0.03 mg/kg/infusion, 5 seconds CS presentation. Sixty seconds TO period was included. Session duration lasted up to 25 infusions or 60 minutes were elapsed. Lever pressing during the 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 (S.E.M.) associations between nicotine infusion and CS (i.e., total number of nicotine reinforcement/CS associations = 200).

### *2.7.5. Instrumental learning extinction phase (ILEXT)*

Following the nicotine S/A phase, NPL responding was extinguished during an instrumental learning extinction phase. On these daily 60 minutes sessions, subjects were placed in the operant chamber and responding on either lever had no programmed consequences. Instrumental learning extinction criterion was reached when NPL presses/session were < 50% of NPL at the first instrumental learning extinction session, for at least three consecutive sessions (Chiamulera et al., 2010). The inclusion in the study protocol 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- extinction effect on nicotine Pavlovian conditioning.

### *2.7.6 Retrieval*

After the ILEXT phase, rats were divided into two groups respectively exposed to retrieval (Ret) or not (No-Ret). On the retrieval session, both groups were placed for 3 minutes in a novel context (CxB; Skinner box with thick blank striped sheets on the

wall and a 1 cm grid on the floor). The Ret, but not the No-Ret, group was exposed to 3 non-contingent CS presentation (3[FI 55 s: 5-second CS]). Both groups were further divided into three sub-groups, respectively treated with MK-801 (MK801) or saline (Veh) or exposed to CS-extinction (CS-Ext).

### *2.7.7 Treatments*

Immediately after the retrieval phase Ret/MK801 and No-Ret/MK801 groups received an IP injection of MK-801 0.1 mg/Kg; while Ret/Veh and No-Ret/Veh groups received an IP injection of water Milli-Q. After retrieval phase Ret/CS-Ext and No-Ret/CS-Ext groups were placed back in their home cage for 1 hour, then they were moved in the CxB and underwent a CS-extinction session in which the each NPL press resulted in 5 seconds CS presentation. There was no nicotine delivery as consequence of NPL presses. Session duration lasted up to extinction of responding on NPL (no NPL presses for 30 minutes), or after 6 hours elapsed.

### *2.7.8 Renewal*

The day after the retrieval and the post-retrieval treatment, all the subjects were re-exposed to CxA (the context previously associated to nicotine Self-Administration), and CS presentation was made contingent upon responding on NPL (renewal session)(FR1: 5-seconds CS). Session duration was 180 minutes.

## 2.8 Data Analyses.

NPL or FLP responding on renewal session was compared among groups in order to test the efficacy of treatments (propranolol, MK-801, CS-extinction, vehicle). First of all, a two factor ANOVA was performed on total NPL or FLP /180 minutes on renewal session for factor retrieval (two levels: Ret, No-Ret) and factor treatment (two to four levels; e.g.: propranolol, MK-801, CS-extinction, vehicle). Since cumulative NPL or FPL were recorded at different time-points during the renewal session (at 15, 30, 45, 60, 120 and 180 minutes during session duration), a series of two factor ANOVA were performed for factors time-point at different levels (15, 30, 45, 60, 120 and 180 minutes), and treatment (two to four levels; e.g.: propranolol, MK-801, CS-extinction, vehicle). Each series of ANOVA was separately performed for Ret and No-Ret groups. Statistical analysis was performed by using Prism 4 (Graph Pad, U.S.A.).

## 3. RESULTS

### 3.1. The Model

#### *3.1.1. Food self-administration acquisition.*

In Experiment #2 rats were trained to self-administer food. Seventy-eight out of eighty rats reached the criteria of food self-administration, the training lasts 16 ± 1 daily sessions (mean ± S.D.). The average numbers of food-paired lever (FPL) and inactive lever (IL) presses across last three sessions were 277.3 ± 9.4 and 27.5 ± 4.7 respectively (mean ± S.E.M.) (Figure 8, panel A). The specificity of food seeking behaviour by rats is confirmed by the discrimination between FPL and IL presses (Figure 8, panel A). Moreover in Figure 8, panel B the stability of the responding can be observed.

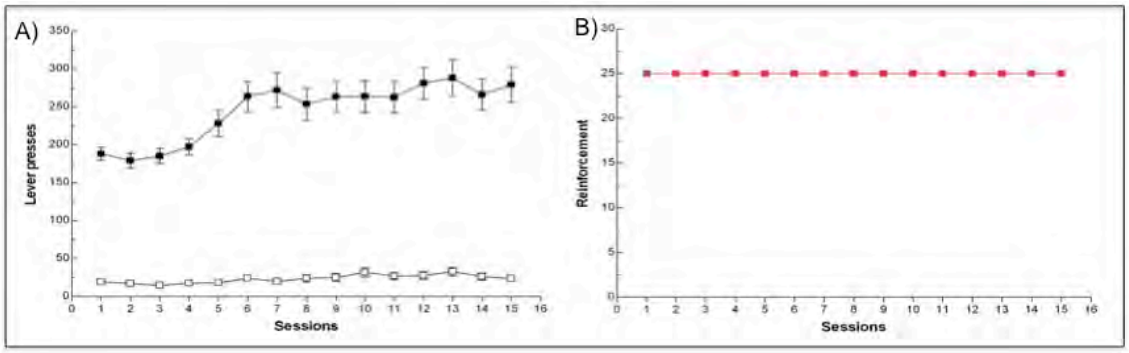


Figure 8: Food self-administration acquisition. A) Mean number of food-paired lever (FPL) presses and inactive lever (IL) presses (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n=78). Discrimination between FPL and IL can be observed across each session. B) Mean number of reinforcements (sugar pellet delivery) across daily session. All the animals reached the criteria of maximum number of reinforcement (25) at each session. (FPL: food paired lever; IL: inactive lever).

### *3.1.2 Nicotine self-administration acquisition*

In Experiment #1, #3, #4 and #5 rats were trained to self-administer nicotine.

In Experiment #1 and #3 (see experimental design in Figure 16 and 23 respectively) were trained until the number of reinforcements/session did not vary more than 20% between three consecutive sessions (criteria of stability).

In Experiment #1 eleven out of 20 rats reached the criteria of stability after the average number of 20.5 ± 1.4 (mean ± SEM) sessions. At stability, the average number of nicotine infusions was 17.8 ± 0.5 (mean ± SEM of the last three self-administration session) (Figure 9, panel B). The average number of nicotine-paired lever (NPL) and inactive lever (IL) presses across the last three sessions were 107,3 ± 12,7 and 13,2 ± 1,8 respectively (mean ± SEM). The specificity of nicotine seeking behaviour is confirmed by the discrimination between NPL and IL (Figure 9, panel B).

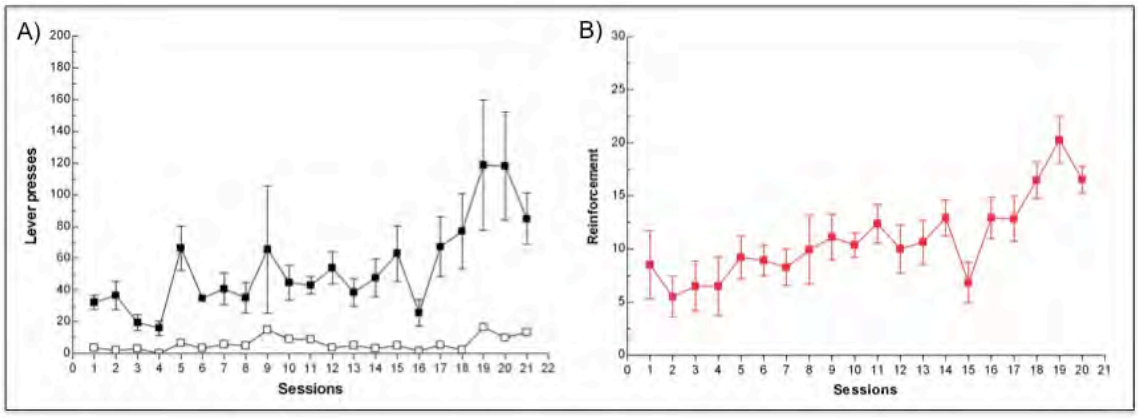


Figure 9: Nicotine self-administration acquisition in Experiment #1. A) Mean number of NPL and IL (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n=11). Discrimination between NPL and IL can be observed across each session. B) Mean number of reinforcement (nicotine infusion) across daily session. Stability of the response can be observed across last three sessions. (NPL: nicotine paired lever; IL: inactive lever).

In Experiment #3, 26 out of 32 rats reached the criteria of stability after the average number of 13.7 ± 0.3 (mean ± SEM) sessions. At stability, the average number of nicotine infusions was 14.1 ± 0.4 (mean ± SEM of the last three self-administration session) (Figure 10, panel B). The average number of NPL and IL presses across the last three sessions was 41.3 ± 0.9 and 11.9 ± 0.9 respectively (mean ± SEM). The discrimination between NPL and IL provides goal-directed evidence towards nicotine-seeking behaviour (Figure 10, panel A).

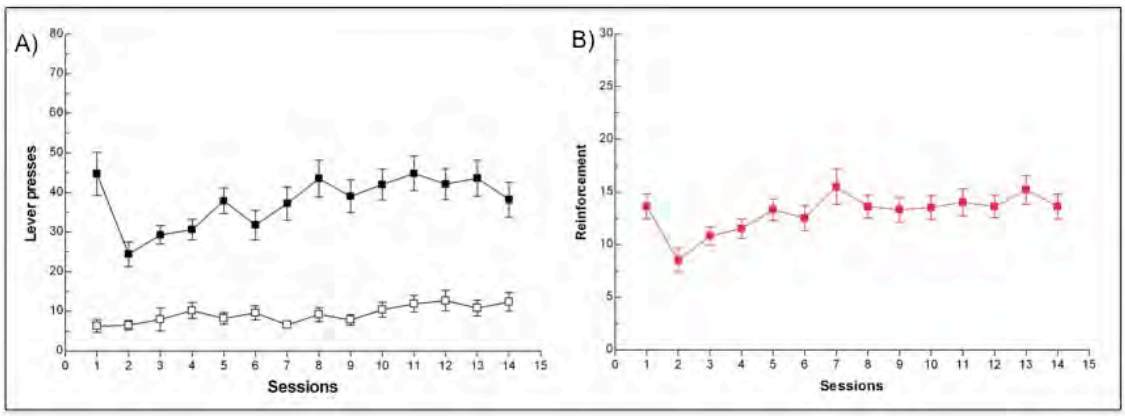


Figure 10: Nicotine self-administration acquisition in Experiment #3. A) Mean number of NPL and IL (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n=26). Discrimination between NPL and IL can be observed across each session B) Mean number of reinforcement (nicotine infusion) across daily session. Stability of the response can be observed across the last three sessions. (NPL: nicotine paired lever; IL: inactive lever).

In Experiments #4 and # 5 (For experimental design see Figure 27 and 30 respectively) rats were considered to meet the criteria of nicotine S/A training once they reached the value of 200 ± 15 (S.E.M.) associations between nicotine infusion and CS (i.e., total number of nicotine reinforcement/CS associations = 200).

In Experiment #4 training to nicotine self administration lasted 13, 7 ± 0,5 daily session (mean ± S.E.M). All the rats met the criteria of total number of CS/nicotine reinforcement associations = 200 (± 15; S.E.M.) at the end of the nicotine self-administration training phase: 190 ± 5,8, 209 ± 8,5, 195.2 ± 5.9 and 196.2 ± 8.1 total number of CS/nicotine reinforcement associations, respectively for 0CS, 3CS, 30CS, 300CS groups (mean ± S.E.M.). Mean (± S.E.M.) number of NPL presses over the last three nicotine S/A sessions was 21.8 ± 2.6, 52.8 ± 13.4, 23.7 ± 2..4 and 30.6 ± 4.4 NPL presses/60 minutes session (4.5 ± 0.9, 10.6 ± 2.2, 6.6 ± 2.3 and 4.6 ± 2.7 IL presses/60 min session), respectively for 0CS, 3CS, 30CS, 300CS groups. The discrimination between NPL and IL provides goal-directed evidence towards nicotine-seeking behaviour (Figure 11, panel A)

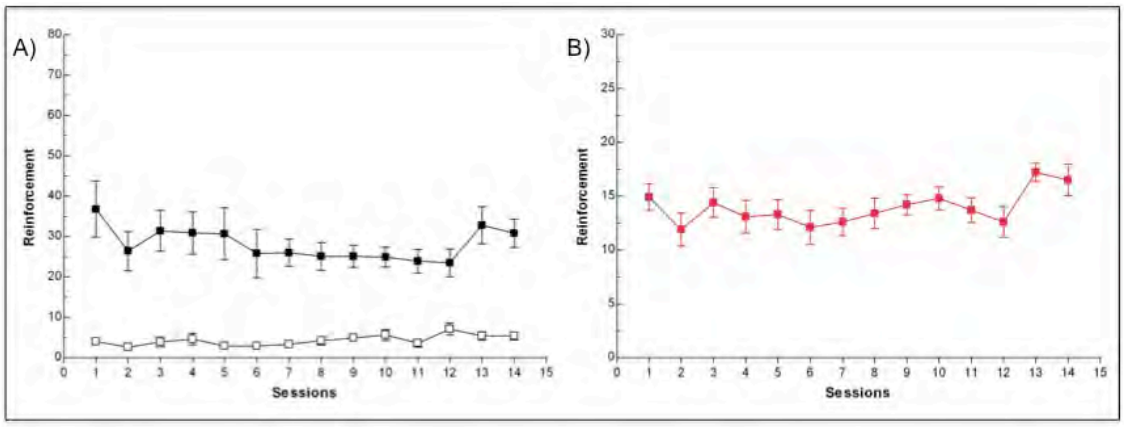


Figure 11: Nicotine self-administration acquisition in Experiment #4. A) Mean number of NPL and IL (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n=16). Discrimination between NPL and IL can be observed across each session. B) Mean number of reinforcement (nicotine infusion) across daily session. (NPL: nicotine paired lever; IL: inactive lever).

In Experiment #5 training to nicotine self administration lasted 13. 4 ± 0.4 daily session (mean ± S.E.M). All the rats met the criterion of total number of CS/nicotine reinforcement associations = 200 ± 15 (mean ± S.E.M.) at the end of the nicotine self-administration training phase: 203.9 ± 8.2, 205.7 ± 80, 200.5 ± 7.7 and 200.3 ± 5.9 total number of CS/nicotine reinforcement associations, respectively for Ret/Sal, Ret/CS-Ext, No-Ret/Sal, No-Ret/CS-Ext groups (mean ± S.E.M.). Mean (± S.E.M.) number of NPL presses over the last three nicotine self-administration sessions was 26.7 ± 2.6, 30.3 ± 2,0, 31.6 ± 3.8 and 28.2 ± 2.8 NPL presses/60 minutes session (5.4 ± 0.9, 7.2 ± 1.1, 6.0 ± 0.9 and 6.8 ± 1.2 IL presses/60 min session), respectively for Ret/Sal, Ret/CS-Ext, No-Ret/Sal, No-Ret/CS-Ext groups. The discrimination between NPL and IL provides goal-directed evidence towards nicotine-seeking behaviour (Figure 12, panel A)

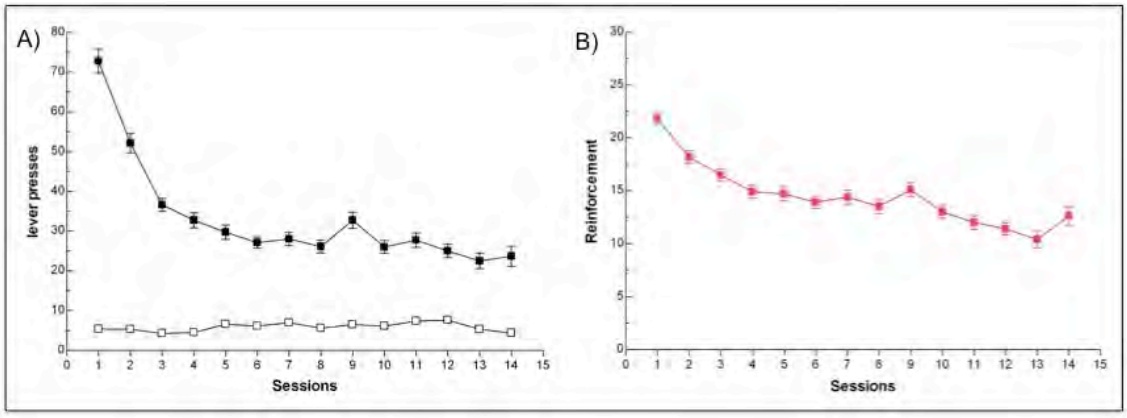


Figure 12: Nicotine self-administration acquisition in Experiment #5. A) Mean number of NPL and IL (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n=88). Discrimination between NPL and IL can be observed across each session. B) Mean number of reinforcement (nicotine infusion) across daily session. Stability of the response can be observed across last three sessions. (NPL: nicotine paired lever; IL: inactive lever).

### *3.1.3 Instrumental learning extinction phase*

In experiments #4 and #5 rats underwent an instrumental learning extinction phase in order to extinguish the operant component of conditioning.

In experiment #4 all the groups met the criteria of extinguished responding (less than 50% of NPL presses at the first instrumental learning extinction phase session, for three consecutive session): 6.4 ± 0.4, 6.1 ± 0.8, 3.6 ± 0.7 and 6.2 ± 0.8 NPL presses/60 min session (3.3 ± 1.8, 3.2 ± 2.0, 1.6 ± 0.5 and 2.6 ± 1.5 inactive lever presses/60 min session) (mean ± S.E.M.) respectively for CS0, CS3, CS30 and CS groups (Figure 13). The criteria of instrumental learning extinction was met after an average number of 11 ± 0,6 daily sessions (mean ± S.E.M.).

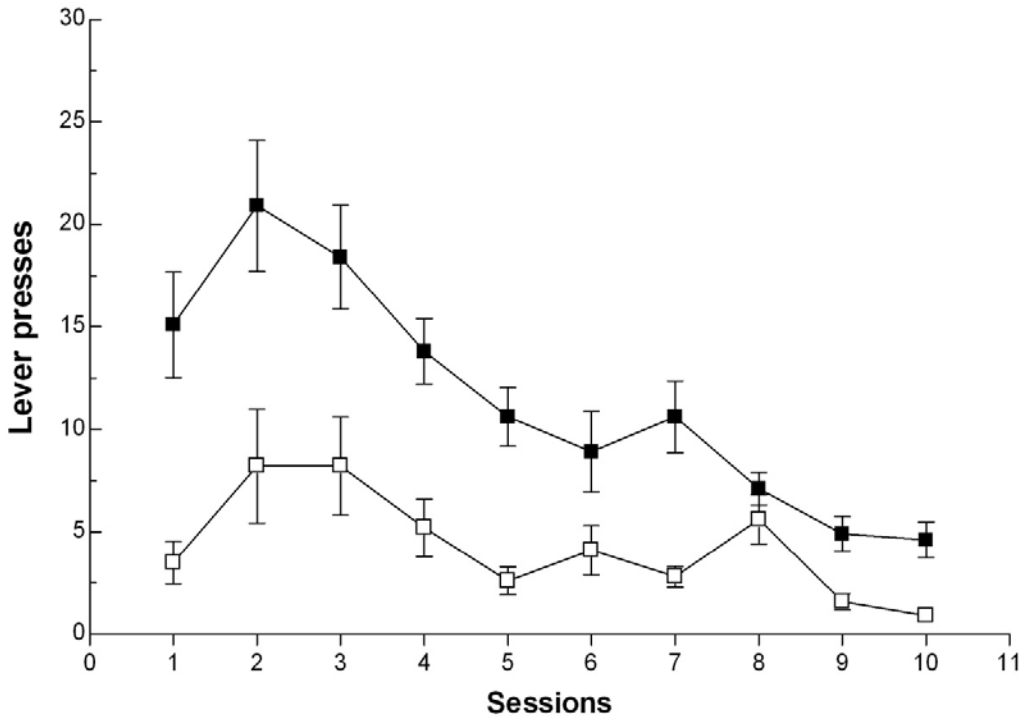


Figure 13: Instrumental learning extinction in Experiment #4. Mean number of NPL and IL presses (± S.E.M.) across daily sessions are represented by black open solid and open squares respectively (n= 16). Across last three session the number of NPL presses were less than 50% of the number of NPL presses on the first session. (NPL: nicotine paired lever; IL: inactive lever).

In experiment #5 all the groups met the criteria of extinguished responding (less than 50% of NPL presses at the first instrumental learning extinction phase session, for three consecutive session): 6.2 ± 0.8, 5.8 ± 0.6, 7.0 ± 0.6, 4.6 ± 0.5, 5.9 ± 0.8 and 6.0 ± 0.4 NPL presses/60 min session (2.2 ± 0.6, 1.8 ± 0.3, 2.4 ± 0.4, 2.6 ± 0.5, 2.1 ± 0.3, 2.5 ± 0.5 inactive lever presses/60 min session) (mean ± S.E.M.) respectively for Ret/Sal, Ret/CS-Ext, Ret/MK801, No-Ret/Veh, No-Ret/CS-Ext and No-Ret/MK801 groups (Figure 14). The criteria of instrumental learning extinction were met after an average number of 9,5±0,6 daily sessions (mean ± S.E.M.).

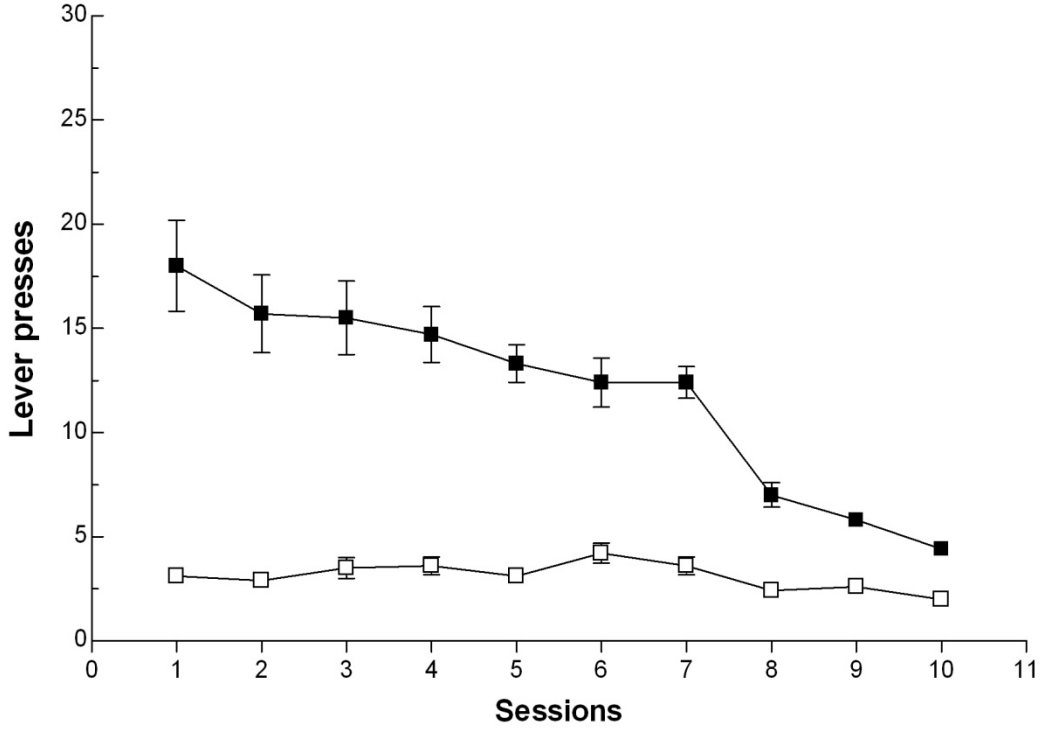


Figure 14: Instrumental learning extinction in Experiment #5. Mean number of NPL and IL presses (± S.E.M.) across daily sessions are represented by solid and open squares respectively (n=88). Across last three sessions the number of NPL presses were less than 50% of the number of NPL presses on the first session. (NPL: nicotine paired lever; IL: inactive lever).

### *3.1.4 Renewal*

Two days after the end of self-administration training (experiments #1,#2 and #3) or instrumental learning extinction phase (experiments #4 and #5), a renewal session was performed. 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. Reinstatement of nicotine seeking behaviour is revealed by a significantly higher responding/lever presses on lever previously paired with nicotine compared (Figure 15, panel B) to the responding during the last Instrumental learning extinction session (Figure 15, panel A).

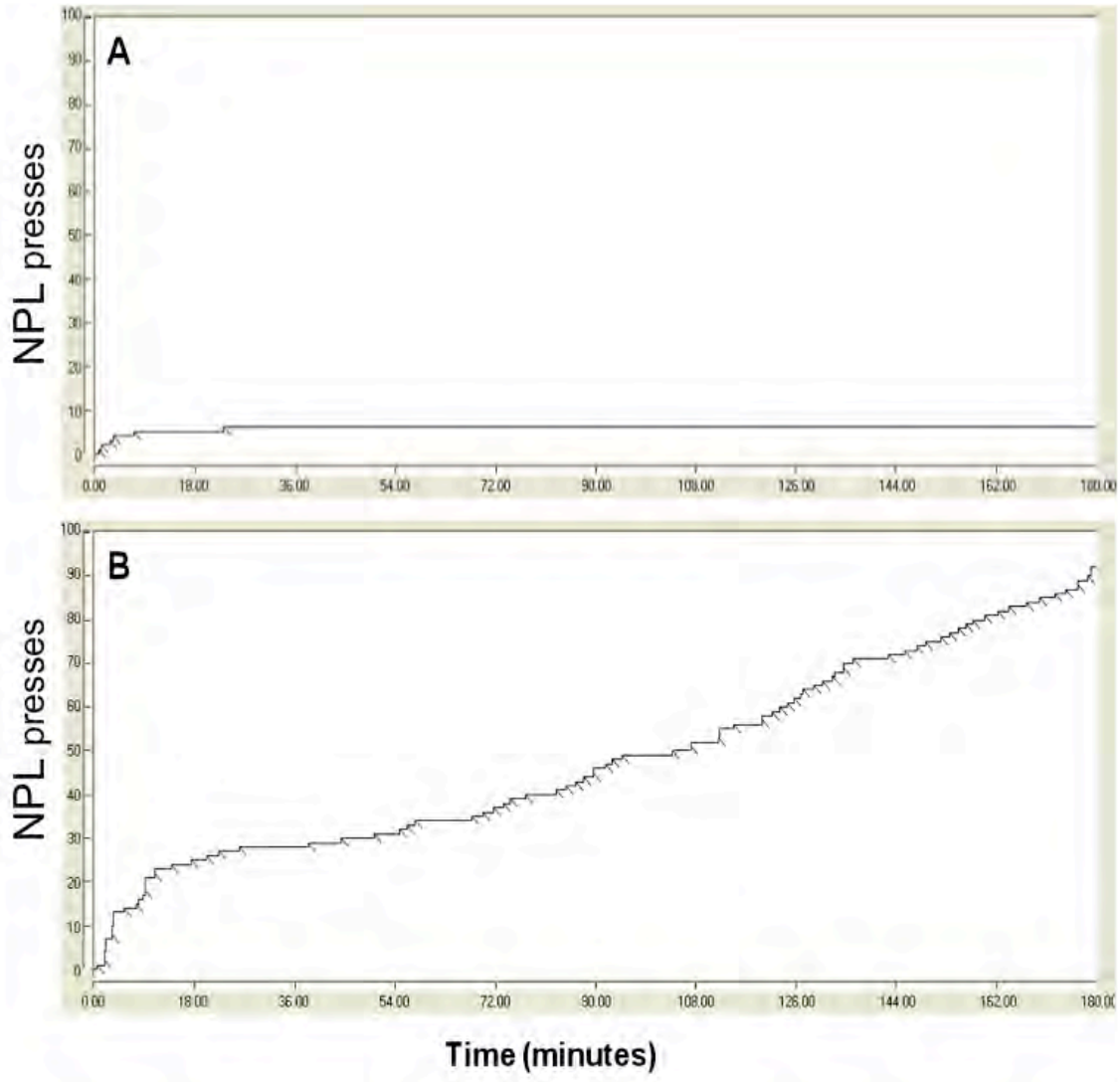


Figure 15: Example of Renewal of nicotine-seeking behaviour (animal code: TOM21). Graphs represent number of NPL (ordinates) across minutes (abscissa) during the last instrumental learning extinction session (panel A) and renewal session (panel B). Each step represents a lever press. On renewal a reinstatement of nicotine seeking behaviour (lever presses) can be observed.

## 3.2. The Project

### *3.2.1. Experiment #1*

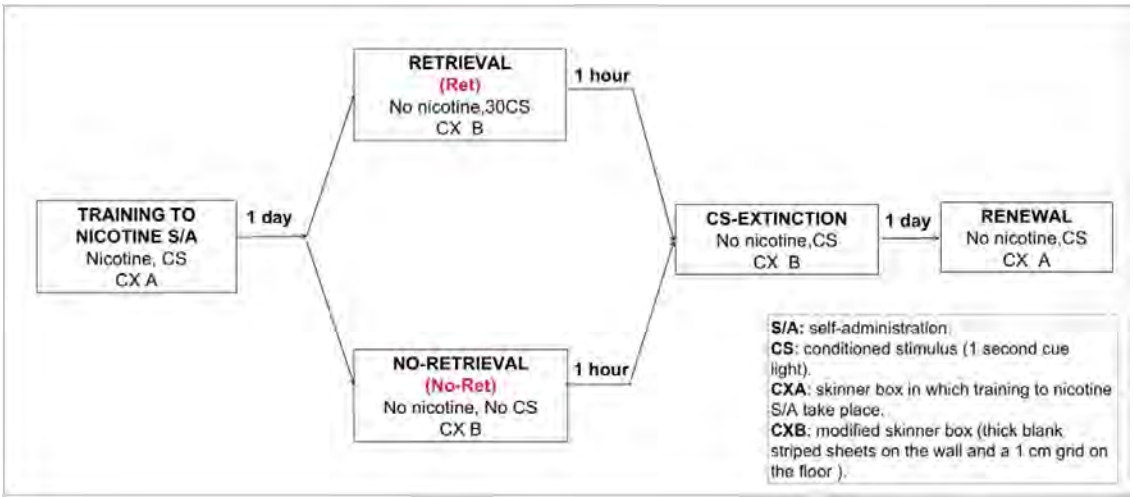


Figure 16: Schematic diagram of experimental design in experiment #1. Rats were trained to nicotine self-administration (S/A)(approximately 20 sessions). One day after last S/A session, they were divided into two groups that underwent a retrieval or no-retrieval session respectively in a context other then the S/A one (CX B). One hour after the end of retrieval (or no-retrieval) both groups (Ret/CS-Ext and No-Ret/CS-Ext) underwent a CS-extinction session in which CS, but not nicotine was presented upon NPL press. Session lasted until the extinction of response (no NPL presses for 30 minutes) and took place in CX B. The day after memory was tested in a renewal session that consisted in placing the rats in the S/A training context (CX A) and during which each NPL press resulted in a CS presentation.

The total NPL presses at the end of the 180 minutes renewal session was 113.0 ± 73.3, 69.8 ± 51.7 (5.6 ± 5.4, 2.2 ± 1.8 ILP) (mean ± S.E.M.) respectively for Ret/CS-Ext (n = 5), No-Ret/CS-Ext (n = 5) (Figure 17). There is no statistically significant difference in NPL between groups.

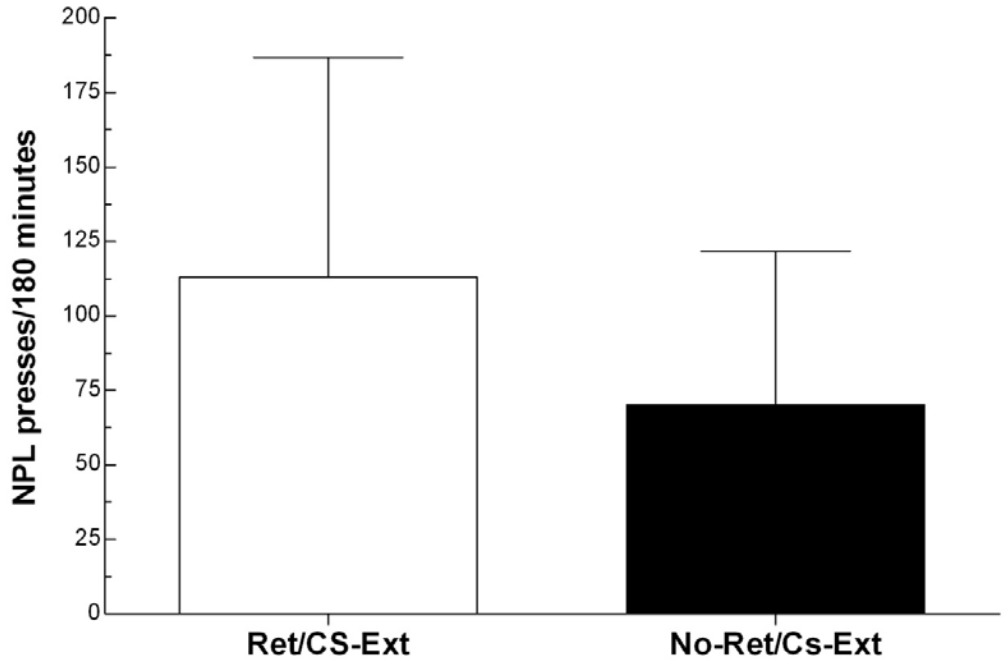


Figure 17: Renewal test in Experiment #1. Retrieval (30 CS presentation) had no effect on Cs-extinction in a renewal test (Experiment #1). Data are expressed as total number of nicotine-paired (NPL) lever presses at the end of renewal session (mean ± S.E.M.) for Ret/Cs-Ext group (open column, n=5) and No-Ret/Cs-Ext group (black column, n=6). No statistically significant difference between Ret/CS-Ext and No-Ret/CS-Ext groups.

Temporal analyses of cumulative NPL responding during the renewal session were performed at different time-points (15, 30, 45, 60, 120, 180 minutes) (Figure 18). Two way ANOVA analyses for factor retrieval at two levels (Ret, No-Ret) and time-point at six levels (i.e., at 180, 120, 60, 45, 30, 15 min) showed statistically significant main effects of time-point ($F_{[5,45]}$ = 0.42; $p$ <0.001) but not of retrieval ($F_{[1,45]}$ = 0.60 ; $p$ = 0.45) neither for the interaction between retrieval and time-point ($F_{[5,45]}$ = 0.91; $p$ = 0.29.).

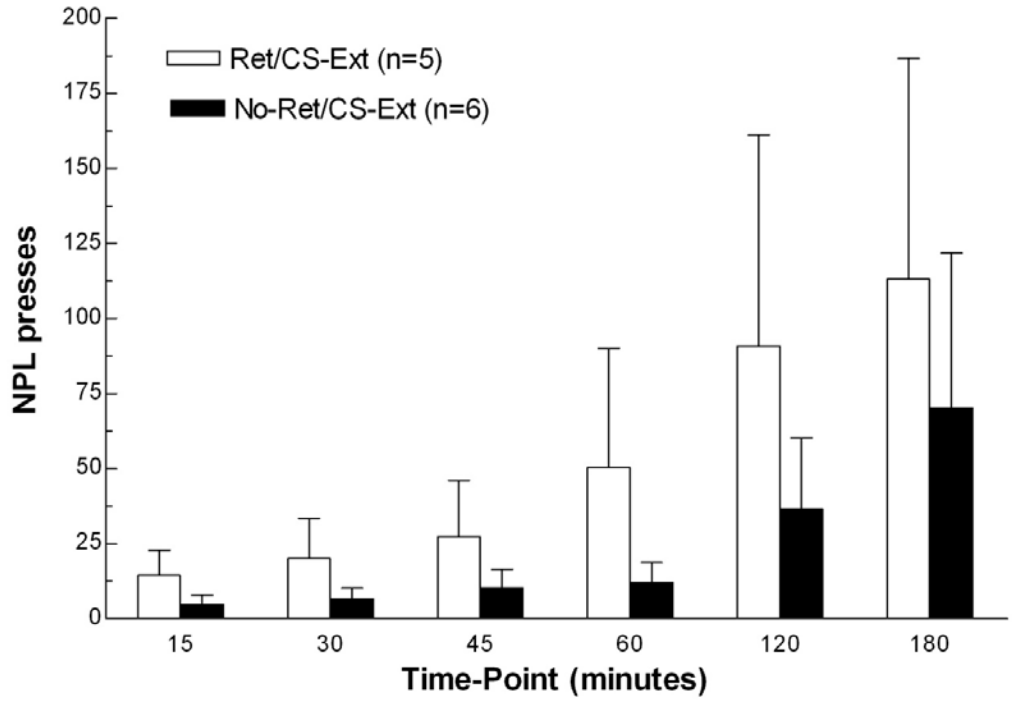


Figure 18: Temporal analysis of renewal (Experiment #1). Data are expressed as number of NPL (mean ± S.E.M) at different time-points of the renewal session. A significant effect of time-point have been observed but not of retrieval (see text).

In summary no difference between Ret/CS-Ext and NoRet/CS-Ext was observed on renewal test. At all time-points during the 180 minutes session, the two groups exhibited a significantly greater responding on NPL than on inactive lever (statistical analysis not shown).

### *3.2.2. Experiment #2*

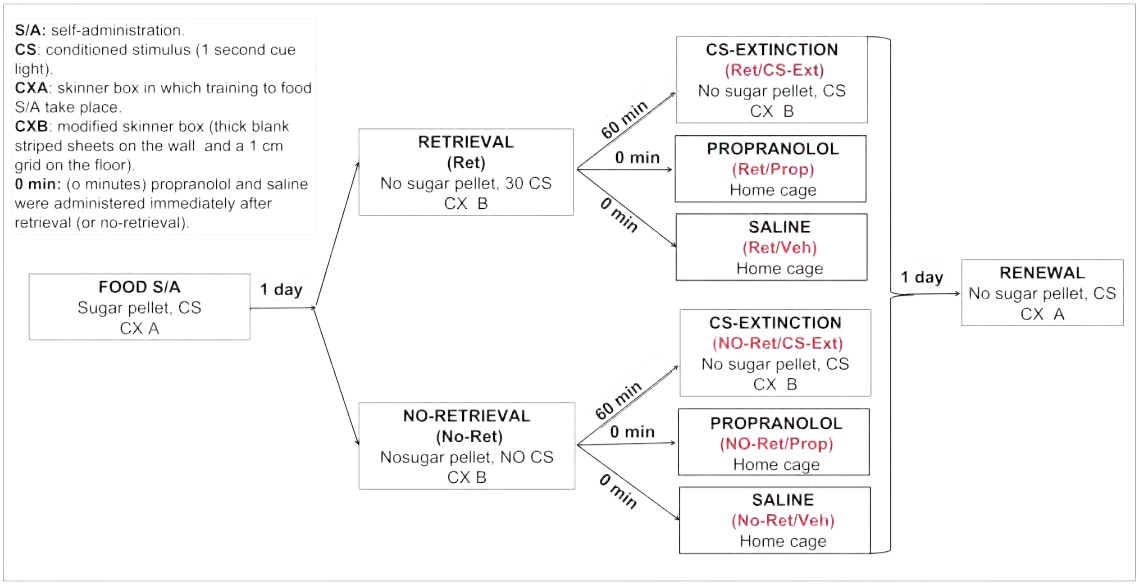


Figure 19: Schematic diagram of experimental design in experiment #2. Rats were trained to food self-administration (S/A) (approximately 16 sessions). One day after the end of S/A phase they were divided into two groups that underwent a retrieval or no-retrieval session respectively, in a context other then the S/A training one (CX B). Then both Ret and No-Ret groups were divided into three subgroups. Ret/CS Ext and No-Ret/CS-Ext, one hour after the end of retrieval, underwent a CS-Extinction session in which CS, but not food was presented upon FPL press. Session lasted until the extinction of response (no FPL presses for 30 minutes) and took place in CX B. Ret/Prop and No-Ret/Prop immediately after retrieval received a propranolol injection. Ret/Veh and No-Ret/Veh received a saline injection. The day after, memory was tested in a renewal session that consisted in placing the rats in the S/A training context (CX A) and during which each FPL press resulted in a CS presentation.

The total food paired lever (FPL) presses active lever presses at the end of the 180 minutes renewal session was 196.8 ± 42.4, 85.8 ± 24, 183.4 ± 26.6, 246 ± 30.3 and 90.6 ± 19.1, 192.8 ± 17.7 (36.9 ± 10.6, 15.0 ± 4.9, 25.9 ± 5.1, 32.9 ± 5.9, 21.4 ± 5.5 and 30.0 ± 5.9 inactive lever presses) (mean ± S.E.M.) respectively for Ret/Veh (n = 14), Ret/CS-Ext (n = 12), Ret/Prop (n= 10), No-Ret/Veh (n = 14), No-Ret/CS-Ext (n = 10), No-Ret/Prop (n=9) groups (Figure 20). To compare the results of CS-extinction or propranolol vs. vehicle we run two separate two way ANOVA with factor retrieval (Ret, No-Ret) and factor treatment (CS-Ext or Prop and Veh). In the analysis comparing CS-

Ext vs. Veh two way ANOVA showed no significant main effect for factors retrieval (Ret, No-Ret) ($F_{[1,27]}$ = 0.87 ; $p$ = 0.35) neither for the interaction between retrieval and treatment (CS-Ext, Veh,) ($F_{[2,27]}$ = 0.59; $p$ = 0.59) but a significant main effect of treatment ($F_{[1,27]}$ = 16.77; $p$ <0.001). Bonferroni post-hoc test revealed a statistically significant difference between Ret/Veh and Ret/CS-Ext (p<0.05), and between No-Ret/Veh and No-Ret/CS-Ext (p<0.01) (Figure 20). In the analysis comparing Prop vs. Veh two way ANOVA showed no significant main effect for factors retrieval (Ret, No-Ret) ($F_{[1,23]}$ = 0.84 ; $p$ = 0.36), neither for factor treatment ($F_{[1,23]}$ = 0.80; $p$ = 0.38), or for the interaction between retrieval and treatment ($F_{[1,23]}$ = 0.39; $p$ = 0.54).

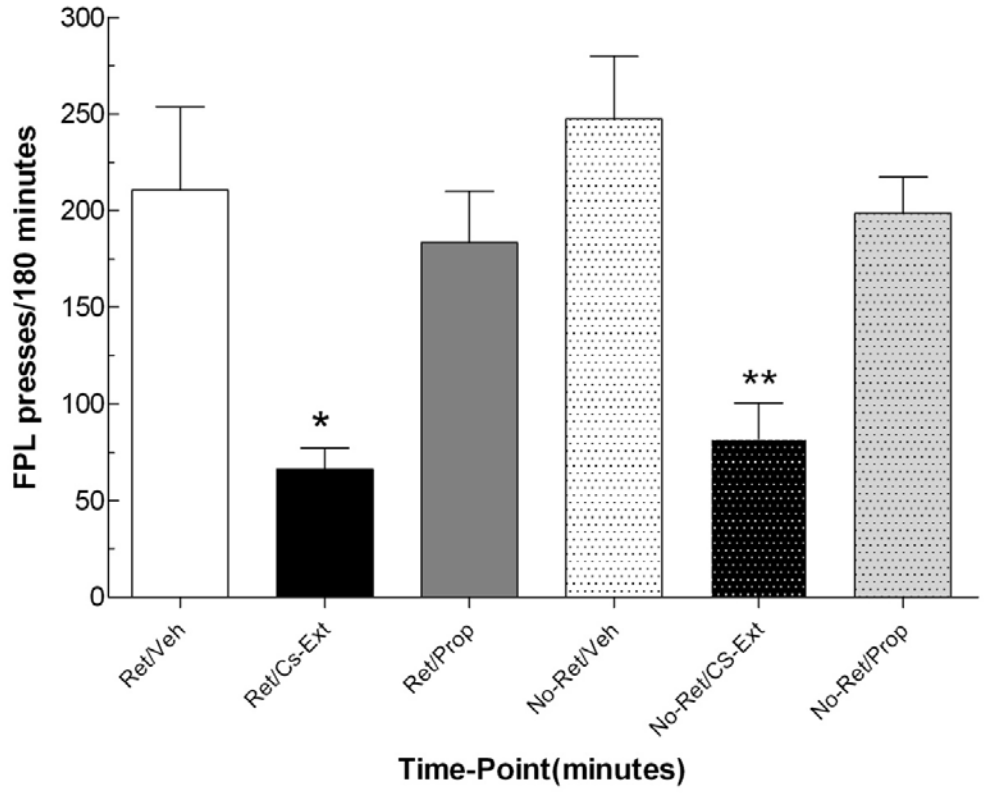


Figure 20: Renewal test in Experiment #2. Total number of food-paired (FPL) lever presses at the end of renewal session in Experiment #2. Data are expressed as mean number of FPL presses ± S.E.M. CS-extinction decreased the renewal of food seeking behaviour (FPL presses) both after retrieval or no-retrieval (Ret/Veh n=14; Ret/CS-Ext n=12; Ret/Prop n=10; No-Ret/Veh n=13; No-Ret/CS-Ext 10; No-Ret/Prop n=9). *= p< 0.05 Ret/CS-Ext vs. Ret/Sal (Bonferroni post-hoc test) ; **= p< 0.01 No-Ret/CS-Ext vs. No-Ret/Sal (Bonferroni post-hoc test ).

Temporal analyses of cumulative active lever responding during the renewal session were performed at different time-points (15, 30, 45, 60, 120, 180 minutes) separately for each Ret and No-Ret groups. For Ret groups (Figure 21), two way ANOVA analyses for factor treatment at two levels (CS-Ext, Veh,) and time-point at six levels (i.e., at 180, 120, 60, 45, 30, 15 minutes) showed statistically significant main effects of both time-point ($F_{[1,120]}$ = 31,72; $p < 0.0001$) and treatment ($F_{[1,120]}$ = 8.27; p = 0.008). Moreover significant effect for interaction between factors was observed ($F_{[5,120]}$ = 11.21; $p < 0.0001$). Bonferroni post-hoc test did not show any significant difference between groups. For Ret groups (Figure 21), two way ANOVA analyses for factor treatment at two levels (Prop, Veh,) and time-point at six levels (i.e., at 180, 120, 60, 45, 30, 15 minutes) showed statistically significant main effects of time-point ($F_{[1,110]}$ = 46.38; $p < 0.0001$) but not for treatment ($F_{[1,110]}$ = 0.23; p = 0.63), neither for interaction between factors was observed ($F_{[5,110]}$ = 0.64; p = 0.67). Bonferroni post-hoc test did not show any significant difference between groups. (Figure 21).

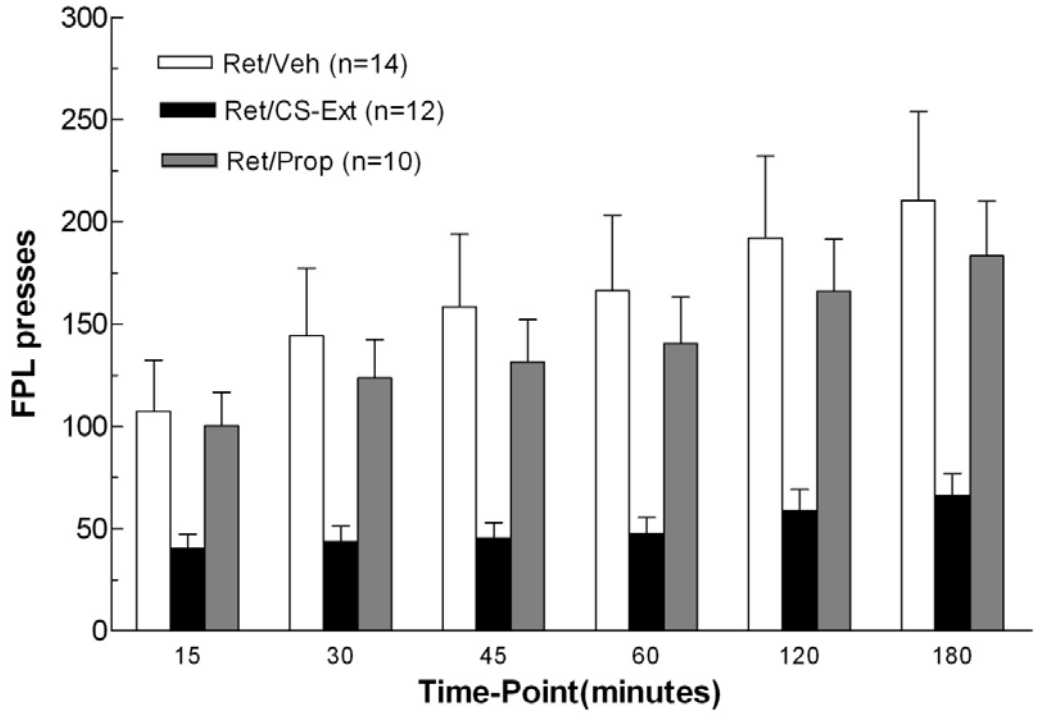


Figure 21: Temporal analysis of renewal for Ret groups in Experiment #2. Data are expressed as number of FPL (mean ± S.E.M) at different time-points of the renewal session. Two way ANOVA have revealed a main effect of CS-extinction but not of propranolol (see text). Bonferroni post-hoc analyses did not show any differences between groups.

For No-Ret group (Figure 22) Two way ANOVA analyses for factor treatment at two levels (CS-Ext, Veh) and time-point (i.e., at 180, 120, 60, 45, 30, 15 min) showed statistically significant main effects of both time-point ($F_{[5,105]} = 30.67$; $p < 0.0001$) and treatment ($F_{[1,105]} = 13.06$; $p = 0.002$). Moreover significant effect for interaction between factors was observed ($F_{[5,105]} = 12.34$; $p < 0.001$). Bonferroni post-hoc test did not show any significant difference between groups. Two way ANOVA analyses for factor treatment at two levels (Veh, Prop) and time-point (i.e., at 180, 120, 60, 45, 30, 15 minutes) showed statistically significant main effects of time-point ($F_{[5,100]} = 44.97$; $p < 0.0001$)but not for treatment ($F_{[1,100]} = 0.84$; $p = 0.37$)neither for interaction between factors was observed ($F_{[5,100]} = 2.30$; $p = 0.05$). Bonferroni post-hoc test did not show any significant difference between groups.

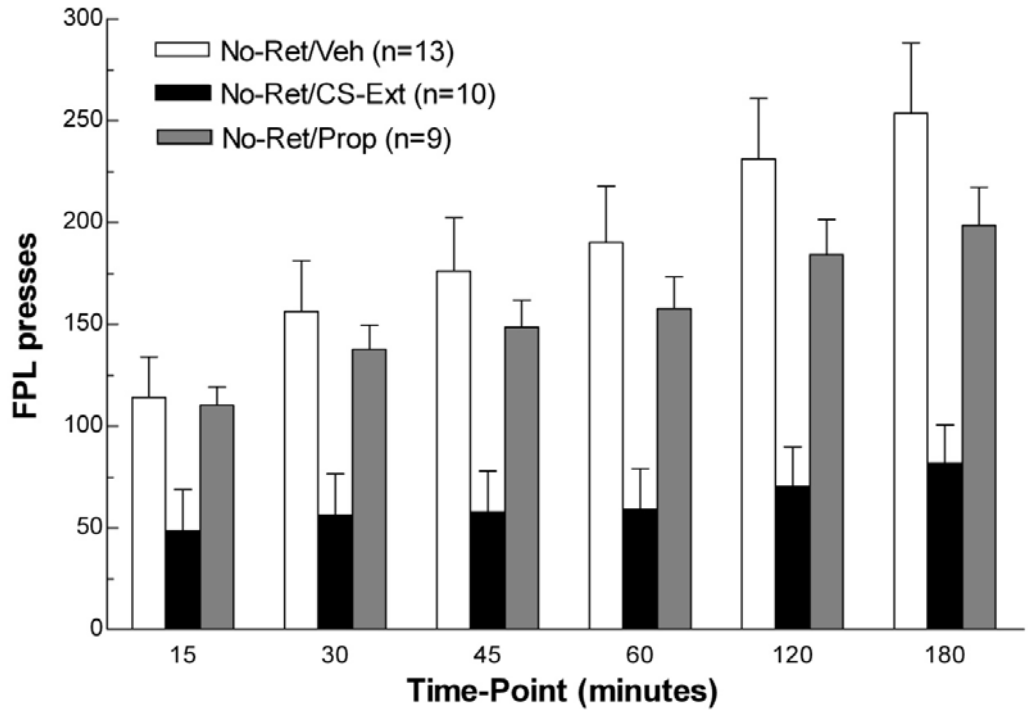


Figure 22: Temporal analysis of renewal for No-Ret groups in Experiment #2. Data are expressed as number of FPL (mean ± S.E.M) at different time-points of the renewal session. Two way ANOVA have revealed a main effect of CS-extinction but not of propranolol (see text). Bonferroni post-hoc analyses did not show any differences between groups.

In summary in the analysis taking in consideration the retrieved group (Ret/Veh and Ret/CS-Ext), as well as in the analysis taking in consideration the no-retrieved group (No-Ret/Veh and No-Ret/CS-Ext), two way ANOVA for factor time point and factor treatment (CS-extinction, vehicle) showed a main effect of treatment. No effect of propranolol was observed across either in retrieved and in no-retrieved groups (Ret/Veh, Ret/Prop or No-Ret/Veh, No-Ret/Prop).

At all time-points during the 180 min session, the six groups exhibited a significantly greater responding on active lever than on inactive lever (statistical analysis not shown).

### *3.2.3 Experiment #3*

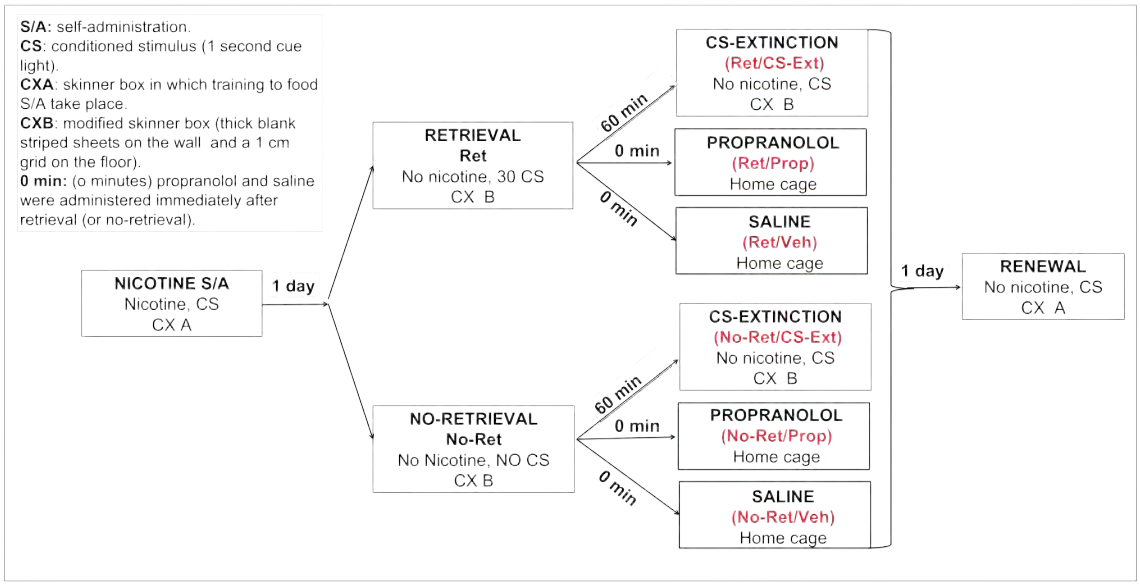


Figure 23: Schematic diagram of experimental design in experiment #3. Rats were trained to nicotine self-administration (S/A) (approximately 14 sessions). One day after the end the S/A phase, rats were divided into two groups that underwent a retrieval or no-retrieval session respectively, in a context other then the S/A training one (CX B). Then both Ret and No-Ret groups were divided into three subgroups. Ret/CS Ext and No-Ret/CS-Ext, one hour after the end of retrieval, underwent a CS-Extinction session in which CS, but not nicotine was presented upon NPL press. Session lasted until the extinction of response (no NPL presses for 30 minutes) and took place in CX B. Ret/Prop and No-Ret/Prop immediately after retrieval received a propranolol injection. Ret/Veh and No-Ret/Veh received a saline injection. The day after memory was tested in a renewal session that consisted in placing the rats in the S/A training context (CX A) and during which each NPL press resulted in a CS presentation.

The total NPL presses at the end of the 180 minutes renewal session was 68.0 ± 0.6, 51.5 ± 10.9, 55.6 ± 9.8, 53.7 ± 5.9 and 48.0 ± 2.0, 42.2 ± 10.9 (11.3 ± 2.0, 12.8 ± 4.1, 20.0 ± 2.0, 9.3 ± 2.7, 12.0 ± 6.0 and 15.2 ± 5.2 inactive lever presses) (mean ± S.E.M.) respectively for Ret/Veh (n = 3), Ret/CS-Ext (n = 4), Ret/Prop (n= 5), No-Ret/Veh (n = 3), No-Ret/CS-Ext (n = 2), No-Ret/Prop (n=5) groups (Figure 24). To compare the results of CS-extinction or propranolol vs. vehicle we run two separate two way ANOVA with factor retrieval (Ret, No-Ret) and factor treatment (CS-Ext or Prop and Veh). In the analysis comparing Cs-Ext vs.Veh two way ANOVA showed main effect

for factor treatment (CS-Ext, Veh) ($F_{[1,3]}$ = 10.98; $p$ = 0.04)but not for factors retrieval (Ret, No-Ret) ($F_{[1,3]}$ = 0.49 ; $p$ = 0.53) neither for interaction between retrieval and treatment ($F_{[1,3]}$ = 2.90; $p$ = 0.19). Bonferroni post-hoc test did not revealed any difference between groups. In the analysis comparing Prop vs.Veh two way ANOVA showed no effect for factor treatment (Prop, Veh) ($F_{[1,6]}$ = 3.36; $p$ = 0.11)for factors retrieval (Ret, No-Ret) ($F_{[1,6]}$ = 1.22 ; $p$ = 0.31) neither for interaction between retrieval and treatment ($F_{[1,6]}$ = 0; $p$ = 0.97). Bonferroni post-hoc test did not revealed any difference between groups.

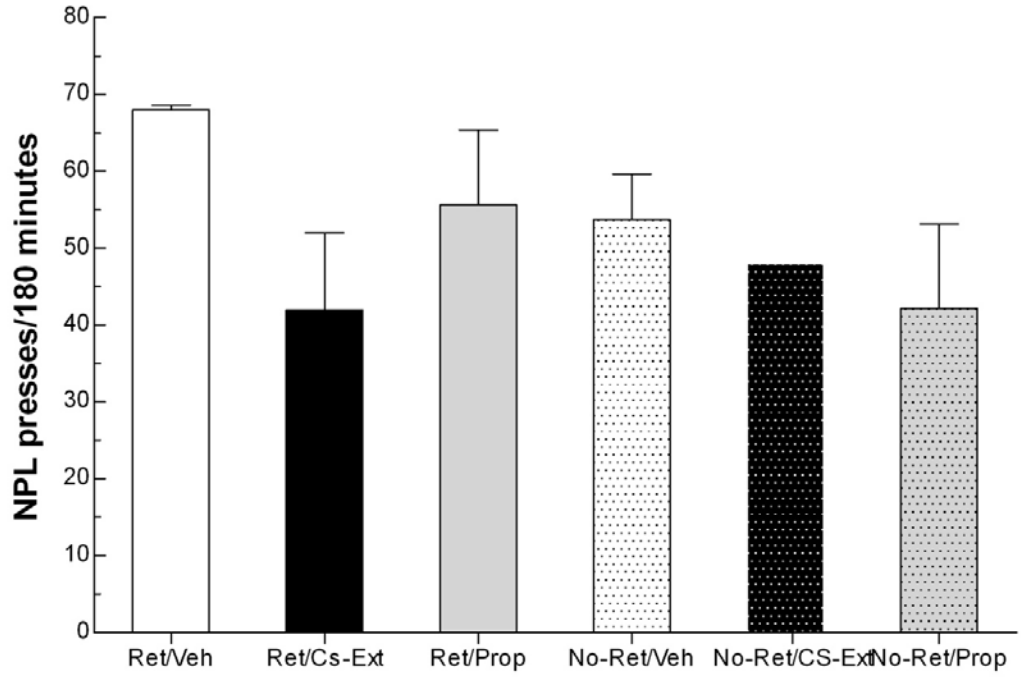


Figure 24: Total number of food-paired (NPL) lever presses at the end of renewal session in Experiment #3. Data are expressed as mean number of NPL presses ± S.E.M (Ret/Veh n=3; Ret/CS-Ext n=4; Ret/Prop n=5; No-Ret/Veh n=3; No-Ret/CS-Ext n=3; No-Ret/Prop n=4). Two way ANOVA revealed a main effect of CS-extinction but not of propranolol. Bonferroni post-hoc test did not revealed any significant difference between groups.

Temporal analyses of cumulative NPL responding during the renewal session were performed at different time-points separately for each Ret and No-Ret groups. For Ret groups (Figure 25), two way ANOVA analyses for factor treatment at two levels (CS-

Ext, Veh) and time-point (i.e., at 180, 120, 60, 45, 30 min) showed statistically significant main effects of both time-point ($F_{[5,25]} = 48.19$; $p < 0.0001$) and treatment ($F_{[1,25]} = 6.72$; $p = 0.048$). No significant effect for interaction between factors was observed ($F_{[5,25]} = 1.35$; $p = 0.28$). Bonferroni post-hoc test did not show any difference between groups. Two way ANOVA analyses for factor treatment at two levels (Prop, Veh) and time-point (i.e., at 180, 120, 60, 45, 30 min) showed statistically significant main effects of both time-point ($F_{[5,30]} = 45.97$; $p < 0.0001$). No significant effect was observed for factor treatment ($F_{[1,30]} = 0.23$; $p = 0.64$) and for interaction between factors was observed ($F_{[2,30]} = 2.30$; $p = 0.07$). Bonferroni post-hoc test did not show any difference between groups.

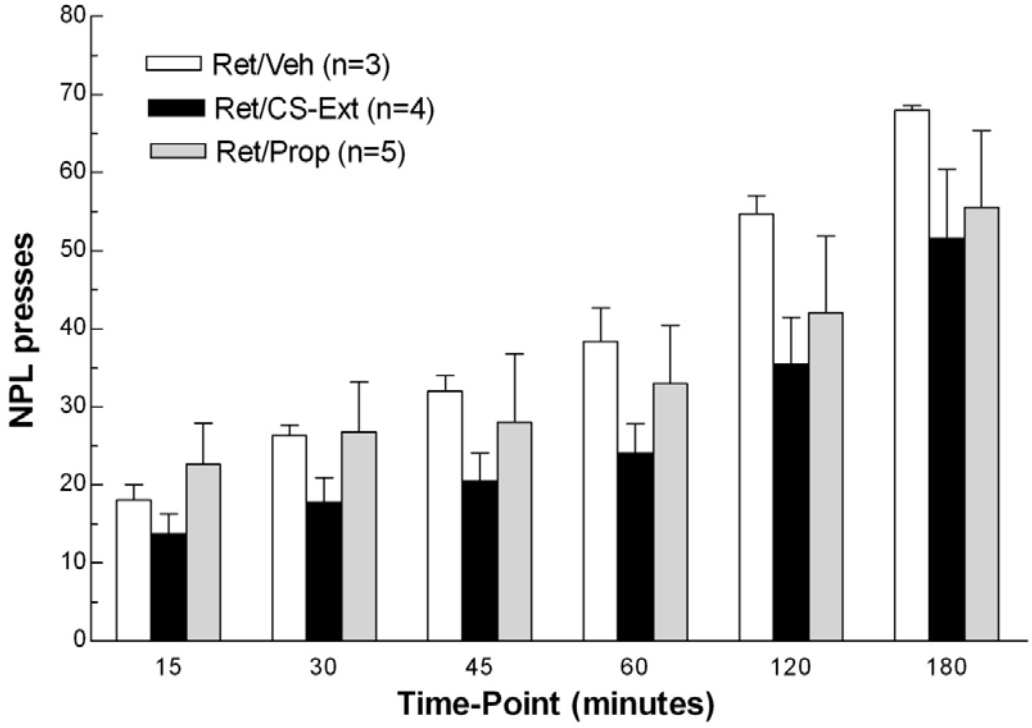


Figure 25: Temporal analysis of renewal for Ret groups in Experiment #3. Data are expressed as number of NPL (mean ± S.E.M) at different time-points of the renewal session. Two way ANOVA revealed a main effect of CS-extinction but not of propranolol. Bonferroni post-hoc test did not revealed any significant difference between groups.

For No-Ret group (Figure 26), two way ANOVA analyses for factor treatment at two levels (CS-Ext, Veh) and time-point (i.e., at 180, 120, 60, 45, 30 min) showed statistically significant main effects of time-point ($F_{[5,20]} = 27.11$; $p < 0.0001$) but not for factor treatment ($F_{[1,20]} = 2.81$; $p = 0.17$) neither for interaction between factors ($F_{[5,20]} = 1.41$; $p = 0.26$). Bonferroni post-hoc test did not show any difference between groups. Two way ANOVA analyses for factor treatment at two levels (Prop, Veh) and time-point (i.e., at 180, 120, 60, 45, 30 min) showed statistically significant main effects of time-point ($F_{[5,25]} = 4.97$; $p < 0.003$) but not for factor treatment ($F_{[1,25]} = 0.20$; $p = 0.67$) neither for interaction between factors ($F_{[5,25]} = 0.70$; $p = 0.63$). Bonferroni post-hoc test did not show any difference between groups

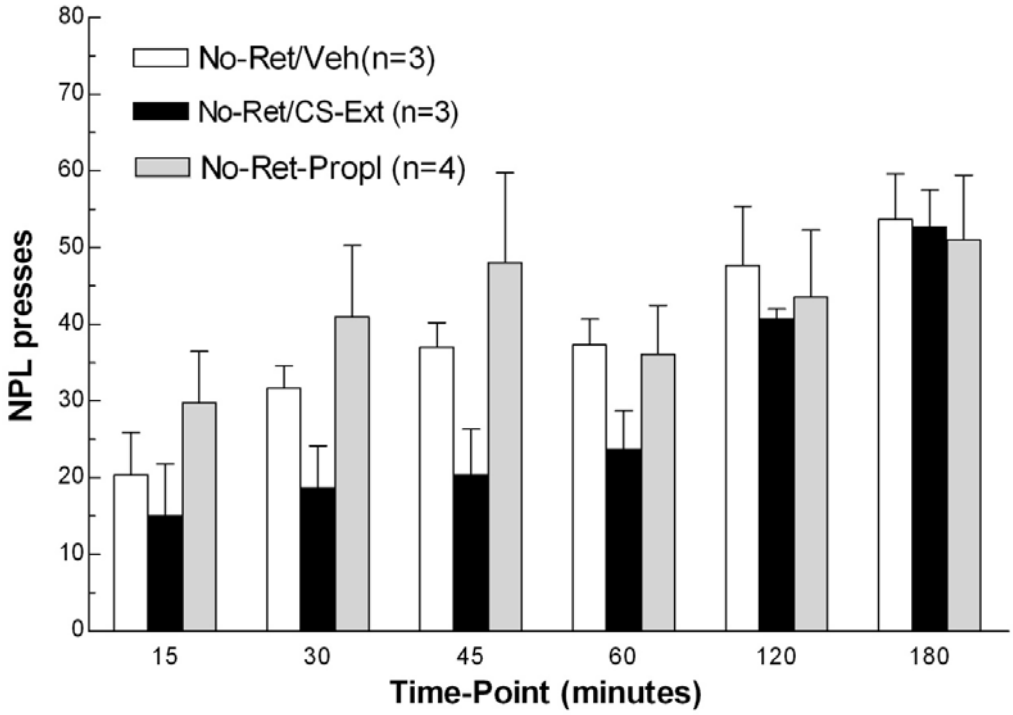


Figure 26: Temporal analysis of renewal for No-Ret groups in Experiment #3. Data are expressed as number of NPL (mean ± S.E.M) at different time-points of the renewal session. No effect CS-extinction or 13propranolol have been observed.

In summary in the analysis taking in consideration the retrieved group (Ret/Veh and Ret/CS-Ext) two way ANOVA for factor time point and factor treatment (CS-extinction, vehicle) showed a main effect of treatment. On the other hand in the analysis

taking in consideration the no-retrieved group (No-Ret/Veh and No-Ret/CS-Ext) two way ANOVA for factor time point at different levels (30, 45, 60, 120 and 180 minutes) and factor treatment (CS-extinction, vehicle) showed no effect of treatment. No effect of propranolol was observed across either in retrieved and in no-retrieved groups (Ret/Veh, Ret/Prop or No-Ret/Veh, No-Ret/Prop).

At all time-points during the 180 minutes session, the six groups exhibited a significantly greater responding on NPL than on inactive lever (statistical analysis not shown).

### *3.2.4. Experiment #4*

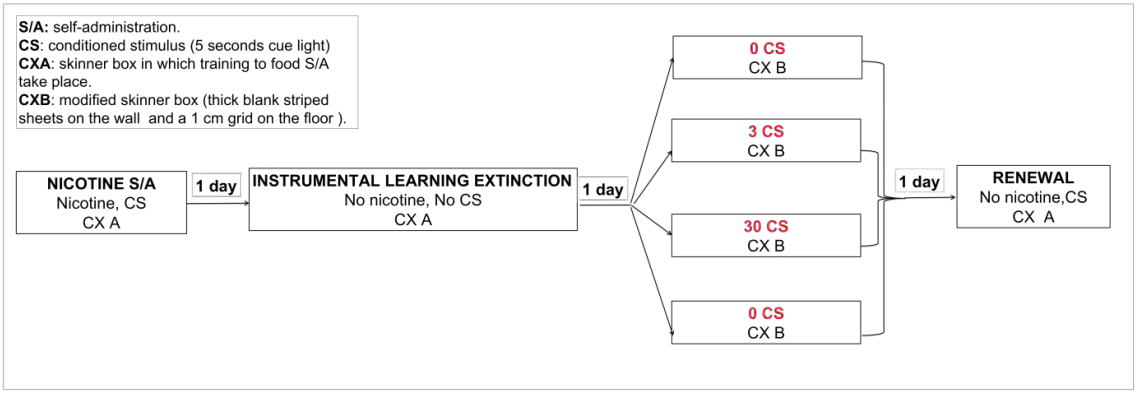


Figure 27: Schematic diagram of experimental design in experiment #4. Rats were trained to nicotine self-administration (S/A) (approximately 14 sessions) then they underwent an instrumental extinction (ILEXT) phase in order to extinguish the instrumental learning component of conditioning (approximately 11 sessions). 1 day after the end ILEXT rats were divided into four groups that were exposed to 0, 3, 30 or 300 CS presentations respectively in a context other to the S/A and ILEXT phases (CX B). The day after, memory were tested in a renewal session that consisted in placing the rats in the S/A and ILEXT context (CX A) and during which each NPL press resulted in a CS presentation.

The total NPL presses at the end of the 180 minutes renewal session was 81.0 ± 29.0, 144.0 ± 19.4, 58.2 ± 24.7 and 47.0 ± 9.0 (4.5 ± 2.0, 6.5 ± 0.8, 7.5 ± 3.2 and 12.5 ± 1.9 inactive lever presses/session) (mean ± S.E.M.) respectively for 0CS (n = 4), 3CS (n = 4), 30CS (n= 4) and 300CS (n = 4) groups (Figure 28). One way ANOVA showed no significant main effect for factors CS presentation (0CS, 3CS, 30CS an 300CS).

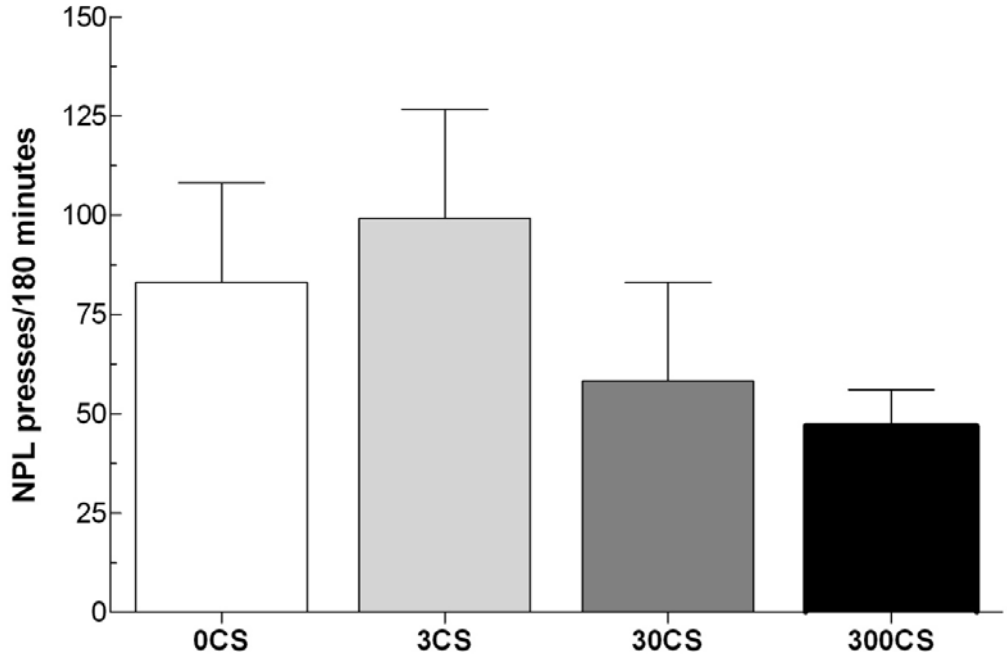


Figure 28: Total number of nicotine-paired (NPL) at the end of renewal session in Experiment #4. Data are expressed as mean number of NPL presses ± S.E.M. No difference between groups has been observed. (n=4 per group).

Temporal analyses of cumulative NPL responding during the renewal session were performed at different time-points (Figure 29). Two way ANOVA analyses for factor CS presentations at four levels (0CS, 3CS, 30CS an 300CS) and time-point (i.e., at 15, 30, 45, 60, 120, 180 minutes) showed statistically significant main effects of time-point ($F_{[5,55]} = 12.89$; $p < 0.0001$), but not of and CS presentations ($F_{[3,55]} = 2.02$; $p = 1.69$), neither for interaction between factors was observed ($F_{[15,55]} = 1.12$; $p = 0.36$). Bonferroni post-hoc test did not show any difference between groups.

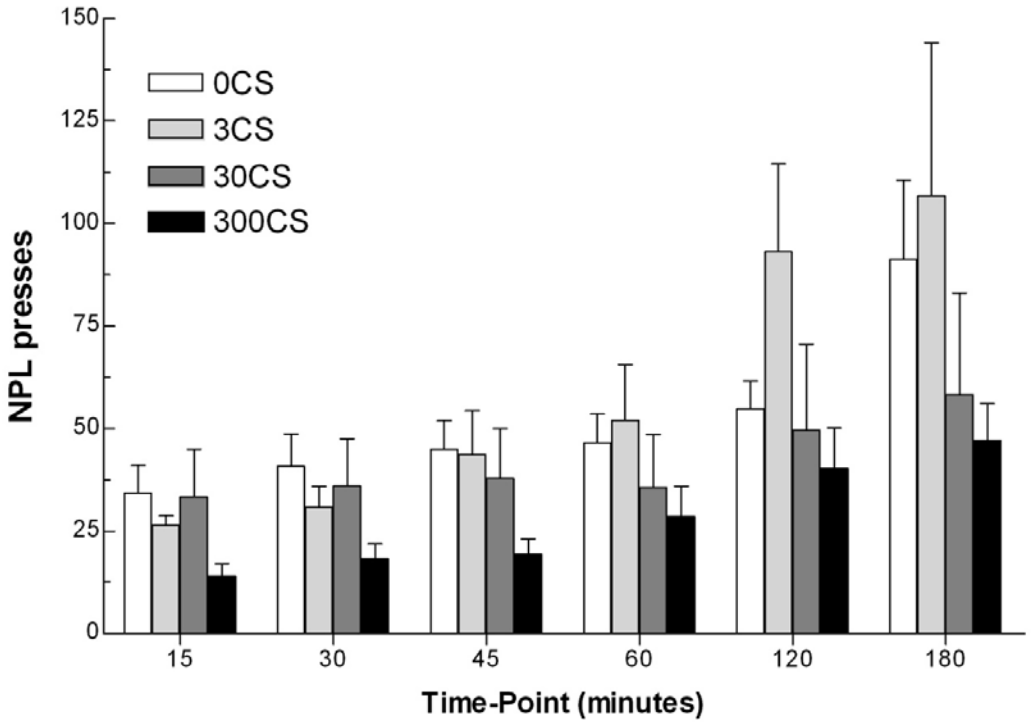


Figure 29: Temporal analysis of renewal in Experiment #4. Data are expressed as number of NPL (mean ± S.E.M) at different time-points of the renewal session. No difference between groups has been observed. However 300 CS presentations tend to decrease the number of NPL presses across the entire session. (n=4 per group).

In summary no statistically significant effect was observed. However 300CS presentation tend to decrease the number of NPL presses during the entire renewal session. At all time-points during the 180 min session, the four groups exhibited a significantly greater responding on NPL than on inactive lever (statistical analysis not shown).

### *3.2.5 Experiment #5*

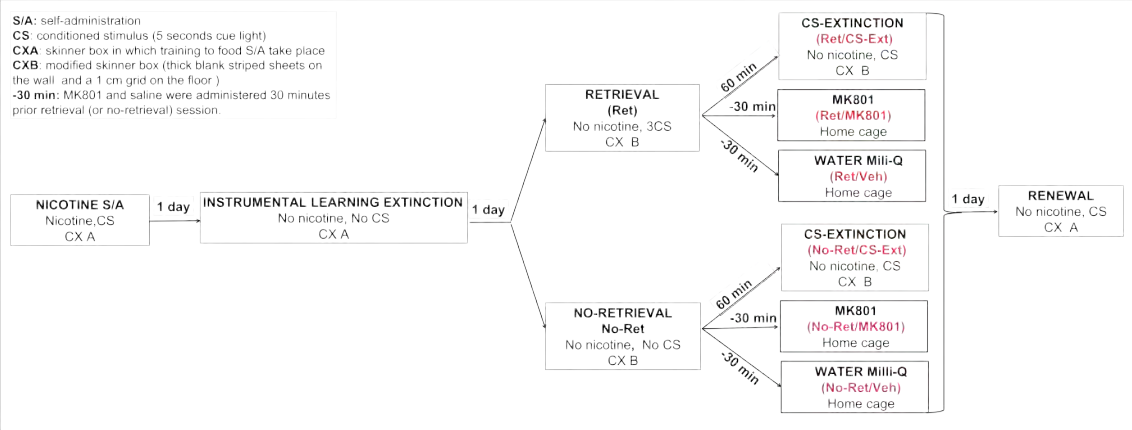


Figure 30: Schematic diagram of experimental design in experiment #5. Rats were trained to nicotine self-administration (S/A) (approximately 13 sessions), and then they underwent an instrumental extinction phase (ILEXT, approximately 12 sessions) in order to extinguish the instrumental component of conditioning. One day after the end of ILEXT, rats were divided into two groups that underwent a retrieval or no-retrieval session respectively in a context other then the S/A and ILEXT one (CX B). Then both Ret and No-Ret groups were divided into three subgroups. Ret/CS Ext and No-Ret/CS-Ext, one hour after the end of retrieval, underwent a CS-extinction session in which CS, but not nicotine was presented upon NPL press. Session lasted until the extinction of response (no NPL presses for 30 minutes) and took place in CXB. Ret/MK801 and No-Ret/MK801 30 minutes before the retrieval or no-retrieval received a MK801 injection. Ret/Veh and No-Ret/Veh 30 minutes before retrieval or no-retrieval received an injection of water Milli-Q (vehicle). The day after, memory were tested in a renewal session that consisted in placing the rats in the S/A training context (CXA) and during which each NPL press resulted in a CS presentation.

The total NPL presses at the end of the 180 min renewal session was 46.0 ± 10.7, 38.7 ± 6.2, 81,2±15, 52.5 ± 8.4, 45.5 ± 10.8 and 60 ± 13 NPL presses/180 min (3.8 ± 0.5, 5.2 ± 1.5,3.6 ± 0.9, 8.2 ± 2.3, 6.0 ± 0.9 and 5.4 ± 1.8 inactive lever presses/180 min session) (mean ± S.E.M.) respectively for Ret/Veh (n = 9), Ret/CS-Ext (n = 13), Ret/MK801 (n=13) No-Ret/Veh (n = 13), No-Ret/CS-Ext (n = 12) and No-Ret/MK801 (n=13) groups (Figure 31). To compare the results of CS-extinction or MK801 vs. vehicle separately we run two separate two way ANOVA with factor retrieval (Ret, No-Ret)

and factor treatment (CS-Ext or MK801 and Veh). In the analysis comparing CS-Ext vs.Veh, two way ANOVA did not showed any significant effect for factor treatment (Veh, CS-Ext) ($F_{[1,43]} = 0.64$; $p = 0.43$), not for factors retrieval (Ret, No-Ret) ($F_{[1,67]} = 0.01$; $p = 0.91$) neither for the interaction between retrieval and treatment ($F_{[1,43]} = 0.00$; $p = 0.99$). In the analysis comparing MK801 vs.Veh, two way ANOVA did not showed any significant effect for factor treatment (Veh, MK801) ($F_{[1,44]} = 2.82$; $p = 0.10$), not for factors retrieval (Ret, No-Ret) ($F_{[1,44]} = 0.14$; $p = 0.71$) neither for the interaction between retrieval and treatment ($F_{[1,44]} = 0.77$; $p = 0.38$).

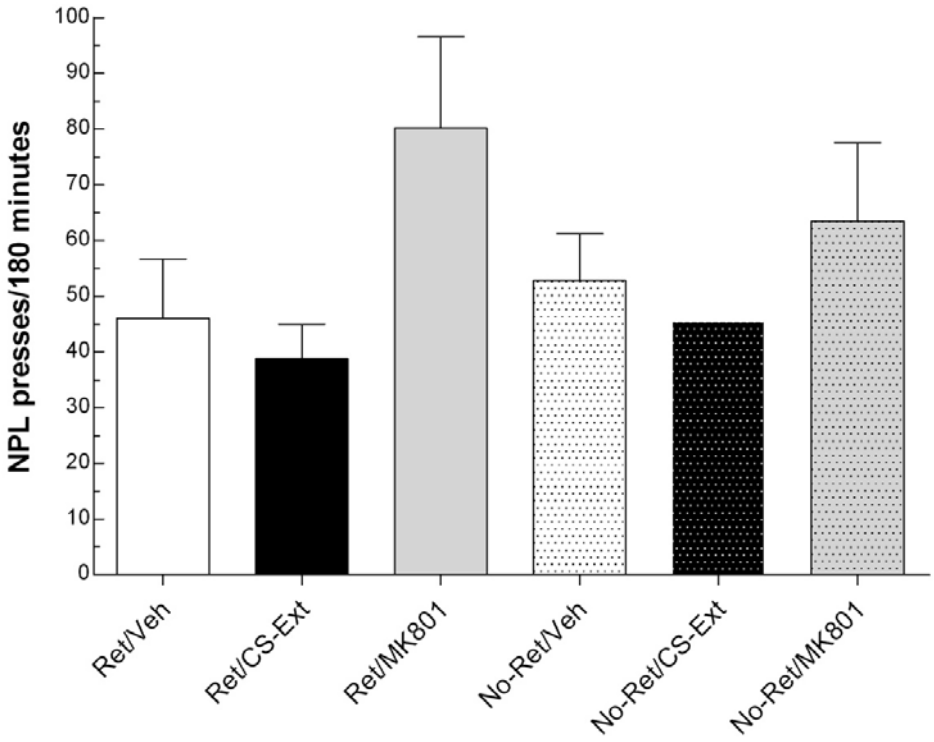


Figure 31: Total number of nicotine-paired (NPL) lever presses at the end of renewal session in Experimen #5. Data are expressed as mean number of NPL presses ± S.E.M. No difference between groups has been observed. (Ret/Veh n=9; Ret/CS-Ext n=13; Ret/MK801 n=13; No-Ret/Veh n=13; No-Ret/CS-Ext n=12; No-Ret/MK801 n=13).

Temporal analyses of cumulative NPL responding during the renewal session were performed at different time-points separately for each Ret and No-Ret groups. For Ret groups (Figure 32), two way ANOVA analyses for factor treatment at two levels (CS-Ext, Veh) and time-point at different levels (i.e., at 180, 120, 60, 45, 30 minutes) showed a statistically significant main effect of time-point ($F_{[5,100]} = 27.29$; $p < 0.0001$;

$F_{[4,80]} = 19.77$; $p < 0.0001$; $F_{[3,60]} = 13.53$; $p < 0.0001$; $F_{[2,40]} = 10.34$; $p = 0.0002$; $F_{[1,20]} = 9.16$; $p = 0.0067$) respectively, whereas a statistically significant main effect for factor treatment was observed only during the early part of the session, i.e. at 60, 45 and 30 minutes ($F_{[1,60]} = 5.84$; $p = 0.02$; $F_{[1,40]} = 7.70$; $p = 0.01$; $F_{[1,20]} = 8.12$; $p = 0.0099$) respectively. No significant effect for interaction between factors was observed at any time-point level. For Ret groups (Figure 32), two way ANOVA analyses for factor treatment at two levels (MK801, Veh) and time-point at different levels (i.e., at 180, 120, 60, 45, 30 minutes) analyses showed a significant main effect for factor time-point at each level ($F_{[5,100]} = 21.55$; $p < 0.0001$; $F_{[4,80]} = 18.06$; $p < 0.0001$; $F_{[3,65]} = 15.75$, $p < 0.0001$; $F_{[2,40]} = 14.70$; $p < 0.0001$; $F_{[1,20]} = 11.57$; $p = 0.003$). On the other hand, treatment main effect was not observed when two way ANOVA analyses were performed at different time-point levels (6, 5, 4, 3 and 2 levels, i.e., at 180, 120, 60, 45, 30 min) ($F_{[1,100]} = 0,60$; $p = 0.45$; $F_{[1,80]} = 0.05$; $p = 0.82$; $F_{[1,60]} = 0.02$, $p = 0.88$; $F_{[1,40]} = 0.11$; $p = 0.75$; $F_{[1,20]} = 0.19$; $p = 0.67$) respectively. No significant effect for interaction between factors was observed at any time-point level.

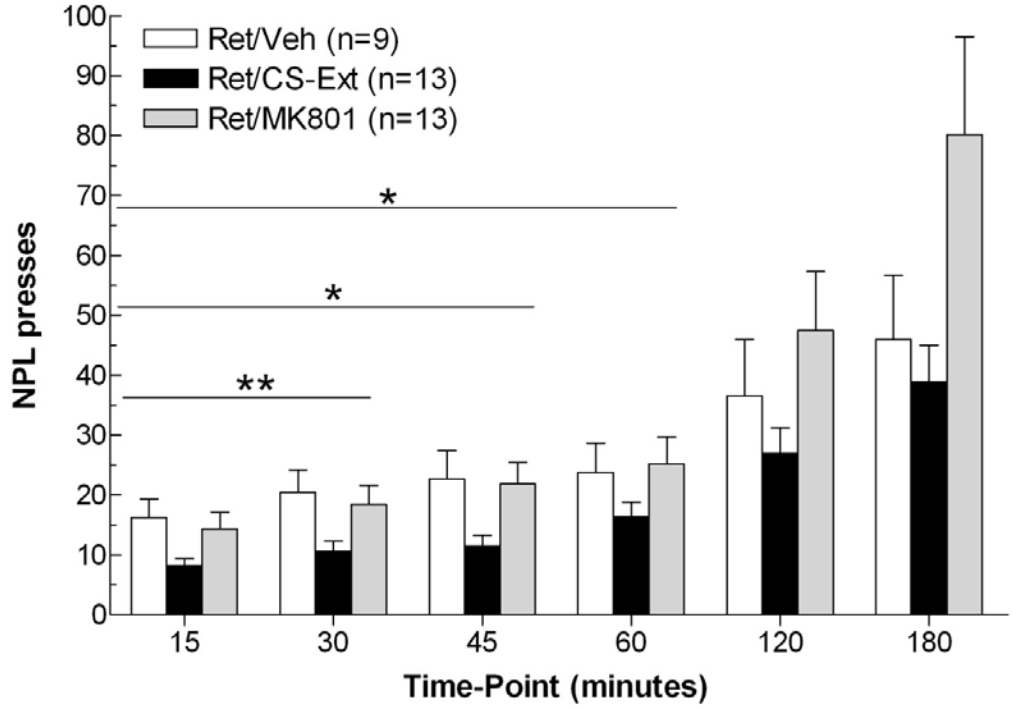


Figure 32: Temporal analysis of renewal for Ret groups in Experiment #5. Data are expressed as number of NPL (mean ± S.E.M) at different time-points of the renewal session. Two way ANOVA for factor time point at 4, 3 and 2 levels (60, 45 and 30,15 minutes; 45, 30, 15 minutes; 30, 15 minutes, respectively) and factor treatment at two levels (CS-Ext, Veh or MK801, Veh) revealed a main effect of CS-extinction in the early part of the session. No effect of MK801 was observed. *p<0.05 main effect of treatment, two way ANOVA with factor time-point and treatment at two levels (Cs-EXT, Veh), **p<0.01 main effect of treatment, two way ANOVA with factor time-point and treatment at two levels (Cs-EXT, Veh).

For No-Ret groups (Figure 33), analyses for factor treatment at two levels (CS-Ext, Veh) and time-point at different levels (i.e., at 180, 120, 60, 45, 30 minutes) showed a significant main effect for factor time-point at each level ($F_{[5,115]} = 40.45$; $p < 0.0001$; $F_{[4,92]} = 35.46$; $p < 0.0001$; $F_{[3,69]} = 19.28$, $p < 0.0001$; $F_{[2,46]} = 150.24$; $p < 0.0001$; $F_{[1,23]} = 13.21$; $p = 0.0014$). On the other hand, treatment main effect was not observed when two way ANOVA analyses were performed at different time-point levels (6, 5, 4, 3 and 2 levels, i.e., at 180, 120, 60, 45, 30 minutes) ($F_{[1,115]} = 0.41$; $p = 0.53$; $F_{[1,92]} = 0.46$; $p = 0.50$; $F_{[1,69]} = 0.68$, $p = 0.42$; $F_{[1,46]} = 0.66$; $p = 0.42$; $F_{[1,23]} = 0.58$; $p = 0.45$)

respectively. No significant effect for interaction between factors was observed at any time-point level.

For No-Ret groups (Figure 33), two way ANOVA analyses for factor treatment at two levels (MK801, Veh) and time-point at different levels (i.e., at 180, 120, 60, 45, 30 minutes) showed a significant main effect for factor time-point at each level ($F_{[5,120]}$ = 32.67; $p < 0.0001$; $F_{[4,96]} = 29.68$; $p < 0.0001$; $F_{[3,72]} = 22.87$, $p < 0.0001$; $F_{[2,48}] = 18.92$; $p < 0.0001$; $F_{[1,24]} = 18.51$; $p = 0.0002$). On the other hand, treatment main effect was not observed when two way ANOVA analyses were performed at different time-point levels (6, 5, 4, 3 and 2 levels, i.e., at 180, 120, 60, 45, 30 min) ($F_{[1,120]} = 0.14$; $p = 0.71$; $F_{[1,96]} = 0.03$; $p = 0.86$; $F_{[1,722]} = 0.00$, $p = 0.98$; $F_{[1,48]} = 0.00$; p =0.98; $F_{[1,24]} = 0.00$; $p$ = 0.95) respectively. No significant effect for interaction between factors was observed at any time-point level.

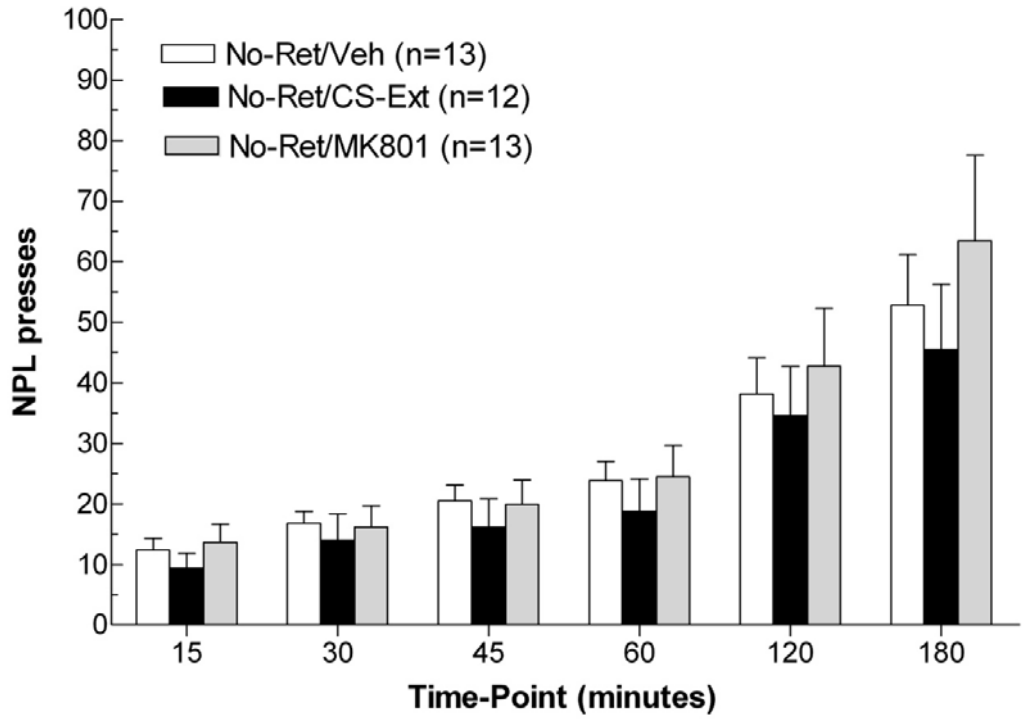


Figure 33: Temporal analysis of renewal for No-Ret groups in Experiment #5. Data are expressed as number of NPL (mean ± S.E.M) at different time-points of the renewal session. No effect of Cs-extinction or MK801 has been observed.

In summary in the analysis taking in consideration the retrieved group (Ret/Veh and Ret/CS-Ext) two way ANOVA for factor time point at different levels (30, 45, 60, 120 and 180 minutes) and factor treatment (CS-extinction, vehicle) showed a main effect of treatment in the early phase of renewal session (60, 45 and 30 minutes). On the other hand in the analysis taking in consideration the no-retrieved group (No-Ret/Veh and No-Ret/CS-Ext) two way ANOVA for factor time point at different levels (30, 45, 60, 120 and 180 minutes) and factor treatment (CS-extinction, vehicle) showed no effect of treatment. No effect of MK801 was observed across either in retrieved and in no-retrieved groups (Ret/Veh, Ret/MK801 or No-Ret/Veh, No-Ret/MK801).

At all time-points during the 180 min session, the six groups exhibited a significantly greater responding on NPL than on inactive lever (statistical analysis not shown).

## 4. DISCUSSION

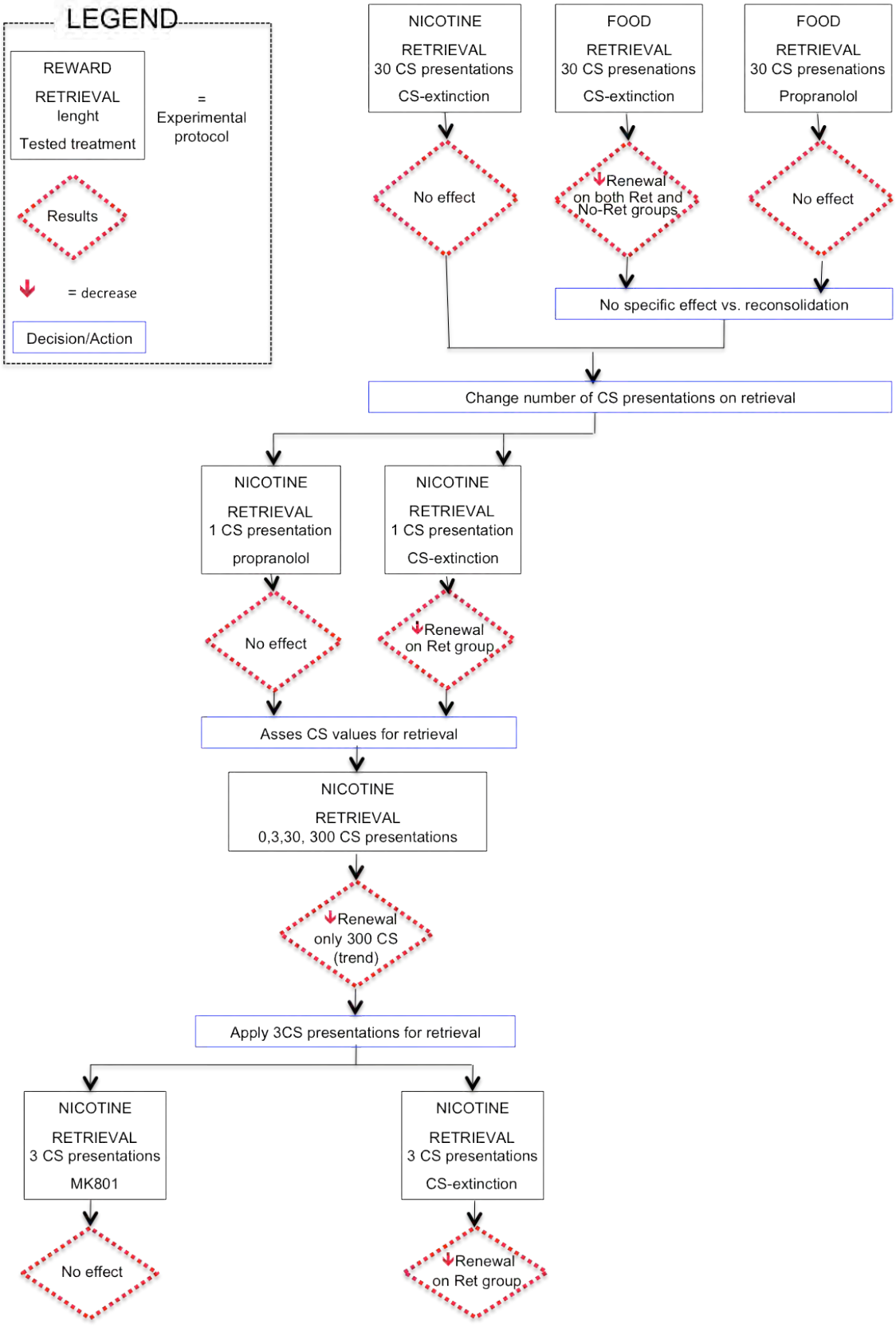


Figura 34: Schematic diagram of project design and main results.

In this project we have investigated whether post-retrieval treatment, such as propranolol, MK-801 or CS-extinction were able to prevent the renewal of food or nicotine-seeking behaviour in an animal model of nicotine addictive behaviour. We have applied these treatments after different retrieval lengths (30, 1 and 3 CS presentations in Experiment #1 and #2, Experiment #3, Experiment #5 respectively). The results can be summarized as follows: i) renewal of nicotine-seeking behaviour have been impaired by the administration of CS-extinction after a short retrieval (1 or 3 CS presentations), on the other hand no effect of CS-extinction has been observed in those subjects without retrieval; ii) renewal of food seeking behaviour have been impaired by the administration of CS-extinction either with or without a previous long retrieval (30 CS presentations); iii) propranolol and MK-801 have had no effect on of food or nicotine seeking behaviour.

In the first experiments we were interested to assess whether the update of the nicotine memories by their retrieval could enhance the efficacy of CS-extinction procedure in inhibiting the renewal of nicotine seeking behaviour. This is based on the idea that new information, provided while memories are labile due to retrieval, could be incorporated in the original memory trace. Therefore we hypothesized that the CS-No US associative information provided through CS-extinction would be integrated in the original CS-US associative memories, leading to a disruption of the conditioned values of CS, and therefore resulting in inhibition of renewal of nicotine-seeking behaviour triggered by CS presentation. CS-extinction per se is a new learning process, and does not delete the original memory but compete with it. It has been argued that updating the original memories trace, instead of creating a new one, could be more effective in preventing the re-expression the original memory (Monfils et al, 2009 and Schiller et al, 2011). However our results showed that the administration of the CS-extinction after retrieval did not increase the effect of CS-extinction when applied alone. This is in contrast with the data obtained by Monfils et al and Schiller et al. They have show in an animal and human model of fear conditioning respectively, that the CS-extinction provided within the reconsolidation window of fear related memories could disrupt their reconsolidation and prevent the return of fear. On the other hand our results are in line with the finding that post-retrieval CS-extinction do not prevent the return of fear both in animal and human model of fear conditioning (Chan et al, 2011; Costanzi et al, 2011; Soeter & Kindt 2011). Recently also Flavell and colleagues (2011) showed that the combination of memory retrieval and extinction did not affect subsequent conditioned freezing

compared with the control group given extinction alone in an auditory fear conditioning paradigm. However they pointed out that some methodological issues might explain the contrasting results with the original finding of Monfils et al (such as training length). However, in the same study, Flavell et al showed that post-retrieval extinction significantly impaired the contextual fear memory, compared to extinction alone. It should be taken in consideration that emotional and appetitive memories underlie on different memory system and mechanisms (Honjio et al., 2009), therefore this could explain the different results we obtained in nicotine self-administration paradigm, involving appetitive memories, and those obtained in fear conditioning paradigm (Chan et al, 2011; Costanzi et al, 2011; Flavell et al, 2011; Soeter & Kindt, 2011), involving emotional memories. However, Flavell et al have recently shown that post-retrieval-extinction procedure is effective in disrupting appetitive (i.e. food-related) Pavlovian memories in rats: they have demonstrated that the combination of retrieval and extinction reduced the conditioned reinforcing values of the food-related memories when compared to extinction alone. It remains to be determined at which extent Pavlovian appetitive food memories undergo reconsolidation in a similar way to Pavlovian appetitive drug memories.

It should be pointed out that, since Experiment #1 was a preliminary experiment, the number of replications was very low, therefore we decided to further investigate the effect of post-retrieval CS-extinction on renewal of food-seeking behaviour (Experiment #2). In the second experiment we included two groups of subjects treated with propranolol with or without previous retrieval of nicotine related memories, and two control groups treated with vehicle (retrieved and no-retrieved). Previous studies showed that propranolol administered in concomitance with appetitive memories retrieval may disrupt their reconsolidation (Diergaarde et al, 2006; Milton et al, 2008). We therefore have included propranolol as a pharmacological standard to assess whether 30 CS presentations at retrieval was able to induce reconsolidation. No effect of propranolol was observed; moreover CS-extinction reduced the renewal of food seeking behaviour both in retrieved and in no-retrieved subjects. These data suggest that our retrieval protocol was not inducing reconsolidation of food-memories.

Flavell et al. have found that either retrieval consisting in 10-minute exposure to the context previously paired with food self-administration, and to the light CS in combination were both able to reactivate food related memories and, in combination with extinction, to produce a deficit in conditioned reinforcement compared to the

extinction controls. They argued that context alone might retrieve and destabilize the light (CS)-food memories. These data are consistent with our finding that CS-extinction reduce renewal of food seeking behaviour both with or without previous retrieval of food-seeking behaviour, considering that no-retrieval procedure consisted in placing the animal for 20 minutes in the "retrieval" context. The hypothetical context-induced retrieval of CS might explain the efficacy of CS-extinction in preventing the renewal of food-seeking behaviour both in retrieved subjects and in no-retrieved subjects. However our experiments retrieval and no-retrieval procedure were performed in a context other than the food-associated one, therefore is unlikely that context may retrieve light (CS)-food association. Given the lack of effect of propranolol and the no specificity of CS-extinction we hypothesized that retrieval protocol used (30 non contingent CS presentations) was not actually inducing destabilization of the memory and subsequent reconsolidation. The retrieval length (i.e. the number of CS presentations) is a critical factor on whether reconsolidation or extinction occurs: a short exposure to a previously conditioned stimulus act as retrieval of the CS-related memory, it induces the destabilization and subsequent reconsolidation of this memory trace, a long exposure to CS induce the extinction of this CS (Monfils et al., 2009; Pedreira & Maldonado 2003). We speculated that 30 CS presentations could induce extinction, instead of reconsolidation; therefore we decided to test the effect of CS-extinction and propranolol applied after a shorter retrieval (1 CS presentation) on renewal of nicotine-seeking behaviour (Experiment #3). As for the previous experiment we observed that retrieval before CS-extinction did not potentiate the effect of CS-extinction alone (after no-retrieval session) and propranolol did not prevent renewal either with or without previous retrieval of the memory. The lack of effect of propranolol on food or nicotine-related memories is in contrast with previous studies in which it has been shown that propranolol disrupt the reconsolidation of food and drug related memory in rats (Diergaarde et al, 2006; Milton et al, 2008). Some protocols differences between these studies should be pointed out. The study carried out by Milton et al. was conducted in a procedure (namely acquisition of new response for conditioned reinforcer, described in Lee et al., 2005) that isolates the acquired Pavlovian conditioned reinforcing properties of appetitive conditioned stimuli (i.e. the association CS-US), from the instrumental component of the conditioning (i.e. the association conditioned response-US). A limitation of our study is that, during retrieval, instrumental component of appetitive memories were not be reactivated (and then supposed to be disrupted by propranolol or

CS-extinction), since the CS were presented not contingently upon response and levers were not present in the retrieval context, therefore it cannot be excluded that renewal of food and nicotine related memories were triggered by instrumental memories instead of Pavlovian memories.

In study carried out by Diergaarde et al, retrieval consisted in the exposure to context previously associated to the sucrose self-administration without the presentation of the tone and cue-light (CSs) previously associated to sucrose availability, therefore only the contextual memory was destabilized at retrieval. Propranolol was administered at the same dose used in our protocol immediately after retrieval. It can be argued that propranolol have selectively disrupted the reconsolidation of the contextual memory resulting in the prevention of context-induced reinstatement of sucrose seeking-behaviour. In our experiment, retrieval consisted in the presentation of the cue-light (CS) previously paired with food delivery and took place in a context other than the conditioned one, therefore the context related memories could not be destabilized by retrieval and subsequently disrupted by propranolol. Therefore it is possible that contextual memories, instead of the retrieved CS-memories, were responsible for the renewal of food or nicotine seeking behaviour in our experiments. We choose to apply retrieval (and also CS-extinction) in a new context to improve the face validity of our experimental design, in a translational perspective. In the real life smoking-cessation treatments are provided by doctors at the hospital, therefore in a context different from that associated with the drug intake. It is well known that drug-related context may trigger the craving for smoking and precipitate relapse (Conklin & Tiffany, 2001). Find a treatment that generalize from clinical setting to real life would be fundamental for the development of new therapeutic intervention for smoking cession, therefore we decided to test the efficacy of retrieval-CS-extinction when it is applied in a context other then the drug-related one.

The efficacy of propranolol in the memory reconsolidation blockade has demonstrated also in different experimental paradigms: Zhao et al (2011) have shown that the administration of propranolol prior to retrieval of heroin-related declarative memories may inhibit their reconsolidation in heroin addicts. Debiec & LeDoux (2004) and Abrari et al (2008) showed that propranolol administered in combination to retrieval disrupts both auditory and contextual fear conditioning respectively; Kindt et al in two studies (Soeter & Kindt, 2011; Kindt & Soeter, 2011) have shown that fear related memories could be disrupted by the administration of propranolol upon retrieval in a

human model of PTDS. On the other hand, other Authors reported a limited efficacy of propranolol on the reconsolidation of fear memories (Muriavieva & Alberini, 2010: Tollenaar 2008). However, as stated above, in comparing different studies analyzing the effect of propranolol on memory reconsolidation, we should keep in mind the different nature of emotional, declarative and appetitive memories.

Our data showing no effect of post-retrieval propranolol administration on appetitive memories reconsolidation are in agreement to findings of Lee et al (2008) and Milton et al (2011). They have demonstrated that propranolol did not disrupt the reconsolidation of Pavlovian conditioned approach (the phenomenon by which animals will approach a CS that has been associated with the presentation of the appetitive reinforce; Brown & Jenkins, 1968) and conditioned motivation (the phenomenon by which CS is also capable of invigorating instrumental behaviour) for the conditioned stimuli associated to sucrose or ethanol reinforcement respectively (for review see Milton & Everitt, 2010) .

Previous studies by Bustos et al (2009) and Suzuki et al (2004) have suggested that a very brief retrieval may not result in the memory destabilization and consequent reconsolidation, therefore we hypothesized that 1 CS presentation was to low for inducing retrieval of the memory. It is also possible the rat might miss the only CS presentation (1 second illumination of the cue-light), for example if they are looking on side of the cage opposite to the cue-light side.

We therefore tested the effect of different retrieval length on renewal of nicotine-seeking behaviour, in order to assess how many CS presentations induced extinction and how many did not induce any change in renewal compared to vehicle. Retrieval alone generally did not affect the conditioned response and the existence of a reconsolidation process is largely revealed by its absence, generally by its inhibition. Therefore, we hypothesized that 300 CS presentations would work as CS-extinction leading to a decrease of the conditioned response on renewal test, 30 CS presentations would lead to a lower decrease of the conditioned response and 3CS presentations would work as retrieval and would not have any effect on renewal. Moreover, since we were interested in study the Pavlovian CS-US association memory, we decided to include in the study protocol design an instrumental learning extinction phase prior of retrieval that allowed to control for the operant conditioning component of nicotine S/A and for the specificity of the CS-extinction and propranolol effect on nicotine Pavlovian memory. 300 CS presentations showed a trend to decrease (even if the effect is not statistically significant) the conditioned response during renewal compared to the 0 CS

presentation, suggesting that it worked as CS-extinction. 30 or 3 CS presentations did not affect the renewal of nicotine-seeking behaviour. On the basis of the data from these experiments (Experiment #1, #2, #3, #4) we decided to use 3 CS presentations as retrieval protocol in the next experiment.

In Experiment #5 we assessed whether CS-extinction and MK-801 block the reconsolidation of nicotine-related memory when applied after retrieval constituted by 3 non-contingent CS presentations. It has been shown that MK-801, an NMDA receptor antagonist, administered in concomitance of retrieval inhibit the reconsolidation of drug-related memories (van der Golz et al, 2009; Milton et al, 2008, 2011), therefore we have included MK-801 as a pharmacological standard to assess whether our retrieval condition induce destabilization of the memories.

Post-retrieval nicotine CS-extinction significantly reduced renewal of nicotine-seeking behaviour compared to the control subjects that did not receive CS-extinction (Ret/Sal). On the other hand, no effect of CS-extinction was observed in those subjects without nicotine Pavlovian memory retrieval. Considering that the instrumental learning component of nicotine self-administration conditioning was extinguished, these findings suggest that the effect of post-retrieval nicotine CS-extinction on renewal was specifically due to inhibition of nicotine Pavlovian memory reconsolidation. To our knowledge, this is the first evidence of post-retrieval CS-extinction effect on drug-seeking behaviour. Considering that this is an indirect demonstration of the occurrence of memory reconsolidation process, we would also consider the result of this experiment as the first evidence of nicotine Pavlovian memory reconsolidation. We have two lines of evidence that allow speculating that post-retrieval CS extinction interfered with the reconsolidation of nicotine CS-memory. First, in the groups where nicotine CS was not reactivated, the exposure to CS-extinction did not modify the nicotine seeking behaviour (i.e. NPL presses) compared to the control group no-retrieved/vehicle (NoRet/Veh). Second, CS-extinction was applied within one 1 h from retrieval, during the so-called ‘reconsolidation window’ when memory enters in a vulnerable state (Duvarci & Nader, 2003). These data therefore suggest that there is a temporal labile window during which reactivated reinforcing drug memory could be inhibited by CS-extinction. The effects of this inhibition may reduce drug-seeking behaviour under renewal (CS re-presentation in the conditioning context after CS-reactivation done in a different context), but also suggest that a similar effect may occur under other conditions such as reinstatement (when the US is unexpectedly re-

presented) and spontaneous recovery (when CS is re-presented after a certain period of time), as reported for fear conditioning in rats (Monfils et al., 2009).

Post-retrieval exposure to CS-extinction significantly reduced NPL responding on renewal session compared to the non CS-extinction condition. The effect was significant during the first hour of the renewal session and highly significant during the early 30 min phase. While the effects were only transient, the finding that renewal early in the session was suppressed only subject given CS-extinction in combination of retrieval suggests that processes that occur in early relapse to nicotine may be impaired.

The mechanism through which post-reactivation CS-extinction interferes with reconsolidation is not clear yet. Although extinction is a learning process by which the CS becomes newly associated with no-outcomes leading to a decrease in the previously established response, in our study CS-extinction, without previous retrieval of the memory, is not however effective on renewal responding in the non-retrieved group. Thus, it seems that the new CS-extinction learning specifically affects the process activated after nicotine CS-reactivation. It is still unclear which mechanisms are involved in the 'extinction vs. reconsolidation' interaction. Some hypothesis have been proposed such as, i) CS-memory is updated during the labile phase with the new information acquired with CS-extinction ('persistent revaluation') or, ii) CS-extinction learning weakens and substitutes for the reactivated CS-memory ('progressive deconsolidation') (Monfils et al., 2009; Schiller et al. 2010). These two possible mechanisms have been demonstrated in human studies for forms of memory (Walker et al., 2003; Forcato et al., 2007; Hupbach et al., 2007) other than the appetitive memory investigated in our study. Therefore, further studies are needed in order to clarify the mechanism through which post-reactivation CS-extinction interferes with the reconsolidation of appetitive memory.

However in this experiment (Experiment #5) MK-801 failed in inhibiting the reconsolidation of nicotine related memory. This result is in contrast with previous studies demonstrating that MK-801, given in conjunction with retrieval, disrupt the reconsolidation of conditioned place preference conditioned to cocaine (Kelley at al., 2007; Brown et al., 2008; Itzhak, 2008), amphetamine (Sadler et al., 2007; Sakuarai et al., 2007) and morphine (Zhai et al., 2008). However it should be taken in consideration that self-administration likely produce a "stronger" memory then does conditioned place preference training because of the amount of number of pairing of drug with context and or CS in rats undergoing self-administration compared with conditioned place

preference, e.g. in conditioned place preference training described by Brown et al. (2008) rats received 4 cocaine-context pairing, in our self-administration training rates receive 200 nicotine-CS pairing. Several authors suggest that strong and old memories are less susceptible to post-retrieval manipulation (Frankland et al., 2006, Inda et al., 2011; Suzuki et al., 2004) therefore the different memory strength between rats trained to self-administration and conditioned place preference could explained the contrasting results. However administration of MK-801 in combination to retrieval has been shown to disrupt drug-related memories reconsolidation also in tasks involving strong memories, such as cocaine, sucrose or alcohol self-administration. van der Goltz et al. (2009) showed that the systemic administration of MK-801 given in conjunction of retrieval, reduced alcohol seeking behaviour in animal trained to alcohol self-administration, compared to a vehicle-treated control group. This effect was not observed when MK-801 was administered without retrieval of alcohol-related memories. The retrieval consisted in presenting the CS and the context previously paired with alcohol self-administration, whereas in our experiment retrieval consisted in the presentation CS and not of the context previously paired with nicotine self-administration. Therefore, as discussed above, it is possible that contextual memories, instead of the retrieved CS-memories, were responsible for the renewal of nicotine seeking behaviour in our experiments.

On the other hand our data are in agreement with those obtained by Wouda et al. (2010) and Brown et al. (2010). Wouda et al. showed that MK-801, administered after a single retrieval session did not reduce the reinstatement of alcohol seeking behaviour in rats trained to alcohol self-administration. Notably they administered MK-801immediately following the retrieval procedure, while we gave MK-801 30 minutes prior retrieval. However, alcohol-seeking behaviour was significantly reduced after repeated post-reactivation treatment (repeated reactivation followed by MK-801 injection). Brown et al. showed that post-retrieval MK-801 at the dose of 0.05 mg/Kg and also 0.2 mg/kg did not disrupt the reconsolidation of cocaine-associated memory in rats trained for cocaine self-administration.

Notably our data are consistent with the previous finding of Lee & Everitt (2008) showed that administration of MK-801 contingently upon retrieval (0,1 mg/kg, 30 minutes prior retrieval as in our experiment) reduced sucrose seeking behaviour in a reinstatement test if, and only if, the CS were contingently presented, during the retrieval procedure. On the contrary no effect of MK-801 was observed when

administered in concomitance with non-contingent CS presentations. The latter data is consistent with our findings. Indeed in our retrieval protocol, CS was non-contingently presented.

It has been recently pointed out that drug-related stimuli (CS) can influence instrumental drug-seeking (relapse) behaviour in at least three psychologically and neurobiologically distinct ways: by i), acting as conditioned reinforcers, ii), supporting Pavlovian conditioned approach (autoshaping or 'sign tracking') which engages attention to, and brings the individual into proximity with, locations where successful drug seeking and taking have occurred and iii), invigorating responding for drugs through their conditioned motivational properties (Pavlovian–instrumental transfer or PIT; Everitt et al. 2001; Milton and Everitt 2010). Systemic MK-801 have been shown to disrupt the reconsolidation of Pavlovian conditioned approach for CS associated to sucrose, cocaine or ethanol (Lee & Everitt, 2008b; Milton et al., 2008b; Milton et al., 2011) and also conditioned motivation (PIT) for CS associated to cocaine or ethanol (Lee & Everitt, 2008b; Milton et al., 2011). As far as concerns the reconsolidation disruption of conditioned reinforcement, in the paradigm of acquisition of new instrumental response for conditioned reinforce, it has been shown that systemic MK-801disrupted the conditioned reinforcing properties of CS previously paired to sucrose, whereas only intra-amygdala injection of MK-801 have been demonstrated to impair the reconsolidation of conditioned reinforcing values of CS previously associated to cocaine (Milton et al., 2008b).

Literature reports on disruption of drugs of abuse memory reconsolidation have identified specific molecular and neuroanatomical mechanisms as targets of drug treatment, i.e. NMDA receptor and β-adrenergic receptor (Diergaarde et al., 2008; Milton & Everitt, 2010). Similar future research should identify the potential mechanisms for post-retrieval extinction effect on memory reconsolidation and may therefore suggest extinction as an alternative or a co-adjuvant to pharmacological disruption of appetitive memory reconsolidation. Given the importance of NMDA receptor in learning and memory it would worth to further investigate its potential role in mediating the effect of post-retrieval extinction on this receptor. It has been demonstrated that NMDA antagonists, such as MK-801 and D-APV, may block the reconsolidation of the previously retrieved memory therefore we can speculate that also the effect of post-retrieval CS-extinction might be mediated by NMDA receptor. More

research is needed to better understand the molecular mechanisms underlying the effect of post-retrieval CS-extinction.

### *4.1.Conclusion*

Our findings suggest that the exposure to nicotine CS-extinction, after a short retrieval of the same nicotine CS memory, may inhibit CS-induced conditioned responses of nicotine-seeking behaviour.

The overall project has some limitations. First of all, as suggested above, it would be important to extend the assessment of post-reactivation nicotine CS-extinction effects to other tests of nicotine-seeking behaviour such as reinstatement and spontaneous recovery. More than one test session on different days following the reactivation may test the time persistence of reconsolidation inhibition (e.g., Lee at al., 2006; Milton et al. 2008a).

Secondly, more evidences are needed to confirm that the effect of post-retrieval CS-extinction were due to interference of CS-extinction with reconsolidation process. Further studies will investigate the effect of CS-extinction applied 6 hours after retrieval, a delay time that allow to apply CS-extinction outside the reconsolidation window. In a group in which CS-extinction is applied outside the reconsolidation window, subject will have equal handling and experience in the retrieval and CS-extinction associated context compared to the group in which CS-extinction is applied within the reconsolidation window (1 hour after retrieval). This will allow us to better assess whether the effect of CS-extinction is retrieval-dependent or not. Moreover it would be fundamental to identify specific molecular markers of reconsolidation or extinction. To find a selective molecular correlate of reconsolidation will allow to disentangle the point of whether our retrieval protocols is inducing reconsolidation or extinction and will provide further evidence that post-retrieval CS-extinction interfere with reconsolidation of CS-memory.

Third, it remains unclear the fact that MK-801 administered in concomitance of retrieval, failed to reduce the renewal of nicotine seeking behaviour. Methodological issues might be responsible of the different data found in literature. Identify a molecular marker specifically activated at retrieval might be useful to better understand the lack of effect of MK-801.

Fourth, as pointed out by Lee & Everitt (2008), to successfully reactivate a memory acquired instrumentally (e.g. lever press) the CS should be presented contingently upon

acquired response. We can then hypothesized that presenting the CS contingently upon lever presses during retrieval session, would lead to a more complete retrieval and destabilization of the memories, and to a stronger effect of CS-extinction (and MK-801) on the reconsolidation of that memory.

Finally, it remains unexplained the fact that the inhibition of renewal responding in the retrieved CS-extinction group is not greater than 50%. This may be due to i), limited efficacy of post-retrieval CS-extinction, ii), partial activation of reconsolidation mechanisms by our retrieval conditions (Lee, 2008; Wang et al., 2009), and/or the existence of other conditions under which reconsolidation does not fully take place ('boundary' conditions; Lee, 2009), iv), incomplete extinction of the instrumental learning component of nicotine S/A conditioning (e.g., Hernandez & Kelley, 2004), or, v), combinations of the conditions above. Notably, other factors may be taken into consideration such as the nicotine memory strength and age (Suzuki et al., 2004). On the latter issue, we however used experimental conditions for reinforcing drug/CS memory strength (total number of nicotine/CS associations) and age (duration of nicotine S/A training phase) similar to other papers (Sanchez et al., 2010).

Therefore further studies are needed to better understand the mechanisms underlying the effect of CS-extinction on nicotine related memories and to optimize its efficacy in preventing the relapse of nicotine seeking behaviour. To achieve these objectives we will perform three sets of experiments.

First of all the effect of CS-extinction, applied 6 hours after retrieval (outside the reconsolidation window), on renewal of nicotine seeking behaviour will be tested. Moreover this effect will be investigated on reinstatement and spontaneous recovery.

Second, the effect of Cs-extinction (and MK-801), applied after a contingent CS presentation at retrieval, will be assessed.

Finally we will perform ex-vivo molecular experiments after retrieval session. Molecular markers correlating reconsolidation needs to be assessed to confirm memory reconsolidation occurrence and its disruption. rpS6P is a new molecular marker taken in consideration in memory reconsolidation field. Recent studies from Hoeffler and Klann (REF) suggest that 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 very specific in the identification of memory reconsolidation process. rpS6P is part of the ribosome and is ubiquitous so double labelled immunofluorescence allows to identify

neurons expressing rpS6P. In our laboratory we are going to standardize immunofluorescence as a qualitative assay and immunohistochemistry as a quantitative assay for rpS6P**.**

In conclusion, our findings suggest that the exposure to nicotine CS-extinction, after a short retrieval of the same nicotine CS, may inhibit CS-induced conditioned responses of nicotine seeking and offer a potential co-adjuvant to current therapeutic interventions.

Current pharmacotherapy and behavioural support to aid smoking cessation are targeted at the early weeks post-quit when withdrawal symptoms are at a peak and individuals are particularly vulnerable to relapse. However, many smokers who successfully maintain abstinence through this early phase of a quit attempt, relapse at a later date. This highlights the importance of developing new interventions targeted at relapse prevention beyond the current treatment phase. Exposure to drug-related cues and retrieval of drug-related memories are potent triggers of relapse, even after months of abstinence (Abrams, 1999) therefore there is an increasing interest across authors in developing novel treatments for addiction through manipulations of drug related memories. The first strategy to achieve this objective is to facilitate extinction; the second is to disrupt drug related memories reconsolidation. Although preclinical studies show promising results in the efficacy of reconsolidation-targeted treatment in preventing the relapse to drug-seeking behaviour, the reconsolidation of drug related memories in human have not yet been investigated. This is probably due to the fact that most of the compound used in animal studies to block memory reconsolidation can not be used in human, since they are toxic. Therefore the identification of a safe drug free paradigm, such that proposed in the present research project that inhibit reconsolidation of drug related memories would be critical for the future development of reconsolidation targeted therapy for smoking cessation.

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